The Role of the Tumour Suppressor p53 in Cooperation with Ras and Raf Oncogenes

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Abstract

To study the molecular mechanisms of oncogene cooperation, rat Schwann cells were used in which oncogenic Ras or Raf alone cause a cell cycle arrest, whereas cooperating oncogenes lead to transformation (Ridley et al., 1988; Lloyd et al., 1997). This model system was used to search for cooperating oncogenes and to investigate the underlying biochemical mechanisms of oncogene cooperation.

Interestingly, mutants of the tumour suppressor p53 efficiently cooperate with oncogenic Ras or Raf to transform Schwann cells. This result prompted me to further investigate the role of p53 in Schwann cells expressing an inducible activated Raf (ΔRafER). Activation of ΔRafER led to the induction of the cell cycle inhibitor p21WAF1/Cip1. Induction of p21WAF1/Cip1 is p53-dependent, since dominant negative mutants of p53 abolish this induction and subsequently the G1 cell cycle arrest.

The pathway leading to the p53-dependent induction of p21WAF1/Cip1 by activated Raf was investigated by addressing the possible role of the MAP-kinase pathway. The contribution of MAP-kinase kinase and MAP-kinase in the signalling pathway triggering the G1 cell cycle arrest were examined using dominant negative and constitutively active mutants as well as a specific chemical inhibitor. My results indicate that MAP-kinase kinase is necessary for p21WAF1/Cip1 induction in Schwann cells. The contribution of the JNK-kinase pathway in the Raf-initiated cell cycle arrest was addressed.
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<td>aa</td>
<td>amino acids</td>
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<tr>
<td>APS</td>
<td>ammonium persulfate</td>
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<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>AC</td>
<td>anchor cell</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>base pairs</td>
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MBP       myelin basic protein
MOPS      (3-(N-morpholino) propanesulfonic acid
mA        milliampere
mt        mutant
nt        nucleotide
dNTP      desoxypynucleotides (all four)
NGF       nerve growth factor
PLL       poly-L-lysine
PAGE      polyacrylamide gel electrophoresis
PMSF      phenylmethylsulfonylfluoride
PBS A     phosphate buffered saline A
PIPIPES    piperazine-N,N′-bis (2 ethane-sulfonic acid)
PCR       polymerase chain reaction
PDGF      platelet-derived growth factor
Py        pyrimidine
Pu        purine
RT        room temperature
rpm       rounds per minute
Rb        retinoblastoma protein
sec       seconds
SDS       sodium dodecyl sulfate
SRE       serum response element
Tris      2-amino-2 (hydroxymethyl)-1,3 propandiol
TCA       trichloroacetic acid
TEMED     N,N,N′,N′-tetramethylethylenediamine
UTR       untranslated region
V         volt
VPC       vulval precursor cell
CHAPTER 1.0

INTRODUCTION
Molecular Mechanisms of Oncogene Cooperation
1.0 Molecular mechanisms of oncogene cooperation

Tumorigenesis is a multistep process in which multiple genetic defects are acquired over time. The requirement for multiple genetic changes in tumorigenesis has been indicated by many studies in vitro and in vivo (Hunter, 1991). Furthermore, in humans the correlation between the increase of cancer incidences and age has supported this notion (Peto et al., 1975). In multistep tumorigenesis, oncogenes cooperate towards cellular transformation (Land et al., 1983; Ruley, 1983).

1.0.1 Oncogene cooperation in vitro

First evidence that more than one oncogene is necessary for cellular transformation was provided by experiments with DNA tumour viruses. The viral functions of DNA tumour viruses which are required for transforming primary non-established cells differed from the viral functions required to transform already established cells. For instance, the adenovirus E1A gene immortalises primary cells. The action of additional viral oncogenes such as E1B leads to full transformation in these immortalised cells (Houweling et al., 1980). Similarly, large T antigen on its own is able to immortalise non-established fibroblasts (Rassoulzadegan et al., 1983b). Likewise, the polyoma virus middle T antigen is only capable of transforming established fibroblasts (Treisman et al., 1981), while the joined action of small-, middle- and large T antigen is required to transform non-established fibroblasts (Rassoulzadegan et al., 1983a).

The necessity for oncogene cooperation in cellular transformation has also been shown for cellular oncogenes. The introduction of ras oncogenes into non-established primary fibroblasts did not result in transformation (Newbold & Overell, 1983; Land et al., 1983; Ruley 1983; Sager et al., 1983, Franz et al., 1986), whereas activated Ras clearly leads
to a transformed phenotype in established NIH3T3 fibroblasts (Stacey & Kung, 1984; Pulciani et al., 1985). However, the transformation of non-established primary cells can be achieved when an activated ras gene is introduced together with a cellular oncogene such as c-myc (Land et al., 1983) or the adenovirus E1A gene (Ruley, 1983). These results clearly indicate that at least two or more oncogenes are required for cellular transformation of non-established cells.

1.0.2 Oncogene cooperation in vivo

The cooperative effect of oncogenes in vivo has been widely observed in transgenic mice. Co-expression of v-Ha ras and c-myc in the mammary gland of transgenic mice led to a dramatic acceleration in the formation of carcinomas compared to transgenic mice with only a single oncogene (Sinn et al., 1987). A similar cooperative effect in tumorigenesis of leukemias was observed in transgenic mice expressing the Eμ-myc gene, under control of the immunoglobulin heavy chain enhancer, infected with a retrovirus harbouring v-Ha ras (Langdon et al., 1989). Cooperation of oncogenes in vivo is not only characterised by an enhanced initiation of tumorigenesis. Furthermore, increased tumour invasiveness and the appearance of tumours in tissue originally not affected in parental mice have been reported in many transgenic mice studies (Donehower et al., 1995).

Transgenic mice have also been employed in the search of cooperating oncogenes. For instance, transgenic Eμ-myc mice were infected with the Moloney murine leukaemia virus (MuLV) acting as an insertional mutagen to activate protooncogenes (van Lohuizen et al., 1991; Haupt et al., 1991). Given that the retroviral insertion of MuLV is random, any repetitive integration in a specific locus ("proviral tagging") found within
the early stages of an outgrowing tumour would suggest that the affected
gene cooperates with the $E\mu$-myc transgene. The analysis of loci with
repetitive integrations, therefore, should reveal genes capable of
cooperating with the $E\mu$-myc transgene (Berns 1991; Allen & Berns, 1996;
Jonkers & Berns, 1996). Using this approach, it was discovered that the
serine/threonine kinase $pim-1$ is activated with high frequency in $E\mu$-myc
transgenic mice (van Lohuizen et al., 1991; Haupt et al., 1991). This result
was confirmed in double transgenic mice carrying oncogenic $myc$ and $pim-
1$ genes which develop tumours in a very short time compared to single
transgenic parental mice (Verbeek et al., 1991; Môrôy et al., 1991). Reversibly,
$L$-myc and $N$-myc genes have been identified which cooperate
with $pim-1$ transgenic mice (van Lohuizen et al., 1989). Similarly, proviral
tagging with MuLV in mice carrying an $E\mu$-myc transgene in a $pim-1$
nullizygous genetic background results in the activation of a related $pim-2$
gene in 80% of observed tumours (van der Lugt et al., 1995).

Despite the value of transgenic mice in studying tumorigenesis and
oncogene cooperation, homogenous expression of oncogenes in targeted
tissues does not appropriately mimic an in vivo situation of early onset of
tumorigenesis. Oncogenic mutations will occur most likely in single cells
surrounded by normal cells. Such a scenario was modelled by introducing
a few retrovirally infected cells into a reconstituted murine prostate
(Thompson et al., 1989). Interestingly, incorporation of prostate gland cells
containing oncogenic ras causes only dysplasia, whereas cells expressing
an activated myc gene alone cause a hyperplastic phenotype (Thompson
et al., 1989). This finding suggests that single oncogenes can induce
different phenotypes in premalignant stages of tumour development. In
contrast, both oncogenes jointly expressed, gave raise to prostate
carcinomas, although additional genetic events are likely to participate in the formation of these tumours (Thompson et al., 1989).

### 1.0.3 Loss of tumour suppressor function cooperates in tumorigenesis

Synergestic oncogenic effects towards cellular transformation have also been observed between a growth promoting gene and the loss of tumour suppressor gene function. In contrast to oncogenes, mutations in tumour suppressor genes are mainly recessive. Therefore, the loss of function is often associated with the loss of both alleles as in case of the tumour suppressors Rb (Levine, 1990; Cowell, 1990) and p53 (Levine, 1992). However, the function of the tumour suppressor p53 can be strongly impaired by mutations in only one allele, since p53 mutants exhibit a dominant negative effect on the function of the remaining wild type p53 (Milner et al., 1991a; 1991b; Martinez et al., 1991). Mutants of the tumour suppressor gene p53 cooperate with an activated ras gene in transforming primary rat embryo fibroblasts as measured by foci formation and tumorigenicity in nude mice (Parada et al., 1984; Eliayahu et al., 1984; Hinds et al., 1990). Similarly, p53 mutants can release the ras-induced cell cycle arrest in REF52 fibroblasts and cooperate in transformation (Hicks et al., 1991). In p53 nullizygous mice, the introduction of oncogenes such as Ras (Kemp et al., 1994; Hundley et al., 1997), myc (Elson et al., 1995), E7 (Howes et al., 1994) and SV40 large T (Symonds et al., 1994) can significantly enhance tumorigenesis confirming that the functional loss of p53 cooperates with various oncogenes in vivo.

Apart from a cooperative effect between a growth promoting gene and the loss of tumour suppressor function, loss of the two tumour suppressor genes p53 and the retinoblastoma (Rb) can also have a
synergistic effect on tumorigenesis (Williams et al., 1994; Harvey et al., 1995). Mice deficient for p53<sup>-/-</sup> and heterozygous for Rb<sup>-/-+</sup> exhibit a reduced viability due to extensive tumour growth (Williams et al., 1994). Furthermore, p53<sup>-/-</sup>/Rb<sup>-/-+</sup> mice have a wider tumour spectrum compared to the parental p53 nullizygous or Rb heterozygous mice. In addition, some tumours in p53<sup>-/-</sup>/Rb<sup>-/-+</sup> mice did lose the remaining Rb-allele, arguing for a selective pressure towards the complete loss of Rb function (Williams et al., 1994).

The inactivation of tumour suppressor genes also appears to be the underlying principle for the cooperation of many viral oncogenes. A number of viral genes abrogate the function of p53 and Rb. For instance, SV40 LT inactivates p53 by physical interaction (Gannon & Lane, 1987) and the adenovirus E1A protein can inactivate Rb or family members p300 and p107 (Barbean et al., 1992; Ludlow & Skuse, 1995). Similarly, the human papilloma virus 16/18 (HPV) E6 and E7 proteins inactivate p53 (Scheffner et al., 1990) and Rb function (Scheffner et al., 1992), respectively. The transgenic expression of both viral proteins (Pan & Griep, 1994) or the Rb inactivating HPV E7 protein in a p53 nullizygous genetic background (Howes et al., 1994) lead to tumorigenesis in murine ocular lens. The functional loss of both p53 and Rb has also been frequently observed in human tumours (Lin et al., 1996; Paggi et al., 1996), arguing that this form of cooperation may play an important role in human carcinogenesis.

1.0.4 Cooperation of oncogenes affect G1-S phase transition

Cooperating oncogenes enable non-proliferative cells to proceed through the cell cycle in the absence of external growth signals. At the restriction point, the regulation of the Rb-protein is instrumental for the
progression into S-phase (Lin et al., 1996; Paggi et al., 1996). The regulation of the Rb-protein can be influenced by cooperating oncogenes. For instance, cyclin D forms active cyclin-dependent kinase complex with cyclin-dependent kinases 4 and 6 (CDKs) which are responsible for the phosphorylation-dependent regulation of Rb (Lin et al., 1996; Paggi et al., 1996). Overexpressed cyclin D can cooperate with oncogenic Ras in cellular transformation of rat embryo fibroblasts indicated by immortalisation, anchorage-independent growth and tumour formation after injection of activated Ha-Ras/cyclin D expressing fibroblasts into nude mice (Lovec et al., 1994a; Hinds et al., 1994). In transgenic mice, the cyclin D1 gene can cooperate with L-myc and N-myc genes in forming B-cell lymphomas (Lovec et al., 1994b; Bodrug et al., 1994). In support of these findings, overexpression or amplification of cyclin D has been widely observed in various tumours (Lammie et al., 1992; Motokura et al., 1991; Schuuring et al., 1992; Leach et al., 1993; Hall & Peters, 1996).

Oncogene cooperation can also occur between activated Ha-Ras and Cdc25A and B phosphatases (Galaktionov et al., 1995a). The Cdc25A and B phosphatases activate cyclin-dependent kinases through dephosphorylation of inhibitory threonine and tyrosine phosphorylation sites (Dunphy & Kumagai, 1991; Galaktinonov & Beach, 1991; Gautier et al., 1991; Strausfeld et al., 1991) and thereby regulate cell cycle progression positively. Moreover, the loss of the retinoblastoma gene (Rb) enables Cdc25A to transform primary mouse embryo fibroblasts (Galaktionov et al., 1995), indicating a cooperative effect between the loss of tumour suppressor function and a phosphatase which activates cyclin-dependent kinase complexes.
1.0.5 Oncogene cooperation and apoptosis

Genes which regulate apoptosis have been found to cooperate with genes regulating cell proliferation. The overexpression or deregulation of \textit{c-myc} is often associated with neoplastic growth (Spencer & Groudine, 1991; Marcu et al., 1992). However, \textit{c-myc} overexpression in the absence of survival factors can also cause apoptosis (Askew et al., 1991; Evan et al., 1992; Shi et al., 1992). The anti-apoptotic gene \textit{bcl-2}, on the other hand, either prolongs cell survival (McDonnell et al., 1989; Nunez et al., 1991) or inhibits apoptosis depending on the cell type (Sentman et al., 1991; Henderson et al., 1991). In the absence of survival factors, the coexpression of c-Myc and Bcl-2 abrogates c-Myc-induced apoptosis (Fanidi et al., 1992; Wagner et al., 1993). The expression of Bcl-2 does, however, not influence c-Myc's mitogenic properties (Fanidi et al., 1992). Therefore, c-Myc and Bcl-2 can cooperate in cellular transformation. Furthermore, cell proliferation in c-Myc and Bcl-2 expressing fibroblasts occurs in the absence of mitogens (Fanidi et al., 1992). Other features of transformation, however, such as the formation of foci or morphological transformation are absent in c-Myc and Bcl-2 expressing fibroblasts (Reed et al., 1990; Wagner et al., 1993). This cooperative effect \textit{in vitro} has also be shown in \textit{in vivo}-studies. Bcl-2 transgenic mice exhibit a small number of malignant lymphomas. Coexpression of the \textit{c-myc} gene dramatically increases the number of tumours compared with the number of tumours in either transgenic parental mice (Vaux et al., 1988; Strasser et al., 1990).

Oncogenes can also cooperate with survival factors such as Insulin-like growth factor I and II (IGF-I/II) in order to abolish an apoptotic response and enable tumour progression. A subset of hyperproliferative islets expressing SV40 large T in a transgenic mouse model (Hanahan 1985), show secretion of IGF-II, whereas normal non-proliferative islets did not
secret this factor. The secretion of IGF-II appears to be responsible for an abolition of SV40-large T-induced apoptosis in this system (Christofori et al., 1995), since expression of SV40 LT in IGF-II nullizygous mice exhibit a greatly reduced number of smaller and more structured tumours compared with tumours which develop in SV40 large T/IGF-II+/+ mice. Furthermore, tumours arising in IGF-II-deficient mice exhibit a five-fold higher rate of apoptosis, suggesting that the absence of the survival factor IGF-II reduces the tumour growth due to a high apoptosis rate (Christofori et al., 1994; Ueda & Ganem 1996). In agreement with these findings, the IGF-I receptor has been shown to promote survival in tissue culture and animals (Resnicoff et al., 1995a, b; Harrington et al., 1994).

Cooperating oncogenes can also influence apoptosis through the functional inactivation of apoptosis-inducing regulators such as p53. Apoptosis which occurs in the development of ocular lenses in Rb-heterozygous mice is lost in a p53-deficient background (Morgenbesser et al., 1994). Retinal tumours develop in Rb heterozygous/ p53+/− mice but do not develop in a p53+/-/+ background (Howes et al., 1994; Pan & Griep, 1994). Similarly, the slow tumour growth due to apoptosis in transgenic mice expressing a SV40 large T antigen mutant inactivating Rb (Symonds et al., 1994) was dramatically accelerated in a p53 nullizygous background and tumour growth was indistinguishable from cells expressing wild type SV40 large T-antigen capable of inactivating both Rb and p53. In agreement with this finding, a strong decrease of apoptosis accompanies the development of malignant tumours (Symonds et al., 1994). In summary, the cooperation of oncogenes can occur between a proliferative oncogene and the loss of cellular apoptotic function through either overexpression of an anti-apoptotic protein, secretion of a survival factor or inactivation of an apoptosis inducing protein.
1.0.6 Oncogene cooperation and human tumorigenesis

In human tumorigenesis sequential mutations in proto-oncogenes are required before cells become fully transformed. Intensive studies into the initiation of colorectal tumourigenesis as a model system have established that seven to ten mutations have to occur before epithelial cells become transformed (Fearon & Vogelstein, 1990; Kinzler & Vogelstein, 1996).

In colon carcinogenesis, the effect of cooperating oncogenes on tumour initiation and progression can be analysed in morphological well-defined stages (Vogelstein & Kinzler, 1993). Although the genes affected and the overall number of genetic lesions appear to be crucial for the onset of tumorigenesis (Fearon & Vogelstein, 1990), defects in specific genes can be correlated with specific stages of tumorigenesis. For instance, loss of the APC gene are correlated with early hyperproliferation of normal epithelium (Levy et al., 1994; Luongo et al., 1994; Jen et al., 1994). Mutations in the ras-gene are often associated with further progression of early premalignant adenomas (Vogelstein et al., 1988; Shibata et al., 1993) and the loss of the tumour suppressor genes DCC and p53 accompanies the development towards malignant carcinomas (Fearon & Vogelstein, 1990).

The progression from hyperproliferation towards malignant tumours is understood to occur via clonal expansion. Clonal expansion describes a process by which the first mutation in a single cell results in limited cell proliferation within a tissue. These hyperproliferative cells may acquire a second mutation resulting in a small benign tumour. Cells within the benign tumour which acquire further mutations clonally expand and may outgrow the original benign tumour towards malignant stages (Fearon & Vogelstein,
1990; Kinzler & Vogelstein, 1996). This hypothesis has been supported by the finding that malignant cells within a tumour exhibit the same set of mutations as found in benign sections, but with additional mutations (Fearon & Vogelstein, 1990; Kinzler & Vogelstein, 1996).
CHAPTER 1.1

INTRODUCTION

The Ras-pathway in signal transduction and transformation
1.1 The Ras pathway in signal transduction and transformation

The Ras signalling pathway is of central importance in mediating cellular growth and differentiation signals from the cell surface to the nucleus. The cellular ras genes H-ras, K-ras and N-ras belong to the family of small GTPases which include other members such as Rap, Rho and Rac (Boguski & McCormick, 1993). Ras has also been widely implicated in tumorigenesis, and mutations of the ras gene have been found in 10-15% of human tumours (Bos, 1988).

1.1.1 The Ras signalling pathway

The Ras signalling pathway mediates growth and differentiation signals, initiated by receptor and non-receptor tyrosine kinases, from the cell surface to the nucleus. The importance of the Ras signalling pathway was initially shown by the requirement of Ras-activity for cell proliferation (Mulcahy et al., 1985; Feig & Cooper, 1988). Blocking the Ras-MAP-kinase pathway via a dominant negative mutant of Ras (Feig & Cooper, 1988) or an interfering antibody to Ras (Mulcahy et al., 1985; Stacey et al., 1991; Moodie et al., 1993) is sufficient to abrogate S-phase entry after mitogenic stimulation.

The stimulation of various growth-factor receptors, such as the Insulin receptor (Skolnik et al., 1993a, 1993b; Baltensperger et al., 1993), the Nerve Growth Factor (NGF) receptor (Li et al., 1992) and the Epidermal Growth Factor (EGF) receptor (Buday et al., 1993a, 1993b) triggers p21Ras activation. Activation of these tyrosine kinase receptors leads to the autophosphorylation of tyrosine residues within their cytoplasmic domains. These specific phosphorylations provide an anchor for adapter molecules
such as Grb2 (Rozakis-Adcock et al., 1993). Grb2 consists of protein-interaction domains, src-homology domains 2 and 3 (SH2/SH3) (Clark et al., 1992) and has no enzymatic activity (Suen et al., 1993; Bar-Sagi et al., 1993). Grb2 bound to the receptor via an SH2 domain (Lowenstein et al., 1992) attracts the Ras guanine nucleotide exchange protein, mSOS-1, to the multi-protein complex via its SH3 domains (Batzer et al., 1993; Egan et al., 1993; Gale et al., 1993). This physical interaction between the phosphorylated receptor, Grb2 and mSOS-1 increases the guanine nucleotide exchange activity of mSOS-1 on Ras (Aronheim et al., 1994). G-protein coupled receptors signal also towards Ras. This involves the Gβγ-mediated tyrosine phosphorylation of Shc, which leads to an increase in Shc-Grb2-Sos association and subsequent Ras-dependent activation of downstream targets (Crespo et al., 1994; van Biesen et al., 1995).

1.1.1.1 Mechanism of Ras activation

Ras is a small signal transducer protein whose activation is regulated by the binding to guanine nucleotide phosphate (Lowy & Willumsen, 1993). In unstimulated mammalian cells the majority of Ras molecules are found in the inactive conformation bound to guanine nucleotide diphosphate (GDP). Upon activation of p21^{RAS}, GDP is exchanged for guanine nucleotide triphosphate (GTP). Activated GTP-bound Ras is slowly hydrolysed by an intrinsic GTPase activity to GDP and free phosphate (Lowy & Willumsen, 1993). Both steps, the activation of Ras through the exchange of GDP to GTP and its inactivation through hydrolysis of GTP to GDP and phosphate, are regulated by a number of accessory proteins. Ras is positively regulated by guanine nucleotide-exchange factors (GEF) (Boguski & McCormick, 1993) such as mSos (Chardin et al., 1993; Simon et al., 1993), GRF/CDC25
(Crechet et al., 1990), Vav (Margolis et al., 1992; Gulbins et al., 1994), Dbl (Shou et al., 1992), which facilitate GTP binding to Ras (Egan et al., 1993). Contrary to GEFs, GTPase-activating proteins (e.g. GAP and NF1) act as negative regulators by increasing the hydrolysis of GTP bound to Ras (Boguski & McCormick, 1993), thereby shifting the equilibrium towards inactive Ras. Oncogenic Ras, however, remains in a constitutively active GTP-bound conformation through mutations that either inhibit the hydrolysis of bound GTP or increase the rate of GDP to GTP exchange, thus resulting in higher levels of GTP-bound Ras (Lowy & Willumsen, 1993).

In addition to their role as negative regulators of Ras, some GTPase-activating proteins may also be effectors of Ras. For instance, the Ras GTPase-activating protein p120GAP contains SH2 and SH3 domains in the amino-terminus which, when expressed separately, can induce transcription from the promoter of the c-fos-gene in a Ras-dependent manner (Medema et al., 1992). The amino-terminus of p120GAP can also increase transformation when co-transfected with v-src into NIH3T3 cells (De Clue et al., 1993). Further evidence that p120GAP may be a Ras effector has come from the observation that the amino-terminus of p120GAP binds to two proteins, p62 (Carpino et al., 1997; Yamanashi & Baltimore, 1997) and p190 (Bryant et al., 1995). This binding is facilitated via SH2/SH3 domains of p120GAP in cells stimulated by mitogens (Boguski & McCormick, 1993). The cytoplasmic protein p62 can also bind to other proteins such as phospholipase Cγ, Grb2 and Src (Richard et al., 1995). The second GAP-binding protein p190 (Settleman et al., 1992a), exhibits GAP-activity towards Rho (Foster et al., 1994) and can inhibit Rho-mediated formation of stress fibers (Ridley et al., 1993). These findings suggest that p190 is a RhoGAP and that Ras activation may influence Rho via the RasGAP-p190RhoGAP interaction (Ridley et al., 1993). However, the formation of a
complex with p120\textsuperscript{GAP}/p62/p190 and its association with the plasma membrane does not require activated Ras, thus raising doubts whether p120\textsuperscript{GAP} is indeed a Ras effector (Pronk et al., 1993).

Neurofibromin (NF-1), another mammalian GTPase activating protein, binds to Ras via its GAP-homology domain and negatively regulates Ras activity (Martin et al., 1990; Xu et al., 1990a). Oncogenic Ras, however, is insensitive to negative regulation by neurofibromin (Martin et al., 1990; Xu et al., 1990a). Neurofibromin shows sequence homology with the \textit{S.cerevisiae} Ras-regulatory proteins, IRA1 and IRA2 (Ballester et al., 1990). The catalytic domain of neurofibromin can functionally replace IRA proteins in regulating Ras in yeast (Xu et al., 1990b; Ballester et al., 1990). IRA1 and IRA2 bind to Ras but exhibit only negative regulatory functions (Tanaka et al., 1990; 1991). Similarly, there is no evidence that neurofibromin is an effector of Ras. In support of this notion, mammalian neurofibromin appears to have no additional known motifs besides its GAP-related domain (Boguski & McCormick, 1993). Furthermore, apart from Ras, the only other protein known to bind to neurofibromin is tubulin (Bollag et al., 1993). Nevertheless, neurofibromin can inhibit Ras-dependent growth in NIH3T3 cells by a mechanism apparently independent of its ability to accelerate the intrinsic GTPase activity of Ras (Johnson et al., 1994).

1.1.1.2 Downstream effectors of Ras

Active Ras can bind to several effector proteins which mediate signals into distinct pathways. The interaction of Ras with its different effectors can occur through two distinct regions of the Ras protein: The first region comprises amino acids 32-38, designated Switch I and a second, the Switch II region of residues 60-76. Both regions dramatically change their conformation in the active GTP-bound state (Krengel et al., 1990; Pai et al.,
1990; Schlichting et al., 1990). Furthermore, these two regions may determine the specificity of Ras signalling towards different cellular target proteins (Moodie et al., 1995).

The best characterised Ras effector so far is the Raf serine/threonine kinase (Kyriakis et al., 1992, 1993; Rapp et al., 1983, 1991) which is part of the Ras-MAP-kinase signalling pathway. Raf-1 kinase is associated through its amino-terminus with GTP-bound Ras as well as oncogenic Ras (Moodie et al., 1993; Van Aelst et al., 1993; Warne et al., 1993; Zhang XF. et al., 1993; Vojtek et al., 1993).

Another Ras effector, phosphatidylinositol-3-OH kinase (PI3K) (Rodriguez-Viciana et al., 1994), consists of two subunits, a 110 kDa catalytic subunit and an 85 kDa regulatory subunit. Ras can bind to the catalytic subunit in vitro (Rodriguez-Viciana et al., 1994), and the expression of both PI3K subunits together with Ras rapidly increase the generation of the second messenger phosphatidylinositol-3,4-bisphosphate. Furthermore, the increase in phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate after the addition of mitogens can be inhibited by coexpression of a dominant negative Ras(N17) mutant, arguing for the Ras-dependency of this process (Rodriguez-Viciana et al., 1994). The activation of phosphatidylinositol-3-OH kinase, and subsequent generation of phosphatidylinositol-3,4-bisphosphate triggers the specific activation of the PKB/Akt kinase (Burgering & Coffer, 1995; Franke et al., 1995; 1997a) and has also been shown to activate the serine/threonine kinase p70S6 (Chung et al., 1994).

MEK-kinase, a homologue of the yeast protein kinases Bry2 and Ste 11 (Lange-Carter et al., 1993), binds directly via its catalytic domain to GTP-bound Ras and was therefore suggested as a possible Ras effector (Russell et al., 1995). Initially identified as a kinase able to activate MAP-kinase
kinase in a Raf-independent manner (Lange-Carter et al., 1993), MEK-kinase, however, has been shown to be a member of the JNK-1 kinase pathway rather than the Ras-MAP-kinase pathway (Sanchez et al., 1994; Yan et al., 1994). Thus, MEK-kinase links Ras with the JNK-kinase pathway (Minden et al., 1994a).

Other proteins, initially identified as Ras effector candidates by their binding capacity to Ras in vitro or by their interaction in yeast two-hybrid system screens, have not yet been clearly characterised as Ras effectors. For instance, the association of Ras with the Ral guanine nucleotide dissociation stimulator (Ral-GDS), an exchange factor for the Ras-related protein Ral has been observed in a yeast two-hybrid system screen (Hofer et al., 1994; Spaargaren et al., 1994). To date, it is not clear whether Ral-GDS is a direct Ras effector, although it does appear that Ral-GDS is involved in the Ras-dependent activation of phospholipase D (Carnero et al., 1994; Jiang et al., 1995). Activated phospholipase D generates various second messengers such as phosphatidic acid, lysophosphatidic acid and diacylglycerol (Jiang et al., 1995). Other Ral-GDS, such as RGL (Kikuchi et al., 1994; Peterson et al., 1996) have been found to bind to GTP-bound Ras in vitro (Van Aelst et al., 1994).

1.1.2 The Ras-MAP-kinase signalling pathway

Ras can activate several different signalling pathways (Denhard, 1996; Marshall, 1996). One of these pathways branching from Ras, the Ras-MAP-kinase signalling pathway has been intensively studied since the activation of this pathway is required for cell proliferation in many cell types. As an initial step in this kinase cascade, Ras in its active conformation as well as oncogenic Ras bind to the amino-terminus of the serine/threonine
kinase c-Raf 1 (Moodie et al., 1993; Van Aelst et al., 1993; Warne et al., 1993; Zhang XF. et al., 1993; Vojtek et al., 1993; Koide et al., 1993). However, the physical interaction between activated Ras and the regulatory domain of Raf (amino acids 51-131) is not sufficient to stimulate Raf-kinase activity. The activation of Raf appears to require multiple activation steps within a multi-protein complex, localised at the cell membrane (Wartmann & Davis, 1994; Morrison & Cutler, 1997), with the binding of GTP-bound Ras to Raf only being involved in a first activation step of localising Raf to the plasma membrane (Traverse et al., 1993; Marais et al., 1995). This localisation appears to be the only requirement of activated Ras in the activation of Raf, since Raf targeted to the membrane does not require Ras for activation (Leevers et al., 1994; Stokoe et al., 1994).

Several additional steps have been proposed, although the exact mechanism of Raf activation is not clear. Further activation steps have been suggested involving the possible binding of phosphatidylserine and ceramide directly to Raf (Ghosh et al., 1996; Huwiler et al, 1996), phosphorylation events, especially on tyrosine residues for the increase of kinase activity (Fabian et al., 1993, Jelinek et al., 1996), triggered by a ceramide-activated protein kinase (CAP-kinase) (Yao B. et al., 1995) or the involvement of 14-3-3 proteins (Li et al., 1995) as part of the multi-protein complex. The CAP-kinase was recently identified as the kinase suppressor of Ras (KSR) protein (Zhang et al., 1997), having been initially discovered in genetic screens in Drosophila (Therrien et al., 1995) and C.elegans (Kornfeld et al., 1995a; Sundaram & Han, 1995). In Drosophila, loss-of-function ksr alleles were recovered as a dominant suppressor of an activate Ras1-induced rough-eye phenotype (Therrien et al., 1995). The KSR-protein appears to function in parallel or upstream of Raf in Drosophila and C.elegans (Downward, 1995). Biochemical studies have revealed that
KSR/CAP-kinase binds to Raf in a ceramide-dependent manner at the plasma membrane, and can activate Raf-kinase activity through phosphorylation on two adjacent threonine residues (Zhang et al., 1997). CAP-kinase also increases the effects of activated Ras in *Xenopus laevis* oocytes and cell transformation (Therrien et al., 1996).

Dimerisation of c-Raf 1, as facilitated by chemicals, activates Raf-kinase activity and the MAP-kinase cascade in the absence of membrane compounds (Farrar et al., 1996; Luo et al., 1996). The Ras-dependency of this process is, however, unclear since activation of dimerised Raf has been observed in a Ras-dependent (Luo et al., 1996) and Ras-independent manner (Farrar et al., 1996). It has been speculated that *in vivo* 14-3-3 proteins may facilitate dimerisation of Raf (Farrar et al., 1996; Luo et al., 1996), although the evidence for a role of 14-3-3 proteins in Raf activation is controversial.

Various 14-3-3 proteins appear to be involved in Raf activation, since the expression of 14-3-3 proteins in *Xenopus* oocytes enhances Raf-1 activity and oocyte maturation (Irie et al., 1994; Fantl et al., 1994) and in *Drosophila* 14-3-3 proteins are positively modulating Ras/Raf signalling (Chang & Rubin, 1997; Kockel et al., 1997). In contrast, some groups have reported a constitutive association between Raf-1 and 14-3-3 proteins independent of Raf-1 activation (Freed et al., 1994a, 1994b; Suen et al., 1995), while others have indicated that Raf-1 kinase activity is suppressed by 14-3-3 proteins (Michaud et al., 1995).

The main target of Raf appears to be the dual specific tyrosine/threonine kinase, MAP-kinase kinase (MKK) also designated MAP-kinase/ERK-kinase kinase (MEK) (Crews et al., 1992; Huang et al., 1993). Activated Raf-1 binds and activates MAP-kinase kinase (Dent et al., 1992; Kyriakis et al., 1992, 1993; Howe et al., 1992) through phosphorylation of
two adjacent serine residues (Alessi et al., 1994; Zheng CF. & Guan, 1994; Papin et al., 1995). These two phosphorylation steps are necessary and sufficient to activate MAP-kinase kinase (Alessi et al., 1994), and point mutations of these phosphorylation sites have led to the generation of a constitutively active MAP-kinase kinase (Cowley et al., 1994; Mansour et al., 1994).

Activated MAP-kinase kinase phosphorylates various isoforms of Mitogen-activated-protein kinases (MAPKs) or extracellular signal-regulated kinases (ERKs) (Boulton et al., 1991; Crewes et al., 1992a; Wu J. et al., 1993; Zheng CF. & Guan, 1993a, 1993b). Upon phosphorylation of both threonine 183 and tyrosine 185 residues MAP-kinase activity increases dramatically (Payne et al., 1991; Haystead et al., 1992). The phosphorylation of threonine 183 facilitates the second phosphorylation step on tyrosine 185 (Payne et al., 1991; Haystead et al., 1992), but both phosphorylation events are required for the increase of kinase activity (Anderson et al., 1990). Removal of either of these phosphorylations through the action of specific phosphatases (Anderson et al., 1990) or site-specific mutagenesis (Cowley et al., 1994) leads to inactivation. The position of MAP-kinases downstream of Ras and Raf was confirmed by the observation that blocking of upstream members such as Ras through coexpression of a dominant negative mutant, abolishes MAP-kinase activity (de Vries-Smits et al., 1992; Wood et al., 1992; Schaap et al., 1993). Furthermore, MAP-kinases are found constitutively activated in v-ras (Leevers et al., 1992) and v-raf transformed cells (Howe et al., 1992).
1.1.2.1 MAP-kinase target proteins

In response to various stimuli, activated MAP-kinase translocates to the nucleus (Chen et al., 1992; Lenormand et al., 1993; Gonzalez et al., 1993), where the MAP-kinase-dependent phosphorylation of transcription factors is understood to mediate immediate early gene expression (Treisman, 1996). A clear effect of MAP-kinase phosphorylation on its ability to transactivate genes has been observed for some of these transcription factors. For instance, proteins in the ternary complex bound to the serum response element (SRE) such as ELK1/p62TCF are regulated in part through the phosphorylation of MAP-kinase (Gille et al., 1992; Janknecht et al., 1993; Marais et al., 1993). The MAP-kinase-dependent phosphorylation of ELK1 increases the ELK1-mediated formation of the ternary complex (Gille et al., 1995a, 1995b) and subsequent transactivation from the serum response element (Gille et al., 1995a, 1995b; Janknecht et al., 1993). As another example, the phosphorylation of the c-Fos transcription factor by MAP-kinase (Chen RH. et al., 1993) has been associated with enhanced stability of the protein and an increase in transactivation activity (Chen RH. et al., 1996; Okazaki & Sagata, 1995). The phosphorylation of other Fos family members, in particular Fra-1 and Fra-2, also increased the DNA-binding affinity of these transcription factors towards target genes (Gruda et al., 1994). The MAP-kinase-dependent phosphorylation of the amino-terminus of c-Myc at serine 62, as observed in vitro has been proposed to increase the transactivation activity of c-Myc in vivo (Seth et al., 1991; Seth et al., 1992). The transcription factors Ets-1 and Ets-2 are direct targets of MAP-kinase in vitro and in vivo and positively influence transactivation activity (Rabault et al., 1996; Yang et al., 1996). The c-Jun transcription factor is also phosphorylated by MAP-kinase (Pulverer et al., 1991, Alvarez et al., 1991). The major phosphorylation site in vitro is serine 243 (Chou et
al., 1992), a carboxy-terminal site which inhibits the DNA-binding capacity of c-Jun (Alvarez et al., 1991; Baker et al., 1992; Chou et al., 1992). MAP-kinase-dependent phosphorylation therefore appears to have an inhibitory effect (Minden et al., 1994a, 1994b), whereas the related JNK-kinases can phosphorylate c-Jun N-terminal phosphorylation sites and enhance c-Jun’s transcriptional activity (Minden et al., 1994a, 1994b), suggesting a coordinated control of transactivation activity converging from at least two signalling pathways.

The phosphorylation of a number of additional transcription factors in vitro has been reported for p53 (Milne et al., 1994), c-Myb (Aziz et al., 1993) and oncoprotein 18 (Marklund et al., 1993), but a clear physiological effect in vivo has not yet been demonstrated.

Activated MAP-kinases also phosphorylate a great number of other proteins at serine or threonine residues in the consensus motif P/LXT/SP (X representing any amino acid) (Alvarez et al., 1991; Gonzalez et al., 1991; Haycock et al., 1992). Apart from cytoskeletal proteins (Drewes et al., 1992; Drechsel et al., 1992), other downstream kinases such as S6 kinases p90\textsuperscript{rsk1/2} (Chung et al., 1991; Erikson et al., 1991; Sutherland et al., 1993) and MAPKAP kinase-2 (Stokoe et al., 1992) have been found up-regulated in their activity upon phosphorylation by MAP-kinase. Furthermore, the cytoplasmic phospholipase A\textsubscript{2} (cPLA\textsubscript{2}) has been convincingly shown to be activated through phosphorylation by MAP-kinase (Lin et al., 1993). The activation of cytoplasmic phospholipase A\textsubscript{2} results in the release of fatty acids from phospholipids which generates various second messengers (Lin et al., 1993).
1.1.3 Negative regulation of the Ras-MAP-kinase signalling pathway

The Ras-MAP-kinase pathway is negatively regulated at various points and through a number of mechanisms. For instance, the phosphorylation of upstream members such as Sos by MAP-kinase, which inhibits the binding of the Sos/Grb2 complex to phosphorylated tyrosines of the EGF-receptor and thereby provide a negative feedback loop (Cherniack et al., 1994; Waters et al., 1996; Buday et al., 1995; Porfiri & McCormick, 1996; Corbalan-Gracia et al., 1996). The phosphorylation of the EGF-receptor itself by MAP-kinase (Alvarez et al., 1991; Northwood et al., 1991; Takishima et al., 1991) may be required for internalisation since mutants had an impaired uptake (Heisermann et al., 1990). This mechanism could reduce the number of EGF-receptors available at the cell membrane after prolonged stimulation. Furthermore, MAP-kinase-dependent phosphorylation of MAP-kinase kinase (Matsuda et al., 1993; Brunet et al., 1994b; Gardner et al., 1994) and c-Raf-1 (Lee et al., 1992; Anderson et al., 1991; Ueki et al., 1994) has been observed, although it is currently not clear whether these phosphorylation events were sufficient to reduce kinase activity in all cases.

Further negative regulation of the Ras-MAP-kinase signalling pathway can be provided by the regulated dephosphorylation of activated protein kinases by either cytoplasmic protein phosphatases or MAP-kinase-specific phosphatases. The protein phosphatase 1 (PP1) and the protein phosphatase 2A (PP2A) can dephosphorylate Raf-kinase (Dent et al., 1995) and MAP-kinase kinase 1/2 in vitro (Nakielny et al., 1992). PP2A also dephosphorylates the activating phosphorylation at threonine 183 in MAP-kinase 1 and 2 in vitro (Anderson et al., 1990), while the other phosphorylation site at tyrosine 185 can be dephosphorylated by several
protein phosphatases (Anderson et al., 1990). The importance of these in vitro results was strengthened by the fact that SV40 small T expression, which inhibits PP2A protein phosphatase 2A, led to the up-regulation of MAP-kinase kinase and MAP-kinase activity followed by stimulation of cell proliferation (Sontag et al., 1993).

MAP-kinase is specifically inactivated by a family of phosphatases consisting of PAC-1 (Rohan et al., 1993; Ward et al., 1994), HVH1 (Zheng & Guan, 1993c), HVH2 (Guan & Butch, 1995), MKP-1/3CH134/ERP (Charles et al., 1993; Sun et al., 1993; Noguchi et al., 1993) and a human homologue of MKP-1/3CH134, CL-100 phosphatase (Keyse & Emsilie, 1992; Alessi et al., 1993; Lewis et al., 1995). Most of the MAP-kinase specific phosphatases are localised in the nucleus (Rohan et al., 1993, Brondello et al., 1995) where they are understood to act on translocated and activated MAP-kinase. These dual-specific tyrosine/threonine phosphatases are mainly regulated at transcriptional level (Charles et al., 1993; Noguchi et al., 1993). Mitogens as well as oxidative stress and heat shock, which activate MAP-kinase, also rapidly induce the transcription of these phosphatases (Sun et al., 1993; Charles et al., 1992; Keyes & Emslie, 1992), thereby providing a negative feedback loop. However, individual growth factors have a different potential to induce these phosphatases (Noguchi et al., 1993) and the protein phosphatase CL-100 dephosphorylates activated MAP-kinase in response to EGF in a cell-type specific manner (Alessi et al., 1995b).

The action of MAP-kinase-dependent phosphatases have clear physiological effects in a number of cell types. The expression of the MAP-kinase-specific phosphatase MKP-1 inhibits Ras induced DNA-synthesis (Sun et al., 1994), S-phase entry after serum stimulation (Brondello et al., 1995) as well as the serum stimulated transcription from the c-fos promoter in fibroblasts (Brondello et al., 1995). The constitutive expression of the
MAP-kinase specific phosphatase PAC-1 inhibits phorbol ester-induced transcriptional activity from the c-fos serum response element (Ward et al., 1994). Moreover, the CL-100 phosphatase inhibits the activated Ras-induced MAP-kinase activation in a cell-free system derived from Xenopus laevis oocytes (Alessi et al., 1993).

1.1.4 The Ras-MAP-kinase pathway in Drosophila melanogaster

In Drosophila melanogaster homologues of mammalian proteins of the Ras-MAP-kinase signalling pathways regulate development and differentiation, for instance, the cell fate of the R7 photoreceptor cell and the termini via the torso-pathway.

The adult Drosophila eye consists of approximately 800 ommatidia which are each made up of twenty cells. Each ommatidium contains eight photoreceptor cells, designated R1-R8, four lens-secreting cone cells and eight accessory cells. The fate of each cell appears to be decided by locally restricted cell-cell interactions (Perrimon, 1994; Zipursky & Rubin, 1994). The ligand Boss protein exclusively expressed on the differentiating R8 photoreceptor cell (Kramer et al., 1991) induces the R7 cell to become a photoreceptor cell via activation of the sevenless receptor (Van Vactor et al., 1991). The sevenless (sev) receptor tyrosine kinase is part of a signalling pathway consisting of homologous proteins to the mammalian Ras-MAP-kinase pathway which are both necessary and sufficient to determine the cellular fate of R7 (Zipursky & Rubin, 1994). In agreement with this model, mutations in various genes such as sevenless (Ready, 1989), boss (Reinke & Zipursky, 1988) and the transcription factor sina (Carthew & Rubin, 1990) disrupt the recruitment of the R7 cell to the ommatidium. The signal for
determining the cell fate in R7 cells is further mediated by the Drk protein, a homologue of the mammalian Grb2 (Olivier et al., 1993). Drk binds to the sevenless receptor as well as to Sos and is required for Ras activation (Simon et al., 1993). Furthermore, D-raf (Dickson et al., 1992) and a MAP-kinase homologue encoded by the rolled-gene (Biggs et al., 1994) have been genetically identified to mediate the differentiation of precursor cells into R7 photoreceptor cells. Target proteins for the MAP-kinase Rolled, such as the transcription factors Sina/Yan and Phyllopod are involved in translating the differentiation signal into specific gene expression patterns (Brunner et al., 1994; O’Neill et al., 1994; Rebay & Rubin, 1995; Chang et al., 1994; Dickson et al., 1995).

The torso receptor pathway in Drosophila determines cell fate at the posterior termini of the Drosophila. Ras (Lu X. et al., 1993) and a MAP-kinase kinase homologue D-MEK (Tsuda et al., 1993) are required for this differentiation process. The generation of gain-of-function mutations in the MAP-kinase Rolled and MAP-kinase kinase homologue D-MEK have indicated that both kinases are members of three pathways in Drosophila: the sevenless pathway, the torso pathway and the EGF receptor (DER) tyrosine kinase pathway (Brunner et al., 1994; Hsu & Perrimon, 1994).

1.1.5 The Ras pathway in Caenorhabditis elegans

The nematode Caenorhabditis elegans requires the activity of various proteins homologous to the mammalian Ras-MAP-kinase pathway proteins for vulva development (Kornfeld, 1997; Sundaram & Han, 1996). Vulval induction is initiated by the anchor cell (AC) of the gonad which induces three of six vulval precursor cells (VPC) to form vulval tissue, while the remaining three VPCs develop into epidermal cells (Kornfeld, 1997;
Sundaram & Han, 1996). Several genes involved in vulval induction are homologous to members of the mammalian Ras-MAP-kinase pathway such as the lin-3 gene which encodes an EGF-like molecule, secreted from the anchor cell (Hill & Sternberg, 1992), the let-23 gene which encodes a tyrosine kinase receptor of vulval precursor cells (Aroian et al., 1990), the sem-5 gene encoding a Grb-2 homologue (Clark et al., 1992) and the let-60 and lin-45 genes which encode a ras and raf homologues, respectively (Han & Sternberg, 1990; Beitel et al., 1990). Deregulation of this pathway by reducing activity causes a vulvaless phenotype, whereas an increased activity of the Ras-homologue let-60 results in a multivulva phenotype (Han et al., 1990a, 1990b).

1.1.6 MAP-kinase pathways in Saccharomyces sp.

In the yeast S. cerevisiae a number of pathways with mammalian MAP-kinase pathway member homologues have been described (Ammerer, 1994) which regulate various aspects such as the pheromone response, invasiveness in haploid strains, pseudohyphal development and spore formation in diploid strains (Ammerer, 1994; Herskowitz, 1995).

The pheromone response signalling pathway consists of homologous members of the mammalian MAP-kinase pathway and is important for the preparation of α- and α-cells prior mating. Haploid cells of S. cerevisiae occur in two distinct phenotypes, a or α-cells. Both haploid cell types grow via mitotic cell division but can also mate to form a diploid a/α cell. In order to prepare cells for mating, a-cells secrete an a-factor which binds to a specific receptor on α-cells, whereas α-cells secrete α-factors, a peptide which binds to a specific receptor on a-cells (Herskowitz, 1995). The pheromone signalling pathway activated by these peptide factors
consist of various yeast homologues of the mammalian MAP-kinase pathway which appear to be identical with the exception of the respective receptors, the α-factor receptor (STE2) or the α-factor receptor (STE3). Both receptors are coupled to G-proteins and it is understood that the Gβγ-proteins mediate the activation of the MEK-kinase STE11 possibly via protein kinase STE20, although the exact mechanism as to how Gβγ-proteins mediate STE11 activation is not yet clear. Activated STE11-kinase phosphorylates and activates STE7, a MAP-kinase kinase homologue (Neiman & Herskowitz, 1994). Activated STE7 phosphorylates and thereby activates the MAP-kinase homologues FUS3 or KSS1 (Errede et al., 1993). It has been suggested that these three kinases are bound to a "scaffolding" protein, STE5 which thereby creates a kinase module (Whiteway et al., 1995). The activated MAP-kinases FUS3 and KSS1 appear to phosphorylate the transcription factor STE12 which leads to its activation (Errede & Ammerer, 1989). The transcription factor STE12 induces transcription of genes required for the fusion between α- and α-cells as well as genes encoding members of the pheromone response signalling pathway itself (Fields & Herskowitz, 1985).

The activation of this pathway also leads to a G1 cell cycle arrest mediated by the FAR-1 protein, which binds and inhibits the cyclin-dependent kinase complexes CDC28-CLN1 and CDC28-CLN2 (Peter & Herskowitz, 1994). The protein level of FAR-1 is elevated by pheromones (Peter et al., 1993; Chang & Herskowitz, 1992). It seems that the activity of FAR-1 is modulated through phosphorylation by FUS3 MAP-kinase, thereby establishing a connection between the activation of the pheromone signalling pathway and the cell cycle machinery (Peter et al., 1993).
1.1.7 The Ras-MAP-kinase pathway in apoptosis

Serum and a set of growth factors can inhibit programmed cell death (apoptosis) and increase the survival rate of cells, whereas the removal of these factors can result in apoptosis (Askew et al., 1993; Raff et al., 1993; Harrington et al., 1994). For example, IGF-1, insulin and PDGF increase survival and inhibit cell death, whereas EGF or FGF are poor survival factors in fibroblasts (Harrington et al., 1994). Both classes of factors can stimulate the Ras-MAP-kinase pathway to various degrees in different cell types, but it is unclear how a Ras-dependent pathway mediates the inhibition of apoptosis. Suppression of apoptosis has been correlated with the activation of the Ras effector PI3-kinase (Yao R. & Cooper, 1995) which is mediated via the Ras and PI3 kinase-dependent activation of PKB/Akt (Franke et al., 1995, 1997a, 1997b) but not the activation of p70S6 kinase (Kauffmann-Zeh et al., 1997; Dudek et al., 1997). Corresponding with these results, the inhibition of PI3-kinase accelerated apoptosis and an activated form of PKB/Akt blocked apoptosis (Franke et al., 1997b). Activated PKB/Akt did not modulate the expression of negative apoptosis regulators Bcl-2 or Bcl-XL, but blocked Ced3/ICE-like protease activity (Kennedy et al., 1997), required for the execution of programmed cell death (Ellis et al., 1991; Nicholson et al., 1995; Korsmeyer, 1995).

1.1.8 The Ras-MAP-kinase pathway and tumorigenesis

The significance of Ras in tumorigenesis was initially elucidated by the discovery that retroviruses with an acquired activated ras-gene were able to transform mammalian cells (Simons et al., 1964; Kirsten et al., 1968; Harvey & East, 1969). In support of this finding, mutations of cellular Ras,
constitutively activating the protein, were found in 10-15% of human tumours examined. In particular, in up to 95% of pancreatic carcinomas mutations of the ras gene occurred (Bos, 1989; Almoguera et al., 1988).

Ras-dependent transformation has mainly been attributed to the continuous activation of members of the Ras-MAP-kinase pathways and the resulting prolonged activation of downstream transcription factors. This notion is supported by the fact that dominant negative mutants of Raf-1 (Kolch et al., 1991), MAP-kinase kinase (Cowley et al., 1994) and MAP-kinase (Khosravi-Far et al., 1995) are all inhibitory to Ras-dependent cell transformation. Moreover, constitutively active Raf (Rapp et al., 1988), Raf targeted to the membrane (Leevers et al., 1994) and constitutively active MAP-kinase kinase (Cowley et al., 1994; Mansour et al., 1994) can transform fibroblasts indistinguishable from cellular transformation caused by oncogenic Ras.

Despite the evidence of the important role of the Ras-MAP-kinase pathway in transformation, other Ras-dependent but MAP-kinase-independent signalling pathways appear to contribute or are even sufficient for cellular transformation (White et al., 1995; Qiu et al., 1995; Khosravi-Far et al., 1995; 1996; Rodriguez-Viciana et al., 1997; Olson et al., 1995). Experimental evidence which implies that multiple pathways were required for Ras transformation was provided by cooperation assays with Ras effector domain mutants. The effector domain mutant Ha-Ras (V12,T35S), although capable of binding Raf and stimulating MAP-kinase activity, is reduced in its transforming activity, suggesting that additional Ha-Ras effectors may contribute towards cellular transformation (White et al., 1995). However, mutant Ha-Ras (V12,T35S) is able to cooperate with another mutant Ha-Ras (G12V, E37G) to effectively transform fibroblasts as measured in a focus assay (White et al., 1995). The mutant Ha-Ras (G12V,
E37G) is unable to transform on its own, because it has lost the capability of binding to Raf but can still bind other Ras effectors. The requirement for both Ha-Ras mutants to efficiently cooperate towards cellular transformation suggests that multiple Ras functions may be involved in the transformation of mammalian cells (White et al., 1995). Similarly, two Ras mutants, Ha-Ras (V12, 37G) and Ha-Ras (V12, 40C), both defective in activating the Raf/MAP-kinase pathway, cause oncogenic transformation when expressed together, indicated by growth in low serum, anchorage-independent growth and formation of tumours after injection of transformed cells into nude mice (Khosravi-Far et al., 1996).

Investigations into additional Ras-dependent signalling pathways required for Ras transformation have focused on the role of Rac1, RhoA and PI3-kinase (Qiu et al., 1995; Khosravi-Far et al., 1995, 1996; Rodriguez-Viciana et al., 1997). Activated Rac1, Rac1(Val12) transforms Rat-1 fibroblasts cells as indicated by the partial loss of contact inhibition, anchorage-independent growth and in vivo tumour growth of Rac1(V12) expressing cells in athymic nude mice (Qiu et al., 1995). Furthermore, dominant negative Rac1(N17) reduces foci formation by oncogenic Ras in NIH3T3 fibroblasts (Qiu et al., 1995; Khosravi-Far et al., 1995) but not by a constitutively active form of Raf, RafCAAX (Leevers et al., 1994) which activates the MAP-kinase signalling pathway independently of Ras, suggesting that Rac1 activation is an essential step in Ras transformation (Qiu et al., 1995). It is interesting to note that RafCAAX or a weakly transforming Raf mutant, Raf(340D) cooperates with activated Rac1 in a focus formation assay (Qiu et al., 1995; Khosravi-Far et al., 1995), suggesting that the Rac1-dependent pathway is independent of the Ras-MAP-kinase signalling pathway and that both pathways are required for strong cellular transformation.
The Ras effector phosphoinositide 3-OH kinase (PI3K) (Sjolander et al., 1991; Rodriguez-Viciana et al., 1994) also appears to be involved in Ras transformation. The coexpression of a dominant negative PI3-kinase mutant (Dhand et al., 1994) strongly inhibits the cellular transformation by oncogenic Ras as measured in a focus formation assay (Rodriguez-Viciana et al., 1997). Constitutively active mutants of PI3-kinase cooperate with Raf and Rho but not Rac1, suggesting that PI3-kinase and Rac1 are members of the same signalling pathway (Rodriguez-Viciana et al., 1997).

Experiments with Ras mutants which selectively bind to various Ras effectors have confirmed these results. For instance, the Ha-Ras mutant (V12, T35S) can still activate Raf cooperates in a focus formation assay with activated mutants of PI3-kinase, Rac1 and Rho, but not with Raf itself. Similarly, Ha-Ras (V12, C40) activates only the PI3-kinase/Rac pathway cooperates with activated Raf and Rho, but not with activated PI3-kinase or Rac1, respectively (Rodriguez-Viciana et al., 1997). Taken together, these findings suggest the Raf/MAP-kinase pathway and the PI3-kinase/Rac pathway downstream of Ras can cooperate in cellular transformation.

The involvement of Rho in Ras transformation has been suggested since the expression of dominant-negative versions of Rho(N19) mitigate transformation by oncogenic Ras (Qiu et al., 1995; Khosravi-Far et al., 1995). Furthermore, activated forms of Rho and Rac, although not transforming on their own, can cooperate in a focus formation assay suggesting that they are acting on different signalling pathways (Khosravi-Far et al., 1995; Rodriguez-Viciana et al., 1997). Thus, despite the fact that many of these oncogene cooperation studies were carried out in established cells, they, nevertheless, have provided first evidence that apart from the Ras-Raf-MAP-kinase pathway, a number of Ras-dependent
downstream pathways, explicitly Rho- and PI3-kinase/Rac1-dependent pathways, may contribute towards cellular transformation by activated Ras.
CHAPTER 1.2

INTRODUCTION

The p53 tumour suppressor gene
1.2 The p53 tumour suppressor gene

The tumour suppressor p53 was first identified as a 53 kiloDalton protein associated with a transforming protein of the SV40 virus, large T (SV40 LT) (Lane & Crawford, 1979; De Leo et al., 1979; Linzer & Levine, 1979a, 1979b). The genomic p53 clones and p53 cDNA clones were subsequently isolated by various groups (Oren & Levine, 1983; Bienz et al., 1984; Pennica et al., 1984; Harlow et al., 1985). p53 is a transcription factor which mediates various cellular activities such as G1 cell cycle arrest (Michalovitz et al., 1990; Martinez et al., 1991), apoptosis (Lowe et al., 1993a, 1993b), the maintenance of genetic stability (Cross et al., 1995; Fukasawa et al., 1996) and the suppression of tumorigenesis (Baker et al., 1990). The p53 protein is mutated in many human tumours and mutant p53 can transform eukaryotic cells in cooperation with oncogenic ras (Eliyahu et al., 1984; Parada et al., 1984). Wild type p53, however, is unable to transform eukaryotic cells (Eliyahu et al., 1989; Hinds et al., 1989) and suppresses cellular transformation by other oncogenes in vitro (Finlay et al., 1989) as well as the formation of tumours in nude mice (Chen et al., 1990). Due to these features, p53 was classified as a tumour suppressor gene (Finlay et al., 1989; Eliyahu et al., 1989; Lane & Benchimol, 1990).

1.2.1 The tumour suppressor p53 in human cancer

Somatic mutations of the tumour suppressor gene p53 are the single most common genetic alteration in human tumours (Levine, 1992). The p53 gene is lost or functionally inactivated by mutations in more than half of all human tumours (Hollstein et al., 1994, 1996; Hainaut et al., 1997). 75-80% of colon tumours and 50% of lung and breast cancers in particular and many other tumours arising from various tissues exhibit somatic mutations.
in the p53 gene (Greenblatt et al., 1994). Most somatic mutations of the p53 gene are missense mutations (Greenblatt et al., 1994) which are not randomly distributed throughout the p53 gene, but occur in specific clusters located in the region encoding amino-acid residues 130-290 (based on 393 amino acids in human p53). Four regions of p53, residues amino acids 117-142, amino acids 171-181, amino acids 234-258 and amino acids 270-286, which are highly conserved across p53 from different species are the target for most of these mutations (Levine et al., 1990). The DNA-binding domain of p53 (Pavletich et al., 1993) carries most mutations, "hot-spots" in particular involving amino acid residues 175, 248 and 273 (Levine et al., 1991; Hollstein et al., 1994). Mutations of p53 in this region dramatically alter the overall structure of the p53 protein (Cho et al., 1994; Gannon et al., 1990). Interestingly, the distribution of "hot-spot" mutations in human tumours appear to be tissue-specific which has been speculated to be either due to the type of mutagen a particular cell-type was exposed to or a selective growth advantage of certain p53 mutations in a tissue-specific context (Levine, 1992).

Besides somatic p53 mutations, a genetic disorder has been reported in which p53 mutations are inherited. The Li-Fraumeni syndrome (Li & Fraumeni, 1969) is an autosomal dominant genetic disorder which is characterised by the high risk of an early onset of tumours and the development of many independent tumours. Half of individuals affected by Li-Fraumeni syndrome develop tumours by the age of 30 compared with 1% of the normal population (Malkin et al., 1990). A number of patients have presented heterozygously inherited p53 missense mutations (Malkin et al., 1990; Srivastava et al., 1990). This heterozygosity for p53 increases the risk for loss of both p53 alleles.
The homozygous loss of p53 has a dramatic effect in mice which become highly prone to acquiring a wide variety of malignant tumours at an early stage of their life (Donehower et al., 1992; Jacks et al., 1994). Within six months, 74% of p53\(^{-/-}\) mice develop various tumours (Donehower et al., 1992). By comparison, mice heterozygous for p53 (p53\(^{+/}\)) exhibit a delayed onset of an altered spectrum of tumours (Jacks et al., 1994) which can be accelerated by the application of the carcinogen dimethylnitrosamine (Harvey et al., 1993). Mice nullizygous for p53 do not reveal substantial developmental alterations, except for a small percentage of female embryos exhibiting exencephaly (Sah et al., 1995).

1.2.2 Cellular functions of p53

1.2.2.1 Wild type p53 in tumour suppression

Wild type p53's function as a potent tumour suppressor was first discovered when wild type p53 was introduced into colorectal carcinoma cell lines resulting in a cell cycle arrest (Baker et al., 1990). Additionally, the overexpression of wild type p53 suppresses transformation by cooperating oncogenes in rat embryo fibroblasts (Chen et al., 1990). Furthermore, the introduction of p53 re-establish the G1 cell cycle arrest point in response to DNA damage and reduces the rate of gene amplifications of transformed cells (Yin et al., 1992). Therefore, wild type p53 can suppress oncogenic transformation which is surprisingly independent from any p53-dependent transcriptional transactivation activity (Crook et al., 1994) which is required for p53-dependent growth suppression (Pietenpool et al., 1994). One mechanism of transformation suppression may be p53-dependent apoptosis (Lowe et al., 1994), especially since p53-dependent apoptosis has been observed which is independent of transcriptional transactivation (Haupt et al., 1995).
1.2.2.2 Wild type p53 and cell cycle arrest

Following DNA damage by a wide range of agents including UV-radiation, γ-irradiation and exposure to DNA damaging agents, p53 protein levels increase and cells arrest in G1 (Fritsche et al., 1993; Hall et al., 1993; Kastan et al., 1992; Kuberbitz et al., 1992). It has been proposed that this G1 arrest allows cells to repair damaged DNA before they proceed to replicate their DNA (Kastan et al., 1991). On the other hand, DNA damage in normal human fibroblasts induces an irreversible cell cycle arrest in G1 (DiLeonardo et al., 1994), questioning the generality of this hypothesis. In addition to p53 playing a role in the G1 cell cycle arrest, recent evidence suggests an involvement of p53 in a cell cycle arrest at the G2/M boundary (Agrawal et al., 1995; Stewart et al., 1995; Guillouf et al., 1995).

The ability of p53 to arrest cells after DNA damage has been implicated in maintaining stability and integrity of the genome. For instance, gene amplification is below detectable levels in normal diploid fibroblasts (<10^-9), but has been observed at much higher frequencies in transformed cells (10^-3-10^-5) (Livingstone et al., 1992), resulting in chromosomal abnormalities in tumour cells. This increase of gene amplification has been associated with a parallel loss of the ability of p53 to arrest cells in response to the uridine biosynthesis inhibitor PALA (Livingstone et al., 1992; Yin et al., 1992). Interestingly, expression of exogenous wild type p53 re-established the G1 arrest and reduces the frequency of gene amplification to the same low levels as observed in normal diploid fibroblasts (Yin et al., 1992).

The p53 molecule has also been implicated in the control of a mitotic cell cycle checkpoint (Cross et al., 1995; Fukasawa et al., 1996). Application of spindle inhibitors or the loss of wild type p53 function in vitro and in vivo...
lead to an increased population of tetraploid cells, suggesting that p53 has an important function in maintaining the diploid status of cells (Cross et al., 1995). In addition, cells deficient in functional p53 or the p21WAF1/Cip1-inhibitor exhibit a substantial lack of coordination between S-phase and mitosis after DNA damage (Waldman et al., 1995). This lack of coordination causes additional rounds of S-phase without a following mitosis. A high proportion of these cells exhibit aneuploidy and are eliminated by apoptosis (Waldman et al., 1995). Further studies suggest that p53 is involved in the regulation of centrosome duplication (Fukasawa et al., 1996). In the absence of p53, the number of centrosomes already increases dramatically with the second passage. This abnormal number of centrosomes results in an unequal segregation of chromosomes which suggests one possible mechanism by which the loss of p53 may cause genomic instability (Fukasawa et al., 1996). These reports support the idea that p53 has the ability to prevent DNA-damaged cells proceeding through the cell cycle, thereby controlling the integrity of the genome.

1.2.3.3 p53-dependent apoptosis

Apoptosis is a mechanism for eliminating proliferating cells damaged beyond DNA repair or affected by oncogenes which are susceptible to malignant progression. Programmed cell death is characterised by cell shrinkage, genomic DNA fragmentation and fast removal of the cell debris from the surrounding tissue (Hale et al., 1996). In response to various stimuli such as DNA damage (Clarke et al., 1993; Lowe et al., 1993a), UV-irradiation (Ziegler et al., 1994), γ-irradiation (Lowe et al., 1993b), the expression of c-myc (Hermeking & Eick, 1994; Wagner et al., 1994) and adenovirus E1A (Debbas & White, 1993; Sabbatini et al., 1995a, 1995b), p53 can induce apoptosis.
The decision, whether a cell undergoes G1 growth arrest or apoptosis depends on various factors which are only partly understood. In response to γ-irradiation the cellular decision between induction of growth arrest or p53-dependent apoptosis appears to be cell-type specific with fibroblasts arresting in G1 phase of the cell cycle and apoptosis occurring in hematopoietic and lymphoid cells (Midgley et al., 1995; Haupt et al., 1996; Canman et al., 1995). Apart from cell specificity, other factors such as the presence of growth factors (Canman et al., 1995), the expression of viral proteins (Howes et al., 1994; Sabbatini et al., 1995) and the protein levels of the retinoblastoma protein (Rb) (Morgenbesser et al., 1994; Pan & Griep, 1994) and the transcription factor E2F (Qin et al., 1994) appear to determine the decision between p53-dependent growth arrest or p53-dependent apoptosis as response.

The presence of survival factors, for instance, leads to a p53-dependent G1 cell cycle arrest after γ-irradiation, whereas their absence can trigger programmed cell death (Canman et al., 1995). Interestingly, expression of v-src could mimic the shift towards G1 cell cycle arrest. It has been speculated, that changes in p21WAF1/Cip1 and GADD45 protein levels are involved in this decision (Canman et al., 1995).

The co-expression of viral proteins is another factor which can influence the decision between growth arrest and apoptosis. The expression of adenovirus protein E1A shifts the cellular response after γ-irradiation from a p53-dependent growth arrest towards induction of apoptosis (Debbas & White, 1993; Lowe & Ruley, 1993; Lowe et al., 1994). Inactivation of retinoblastoma protein (Rb) by adenovirus E1A and E7 protein results in unregulated E2F-DP1 activity. Similarly, overexpression of E2F causes S-phase entry and induction of apoptosis (Qin et al., 1994; Wu & Levine 1994), whereas the overexpression of Rb blocks p53-dependent
apoptosis (Haupt et al., 1995). Thus, a model emerges in which the decision for p53-dependent growth arrest or apoptosis depends on the availability of survival factors and whether activated oncogenes enable cells with DNA damage to enter the cell cycle, leading to apoptosis in the presence of wild type p53 (Levine, 1997).

Both p53-dependent G1 cell cycle arrest and apoptosis, are mainly regulated via p53’s transactivation activity. The inhibition of cell proliferation by p53 is mediated through up-regulation of transcription of target genes such as p21\(^\text{WAF1/Cip1}\) (El-Deiry et al., 1993). Similarly, the regulation of p53-dependent apoptosis is understood to be mediated via the transactivation of genes responsible for the induction of apoptosis (Miyashita & Reed, 1995; McCurrach et al., 1997; Sabbatini et al., 1995). However, a p53 molecule with two mutations in amino acids critical for transcriptional activity (Lin et al., 1995) was nevertheless capable of triggering apoptosis without inducing any sequence-specific transactivation (Haupt et al., 1995). This unexpected result was confirmed by the observation of p53-dependent apoptosis in the presence of RNA or protein synthesis inhibitors (Caelles et al., 1994). These results indicate that p53-dependent induction of apoptosis may occur in a cell type-specific manner (Haupt et al., 1996) by either an unidentified transactivation-independent mechanism or via transactivation of p53 target genes which are involved in the apoptotic process (Sabbatini et al., 1995; Levine, 1997).

### 1.2.4 Functional domains of wild type p53

The human p53 protein can be separated into four functional domains: the N-terminal transcriptional activation domain (amino acids 1-42) (Unger et al., 1992), the sequence-specific DNA-binding domain (amino
acids 102-292), the oligomerisation domain (amino acids 324-355) and the very C-terminal activation domain (amino acids 363-393) (Levine, 1997; Ko & Prives, 1996).

The N-terminal transactivation domain interacts with basal transcription factors in regulating transcription from p53 target genes (Lin et al., 1994). Particularly, the physical interaction with TATA-associated factors of the TFIID-complex are understood to mediate the p53-dependent increase in transcription (Thut et al., 1995; Lu H. & Levine, 1995). Amino acid residues 19, 22 and 23 have been shown to be crucial for p53’s transcriptional activation ability (Lin et al., 1995). The interaction of the TATA-binding protein with p53 has also been mapped to this region (Liu X. et al., 1993; Truant et al., 1993, Liu X. & Berk, 1995). In addition, the cellular protein Mdm-2 and the viral protein E1B-55 kDa abrogate p53’s transactivation activity by binding to this domain (Chen J. et al., 1993; Braithwaite et al., 1991).

The central domain of p53 confers sequence-specific DNA binding (Wang et al., 1993a, 1994a; Pavletich et al., 1993; Bargonetti et al., 1993). The importance of the specific DNA-binding domain has been illustrated by the fact that most p53 mutations in tumours negatively affect this domain through changes to the structural integrity rendering the molecule unable to bind DNA specifically (Cho et al., 1994; Bargonetti et al., 1993). The DNA-binding domain alone exhibits sequence-specific DNA-binding affinity in vitro (Bargonetti et al., 1993; Pavletich et al., 1993; Wang et al., 1993). This specific DNA-binding affinity can be abrogated by SV40 LT whose binding to p53 has been mapped to the DNA-binding domain (Jenkins et al., 1989).

The C-terminal oligomerisation domain mediates dimerisation and tetramerisation of p53 (Stürzbecher et al., 1992; Pavletich et al., 1993; Reed et al., 1993; Lee et al., 1994; Cho et al., 1995; Jeffrey et al., 1995).
minimal domain required for transformation lies within the oligomerisation domain (Shaulian et al., 1992), although the significance of oligomerisation for the various biological functions of p53 is unclear. It has been suggested that oligomerisation of p53 is required for DNA binding (Halazonetis & Kandil, 1993; Shaulian et al., 1993; Pietenpol et al., 1994) and that the C-terminal oligomerisation domain is required for p53 transactivation activity (Halazonetis & Kandil, 1993; Pietenpol et al., 1994; Zhang W. et al., 1994). However, transfected monomeric p53 molecules resulting from a deletion in the oligomerisation domain and lacking sequence-specific DNA-binding capacity, still exhibit transactivation activity in transient transfection assays (Shaulian et al., 1993; Tarunina & Jenkins, 1993) and the ability to suppress oncogene-induced transformation (Shaulian et al., 1993).

The p53 activation domain induces the latent p53 sequence-specific DNA-binding activity in response to a number of modification at the C-terminus. The deletion of the last 30 amino acid of p53 as well as binding of the monoclonal antibody 421 to residues amino acids 370-378 activate p53's sequence-specific DNA binding activity (Hupp & Lane, 1994). More physiological stimuli, such as phosphorylation at serine 378 by protein kinase C and serine 392 by casein kinase II, or short single DNA strands can also activate the sequence-specific DNA binding of p53 (Hupp & Lane, 1994; Jayaraman & Prives, 1995). The C-terminal domain contains residues that enable p53 to oligomerise and to bind both single-stranded DNA and RNA (Pavletich et al., 1993; Clore et al., 1994; Wu et al., 1995).
1.2.5 Regulation of transcription by p53

Wild type p53 regulates transcription in two ways. Firstly, activated p53 positively regulates transcription from promoters or introns of target genes that contain p53-specific DNA-binding sites (Farmer et al., 1992; Zambetti et al., 1992). Although the exact sequence of p53 DNA-binding sites varies, a palindromic consensus site consisting of two copies of a 10 base pair sequence 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3' has been defined (El-Deiry et al., 1992; Funk et al., 1992).

Secondly, wild type p53 can repress transcription from promoters with a TATA-binding site but without a p53 consensus site, probably by interacting with the basal transcription machinery (Subler et al., 1992, 1994; Jackson et al., 1993; Seto et al., 1992).

1.2.5.1 Transcriptional activation of p53 target genes mediating growth inhibition

Wild type p53 mediates growth inhibition mainly through the activation of target genes. Activated p53 strongly induces the expression of the wild type p53-activated fragment 1 gene (WAF1) (El-Deiry et al., 1993). The same protein was independently isolated as a Cdk-interacting protein 1 (Cip1) which was found to bind to Cdk2/cyclin complexes and to inhibit their kinase activity (Harper et al., 1993). This parallel discovery immediately led to the suggestion that p21WAF1/Cip1 could mediate the G1 growth inhibitory function of p53 since, in particular cyclin A-Cdk2, cyclin E-Cdk2 and cyclin D-Cdk4 complexes are essential for the G1-S phase transition of the cell cycle (El-Deiry et al., 1993; Harper et al., 1993). In fact, it was shown that p21WAF1/Cip1 can inhibit a wide range of cyclin/Cdk complexes (Xiong et al., 1993b; Gu et al., 1993) and the overexpression of p21WAF1/Cip1 leads to
growth inhibition in human tumour cell lines (El-Deiry et al., 1993) as well as in normal diploid human fibroblasts (Harper et al., 1993). Conversely, a decrease of \( p21^{WAF1/Cip1} \) protein levels by antisense RNA allows arrested fibroblasts to progress into the cell cycle (Nakanishi et al., 1995). Moreover, the p53-dependent inhibition of cyclin/Cdk kinase activity after \( \gamma \)-irradiation is due to induction of \( p21^{WAF1/Cip1} \) (Dulic et al., 1994) and the ability to arrest in G1 is impaired in \( p21^{WAF1/Cip1} \) nullizygous mice (Brugarolas et al., 1995; Deng et al., 1995). However, further investigations revealed that \( p21^{WAF1/Cip1} \) is only partly responsible for mediating the growth inhibitory functions of p53, since \( p21 \) nullizygous mouse fibroblasts exhibited only a partial loss of G1 arrest in response to \( \gamma \)-irradiation (Deng et al., 1995). In contrast, nullizygous p53 fibroblasts exhibit a complete loss of the G1 arrest after \( \gamma \)-irradiation (Deng et al., 1995), indicating the existence of p53-dependent, but \( p21^{WAF1/Cip1} \)-independent ways of cell cycle arrest. However, the G1 arrest in response to a specific inhibitor of the \textit{de novo} uridine synthesis, PALA, was absent in both \( p21^{-/-} \) and in \( p53^{-/-} \) cells (Deng et al., 1995), arguing for an important role of \( p21^{WAF1/Cip1} \) in p53-dependent cell cycle arrest.

Many well-characterised cellular functions of p53 were not affected in \( p21^{-/-} \) mouse fibroblasts. Wild type p53 has been implicated in tumour suppression (Baker et al., 1990), emphasised by the fact that p53-nullizygous mice displayed a wide variety of tumours at an early stage (Donehower et al., 1992; Jacks et al., 1994), whereas \( p21^{-/-} \) mice develop normally without any malignancies for the first 7 months (Deng et al., 1995). Two other functions of wild type p53 in controlling a mitotic spindle checkpoint and thereby cell ploidy (Cross et al., 1995) as well as apoptosis in response to DNA damage in thymocytes (Lowe et al., 1993b; Clarke et al., 1993) were not negatively affected in \( p21^{-/-} \) mice (Deng et al., 1995).
Thus, these results indicate that $p21^{\text{WAF1/Cip1}}$ mediates only certain functions of p53.

The function of $p21^{\text{WAF1/Cip1}}$ depends on protein concentration within the cell. In growing diploid human fibroblasts, $p21^{\text{WAF1/Cip1}}$ is associated in a complex with the proliferating cell nuclear antigen (PCNA), cyclin-dependent kinases (Cdks) and their respective cyclin partners of type A, E and D (Zhang H. et al., 1994a). Nevertheless, $p21^{\text{WAF1/Cip1}}$ did not appear to inhibit the kinase activity of these complexes (Xiong et al., 1993a). As an explanation for this observation, it was proposed that $p21^{\text{WAF1/Cip1}}$ containing cyclin-Cdk-PCNA complexes can exist in active and inactive states, depending on the stoichiometry of $p21^{\text{WAF1/Cip1}}$ molecules in the complex (Zhang H. et al., 1994b). Indeed, a low protein concentration of $p21^{\text{WAF1/Cip1}}$ facilitates the assembly of cyclin D-Cdk4 complexes by stabilising the formed complexes. At higher concentrations, $p21^{\text{WAF1/Cip1}}$ inhibits the kinase activity of these complexes (LaBaer et al., 1997). In addition, $p21^{\text{WAF1/Cip1}}$ targets cyclin D1 and Cdk4 protein to the nucleus. This function is independent of its function to promote the assembly of cyclin D1-Cdk4 complexes (LaBaer et al., 1997).

The elevation of $p21^{\text{WAF1/Cip1}}$ protein level during development, in senescent and differentiated cells is mostly regulated in a p53-independent manner (Huppi et al., 1994; Macleod et al., 1995; Parker et al., 1995; Jiang et al., 1994). The cyclin-dependent kinase inhibitor $p21^{\text{WAF1/Cip1}}$ has been implicated in cell senescence and differentiation (Noda et al., 1994; Parker et al., 1995; Macleod et al., 1995; Halevy et al., 1995; Steinman et al., 1994). In fact, $p21^{\text{WAF1/Cip1}}$ was cloned as a protein induced in senescent cells (Noda et al., 1994). A $p21^{\text{WAF1/Cip1}}$ related p24 protein has also been implicated in senescence (Mazars & Jat, 1997). In addition to its role in senescence and differentiation, $p21^{\text{WAF1/Cip1}}$ blocks proliferating cell
nuclear antigen (PCNA) activity to activate DNA polymerase δ in vitro, which is required for DNA replication (Flores-Rozas et al., 1994), through direct interaction (Waga et al., 1994).

Apart from the p21WAF1/CIP1 gene, the Insulin-like growth factor binding protein 3 (IGF-BP3) gene has been described as a p53 target gene involved in growth inhibition (Buckbinder et al., 1995). In response to DNA damage, IGF-BP3 expression and secretion are induced. The secreted IGF-BP3 inhibits signalling initiated by insulin growth factor (IGF-1), presumably through complex formation (Buckbinder et al., 1995). Since IGF-1 can act as a mitogen or as a survival factor (Harrington et al., 1994), it may be a possibility that p53 can activate an autocrine/paracrine loop to negatively regulate mitogenic or survival signals.

Another transcriptional target of p53 is cyclin G (Okamoto & Beach, 1994; Zauberman et al., 1995). Its role in mediating p53 function, however, has remained unclear, since overexpression of cyclin G did not lead to growth arrest making it unlikely that cyclin G mediates p53’s growth-inhibitory function directly (Okamoto & Beach, 1994). However, the regulatory subunit of the protein phosphatase 2A has been found to bind in a p53-dependent way to cyclin G, although the biological implications of this interaction remain unclear as well (Okamoto et al., 1996).

1.2.5.2 Genes with other functions

Wild type p53 can regulate its own transcriptional transactivation activity through the transcriptional induction of the murine double minute 2 gene (mdm-2) (Barak et al., 1993; 1994; Wu X. et al., 1993). The mdm-2 gene has been identified as a gene amplified in a spontaneously transformed mouse BALB/c 3T3 cell line (Fakharzadeh et al., 1991) and shown to be amplified in a number of sarcomas (Oliner et al., 1992). Wild
type p53 induces transcription from the \textit{mdm-2} gene (Barak et al., 1993) through a p53 DNA-binding site of an internal promoter (Juven et al., 1993). The relationship between Mdm-2 and p53 represents an autoregulatory negative feedback loop to regulate p53 activity (Wu X. et al., 1993; Barak et al., 1993, 1994). The Mdm-2 protein binds to the amino terminal activation domain of p53 (Momand et al., 1992; Picksley et al., 1995) and thereby inhibits its function (Momand et al., 1992; Oliner et al., 1993; Chen J. et al., 1995). Furthermore, Mdm-2 binding to p53 enhances the degradation of p53 (Haupt et al., 1997; Kubbutat et al., 1997) via a ubiquitin-dependent pathway (Haupt et al., 1997).

The critical importance of the Mdm-2 protein in regulating p53 activity was confirmed by experiments with transgenic mice. \textit{Mdm-2} nullizygous mice are not viable due to early embryonic lethality (Montes de Oca Luna et al., 1995; Jones et al., 1995). However, in a p53-deficient genetic background \textit{mdm-2} nullizygous mice become viable and develop normally (Montes de Oca Luna et al., 1995; Jones et al., 1995). This surprising result shows the important role of the Mdm-2 protein in negatively regulating p53’s function at different stages of development. These experiments confirmed that overexpression of wild type p53 is not compatible with normal development (Godley et al., 1996; Nakamura et al., 1995; Hoever et al., 1994).

Wild type p53 activates the thrombospondin-1 (TSP-1) gene (Dameron et al., 1994), which is a strong inhibitor of angiogenesis. The absence of functional p53 causes a rapid decline of thrombospondin-1 and formation of an angiogenic phenotype in fibroblasts (Dameron et al., 1994).

A number of other genes have been described as possible p53 targets and these include the muscle creatine kinase (Weintraub et al., 1991; Zambetti et al., 1992; Zhao et al., 1994, 1996), the p53 gene itself.
(Deffie et al., 1993), the transforming growth factor α (TGF-α) (Shin et al., 1995), the hsp70 gene (Agoff et al., 1993), the retinoblasoma gene (Olsichin et al., 1994), the epidermal growth factor (EGF) receptor (Deb et al., 1994; Ludes-Meyer et al., 1996) and proliferating cell nuclear antigen (PCNA) (Shivakumar et al., 1995; Morris et al., 1996). However, the biological significance of p53-dependent induction of these genes are currently not understood.

1.2.6 General suppression of transcription by p53

Elevated p53 protein levels have been shown to generally repress transcription from viral and cellular promoters (Ginsberg et al., 1991; Subler et al., 1992). Those promoters which contain a TATA-box but lack a specific p53 DNA-binding site, in particular, can be suppressed by wild type p53 (Mack et al., 1993). This suppression of transcription is mediated through the interaction of p53 with basal transcription factors, required for the initiation from TATA-box containing promoters (Mack et al., 1993). Although p53 physically interacts with two TBP-associated factors (TAFs) TAF\textsubscript{II}40 and TAF\textsubscript{II}60 in the basal transcriptional machinery, this interaction is required for the transcriptional activation by p53 but not for repression of transcription (Thut et al., 1995). However, p53 also binds to the TATA-binding protein (TBP) another important component of the transcriptional machinery (Seto et al., 1992; Liu et al., 1993; Truant et al., 1993; Martin et al., 1993; Chen et al., 1993). It has been suggested that p53 binding to TBP is responsible for the observed general suppression of transcription (Seto et al., 1992; Horikoshi et al., 1995). In contrast to this hypothesis, however, a p53 mutant which fails to interact with TAF\textsubscript{II}40 and TAF\textsubscript{II}60 but binds to TBP is incapable of repressing transcription (Sabbatini B. et al., 1995). The exact molecular
mechanism and the proteins through which p53 mediates its general suppression of transcription therefore remain to be identified.

1.2.7 Genes regulating p53-dependent apoptosis

The special significance of p53’s apoptosis-inducing function has been clearly established by the effect of its inactivation in human tumours (Bardeesy et al., 1995) as well as by its role in growth and progression of tumours in an animal model (Symonds et al., 1994). The p53-dependent expression of the \textit{bax} gene is an important step in the induction of apoptosis (Selvakumaran et al., 1994; Miyashita & Reed, 1995; McCurrach et al., 1997). Bax encodes a protein with homology to the survival factor Bcl-2. Both proteins Bcl-2 and Bax can heterodimerize (Oltvai et al., 1993) and the equilibrium between these two proteins influences apoptosis (Reed, 1994), although other p53 effectors may modulate this decision (McCurrach et al., 1997). The induction of Bax in response to \(\gamma\)-irradiation correlates with the p53 status in human cells (Zhan et al., 1994), and in p53\(^{-/-}\) fibroblasts expression of the Bax protein can not be detected (McCurrach et al., 1997). Alternatively, the proposed p53-dependent induction of the fas/APO-1 gene (Owen-Schaub et al., 1995) may be another way for p53 to mediate apoptosis, since the fas/APO-1 factor triggers apoptosis.

1.2.8 p53 function in DNA replication and DNA repair

Wild type p53 may also regulate DNA replication and DNA repair via its own enzymatic activity (Mummenbrauer et al., 1996), by protein-protein interactions (Dutta et al., 1993; Waga et al., 1994; Flores-Rozas et al., 1994) or by regulating transcription from target genes (El-Deiry et al., 1994; Harper et al., 1994; Kastan et al., 1992).
The regulation of DNA replication by p53 appears to occur in multiple ways. The p53-dependent transcriptional activation of the cyclin-dependent kinase inhibitor p21WAF1/Cip1 arrests cells before entering S-phase (El-Deiry et al., 1993; Harper et al., 1993; Dulic et al., 1994). Cells in S-phase could be prevented from replicating DNA due to p21WAF1/Cip1 binding to proliferating-cell nuclear antigen (PCNA) and blocking its ability to activate DNA polymerase δ (Flores-Rozas et al., 1994; Waga et al., 1994; Warbrick et al., 1995). Interestingly, the PCNA-gene can also be transcriptionally activated by low p53 protein levels (Shivakumar et al., 1995; Morris et al., 1996). In addition to transcriptional regulation, wild type p53 can directly inhibit viral SV40 DNA replication in vitro (Braithwaite et al., 1987; Wang E. et al., 1989; Friedman et al., 1990), and p53 constitutively active in DNA binding is able to inhibit eukaryotic DNA replication in the absence of any transcription, suggesting that there is a direct involvement of p53 (Cox et al., 1995). Moreover, wild type p53 binds to the replication factor RPA and inhibits its binding to single-stranded DNA, an essential step for the initiation of DNA replication, although certain p53 mutants also bind and inhibit RPA, so that the biological significance of this interaction is not yet fully understood (Dutta et al., 1993; He et al., 1993).

The possible role of wild type p53 in DNA repair has been strengthened by the recent findings that the p53 protein itself exhibits 3′→5′ exonuclease activity which is absent in mutant p53 (Mummenbrauer et al., 1996). Furthermore, wild type p53 can recognise DNA structures resulting from damage-induced lesions such as insertion/deletion mismatches. Wild type p53 binds as tetramers to these lesions (Lee et al., 1995). This specific binding capacity is mediated via the C-terminus (Lee et al., 1995) which regulates the DNA-binding activity of p53 (Hupp et al., 1992, 1995; Bayle et al., 1995; Jayamaraman & Prives, 1995). In addition to these findings, wild
type p53 interacts with a number of proteins involved in DNA repair. Wild
type p53 binds to ERCC3, a transcription factor involved in nucleotide
excision repair (Wang X. et al., 1994), and binds the DNA repair factors XPD
(RAD3) and XPB, as well as inhibiting DNA helicase activity (Wang X. et al.,
1995). Although many reports suggest that the p53-dependent modulation
of DNA repair occurs via protein-protein interaction, the p53-dependent
induction of a Growth arrest and DNA damage gene, GADD45 (Zhan et al.,
1994; Kastan et al., 1992), appears to contribute to DNA repair. The
GADD45 protein has been shown to interact with the proliferating cell
nuclear antigen (PCNA) and to stimulate nucleotide excision repair activity
in vitro and inhibited S-phase entry (Smith et al., 1994; Hall et al., 1995).

1.2.9 Mechanisms of p53 activation and regulation
1.2.9.1 Translational regulation and protein
degradation of p53

Wild type p53 activity is highly regulated by various mechanisms. In
normal cells wild type p53 is in an inactive state and only detectable at very
low concentrations. Following DNA damage, the activation of p53 has been
correlated with an increase of p53 protein levels (Maltzman & Czyzyk, 1984;
Kastan et al., 1991; Hall et al., 1993; Midgley et al., 1995), although no
increase in p53 mRNA can usually be detected (Kastan et al., 1991).
Moreover, the addition of the RNA polymerase II and III inhibitor α-amanitin
does not inhibit the accumulation of wild type p53 (Maltzman & Czyzyk,
1984). The enhanced translation of existing mRNA molecules may be
partially responsible for the increase of p53 protein levels since the
translation inhibitor cyclohexamide blocks an increase in p53 protein, and
at the same time partially abrogates the G1 arrest in response to DNA
damage (Kastan et al., 1991). It has been suggested that p53 3’UTR may play a role in the translational control of p53 protein expression (Fu et al., 1996). Interestingly, elevated p53 protein levels show an increase both in DNA-binding activity and sequence-specific transactivation activity (Hupp et al., 1992; Lu X. & Lane, 1993; Zhan Q. et al., 1993; Hupp & Lane, 1994; Hupp et al., 1995).

Altering the half-life of p53 from approximately 35 minutes to around 150 minutes after γ-irradiation (Maltzman & Czyzyk, 1984) also contributes to the increase in p53 protein. Moreover, since p53 is specifically degraded via the ubiquitin-dependent degradation pathway (Scheffner et al., 1990; 1993; Maki et al., 1996), changes in this pathway appear to regulate p53’s steady-state levels. As an illustration of this regulation, the expression of the HPV E6-protein together with the cellular ubiquitin-ligase E6AP-protein lowers p53 protein levels (Scheffner et al., 1990, 1993; Huibregtse et al., 1991; Hubbert et al., 1992), while an inactivating mutation in the ubiquitin-dependent degradation pathway leads to an increase in p53 protein levels (Chowdary et al., 1994). Recently, the proteolytic cleavage of p53 at the amino terminus by calpain has been correlated with an increase in protein stability (Kubbutat & Vousden, 1997), although the significance for p53 regulation requires further investigations.

The regulation of p53 protein degradation also contributes to the down-regulation of activated p53. The down-regulation of p53 steady-state levels as well as p53’s activity can be regulated by the Mdm-2 protein. The mdm-2 gene itself is induced by wild type p53 (Barak et al., 1993). The binding of Mdm-2 to p53 enhances the ubiquitin-dependent protein degradation of p53 (Haupt et al., 1997; Kubbutat et al., 1997). In addition to Mdm-2 enhanced degradation of p53, Mdm-2 binds to the amino-terminus of p53 (Momand et al., 1992; Picksley et al., 1994) thereby abolishing its
transactivation activity (Barak et al., 1993; 1994; Wu X. et al., 1993). Thus, the Mdm-2 protein provides a negative feedback and modulates the duration of p53 activity in response to DNA damage.

1.2.9.2 Phosphorylation of p53

Wild type p53 is extensively phosphorylated at amino- and carboxy-terminal sites in vivo and can be phosphorylated at these sites by several protein kinases in vitro (Meek, 1994). For example, the p53 protein is phosphorylated by the double-stranded DNA activated protein kinase (DNA-PK) (Lees-Miller et al., 1992; Wang & Eckhart, 1992), Casein kinase I (CKI) (Milne et al., 1992b), MAP-kinase (Milne et al., 1994) and c-Jun kinase (JNK) (Milne et al., 1995) at the amino-terminus. Cyclin A and cyclin B-associated forms of p34cdc2 (Bischoff et al., 1990; Sturzbecher et al., 1990), protein kinase C (PKC) (Baudier et al., 1992; Milne et al., 1996) and cyclic nucleotide-independent serine/threonine protein kinase, casein kinase II (CKII) (Meek et al., 1990b; Hupp et al., 1992) and PAK1 (Thiagalingam et al., 1995) have been reported to phosphorylate the p53 C-terminus.

Despite the number of kinases capable to phosphorylate p53 in vitro, a clear effect of individual phosphorylations in modulating p53 function has only been shown in a few cases. The phosphorylation of the PKC site has been suggested to enhance p53’s DNA-binding affinity (Hupp et al., 1993) and to increase its transactivation and suppression ability (Takenaka et al., 1995). Similarly, phosphorylation of p53 by casein kinase II in vitro potentially activates p53’s specific DNA-binding activity (Hupp et al., 1992). However, the mutational analysis of the CKII site has led to contradictory results since an inactivating mutation resulted in either a reduction of p53-dependent growth inhibition (Milne et al., 1992) or in no measurable physiological effect whatsoever (Fiscella et al., 1994). P53 has also been
reported to be specifically phosphorylated by S and G2/M-Cyclin-dependent kinases. The phosphorylation of p53 results in increased sequence-specific DNA-binding activity and confers a preference for specific p53 binding sites (Wang & Prives, 1995). The effect of phosphorylation on p53’s activity is apparently complex because individual phosphorylation sites have different effects on p53’s sequence-specific DNA-binding activity which depends on the specific nature of p53 binding sites in target genes (Fuchs et al., 1995; Wang & Prives, 1995; Lohrum & Scheidtmann, 1996; Hecker et al., 1996).

1.2.9.3 Posttranslational modifications of the p53 C-terminus

The p53 C-terminal region (amino acid residues 363-393) has been shown to be essential in activating the sequence-specific DNA-binding activity of p53. The carboxy-terminal domain contains residues that confer on p53 the ability to oligomerise and to bind both single-stranded DNA and RNA (Pavletich et al., 1993; Clore et al., 1994; Wu et al., 1995). The very C-terminus of wild type p53 recognises products of DNA damage such as short single DNA strands, DNA insertion/deletion mismatches (Jayaraman & Prives, 1995; Lee et al., 1995). In contrast, low levels of UV radiation can induce the sequence-specific activation of p53 without an accompanied increase in protein levels (Lu X. & Lane, 1993). The finding that lesion binding is mediated via the C-terminal domain (Hupp et al., 1992; Bayle et al., 1995) shows that the C-terminal domain communicates with the core DNA-binding domain of p53.

This activation of p53’s sequence-specific DNA-binding activity can be achieved through phosphorylation at the carboxy-terminus. Examples which activate DNA-binding are phosphorylations at serine 378 by protein
kinase C and at serine 392 by casein kinase II (Hupp & Lane, 1994). Other modifications, such as O-glycosylation, occur at the carboxy-terminus although it is unclear whether this modification can activate p53 (Shaw et al., 1996). Deleting the last 30 amino acid residues of the p53 molecule (Hupp & Lane, 1994) and binding of the monoclonal antibody 421 to amino acid 370-378 activates sequence-specific DNA-binding activity of p53 (Hupp & Lane, 1994) as well as short single DNA strands which can activate the sequence-specific DNA binding of p53 (Jayaraman & Prives, 1995; Hupp et al., 1995). Recent findings have convincingly shown that the p53 sequence-specific DNA binding activity can also be activated by acetylation of the p53 C-terminal domain (Gu & Roeder, 1997).

### 1.2.9.4 Redox regulation

The conformation of *in vitro* translated p53 and its sequence-specific DNA binding can be modulated by metal chelators and oxidising agents. Oxidation disrupts the protein conformation and inhibits p53’s sequence-specific DNA-binding activity, whereas reduction restores this activity as well as protein conformation (Hainaut & Milner, 1993; Hupp et al., 1993). This redox regulation can be partly attributed to the central Zn$^{2+}$ atom in the p53 molecule and to cysteine residues implicated in sequence-specific DNA binding (Rainwater et al., 1995). However, the biological relevance of the redox regulation of p53 *in vivo* was questioned, since a metal chelator which inactivates p53 *in vitro* unexpectedly activates p53’s transactivation activity *in vivo* (Sun et al., 1997).

### 1.2.9.5 Alternative splicing of p53

Alternative splicing involving the p53 carboxy-terminus has been reported for murine and human p53 leading to the shorter p53as protein
(Han & Kulesz-Martin, 1992; Kulez-Martin et al., 1994; Will et al., 1995; Flaman et al., 1996). The murine p53as mRNA, which contains an additional 96 bp derived from intron 10, represents 25-30% of regularly spliced p53 mRNA (Han & Kulesz-Martin, 1992) and is predominantly found in G2 of the cell cycle (Wu Y. et al., 1994). The predicted protein from p53as mRNA is 9 amino acids shorter and exhibits 17 different amino acid between residues 364-390 at the carboxy-terminus (Kulesz-Martin et al., 1994; Wu L. et al., 1995). The human p53as protein which is truncated at amino acids 341 includes 10 new amino acids and is mainly detected in quiescent cells (Flaman et al., 1996).

In contrast to p53, the p53as protein lacks non-specific DNA-binding activity (Bayle et al., 1995) but exhibits strong constitutive sequence-specific DNA-binding ability compared to p53. The DNA-binding activity of p53as does not require activation and can be inactivated through the formation of heterodimers with p53 in vitro (Wu Y. et al., 1994; Bayle et al., 1995; Wolkowicz et al., 1995). This strong constitutive sequence-specific DNA-binding ability of p53as has been attributed to the changes in the C-terminus, which in full-length p53 has a negative effect on sequence-specific DNA-binding (Bayle et al., 1995; Wolkowicz et al., 1995).

The alternatively spliced p53 protein has been implicated in the delayed induction of apoptosis compared with p53 (Almog et al., 1997). Furthermore, p53 promotes the association of single-stranded RNA or DNA into duplex hybrids (Pavletich et al., 1993; Clore et al., 1994). The p53as protein lacks this function of normal p53 (Wu L. et al., 1995). These first examples point to alternative splicing as part of the regulation of p53.
1.2.10 Properties of wild type and mutant p53 protein

The vast majority of p53 mutants can be characterised by functional and biochemical features which clearly distinguish them from wild type p53. Firstly, most mutants have an extended protein half-life compared to the wild type p53. Whereas wild type p53 has a protein half-life of approximately 30 minutes, p53 mutants exhibit an extended half-life of up to several hours (Finlay et al., 1988; Hinds et al., 1990). P53 mutant protein therefore accumulates at higher concentrations than wild type p53 within the cell (Iggo et al., 1990). Wild type p53 inhibits the growth of tumour cells in vitro (Michalovitz et al., 1990; Baker et al., 1990; Diller et al., 1990; Chen et al., 1990; Mercer et al., 1990a; 1990b) and can inhibit the transformation of primary fibroblasts by cooperating oncogenes (Finlay et al., 1989; Eliyahu et al., 1989). Conversely, mutant p53 has lost the ability to suppress growth of tumour cells and lacks the ability to inhibit transformation by cooperating oncogenes (Finlay et al., 1989; Eliyahu et al., 1989). These biological functions of p53 are mediated through the specific DNA-binding activity of wild type p53 (Kern et al., 1991a; Bargonetti et al., 1991) which is strongly impaired in p53 mutants. As a result, the induction of essential cellular p53 target genes is abrogated (Kern et al., 1992). Many p53 mutants exhibit an altered protein conformation which can be detected through altered affinity towards certain monoclonal antibodies (Gannon et al., 1990) and by crystallographical studies (Cho et al., 1994).

1.2.10.1 Mutated p53 transdominantly Inactivates wild type p53

The functional inactivation of wild type p53 through co-expression of mutant p53 is a common mechanism of p53 inactivation. In general, missense mutations in one p53 allele not only result in high cellular
concentrations of mutant p53 protein; mutant p53 also binds to DNA with a much lower affinity than wild type p53 both in vitro and in vivo (Kern et al., 1991; Bargonetti et al., 1993). Moreover, complexes between wild type and co-expressed mutant p53 can be formed (Milner et al., 1991a; 1991b; Martinez et al., 1991). This stable interaction abrogates wild type p53-mediated transcriptional activation of target genes (Kern et al., 1992). Co-expression of p53 mutants from all evolutionary conserved regions of "hot spot" mutations are capable of abolishing wild type p53-dependent transactivation from p53 DNA-binding sites (Kern et al., 1992). Therefore, mutant p53 is believed to act in a negative trans-dominant fashion by sequestering wild type p53 into inactive complexes (Finlay et al., 1988; Martinez et al., 1990; Milner & Metcalf, 1991; Kern et al., 1992; Bargonetti et al., 1992). This idea is supported by recent findings in transgenic mice where the expression of a mutant p53 transgene accelerate tumorigenesis in a heterozygous p53 genetic background but not in a homozygous p53 deficient background (Harvey et al., 1995).

1.2.10.2 Inactivation of p53 by viral proteins

The transactivation function of p53 can also be inactivated by a number of DNA tumour virus proteins. For instance, the SV40 large T antigen blocks p53-dependent transactivation by binding to the p53 DNA-binding domain (Mietz et al., 1992). In addition to SV40 LT protein, the E1B 55 kDa proteins encoded by adenovirus 2 and 5 inactivate p53-dependent transcriptional activation by binding to the N-terminal transcriptional activation domain within the p53 protein (Braithwaite et al., 1991).

The inactivation of wild type p53 function by the human papillomavirus 16 & 18 (HPV16 & 18) E6 protein appears to be an important contributing factor in the development of cervical carcinomas. The
papillomavirus E6 protein binds to p53 protein (Werness et al., 1990) and accelerates the degradation of the p53 protein through the ubiquination degradation pathway (Scheffner et al., 1990; Mietz et al., 1992). As a result of increased p53 degradation, the expression of the E6 protein abrogates the G1 arrest in response to DNA damage (Kessis et al., 1993).

Proteins of two other viruses, the Hepatitis B virus protein X (HBX) and the Epstein-Barr virus proteins BZLF1 have been implicated in the inactivation of p53 (Wang et al., 1994; Zhang et al., 1994). The Hepatitis B virus protein X (HBX) is capable of inactivating the transactivation activity of wild type p53 (Wang et al., 1994) as well as possibly preventing the translocation of p53 into the nucleus by direct binding (Ueda et al., 1995).

### 1.2.10.3 Gain-of-function of p53 mutants

The current understanding of p53 inactivation by most p53 mutants is that wild type p53 and mutant p53 form stable complexes which are non-functional in transactivating target genes (Milner & Metcalf, 1991; Kern et al., 1992; Barnonetti et al., 1992). However, since there seems to be a positive selection for the presence of a mutated p53 gene in human tumours even at later stages of tumorigenesis, it has been proposed that a gain-of-function is associated with certain p53 mutations (Levine, 1992). Indeed, some p53 mutants exhibit a gain-of-function in p53-null cell lines reportedly capable of transactivating a specific target gene (Dittmer et al., 1993, Chin et al., 1992; Crook et al., 1994). However, experimental evidence suggests that a gain-of-function of p53 mutants strongly depends on the nature of the p53 mutation (Crook et al., 1994), the p53 binding site of the reporter construct (Chen et al., 1993) and the cell line used for the investigation (Dittmer et al., 1993; Sehgal & Margulies, 1993). The relevance of these gain-of-function mutations in tumorigenesis in vivo is therefore difficult to assess, although
positive modulation of the proliferating-cell nuclear antigen (PCNA) promoter (Deb et al., 1992) and the multi-drug resistance gene expression (Chin et al., 1992; Dittmer et al., 1993) by p53 gain-of function mutations may confer some growth advantage.

1.2.10.4 Exclusion of p53 from the nucleus

Wild type p53 is a nuclear protein and requires localisation within the nucleus in order to function (Shaulsky et al., 1991). Translocation has been attributed to three nuclear localisation signals (NLS I, II, III) in the C-terminus of p53 (Addison et al., 1990; Shaulsky et al., 1990b). A pattern of cell cycle specific regulation of the localisation of wild type p53 has been observed with p53 accumulating in the cytoplasm during G1 and entering the nucleus for a short period at early S-phase (Shaulsky et al., 1990a). Wild type p53, as well as mutant p53, require intact nuclear localisation signals to suppress transformation or enhance transformation in cooperation with activated ras, respectively (Shaulsky et al., 1991). Thus, the exclusion of wild type p53 from the nucleus may be another mechanism of inactivating the function of p53 and thereby increasing the risk of genetic instability of cells affected (Knippschild et al., 1996). In this context, it is interesting to note that in some tumours wild type p53 is only found in the cytoplasm (Moll et al., 1995) and that functional inactivation by binding to a viral protein, such as HBX, in the absence of any p53 mutations can exclude p53 from the nucleus and increase the risk of tumour formation (Ueda et al., 1995).

1.2.11 The role of p53 in tumorigenesis

The important role of p53 in protecting eukaryotic cells from tumorigenesis has been indirectly emphasised through the high incidences of loss or p53 mutation in tumours (Hollstein et al., 1991, 1996, 1997). By
investigating the functions of wild type p53, it has become clear why loss of functional p53 dramatically increases the risk of tumorigenesis. Although p53 appears not to be directly involved in cell cycle progression, p53 regulates the G1 cell cycle arrest in response to DNA damage. The p53-dependent G1 arrest provides the time for DNA to be repaired. Experimental evidence suggests that p53 is also involved in the coordination of DNA-repair mechanisms in damaged and arrested cells and recently p53 has been shown to have an intrinsic 3'-5' exonuclease activity (Mummenbrauer et al., 1996). The loss of functional p53 would not only abolish the G1 cell cycle arrest but also impair essential DNA repair mechanisms. Severely DNA damaged cells are usually eliminated by p53-dependent apoptosis. P53-dependent control of apoptosis is an important part of p53 function in normal cell growth. The abrogation of p53’s apoptotic function would also allow these cells to proliferate.

Finally, a very interesting p53 function in spindle checkpoint control has been reported which monitors the ploidy of cells (Cross et al., 1995). Tumours and tumour-derived cell lines are very often characterised by a degree of aneupody and, coincidentally, a lack of functional p53. The inactivation or abolition of any of these described p53 functions can lead to severe damage of the integrity of the genome, with the risk of mutations and aneuploidy being established after further cell division.

Another important factor for progression of tumours is angiogenesis. Wild type p53 expression results in secretion of inhibitors of angiogenesis (Dameron et al., 1994). The mutation of wild type p53 would not only reduce apoptosis within the tumour dramatically but also reduces the secretion of angiogenesis inhibitors.
1.3 Aim of my project

The aim of my project was to further explore the mechanisms of oncogene cooperation in Schwann cells. Based on previous results with SV40 larger T (Ridley et al., 1988), I sought to find a cellular oncogene which would be able to cooperate with Ras. Furthermore, my aim was it to delineate the pathway for the growth inhibitory signal initiated by Ras in Schwann cells and to understand the mechanism by which a cooperating oncogenes could abrogate this response leading to cell proliferation.
CHAPTER 2.0

MATERIALS
2.0 Materials

2.1 Stock solutions

**PBSA**
- 100 mM NaCl
- 4.5 mM KCl
- 7 mM Na₂HPO₄
- 3 mM KH₂PO₄

**TE-buffer**
- 10 mM Tris pH 8.0;
- 1 mM EDTA pH 8.0

**TAE-buffer**
- 40 mM TrisOAc,
- 2 mM EDTA

**TBE-buffer**
- 89 mM Tris base,
- 89 mM boric acid,
- 2 mM EDTA

**LB-medium**
- 10 g/l Bacto-tryptone
- 5 g/l yeast extract
- 10 g/l sodium chloride
  Adjusted to pH to 7.5 with 5 N sodium hydroxide
**LB-agar:**
LB-medium, supplemented with 1.5% (w/v) bacto agar

**BHI-medium**
Brain heart infusion broth powder
37g/l, dissolved in destilled H$_2$O, autoclaved

**2x Ca$^{2+}$-Transfection Buffer**
50 mM HEPES, pH 7.1
280 mM NaCl
1.5 mM Na$_2$HPO$_4$
KCI
Dextrose

**DNA loading buffer**
0.25% bromphenol blue
0.25% xylene cyanol FF
30% glycerol in water

**Protein loading buffer (4x)**
200 mM Tris/HCl pH 6.8
8% (w/v) SDS
0.4% (w/v) bromophenol blue
40% (v/v) glycerol
400 mM DTT (added shortly before use)
**Luciferase buffer**
25 mM glycyglycine pH 7.8
5 mM ATP pH 8.0
15 mM MgSO₄

**SDS-running buffer**
25 mM Tris
192 mM glycine
0.1% (w/v) SDS

**Tfb I-Buffer**
30 mM KOAc
50 mM MnCl₂
100 mM KCl pH 5.8
10 mM CaCl₂
15% glycerol
adjusted to pH 5.8 with 0.2 M acetic acid,
filtered and stored at 4°C

**Tfllb-buffer**
10 mM Na-MOPS pH 7.0
75 mM CaCl₂
10 mM KCl
15% glycerol
filtered and stored at 4°C
# 2.2 Chemicals

<table>
<thead>
<tr>
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<th>Supplier</th>
</tr>
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</tr>
<tr>
<td>Acrylamide solution</td>
<td>Scotlab</td>
</tr>
<tr>
<td>N,N'-methylene bisacrylamide solution</td>
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<td>SeaKemFMCBioProducts</td>
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<td>Agarose, low gelling temperature</td>
<td>SeaPlaque FMC BioProducts</td>
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<td>HEPES</td>
<td>Sigma</td>
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<td>FSA</td>
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<td>Film</td>
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<td>Amersham</td>
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<td>O-nitrophenyl-β-D-Galactopyranoside</td>
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<td>Sigma</td>
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<tr>
<td>Poly-L-lysine</td>
<td>Sigma</td>
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<tr>
<td>Aprotinin</td>
<td>Sigma</td>
</tr>
<tr>
<td>Protein Assay Mix</td>
<td>BioRad</td>
</tr>
<tr>
<td>SDS</td>
<td>Sigma</td>
</tr>
<tr>
<td>TEMED</td>
<td>BioRad</td>
</tr>
<tr>
<td>Tissue culture dishes</td>
<td>Falcon</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

### 2.3 Radiochemicals

All radiochemicals were purchased from Amersham Life Science and the most recently dated radiochemicals were employed for experiments. In particular, the following radiochemicals; Redivue ($\gamma^{32}$ P) dATP, aqueous solution >5000 Ci/mmol and $^3$H-thymidine 25Ci/mmol, 1mCi/ml were employed.

### 2.4 Enzymes

| Calf Intestinal Alkaline Phosphatase     | Boehringer Mannheim       |
| Taq DNA polymerase                      | Boehringer Mannheim       |
| DNA polymerase I, Klenow Fragment       | Pharmacia                 |
| Proteinase K                            | Boehringer Mannheim       |
| Restriction endonucleases               | New England Biolabs       |
| RNase A                                 | Sigma                     |
| DNA T4-ligase                           | Boehringer Mannheim       |
2.5 Media and sera

Low glucose Dulbecco's modified Eagle medium (DMEM) minus phenol red containing 1 mg/ml glucose and 0.11 mg/ml sodium pyruvate was supplemented with 4 mM glutamine and used for Schwann cells. High glucose DMEM containing 4.5 mg/ml glucose without sodium pyruvate was used for all other cell types, provided by Media Production, ICRF.

Foetal calf serum (FCS), batch 2321 (Globepharm) was used for Schwann cells, whereas foetal calf serum batch 940278 (Autogen Bioclear) was used for all other cells.

2.6 Medium supplements

Low glucose DMEM for Schwann cells was supplemented with forskolin, Glial Growth Factor (GGF), gentamycin and kanamycin. Forskolin (Calbiochem) was solubilised at 25 mM in ethanol and stored at -20°C. A final concentration of 2 mM was added to the media. GGF was produced using a baculovirus-system and an empirically measured dilution of 1:5000 of the supernatant was added for growth promotion. Gentamycin and kanamycin were solubilised in water, sterile filtered and kept at 4°C. A final concentration of 2 mg/ml gentamycin and 100 mg/ml kanamycin were added to low glucose DMEM. High glucose DMEM for all other cells was supplemented with gentamycin and kanamycin at the same final concentration.
2.7 **Cell lines**

All Schwann cell had to be infected via co-cultivation with GPE-virus producer cells. The following table shows the generated GPE-virus producer cell lines, introduced retroviral vector and their resistance.

**Tab. II.1: Generated GPE-virus producing cell lines**

<table>
<thead>
<tr>
<th>GPE virus-producing cell line</th>
<th>retroviral vector</th>
<th>resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPE Puro</td>
<td>pBabe Puro</td>
<td>puromycin</td>
</tr>
<tr>
<td>GPE P175</td>
<td>pBabe Puro p53R175H</td>
<td>puromycin</td>
</tr>
<tr>
<td>GPE p53CT</td>
<td>pBabe Puro p53CT302-90</td>
<td>puromycin</td>
</tr>
<tr>
<td>GPE P248</td>
<td>pBabe Puro p53R248W</td>
<td>puromycin</td>
</tr>
<tr>
<td>GPE ΔMEK1:ERTM</td>
<td>pBabe PuroΔMEK1:ERTM</td>
<td>puromycin</td>
</tr>
<tr>
<td>GPE MKK</td>
<td>pBabe Puro MKK</td>
<td>puromycin</td>
</tr>
<tr>
<td>GPE MANA</td>
<td>pBabe Puro MKK (MANA)</td>
<td>puromycin</td>
</tr>
<tr>
<td>GPE LIDA</td>
<td>pBabe Puro MKK (LIDA)</td>
<td>puromycin</td>
</tr>
<tr>
<td>GPE ERK2</td>
<td>pBabe Puro ERK2</td>
<td>puromycin</td>
</tr>
<tr>
<td>GPE ERK2 (AF)</td>
<td>pBabe Puro ERK2 (AF)</td>
<td>puromycin</td>
</tr>
<tr>
<td>GPE ERK2 (A52)</td>
<td>pBabe Puro ERK2 (A52)</td>
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<tr>
<td>GPE Bleo</td>
<td>pBabe Bleo</td>
<td>phleomycin</td>
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<td>pBabe Bleo SEK1</td>
<td>phleomycin</td>
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<tr>
<td>GPE B/SEK1/AL</td>
<td>pBabe Bleo B/SEK1-AL</td>
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<td>GPE ΔMEK1: ER™/H</td>
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<td>GPE LXSN-Raf-CAAX</td>
<td>LXSN-Raf-CAAX</td>
<td>neomycin</td>
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</table>
Generated cell lines as listed in Tab. II.2 are sorted into groups according to their parental cell line. Cell lines which harbour more than one retroviral vector are separated by a dash showing both introduced genes. Additionally all resistances of a cell line are indicated. Cell lines with an asteriks were generated by Alison Lloyd.

Tab.II.2 : Generated cell lines and introduced genes

<table>
<thead>
<tr>
<th>Cell line</th>
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<th>Resistance markers</th>
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<tr>
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<td>pBabePuro</td>
<td>neomycin/puromycin</td>
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<td>pBabePuro p53R175H</td>
<td>neomycin/puromycin</td>
</tr>
<tr>
<td>11ras2 p53248</td>
<td>pBabePuro p53R248W</td>
<td>neomycin/puromycin</td>
</tr>
<tr>
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<td>pBabePuro</td>
<td>neomycin/puromycin</td>
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<td>pBabePuro p53R175H</td>
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<tr>
<td>NSp53 CT</td>
<td>pBabePurop53CT302-90</td>
<td>puromycin</td>
</tr>
<tr>
<td>NSPuro/LXSN-cHaRasVal12</td>
<td>pBabePuro</td>
<td>puromycin/</td>
</tr>
<tr>
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<td>pBabePuro p53R175H/</td>
<td>puromycin/</td>
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<tr>
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<td>pBabePurop53CT302-90</td>
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</tr>
<tr>
<td>cHaRasVal12</td>
<td>LXSN-cHa Ras Val12</td>
<td>neomycin</td>
</tr>
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<td>LXSN-cHa Ras Val12</td>
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<td>/LXSN-cHa Ras Val12</td>
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<tr>
<td>-----------------------------</td>
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<tr>
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<td>pBabePuro p53R175H/</td>
<td>puromycin/</td>
</tr>
<tr>
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<td>pBabePurop53CT&lt;sup&gt;302-90&lt;/sup&gt;</td>
<td>puromycin/</td>
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<td>neomycin,</td>
</tr>
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<td></td>
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</tr>
<tr>
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<td>neomycin,</td>
</tr>
<tr>
<td></td>
<td>pBabe Puro MKK</td>
<td>puromycin</td>
</tr>
<tr>
<td>NSΔRafER/MANA</td>
<td>LXSN ΔRafER, neomycin, pBabe Puro MANA puromycin</td>
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</tr>
<tr>
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<tr>
<td>NSΔRafER/B/AL</td>
<td>LXSN ΔRafER, neomycin, pBabe Bleo SEK1-AL phleomycin</td>
<td></td>
</tr>
</tbody>
</table>

### 2.8 DNA constructs

pBabe Puro CT\(^{90}\) was constructed by Trevor Littlewood (ICRF) by PCR and contains the murine carboxyterminus of murine p53 from aa 302-390, cloned BamH I- EcoR I into pBabe Puro (Morgenstern & Land, 1990).

pBabe Puro MKK, MANA (221S to A), LIDA (217S to A) were kind gifts from Sally Cowley in Chris Marshalls Laboratory (Cowley et al., 1994). The inserts, rabbit MAP-kinase kinase, were cloned into BamH I- EcoR I of pBabe Puro (Morgenstern & Land, 1990).

pBabe Puro ERK2, AF, A52 were constructed by inserting the murine ERK2, the mutation of murine ERK2 designated AF (183T to A, 185Y to F) and A52 (52K to A) into the BamH I- restriction site of pBabe Puro.
pBabe Puro SV40 LT was constructed by excising the cDNA of SV40 LT from the plasmid pZipSVLT (a kind gift by Parmjit Jat) with BamH I. The plasmid pZIPSV40 LT contains the cDNA of SV40 LT from Bgl I position nt 5235 to Hpa I at position nt 2666 cloned into the BamH I site with BamH I - linkers. The BamH I-fragment was cloned into the polylinker BamH I site of pBabe Puro.

pBabe Puro p53R175H argenine to histidine at position aa175 was constructed by excision of mt p53R175H BamH I from pBluescript and cloned into BamH I site of pBabe Puro.

pBabe Bleo p53R175H argenine to histidine at position aa175 was constructed by excision of mt p53R175H BamH I from pBluescript and cloned into BamH I site of pBabe Bleo.

pBPSTR wt p53 tk luc 2F
pBPSTR1 was cut Sal I-BamH I, thereby removing a total of 1.5 kb containing the TET- system elements. The luciferase gene from pGL3 basic vector (Promega) was excised through Bgl II-Sal I and ligated into pBPSTR1 with Sal I -BamH I, leading to pBPSTR1 luc 2 F. pBPSTR1 luc 2F was cut Bgl II - Not I and the p53 DNA binding site, previous ligated into BamH I-Not I into pcDNA 3, was cut and ligated into Bgl II-Not I. The HSV-tk minimal promoter was cut Hind III - Not I and ligated into pBPSTR1 p53 luc 2F.

pGEX2T c-Jun is a GST-expression vector with the amino acids 1-257 of c-Jun (a kind gift by Jim Woodgett).
pBabe Puro ΔMEK1:ERTM and pBabe Puro ΔMEK1:ER were generated by Martin McMahon (DNAX) and contain a constitutively active form of MEK1 (Masour et al., 1994) fused to the hormone binding region of the human oestrogen receptor cloned BamH I-Sal I. pBabe Puro ΔMEK1: ERTM is not responsive to oestrogen concentrations up to more than 250 nM (M. McMahon, pers. comm.).

pBabe Hygro ΔMEK1: ERTM was constructed by subcloning the BamH I-Sal I fragment from pBabe Puro ΔMEK1: ERTM into pBabe Hygro.

pBabe Bleo SEK1 and pBabe Bleo SEK1-AL were constructed by excising SEK1 and SEK1-AL from pcDNAS with Kpn I and Xba I, blunted (kind gifts by Jim Woodgett). These inserts were cloned into the SnaBl site of pBabe Puro.

2.8.1 Oligomers
p53 RGC oligomer flanked by BamHI and Not I sites
5'AGG CCA GAC CTG CCC GGG CAA GCC TTG GCA 3'

2.9. Antibodies
2.9.1 Primary antibodies
Antibody 122 against p42ERK2 (Leevers et al., 1992)
Ab 122 is a rabbit polyclonal antibody and recognises both the phosphorylated and unphosphorylated form of p42ERK2 MAP-kinase.

Antibody erk2 against p42ERK2 (UBI Ltd.)
Ab erk2 is a monoclonal antibody, specific for MAP-kinase ERK2.
Antibody 240 against mutant p53 protein (Oncogene Science Ltd.)
Ab 240 is a monoclonal mouse antibody, raised against mouse epitope aa 206-211 of p53 (Gannon et al, 1990). This antibody specifically recognises mutant human and rat p53 in immunoprecipitation.

Antibody 423 against SV40 large T
Ab 423 is a monoclonal mouse antibody, raised against SV40 large T (Harlow et al., 1981). This antibody recognises SV40 large T on western blots. Ab 423 was provided by the Monoclonal Antibodies Unit (ICRF, Clare Hall).

Antibody 9E10 against c-myc tag
Ab 9E10 is a monoclonal mouse antibody raised against c-myc (Evan et al., 1985) and is used for the detection of various tagged proteins. Ab 9E10 was a kind gift by Gerad Evan (ICRF, London).

Antibody JNK-1 (C-17) (Santa Cruz Ltd.)
Antibody JNK-1 (C-17) is a rabbit polyclonal antibody and can be used for western blot analysis and immunoprecipitation with subsequent in vitro kinase assay of Jun-N-terminal kinase.

Antibody MKK-1 and MKK-2 (Transduction Laboratories Ltd.)
Antibodies MKK-1 and MKK-2 are monoclonal antibodies which recognise the amino-terminus of MAP-kinase kinase 1 or 2 in western blot analysis and immunoprecipitation prior to in-vitro kinase assays.
2.9.2 Secondary Antibody

Secondary antibody against rabbit immunoglobulins coupled to horseradish peroxidase (Amersham Life Science).

Secondary antibody against mouse immunoglobulins coupled to horseradish peroxidase (Amersham Life Science).

Secondary antibody against mouse immunoglobulin (Amersham Ltd.)
CHAPTER 3.0

METHODS
3.0. Methods

3.1 Tissue culture techniques

3.1.1 Growth conditions for Schwann cells

All eukaryotic cells were grown in humidified incubators in 10% CO₂ : 90% air at 37°C. Normal Schwann cells and all cell lines derived from Schwann cells were maintained in low-glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco Ltd.) minus phenol-red, containing 3% stripped foetal calf serum (FCS) (batch no. 2321, BRL-Gibco Ltd.), 4 mM glutamine, 100 mg/ml kanamycin, 2 mg/ml gentamycin, 2 mM forskolin (Calbiochem Ltd.) and 100 µl recombinant glial growth factor (GGF) solution. Medium was changed every 2-3 days.

Schwann cells containing proteins fused to the hormone-binding domain of the oestrogen receptor were grown in medium without phenol-red and stripped foetal calf serum, since it had been reported that phenol-red as well as components of foetal calf serum can activate fusion proteins with the oestrogen receptor leading to an undesired background activity (Littlewood et al., 1995). Stripping of foetal calf serum was carried out by stirring serum with 0.1% (w/v) charcoal and 0.01% (w/v) dextran T-70 for three hours at 37°C. After this time serum was filtered (0.45 µm), aliquoted and stored at -20°C until further use.

All Schwann cells were grown on poly-L-lysine coated dishes. Poly-L-lysine (PLL) was solubilised in destilled water to give a 0.4 mg/ml stock solution. Dishes were coated with 2.5 ml of PLL-solution (13.3 mg/ml in destilled water) for 10 minutes at room temperature, drained for 5 minutes, washed twice with sterile destilled water and allowed to dry for 5-10 minutes.
Cells were passaged by first washing them in PBSA and then briefly incubating them with 1:5 (v/v) mix of trypsin/versene at 37 °C. Once the cells detached from the dish surface, they were diluted with fresh medium and replated. Schwann cells were routinely split 1:3 and cells with low passage numbers (below passage number 10) were used in all experiments.

3.1.2 General growth conditions for eukaryotic cells

Unless otherwise stated in individual experiments, all other cells were maintained in Dulbecco's modified eagle medium (DMEM) containing 10% foetal calf serum (FCS) (Autogen, batch no. 940278) plus 100 mg/ml kanamycin, 2 mg/ml gentamycin. Medium was changed every 2-3 days and cells were routinely split between 1:3 to 1:5.

3.1.3 Freezing and storage of eukaryotic cells

In order to maintain stocks of eukaryotic cells, the following freezing procedure was employed. Cells were trypsinized as previously described (see section 3.1.1) and resuspended in a pre-cooled freezing mix containing the specific medium required for these cells, mixed 1:1 with foetal calf serum. To this freezing mix 10% (v/v) dimethyl sulfoxide (DMSO) was slowly added. Each freezing vial (NUNC) was filled with 1 ml of cell suspension and immediately placed on ice. The vials were transferred into styrofoam freezing boxes into the -80 °C freezer for two days and then stored under liquid nitrogen. Frozen cells were recovered by rapidly thawing them in a 37°C water bath. The cell suspension was slowly mixed on a plate with 10 ml of prewarmed medium. After 2 hours, the medium was changed again to remove remaining traces of DMSO.
3.1.4 Retroviral infection of eukaryotic cells

3.1.4.1 Transfection and selection of GPE-virus producer cells

Transfection procedures were based on the protocol by Wigler et al., 1979. Prior to transfection, medium was changed to fresh 10 ml medium supplemented with 10% foetal calf serum. For every 90 mm-dish of GPE-cells (Markowitz et al., 1990) to be transfected, up to 10 μg plasmid DNA was mixed in 500 μl 1x transfection buffer followed by the addition of 23 μl of 2.5 M sterile CaCl₂ solution. A white translucent precipitate formed after approximately 10 minutes, and this transfection mixture was added dropwise to the cells. Cells were incubated with the DNA-Ca²⁺-precipitate for 16-20 hours and medium was changed afterwards. Two days after transfection, GPE-cells were split 1:5, 1:10 and 1:20 into the appropriate selective medium. Cells were maintained under selection pressure with medium changed every 2-3 days until single colonies appeared. Single colonies usually appeared after 1-3 weeks depending on the drug selection used. For the following co-cultivation experiments, these selected GPE-virus producing cells were pooled.

All cell lines with retrovirally introduced genes were grown under constant drug selection. The table below gives the parental cell line and various drug concentrations routinely used for this purpose.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>selective drug</th>
<th>final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schwann cells (NS)</td>
<td>neomycin, gentamycin</td>
<td>400 μg/ml</td>
</tr>
<tr>
<td></td>
<td>puromycin</td>
<td>0.4 μg/ml</td>
</tr>
<tr>
<td></td>
<td>hygromycin</td>
<td>75 μg/ml</td>
</tr>
<tr>
<td></td>
<td>phleomycin</td>
<td>0.5 μg/ml</td>
</tr>
<tr>
<td>---------------------</td>
<td>------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NSARafER cells</td>
<td>neomycin</td>
<td>0.4 μg/ml</td>
</tr>
<tr>
<td></td>
<td>puromycin</td>
<td>0.4 μg/ml</td>
</tr>
<tr>
<td>GPE cells</td>
<td>puromycin</td>
<td>5 μg/ml</td>
</tr>
<tr>
<td></td>
<td>hygromycin</td>
<td>150 μg/ml</td>
</tr>
<tr>
<td></td>
<td>neomycin</td>
<td>400 μg/ml</td>
</tr>
<tr>
<td></td>
<td>phleomycin</td>
<td>25 μg/ml</td>
</tr>
</tbody>
</table>

Neomycin (G418) was prepared as a 100 mg/ml stock solutions in 10 mM HEPES pH 7.5. A puromycin stock solution of 25 mg/ml in sterile destilled water was used. Hygromycin was directly used as provided by the supplier. Bleomycin (=phleomycin) was prepared as a 25 mg/ml solution. All stock solution were sterile filtered and stored at 4°C.

3.1.4.2 Co-cultivation of Schwann cells with GPE-producer cells

Selected pools of GPE-producer cells were trypsinised and cells were collected in DMEM medium containing 10% FCS, washed once with PBSA and placed on ice. An aliquot of cells was counted with the coulter counter model ZF (Coulter Electronics Ltd.). Subsequently 2 x 10^6 GPE-cells were seeded per 90 mm-dish. Following an overnight incubation, 125 μl mitomycin C (final concentration 25 μg/ml) was added to the medium and cells were incubated for a further 2 hours. After this time cells were trypsinnised and spun for 5 minutes at 1500 rpm in a bench-top centrifuge (Centaur 2/MSE) and washed once with PBSA. Cells were re-suspended...
in medium used for the co-cultivation and an aliquot of the cell suspension was counted as previously described. For the co-cultivation, 7.5 x 10^5 cells of both cell types (i.e. GPE Puro + Schwann cells) were seeded onto a PLL-coated 90 mm-dish and incubated for 2-3 days. After this period cells were usually split 1:5, 1:10, 1:20 into the appropriate selection medium until resistant colonies appeared.

3.2 Proliferation assays

3.2.1 Growth assay without growth-promoting factors

Normal Schwann cells or those cell lines derived from Schwann cells were seeded at 2 x 10^4 cell per well in a 6-well dish in 2% low glucose DMEM without any growth promoting supplements. Schwann cells were trypsinised daily with 500 µl of 1:5 (v/v) trypsine/versine for 5-10 minutes at 37°C. Subsequently 500 µl medium was added. 100 µl of this cell suspension was diluted in 10 ml of isotone and counted with the Coulter Counter. The number of cells of two wells were counted in triplicates.

3.2.2 ^3H-thymidine DNA-synthesis assay

In order to measure DNA-synthesis as an indication of S-phase entry of cells, to 2 x 10^4 cells in a 1 ml well 0.5 µCi/ml ^3H-thymidine (used 2.5 µl/ml medium) was added for the indicated times. Cells were washed once with PBSA and 0.5 ml of 1% SDS solution was added. After 10 minutes at room temperature the lysate was transfered and the well washed once with 500 µl PBSA. 1 ml 15% (w/v) trichloroacetic acid was then added. After 10 minutes on ice the solution was filtered through a 2.5 µm glass fibre filter (Whatman). These filters were washed twice with 10 ml 5% TCA solution and once with 5 ml 100% ice-cold ethanol, air-dried and scintillation counted.
3.2.3 FACS-analysis

FACS-analysis was performed to establish the distribution of cells in the various phases of the cell-cycle. Bromodeoxyuridine (BrdU) with a final concentration of 10 μM was added to cells for times as indicated in the individual experiment. Thereafter cells were trypsinised and transferred into complete medium to inactivate trypsin. Cells were spun for 5 minutes at 1500 rpm in centrifuge Centaur 2, washed once with cold PBSA and resuspended in 200 μl PBSA. Whilst vortexing, 2 ml 80% (v/v) cold ethanol was slowly added to fix cells. Samples were stored for 30 minutes on ice and afterwards at 4 °C in the dark. The fixed cells were then incubated with fluorescein isothiocyanate-conjugated anti-BrdU antibodies (Becton-Dickinson) and stained with propidium iodide containing RNase (20 μg/ml). Replicative DNA synthesis and DNA content were analysed using bivariate flow cytometry. FACS-analysis was carried out by Derek Davies and Clare Hughes in the ICRF FACS-laboratory.

3.3 DNA-techniques

3.3.1 Isolation of plasmid DNA through alkaline lysis

BHI-medium (2-3 ml) containing 100 μg/ml ampicillin was inoculated with a single bacterial colony from a BHI-agar plate. After an incubation period of between 5 hours to overnight, 1.5 ml of the bacterial suspension was spun for 5 minutes at 13000 rpm in a microfuge. The pellet was re-suspended in 200 μl resuspension buffer (25 mM Tris/HCl pH 8.0, 10 mM EDTA pH 8.0). 100 μl lysis buffer (0.2 M NaOH, 1% SDS) was added. After 5 minutes at room temperature, 150 μl of neutralisation buffer (3 M potassium acetate, pH 5.5) was added and the tubes were incubated on ice for 5 minutes. After a centrifugation step for 10 minutes at 13000 rpm, the
supernatant was mixed with 2.5x volumes of 100% (v/v) ice-cold ethanol and precipitated by spinning at 13000 rpm for 2 minutes at room temperature. The DNA-pellet was washed once with 70% (v/v) ethanol and then solubilised in 20 µl TE-buffer plus 100 µg/ml RNase A.

3.3.2 Plasmid DNA-preparation with Qiagen resin column

Bacteria were grown in 100 ml LB-medium containing 100 µg/ml ampicilin at 37°C overnight. The bacterial cells were harvested by centrifugation at 5500 rpm for 7 minutes. Isolation of plasmid DNA was carried out according to the manufacture's protocol. Bacterial pellets were completely re-suspended in 10 ml resuspension buffer P1 (100 µg/ml RNase A, 50 mM Tris/HCl, 10 mM EDTA pH 8.0) and 10 ml of lysis buffer P2 (200 mM NaOH, 1% SDS) were added. The suspension was neutralised with buffer P3 (3 M potassium acetate, pH 5.5). Samples were centrifuged at 5800 rpm for 30 minutes. In the meantime a Quiagen-tip 500 column was equilibrated with 10 ml of buffer QBT (750 mM NaCl, 50 mM MOPS, 15% (v/v) ethanol, pH 7.0, 0.15% Triton X-100). After the centrifugation the supernatant was immediately added onto the equilibrated column. The column was washed twice with 30 ml of wash buffer (1.0 M NaCl, 50 mM MOPS, 15% ethanol, pH 7.0) and the DNA was eluted by applying 15 ml buffer QF (1.25 M NaCl, 50 mM Tris/HCl, 15% ethanol, pH 8.5) onto the column. The eluted DNA was precipitated after addition of 0.7x volumes of isopropanol (10.5 ml). After a centrifugation step at 5800 rpm for 30 minutes at 4°C, the precipitated DNA was solubilised in 200 µl TE-buffer. This protocol routinely led to 0.5-3.0 mg/ml DNA depending on the plasmid used.
3.3.2.1 Quantification of DNA

The concentration and purity of DNA solutions was assessed on the basis of absorption at wavelength of 260 nm and 280 nm. One unit of optical density $A_{260}$ corresponds to the concentration of 50 µg/ml for dsDNA, 40 µg/ml for RNA or 33 µg/ml for ssDNA including oligonucleotides. With proteins generating peak absorbance at 280 nm of the spectrum, the ratio $A_{260}/A_{280}$ is indicative of the extent of protein contamination in nucleic acids solution. A ratio of 1.7 or bigger was considered acceptable.

3.3.3 Restriction analysis, modification and ligation of DNA

Restriction digests were performed using 0.5 to 1 µg of DNA and a 3-5x units excess of restriction enzyme in 20 µl total volume. Multiple restriction digests with incompatible buffer conditions were subsequently carried out with a phenol/chloroform extraction and ethanol precipitation between reactions. DNA molecules of 0.1-20 kb length were resolved on agarose gel matrixes. 0.5%-2% (w/v) agarose powder (SeaKem) was dissolved in 1x TAE-buffer and then melted in a microwave oven. Ethidium bromide (0.5 µg/ml) was added to the pre-cooled gel mix and poured into prepared electrophoresis trays. DNA samples and 0.5 µg 1 kb marker were mixed 1:5 with loading dye (50 mM EDTA pH 8.0, 100 mM Tris pH 8.0, 50% (v/v) glycerol, 0.4% (w/v) bromphenol blue) and loaded into the wells. DNA molecules were separated with a constant voltage electric field of 5 V/cm gel. Photographs of gels were taken under UV-illumination using Polaroid type 57 film.

DNA fragments used in ligations were prepared by digesting plasmid DNA with the appropriate restriction endonucleases. Modification of 5' or 3'
DNA ends were carried out with T4-DNA polymerase I, Klenow fragment, and T4-DNA polymerase according to the supplier's standard protocols. To obtain blunt ends, 1 µl (10 units) of T4-DNA polymerase was added at the end of a restriction digestion. After 15 minutes of incubation at room temperature, the reaction tube was heated at 65°C for 20 minutes. To fill recessed 3’ ends, DNA polymerase Klenow fragment was employed along with 33 nM dNTP. Self-ligation of DNA-vectors with compatible ends was prevented by dephosphorylation with calf intestinal alkaline phosphatase (CIP). 3 units per µg restricted DNA were added to the restriction reaction and incubated at 37°C for 30 minutes.

Analytical separation of DNA molecules for ligations were carried out by resolving DNA in up to 1% low-melting-point agarose gels (LMP-agarose) in 1x TAE running buffer. DNA bands were visualised under long wave UV-light (340 nm) and excised with a clean razor blade. Standard preparations provided with about 1 µg of an isolated fragment in 50 µl LMP-agarose. The LMP-agarose slices were transferred to sterile tubes. The LMP-agarose was melted for 10 minutes at 68°C and cooled down in a 37°C water bath. In a ligation of compatible and overhanging ends, 1 µl (20 ng) vector DNA was combined with 4 µl (100 ng) insert DNA in 50 mM Tris pH 7.8, 10 mM MgCl₂, 1 mM ATP, 20 mM DTT, 1 µl T4-DNA ligase (6 Weiss units) in a total volume of 20 µl. Ligations were incubated for between 4 hours to overnight at 14-16°C depending on the generation of cohesive or blunt ends. To stop the ligation, tubes were heated for 5 minutes to 68°C, cooled down to 37°C and a 10 µl aliquot was used to transform 100 µl freshly thawed competent DH5α bacteria.
3.3.4 Preparation and transformation of competent bacteria

A single colony of DH5α bacteria from a BHI-agar plate was inoculated into 3 ml BHI-medium and grown overnight at 37°C. 1 ml of overnight culture was further diluted in BHI-medium and left to grow at 37°C for approximately 2-3 hours until the density OD$_{550}$ nm = 0.5. Bacteria were pelleted at 3.5 k for 10 minutes at 4°C. The supernatant was discarded and the remaining bacteria pellet were gently re-suspended in 20 ml ice-cold Tfb I-buffer for each 50 ml volume of bacterial suspension. After 15 minutes incubation on ice, bacteria were pelleted again at 3.5 k for 10 minutes at 4°C. The supernatant was discarded and the pellet re-suspended in 4 ml sterile ice-cold Tfb II-buffer by gentle swirling on ice. It was important to re-suspend the bacteria slowly to obtain a high degree of competence. 100 µl aliquots of competent DH5α bacteria were frozen at -80°C.

To transform ligated DNA into bacteria, a frozen aliquot of DH5α was thawed on ice. Purified DNA or melted LMP-agarose containing DNA was added and gently mixed with bacteria suspension. Bacteria were incubated for 20 minutes on ice. After this incubation period bacteria were incubated for 120 sec in a 37°C water bath and then placed on ice again for a further 3 minutes. After a further 3 minute-incubation at room temperature, 1 ml of BHI-medium was added. Bacteria were incubated for 1 hour at 37°C with occasional inversion of the tube. Bacteria were then spun down at 6500 rpm in minifuge, re-suspended in 200 µl BHI-medium and plated onto BHI-agar plates containing ampicilin (100 µg/ml). Incubations were carried out at 37°C overnight until colonies were clearly visible.
3.4 Protein techniques

3.4.1 Preparation of protein extracts for Western blotting

Prior to cells lysis, cells were rinsed twice with PBSA to remove last traces of tissue-culture medium. Cells were then scraped with a rubber policeman and transferred into an 1.5 ml-tube. Cells were spun at 1000 rpm for 5 to 10 minutes. The pellet was then re-suspended in 1x EDTA sample buffer, boiled for 2 minutes, vortexed and boiled again for 8 minutes. After 5 minutes on ice samples were spun for 5 minutes at 13000 rpm at 4°C and the supernatant was transferred into a new tube and immediately frozen at -70°C.

Prior to separation on an SDS-PAGE gel, protein samples were thawed on ice. The protein concentration was determined as described below. Aliquots were mixed with 4x protein sample buffer (0.4 M Tris pH 6.8, 10% (w/v) SDS, 4 M DTT, 50% glycerol, 10 mg bromophenol blue), boiled at 95°C for 5 minutes and loaded onto an SDS-PAGE gel.

3.4.1.1 Quantification of protein concentration

The protein concentration of a total cell lysate was measured with the BioRad Protein Assay kit following the manufacturer's recommendations. The stock solution was diluted 1:5. Aliquots of 1 ml were filled into disposable plastic cuvettes. The cell extract (1.0 μl) was added and after 5 minutes the samples were measured at OD_{595nm}. A standard curve for 1 to 10 μg BSA (BioRad) was used to calibrate the sample readings.
3.4.2 Protein analysis by SDS-PAGE

Different polyacrylamide gel concentrations were used to analyse various sizes of proteins. The following table shows the standard mixtures used in all experiments unless not otherwise stated:

Table III.2: SDS polyamide/acrylamide gels

<table>
<thead>
<tr>
<th></th>
<th>7.5%</th>
<th>10%</th>
<th>12.5%</th>
<th>15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>acrylamide/</td>
<td>3.8 ml</td>
<td>5.0 ml</td>
<td>6.3 ml</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>bisacrylamide 30% : 0.8%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 M Tris pH 8.8</td>
<td>5.6 ml</td>
<td>5.6 ml</td>
<td>5.6 ml</td>
<td>5.6 ml</td>
</tr>
<tr>
<td>water</td>
<td>5.5 ml</td>
<td>4.3 ml</td>
<td>3.0 ml</td>
<td>1.8 ml</td>
</tr>
<tr>
<td>20% SDS</td>
<td>0.075 ml</td>
<td>0.075 ml</td>
<td>0.075 ml</td>
<td>0.075 ml</td>
</tr>
<tr>
<td>20% APS</td>
<td>0.05 ml</td>
<td>0.05 ml</td>
<td>0.05 ml</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.005 ml</td>
<td>0.005 ml</td>
<td>0.005 ml</td>
<td>0.005 ml</td>
</tr>
</tbody>
</table>

For the analysis of the MAP-kinase phosphorylation shift, a special ratio of acrylamide to bisacrylamide was used in order to separate the phosphorylated and non-phosphorylated form of these protein.

Tab.III.3: Running gel for MAPK-phosphorylation shift
(15% acrylamide/0.075% bisacrylamide)

<table>
<thead>
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<th>volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide</td>
<td>15 ml</td>
</tr>
<tr>
<td>2% bisacrylamide</td>
<td>1.126 ml</td>
</tr>
<tr>
<td>1 M Tris pH 8.8</td>
<td>11.2 ml</td>
</tr>
</tbody>
</table>
A 5% acrylamide/bisacrylamide stacking gel was universally used for all gels. The exact specifications are listed in the table below.

### Tab. III.4: Concentrations of stacking gel

<table>
<thead>
<tr>
<th>compound</th>
<th>volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide/Bis</td>
<td></td>
</tr>
<tr>
<td>30%: 0.8%</td>
<td>0.835 ml</td>
</tr>
<tr>
<td>1 M Tris pH 6.8</td>
<td>0.625 ml</td>
</tr>
<tr>
<td>water</td>
<td>3.49 ml</td>
</tr>
<tr>
<td>20% SDS</td>
<td>0.025 ml</td>
</tr>
<tr>
<td>20% APS</td>
<td>0.025 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.005 ml</td>
</tr>
</tbody>
</table>

Gels were run with SDS-running buffer (50 mM Tris base, 380 mM glycine, 0.1% SDS) at a constant 0.5 mA.

### 3.4.3 Western blotting

After separating the protein of interest on an SDS-PAGE gel, the gel was electroblotted onto PVDF-membrane using the BioRad semi-dry transfer system (15 V for 30 minutes to 1 hour, depending on the size of protein and thickness of gel) using transfer buffer (50 mM Tris base, 380 mM glycine, 0.1% (w/v) SDS, 20% (v/v) methanol). All the following steps were
carried out at room temperature on a shaker. The PVDF-membrane was blocked with 5% non-fat dried milk in TBST (20 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween 20) for one hour up to overnight at 4°C. The primary antibody was diluted in blocking buffer with 5% non-fat dried milk (for dilutions of individual antibodies, see Chapter 2.0) and incubated for one hour in a sealed plastic bag. To remove unspecifically bound primary antibody, the blot was rinsed twice and then washed with TBST. The secondary antibody (sheep anti-rabbit antibody, coupled to horse raddish peroxidase) was diluted 1:2000 in TBST + 5% (w/v) milk powder and incubated for one hour. The blot was washed again for 3 x 20 minutes in TBST plus 25 mM EDTA. Subsequently proteins were detected using the enhanced chemiluminescent system (ECL, Amersham Ltd.), according to the manufacture's protocol.

3.4.3.1 Stripping of Western blots

Western blot membranes were stripped of bound antibodies to allow detection of other proteins on the same filter. To strip filters, these filters were washed for 3 x 10 minutes in 0.1 M glycine pH 2.5. Thereafter, the blot was rinsed with 1 M Tris pH 8.0 and washed 3 x 10 minutes with a large volume of PBSA. The blot was then blocked in 5% milk in PBSA for 1 hour or overnight at 4°C and incubated with a different antibody as described. Prior the storage at -20°C western blots were washed with PBSA once and then wrapped in foil.

3.4.4 Immunoprecipitation

3.4.4.1 In vivo labelling with 35S-methionine

Various Schwann cells were grown in 3% FCS in low glucose DMEM plus glial growth factor (GGF) and forskolin up to 80% confluency on 90 mm
dishes. Schwann cells were washed once in prewarmed DMEM-medium without methionine. 2.5 ml of DMEM-medium without methionine containing 2% dialysed foetal calf serum and $^{35}\text{S-L}$-methionine (300 $\mu$Ci per 90 mm dish) were added for times as indicated in the individual experiment.

3.4.4.2 **Harvest of radioactive labelled cells**

After this time cells were washed twice with cold PBSA and scraped from the dish. All cells were lysed for 15 minutes on ice in reaction tubes with 0.5 ml of lysis buffer (150 mM NaCl, 50 mM Tris pH 8.0, 5 mM EDTA, 1% NP-40, 1 mM PMSF, 1 $\mu$g/ml aprotinin, 1 $\mu$g/ml leupeptin). After a spin for 5 minutes at 13000 rpm at 4°C the supernatant was transferred into new reaction tubes. To the radioactive cell lysates 20 $\mu$l of beads (50% (v/v)) were added to preclear non-specific interactions prior to immunoprecipitation. After an incubation period of 1 hour on a rotating wheel at 4°C, reaction tubes were spun for 30 seconds at 10000 rpm at 4°C and the supernatant was transferred into new tubes.

As normalisation for the following immunoprecipitation, cell lysate with the same number of TCA-precipitable counts was used. 1$\mu$g of mAb 240 was added to the cell lysates and after incubation on a rotating wheel for 1 hour at 4°C, 20 $\mu$l of protein G -beads (50% (v/v) lysis buffer) were added. After a further incubation for 1 hour at 4°C, the immunoprecipitates were pelleted by centrifugation for 30 seconds at 10000 rpm at 4°C and subsequently washed 4x with lysis buffer at 4°C. After the final wash the remaining liquid was removed completely and equal volumes of 2x protein sample buffer were added to the pellet. Tubes were heated to 95°C for 10 minutes, spun and the supernatant was loaded onto an SDS-PAGE gel.

SDS-PAGE gels were fixed by soaking them twice for 5-10 minutes in methanol/acetic acid (40:10). Thereafter twice for 5-10 minutes in
methanol/acetic acid (10:5) followed by washing with aqua dest. for 15 minutes.

To further enhance the detection of radioactive labelled protein, fixed gels were soaked in a 1 M salicylic acid solution and then dried for an hour before being subjected to autoradiography.

Protein G sepharose (Sigma) was supplied as a suspension. After 3 washes with the buffer used in the specific experiment, protein G sepharose beads were used for immunoprecipitation. Protein A sepharose (Sigma), supplied as a powder, was re-suspended in PBSA according to the manufacturer's instructions. Before usage an aliquot of this suspension was washed several times with the specific buffer used for the immunoprecipitation.

3.5  In vitro kinase assays

3.5.1  In vitro kinase assay for MAP-kinase p42ERK2

All the following steps were carried out at 4°C. Nearly confluent cells were washed once in cold PBSA. Cells were scraped off the dish with a rubber policeman and spun for 1 minute at 6500 rpm in a microfuge. Cells from a 150-mm dish were re-suspended in 150 µl buffer (10 mM Tris pH 8.0, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 µM Na3VO4, 1% Triton X-100 containing 1mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin, 10 µg/ml leupeptin, pH 7.6). After an incubation of 10 minutes on ice, lysates were spun for 5 minutes at 13000 rpm. The supernatant was aliquoted into new tubes and snap frozen. Due to the lack of SDS in the lysis buffer which strongly inhibits phosphatase activity, these cell lysates could be stored for approximately one month at -20°C without loss of kinase activity. Prior to immunoprecipitation the protein concentration was estimated using the Biorad protein assay. For each
immunoprecipitation 30 μl protein A sepharose beads were incubated with
5 μl of antibody 122 (Leavers & Marshall, 1992) in 200 μl lysis buffer for 1
hour at 4°C. After a centrifugation step and aspiration of the supernatant,
100 μg total protein in 200 μl lysis buffer were added and rotated for 1 hour
at 4°C. Beads were washed twice in lysis buffer and twice in ice-cold kinase
buffer (15 mM Tris/HCl pH 7.5, 15 mM MgCl₂) prior to the in vitro kinase assay.

Following immunoprecipitation, beads were incubated in 25 μl of
kinase assay mix containing kinase buffer with 0.5 mM EGTA, bovine myelin
basic protein (MBP) at 7.5 μg per reaction as substrate, 50 μM ATP, 50
μCi/ml of (γ-32P) ATP, 2 μM cAMP-dependent protein kinase inhibitor peptide
and 100 nM microcysteine LR at 30°C for 30 minutes by shaking on the
Eppendorf Thermomixer. The kinase reaction was terminated by adding 1/4
volume 4x protein gel sample loading buffer (SB) and reactions were
loaded onto a 15% SDS-PAGE gel. The gel was fixed, dried and exposed
to X-OMAT-film (Kodak) overnight.

3.5.2 Production and purification of GST-fusion proteins

Bacteria transformed with a GST-fusion protein construct were grown
overnight and diluted 1:10 into a 100 ml BHI-medium culture plus 100 μg/ml
ampicillin. At an optical density of OD₅₅₀ of 0.8 protein production was
induced with 1 mM IPTG for 4 hours. After this incubation time the
suspension was spun down and re-suspended in 1/10 vol. PBSA + 1% (v/v)
triton x-100 and 10 μg/ml aprotinin and 10 μg/ml leupeptin. The re-
suspended bacteria were transferred into 1.5 ml tubes and sonicated for 3 x
15 seconds on half maximal power. Tubes were kept on ice in between the
sonication steps. After a spin of 10 minutes at 14000 rpm the supernatant
was pooled and filtered through a 0.45 μm filter. Supernatant was incubated overnight with GST-sepharose beads on a rotating wheel at 4°C and washed 5 x with PBSA + Triton X-100 (1%). The bound GST-fusion protein was eluted with freshly prepared 10 mM glutathione (Sigma) in 50 mM Tris pH 8.0. At least 5 washes were performed and eluted fractions were kept separately, run on a gel and stained.

Overnight cultures of DH5α were diluted 1:10 and grown for 1 hour. GST-ERK2 was induced with 1 mM IPTG for 4 hours at 37°C and GST-MEK induced with 30 μM IPTG at 27°C overnight. The bacteria were then pelleted, frozen and thawed. Bacteria were sonicated on ice 3 x for 15 seconds with Sonicator model Soniprep 150 (MSE) at 14 microm. Cell debris was removed by centrifugation and GST-fusion proteins were purified by adding 0.5 ml glutathione-Sepharose beads and rotating for 30 minutes at 4°C. The GST-ERK2 was cleaved from the GST in thrombin buffer while the GST-MEK was eluted from the glutathionine-sepharose beads with 5 mM glutathione in 50 mM HEPES.

3.5.3  In vitro  kinase assay for MAP-kinase kinase

MAP-kinase kinase activity was measured in a coupled assay with recombinant MAP-kinase and myelin basic protein as final substrate. Initially MAP-kinase kinase was precipitated with a 1:1 mixture of monoclonal anti-MEK-1 and anti-MEK-2 antibodies for 2 hours with 40 μl of protein A-agarose (1:1 slurry) added for the another hour. Immune complexes were collected by centrifugation and washed three times in lysis buffer without PMSF and once with buffer A (50 mM Tris-HCL, pH 7.5, 0,1 mM EGTA, 0.5 mM sodium vanadate and 0.1% 2-mercaptoethanol). Pellets were resuspended in 30 μl of MEK/MAPK buffer (30 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1% 3-mercaptoethanol, 100μg/ml GST-MAPK (a kind gift of
Thomas Seufferlein, ICRF), 0.03% Brij-35, 10 mM Mg-ATP and 20 mM N-octyl-β-D-glucopyranoside) and incubated at 30 °C for 30 minutes. The reaction was stopped by addition of 4X protein loading buffer.

3.5.4 In vitro kinase assay for JNK-1 kinase

Cells were washed twice with cold PBSA and left to drain. 600 µl lysis buffer was added to a large tissue-culture dish. The large tissue-culture dish was left on ice for 5 minutes. Cells were scraped with a rubber policeman, resuspended in JNK-lysis buffer (20 mM HEPES pH 7.5, 2 mM EGTA, 50mM β-glycerolphosphate, 1 mM DTT, 1 mM Na₃ VO₄, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 400 µM PMSF, 5 µg/ml leupeptin, 5 µg/ml aprotinin, 0.1 ng/µl ocadaic acid) and after another 5 minutes on ice, spun for 10 minutes at 4°C. The supernatant was transferred to new tubes. The protein concentration was immediately measured using the BioRad protein system. The indicated concentration of total cell protein lysate was subjected to immunoprecipitation. After incubation with 1 µg of antibody JNK1 (C-17, Santa Cruz Ltd.) for 2 hours, immunoprecipitates were washed 3x in lysis buffer, 3x in LiCl-buffer (500 mM LiCl, 100 mM Tris pH 7.5, 0.1% (v/v) Triton x-100, 1 mM DTT) and 3x in JNK-kinase buffer (20 mM MOPS pH 7.2, 2 mM EGTA, 10 mM MgCl₂, 1 mM DTT, 0.1% (v/v)Triton x-100), before a kinase reaction was carried out. For the JNK-1 kinase reaction, an ATP-MIX was prepared consisting of 2 µl of 0.1 mM ATP, 5 µl of 50 mM MgCl₂, 2.66 µl of ³²γ-ATP (= 26,6 μCi) and 90.34 µl water. 7.5 µl of the ATP-MIX was added to the protein A sepharose beads together with 20 µl JNK-1 kinase buffer and 3 µl GST-Jun aa 1-257 (1 mg/ml). The kinase reaction was incubated at 30°C for 20 minutes and stopped with 4x protein loading dye.
3.6 Luciferase activity assay

Prior to harvest cells were washed twice with ice-cold PBSA and drained for 5 minutes. 100 µl 0.25 M Tris pH 7.5 was added and cells were scraped with a rubber cell scraper. After transfer of the cell suspension into reaction tubes, the cells were lysed by snap-freeze and thaw three times. Tubes were subsequently spun for 5 minutes. The supernatant was removed and used for the following luciferase assay. 30 µl of supernatant were added to luminator cuvettes containing 350 µl luciferase reaction buffer (25 mM glycylglycine pH 7.8, 5 mM ATP (pH corrected to 8.0), 15 mM MgSO4). Samples were loaded into an LKB 1251-luminator and luciferase activity was measured after injection of 30 µl of 3 mM luciferin into the individual sample. Luciferase activities were normalised against protein concentration measured by the BioRad protein assay as previously described (see section 3.4.1.1).
CHAPTER 4.0

Mutants of the tumour suppressor gene p53 cooperate with oncogenic Ras and oncogenic Raf in Schwann cells
4.0 Mutants of the tumour suppressor gene p53 cooperate with oncogenic Ras and oncogenic Raf in Schwann cells

4.1 Introduction

Oncogenic Ras mutants introduced into Schwann cells cause a cell-cycle arrest, which can be rescued by a second cooperating oncogene such as the Simian virus 40 large T antigen (SV40 LT) (Ridley et al., 1988). To facilitate the investigation of oncogene cooperation, Schwann cell clones contain v-Ha Ras and a temperature-sensitive mutant of SV40 large T antigen (tsA58 LT) (Tegtmeyer et al., 1975) were generated (Ridley et al., 1988). Cells shifted to the non-permissive temperature (39°C) arrested in the G1-phase of the cell-cycle, revealing the growth inhibitory effect of oncogenic Ras in Schwann cells. In contrast, at the permissive temperature (33°C) Schwann cell clones 10ras3 and 11ras2 proliferated rapidly, exhibit a transformed morphology and a reduced requirement for growth factors due to v-Ha Ras and SV40 LT cooperativity (Ridley et al., 1988). Since a viral protein had been convincingly shown to cooperate with activated Ras in transformation of Schwann cells, I was interested to find a cellular oncogene which could be utilised for my investigations into the mechanism of oncogene cooperation.

SV40 LT is a multifunctional protein which interacts with a number of cellular target proteins. One important cellular target of SV40 LT is the tumour suppressor protein p53 which is inactivated in its function through the interaction with SV40 LT (Lane & Crawford, 1979). Therefore, I investigated whether mutants of p53 itself were able to cooperate with activated Ras in our Schwann cells model.
4.2 Mutant p53 cooperates with v-Ha Ras in 10ras3 and 11ras2 Schwann cells

To address the question whether mutants of the tumour suppressor p53 were capable of cooperating with oncogenic Ras in the transformation of Schwann cells, I introduced two mutants of the human p53 gene into the described Schwann cell clones 10ras3 and 11ras2 (Ridley et al., 1988). Based on previous results with Ras and SV40LT (Ridley et al., 1988), I reasoned that Schwann cells at the non-permissive temperature should be enabled to overcome the ras-induced growth arrest, if mutants of p53 cooperate with oncogenic Ras. To test this hypothesis, two missense mutants of the human p53 gene (R175H and R248W) in the DNA binding domain (Baker et al., 1990) which naturally occurred with high frequency in various human tumours (Hollstein et al., 1996) were used. These two p53 mutants were cloned into the retroviral vectors pBabe Puro (Figure IV.1) or, alternatively into pBabe Bleo (Morgenstern & Land, 1990).

To derive Schwann cells expressing these mutant p53 proteins, Schwann cells were infected by co-cultivation with GPE-virus producer cells for pBabe Puro p53\(^{R175H}\) and pBabe Puro p53\(^{R248W}\) including pBabe Puro vector as control. After infection of 10ras3 and 11ras2 cells with these viruses, Schwann cells were split and selected for two days at the permissive temperature. Then, half of the dishes were shifted to the non-permissive temperature (39°C), whereas the other half were kept at the permissive temperature (33°C). Three weeks after co-cultivation, outgrowing colonies at both temperatures were stained and counted. Interestingly, at the non-permissive temperature both introduced mutant p53 cDNAs could rescue the ras-induced cell-cycle arrest in 10ras3 and 11ras2 cells (Figure IV.2A and IV.3A). Between 60-100% of colonies found at the permissive temperature were also able to grow at the non-permissive
Figure IV.1: Mutant p53$^{R175H}$ and mutant p53$^{R248W}$ in pBabe Puro vector

Two missense mutations of the human p53 gene, mutant p53$^{R175H}$ and mutant p53$^{R248W}$ were cloned into the BamH I-restriction site of the retroviral vector pBabe Puro (Morgenstein & Land, 1990).
Cap Site

poly A site

BamHI

BglII

SmaI

BstXI

EcoRI

SnaBI

amp

p53

w

poly A site
Figure IV. 2A: Mutant p53 can rescue ras-induced growth arrest in 11ras2 cells

Schwann cells 11ras2 were co-cultivated with GPE-virus producer cells shedding pBabe Puro, pBabe Puro p53R175H and pBabe Puro p53R248W viruses over a period of two days as described (see Methods). Cells were split 1:10 and kept for two days under puromycin selection. Half of the dishes were shifted to the non-permissive temperature (39°C), whereas the other half was kept at the permissive temperature (33°C). Outgrowing colonies were stained and counted three weeks after infection. The standard deviation of the number of 11ras2 Puro colonies at 39 °C was too small to be seen in the presented graph.

Figure IV.2B: Mutant p53 is expressed in 11ras2 cells

11ras2 Puro, 11ras2 p53175 and 11ras2 p53248 cells were grown at indicated temperatures. After addition of 35S-methionine for four hours, cells were lysed and equal numbers of TCA-precipitable counts were immunoprecipitated with the mutant p53 specific antibody 240 or a control antibody as described (see Methods). Immunoprecipitates were separated on a 10% SDS-PAGE gel and exposed to film.

1: 11ras2 Puro, 33°C, Ab 9E10
2: 11ras2 Puro 33°C, Ab 240
3: 11ras2 p53175, 39°C, Ab 9E10
4: 11ras2 p53175, 39°C, Ab 240
5: 11ras2 p53248, 39°C, Ab 9E10
6: 11ras2 p53248, 39°C, Ab 240
7: radioactive size-marker
A. 

Number of colonies

<table>
<thead>
<tr>
<th>Temperature</th>
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<th>39°C</th>
</tr>
</thead>
<tbody>
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<td>100</td>
</tr>
<tr>
<td>11ras2 mt p53&lt;sup&gt;175&lt;/sup&gt;</td>
<td>500</td>
<td>700</td>
</tr>
<tr>
<td>11ras2 mt p53&lt;sup&gt;348&lt;/sup&gt;</td>
<td>750</td>
<td>900</td>
</tr>
</tbody>
</table>

B. 

mt p53

<table>
<thead>
<tr>
<th>Lane</th>
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</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>5</td>
<td>348</td>
</tr>
<tr>
<td>6</td>
<td>175</td>
</tr>
<tr>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>
Figure IV.3A: Mutant p53 can rescue ras-induced growth arrest in 10ras3 cells

10ras3 cells were co-cultivated with GPE-virus producer cells shedding pBabe Puro, pBabe Puro p53R175H and pBabe PuroR248W viruses over a period of two days (see Methods). Cells were split 1:10 and kept for two days under puromycin selection. Half of all dishes were shifted to the non-permissive temperature (39°C), whereas the other half remained at the permissive temperature (33°C). Colonies were stained and counted three weeks after co-cultivation. The standard deviation of the number of 10ras3 Puro colonies at 39 °C was too small to be seen in the presented graph.

Figure IV.3B: Mutant p53 is expressed in 10ras3 cells

10ras3 Puro, 10ras3 p53R175H and 10ras3 p53R248W cells were grown at indicated temperatures. After labelling with 35S-methionine for four hours, cells were lysed and equal numbers of TCA-precipitable counts were immunoprecipitated with the mutant p53 specific antibody 240 or a control antibody as described (see Methods). Immunoprecipitates were separated on a 10% SDS-PAGE and exposed to film.

1: 10ras3 Puro, 33°C, Ab 9E10
2: 10ras3 Puro, 33°C, Ab 240
3: 10ras3 Puro, 39°C, Ab 9E10
4: 10ras3 Puro, 39°C, Ab 240
5: 10ras3 p53R175, 39°C, Ab 9E10
6: 10ras3 p53R175, 39°C, Ab 240
7: 10ras3 p53248, 39°C, Ab 9E10
8: 10ras3 p53248, 39°C, Ab 240
9: radioactive size-marker
temperature whereas the infection with the retroviral vector alone did not result in colony growth at the non-permissive temperature. Similar results were obtained in several independent experiments. In addition, experiments with other Schwann cell clones as well as with the retroviral vector pBabe Bleo, containing mutant p53 confirmed the presented results that up to 100% of infected cells were able to overcome the ras-induced growth arrest in Schwann cells. These results show that the effect of mutant p53 expression was neither dependent on the Schwann cell clone nor on the retroviral vector used.

As illustrated in Figure IV.2B and IV.3B, 10ras3 and 11ras2 cells expressed mutant p53R175H or mutant p53R248W protein at the permissive and non-permissive temperature. The apparent difference in the amount of mutant p53R175H protein compared to mutant p53R248W protein after immunoprecipitation was very likely due to a reduced affinity of the antibody Ab240 used as reported by Gannon et al., 1990 and did not necessarily reflect differences in expression levels.

4.3 A carboxy-terminal region of p53 is sufficient to cooperate with oncogenic Ras in Schwann cells

Mutants of the p53 tumour suppressor can overcome the ras-induced growth arrest in 10ras3 and 11ras2 cells as shown in the previous section. However, the mechanism by which p53 mutants can cooperate with oncogenic Ras remained unclear. Despite ample supportive evidence that mutant p53 binds to wild-type p53 leading to the formation of stable non-functional complexes (Milner & Metcalf, 1991; Kern et al., 1992; Bargnonetti et al., 1992), this explanation may not be fully satisfactory, since certain p53 mutants have been reported to transactivate a specific target gene when
introduced into p53-null cell lines (Dittmer et al., 1993, Chin et al., 1992; Crook et al., 1994). This indicated the possibility that p53 mutations could also lead to the gain of certain functions. The two p53 mutants (R175H, R248W) employed in my experiments have been reported to show a gain-of-function in p53-null cells in terms of transactivation of a reporter construct containing the promoter region of the multi-drug resistant gene and tumourigenicity after injection into nude mice. However, in p53 negative SAOS-2 cells the same p53 mutants displayed no gain-of-function (Dittmer et al., 1993). In contrast to this report, mutant p53R175H has been shown to be transcriptionally inactive towards a different p53 binding site, p53CON (Funk et al., 1992) in p53-null K562 chronic myelogenous leukemia (CML) cells (Zhang et al., 1993) and in a p53-null ovarian carcinoma cell line (SKOV-3) (Chumakov et al., 1993). Nevertheless, a missense mutation at the same position substituting arginine to a proline, p53R175P, resulted in a clearly transcriptional active p53 mutant (Crook et al., 1994). Some experimental evidence suggested that the reported observation of a gain-of-function of p53 mutants depended strongly on the specific mutant (Crook et al., 1994), the nature of the p53 binding site of the reporter construct (Chen et al., 1993) and the cell line used for investigation (Dittmer et al., 1993, Sehgal & Margulies, 1994).

Intensive studies into the functions of the p53 protein have established a minimal carboxy-terminal domain which is required for transformation in primary cells (Shaulian et al., 1992). The transforming activity of this C-terminal region is not due to any mutation within but has been correlated with the ability to form stable oligomers with wild-type p53 (Shaulian et al., 1992; Bowman et al., 1996). This minimal transformation domain of p53 (Figure IV.4A) widely overlaps with the oligomerisation domain of amino acids 330-390 in murine p53 (Shaulian et al., 1992;
Stürzbecher et al., 1992; Wang et al., 1994) which is required for the formation of functional p53 complexes and, in particular, tetramers (Stürzbecher et al., 1992). Similarly to stable complexes formed between full-length mutants of p53 with wild-type p53, oligomers between wild-type p53 and the carboxy-terminal domain abrogate any sequence-specific DNA-binding of wild-type p53 (Shaulian et al., 1992) and the transactivation from p53 reporter genes (Reed et al., 1993; Shaulian et al., 1995). Consistent with this notion, a full-length mutant of p53 with a truncation of this C-terminal domain did not inhibit the ability of wild-type p53 to activate transcription (Unger et al., 1993). Conversely, the carboxy-terminal region of p53 cooperated with activated Ras in the transformation of rat embryo fibroblasts (Shaulian et al., 1992) and enhanced the transformation by oncogenic ras and E1A (Reed et al., 1993). Moreover, overexpression of the p53 carboxy-terminus interfered with p53-mediated apoptosis in response to cytokine withdrawal in haematopoietic cells (Gottlieb et al., 1994). The effectiveness of the p53 carboxy-terminus in inactivating wild-type p53 was confirmed in a transgenic mouse model for brain tumour development in which tissue-specific expression of the p53 carboxy-terminus resulted in a reduction of apoptosis and accelerated tumourigenesis (Bowman et al., 1996). Thus, the carboxy-terminal domain provides a way to investigate the cooperation of oncogenic Ras and a functional defective p53 protein in the absence of any possible gain-of-function activity.

To test whether the rescue of the ras-induced growth arrest by p53 mutants required a gain-of-function of these mutants, I repeated the experiments described above using the carboxy-terminal region of wild-type p53 rather than the full-length mutant protein. The murine p53 carboxy-terminus from amino acid 302 to 390 (Figure IV.4A) expressed in pBabe Puro designated pBabe Puro p53CT was introduced into Schwann cell
Figure IV.4A: Schematic representation of p53 carboxy-terminus in comparison to full-length murine p53 protein

The murine p53 carboxy-terminus amino acids 302-390 is aligned to the major functional regions of p53 after Osbun & Butel, 1995, indicating the overlapping region in particular with the oligomerisation domain of p53.

Figure IV.4B: p53CT^aa302-390 rescues ras-induced growth arrest in 10ras3 cells

10ras3 cells were co-cultivated with GPE Puro and GPE p53 CT^aa302-90 cells for two days as described (see Methods). Cells were split and kept under puromycin selection for three days. Half of all dishes were then shifted to the non-permissive temperature of 39°C, whereas the other half remained at the permissive temperature of 33°C. Colonies were stained and counted three weeks after co-cultivation. The standard deviation of the number of 10ras3 Puro colonies at 39 °C was too small to be seen in the presented graph.
A.

![Graph showing protein domains and regions](image)

- **wild-type p53**
- **transactivation domain**
- **DNA-binding region**
- **oligomerisation domain**

B.

![Bar graph showing temperature effects](image)

- **Number of colonies**
- **Temperature**:
  - 33°C
  - 39°C

- **Strains**:
  - 10ras3 Puro
  - 10ras3 p53 CT

Additional Note: The graph on the right compares the number of colonies at different temperatures and between different strains.
clone 10ras3. This carboxy-terminal region of the p53 mouse and rat sequence differ in only a very few amino acid positions (Stürzebecher et al., 1992). Following infection, Schwann cells were split into drug-selective medium. After two days at the permissive temperature (33°C) half of the dishes were shifted to the non-permissive temperature (39°C). Three weeks after infection, outgrowing colonies at both temperatures were stained and counted.

The p53 minimal transformation domain enables Schwann cell clone 10ras3 to overcome the ras-induced growth arrest and to form growing colonies at the non-permissive temperature. The number of colonies obtained at the permissive (33°C) and non-permissive temperature (39°C) indicated that up to 100% of infected cells were enabled to overcome the ras-induced growth arrest by expression of the p53 carboxy-terminus (Figure IV.4B). These results showed that the inactivation of wild-type p53 is required for the abrogation of the ras-induced growth arrest. It therefore seemed unlikely that a possible gain-of-function of the full-length p53 mutants contributed to the rescue of the ras-induced growth arrest described in previous experiments.

4.4 Mutant p53 and p53CT cooperate with oncogenic Ras in normal Schwann cells

The results presented in the previous sections have established that p53 mutants as well as a p53 carboxy-terminal region can cooperate with oncogenic Ras in 10ras3/10ras3-1 and 11ras2 cells. However, it has been reported, that various cells expressing the temperature-sensitive large T (tsLT) arrest or enter senescence after withdrawal of the LT antigen which indicates that proliferation had become dependent on the presence of LT
(Ikram et al., 1994; Gonos et al., 1996). If this phenomenon also occurs in Schwann cells used in previous experiments, it would make it impossible to distinguish between growth arrest initiated by activated Ras or due to the withdrawal of LT at the non-permissive temperature. As a consequence, the abrogation of the growth arrest by mutant p53 may not necessarily have directly contributed to the cooperation with activated Ras.

To investigate whether mutant p53 and activated Ras could cooperate in the absence of any LT protein, I introduced the retroviral vectors pBabe Puro p53R175H or pBabe Puro p53CT into normal Schwann cells. After selection, Schwann cells with empty vector (NS Puro), p53R175H (NS mt p53175) and the p53 carboxy-terminus (NSp53CT) were pooled and subsequently infected with oncogenic c-Ha Ras Val12-LXSN or the empty vector LXSN virus (Miller & Rosman, 1989).

In line with previous findings (Ridley et al., 1988), c-Ha Ras Val12 introduced alone did not result in any sustainable Schwann cells colonies. Therefore, Schwann cells expressing c-Ha Ras Val12 were not available for further experimentation. Similar numbers of resistant control and mutant p53 or p53CT expressing colonies were recovered, although colonies expressing mutant p53 or the p53 carboxy-terminus were larger compared with controls. Colonies resistant to both selective markers were pooled.

Cooperation of c-Ha Ras Val12 and p53R175H or p53CT was measured as growth factor-independent proliferation. The cell proliferation rate was determined to establish whether p53 mutants and oncogenic Ras were able to alter the growth factor requirements of Schwann cells since growth factor-independent cell proliferation is a hallmark of Schwann cell transformation (Ridley et al., 1988). Generally, proliferation of normal Schwann cell is weakly stimulated by foetal calf serum and required
Figure IV.5: Growth curve of NS p53\textsuperscript{175}/c-Ha Ras Val12 cells
Schwann cells were infected with either pBabePuro or pBabePuro p53\textsuperscript{R175H} as described (Methods). After selection, these cells were infected with c-Ha Ras Val12-LXSN or the retroviral vector LXSN alone and selected. Drug-resistant colonies were pooled and 2x10\textsuperscript{4} cells were seeded into 2\% FCS containing DMEM in the absence of glial growth factor and forskolin. Cells from two wells of a 6-well dish were trypsinised daily and counted three times for six days. The average cell number was plotted against time. The standard deviation of some points was too small to be seen in the presented graph. Growth curves presented in Figure IV.5 & 6 were obtained in the same experiment. Therefore, the control growth curves are identical in both figures.

Figure IV.6: Growth curve of NS p53 CT\textsuperscript{aa302-390}/c-Ha Ras Val12 cells
Schwann cells were infected with either pBabe Puro or pBabe Puro p53CT\textsuperscript{aa302-290} as described (Methods). After selection, these cells were infected with c-Ha Ras Val12-LXSN or the retroviral vector LXSN alone and selected. Drug-resistant colonies were pooled and 2x10\textsuperscript{4} cells were seeded into 2\% FCS containing DMEM in the absence of glial growth factor and forskolin. Cells from two wells of a 6-well dish were trypsinised daily and counted three times for six days. The average cell number was plotted against time. The standard deviation of some points was too small to be seen in the presented graph.
Figure IV.7: Morphological transformation of Schwann cells by mutant p53 or p53CT and c-Ha Ras Val12

Schwann cells were infected with pBabe Puro, pBabe Puro p53R175H or pBabe Puro p53 CTaa302-90 as described (Methods). After selection, these cells were infected with c-Ha Ras Val12-LXSN or the retroviral vector LXSN alone and selected for drug-resistant colonies. Fotos were taken at a magnification of 400x directly after outgrowth of colonies.
additional growth promoting factors, in particular glial growth factor and forskolin (Ridley et al., 1988; my own observation). In the absence of these growth promoting factors, untransformed Schwann cells did not grow at all or extremely slowly probably due to traces of growth promoting factors in serum.

Schwann cells NS Puro, NS mtp53^{175} or NS p53CT, or the same cells infected with c-Ha Ras Val12-LXSN, or the empty vector LXSN respectively, were seeded at 2x10^4 cells/well in medium without growth promoting factors. Cells were counted daily over a period of five days.

Schwann cells with c-Ha Ras Val12 and either mutant p53^{R175H} or the carboxy-terminus of p53 proliferated more rapidly compared to control cells (Figure IV.5 and IV.6). Cells with full-length mutant p53^{R175H}/c-Ha Ras Val12 had an increased proliferation rate compared to NS p53CT/c-Ha Ras Val12 cells. Schwann cells expressing either mutant p53^{R175H} or p53CT alone exhibited a similar growth rate to vector alone control cells (Figure IV.5 and IV.6). Schwann cells harbouring both mutant p53^{R175H} or p53CT and c-Ha Ras Val12 presented a highly refractile, spindular morphology as compared to cells containing only mutant p53^{R175H}/p53CT or vector alone (Figure IV.7). These data indicate that oncogenic Ras and mutant p53^{R175H} or the p53 carboxy-terminus are able to cooperate in growth factor-independent cell proliferation and morphological transformation (Figure IV.5, 6, 7) and thus, behave very similarly to Schwann cells expressing activated Ras and SV40 large T antigen (Ridley et al., 1988).

4.5 Mutant p53 cooperates with oncogenic Raf in Schwann cells
Oncogenic Ras and mutants of the tumour suppressor gene p53 cooperate in Schwann cells as has been established in the previous sections. To further elucidate the cooperation between mutant p53 and oncogenic Ras, I sought to determine the signalling pathway downstream of Ras which was required for the cooperative action with mutant p53. Since oncogenic Ras connects to various downstream signalling pathways, I was interested in investigating whether Raf, one of the major downstream targets of Ras (Moodie et al., 1993; Van Aelst et al., 1993; Warne et al., 1993; Zhang et al., 1993; Vojtek et al., 1993), can also cooperate with mutant p53 in Schwann cell transformation. If activated Ras signals through Raf in order to cooperate with mutant p53, then constitutively active Raf molecules should also be capable of cooperating with mutant p53. The c-Raf mutant ΔRaf which has a deletion of the regulatory region-1 (CR-1) of Raf reportedly exhibited constitutive kinase activity (Leevers et al., 1994). Therefore, I used this constitutively active molecule expressed in the retroviral vector LNSX (Miller & Rosman, 1989) to explore the cooperation of mutant p53R175H and oncogenic Raf as a downstream target of Ras in transforming Schwann cells. To test this hypothesis, the retroviral vectors ΔRaf-LXSN (Leevers et al., 1994) were introduced into low passage Schwann cells NSPuro and NSmtp53175. Outgrowing colonies infected with ΔRaf alone were very small compared to colonies infected with ΔRaf and mutant p53175. Furthermore, Schwann cells expressing ΔRaf on its own grew very poorly so that after more than three weeks not enough cells were available for the following experiment.

Transformation by cooperating ΔRaf and mutant p53R175H were again measured as a function of cell proliferation of the pooled Schwann cell colonies in the absence of any growth-promoting factors. NSPuro and
Schwann cells were infected with either pBabe Puro or pBabe Puro mtp53^{R175H} as described (Methods). After selection, these cells were infected with either ΔRaf-LXSN or the retroviral vector LXSN alone and selected. Drug-resistant colonies were pooled and 2x10^4 cells were seeded into DMEM supplemented with 2% FCS but without the growth-promoting factors GGF and forskolin. Cells from two wells of a 6-well dish were trypsinised daily for six days and counted. The average cell number is plotted against time. The standard deviation of some points was too small to be seen in the presented graph.
NSmtp53175 or the same cells infected with ΔRaf or the empty vector LXSN respectively were seeded at 2×10^4 cells/well in medium without growth promoting-factors. Cells were counted daily over a period of six days.

Schwann cells harbouring ΔRaf and mutant p53R175H had an increased proliferation rate compared to control cells (Figure IV.8). Schwann cells expressing mutant p53 and an empty LXSN vector alone also showed an increased rate of cell proliferation, indicating that mutant p53 alone reduces the growth factor requirements in Schwann cells dramatically. As mentioned previously, Schwann cells infected with ΔRaf and the empty vector pBabe Puro were growing very slowly and cell confluency could not be achieved, indicating that ΔRaf had a growth-inhibitory effect on these cells. In comparison to Schwann cells expressing oncogenic Ras and mutant p53, the morphology of ΔRaf and mutant p53 expressing cells was less refractile.

In summary, these results indicated that constitutively active Raf strongly impeded cell growth and this effect was abolished by co-infection with mutant p53 in this cell proliferation assay. However, the difference in growth rate between NSp53175/ΔRaf and NSp53175/LXSN cells were not as profound as between Ras and mutant p53 which could be an indication for the involvement of additional Ras-dependent but Raf-independent pathways.

4.6 Mutant p53 abolishes ΔRafER induced growth arrest in Schwann cells

The previous experiments have provided evidence that mutants of the tumour suppressor gene p53 as well as the carboxy-terminus of p53 cooperate with activated Ras and activated Raf to transform Schwann cells.
However, to further investigate the mechanisms of cooperation between oncogenic Ras/Raf and mutant p53, these cells were only of very restricted use. Schwann cell clones 11ras2 and 10ras3 had to be grown in the presence of a cooperating oncogene at the permissive temperature prone to additional mutagenesis under these conditions. On the other hand, the generated Schwann cells harbouring mutant p53 and oncogenic Ras/Raf could not be regulated at all and a biochemical analysis of the same cells in the transformed and arrested state was impossible.

These restrictions did not apply to Schwann cells with an inducible form of ΔRaf (Lloyd et al., 1997). Oncogenic ΔRaf fused to the hormone-binding domain of the human oestrogen receptor (Samuels et al., 1993) was cloned into the high-titre retroviral vector LXSN (Miller & Rosman, 1989) in order to infect low-passage normal Schwann cells. In these Schwann cells, designated NSΔRafER, constitutive Raf-kinase activity had become strictly dependent on oestrogen or alternatively on the oestrogen analogue, 4-hydroxy tamoxifen (Lloyd et al., 1997).

NSΔRafER cells harbouring the inducible Raf-molecule offered various advantages for further investigation into the signalling pathways for the Ras/Raf cell-cycle arrest. Firstly, these Schwann cells could be grown in the absence of any cooperating oncogene and, secondly, induction of ΔRafER-dependent kinase activity was rapid, since induction occurred at the protein level. Kinetic studies regarding the activation of downstream targets of Raf and their contribution towards the growth arrest became technically possible.

Similar to Ras, activation of ΔRafER induces Schwann cells to arrest in G1 (Figure IV.9). This G1-phase arrest was independent of specific growth conditions (Lloyd et al., 1997). Furthermore the changes in cell morphology upon activation of Raf were indistinguishable from those
Figure IV.9: Growth arrest in NSΔRafER cells is abrogated by mutant p53

Subconfluent NSΔRafER, NSΔRafERp53 and NSempty control cells were assynchronously grown in the presence or absence of 1μM 4-hydroxy tamoxifen for 30 hours. Four hours prior trypsinisation, 10 μM bromo-desoxyuridine (BrdU) were added. Cells were examined for DNA content by propidium iodide staining and flow cytometry and analysed for BrdU uptake.
observed in Schwann cells expressing oncogenic Ras protein (Lloyd et al., 1997; Ridley et al., 1988). These findings indicated that the newly generated NSΔRafER cells could be employed for further investigation into the nature of the Ras and Raf induced growth arrest.

Using NSΔRafER cells, I therefore further investigated whether mutant p53R175H was capable of cooperating with constitutively active Raf to overcome the growth arrest. This question was of particular interest in order to establish whether these cells could be used for a biochemical analysis of the mechanism of cooperation of mutant p53 and ΔRaf (described in chapter 5.0). Initially I infected NSΔRafER cells with the retroviral vector pBabe Puro p53R175H and the empty vector pBabe Puro. Puromycin-resistant colonies were expanded in the absence of the ΔRafER inducer 4-hydroxy tamoxifen and subsequently pooled.

To test the proliferative behaviour of these cells in the presence and absence of active RafER, FACS analysis was carried out to determine the cell cycle distribution. NSempty cells containing only the vector LXSN, NSΔRafER and NSΔRafER p53R175H cells were grown asynchronously in the presence or absence of 4-hydroxy tamoxifen. As indicated in Figure IV.9, induced NSΔRafER cells were arrested in G1 whereas uninduced NSΔRafER cells continued to proliferate. Interestingly, the coexpression of mutant p53R175H in induced NSΔRafER cells enabled these cells to continue proliferating. Thus, mutant p53R175H was also capable of overcoming the ΔRaf-induced arrest in NSΔRafER cells.

4.7 ΔRaf arrests Schwann cells through p53-dependent induction of p21WAF1/Cip1

4.7.1 Introduction
The generation of Schwann cells which arrested in a strictly hormone-dependent manner (NSΔRafER) (Figure IV.9) allowed investigations into the effects of activated Raf on members of the cell cycle machinery in particular cyclin/cdk complex kinase activity. Investigations into cyclin E, cyclin A and cdk2-dependent kinase activity revealed a strong decrease of activity in arrested Schwann cells, despite the fact that CDK2 complexed with cyclin A or cyclin E was mostly found in the CAK phosphorylated form understood to be the active form of these complexes (Lloyd et al., 1997). Since cyclin E, cyclin A and CDK2-dependent kinase activity is required for S-phase entry (Girard et al., 1991; Pagano et al., 1992; Zindy et al., 1992; Tsai et al., 1993; van den Heuvel & Harlow, 1993; Ohtsubo et al., 1995), the observed inhibition of cyclin/cdk kinase activity appeared to be sufficient to arrest Schwann cells in G1. In addition, my results showing that p53 mutants were capable of abrogating the Raf-induced cell cycle arrest suggested a direct or indirect involvement of wild type p53 in mediating this cell cycle arrest. The nature of this interaction was however unclear at the time.

We considered the Raf-dependent and p53-dependent induction of a cdk-inhibitor as a possible explanation for our observations. As one possible candidate, the cdk-inhibitor p21WAF1/Cip1 (Harper et al., 1993; Xiong et al., 1993; el-Deiry et al., 1993; Noda et al., 1994) not only binds to cyclin E/cdk2 complexes, cyclin A/cdk2 and cyclin A/cdc2 complexes and inhibits their specific kinase activity (Harper et al., 1993; Xiong et al., 1993; Gu et al., 1993). Moreover, p21WAF1/Cip1 is also transcriptionally induced by wild type p53 (el-Deiry et al., 1993) and is able to mediate p53’s growth-inhibitory function in various cell types. It was therefore intriguing to test the possibility that the Raf and p53-dependent induction of p21WAF1/Cip1 was the underlying cause for the G1 cell cycle arrest in Schwann cells.
4.7.2 ΔRafER induces cell cycle inhibitor $p21^{WAF1/Cip1}$ in Schwann cells

To analyse $p21^{WAF1/Cip1}$ protein level in Schwann cells, NSΔRafER cells were quiesced for two days prior to the addition of 4-hydroxy tamoxifen and mitogens. Protein samples were taken at indicated time points after induction of ΔRaf. The activation of ΔRafER in Schwann cells led to a strong induction of the cyclin/cdk inhibitor $p21^{WAF1/Cip1}$ initially visible at 10 hours and increasing over time (Figure IV.10). This induction coincided with the decrease of cyclinE/Cdk2 kinase activity at approximately 10 hours after activation of Raf (Figure IV.11). Furthermore, in parallel to the induction of $p21^{WAF1/Cip1}$ induction and the reduction of cyclin E-dependent kinase activity, the rate of DNA synthesis decreased steadily, indicating that cells were arrested in G1 (Figure IV.11). These findings provided first evidence that the induction of $p21^{WAF1/Cip1}$ could be the underlying cause for the Raf-induced G1 cell cycle arrest in Schwann cells.
Figure IV.10: ARafER induces p21\textsuperscript{WAF1/Cip1} in Schwann cells
Asynchronously growing NS\textDelta RafER cells were induced with 1 μM 4-hydroxy tamoxifen. Protein samples were prepared from quiescent NS\textDelta RafER cells or induced or uninduced NS\textDelta RafER cells at the indicated time points. 50 μg protein were analysed by western blot with the p21\textsuperscript{WAF1/Cip1} specific antibody C36 (a kind gift by Wade Harper) and the ECL-detection system (Amersham International Ltd.).

Figure IV.11: Cyclin E dependent kinase activity and DNA synthesis decreases in response to constitutive Raf-activity
Asynchronously growing NS\textDelta RafER cells were incubated in the presence of 1 μM 4-hydroxy tamoxifen or as a control ethanol. Protein lysates were prepared at indicated times and 300 μg protein were immunoprecipitated with an cyclin E antibody and assayed for histone H1 kinase activity. Incorporated γ\textsuperscript{-32P} was quantified using ImageQuant-software (Molecular Dynamics Ltd.) and are expressed as percentage of cyclin E-dependent kinase activity in untreated growing NS\textDelta RafER cells. DNA synthesis was measured in a thymidine assay as described in Lloyd et al., 1997 and expressed as percentage of DNA synthesis in untreated cells.

I am indebt to Alison Lloyd for this figure.
time after 4-hydroxy tamoxifen addition

<table>
<thead>
<tr>
<th>Q</th>
<th>0</th>
<th>4 h</th>
<th>10 h</th>
<th>14 h</th>
<th>18 h</th>
<th>22 h</th>
<th>30 h</th>
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Relative activity over time:
- Cyclin E-dependent kinase activity
- DNA synthesis

Graph showing relative activity over time after 4-hydroxy tamoxifen addition.
4.8 Mutant p53 abolishes p21WAF1/Cip1 induction

The cell-cycle inhibitor p21WAF1/Cip1 was discovered as a gene induced after activation of wild type p53 (el-Deiry et al., 1993). In NSΔRafER cells, the coexpression of a dominant negative p53 mutant understood to abolish wild-type p53 transcriptional activity towards specific targets was able to overcome the Raf-induced cell cycle arrest (see chapter 4.0, Figure IV.9). If the induction of p21WAF1/Cip1 in response to activated Raf was p53-dependent, then NSΔRafER cells coexpressing mutant p53 should exhibit a clear decrease of p21WAF1/Cip1 protein levels compared to arrested NSΔRafER cells.

To examine p21WAF1/Cip1 protein levels in Schwann cells coexpressing dominant negative p53, NSΔRafER mtp53^{175}, NSΔRafER Puro and NSΔRafER SV40 LT cells were induced with 4-hydroxy tamoxifen. Protein lysates were prepared from induced and uninduced control cells 26 hours after induction, a time point at which the p21WAF1/Cip1 protein level peaks (see Figure IV.10). Activated Raf in Schwann cells coexpressing p53^{R175H} or SV40 LT cells did, however, not induce p21WAF1/Cip1 (Figure IV.12). In NSΔRafER Puro control cells the protein levels p21WAF1/Cip1 were clearly induced 26 hours after 4-hydroxy tamoxifen induction as previously observed (Figure IV.12). Thus, the induction of p21WAF1/Cip1 by activated ∆Raf occurred in a p53-dependent manner. This finding further established a correlation of p21WAF1/Cip1 induction with growth arrest and the decrease of p21WAF1/Cip1 with cell cycle progression since Schwann cells coexpressing mutant p53 continued to progress through the cell cycle regardless of Raf activation (see Figure IV.10).
Figure IV.12: Raf induces p21WAF1/Cip1 by a p53-dependent mechanism
NSARafER Puro, NSARafER p53175 or NSARafER LT were grown for 30 hours in the presence or absence of 1 μM 4-hydroxy tamoxifen. Subsequently protein lysates were prepared. Cells were harvested after this time period and 50 μg of total cell protein were analysed on a SDS-PAGE. Western blotting analysis with an p21WAF1/Cip1 specific antibody was carried out.

Figure IV.13: Cyclin D1 is induced in NSARafER p53175 cells in response to constitutively active Raf
NSARafER Puro, NSARafER p53175 and NSARafER LT cells were grown as specified above in the presence (+) or absence (-) of 1 μM 4-hydroxy tamoxifen. Cells were harvested after this time period and 50 μg of total cell protein were analysed on a SDS-PAGE. Western blotting analysis with an cyclin D1 specific antibody was carried out.
4.9 Cyclin D1 induction by Raf does not contribute to G1 cell cycle arrest in Schwann cells

The superinduction of cyclin D1 in response to Ras expression and transformation has been previously reported (Liu et al., 1995; Winston et al., 1996) and it has been suggested that superinduction of cyclin D1 can result in cell cycle arrest in some cell types. To test whether activated Raf can also superinduce cyclin D1 protein and whether this possible superinduction is correlated with cell cycle arrest in Schwann cells, cyclin D1 protein levels were examined in arrested and proliferating Schwann cells. NSARafER Puro, NSARafER p53<sup>175</sup> and NSARafER LT cells were grown in the presence or absence of 1 µM 4-hydroxy tamoxifen for 30 hours. Protein lysates were prepared and subsequently analysed by western blotting with a cyclin D1 specific antibody. Cyclin D1 protein levels are induced in response to activated Raf (Figure IV.13). However, the induction of cyclin D1 appears not to contribute to the G1 cell cycle arrest, since protein levels of cyclin D1 are similar in arrested NSARafER Puro and proliferating NSARafER p53<sup>175</sup> and NSARafER LT cells (Figure IV.13).

4.10 Raf induction and p53 transactivation activity

The induction of the cell-cycle inhibitor p21<sup>WAF<sub>1/Cip1</sub></sup> is necessary and sufficient to arrest Schwann cells in the G1 phase of the cell-cycle (this thesis; Lloyd et al., 1997). This induction depends on p53 transactivation activity, since a dominant negative p53 mutant abrogates the p21<sup>WAF<sub>1/Cip1</sub></sup> induction (this thesis; Lloyd et al., 1997). However, as the evidence was indirect, I sought a more direct approach to investigate whether p53's transactivation activity was actually increased after Raf activation.
To investigate a possible change in p53 transactivation activity in response to activated Raf, transient transfection experiments introducing p53-responsive reporter genes were performed. These experiments were not successful. Schwann cells can be only transfected with very low efficiency and did not grow any further after transfection. It has been reported that in eukaryotic cells p53 is activated after transient transfections and can cause growth arrest which may also occur in Schwann cells. To overcome these difficulties, I decided to construct a retroviral vector containing a p53-responsive reporter gene and infect NSΔRafER cells.

4.10.1 Construction of retroviral vector containing a p53 binding site reporter gene

To directly measure p53 transactivation activity in NSΔRafER cells after activation of Raf, I constructed a reporter gene with a p53 binding consensus sequence. Since Schwann cells have to be infected for a successful introduction of genes, the luciferase reporter gene was cloned into a modified version of retroviral vector pBPSTR1 (Paulus et al., 1995). The DNA-sequences in pBPSTR1 responsible for the tetracycline regulation of the pBPSTR1 vector were substituted by a minimal thymidine kinase promoter and an adjacent luciferase gene. Oligomers containing a p53 consensus binding sequence (Chen et al., 1993) were cloned in front of the minimal thymidine kinase promoter and luciferase reporter gene (Figure IV.14) to measure luciferase gene expression in a p53-dependent way.
Figure IV.14: Retroviral P53-reporter construct pBPSTR WTp53-tk-luc2F

To test p53 transactivation activity directly, the retroviral p53-reporter construct pBPSTR WTp53-tk-luc2F was constructed as described in Materials and Methods. A p53 DNA binding site from the Ribosomal Gene Cluster was integrated in front of a tk promoter and luciferase reporter gene into the retrovirus pBSTR.
pBSTR p53 tk-luc2F

LTR → gag → Puromycin → SV40 → polyA → luciferase → tk → p53 binding site → LTR

AGG CCA GAC CTG CCC GGG CAA GCC TTG GCA

ATG.
Figure IV.15A: p21WAF1/Cip1 protein level in NSΔRafER wtp53 luc cells after UV-Irradiation and addition of 4-hydroxy tamoxifen

NSΔRafER wtp53 luc cells were assynchronously grown. Cells were irradiated with UV for 20 seconds or alternatively 4-hydroxy tamoxifen was added. Cells were harvested 20 hours after addition of 4-hydroxy tamoxifen or UV-irradiation. 30µg total cell protein were separated on SDS-PAGE. Western blot analysis was carried out with the p21 specific antibody.

Figure IV.15B: Luciferase activity in NSΔRafER wtp53 luc cells

Growing NSΔRafER wtp53 luc cells were either radiated with UV for 20 seconds or 1 µM 4-hydroxy tamoxifen was added. Cells were harvested after 20 hours and luciferase activity was determined as described in Materials.
4.10.2 p53 transactivation activity after activation of Raf

To test whether Raf induces p53 transactivation activity in NSΔRafER cells, Schwann cells infected with the retrovirus containing the luciferase gene under the control of a p53 binding site were tested for luciferase activity. NSΔRafER wtp53 luc cells were subjected to UV-irradiation or alternatively 4-hydroxy tamoxifen was added to activate Raf. Cells were harvested 20 hours later and luciferase activity was determined. In parallel to measuring luciferase activity, I examined the induction of p21WAF1/Cip1 protein.

The addition of 4-hydroxy tamoxifen as well as UV-irradiation which should clearly activated p53 did not result in measurable luciferase activity (Figure IV.15). Nevertheless, the chosen conditions activated the induction of p21WAF1/Cip1 (Figure IV.15) which has been previously shown to be p53-dependent. Therefore, it seems likely that the retroviral p53 reporter gene construct is not functional. Thus, the p53-dependency of p21WAF1/Cip1 induction in response to constitutively active Raf remains to be investigated.
CHAPTER 5.0

Involvement of MAP-kinase kinase and MAP-kinase
in Raf-induced growth arrest
in Schwann cells
5.0 Signalling pathway for \( \Delta \text{Raf-induced growth arrest} \) in Schwann cells

5.1 Introduction

Activated Ras and Raf are sufficient to induce growth arrest in Schwann cells (Ridley et al., 1988; Lloyd et al., 1997). However, the pathway mediating the growth inhibitory signal in Schwann cells downstream of Ras and Raf is unknown.

The same members of the Ras-MAP-kinase signalling pathway can be utilised to mediate a mitogenic or alternatively a growth inhibitory signal. The activation of the Ras-MAP-kinase pathway has been implicated in mitogenesis in most cells (Feramisco et al., 1984; Morris et al., 1989; Meloche et al., 1992; Cowley et al., 1994; Mansour et al., 1994). Nevertheless, the activation of the MAP-kinase pathway has also been reported to be involved in differentiation (Cowley et al., 1994) in other cells. The mediation of different signals via the same signalling pathway could occur through differences in the kinetics of activation. Such a model has been proposed for phaeocytochroma PC12 cells (Traverse et al., 1992), in which members of the Ras-MAP-kinase pathway are involved in mitogenesis as well as differentiation. In PC12 cells the different biological responses after addition of epidermal growth factor (EGF) (mitogenesis) (Huff et al., 1981) or nerve growth factor (NGF) (growth arrest and differentiation) (Greene & Tischler, 1976) have been correlated with a difference in duration of the activation of the Ras-MAP-kinase pathway. The application of NGF led to a persistent increase of GTP-loaded Ras, in contrast to a much shorter increase in response to EGF (Muroya et al., 1992). Furthermore, a transient activation of MAP-kinase was observed after the addition of mitogens such as EGF, whereas a prolonged activation could be seen after addition of NGF, correlated with growth arrest and
differentiation (Heasley & Johnson, 1992; Traverse et al., 1992; Nguyen et al., 1993). In support of this idea of a prolonged versus transient activation of the Ras-MAP-kinase pathway being required for different biological responses, the overexpression of the insulin receptor, usually not involved in MAP-kinase activation and differentiation in PC12 cells (Ohmichi et al., 1993), resulted in MAP-kinase activation and differentiation (Dikic et al., 1994). Conversely, PC12 cells with a reduced number of the NGF-receptor TrkA responded to the addition of NGF with a transient MAP-kinase activation and proliferation instead of differentiation usually observed after NGF addition (Schlessinger & Bar-Sagi, 1995). Moreover, oncogenic Ras and Raf which cause the prolonged activation of the MAP-kinase pathway (Leevers & Marshall, 1992), can also stimulate neurite outgrowth, indicative for differentiation in PC12 cells (Noda et al., 1985; Wood et al., 1993).

Given the large body of evidence for an involvement of activated Ras and Raf in growth arrest and subsequent differentiation in PC12 cells, it was conceivable that a similar mechanism of transient versus prolonged signalling of the MAP-kinase pathway was the underlying mechanism for the Ras/Raf induced cell-cycle arrest in Schwann cells.

5.2 MAP-kinase activation in ΔRaf-induced growth arrest

To investigate whether MAP-kinase was indeed activated in the ΔRaf-induced growth arrest and whether a difference in the duration of MAP-kinase activation after mitogenic stimulation or Raf activation could explain the different biological responses, MAP-kinase p42ERK2 activation after induction of ΔRaf or addition of mitogens was examined. NSΔRafER cells were kept in the absence of growth promoting factors for two days and then induced with foetal calf serum (FCS) plus glial growth factor and forskolin
containing medium. Addition of mitogens to quiescent NS\Delta RafER cells resulted in a transient activation of MAP-kinase p42\textsuperscript{ERK2}. The activation of MAP-kinase which strictly depends on two phosphorylations within the kinase domain (Payne et al., 1991) was indicated by the appearance of a characteristic phosphorylation-dependent mobility shift (de Vries-Smits et al., 1992; Leevers et al., 1992). MAP-kinase activation was detected as early as ten minutes after addition of mitogens and remained elevated for up to four hours after stimulation (Figure V.1A). Individual experiments varied in that activation was observed between up to two to four hours after foetal calf serum addition. This showed that after stimulation with mitogens MAP-kinase p42\textsuperscript{ERK2} was transiently activated. In contrast, the induction of \Delta RafER in the absence of added mitogens resulted in the prolonged activation of MAP-kinase p42\textsuperscript{ERK2}. In quiescent cells the induction of \Delta RafER led to a continuous activation of MAP-kinase from fifteen minutes until the end of the observation period 48 hours after addition of 4-hydroxy tamoxifen (Figure V.1B).

The activation of p42\textsuperscript{ERK2} by activated \Delta Raf was dominant over activation by mitogens. When both stimuli, mitogens and 4-hydroxy tamoxifen were added simultaneously, p42\textsuperscript{ERK2} remained activated over 48 hours (Figure V.1C), indicating that the reported induction of MAP-kinase specific phosphatases by foetal calf serum (Charles et al., 1993; Noguchi et al., 1993) was not sufficient to down-regulate the constitutive signal from \Delta Raf. As a control, NSempty cells harbouring the retroviral vector LXSN showed only a very transient activation of MAP-kinase p42\textsuperscript{ERK2} upon addition of 4-hydroxy tamoxifen which disappeared already after 30 minutes (Figure V.1D) and these cells do not arrest (Lloyd et al., 1997). This result confirmed that activation of MAP-kinase after addition of 4-hydroxy
Figure V.1A: Stimulation of p42\textsuperscript{ERK2} kinase activation by mitogens in NS\textDelta RafER cells

Nearly confluent NS\textDelta RafER cells were kept in DMEM medium plus 2% fetal calf serum for two days. Cells were stimulated with DMEM containing 3% fetal calf serum as well as GGF and forskolin. Cells were lysed at times indicated and 30 \mu g cell lysate were separated on a 15%SDS-PAGE. After transfer onto PVDF-membrane, the western blot was incubated with a 1:15000 dilution of Ab122, 1:2000 secondary antibody against rabbit IgGs coupled to horse raddish peroxidase. Immunoreactive bands were visualised using the ECL-system (Amersham International).

Figure V.1B: p42\textsuperscript{ERK2} kinase activation in induced NS\textDelta RafER cells

Nearly confluent NS\textDelta RafER cells were kept in DMEM medium plus 2% fetal calf serum for two days and then 1 \mu M 4-hydroxy tamoxifen was added. Western blotting was carried out as described in figure V.1A.

Figure V.1C: p42\textsuperscript{ERK2} kinase activation after addition of mitogens and 4-hydroxy tamoxifen

Nearly confluent NS\textDelta RafER cells were kept in DMEM medium plus 2% fetal calf serum for two days. DMEM medium with mitogens as described in figure V.1A was added as well as 1 \mu M 4-hydroxy tamoxifen. Cells were harvested at indicated times after stimulation and western blotting was carried out as described in figure V.1A.

Figure V.1D: p42\textsuperscript{ERK2} kinase activation in induced NSempty cells

Nearly confluent NSempty cells were kept in DMEM medium plus 2% fetal calf serum for two days. Then 1 \mu M 4-hydroxy tamoxifen was added. Cells were harvested and analysed as described in figure V.1A.
tamoxifen was specifically due to \(\Delta\text{RafER} \) induction and not due to any unspecific activation of the MAP-kinase pathway.

The described kinetics of MAP-kinase activation after addition of mitogens or \(\Delta\text{Raf} \) induction were congruent with a model in which the duration of the activation of the MAP-kinase pathway would determine the biological response to this signal in Schwann cells. As suggested for PC12 cells (Traverse et al., 1992), such a model for Schwann cells would mean that the prolonged activation of the MAP-kinase pathway by activated Raf would lead to growth arrest, regardless of surrounding growth conditions, whereas a transient activation would correlate with mitogenesis. In accordance with this hypothesis, cells with activated Raf plus added mitogens exhibited a prolonged activation of MAP-kinase and a G1 cell cycle arrest (Figure V.1C). Although it was plausible to explain the observed differences in MAP-kinase activation in Schwann cells through this described model, the presented data did not exclude other explanations.

5.2.1 MAP-kinase activation in NS\(\Delta\text{RafER} \) SV40 LT cells

Co-expression of SV40 large T antigen (SV40 LT) enabled Ras/Raf-arrested Schwann cells to overcome the cell cycle arrest (Ridley et al., 1988; Lloyd et al., 1997). Although it seemed likely that SV40LT rescued the cell-cycle arrest through its function to sequester p53 which then in turn would abolish p53-dependent p21\(^{\text{WAF1/Cip1}} \) induction, I also sought to exclude the possibility that SV40LT had a direct or indirect effect on the prolonged activation of the MAP-kinase pathway upstream. An influence on the MAP-kinase pathway has been reported for SV40 small T antigen which inhibits phosphatases 2A required for the inactivation of the Ras-MAP-kinase pathway (Sontag et al., 1993).
Figure V.2A: p42ERK2 kinase activation in NSΔRafER LT cells
Nearly confluent NSΔRafER LT cells were kept in DMEM plus 2%FCS for two days. 1 μM 4-hydroxy tamoxifen was added at point 0 and cells were harvested at indicated times after stimulation. 30 μg cell lysate were separated on a 15%SDS-PAGE. The western blot was incubated with a 1:15000 dilution of Ab122, 1:2000 secondary antibody against rabbit IgGs coupled to horse raddish peroxidase and detected using the ECL-system.

Figure V.2B: Expression of SV40 LT antigen
50 μg NSΔRafER LT protein cell lysate used in the experiment above was analysed for SV40 large T expression with the monoclonal antibody 423 supernatant (0.5 μg /ml) and 1:2000 secondary antibody against mouse IgGs coupled to horse raddish peroxidase. Immunoreactive bands were visualised using the ECL-system (Amersham International).

Figure V.2C: p42ERK2 kinase activation in mitogenically stimulated NSΔRafER LT cells
Nearly confluent NSΔRafER LT cells were changed into 2% fetal calf serum in DMEM two days prior stimulation with 3% fetal calf serum in DMEM with GGF and forskolin. Cells were harvested at indicated times after stimulation. 30 μg cell lysate were separated on a 15%SDS-PAGE. The western blot was incubated with a 1:15000 dilution of Ab122, 1:2000 secondary antibody against rabbit IgGs coupled to horse raddish peroxidase. Immunoreactive bands were visualised using the ECL-system (Amersham International).

Figure V.2D: Expression of SV40 LT antigen
The expression of SV40 LT antigen in NSΔRafER LT cells used in the experiment figure V.3C were analysed as described for figure V.3B.
To investigate a possible interaction between SV40LT and the activation of Raf, MAP-kinase kinase or MAP-kinase, the kinetics of MAP-kinase activation after addition of mitogens or 4-hydroxy tamoxifen were analysed in NS\(\Delta\)RafER cells co-expressing SV40LT. The activation of \(\Delta\)RafER in cells co-expressing large T-antigen (NS\(\Delta\)RafERLT) led also to the prolonged activation of MAP-kinase p42\(^{ERK2}\) with identical kinetics to those seen in NS\(\Delta\)RafER cells, independent of whether cells were quiesced or stimulated with mitogens (Figure V.2A & 2C). Therefore, the expression of SV40 LT has no effect on the activation of MAP-kinase p42\(^{ERK2}\).

5.3 Dominant negative MAP-kinase kinase in the Raf-induced cell cycle arrest

5.3.1 Introduction

The previously described studies of MAP-kinase activation suggested that MAP-kinase may be a mediator of the \(\Delta\)Raf-mediated growth arrest. Therefore, I examined whether MAP-kinase activity was indeed causally involved in the \(\Delta\)Raf-induced growth arrest. If MAP-kinase activity was instrumental in establishing the cell-cycle arrest, then blocking of MAP-kinase activity should abolish or at least weaken the G1 cell cycle arrest mediated by activated Raf.

Inhibition of MAP-kinase activity could be achieved through either expression of a kinase-deficient MAP-kinase ERK2 protein which has a dominant negative function (Sally Leervers, personal communication) or through the expression of dominant negative mutants of the direct upstream activator MAP-kinase kinase (Cowley et al., 1994). In particular, the expression of dominant negative mutants of MAP-kinase kinase appeared to be promising for my investigations, since experiments in PC12 cells and
NIH3T3 cells did confirmed their potential of inhibiting biological responses mediated via the Ras-MAP-kinase pathway (Cowley et al., 1994).

**5.3.2 Dominant negative MAP-kinase kinase mutants in NSΔRafER cells**

To address whether dominant negative mutants of MAP-kinase kinase were capable of inhibiting activation of MAP-kinase after ΔRaf activation, I subcloned wild-type MAP-kinase kinase (M KK1), MAP-kinase kinase mutant M KK1 L IDA (Ala-217) and MANA (Ala-221) into the retroviral vector pBabe Puro (Figure V.3A). Dominant negative mutants of M KK1, L IDA (Ala-217) and MANA (Ala-221) have point mutations in either one of the two phosphorylation site required for MAP-kinase kinase activity (Alessi et al., 1994).

NSΔRafER cells were infected with the described constructs and drug-selected, leading to pools of NSΔRafER M KK, NSΔRafER L IDA (Ala-217) and NSΔRafER MANA (Ala-221) cells. Initially, these cells were analysed for the expression of the exogenous MAP-kinase kinase proteins (Figure V.3B) and for their potential to block MAP-kinase activity after ΔRafER induction. In order to examine whether dominant negative mutants were sufficient to abrogate MAP-kinase activity after ΔRafER induction, cells were quiesced for two days and ΔRaf was induced. The co-expression of dominant negative mutants of MAP-kinase kinase was not sufficient to reduce the kinase activity of MAP-kinase compared to control cells (Figure V.3C).

In parallel, I examined whether the expression of dominant negative mutants of MAP-kinase kinase was sufficient to abolish the Raf-induced cell cycle arrest using FACS-analysis. As illustrated in Figure V.4A-D, the co-expression of dominant negative MAP-kinase kinase in NSΔRafER cells
Figure V.3A: Schematic representation of MAP-kinase kinase mutants

Figure V.3B: Expression of MAP-kinase kinase mutants in NSARafER cells
NSARafER Puro, NSARafER MKK, NSARafER MANA and NSARafER LIDA cells were examined for protein expression of the introduced MAP-kinase kinase mutants. 100 µg cell lysate were separated on a 12.5% SDS-PAGE gel and western blotted. Immunoreactive proteins with the antibody 9E10 in cells either quiesced (-), after addition of mitogens (+M) or after the addition of 4-hydroxy tamoxifen (+ TM) are indicated by an arrow.

Figure V.3C: MAP-kinase activity in NSARafER cells co-expressing MKK-mutants
NSARafER cells co-expressing MAP-kinase kinase mutants were quiesced prior the addition of 4-hydroxy tamoxifen. Cells were harvested 12 hours after addition of 4-hydroxy tamoxifen and 150 µg of cell lysates were used for an immunoprecipitation of the p42ERK2 specific antibody 122, followed by an *in-vitro* kinase assay with myelin basic protein as a substrate (MBP). 10 µl of the kinase assay were separated on a 15% SDS-PAGE gel.
Figure V.4A-D: FACS-analysis of NSΔRafER cells expressing MAP-kinase kinase mutants

NSΔRafER cells co-expressing mutants of MAP-kinase ERK2, ERK2 (A52) and ERK2 (AF) were assynchronously grown. 1 μM 4-hydroxy tamoxifen or ethanol as control were added. BrdU (10 μM) was added 26 hours after 4-hydroxy tamoxifen addition for 4 hours. The cell cycle distribution of these cells were measured by BrdU incorporation and DNA-content flow cytometry analysis. Cells were stained with FITC-anti-BrdU to detect BrdU incorporation and propidium iodide to detect total DNA. The upper box identifies cells incorporating BrdU (S-phase), the lower left box identifies G0/G1 cells and the lower right box displays G2/M cells.
was not sufficient to abolish the Raf-induced G1 cell cycle arrest. Most cells expressing mutants showed a G1 cell cycle arrest upon addition of 4-hydroxy tamoxifen very similar to control cells expressing either wild-type MAP-kinase kinase or the vector alone. However, NShaRafER cells expressing MAP-kinase kinase LIDA (Ala-217) showed only a slight reduction of cells in S-phase, although the number of S-phase cells in the absence of 4-hydroxy tamoxifen is only half of all other cells examined. This result may be explained by the effect of dominant negative MAP-kinase kinase in both mitogenesis and in blocking the growth arrest. In both situations, dominant negative MAP-kinase kinase negatively influence endogenous MAP-kinase kinase activity which could lead to a reduction of cells in S-phase in mitogenesis, whereas the inhibition of ΔRafER-induced G1 arrest will result in a higher proportion of cells remaining in S-phase. This result pointed to the possible involvement of MAP-kinase kinase in mediating the cell cycle arrest in response to activated Raf.

5.3.3 Dominant negative MAP-kinase mutants in NShaRafER cells

In addition to experiments with dominant negative mutants of MAP-kinase kinase, similar mutants of MAP-kinase were used to investigate the contribution of MAP-kinase in the Raf-induced cell cycle arrest. To address whether dominant negative mutants of MAP-kinase ERK2 were capable of inhibiting the ΔRaf-induced cell cycle arrest, I subcloned wild-type ERK2 and two mutants ERK2 (A52) and ERK2 (AF) into pBabe Puro. Mutant ERK2 (A52) exhibits no kinase activity due to a point mutation in the ATP-binding site of ERK2, whereas in mutant ERK2 (AF) both phosphorylation sites in the catalytic domain required for MAP-kinase activity are exchanged (Sally Leevers, personal communication).
Figure V.5A-C: FACS-analysis of cells expressing MAP-kinase mutants

NSΔRafER cells co-expressing mutants of MAP-kinase ERK2, ERK2 (A52) and ERK2 (AF) were assynchronously grown. 1 μM 4-hydroxy tamoxifen or ethanol as control were added. BrdU (10 μM) was added 26 hours after 4-hydroxy tamoxifen addition for 4 hours. The cell cycle distribution of NSΔRafER cells was measured by BrdU incorporation and DNA-content flow cytometry analysis. Cells were stained with FITC-anti-BrdU to detect BrdU incorporation and propidium iodide to detect total DNA. The upper box identifies cells incorporating BrdU (S-phase), the lower left box identifies G0/G1 cells and the lower right box displays G2/M cells.
NSΔRafER cells were infected with the constructs described and drug-selected, leading to pools of NSΔRafER Puro, NSΔRafER ERK2, NSΔRafER ERK2 (A52), NSΔRafER ERK2 (AF) cells. I examined whether the expression of either of the dominant negative MAP-kinase mutants was sufficient to abolish the ΔRaf induced cell-cycle arrest using FACS-analysis. The coexpression of dominant negative MAP-kinase was not sufficient to abolish the Raf-induced G1 cell cycle arrest as illustrated in Figure V.5A-D. The mutant MAP-kinase expressing cells showed a G1 cell cycle arrest upon addition of 4-hydroxy tamoxifen very similar to control cells expressing wild-type MAP-kinase or the vector alone. However, the co-expression of ERK2 (AF) leaves more cells in S-phase after addition of 4-hydroxy tamoxifen (Figure V.5C) than in induced control cells (Figure V.5A) or ERK2 (A52) mutant cells (Figure V.5B), suggesting that MAP-kinase inhibition of this pathway by ERK2 (AF) is only partial and may be required for the growth inhibitory signalling pathway downstream of activated Raf.

5.3.4 Conclusions

The co-expression of the dominant negative MAP-kinase kinase mutant LIDA resulted in a reduction of cells in G1 phase with a higher number of cells remaining in S-phase in response to constitutive Raf-activity (see Figure V.4). This finding indicates the possibility that MAP-kinase kinase activity could be required for the mediation of the activated Raf-dependent cell cycle arrest. Interestingly, the same mutant reduces the number of cells in S-phase in the absence of constitutive Raf-activity. Constitutively active MAP-kinase kinase activity has been shown to be necessary and sufficient to induce S-phase in some cells (Cowley et al.,
In line with these findings, a dominant negative mutant could negatively effect S-phase entry in Schwann cells.

The co-expression of dominant negative MAP-kinase molecules in NSΔRafER cells was not sufficient to inhibit MAP-kinase activity and subsequently the ΔRaf-induced cell cycle arrest. However, it can not be excluded that the protein expression achievable from a integrated retroviral constructs was too low to inhibit the MAP-kinase pathway. Since dominant negative mutants are understood to exhibit their inhibitory effects through competition with the wild-type molecule for the upstream activator. The possible low expression of these dominant negative mutants could have allowed the activation of the wild type molecule.

In this respect, it is also worth mentioning that the abrogation of biological effects by dominant negative MAP-kinase kinase mutants was achieved through either microinjection of protein into PC12 cells or injection of plasmid DNA into NIH3T3 cells (Cowley et al., 1994). Both methods will provide most certainly a higher protein concentration than achievable by the expression of a gene from an integrated retrovirus. Microinjections of either plasmid DNA or protein into Schwann cells have proven unsuccessful. Thus, it is not absolutely clear whether the lack of inhibition is due a possible low protein expression or reflects the fact that MAP-kinase is not involved in mediating the Raf-induced cell cycle arrest.

5.4 MAP-kinase kinase inhibitor PD98059 blocks 
\( p21^{WAF1/Cip1} \) induction in Schwann cells

5.4.1 Introduction

The results utilising dominant negative mutants of MAP-kinase kinase or MAP-kinase were inconclusive due to the lack of strong inhibition by
these molecules in Schwann cells. Therefore, I tried to find alternative ways to block the MAP-kinase signalling pathway downstream of Raf in order to address the significance of this pathway in mediating the cell cycle arrest.

The recent discovery of a synthetic inhibitor for MAP-kinase kinases (MAPKKs) (Dudley et al., 1995) offered an independent approach to the usage of dominant negative MAP-kinase kinase or MAP-kinase molecules in assessing the contribution of MAP-kinase kinase and MAP-kinase in mediating the growth inhibitory signal in Schwann cells. The synthetic inhibitor for MAP-kinase kinase was isolated by testing a compound library for inhibitory substances in a coupled MAP-kinase cascade assay. The compound, PD98059 \[2-(2'\text{-amino-3'}\text{-methoxyphenyl)}\text{-oxanaphthalen-4-one}] (Figure V.6A) was identified as a specific inhibitor of MAP-kinase kinase 1 and 2 (Dudley et al., 1995). The compound PD98059 inhibits the activation of the unphosphorylated form of MAP-kinase kinase 1 & 2 (MAPKK1/MAPKK2) but was shown to be ineffective in inhibiting already phosphorylated and activated MAP-kinase kinase molecules (Dudley et al., 1995). PD98059 inhibits MAPKK2 at higher concentrations than MAPKK1. Additionally, PD98059 showed an inhibitory effect on a constitutively active mutant of MAPKK1(S217E, S221E) (Alessi et al., 1995; Cowely et al., 1994).

PD98059 has a very restricted specificity towards MAPKK1 and MAPKK2. Extensive studies were unable to reveal any inhibitory effects on a large number of highly related kinases over a wider range of concentrations \textit{in vitro} and \textit{in vivo}. Among other kinases, the activity of 18 protein Ser/Thr kinases, p70^{S6}-kinase, Jun N-terminal kinase (JNK), p38 kinase (RK), MAP-kinase kinase 4 (M KK4), MAP-kinase, MAPKAP kinase-1, Protein kinase A, Cyclin A/cdk2 (Alessi et al., 1995) and cAMP-dependent kinase, protein kinase C, v-src kinase, EGF-kinase, phosphatidylinositol-3-
kinase were unaffected by the inhibitor (Dudley et al., 1995). Most importantly for my investigations, PD98059 did not prevent the activation of c-Raf (Dudley et al., 1995) by such different stimuli as platelet-derived growth factor (PDGF), serum, insulin and phorbol esters in SW3T3 cells (Alessi et al., 1995).

The inhibition of MAP-kinase kinase 1 in *in vitro* assays has been confirmed by the inhibition of biological responses initiated by the Ras-MAP-kinase signalling pathway *in vivo*. For instance, PD98059 indirectly inhibits the activation of MAP-kinase, a direct downstream target of MAPKKs by growth factors *in vivo*. Consistent with the function of MAP-kinase in mediating mitogenic signals and differentiation, PC98059 is capable of blocking both processes. For example, PD98059 is capable of reversible inhibition of Ras-induced transformation in BALB 3T3 mouse fibroblasts and NRK rat kidney cells (Dudley et al., 1995). PD98059 can also inhibit nerve growth factor (NGF)-induced MAP-kinase activity and differentiation in phaeocytochroma cells (PC12) (Pang et al., 1995). These reports convincingly show that the inhibitory effect of PD98059 towards MAPKK1 & 2 *in vitro* can be reproduced *in vivo*.

### 5.4.2 MAP-kinase kinase inhibitor PD98059 in NSΔRafER cells

The inhibitor PD98059 with its well-characterised features in different cells (Dudley et al., 1995; Pang et al., 1995; Alessi et al., 1995, Lazar et al., 1995) offered an experimental tool for investigations into the involvement of MAP-kinase kinase in the Raf-induced growth arrest. The fact that PD98059 is not inhibiting c-Raf itself (Dudley et al., 1995), made PD98059 an interesting tool for my investigations into the MAP-kinase signalling pathway downstream of Raf. If MAP-kinase kinase activity was involved in the
ΔRafER induced growth arrest due to the induction of p21^{WAF1/Cip1}, then the application of PD98059 should abolish or at least delay the induction of p21^{WAF1/Cip1} protein and the onset of the G1 cell cycle arrest. As a consequence, Schwann cells may enter into S-phase in the presence of PD98059.

5.4.3 Inhibitory effects of PD98059 in NSΔRafER cells

Initially I determined the inhibitory concentration of PD98059 for MAP-kinase kinase 1 & 2 activity after 4-hydroxy tamoxifen addition in a coupled assay using MAP-kinase and myeelin basic protein as a substrate in Schwann cells. The inhibitory effects of PD98059 on MAPKK 1 & 2 were measured indirectly by monitoring the activity of MAP-kinase.

The inhibitor PD98059 can only act on unphosphorylated inactive MAPKK 1 & 2. Therefore, Schwann cells were kept in DMEM plus 2%FCS for two days to exclude any stimulation by growth factors and allow maximal inhibition. Prior to ΔRafER activation, various concentrations of PD98059 or as a negative control the solvent DMSO were added. Cells were harvested at indicated times and MAPKK1 & 2 activity was measured. Maximal inhibition of MAP-kinase kinase 1 & 2 activities were achieved at approximately 1.0 μM PD98059 (Figure V.6 B & C). However, the kinase activity of the direct downstream target MAP-kinase was completely inhibited at a higher concentration of PD98059 as indicated by the absence of the characteristic phosphorylation shift on the western blot (Figure V.7) paralleled by the lack of kinase activity (Figure V.7 B & C). This higher inhibitory concentration of 50 μM was used for all following experiments to ensure the blocking of MAP-kinase kinase and MAP-kinase activity. The inhibitor PD98059 showed no visible toxicity on NSΔRafER cells at any concentration tested.
Figure V.6A: Chemical structure of inhibitor PD98059
[2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one]

Figure V.6B: PD98059 inhibits MAP-kinase kinase 1 and 2 in a dose-dependent manner
NSΔRafER cells were quiesced for two days in DMEM supplemented with 2% FCS. 30 minutes prior addition of 4-hydroxytamoxifen, the indicated concentration of PD98059 was added. Cells were harvested three hours after ΔRafER induction. 100 μg total cell lysate were used in a immunoprecipitation with antibodies against M KK1 and M KK2. Immunoprecipitates were used in a coupled assay with MAP kinase measuring kinase activity as radioactive phosphate incorporation into myelin basic protein as described (see Methods).

Figure V.6C: Quantification of MAP kinase kinase 1 & 2 inhibition
Incorporated $^{32}$P phosphate into myelin basic protein was quantified using ImageQuant-software (Molecular Diagnostics Ltd.). The inhibition of MAP-kinase kinase 1 & 2 is shown as percentage of inhibition plotted against concentration.
Inhibition of MKK1/2 kinase activity
Figure V.7A: PD98059 blocks phosphorylation of p42^{ERK2} in a dose-dependent manner

Nearly confluent NS\Delta RaFER cells were washed twice with pre-warmed PBSA and changed into 2% stripped fetal calf serum in DMEM without any growth promoting factors two days before the experiment. Indicated concentrations of PD98059 in DMSO were added 30 min prior induction of \Delta RaFER with 1 \muM 4-hydroxy-tamoxifen. As a control the highest volume of DMSO used was added at point 0. Cells were harvested three hours after induction and 30 \mu g total protein were separated on a SDS-PAGE. The western blot was incubated with a 1:15000 dilution of Ab122, 1:2000 secondary antibody against rabbit IgGs coupled to horse raddish peroxidase. Immunoreactive bands were visualised using the ECL-system (Amersham Life Science).

Figure V.7B: PD98059 inhibits p42^{ERK2} kinase activity after \Delta RaFER induction

p42^{ERK2} was immunoprecipitated with 5 \mu l Ab 122 from 100 \mu g total protein of the same cell lysates used for the western blot above. Immunoprecipitation and the following in-vitro kinase assay were carried out as described (see Methods). 10 \mu l of the in-vitro kinase assay were separated on a 15% minigel and subject to autoradiography.

Figure V.7C: Quantification of p42^{ERK2} kinase activity

\gamma^{32P}-incorporation into myelin basic protein (MBP) from figure V.8B was quantified using Molecular Dynamics phosphoimager and ImageQuant software. Arbitrary units are expressed as percentage inhibition of positive control and plotted against concentration of PD98059.
Inhibition of p42 ERK2 kinase activity
5.4.4 PD98059 delays p21WAF1/Cip1 induction in NS△RafER cells

The induction of △RafER caused a strong induction of the cell-cycle inhibitor p21WAF1/Cip1 (Figure IV.10). Therefore, I was interested in investigating whether MAP-kinase kinase 1 & 2 are required for mediating the induction of p21WAF1/Cip1. If MAP-kinase kinase 1 & 2 are mediating the signal towards p21WAF1/Cip1 induction, the inhibition of MAPKK 1 & 2 by PD98059 should abolish or at least reduce the induction of p21WAF1/Cip1.

To address these questions experimentally, NS△RafER cells were quiesced for two days. Prior to the addition of mitogens and induction of △RafER, cells were treated with or without PD98059. A time-course over 48 hours was performed with cells harvested at indicated times. MAP-kinase p42ERK2 activation as characterised by the appearance of a phosphorylation shift was determined (Figure V.8). The same cell lysates were used to determine p21WAF1/Cip1 induction in the presence and absence of PD98059 (Figure V.9).

Interestingly, p21WAF1/Cip1 induction was strongly reduced at 24 hours in the presence of PD98059 arguing that MAP-kinase kinase was involved in mediating the growth inhibitory signal (Figure V.9). Correspondingly, at the same time the activation of MAP-kinase p42ERK2 was inhibited by PD98059, although the inhibition appeared less effective over a longer time period than 4 hours after Raf induction (Figure V.9). However, at 48 hours after induction no inhibitory effect on MAP-kinase activation nor p21WAF1/Cip1 protein levels by PD98059 could be observed. This decrease of inhibition could be explained for instance through a gradual degradation of the inhibitor or an increased strength of the initiated signal from △RafER which has been previously reported (Samuels et al., 1994).
Figure V.8: Long-term effect of PD98059 on p42ERK2 activation in NSΔRafER cells

NSΔRafER cells were quiesced for two days. 30 minutes prior induction with 1 μM 4-hydroxy tamoxifen, 50 μM PD98059 or equal volumes of DMSO as control were added. Cells were harvested at indicated times after 4-hydroxy tamoxifen addition. 30 μg total cell protein were separated on SDS-PAGE, blotted and incubated with antibody 122. Immunoreactive bands were visualised with the ECL-system (Amersham International).

Figure V.9: PD98059 delays induction of p21WAF1/CIP1 in NSΔRafER cells

30 μg total protein from each sample of the experiment described above were separated on a 15% SDS-PAGE, blotted and incubated with antibody CP36 against p21WAF1/CIP1. Immunoreactive bands were visualised with the ECL-system (Amersham International). As a positive control NSΔRafER Puro cell lysate harvested 26 hours after 4-hydroxy tamoxifen induction was used.
Time after 4-hydroxy tamoxifen addition
5.4.5 PD98059 delays signs of morphological transformation

In NSΔRafER cells the induction of ΔRaf resulted in morphological transformation characterised by highly retractile cells which became increasingly rounded up. The first morphological changes were detectable at approximately 12 hours but became more obvious at 24 hours after induction. Since PD98059 inhibited ΔRaf induced activation of MAP-kinase as a downstream target, I was interested to investigate whether the changes to morphological transformation could also be inhibited.

NSΔRafER cells pre-treated with PD98059 or the solvent DMSO were induced with 4-hydroxy tamoxifen. Photos were taken at indicated time points. The typical morphological changes in induced NSΔRafER cells were inhibited by PD98059 at 24 hours, whereas after 48 hours the morphological differences between PD98059 pre-treated and untreated cells were less obvious (Figure V.10). In conclusion, PD98059 delayed the morphological changes, although it was not able to block these changes fully.

5.5 Constitutively active MAP-kinase kinase in Schwann cells

5.5.1 Introduction

The MAP-kinase kinase inhibitor PD98059 is able to delay the activation of MAP-kinase for up to 24 hours and subsequential the induction of p21^{WAF1/Cip1}, as shown previously (chapter 5.4.4). This finding argued for an involvement of MAP-kinase kinase in the induction of p21^{WAF1/Cip1}. However, these experiments did not address whether activated MAP-kinase kinase is also sufficient in transmitting the signal for growth arrest.
Figure V.10: PD98059 delays morphological changes associated with ΔRafER induction

NSΔRafER cells were quiesced for 2 days. Either DMSO or 50 μM PD98059 were added 30 minutes prior addition of 4-hydroxy tamoxifen to NSΔRafER cells. Photos were taken 24 hours (upper photos) and 48 hours after 4-hydroxy tamoxifen addition (lower photos) (magnification 400x).
The generation of constitutively active mutants of MAP-kinase kinase enabled the analysis of the Ras-signalling pathway and its contribution in differentiation and mitogenesis in mammalian cells (Cowley et al., 1994; Mansour et al., 1994; Brunet et al., 1994). MAP-kinase kinase (MKK) also referred to as MAPK/ERK-kinase (MEK) is phosphorylated by c-Raf-1 (Alessi et al., 1994; Zheng & Guan, 1994), B-Raf (Papin et al., 1995) or v-Mos (Pham et al., 1995) at serine residues 218 and 222. These two phosphorylation events are necessary and sufficient to activate kinase activity towards its substrate, MAP-kinase. Constitutively active MAP-kinase kinase 1 (Glu 217/Glu 221) was generated by substitution of both phosphoacceptor residues to glutamic acid in the catalytic domain. These substitutions resulted in constitutive active MAP-kinase kinase (Cowley et al., 1994). Similarly, another constitutively active MAP kinase kinase was generated by substitution of serine 218 to glutamic acid and serine 222 to aspartic acid and a deletion of a predicted α-helical domain from amino acid 32 to 51 resulting in a even stronger constitutive MAP-kinase kinase, ΔMEK1(S218E,S222D) (Mansour et al., 1994).

The biological effects of constitutively active MAP-kinase kinase 1 (Glu-217/Glu-221) in mitogenesis and differentiation were examined in NIH3T3 cells and PC12 phaeochromocytoma cells, respectively. Constitutive active MAP-kinase kinase (S218D or S218D/S222D) decreased the growth factor requirement for DNA-synthesis in a fibroblast cell line (Brunet et al., 1994) and caused an increase of cells entering S-phase upon injection of constitutively active MAP-kinase kinase protein (Cowley et al., 1994). Furthermore, NIH3T3 cells transfected with MAP-kinase kinase 1 (Glu-217/Glu-221) resulted in about 15-30% of morphological transformed and drug resistant-colonies (Cowley et al., 1994). Individual clones were capable of anchorage-independent growth in...
soft agar (Cowley et al., 1994; Mansour et al., 1994) and proliferated in serum-free conditions similarly to NIH3T3 cells transformed with oncogenic ras (Cowley et al., 1994; Marshall et al., 1985). Rapid tumour growth has been observed after the injection of NIH3T3 cells expressing MAP-kinase kinase 1 (Glu-217/Glu-221) into nude mice (Cowley et al., 1994; Mansour et al., 1994; Brunet et al., 1994) confirming in vitro data. As a model system for differentiation, PC12 phaeochromocytoma cells were employed since they differentiate in response to nerve growth factor (NGF) (Greene & Tischler, 1976), the expression of activated Ras (Noda et al., 1985) or activated Raf (Wood et al., 1993). Microinjections of MAP-kinase kinase 1 Glu-217/Glu-221 protein led to differentiation of more than 75% of injected PC12 cells, as indicated by the outgrowth of neurites (Cowley et al. 1994).

In summary, these extensive investigations established that a constitutive active MAP-kinase kinase was capable of mimicking the biological responses previously observed with activated Ras and Raf. These studies showed further that MAP-kinase kinase was located downstream of Ras in the Ras-MAP-kinase signalling pathway, since blocking of Ras had no effect on the cellular responses initiated by MAP-kinase kinase (Cowley et al., 1994).

5.5.2 Constitutive MAP-kinase kinase does not inhibit cell growth in Schwann cells

The generation of constitutive active mutants of MAP-kinase kinase offered the possibility to further investigate the signalling pathway from Raf to the cell-cycle arrest via the p53-dependent p21WAF1/Cip1 induction in Schwann cells. Provided that MAP-kinase kinase was part of the signalling pathway which led to the induction of p21WAF1/Cip1, constitutive active MAP-kinase kinase should be capable of inducing p21WAF1/Cip1 and arresting
Schwann cells. If a prolonged MAP-kinase signal is not only necessary but also sufficient to cause growth arrest in Schwann cells, a constitutive active MAP-kinase kinase should also be able to arrest Schwann cells.

The constitutive active $\Delta$MEK1(S218E, S220D) mutant (Mansour et al., 1994), was fused to the hormone-binding domain of the mutated oestrogen receptor responsive to 4-hydroxy tamoxifen (a kind gift by Martin MacMahon). Constitutive MAP-kinase kinase 1 ($\Delta$MEK1 (S218E, S222D):ERT$^{TM}$) activity thereby became dependent on the presence of 4-hydroxy tamoxifen (Martin MacMahon, personal communication). This inducible constitutive active $\Delta$MEK (S218E, S222D):ERT$^{TM}$ pBabe Puro construct was introduced through retroviral infection into Schwann cells. After selection puromycin-resistant Schwann cells were pooled.

To test whether the inducible $\Delta$MEK(S218E, S222D):ERT$^{TM}$ was indeed activatable in Schwann cells, I analysed the kinetics of activation of the downstream target MAP-kinase after induction of $\Delta$MEK(S218E, S222D) by 4-hydroxy tamoxifen in starved Schwann cells. NS$\Delta$MEK(S218E, S222D) cells were grown to confluency and starved for any exogenous stimuli for two days. Cells were stimulated with or without 4-hydroxy tamoxifen and tested for MAP-kinase activity at indicated times (Figure VI.11A). MAP-kinase activity was observed from 1 hours to 4 hours after addition of 4-hydroxy tamoxifen. However, in the absence of 4-hydroxy tamoxifen and mitogens MAP kinase activity as indicated by the phosphorylation shift was detectable in NS$\Delta$MEK(S218E, S222D) cells but not in NS$\Delta$RafER cells used as negative control cells. This result showed that the constitutively active MAP-kinase kinase is active even in the absence of 4-hydroxy tamoxifen and therefore is not fully regulatable by 4-hydroxy tamoxifen.
To test whether constitutive active MAP-kinase kinase was able to arrest Schwann cells similarly to activated ΔRafER, NSΔMEK (S218E, S222D):ERTM cells were treated with mitogen plus and minus 4-hydroxy tamoxifen and S-phase entry was measured by $^3$H-thymidine incorporation. As control NSΔRafER cells were also investigated for S-phase entry under identical conditions (Figure VI.11B,C). Induced constitutively active MAP-kinase kinase was not able to arrest Schwann cells as illustrated in Figure VI.11.BC & D. Schwann cells expressing inducible constitutive active MAP-kinase kinase entered S-phase as indicated by the incorporation of $^3$H-thymidine. The increase of $^3$H-thymidine incorporation with time suggested that under conditions which clearly arrested NSΔRafER cells (Figure VI.11B), NSΔMEK1 (S218E, S222D):ERTM cells did not arrest but instead led to a slight stimulation of S-phase entry compared with uninduced control cells (Figure VI.11C). The continuous incorporation of $^3$H-thymidine at all time periods suggested that NSΔMEK1 cells were not arrested in the absence of mitogens at the start of the experiment. These effects may be due to the observed MAP-kinase kinase activity in the absence of 4-hydroxy tamoxifen. Similarly, the FACS-analysis of NSΔMEK1 (S218E, S222D):ERTM cells confirmed that the addition of 4-hydroxy tamoxifen to asynchronous growing cells did not result in a G1 cell cycle arrest but led to a slight increase in the population of S-phase cells (Figure VI.11B). This result was surprising, since the inhibition of Raf-induced MAP-kinase kinase activity by the specific inhibitor PD98059 did clearly delay the induction of p21$^{WAF1/Cip1}$ (Figure VI.9) which was instrumental to the cell cycle arrest (Lloyd et al., 1997). Although it has been reported that ΔMEK1 (S218E, S222D) exhibits a relative strong kinase activity in a number of assays (Mansour et al., 1994), this activity might be not strong enough to induce a cell cycle arrest in Schwann cells. As another possible explanation, due to
Figure V.11A-D : Constitutive active MAP-kinase kinase in Schwann cells

A. ΔMEK1:ERTM is active in the uninduced state

NSΔRafER cells were kept in the absence of mitogens for two days prior to the addition of 1μM 4-hydroxy tamoxifen. Control cells were left untreated in the absence of any mitogens. 30 μg cell lysate was subject to western blotting analysis. Immunoreactive bands with the p42ERK2 specific antibody 122 were detected using the ECL-system.

B. NSΔMEK1:ERTM cells do not arrest in the presence of 4-hydroxy tamoxifen

NSΔMEK1:ERTM cells were seeded at 2x10^4 cells per well. NSΔMEK1:ERTM cells were kept in the absence of mitogens for two days prior to the addition of mitogens. 1 μM 4-hydroxy tamoxifen or ethanol were added at point 0. ^H-thymidine was added for indicated time periods. ^H-thymidine incorporation was measured as described in Methods.

C. NSΔRafER cells arrest in the presence of 4-hydroxy tamoxifen

NSΔRafER cells were seeded at 2x10^4 cells per well. NSΔRafER cells were kept in the absence of mitogens for two days prior to the addition of mitogens. 1 μM 4-hydroxy tamoxifen or ethanol were added at point 0. ^H-thymidine was added for indicated time periods.

D. FACS-analysis of NSΔMEK:ERTM cells

NSΔMEK1:ERTM cells were kept in the absence of mitogens for two days prior to the addition of mitogens. 1 μM 4-hydroxy tamoxifen or ethanol were added at point 0. BrdU was added 26 hours after induction for 4 hours and FACS-analysis was carried out.
A. NS\textunderscore MEK1:ER\textsuperscript{TM}

- PP
- p42\textsuperscript{ERK2}

0 - 4 h

1 h 4 h

time after 4-hydroxy tamoxifen addition

B. NS\textunderscore MEK1:ER\textsuperscript{TM}

\[
\begin{array}{c|c|c|c}
\text{TM} & \text{0-17 h} & \text{17-35 h} & \text{35-52 h} \\
\hline
- & \text{[chart]} & \text{[chart]} & \text{[chart]} \\
+ & \text{[chart]} & \text{[chart]} & \text{[chart]} \\
\end{array}
\]

C. NS\textunderscore RAF1:ER

\[
\begin{array}{c|c|c|c}
\text{TM} & \text{0-17 h} & \text{17-35 h} & \text{35-52 h} \\
\hline
- & \text{[chart]} & \text{[chart]} & \text{[chart]} \\
+ & \text{[chart]} & \text{[chart]} & \text{[chart]} \\
\end{array}
\]

D. NS\textunderscore MEK1:ER\textsuperscript{TM}, - TM

\[
\begin{array}{c|c}
\text{G1: 77.28 \%} & \text{S: 16.61 \%} \\
\text{G2/M: 3.02 \%} & \\
\end{array}
\]

NS\textunderscore MEK1:ER\textsuperscript{TM}, + TM

\[
\begin{array}{c|c}
\text{G1: 72.89 \%} & \text{S: 21.95 \%} \\
\text{G2/M: 2.21 \%} & \\
\end{array}
\]
the activation of ΔMEK1 (S218E, S222D):ER\textsuperscript{TM} in the absence of 4-hydroxy tamoxifen individual cells which might have exhibited a strong kinase activity sufficient to arrest cells could have been selected against, thereby leaving only non-responsive cells. Further experimentation could address this possibility by testing single constitutively active MAP-kinase kinase expressing clones. Differences in inducibility and strength of MAP-kinase kinase activity could then be tested for p21\textsuperscript{WAF1/Cip1} induction.
CHAPTER 6.0
The c-Jun amino-terminal kinase 1 (JNK) in
\(\Delta\)Raf-induced growth arrest in Schwann cells
6.0 The c-Jun NH₂ terminal kinase 1 (JNK) in ΔRaf-induced growth arrest in Schwann cells

6.1 Introduction

The data presented so far have established a link between the prolonged activity of ΔRafER in Schwann cells and the growth arrest due to p53-dependent induction of p21WAF1/Cip1. Inhibition by the MAPKKs specific inhibitor PD98059 (see chapter 5) has provided strong evidence for the mediation of the growth inhibitory signal by the Ras-MAP-kinase pathway downstream of Ras and Raf. However, various lines of evidence suggest a possible involvement of the JNK-kinase pathway in mediating the signal for the Ras/Raf-induced cell cycle arrest in Schwann cells.

The delay of p21WAF1/Cip1 induction 10 hours after Raf induction (Chapter 4.0) could be explained by the secretion and the accumulative effect of a growth factor on Schwann cells. Interestingly, in NIH3T3 cells the induction of ΔRafER resulted in an enhanced expression and secretion of the heparin-binding epidermal growth factor (HB-EGF) (McCarthy et al., 1995). Furthermore, HB-EGF had a stimulatory effect on JNK-1 kinase activity in these cells (McCarthy et al., 1995). In particular, ΔRafER activation led to a delayed activation of JNK-1 kinase after 16 to 24 hours which was shown to be due to the effect of an autocrine loop in NIH3T3 cells (Minden et al., 1994; McCarthy et al., 1995). JNK-1 kinase has also been shown to be activatable by other factors such as the tumour necrosis factor (TNF-α) (Kyriakis et al., 1994) and possibly sphingomyelin-based second messengers (Hannun et al., 1994). Thus, it was a possibility that ΔRaf-dependent production and secretion of a factor or factors could result in a possible activation of the JNK-kinase pathway in Schwann cells. In this respect, the suggestion that UV-activated JNK-1 kinase may phosphorylate
murine p53 \textit{in vitro} and \textit{in vivo} (Milne et al., 1995) was of interest, since the p21\textsuperscript{WAF1/Cip1} induction in NS\Delta RafER cells is p53-dependent (chapter 5.0; Lloyd et al., 1997). Given these results, I sought to investigate whether JNK-1 kinase activity was firstly elevated in response to constitutive Raf-kinase activity in Schwann cells and secondly to establish whether JNK-kinase activity may contribute to the G1 cell cycle arrest.

6.2 \textbf{JNK-1 kinase activity is induced by \Delta RafER}

To explore whether the JNK-pathway is activated in the \Delta RafER-induced growth arrest in Schwann cells, I examined Jun N-terminal kinase 1 (JNK-1) activity in response to activated Raf. Confluent NS\Delta RafER cells were therefore induced with 4-hydroxy tamoxifen. Cells were harvested at indicated times. After immunoprecipitation with a JNK1-specific antibody, an \textit{in-vitro} kinase assay with recombinant c-Jun amino acid 1-257 protein as substrate was performed.

Following induction of \Delta RafER, JNK1-kinase activity increased dramatically (Figure VI.1A), while the addition of the solvent ethanol did not lead to a substantial increase of kinase activity. The quantification of incorporated \textsuperscript{32}P\gamma-phosphate showed a considerable increase of JNK-1 kinase activity 12 hours after induction, peaking at 14 hours with a 10-fold increase of kinase activity compared with uninduced cells. The JNK-1 kinase activity remained approximately 7-fold above background for at least 24 hours (Figure VI.1B). JNK-1 kinase activity after \Delta RafER induction was tested in three independent experiments with very similar results to the results presented (Figure VI.1A). The protein expression of JNK-1 kinase remained relatively unchanged during this time course (Figure VI.2), suggesting that activation occurs via posttranslational modification such as
Figure VI.1A: JNK-1 kinase activity in induced NSΔRafER cells
NSΔRafER were grown in medium containing 3% stripped fetal calf serum, GGF and forskolin to 80% confluence. Cells were washed twice with prewarmed PBSA. Medium without mitogens was added for two days. Thereafter cells were stimulated with 1 μM 4-hydroxyl tamoxifen. Cells were harvested at indicated times. Cell lysates were prepared as described (see Methods) and 500 μg total protein was used for immunoprecipitation with monoclonal antibody C-17 against JNK-1 (Oncogene Science Ltd.). Immunoprecipitates were used in an in-vitro kinase assay with GST-c-Jun amino acids 1-257 as substrate (see Methods). 10 μl of the in-vitro kinase assay were separated on a 15% SDS-PAGE and subject to autoradiography.

Figure VI.1B: Quantification of JNK-1 kinase activity in NSΔRafER cells
The incorporation of radioactive γ32-phosphate was quantified with a PhosphorImager and ImageQuant software (Molecular Dynamics Ltd.). The JNK-1 kinase activity of various time points is referred to 1-fold of time point zero.

Figure VI.2: Elevated JNK-1 kinase activity is independent of protein expression
30 μg total protein from cell lysates used for the kinase assay in figure VI.1A were separated on a 12.5% SDS-PAGE, blotted onto PVDF-membrane and incubated with JNK-1 specific antibody (C-17) (Oncogene Science Ltd.). Immunoreactive bands were visualised using the ECL-detection system (Amersham Ltd.).
### Fold kinase-activity

<table>
<thead>
<tr>
<th>Tm</th>
<th>24 h</th>
<th>18 h</th>
<th>14 h</th>
<th>12 h</th>
<th>2 h</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**A.**

**B.**

### Bar Graph

- **peptide**
  - +
  - -

**Fold kinase-activity (0=1x)**

- 0
- 10
- 20
- 30
phosphorylation by MKK4/SEK1 (Yan et al., 1994) which is likely to be responsible for the increase in kinase activity.

The finding that JNK1-kinase activity strongly increases after ΔRafER induction in Schwann cells is surprising, since the weak activation of JNK-1 kinase by activated Ras has been explained by a Raf-independent pathway (Minden et al., 1994). However, similar to results in NIH3T3 cells (Minden et al., 1994; McCarthy et al., 1995), it is possible that the production of autocrine factors are responsible for the delayed JNK-1 kinase activation, given that high JNK-kinase activity coincides with the beginning of p21WAF1/Cip1 induction (chapter 4.0) in Schwann cells.

6.3 MAP-kinase kinase inhibitor PD98059 indirectly inhibits JNK-1 kinase activity

The increase in JNK-1 kinase activity in response to activated Raf could have been the result of activation by a MAP-kinase kinase-dependent or independent signalling pathway. Previously, I have shown that the inhibition of MAP-kinase kinase by PD98059 led to a delayed induction of p21WAF1/Cip1 (chapter 5.0). MAP-kinase kinase activation was therefore required for the induction of p21WAF1/Cip1. Based on these results, I reasoned that if the activation of JNK-1 kinase was involved in mediating p21WAF1/Cip1 induction, MAP-kinase kinase activation was necessary for enhanced JNK-1 kinase activity to occur in response to Raf.

To distinguish between a MAP-kinase kinase-dependent or independent activation of JNK-1 kinase, the specific MAP-kinase kinase inhibitor PD98059 was used. NSΔRafER cells were treated with or without 50 μM PD98059 prior to induction with 4-hydroxy tamoxifen. Cells were harvested at indicated times after ΔRafER induction. After
Figure VI.3A: PD98059 inhibits JNK1-kinase activity in NSΔRafER cells

NSΔRafER were grown in medium containing 3% stripped fetal calf serum, GGF and forskolin to 80% confluence. Medium with 2% FCS only was added for two days. One hour prior to stimulation with 1 μM 4-hydroxyl tamoxifen, 50 μM PD98059 or the solvent DMSO were added to the cells. Cells were harvested at indicated times and 500 µg total protein was used for immunoprecipitation with monoclonal antibody C-17 against JNK-1 (Oncogene Science Ltd.). Immunoprecipitates were used in an \textit{in-vitro}-kinase assay with GST-c-Jun amino acids 1-257 as substrate (see Methods). 10 μl of the \textit{in-vitro} kinase assay were separated on a 15% SDS-PAGE and subject to autoradiography.

Figure VI.3B: Quantification of JNK-1 kinase activity in PD98059 treated NSΔRafER cells

The incorporation of radioactive $\gamma^{32}$-phosphate was quantified with a PhosphorImager and ImageQuant software (Molecular Dynamics Ltd.).
A.  

![Immunoblot](image.png)

**c-Jun aa 1-257**

**PD98059**

- - - + - + - + - + - +

GST 0 2 h 14 h 24 h UV peptide

**Time after 4-hydroxy tamoxifen addition**

B.  

![Bar chart](image.png)

**arbitrary units**

0 100 200 300 400

**PD98059**

- - + + + + + +

GST 0 2 h 14 h 24 h UV peptide

**Time after 4-hydroxy tamoxifen addition**
immunoprecipitation with a JNK1-specific antibody, an *in-vitro* kinase assay with recombinant c-Jun amino acids 1-257 protein as substrate was performed.

The MAP-kinase kinase inhibitor PD98059 had an inhibitory effect onto JNK-1 kinase activity at 14 hours and 24 hours (Figure VI.3A; VI.3B). This effect was not due to a direct inhibition of JNK-1 by PD98059, since JNK-1 kinase activation through UV-irradiation was not inhibited, confirming the lack of JNK-1 kinase inhibition by PD98059 previously reported (Alessi et al., 1995). Thus, the observed inhibition of JNK-1 kinase activity by PD98059 is likely due to inhibition of MAP-kinase kinase. Importantly, this result suggested the possibility that JNK-1 kinases could indeed be mediating the ΔRaf-induced growth arrest in Schwann cells.

6.4 Dominant negative Stress-activated protein kinase (SEK-1) in NSΔRafER cells

The inhibition of JNK-1 kinase activity by the MAP-kinase kinase inhibitor PD98059 suggests that the JNK-1 kinase is downstream of MAP-kinase kinase in the ΔRafER-induced signalling pathway (Figure VI.2A; VI.2B). Thus, the question arose whether JNK-kinase activity is causally involved in the induction of p21<sup>WAF1/Cip1</sup> and the following cell cycle arrest.

The generation of a dominant negative MAP-kinase kinase 4 (M KK4), also named Stress-activated protein kinase (SEK1) (Yan et al., 1994; Zanke et al., 1996), offered a genetic approach to address this question. It has been shown that M KK4/SEK1 is directly responsible for phosphorylation of JNK-1 kinase after activation by various stimuli (Yan et al., 1994). A dominant negative mutant of SEK1, designated SEK1-AL, was mutated to code for alanine and leucine at positions amino acids 220 and 224,
Figure VII.4A: Dominant negative SEK1-AL mutant reduces \( \Delta \text{RafER} \) induced JNK-1 kinase activity

NS\( \Delta \)RafER, NS\( \Delta \)RafER SEK-1 and NS\( \Delta \)RafER SEK-1-AL cells were grown in medium containing 3% stripped fetal calf serum, GGF and forskolin to 80% confluence. Cells were washed twice with prewarmed PBSA. Medium with 2% FCS only was added for two days. Thereafter, cells were stimulated with either 1 \( \mu \)M 4 hydroxy tamoxifen or ethanol as a control. Cells were harvested at indicated times and 500 \( \mu \)g total protein was used for immunoprecipitation with a monoclonal antibody C-17 against JNK-1 (Oncogene Science). Immunoprecipitates were used in an \textit{in-vitro} kinase assay with GST-c-Jun 1-257 as substrate (see Methods). 15 \( \mu \)l of the \textit{in-vitro} kinase assay were separated on a 15% SDS-PAGE and subjected to autoradiography.

**Figure VII.4B: Quantification of JNK-1 kinase activity**

The incorporation of radioactive \( \gamma^{32} \)-phosphate was quantified with a PhosphorImager and ImageQuant software (Molecular Dynamics Ltd.) and is expressed in arbitrary units.

**Figure VII.5: p21\textsuperscript{WAF1/CIP1} induction in NS\( \Delta \)RafER SEK1-AL cells**

60 \( \mu \)g total protein from cell lysates used for the kinase assay in figure VII.4A were separated on a 15% SDS-PAGE, blotted onto PVDF-membrane and incubated with an antibody against rat p21\textsuperscript{WAF1/CIP1} (Santa Cruz Ltd). Immunoreactive bands were visualised using the ECL-detection system (Amersham Ltd).
respectively (Yan et al., 1994). This mutant SEK1-kinase was capable of substantially reducing JNK-1 kinase activity mediated through SEK1 after heat-shock and the application of the DNA-damaging drug cis-platinum (Zanke et al., 1996).

I reasoned that if JNK-1 kinase activation is required and sufficient to induce p21^{WAF1/CIP1}, then the expression of a dominant negative mutant of SEK1 should inhibit or delay p21^{WAF1/CIP1} induction and overcome the ΔRafER-induced growth arrest. To address this hypothesis, I cloned the dominant negative mutant SEK1-AL and wild type SEK1 (Zanke et al., 1996) into the retroviral vector pBabe Bleo. NSΔRafER cells were infected with pBabe Bleo SEK1 or pBabe Bleo SEK1-AL resulting in NSΔRafER SEK1 and NSΔRafER SEK1-AL cells. After selection, cells were pooled and expanded. Firstly, I tested whether Schwann cells expressing SEK1-AL were capable of reducing ΔRafER induced JNK-1 kinase activity. On the contrary, the expression of exogenous wild-type SEK1 should lead to a clear increase of JNK-1 kinase activity over control cells with only endogenous MKK4/SEK kinase. The JNK-1 kinase activity of these cells was compared to NSΔRafER cells harbouring the empty vector pBabe Bleo. NSΔRafER cells, NSΔRafER SEK1 cells and NSΔRafER SEK1-AL cells were induced with 4-hydroxy tamoxifen and harvested at indicated times (Figure VI.3A). After immunoprecipitation an in-vitro JNK-1 kinase assay was performed.

Expression of the dominant negative kinase (SEK-AL) reduced ΔRafER-induced JNK-1 kinase activity after 24 hours to about 40% of JNK-1 kinase activity observed in NSΔRafER Bleo control cells. Moreover, cells expressing exogenous wild-type SEK1 in addition to endogenous SEK1-kinase showed an approximately 45% increase of JNK-1 kinase activity at 14 and 24 hours after induction (Figure VI.3B). The observed JNK-1 kinase
activity after 4-hydroxy tamoxifen addition was not observed after the addition of the solvent ethanol (Figure VI.3B).

These results confirmed that infection with dominant negative SEK1 was sufficient to partially reduce ΔRafER-dependent JNK-1 kinase activity. As predicted, the introduction of exogenous SEK-1 led to an increase of JNK-1 kinase activity above that observed in control cells, arguing for a role of SEK1 in mediating ΔRafER-dependent induction of JNK-1 kinase activity. Given these results, I was interested to see whether the observed reduction of JNK-1 kinase activity in NSΔRafER SEK1-AL cells had an effect on the induction of $p^{21^{WAF1/Cip1}}$ protein. Conversely, the increase of JNK-1 kinase activity in NSΔRafER SEK1 cells might also be reflected in a stronger induction of $p^{21^{WAF1/Cip1}}$ in these cells.

To test whether such a correlation existed, I examined the $p^{21^{WAF1/Cip1}}$ protein levels from the same cell lysates previously used for the JNK1-kinase assay (Figure VI.3A). Protein lysates were separated on an SDS-PAGE gel and analysed. Western blot analysis revealed that the co-expression of dominant negative SEK1-AL had no effect on the level of induced $p^{21^{WAF1/Cip1}}$ protein. In agreement with this finding, the co-expression of wild-type SEK-1 did not increase the $p^{21^{WAF1/Cip1}}$ protein level compared to that in induced control cells (Figure VI.4). In parallel, I examined whether NSΔRafER cells expressing the dominant negative mutant SEK1-AL were capable of rescuing the Raf-induced growth arrest by FACS-analysis. For this purpose, BrdU was added to asynchronously growing NSΔRafER SEK1-AL and SEK1 or control cells and 26 hours after Raf induction the cell cycle distribution of these cells was analysed. The FACS-analysis of NSΔRafER SEK1 and NSΔRafER SEK1-AL cells was inconclusive in that both NSΔRafER SEK1 and NSΔRafER SEK1-AL cells were inhibited to a similar degree in the presence of 4-hydroxy tamoxifen.
Figure VI.6A-B: FACS-analysis of NSΔRafER SEK1-AL cells and NSΔRafER SEK1

Assynchronous growing NSΔRafER SEK1-AL and NSΔRafER SEK1 cells were induced with 1 μM 4-hydroxy tamoxifen. After 26 hours 10 μM BrdU were added for 4 hours. The cell cycle distribution of NSΔRafER SEK1-AL and NSΔRafER SEK1 cells were measured by BrdU incorporation and DNA-content flow cytometry analysis. The upper box identifies cells incorporating BrdU (S-phase), the lower left-hand box represents Go/G1 cells and the lower right-hand box represents G2/M cells. The percentage of cells in G1, S or G2/M-phase of the cell cycle is given underneath the individual graph.
Surprisingly, the reduction of S-phase cells of the control NSΔRafER SEK1 cells was far less strong than that observed in NSΔRafER Bleo cells (Figure VI.5).

### 6.5 Discussion

The induction of constitutively active Raf-kinase activity led to a delayed MAP-kinase kinase-dependent increase in JNK-1 kinase activity. This enhanced JNK-1 kinase activity appears not to contribute directly to the Raf-induced cell cycle arrest in Schwann cells, since the co-expression of a dominant negative SEK-AL mutant did neither change the amount of p21^{WAF1/Cip1} protein induced by ΔRafER nor did it apparently alter the ability of NSΔRafER cells to arrest in the G1-phase of the cell-cycle arrest. Although these results seemed to indicate that the ΔRafER-dependent activation of JNK-1 kinase was not required for the induction of p21^{WAF1/Cip1}, it can not been excluded that the approximately 40% inhibition of JNK-1 kinase was not sufficient to abrogate the induction of p21^{WAF1/Cip1} and the G1 cell cycle arrest. Alternative ways to inhibit JNK-1 kinase activity through a dominant negative JNK-1 molecule or experiments utilising antisense RNA may result in a stronger inhibition thereby clearly ruling out any possible involvement of JNK1-kinase in the induction of p21^{WAF1/Cip1}. The presented evidence currently suggest that it is unlikely that the observed JNK-1 kinase activity contributes to the induction of p21^{WAF1/Cip1} in Schwann cells.
7.0 Discussion

7.1 Oncogene cooperation in Schwann cells

Mutants of the tumour suppressor p53 cooperate with activated Ras and activated Raf in transforming Schwann cells. Constitutive Raf-activity on its own results in G1 cell cycle arrest mediated by the induction of the cell cycle inhibitor p21^{WAF1/Cip1} and a subsequent decrease in cyclin/cdk activity. The coexpression of dominant inhibitory mutants of p53 suppress the induction of p21^{WAF1/Cip1} and enable Schwann cells to proliferate continuously. The cooperative potential of p53 mutants is unlikely to be due to a gain-of-function of p53 but rather reflects the reported dominant inhibitory function of mutant p53 over endogenous wild type p53 due to direct binding (Milner & Metcalf, 1991; Kern et al., 1992; Barbonetti et al., 1992). Thus, the suppression of the Raf-dependent induction of p21^{WAF1/Cip1} by coexpression of mutant p53 offers a model of how cooperating oncogenes can overcome cell cycle control mechanisms.

7.2 Raf induces the CDK-inhibitor p21^{WAF1/Cip1}

Constitutive Raf-activity induces the cell cycle inhibitor p21^{WAF1/Cip1} in Schwann cells (this thesis, Lloyd et al., 1997). p21^{WAF1/Cip1} has been shown to interact (Nakanishi et al., 1995; Chen et al., 1996; Ball et al., 1997) with a number of G1 cyclin-dependent kinase complexes (Xiong et al., 1993). In particular, CDK2, CDK3, CDK4 and CDK6-dependent kinase activities are inhibited by p21^{WAF1/Cip1} (Xiong et al., 1993). In addition, p21^{WAF1/Cip1} prevents the activating phosphorylation steps of CDK2 and CDK6 by CDK-activating kinase (CAK) (Aprelikova et al., 1995). No direct interaction between CAK and p21^{WAF1/Cip1} could be detected, suggesting
that the binding of p21WAF1/Cip1 to CDK subunits renders CDK inaccessible to CAK-dependent phosphorylation (Aprelikova et al., 1995).

The inhibition of CDK-complex activity requires the binding of several p21WAF1/Cip1 molecules to CDKs (Harper et al., 1995) which is in agreement with the finding that p21WAF1/Cip1 protein levels are significantly increased in arrested Schwann cells and cyclin E and cyclin A-dependent kinase activity strongly reduced (this thesis; Lloyd et al., 1997). Since p21WAF1/Cip1 is part of Cyclin-CDK complexes in growing cells (Xiong et al., 1993; Zhang et al., 1994a, 1994b; Harper et al., 1995), this finding did suggest that the number of bound p21WAF1/Cip1 molecules determines whether CDK-activity is inhibited or not (Harper et al., 1995). Indeed, at low protein concentrations p21WAF1/Cip1 facilitates the assembly of CDK4/Cyclin D complexes by decreasing the dissociation rate of already formed complexes and supporting the localisation of CDK4 and Cyclin D protein to the nucleus (LaBaer et al., 1997).

In arrested human fibroblasts high p21WAF1/Cip1 protein levels can be observed compared to cells in S-phase (Nakanishi et al., 1995; Li et al., 1994) and overexpression of p21WAF1/Cip1 leads to G1 cell cycle arrest (Harper et al., 1995). The coexpression of antisense p21WAF1/Cip1 RNA results in abrogation of the growth arrest and S-phase entry (Nakanishi et al., 1995). Similarly, in arrested Schwann cells, lowering of p21WAF1/Cip1 protein levels by coexpression of p21WAF1/Cip1 antisense RNA leads to the abrogation of G1 cell cycle arrest (Lloyd et al., 1997), arguing that the p21WAF1/Cip1 induction is causal to the G1 cell cycle arrest.
7.3 The p53-dependency of p21\textsuperscript{WAF1/Cip1} induction in Schwann cells

In Schwann cells the abrogation of p53 function abolishes the induction of p21\textsuperscript{WAF1/Cip1} protein levels and allows activated Raf to transform these cells (this thesis). The p21\textsuperscript{WAF1/Cip1} gene has been described as a transcriptional target of p53 (El-Deiry et al., 1993), a notion supported by the existence of two conserved p53-binding sites in the p21\textsuperscript{WAF1/Cip1} promoter (Dulic et al., 1994; Michieli et al., 1994; Macleod et al., 1995). Although abrogation of p21\textsuperscript{WAF1/Cip1} induction by coexpressing mutant p53 suggests that p53 activates the p21\textsuperscript{WAF1/Cip1} gene in Schwann cells, direct evidence for increased p53 transactivation activity could not be obtained. Experiments carried out so far could not clearly establish an increase of p21\textsuperscript{WAF1/Cip1} transcription in arrested Schwann cells (A. Lloyd pers. comm.). Apart from transcriptional mechanisms, posttranslational mechanisms regulating the protein half-life may regulate p21\textsuperscript{WAF1/Cip1}. These mechanisms may also be occurring in response to constitutively active Raf in Schwann cells. In this respect, the recent discovery of a p73 protein which has a strong structural homology with p53, in particular with the DNA-binding domain (Kaghad et al., 1997) may possibly contribute towards Raf-induced p21\textsuperscript{WAF1/Cip1} induction in Schwann cells. The overexpression of p73 led to the induction of p21\textsuperscript{WAF1/Cip1} and effectively blocked colony formation of transformed cells (Kaghad et al., 1997; Jost et al., 1997). Since an interaction between p73\textbeta, a splice variant and p53 has been reported \textit{in vitro} (Kaghad et al., 1997), a possible functional interaction \textit{in vivo} could be negatively intercepted by mutant p53. The exact nature of p53-dependency and possible contribution of p73 in the induction of p21\textsuperscript{WAF1/Cip1} in Schwann cells remains to be further investigated.
Various studies have revealed that the p21WAF1/Cip1 induction is regulated in either p53-dependent or p53-independent ways. The regulation of p21WAF1/Cip1 in a p53-independent manner has been observed in tissue development (Macleod et al., 1995), cell differentiation (Macleod et al., 1995; Steinman et al., 1994; Halevy et al., 1995; Skapek et al., 1995; Parker et al., 1995; Zeng & El-Deiry, 1996; Liu et al., 1996) and senescence (Noda et al., 1994; Serrano et al., 1997). Furthermore, p21WAF1/Cip1 induction has been observed in response to serum stimulation (Macleod et al., 1995; Jiang et al., 1994; Sheikh et al., 1994; Li et al., 1994), interferon γ (Chin et al., 1996) and TGF-β application (Datto et al., 1995). Although it is not in all cases absolutely clear whether p53 is involved or not in p21WAF1/Cip1 induction by the above mentioned stimuli, the induction in a p53-dependent manner has been mainly described after DNA damage by ionizing radiation or DNA-damaging agents (Dulic et al., 1994; Xiong et al., 1993a, b; El-Deiry et al., 1993, 1994; Di Leonardo et al., 1994; Brugarolas et al., 1995).

In Schwann cells is seems unlikely that constitutive Raf-activity results in DNA-damage since there are no signs of apoptosis in G1-arrested cells (Lloyd et al., 1997), although it can not be fully excluded. Furthermore, the p53-dependent induction of p21WAF1/Cip1 and subsequent G1 cell cycle arrest does not appear to resemble the phenotype of differentiated Schwann cells nor is there any evidence for the induction of the P0 protein (A. Lloyd, unpublished observation) which is strictly associated with differentiation in Schwann cells (Owens & Boyd, 1991). It has been reported, that in fibroblasts oncogenic Ras results in a permanent G1 cell cycle arrest and these cells are phenotypically not distinguishable from senescent cells (Serrano et al., 1997). Although senescence markers have not been tested in G1-arrested Schwann cells, cells are viable but
permanently arrested as long as the constitutive Raf signal persists which suggests that premature senescence could have occurred. It has been suggested that since high p21WAF1/Cip1 protein levels occur in senescent as well as DNA damaged cells, the progressively shortening of telomeres in senescence could be interpreted as a form of DNA damage, leading to a similar arrest mechanism (Stein & Dulic, 1995). However, there is evidence for a direct involvement of p53, since mutant p53 directly allows fibroblasts to escape from senescence (Bond et al., 1994; 1995).

The p53-dependent induction of p21WAF1/Cip1 as a response to the constitutive action of a single oncogene may be a new feature of p53’s tumour suppressor function in that mitogenic signals required for cell proliferation are monitored by p53 and aberrant signals provoke a p53-dependent cell cycle arrest or senescence. In this respect, the growth factor-mediated transient stimulation of Raf activity may result in a form of p53 activation which allows cell-cycle progression, whereas continuous Raf-activity could strongly increase p53 transactivation activity which in turn would lead to the induction of target genes such as p21WAF1/Cip1.

Interestingly, the p21WAF1/Cip1 gene itself is rarely lost or mutated in tumours or cancer cell lines from various cell types (Shiohara et al.; 1994; Li et al., 1995), whereas mutations or deletions of the p53 gene occur within tumours with a high frequency (Hollstein et al., 1996). This finding has been supported by investigations of p53 or p21WAF1/Cip1 nullizygous mice, respectively. Mice nullizygous for p21WAF1/Cip1 develop normally (Deng et al., 1995), however, in these mice the G1 cell cycle arrest is strongly impaired in response to DNA damage (Deng et al., 1995). In contrast, p53 nullizygous mice are highly susceptible to tumorigenesis and die due to multiple tumours at an early stage (Donehower et al., 1992). Similarly, primary murine fibroblasts nullizygous for p53 are transformed by activated
Ras alone (Tanaka et al., 1994). Surprisingly, keratinocytes from p21^{WAF1/Cip1} nullizygous mice are also aggressively transformed by activated Ras alone (Missero et al., 1996).

The apparent conflicting result that p21^{WAF1/Cip1} mice can be easily transformed by introduction of activated Ras, although mutations of p21^{WAF1/Cip1} in human cancer are relatively sporadic in comparison with p53 mutations could be explained through the multiple regulatory functions of p53. The inactivation of p53 will not only affect the transactivation activity and thereby the p21^{WAF1/Cip1} induction, moreover, mutant p53 abrogates p53-dependent apoptosis which could be the limiting factor in progression of tumour growth. Other functions regarding the genomic stability such as a p53-dependent spindle checkpoint could also be affected (Cross et al., 1995) offering an explanation for the observed differences.

7.4 Signalling pathways regulating the induction of p21^{WAF1/Cip1}

The result that activated Ras and activated Raf cause cell cycle arrest in the G1 phase of the cell cycle in primary cells (this thesis; Lloyd et al., 1997; Serrano et al., 1997) raises the question of normal Ras and Raf function in cell proliferation and signal specificity. As shown in this thesis, MAP-kinase kinase activity is required to induce p21^{WAF1/Cip1}, arguing for the involvement of the Ras-MAP-kinase module in mediating the G1 cell cycle arrest. However, the activity of the Ras-MAP-kinase kinase module is also required for cell cycle progression in many cells (Mulcahy et al., 1985; Feig & Cooper, 1988; Moodie et al., 1993; Cowley et al., 1994) and appears to be also important for cell cycle progression in Schwann cells, since the expression of a dominant negative MAP-kinase kinase reduces the
percentage of cells in S-phase (this thesis). It is, therefore, surprising that
the same signalling pathway appears to be sufficient for S-phase entry
(Cowely et al., 1994) and can activated p53 and p21WAF1/Cip1 resulting in
G1 cell cycle arrest (this thesis, Lloyd et al., 1997). The question how
presumably the same signalling pathway can mediate a mitogenic and a
growth inhibitory effect could be explained by different kinetics of Raf
activity, in particular duration and strength of the signal.

7.4.1 Duration of the signal initiated by activated Raf

The finding that members of the Ras-MAP-kinase signalling pathway
can mediate a mitogenic or inhibitory cellular response could be explained
by a difference in the duration of Raf-activity. MAP-kinase kinase, a
downstream target of Raf-kinase, is required for the induction of
p21WAF1/Cip1, since inhibition of this kinase effects the induction of
p21WAF1/Cip1 (this thesis). Experiments with the MAP-kinase kinase specific
inhibitor PD98059 suggest that the Raf-dependent induction of
p21WAF1/Cip1 is mediated via MAP-kinase kinase and the downstream
target MAP-kinase. Furthermore, studies of the kinetics of MAP-kinase
activation have shown that the induction of constitutively active Raf results in
a continuous activation of MAP-kinase correlated with the induction of
p21WAF1/Cip1, whereas mitogenic stimulation is associated with a temporary
activation for up to four hours and much lower protein levels of
p21WAF1/Cip1 (this thesis). Although prolonged MAP-kinase activation after
Raf induction coincides with the G1 cell cycle arrest, it can, however, not be
excluded that other so far unknown targets of MAP-kinase kinase mediate
the induction of p21WAF1/Cip1 in Schwann cells.

The phenomenon that a prolonged activation of a signalling pathway
has a different biological outcome compared to a short activation is not
unprecedented. The differences in duration of activation of the Ras-MAP-kinase pathway has been suggested as an explanation for the different biological response of PC12 cells to either epidermal growth factor (EGF) or nerve growth factor (NGF) (Traverse et al., 1992). Addition of NGF or the prolonged activation of the Ras-MAP-kinase pathway by expression of activated Ras (Noda et al., 1985), activated Raf (Wood et al., 1993) and activated MAP-kinase kinase (Cowley et al., 1994) results in growth arrest followed by neurite outgrowth whereas a transient activation led to cell proliferation (Traverse et al., 1992).

7.4.2 Strength of the signal initiated by activated Raf

Differences in signal strength could be another possible explanation for the opposing cellular responses to oncogenic Raf and mitogenically activated Raf. For instance, constitutively active Raf leads to a mitogenic response in NIH3T3 cells (Kolch et al., 1991; Smith et al., 1990; Pritchard et al., 1995). Nevertheless, it seems that the strength of the signal is significant for this cellular response. In NIH3T3 cells, a strong Raf signal leads to G1 cell cycle arrest due to the induction of p21\textsuperscript{WAF1/Cip1} accompanied by inhibition of cyclin E and D-dependent kinase complexes (Sewing et al., 1997; Woods et al., 1997). However, the induction of p21\textsuperscript{WAF1/Cip1} by constitutively active Raf is p53-independent, since it also occurs in p53\textsuperscript{-/-} mouse embryo fibroblasts (Sewing et al., 1997). The strength of the inducible Raf-signal has been correlated with the degree of MAP-kinase activation with a weak signal sufficient for proliferation while a strong Raf-signal causes G1- growth arrest (Sewing et al., 1997). In support for this hypothesis, the induction of ΔA-RafER in NIH3T3 cells led to a weak activation of MAP-kinase activity and entry into DNA synthesis, whereas ΔRaf-1ER and ΔB-RafER strongly activate MAP-kinase but were insufficient
to promote the entry of the NIH3T3 cells into DNA synthesis (Pritchard et al., 1995). Interestingly, it has been reported that the strength of the signal initiated by activated ΔRafER increases 10fold over 16 hours in NIH3T3 cells (Samuels et al., 1993). This increase of signal has been partly attributed to the selective stabilisation of the ΔRaf-1ER fusion protein (Samuels et al., 1993) or the ΔB-Raf and ΔA-RafER fusion proteins (Pritchard et al., 1995).

Different cellular responses to a strong or weak signal have been reported in other cell systems. In A431 human squamous carcinoma cells picomolar EGF concentrations stimulate cell proliferation whereas nanomolar EGF concentrations inhibit cell proliferation (Fan et al., 1995; Jakus & Yeudall, 1996). The EGF-mediated inhibition of cell proliferation is correlated with a rapid p21WAF1/Cip1 induction (Fan et al., 1995), increased binding to cyclin-CDK complexes and subsequent inhibition of CDK2/6-dependent kinase activity as well as cyclin A and cyclin E associated kinase activity (Jakus & Yeudall, 1996; Fan et al., 1995). Since A431 cells express a mutant p53 (Somers et al., 1992), the EGF-mediated p21WAF1/Cip1 induction appears to be p53-independent.

In developmental processes, the strength of a signal can determine the cell fate of precursor cells. The Ras involvement in a number of developmental systems suggests that the strength of Ras activity mediates different responses. For instance, in the fungus Aspergillus nidulans high Ras activity is associated with nuclear division but the development stops at an early step of germ tube formation. At intermediate Ras activity aerial hypha formation is inhibited and low Ras activity inhibits the formation of conidiophores (Som & Kolaparthi, 1994).

The distinction between a strong signal and a prolonged signal may, however, be artificial. The generation of a strong signal, for instance, due to
the overexpression of a receptor may lead to a stronger signal which at the same time may be prolonged when measured downstream of the receptor, since the inactivation mechanisms, for example, through phosphatase activity may not be sufficient to down-regulated a stronger signal. For example, the introduction of exogenous EGF-receptors into NIH3T3 cells increases cell proliferation and the transformation frequency in response to EGF (Velu et al., 1989). In PC12 cells, additional EGF-receptors convert a proliferative response into differentiation, usually only observed after NGF addition (Traverse et al., 1994).

7.5 Cyclin D1 induction in response to activated Raf

In Schwann cells, constitutive active Raf strongly induces cyclin D1 protein levels as early as 3 hours after Raf-induction (Lloyd et al., 1997). The induction of cyclin D has also been previously reported in cells expressing activated Ras (Liu et al., 1995; Winston et al., 1996). Although cyclin D1 induction by activated Ras via the MAP-kinase pathway (Lavoie et al., 1996) has been correlated with cell cycle progression in that it shortens the G1-phase (Filmus et al., 1994; Liu et al., 1995; Winston et al., 1996), in Schwann cells cyclin D1 induction does not prevent the G1 cell cycle arrest (Lloyd et al., 1997).

The strong induction of cyclin D1 on its own (Baldin et al., 1993; Pagano et al., 1994; Atajada et al., 1995) or in conjunction with p21WAF1/Cip1 (DeSal et al., 1996) has also been associated with growth inhibition. In Schwann cells, however, the Raf-induced up-regulation of cyclin D1 is not likely to contribute to the G1 cell cycle arrest, since arrested Schwann cells and proliferating Schwann cells coexpressing mutant p53 exhibit comparable levels of cyclin D1 protein in response to Raf activity.
(this thesis; Lloyd et al., 1997), also indicating that cyclin D1 induction is independent of p53 activity. The G1 cell cycle arrest in Schwann cells is characterised by the inhibition of cyclin E-dependent kinase activity through binding by the CDK-inhibitor p21WAF1/Cip1 (Lloyd et al., 1997). The increase in p21WAF1/Cip1 protein may also be sufficient for the inhibition of cyclin D1-dependent kinase activity so that the induction of cyclin D1 does not result in an increase of cyclin D-dependent kinase activity.

7.6 Autocrine factors and the JNK-kinase pathway

The p53-dependent induction of p21WAF1/Cip1 is characterised by a delayed increase of p21WAF1/Cip1 protein levels (this thesis). This observation may point to the involvement of a feedback loop of a secreted autocrine factor. As observed in Schwann cells, in NIH3T3 cells MAP-kinase kinase and MAP-kinase were rapidly activated by ΔRaf-1ER induction (Samuels et al., 1993), whereas the strong activation of JNK-kinase was delayed (McCarty et al., 1995). In NIH3T3 cells, the delayed activation of JNK-kinase was due to the ΔRafER-dependent increase of the heparin-binding epidermal growth factor (HB-EGF) secretion, since antibodies against HB-EGF abolish the activation of JNK-kinase (McCarthy et al., 1995).

In Schwann cells, my results have shown that JNK-1 kinase activity is strongly enhanced in response to ΔRafER induction and it is possible that this delayed activation of JNK-1 kinase is also due to the secretion of HB-EGF or another factor. In general, Schwann cells have been reported to secrete a number of factors (Rogister et al., 1993; Eccleston et al., 1991, Krystosek et al., 1984) and constitutively active Raf leads to the secretion of
an unknown factor or factors with a positive effect on cell proliferation of Swiss3T3 but not on Schwann cells (A.Lloyd, unpublished observation).

JNK-1 kinase has been reported to phosphorylate p53 (Milne et al., 1995). Furthermore, the p53-dependent induction of p21^{WAF1/Cip1} coincides with the strongest JNK-1 kinase activity in Schwann cells (this thesis). Despite this intriguing coincidence, a direct cause-effect relationship between these two events has not been established. The co-expression of dominant negative JNK-1 molecules reduces endogenous JNK-1 kinase activity but failed to delay or reduce p21^{WAF1/Cip1} induction (this thesis). In summary, it cannot be excluded that the induction of p21^{WAF1/Cip1} is mediated via JNK-1 kinase since the experimental approaches used do not sufficiently address this question.

7.7 Cellular responses to a single oncogene or cooperating oncogenes

The presented results in Schwann cells provide important evidence for a crucial role for CDK-inhibitors in protecting against the growth promoting effects of single oncogenes in primary cells. The induction of a cell cycle inhibitor in response to oncogenes is not restricted to Schwann cells but has been found in other cell types via the induction of different cell cycle inhibitors. For instance, constitutively Ras-activity arrests primary human fibroblasts via the induction of p16 and p53 (Serrano et al., 1997). Interestingly, the observed arrest of human fibroblasts in response to activated Ras is indistinguishable from cellular senescence (Serrano et al., 1997), suggesting that senescence may not entirely dependent on the lifespan of cells (Stein & Dulic, 1995) but can be also occur as a response to a single oncogene such as Ha-ras (Serrano et al., 1997). Similarly,
oncogenic Raf may also lead to senescence in Schwann cells, although the expression of senescence markers has not been tested.

The cooperation between oncogenic Raf and mutant p53 due to the suppression of p21\(^{\text{WAF1/Cip1}}\) protein induction in Schwann cells allows cellular transformation to occur. Similarly, the suppression of other CDK-inhibitors enable cooperating oncogenes to transform other cell types, indicating that the described molecular mechanism of cooperation may be of general relevance. For instance, the adenovirus E1A protein has been found to block the growth inhibitory effects of the CDK-inhibitor p27\(^{\text{Kip1}}\) through direct binding (Mai et al., 1996). The adenovirus E1A protein can also overcome the suppression of cell transformation by the CDK-inhibitor p16\(^{\text{INK4A}}\) (Serrano et al., 1995). Moreover, Ras or Raf alone can transform primary fibroblasts which have lost the INK4A tumour suppressor locus, understood to encode p16\(^{\text{INK4A}}\) (Serrano et al., 1996) or in keratinocytes, the p21\(^{\text{WAF1/Cip1}}\) gene (Missero et al., 1996). These examples suggest that similarly to Raf and mutant p53 in Schwann cells, a reduction in cyclin/CDK inhibitory protein levels reveals the transforming potential of Raf or Ras. Thus, the induction of cellular senescence or cell cycle arrest can be understood as mechanism by which organisms can protect themselves from uncontrolled proliferation initiated by activated oncogenes with additional mutations required for uncontrolled cell proliferation to occur.

7.8 Schwann cells as model for neurofibromatosis

Neurofibromatosis type 1 is a human genetic disorder affecting the peripheral nervous system and various organs (Riccardi 1992). NF-1 patients are susceptible to developing benign neurofibromas which consist mainly of Schwann cells and fibroblasts. The loss of the neurofibromin
gene, a GTPase activating protein of Ras (Ballester et al., 1990; Martin et al., 1990; Xu et al., 1990a, b) has been associated with the development of neurofibromas and neurofibrosarcomas (Wallace et al., 1990; Li et al., 1992; The et al., 1993; Shannon et al., 1994). In fact, malignant Schwann cell lines derived from NF1-patients exhibit very low protein levels of neurofibromin which has been shown to correlate with an increase of Ras in the active state (Basu et al., 1992; DeClue et al., 1992). Furthermore, murine Schwann cells deficient in \( \textit{NF-1} \) are angiogenic and invasive (Sheela et al., 1990; Kim et al., 1997). These studies suggest that the lack of neurofibromin function and increase of Ras activity in Schwann cells contribute to the formation of neurofibromas.

The absence of the \( \textit{NF-1} \) gene in primary murine Schwann cells, however, results in elevated Ras activity and inhibition of cell proliferation (Kim et al., 1995). Similarly, high Ras/Raf activity in primary Schwann cells inhibit their growth (Ridley et al., 1988; Lloyd et al., 1997; this thesis). In contrast, the introduction of activated Ras into the Schwann cell line RN-22 (Kim et al., 1995) as well as high Ras activity malignant Schwannomas in cells from NF-1 patients increase cell proliferation (Basu et al., 1992; DeClue et al., 1992). This increase in cell proliferation was inhibited by lowering the amount of active Ras by injection of an neutralising Ras-antibody or the increase of Ras-GAP activity (Basu et al., 1992; DeClue et al., 1992). The difference in cell growth to constitutively Ras activity could be explained by the accumulation of additional mutations in Schwannomas cells or the Schwann cell line RN-22 which will alter the cellular response to Ras. For instance, mutations in the tumour suppressor gene \( p53 \) alter the cellular response to constitutive Ras or Raf activity towards increased cell proliferation and transformation (this thesis). In support of this idea, p53 mutations have been described in neurofibrosarcomas from patients with
neurofibromatosis type 1 (Menon et al., 1990). Moreover, a cooperative
effect between the loss of the NF-1 gene, leading to high Ras activity (Kim et
al., 1995), and mutants of p53 has been shown in a transgenic mice model
(Cichowski et al., 1996). Likewise, a SV40 LT transgene expressed in
Schwann cells led to the development of neurocristopathies which
resemble neurofibromatosis type 1, indicating that LT’s various function,
such as inactivating p53, can contribute towards tumour development in
Schwann cells (Mazarakis et al., 1996). The expression of SV40 LT in our
Schwann cell model also cooperates strongly with activated Ras and Raf
(Ridley et al., 1988; Lloyd et al., 1997). Thus, Schwann cells expressing
inducible activated Raf will be useful in further investigations addressing the
molecular mechanisms of neurofibromatosis.

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Cooperating oncogenes converge to regulate cyclin/cdk complexes

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The cooperation of oncogenes in the transformation of primary rat Schwann cells is a strikingly synergistic process. We have explored the molecular mechanisms involved. Activation of an inducible Raf kinase results in morphologically transformed cells that are arrested in G1 via the induction of p21CIP1 and subsequent inhibition of cyclin/cdk activity. In contrast, coexpression of SV40 large T (LT) or a dominant-negative mutant of p53 abolishes p21CIP1 induction and alleviates the growth arrest. Moreover in this scenario, Raf activation results in an increase in the specific activity of cyclin/cdk complexes with Raf and LT cooperating to superinduce cyclin A/cdk2 activity and stimulate proliferation in the absence of mitogens. Thus, signaling by Raf and its cooperating partners converges at the regulation of cyclin/cdk complexes, with the cellular responses to Raf modulated by p53.

[Key Words: Cell cycle; oncogenes; cyclin-dependent kinase; p21CIP1; Raf; p53]

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Tumorigenesis is a multistep process involving the accumulation of genetic defects that contribute to the phenotype of the tumor. This is reflected by a large number of in vitro and in vivo studies that have shown that more than one oncogenic lesion is required to fully transform most primary cell types (for review, see Hunter 1991). However, the contribution of each oncogene to the transformed phenotype and the molecular mechanisms that underlie oncogene cooperation are poorly understood.

Primary rat Schwann cells are particularly suitable to study oncogene cooperation as homogenous cultures of these cells isolated from neonatal sciatic nerves can be cultured successfully in vitro for extended periods (Brockes et al. 1979). Previously we have shown that the cooperation of Ras and simian virus 40 large T antigen (LT) in the transformation of primary Schwann cells is a synergistic process in which the cellular response to Ras is dependent on the presence of LT (Ridley et al. 1988). Introduction of activated Ras alone results in morphologically transformed cells that are growth-arrested and only when coexpressed with LT does Ras induce the formation of highly proliferative, anchorage-independent cultures. In contrast, LT alone lowers the growth factor requirement of cells that otherwise exhibit normal behavior.

The Ras signaling pathway in Schwann cells is of particular interest as activation of the Ras pathway has been implicated in the common genetic disorder neurofibromatosis type 1, a disease primarily affecting Schwann cells (Ballester et al. 1990; Martin et al. 1990; Xu et al. 1990a,b). Patients with this disorder frequently develop multiple benign neurofibromas, which are composed mainly of Schwann cells. They also have an increased risk of developing malignant tumors derived from Schwann cells or other neural crest-derived cell types. Inhibition of the Ras pathway in tumor cells derived from these patients, using either neutralizing antibodies to Ras or by increasing Ras-GAP activity, results in a reversion of the tumor cells, confirming the role of Ras in tumor formation in this disease (Basu et al. 1992; DeClue et al. 1992). In addition, transgenic animals in which LT expression is directed to Schwann cells develop neurocristopathies that resemble neurofibromatosis type 1, demonstrating that LT expression can also contribute to the development of tumors in this cell type in vivo (Mazarakis et al. 1996).

To explore the molecular basis for the cooperation between Ras and LT we have used an inducible Raf protein in which an activated Raf kinase has been fused to the estrogen receptor hormone-binding domain (ΔRaf-1:ER) (Samuels et al. 1993). Raf has been shown to act directly upstream of Ras (Moodie et al. 1993; Van Aelst et al. 1993; Vojtek et al. 1993; Warne et al. 1993) and elicits a similar phenotype in some cell types; for example, in PC12 cells both activated Ras and Raf proteins are capable of inducing neurite outgrowth (Wood et al. 1993). In primary Schwann cells we show that the effects of Raf are indistinguishable from those of Ras. The use of ΔRaf-
1:ER has allowed the biochemical analysis of signaling events in Schwann cells following Raf activation in the presence and absence of LT. We show that Raf and LT cooperate to regulate cyclin/cdk activity. Raf alone induces the cdk inhibitor p21<sup>WAF1/CIP1</sup> by a p53-dependent mechanism, resulting in a cell cycle arrest. In cells in which p21<sup>WAF1/CIP1</sup> induction is counteracted by the expression of dominant-negative p53 or LT, Raf increases the specific activity of cyclin/cdk complexes with Raf and LT signals cooperating to superinduce cyclin A/cdk2 activity and stimulate proliferation of the transformed cells.

**Results**

Raf alters Schwann cell morphology and causes G<sub>1</sub> arrest

To facilitate the analysis of Ras and LT cooperation in primary Schwann cells we exploited the ΔRaf-1:ER system. This Raf/estrogen receptor fusion protein (ΔRaf-1:ER) exhibits kinase and transforming activity in a strictly hormone-dependent fashion [Samuels et al. 1993]. Low passage normal rat Schwann cells were infected with the retroviral vector LXSN (Miller and Rosman 1989) or its derivative LXSN-ΔRaf-1:ER. Several hundred G418 resistant colonies infected with the empty vector (NSE) or the vector encoding the inducible Raf protein (NSARafER) were pooled and expanded. The growth properties of these cells were maintained over several months, such that the cells did not appear to enter crisis or show any significant changes in their ability to quiesce or respond to mitogenic signals. Early passage NSARafER cells were then infected with a second retroviral vector, Babe-Puro [Morgenstern and Land 1990] or with the Babe-Puro vector expressing SV40 LT. Puromycin-resistant colonies expressing both ΔRaf-1:ER and LT (NSARafERLT) or ΔRaf-1:ER and the empty vector (NSARafERΔp) were pooled and expanded.

The addition of 4-hydroxy-tamoxifen (TMX) to the NSARafER cells resulted in a dramatic change in cell morphology (Fig. 1A). When viewed by time-lapse microscopy, elongation of the normally flat cells could be seen as early as 6 hr. In addition to the cells exhibiting a highly refractile phenotype, they also extended processes and became more motile. This motility was not inhibited by cell-cell contact as the cells would move across each other. These morphological changes were indistinguishable from those seen when Schwann cells are injected with Ras protein [Ridley et al. 1988] or those reported in Schwann cells isolated from late-stage embryos with a homozygous deletion of the NF1 gene [Kim et al. 1995]. The activation of Raf in the cells coexpressing LT (NSARafERLT) led to similar morphological changes and increased cell motility, whereas the addition of TMX to Schwann cells expressing the empty LXSN vector (NSE) had no detectable effects on the morphology of the cells [data not shown].

In order to address the effects of the activation of ΔRaf-1:ER on the proliferation of normal Schwann cells, the cells were analyzed for bromodeoxyuridine (BrdU) incorporation and DNA content by flow cytometry. The addition of TMX to the NSARafER cells resulted in a cell cycle arrest in the G<sub>1</sub> phase of the cell cycle, whereas TMX had no effect on the cell cycle profile of the control NSE cells (Fig. 1B). This inhibition of cell growth was confirmed by <sup>3</sup>H-thymidine uptake assays, which showed a ~80% decrease in DNA synthesis [Fig. 1C, right]. When the cells were followed by time-lapse microscopy a complete cessation of cell division was observed within 30 hr. This was not associated with any observable cell death during the 72 hr of the experiment [data not shown]. Our previous results showed that Ras induced both a G<sub>1</sub> and G<sub>2</sub> arrest in Schwann cells [Ridley et al. 1988]. However, these experiments were carried out in cells coexpressing a temperature-sensitive LT and it is possible that the G<sub>2</sub> arrest is attributable to the removal of LT from cells cultured habitually in the presence of LT [Gonas et al. 1996]. In contrast to cells expressing ΔRaf-1:ER alone, cells coexpressing LT [NSARafERLT] were not inhibited in response to the activation of ΔRaf-1:ER; indeed, ΔRaf-1:ER increased the rate of DNA synthesis [Fig. 1C, right]. The mitogenic effect of Raf in the NSARafERLT cells was seen more clearly when the cells were incubated in Dulbecco's modified Eagle medium (DMEM) containing 2% FCS without glial growth factor (GGF) or forskolin, conditions in which the NSARafER cells quiesce and the LT cells grow more slowly [Fig. 1C, left]. Under these conditions, addition of TMX to the NSARafERLT cells led to a greater than three-fold increase in the rate of DNA synthesis as measured by <sup>3</sup>H-thymidine uptake. The activation of ΔRaf-1:ER had no significant effect on DNA synthesis in the quiescent NSARafER cells [Fig 1C, left].

Thus, Raf behaves similarly to Ras in Schwann cells by inducing distinct morphological changes and a cell cycle arrest. As with Ras, the cell cycle block induced by Raf is overcome by coexpressing LT and in this scenario the Raf signal is reinterpreted as a mitogenic stimulus. This would suggest that in Schwann cells it is the Raf signaling pathway that is responsible for both Ras-induced morphological transformation and the effects of Ras on the cell cycle.

**Constitutive activation of MAP kinase by ΔRaf-1:ER in Schwann cells**

As an indicator of the activation of signaling pathways downstream from Raf, we compared the kinetics of mitogen-activated protein (MAP) kinase activation following mitogen stimulation in the presence and absence of activated ΔRaf-1:ER. Treatment of quiescent NSARafER cells with mitogens resulted in a transient activation of p42-ERK-2, as measured by either a phosphorylation-dependent mobility shift that has been shown to be characteristic for MAP kinase activity [Fig. 1D] or an in vitro kinase assay [data not shown]. In contrast, the addition of TMX to the NSARafER cells in the presence [Fig. 1D] or absence (not shown) of exogenous mitogens resulted...
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Figure 1. Activation of Raf in primary Schwann cells leads to the constitutive activation of MAP kinase, morphological alterations, and a G1-specific cell cycle arrest. (A) Phase-contrast micrographs of pools of primary Schwann cells infected with a retrovirus encoding ΔRaf-1:ER, grown in the presence or absence of 200 nM TMX for 30 hr. (B) Pools of asynchronously growing NSARafER cells and control NSE cells were cultured in the presence or absence of 200 nM TMX for 30 hr. Cells were trypsinized and examined for DNA content by propidium iodide staining and flow cytometry and also analyzed for BrdU uptake in the 4 hr prior to lysis (see insets). (C) NSE, NSARafER and NSARafERLT were seeded into DMEM supplemented with 2% FCS. Forty-eight hours later the cells were treated with TMX or ethanol [-TMX] in the absence [left] or presence [right] of fresh medium supplemented with 3% FCS, forskolin, and GGF. [3H]Thymidine uptake was measured between 12 and 28 hr following the addition of hormone. (D) NSARafER cells were quiesced for 48 hr in DMEM supplemented with 2% FCS. The cells were lysed at the indicated time points following the addition of fresh medium supplemented with GGF and forskolin and 200 nM TMX. The lysates were equalized for protein content and then Western blotted using the anti-ERK-2 polyclonal antiserum 122.
in the prolonged activation of p42-ERK-2. It therefore appears that the constitutive activity of ΔRaf-1:ER, which results in a cell cycle arrest, is overriding the growth-promoting signals by the mitogens. The activation of ΔRaf-1:ER in cells coexpressing LT (NSARafERLT) also led to the prolonged activation of p42-ERK-2, with kinetics indistinguishable from those seen in the NSARafER cells [not shown]. Thus depending on the presence of LT, a constitutive Raf signal has opposing effects on the cell cycle.

Raf arrests Schwann cells prior to induction of cyclin A

To investigate the mechanism by which Raf causes a cell cycle arrest and how this is overcome in the NSARafERLT cells, we decided to study the effects of Raf on cyclin/cdk activity. In an initial experiment, we addressed whether the activation of Raf was capable of blocking NSARafER cells from entering the first S phase following growth factor stimulation of quiescent cells, as this would enable us to analyze the effects of Raf in a synchronized cell population. Subconfluent NSARafER cells were rendered quiescent by incubating them in medium containing 2% FCS for 2 days. Pulse-labeling with 3H-thymidine showed that entry into S phase occurred 16–20 hr following mitogen addition with a peak of DNA synthesis between 20 and 28 hr [Fig. 2A]. The coaddition of TMX resulted in a ~80% decrease in the proportion of cells reaching the first S phase. This inhibition was not attributable to a delay in the cells entering S phase because further incubation did not lead to a significant increase in the incorporation of 3H-thymidine in cells treated with TMX, whereas the untreated cells continued to cycle [not shown].

To determine whether this ΔRaf-induced arrest was linked to changes in cyclin or cdk expression, cell lysates were prepared at various time points and analyzed by immunoblotting. Cyclin D1 levels were very low in the quiescent cells [Fig. 2B] and were induced as the cells entered G1. In the presence of TMX, the levels of cyclin D1 were superinduced. This induction is not dependent on the presence of growth factors as it can be seen in the absence of factor addition with similar kinetics [data not shown]. Other studies have reported that Ras and Raf expression and transformation leads to a marked elevation of cyclin D1 levels in immortalized rodent fibroblasts [Liu et al. 1995; Winston et al. 1996]. Our results demonstrate that the activation of the Raf pathway is sufficient to induce cyclin D1 in nonestablished cells. Cyclin E levels appeared to be invariant as the cells progressed from the quiescent state through G1 and were unaffected by the activation of ΔRaf-1:ER, suggesting that modulation of cyclin E levels is not the principal mechanism controlling cell cycle progression in primary Schwann cells. Cyclin A protein, although present in significant amounts in the quiescent cells, was induced as the cells progressed toward and entered S phase. However, in the cells treated with TMX, this induction of cyclin A was not seen [Fig. 2B]. Cdk4 expression increased slightly during G0/G1 progression, whereas cdk2 levels remained relatively unchanged. However, neither cdk4 nor cdk2 levels appeared to be affected by ΔRaf-1:ER activation. These results suggest that activation of ΔRaf-1:ER leads to a cell cycle arrest in G1 prior to the induction of cyclin A.

Raf inhibits cyclin E- and cyclin A-dependent kinase activity

The finding that the block in G1 appeared to occur prior to the induction of cyclin A was also reflected by the low levels of cyclin A protein observed when growing cells were arrested by activation of ΔRaf-1:ER [Fig. 3A, top left]. Immunoprecipitation of cyclin A complexes from these cells showed that there was a corresponding decrease in cyclin A/cdk2 complexes [Fig. 3A, top right] and cyclin A-dependent kinase activity [Fig. 3A, bottom]. It appears that in Schwann cells, virtually all the cdk2 associated with cyclin A is the faster migrating isoform of cdk2, which has been shown to correspond to the ac-
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Figure 3. Raf activation inhibits cyclin A- and cyclin E-dependent kinase activity. Asynchronously growing NSARafER cells were cultured in the presence (+) or absence (−) of 200 nM TMX for 30 hr. Protein lysates were prepared and standardized for protein content. (A,B topleft) Western analysis for cyclin A (A) and cyclin E (B). Lysates (100–300 μg) were subjected to immunoprecipitation with a cyclin A monoclonal antibody [A] or a cyclin E antibody [B]. The controls were protein G-Sepharose beads alone for the cyclin A antibody or a peptide block for the cyclin E antibody. The immunoprecipitates were either Western blotted with a cdk2 antibody [top right] or assayed for histone H1 kinase activity [bottom]. (* Alternative spliced form of cdk2 seen in rodent cells (Matsushima et al. 1994).)

rative form phosphorylated on residue Thr-160 (Gu et al. 1992). This may reflect high CDK-activating kinase (CAK) activity in Schwann cells or possibly a preferential association of cyclin A with the activated kinase.

Unlike cyclin A, cyclin E levels were unaffected by activation of ∆Raf-1:ER [Fig. 3B, top left]. Moreover, immunoprecipitation of cyclin E complexes from both growing and arrested NSARafER cells showed that there were similar levels of cyclin E/cdk2 complexes in the growing and arrested cells [Fig. 3B, top right]. The cdk2 complexed to cyclin E, as for the cyclin A complexes, was mostly the CAK-phosphorylated form. Interestingly, however, the cyclin E-dependent kinase activity associated with these complexes was reduced almost to background levels in the arrested cells [Fig. 3B, bottom]. Thus, activation of Raf leads to a dramatic decrease in the specific activity of cyclin E/cdk complexes. The loss of both cyclin E and cyclin A-dependent kinase activities in the arrested cells was reflected by a similar inhibition of cdk2 precipitable H1 kinase activity (data not shown). Cyclin E, cyclin A, and cdk2 activity have each been shown to be required for entry into S phase (Girard et al. 1991; Pagano et al. 1992; Zindy et al. 1992; Tsai et al. 1993; van den Heuvel and Harlow 1993; Ohtsubo et al. 1995). Therefore it is likely that the inhibition of these cyclin/cdk complexes is sufficient to arrest the cells in G1.

Raf induces p21Cip1 expression

Because cyclin E/cdk2 activity was reduced without a concomitant decrease in the expression of the proteins, we investigated whether Raf induction affected the expression of cdk inhibitors, such as p21Cip1 (el-Deiry et al. 1993; Harper et al. 1993; Noda et al. 1994) and p27Kip1 (Polyak et al. 1994; Toyoshima and Hunter 1994). Lysates were prepared from NSARafER cells grown in the presence or absence of TMX and the inhibitor levels were analyzed by Western blotting. Activation of Raf had no effect on p27Kip1 levels [Fig. 4A]. However, Raf activation induced a large increase in the levels of p21Cip1 [Fig. 4B]. The addition of TMX to the control NSE cells had no effect on the levels of p21Cip1 (data not shown). Samples of each lysate were also immunoprecipitated with cyclin E antibodies and the precipitated proteins were immunoblotted with p21Cip1 antibodies. Figure 4C shows that the induction of Raf in the NSARafER cells resulted in a corresponding increase
in the amount of p21\textsuperscript{Cip1} associated with cyclin E complexes.

To further address whether the induction of p21\textsuperscript{Cip1} is responsible for the suppression of cyclin E-dependent kinase activity and the inhibition of DNA synthesis in Raf-arrested cells, we determined the kinetics of these three events. The rate of the \Delta Raf-1:ER-induced inhibition of DNA synthesis was analyzed by measuring the incorporation of \textsuperscript{3}H-thymidine in NSARafER cells pulse-labeled at various time points. These experiments showed that the growth inhibition induced by \Delta Raf-1:ER commenced after a 12-hr lag period with significant decreases in \textsuperscript{3}H-thymidine uptake apparent after 14 hr and complete inhibition after 24 hr (Fig. 5A). The late onset of the growth arrest induced by \Delta Raf-1:ER was paralleled by a similar delay in the induction of p21\textsuperscript{Cip1}, with a small increase seen at 10 hr and maximal induction at -22 hr (Fig. 5B). This delayed appearance of p21\textsuperscript{Cip1} was in contrast to the rapid induction of cyclin D1, which could be seen as early as 3 hr following TMX addition to growing (not shown) or quiescent cells (Fig. 2B). The decrease in cyclin E-dependent kinase activity coincided with the induction of p21\textsuperscript{Cip1} and preceded the inhibition of DNA synthesis [Fig. 5A], arguing that p21\textsuperscript{Cip1} is responsible for the inhibition of the kinase activity that results in the growth arrest of the cells.

**Raf induction of p21\textsuperscript{Cip1} and cell cycle arrest are p53 dependent**

Schwann cells coexpressing \Delta Raf-1:ER and LT (NSARafERTLT) no longer arrest in response to \Delta Raf-1:ER activation. Although it has been reported that LT can counteract the inhibitory effect of p21\textsuperscript{Cip1} in cotransfection experiments (Harper et al. 1993), we were interested to explore whether \Delta Raf-1:ER would induce p21\textsuperscript{Cip1} in these cells. Pools of NSARafERTLT cells and control

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**Figure 4.** Activation of Raf leads to an increase in p21\textsuperscript{Cip1}. NSARafER cells were grown for 30 hr in the presence of TMX (+) or the solvent control (-). Thirty micrograms of protein lysates was subjected to Western blot analysis with an anti-p27 antibody (A) or an anti-p21 antibody (B). Three hundred micrograms of the lysates was immunoprecipitated with the cyclin E antibody and Western blotted with an anti-p21 antibody.

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**Figure 5.** The kinetics of p21\textsuperscript{Cip1} induction, cyclin E-dependent kinase activity, and DNA synthesis following Raf activation. Asynchronously growing NSARafER cells were incubated in the presence of TMX (+) or control solvent (-). (A) Protein lysates were prepared at the times indicated. Three hundred micrograms of the lysates was immunoprecipitated with an anti-cyclin E antibody and assayed for histone H1 kinase activity. (+) DNA synthesis was measured by the uptake of \textsuperscript{3}H-thymidine added between the times indicated. The results are expressed as the amount of activity relative to the corresponding control activity. (B) Protein lysates were prepared at the indicated time points or from quiescent NSARafER cells (Q) and were subjected to immunoblotting analysis with an anti-p21 antibody.
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Figure 6. The induction of p21<sup>Cip1</sup> in Raf-arrested cells is p53-dependent. NSARafER cells were infected with retroviral vectors expressing a dominant-negative form of p53 (p53<sup>3175</sup>). Pools of NSARafER<sub>R</sub>, NSARafER<sub>p53<sup>3175</sup></sub>, and NSARafER<sub>LT</sub> cells were grown in the presence of TMX (+) or the control solvent (−) for 30 hr. (A) Protein lysates were prepared and analyzed by Western blotting with an anti-p21 antibody or an anti-cyclin D1 antibody as indicated. (B, C) Three hundred micrograms of lysates was immunoprecipitated with an anti-cyclin E antibody and then (B) analyzed by Western blotting with an anti-p21<sup>Cip1</sup> antibody or (C) assayed for histone H1 kinase activity. (D) The NSARafER<sub>p53<sup>3175</sup></sub> cells were trypsinized and analyzed for DNA content by propidium iodide staining and flow cytometry and for BrdU incorporation in the 4 hr prior to trypsinization (see insets).

Completely deficient in their ability to arrest in G<sub>1</sub> in response to p53-dependent growth inhibitory signals [Brugarolas et al. 1995; Deng et al. 1995; Waldman et al. 1995]. Therefore, we were interested in whether the induction of p21<sup>Cip1</sup> in response to Raf was p53-dependent and whether inactivation of p53 was capable of abolishing the ΔRaf-1:ER-induced growth arrest. NSARafER cells were infected with a Babe-Puro retroviral vector constructed to encode a dominant negative mutant (dn) of p53 (p53<sup>3175</sup>), which has been shown to inhibit the activity of endogenous p53 [Kern et al. 1992]. Puromycin-resistant colonies were pooled and expanded. Proliferating NSARafER<sub>p53<sup>3175</sup></sub> were incubated in the presence or absence of TMX. Lysates were prepared and FACS analysis was carried out on the cells 30 hr after the addition of hormone. Immunoblot analysis of the lysates showed that as with the LT-expressing cells, the basal levels of p21<sup>Cip1</sup> expression were reduced in the cells expressing a dominant-negative mutant of p53 (dn-p53) and ΔRaf-1:ER activation no longer stimulated a significant induction of p21<sup>Cip1</sup> [Fig. 6A]. Consistent with this finding, equivalent levels of p21<sup>Cip1</sup> were precipitated by cyclin E antibodies from the same lysates [Fig. 6B]. Similar results were obtained with NSARafER cells infected with a retrovirus expressing the carboxy-terminal oligomerization domain of p53 [data not shown]. Thus ΔRaf-1:ER induction of p21<sup>Cip1</sup> in Schwann cells is p53-dependent.

Cyclin D1 induction by ΔRaf-1:ER, unlike p21<sup>Cip1</sup>, does not appear to be p53-dependent. Immunoblot analysis of lysates of the NSARafER<sub>p53<sup>3175</sup></sub> cells showed that Raf stimulated a large induction of cyclin D1, similar to that seen in the parental cells [Fig. 6A]. This observation demonstrates that p53 is required only for a specific subset of Raf signals and suggests that the levels of cyclin D1 induced by Raf are unlikely to be involved in the growth arrest of these cells. Cyclin D1 levels are lower in NSARafER<sub>LT</sub> cells, a finding consistent with other reports that have shown that RB-binding proteins such as LT down-regulate cyclin D1 expression and these cells no longer require cyclin D1 function to cycle [Lukas et al. 1994]. Raf activation is still able to stimulate cyclin D1 expression in these cells but only to levels found in the uninduced NSARafER cells [Fig. 6A], indicating that the pathway required for cyclin D1 induction remains active in LT-expressing cells.
Cyclin E-dependent kinase assays performed on aliquots of lysates from NSARafERp53<sup>−/−</sup> showed that in the presence of dn-p53, ΔRaf-1:ER no longer inhibited the kinase activity but instead stimulated a two-fold increase in kinase activity (Fig. 6C). Likewise, in the NSARafERLT cells Raf also stimulated an increase in cyclin E-dependent kinase activity. Thus, in the absence of p21<sup>Cip1</sup> induction, signals from Raf increase cyclin E-dependent kinase activity.

FACS analysis of the NSARafERp53<sup>−/−</sup> cells showed that their cycling was unaffected by ARaf-l:ER activation (Fig. 6D), demonstrating that the Raf-induced growth arrest is dependent on normal p53 function. This finding indicates that the increase in cyclin E-dependent kinase activity stimulated by Raf is not the result of increased cell cycling and is thus likely to be a more direct effect of Raf activation. These results would also suggest that the elevation in cyclin D levels and the increased cyclin E-dependent kinase activity are not sufficient to effect the cell cycle distribution of these cells.

Induction of p21<sup>Cip1</sup> is necessary for Raf-induced cell cycle arrest

We have shown that the induction of p21<sup>Cip1</sup> by Raf is p53-dependent. As it has been shown previously that p21<sup>Cip1</sup> is at least partly responsible for the G<sub>1</sub> arrest in response to p53-dependent radiation damage (Brugarolas et al. 1995; Deng et al. 1995; Waldman et al. 1995), we compared the levels of p21<sup>Cip1</sup> induced by ΔRaf-1:ER and γ-irradiation in primary Schwann cells. NSARafER cells were treated with either 5 Gy γ-irradiation or TMX. [<sup>3H</sup>]Thymidine-uptake assays showed that this led to a 75% and 80% inhibition of DNA synthesis, respectively. As expected, NSARafERp53<sup>−/−</sup> cells were mostly protected from the inhibition of DNA synthesis induced by γ-irradiation or TMX with only a 24% and 3% inhibition of DNA synthesis seen in these cells, respectively. Western analysis of lysates prepared from parallel plates showed that the levels of p21<sup>Cip1</sup> induced by ΔRaf-1:ER were equivalent to those induced by γ-irradiation [Fig. 7A] and that the induction was reduced greatly in the cells expressing dn-p53. Thus the levels of p21<sup>Cip1</sup> induced by Raf are likely to be sufficient to arrest the cells.

To further test the role of p21<sup>Cip1</sup> in the growth arrest elicited by Raf we attempted to reduce the induction of p21<sup>Cip1</sup> by expression of antisense RNA. NSARafER cells were infected with the Babe-Puro retroviral vector constructed to express antisense rat p21 mRNA. Clones were picked and expanded. [<sup>3H</sup>]Thymidine was added 20 hr following tamoxifen addition. At 30 hr the cells were lysed. TCA precipitated material was filtered and counted. The results for clones 1, 2, 7, and 9 are shown. (C) Cell lysates of clones 1, 2, 7, and 9 were prepared 26 hr following the addition of ethanol (−) or TMX (+) to growing cells. Thirty micrograms of protein was analyzed by Western blotting with an anti-hER, anti-cyclin D1, or anti-p21 antibody as indicated. (D) Cell lysates of clones 1, 2, 7, and 9 (30 μg protein) were analyzed by Western blotting with an anti-p53 antibody.

Figure 7. Induction of p21<sup>Cip1</sup> is responsible for the G<sub>1</sub> arrest. (A) Preconfluent NSARafER and NSARafERp53<sup>−/−</sup> cells were treated with either 200 nM TMX or 5 Gy of γ-irradiation. Lysates were prepared 24 hr following stimulation. Thirty micrograms of protein lysates were subjected to immunoblot analysis with an anti-p21 antibody. (B) NSARafER cells were infected with the Babe-Puro retroviral vector constructed to express antisense rat p21 mRNA. Clones were picked and expanded. [<sup>3H</sup>]Thymidine was added 20 hr following tamoxifen addition. At 30 hr the cells were lysed. TCA precipitated material was filtered and counted. The results for clones 1, 2, 7, and 9 are shown. (C) Cell lysates of clones 1, 2, 7, and 9 were prepared 26 hr following the addition of ethanol (−) or TMX (+) to growing cells. Thirty micrograms of protein was analyzed by Western blotting with an anti-hER, anti-cyclin D1, or anti-p21 antibody as indicated. (D) Cell lysates of clones 1, 2, 7, and 9 (30 μg protein) were analyzed by Western blotting with an anti-p53 antibody.

with the levels being induced to a similar extent by the addition of tamoxifen (Fig. 7C) as reported previously (Samuels et al. 1993). Cyclin D1 expression was also induced to a similar extent in clones 7 and 9 when compared with arresting clones [Fig. 7C] or with cells expressing empty vector (not shown). These results dem-
onstrate that Raf appears to be similarly active in all four clones in response to tamoxifen. However, in both clones 7 and 9 the ability of ΔRaf-1:ER activation to induce p21\(^{CIP1}\) levels was specifically attenuated (Fig. 7C).

This phenotype is similar to cells expressing mutant p53. We therefore compared the p53 status of the four clones. As shown in Figure 7D, the clones express similar levels of p53. Because mutant p53 proteins are more stable than their wild-type counterparts [Finlay et al. 1988; Harvey and Levine 1991], this indicates that all clones express normal p53. Taken together, these results support the idea that p21\(^{CIP1}\) induction is responsible for the G1 cell cycle arrest induced by Raf, although we cannot rule out other mechanisms contributing to the partial inhibition still seen in clones 7 and 9.

Raf and LT cooperate to increase cyclin A/cdk2 activity and induce DNA synthesis

Although Schwann cells expressing dn-p53 do not arrest in response to ΔRaf-1:ER, it is only in LT-expressing Schwann cells that ΔRaf-1:ER causes an increase in cell cycling. This indicates that in addition to inhibiting p53 activity, LT has additional effects on the cell cycle (Fig. 8A). It is unlikely that this involves the ΔRaf-1:ER-induced increase in cyclin E-dependent kinase activity, as similar levels of kinase activity were found in cells expressing dn-p53 or LT (Fig. 6D). In contrast, cyclin A-dependent kinase activity was found to be consistently higher in the LT cells (Fig. 8B). Western blot analysis showed that NSΔRafERLT cells had elevated levels of cyclin A and cdk2 when compared with NSΔRafER or NSΔRafERP53\(^{175}\) cells (Fig. 8B), whereas cyclin E levels were unaffected by LT expression [not shown]. Cyclin A and cdk2 levels were unaffected by the removal of mutants [not shown] or by the activation of Raf [Fig. 7B], demonstrating that the increase in expression levels does not correlate with the proliferative rate of the cells and thus appears to be the direct result of LT expression. These findings are consistent with those reported by Os-shima et al. [1993], showing that the expression of LT in primary rat lung epithelial cells resulted in a dramatic increase in the expression of cyclin A and cdc2 proteins.

To address whether the elevated levels of cyclin A complexes induced by LT may contribute to the ability of Raf to stimulate DNA synthesis in the NSΔRafER cells, we infected NSΔRafERp53\(^{175}\) cells with a Babe-Hygro retroviral vector constructed to encode cyclin A. Consistent with a role of cyclin A in the increased proliferative response to ΔRaf-1:ER, polyclonal populations of these cells showed an increased proliferative response to ΔRaf-1:ER, whereas Babe-Hygro-infected control cells behaved as the parental cells [Fig. 8A, right]. These results show that the induction of cyclin A by LT is likely to contribute to the increased proliferation seen in response to Raf, although as LT is a multifunctional protein, other factors are likely to be involved as well.

When we measured cyclin A-dependent kinase activity in NSΔRafERLT cells we found that in the complete absence of exogenous mitogens, the complexes were mostly inactive, even though the levels of the complexes were similar to those seen in the presence of mitogens [Fig. 8C]. Activation of ΔRaf-1:ER in these conditions stimulated a 3- to 4-fold increase in the proportion of cells in S phase, as measured by FACS analysis or \(^{3}H\)thymidine uptake [not shown] and resulted in a 8- to 20-fold increase in kinase activity [Fig. 8C, top]. This activation was not associated with a detectable change in the levels of cyclin A/tdk2 complexes [Fig. 8C, bottom] and thus represents an increase in the specific activity of the complexes. Addition of mitogens also increased the cyclin A-dependent kinase activity and subsequent activation of Raf resulted in a further two- to fivefold stimulation of kinase activity [Fig. 8C], although a small increase [less than twofold] in cdk2 levels was seen upon Raf activation under these conditions. Activation of cdk2-dependent kinase activity was stimulated to a similar extent [not shown], indicating the increase in kinase activity was at the level of the cyclin A/tdk2 complexes.

Thus Raf signaling and LT function not only converge to regulate cyclin E/tdk2 activity but also cooperate to increase cyclin A/tdk2 activity with LT-inducing cyclin A/tdk2 levels and Raf increasing the specific activity of these complexes. This effect is most apparent in the absence of exogenous mitogens, suggesting that Raf and mitogens may activate the complexes by a similar mechanism. Thus cooperation between Raf and LT involves both the loss of a cell cycle inhibitor protein and the synergistic activation of cyclin A complexes.

Discussion

Cooperating oncogenes target cyclin/cdks

We have shown that cooperating oncogenes can regulate cyclin/cdk complexes by distinct inhibitory and activating mechanisms. Such regulation appears to play a key role in determining the specificity of the cellular response to the Ras/Raf pathway. Activation of an inducible Raf protein alone results in a G1-specific cell cycle arrest mediated by an induction of p21\(^{CIP1}\) and the concomitant inhibition of cyclin/cdk activity. In the presence of LT or dominant-negative mutants of p53, however, the p21\(^{CIP1}\) induction is suppressed, the ability of Raf to increase the specific activity of cyclin-dependent kinases is revealed, and the growth arrest abolished [Fig. 9]. This effect is most evident in cells containing LT, as they express higher levels of cyclin A/tdk2 complexes and in these cells Raf activation is sufficient to stimulate proliferation in the absence of exogenous growth factors.

Our experiments support the idea that the p21\(^{CIP1}\) induction is at least in part responsible for the Raf-dependent cell cycle arrest. As such, its suppression by cooperating oncogenes plays an essential role in cellular transformation. Indeed, it appears that the suppression of cdk inhibitor proteins is a common property of immortalizing oncogenes that cooperate with Ras and/or Raf. E1A is able to bind to and block the inhibitory effects of

DNA damage-independent role of p53

The activity of p53 is pivotal to the switch in Raf from a growth-inhibitory to a stimulatory signal. Previous work has demonstrated a critical role of p53 in G1 arrest and apoptosis induced by DNA damage after UV irradiation (Kastan et al. 1992, Kuhrbittz et al. 1992). Here we show that the activation of the Raf pathway arrests Schwann cells in a p53-dependent fashion in the absence of DNA damage. Thus, it is tempting to speculate that the ability of p53 to modulate the proliferative response to signals such as Ras/Raf activation may play an important role in its function as a tumor suppressor gene. It is possible that the constitutive activation of the Raf pathway may...
The ARaf-induced G1 arrest is preceded by a loss of cyclin A-dependent kinase activity. The ARaf-induced growth arrest is mimicked by inhibitors or activators of the MAP kinase pathway (Liu et al. 1996). The role of the induction of p21Cip1 expression is induced as an immediate early gene, by various mitogens and differentiation agents and by the growth inhibitory peptide transforming growth factor-β (TGF-β), in a p53-independent fashion (Jiang et al. 1994; Li et al. 1994; Michieli et al. 1994; Steinman et al. 1994) and the response to mitogens can be blocked or mimicked by inhibitors or activators of the MAP kinase pathway (Liu et al. 1996). The role of the induction of p21Cip1 as cells enter the cycle is unclear, although it has been proposed that p21Cip1 may act as an assembly factor of cyclin/CDK complexes (Zhang et al. 1994). Elevated levels of p21Cip1 expression are also associated with the differentiated state of specific tissues and have been postulated to be involved in the maintenance of the quiescent differentiated state (Haley et al. 1995; Macleod et al. 1995). The latter has also been shown to be a p53-dependent mechanism. In Schwann cells, we show that the Raf-dependent induction of p21Cip1 is a p53-dependent process and thus differs from the immediate early response seen following mitogen stimulation. It will be of interest to dissect the mechanisms involved in this p53-dependent induction.

In contrast to the rapid induction of cyclin D1 in response to RAF activation, the induction of p21Cip1 is delayed. This suggests that the mechanism by which RAF stimulates p21Cip1 expression may be indirect. The kinetics of the induction, however, may explain how activation of the same pathway, that is, the MAP-kinase pathway, can result in opposing effects on the cell cycle. If constitutive activation of the MAP kinase pathway for several hours is required to induce p21Cip1, this may partly explain why transient activation of the same pathway by mitogens stimulates rather than inhibits the cell cycle.
Raf activates cyclin/cdk activity in presence of a cooperating partner

In the absence of normal p53 function, Raf activation results in an increase in cyclin/cdk activity, presumably by a mechanism that can be suppressed by \( p21^{\text{Cip1}} \). This demonstrates that the effects of an oncogene cannot be gauged solely by the introduction of a single oncogene into a primary cell, as the specificity of the cellular response can depend on the expression of other cooperating genes. In addition to inhibiting p53, LT leads to the constitutive overexpression of cyclin A/cdk2 complexes with Raf activation causing a superinduction of the kinase activity. In this context it is noteworthy that although loss of functional p53 abolishes the Raf-induced cell cycle arrest, only cells expressing LT respond to Raf activation with increased proliferation. The elevation of the cyclin A complexes appears to be at least partly responsible for the ability of Raf to stimulate proliferation in these cells, as p53-defective cells infected with a cyclin A-carrying retrovirus are similarly induced to cycle by Raf. Preliminary experiments indicate that the mechanism of this activation does not involve further alterations in the levels of \( p21^{\text{Cip1}} \) or \( p27^{\text{Kip1}} \) or cdc25-dependent dephosphorylation of cdk2 [A. Lloyd and H. Land, unpubl.]. As the activation of cyclin-dependent kinase activity appears to be induced in a similar fashion by growth factors [Fig. 8C] it will be important to identify the mechanisms involved in this process.

Implications for neurofibromatosis

The model system that we have developed demonstrates how progressive genetic changes contribute to the transformed phenotype and describe some of the molecular mechanisms involved. Raf alone leads to a change in the morphology of the cells, coincident with an increase in cell motility and the induction of growth factor secretion, all of which can be imagined to disrupt the microenvironment of the cell. However, these cells are growth-arrested as a result of the induction of \( p21^{\text{Cip1}} \). Inactivation of p53 abolishes the growth arrest, while retaining the other Raf-dependent properties. The coexpression of LT, in addition, results in the cooperative activation of cyclin A-dependent kinases, and these cells acquire the ability to proliferate in response to the Raf signal in the absence of exogenous mitogens.

In neurofibromatosis type 1, the Ras pathway is activated via inactivation of neurofibromin, a Ras-GAP [Ballester et al. 1990; Martin et al. 1990; Xu et al. 1990a,b]. Inhibition of the Ras pathway in tumor cells derived from these patients results in a reversion of the tumor cells, confirming the role of Ras in tumor formation in this disease [Basu et al. 1992; DeClue et al. 1992]. We have shown that activation of Raf in primary Schwann cells, as reported previously for Ras [Ridley et al. 1988], results in a growth arrest of the cells. In addition, a recent report described the isolation of Schwann cells from NF-/- embryos. These Schwann cells were shown to have elevated levels of Ras–GTP, resembled Ras-infected Schwann cells, and grew much more slowly than the wild-type cells [Kim et al. 1995], demonstrating that the observable NF-/- phenotype is mimicked by activation of the Ras/Raf pathway. The poor growth properties of primary Schwann cells in which the Ras pathway is activated suggest that further genetic events are required for tumor formation in this cell type. It will be of great interest to investigate whether the benign and malignant tumors found in patients suffering from neurofibromatosis type 1 have further genetic defects that result in the suppression of the effects of \( p21^{\text{Cip1}} \). These may involve the loss of \( p21^{\text{Cip1}} \) or p53 expression, mutations that affect cyclin/p21^\text{Cip1} interaction, or increases in the levels of cyclin/cdk complexes. Interestingly, reports of p53 mutations in neurofibrosarcomas from neurofibromatosis type 1 patients have been reported previously [Menon et al. 1990].

Oncogene cooperation in cell regulation

Cooperating oncogenic lesions are expected to increase the proliferative advantage of tumor cells. However, it is remarkable that as part of this process they enhance the oncogenic potential of each other. This applies not only to the cooperation between Raf and LT or loss of p53 function, but also to the cooperation between Myc and Bcl-2 where the latter blocks Myc-induced apoptosis and reveals the ability of Myc to induce cell cycle entry [Fanidi et al. 1992]. The Ras/Raf pathway is involved in many cellular responses to peripheral signals and in the experimental system used here activates two mutually exclusive cellular programs: cell cycle progression and cell cycle arrest. The decision of whether to progress through the cell cycle or to arrest, however, is determined by multiple signals converging at the regulation of cyclin/cdk complexes. How cellular signaling networks specify cellular decisions is of central importance to the understanding of cell regulation. Models in which the cooperation of oncogenes or other signaling molecules can be studied at the molecular level will provide a powerful tool to explore this question.

Materials and methods

Cell culture

Schwann cells were purified from 2- to 3-day old Wistar rats, as described previously [Brookes et al. 1979; Ridley et al. 1988]. The Schwann cells were cultured routinely at 37°C (10% \( \text{CO}_2 \)) in DMEM with 1.5 mg/ml glucose, supplemented with 3% FCS, 1 μM forskolin [Calbiochem] and GGF (a kind gift from Mark Noble, University of Utah, Salt Lake City), on dishes precoated with poly-L-lysine [Sigma]. Throughout all the experiments phenol red-minus medium and charcoal-stripped serum were used. To render the cells quiescent, the cells were washed twice in DMEM and incubated in either DMEM supplemented with 2% FCS or B/S medium, a mitogen-free supplement [Raf et al. 1983], for 48 hr.

Retroviral vectors

The XhoI-Cle[blunted] fragment encoding ΔRaf-1:ER [Samuels et al. 1993] was subcloned into the XhoI-BamHI[blunted] site of...
the retroviral vector (Miller and Rosman 1989). The BamHI fragment encoding SV40LT (Jat et al. 1986) and a BamHI fragment encoding human p53 (provided by David Lane) (Voiteck et al. 1992) were subcloned into the BamHI site of the Babepuro retroviral vector (Morgenstern and Land 1990). A sequenced PCR fragment encoding amino acids 302–390 of murine p53 (p53C') was subcloned into the BamHI-XhoI site of Babepuro (a kind gift of Trevor Littlewood, ICRF, London, UK). A BamHI-SalI fragment encoding the human cyclin A gene [a kind gift from Jonathan Pines, Wellcome/CRC Institute, Cambridge, UK] was subcloned into the BamHI–SalI sites of the Babepuro vector (Morgenstern and Land 1990). A Xhol-BglII fragment encoding rat p21 cDNA (R. Mazars and P. Jat, unpubl.) was subcloned into the Xhol-BamHI sites of the pBabe-Puro/2 vector. Each of the constructs was transfected, using the standard calcium phosphate method, into the packaging cell line GF+ (Markowitz et al. 1990), and G418, hygromycin, or puromycin resistance colonies were pooled and expanded.

Infection of Schwann cells

Schwann cells were infected by cocultivation, at a 1:2 ratio, with the producer cell lines, which had been pretreated for 2 hr with 20 μg/ml of mitomycin C (Sigma). Two to three days after plating the cultures were transferred into selective medium containing 400 μg/ml G418 (GIBCO) or 0.4 μg/ml puromycin (Sigma) as appropriate. Drug-resistant colonies were pooled and expanded.

FACS analysis and DNA synthesis assays

Cells (1 × 10^6 to 2 × 10^6) were preincubated for 4 hr with 10 μM BrdU (Sigma), trypsinized and then fixed in 80% ethanol. The fixed cells were then incubated with fluorescein isothiocyanate-conjugated anti-BrdU antibodies (Becton-Dickinson) and stained with propidium iodide containing RNase (20 μg/ml). Replica-tive DNA synthesis and DNA content were analyzed using bi-variate flow cytometry. For DNA synthesis assays, 5 × 10^5 cells were seeded in triplicate into six-well dishes in conditions as described in the figure legends. [3H]Thymidine was used at a concentration of 0.5 μCi/ml. At the indicated times the cells were lysed in 1% SDS and the trichloroacetic acid (TCA)-precipitable material was filtered and counted.

Western blot analysis

The cells were lysed in buffer A [1% NP-40, 50 mM Tris at pH 8, 150 mM NaCl 10 μg/ml of aprotinin, leupeptin, and pepstatin, 20 mM NaF, 1 mM NaVO₃, 100 μg/ml of PMSF]. Protein concentration was determined using the Biorad protein assay. Thirty micrograms of lysate were resolved by SDS-PAGE and electroblotted onto Immobilon P membranes (Millipore). The following antibodies were used: anti-p42 ERK-2 (122), provided by Gordon Peters (Bates et al. 1994); anti-cyclin E (Santa Cruz, sc-481); anti-cyclin A-E-23, provided by Julian Gannon and Tim Hunt (ICRF, South Mimms, UK); anti-cdk4 (Santa Cruz, sc-749); anti-cdk2 (Santa Cruz, sc-163); anti-p27 (Santa Cruz, sc-528); anti-p21(CFS6), provided by Wade Harper (Baylor College of Medicine, Houston, TX); anti-her (Samuels et al. 1993); and anti-p53 (Santa Cruz, sc-1313). Immunoreactive bands were visualized using enhanced chemiluminescence detection (Amersham International PLC).

Immunoprecipitations and kinase assays

Cells were lysed in buffer A. Three hundred micrograms of lysate was incubated with 10 μg of antibody [cyclin E-Santa Cruz 481, cyclin A-E-72, provided by Julian Gannon and Tim Hunt (Slingerland et al. 1994), cdk-2-Santa Cruz 163] for 1 hr at 37°C, followed by incubation for an additional hour with protein A– or protein G–sephrose, as appropriate. The beads were washed five times in buffer A and subjected either to Western blot analysis or to kinase assays. For kinase assays the beads were washed a further two times in kinase buffer (50 mM Tris at pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol) and then resuspended in 50 μl of kinase buffer supplemented with 50 μM ATP, 5 μCi [γ³²P]ATP and 10 μg of histone H1 (Boehringer Mannheim) for 30 min at 37°C. The samples were resolved by SDS-PAGE and exposed to Kodak X-OMAT AR. The kinase assays were quantified using ImageQuant by Molecular Dynamics.

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