Cellular and Molecular Studies Related to a Cell Line of the Oligodendrocyte-Type-2 Astrocyte Lineage Derived from a Human Glioblastoma Multiforme

by

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A thesis submitted to the Faculty of Medicine and Dentistry of The University of Birmingham for the degree of DOCTOR OF MEDICINE

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October 1995
Abstract

By using techniques from studies of the rodent oligodendrocyte-type-2 astrocyte (O-2A) lineage, the Human O-2A lineage Glioblastoma number 1 (Hu-O-2A/Gb1) cell line was grown from a human glioblastoma multiforme. This thesis describes findings related to this cell line. Receptor tyrosine kinases and other tyrosine kinase complementary DNA fragments were cloned from the cell line to provide information about growth factors to which the cells might respond. Consequently, it was found that the cells showed mitogenic and antigenic changes in several culture conditions, similar to that seen for rodent O-2A progenitor cells. The cloning data also suggested that Vascular Endothelial Growth Factor (VEGF) might affect these cells. It was found that VEGF enhanced oligodendrocyte maturation in the Hu-O-2A/Gb1 cell line and also in rodent oligodendrocytes in vitro. In addition, the HEK2 receptor tyrosine kinase found during initial cloning from the Hu-O-2A/Gb1 cell line appears to be a helpful, but not entirely specific, marker for O-2A lineage cells. A new protein kinase LIMK was cloned from the complementary DNA library of the Hu-O-2A/Gb1 cell line. LIMK is predominantly expressed in the nervous system and high expression was found in malignant glioma cell populations, their original tumours and also during human embryonic development.
To my long-suffering wife and son
and also to my mother and the memory of my father.
Acknowledgements

I would like to thank Professor Mark Noble, in whose laboratory at the Ludwig Institute for Cancer Research this work was performed. Mark provided me with the opportunity to do this work, offered constant support and advice and finally read this thesis. I would also like to thank all the other members of the Neurobiology Group for helping me with advice and interesting discussions on a huge range of various techniques in both cellular and molecular biology. It is therefore a pleasure to acknowledge: Karen Bevan, Marie-José Blouin, Ute Engel, Alan Entwistle, Ellie Landells (who sadly died), Mark Linskey, Bob Ludwig, Clare McFarlane, Margot Mayer, Chris Proschel and Guus Wolswijk, and also the group leaders Parmjit Jat and Alastair Reith.

In the text I acknowledge the contributions by other members of the Neurobiology Group and also my collaborators to the work presented here. In addition, I would like to thank Professors W.I. McDonald and J.R. Heron for their constant support and advice.

This work was supported by a Wellcome Trust Clinical Fellowship grant number 035404 and latterly by funding from the Carrie Rudolph Trust.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACM</td>
<td>50% DMEM-BS/ 50% rodent cortical astrocyte conditioned medium.</td>
</tr>
<tr>
<td>aFGF</td>
<td>Acidic fibroblast growth factor.</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate.</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor.</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs.</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromodeoxyuridine.</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxynucleic acid.</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system.</td>
</tr>
<tr>
<td>CNTF</td>
<td>Ciliary neurotrophic factor.</td>
</tr>
<tr>
<td>dATP</td>
<td>Deoxyadenosine triphosphate.</td>
</tr>
<tr>
<td>dCTP</td>
<td>Deoxycytidine triphosphate.</td>
</tr>
<tr>
<td>DEPC</td>
<td>Di-ethyl-pyro-carbonate.</td>
</tr>
<tr>
<td>ddH2O</td>
<td>Deionized, filtered and autoclaved water.</td>
</tr>
<tr>
<td>dGTP</td>
<td>Deoxyguanosine triphosphate.</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleotide triphosphate mix (dATP, dCTP, dGTP, dTTP).</td>
</tr>
<tr>
<td>dTTP</td>
<td>Deoxythymidine triphosphate.</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dubecco's modified Eagle's medium.</td>
</tr>
<tr>
<td>DMEM-BS</td>
<td>DMEM modified according to Bottenstein and Sato.</td>
</tr>
<tr>
<td>DMEM+10%FCS</td>
<td>DMEM containing 10% FCS.</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide.</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxynucleic acid.</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease.</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence.</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetra-acetic acid.</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor.</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum.</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor.</td>
</tr>
<tr>
<td>FLT</td>
<td>Fms-like tyrosine kinase.</td>
</tr>
<tr>
<td>FN</td>
<td>Fibronectin.</td>
</tr>
<tr>
<td>GalC</td>
<td>Galactocerebroside.</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde phosphate dehydrogenase.</td>
</tr>
<tr>
<td>GFAP</td>
<td>Gial fibrillary acidic protein.</td>
</tr>
<tr>
<td>GGF</td>
<td>Gial growth factor.</td>
</tr>
<tr>
<td>HEK2</td>
<td>Human embryo kinase 2.</td>
</tr>
<tr>
<td>H-NMR</td>
<td>Proton nuclear magnetic resonance.</td>
</tr>
<tr>
<td>Hu-O-2A/Gb1</td>
<td>Human O-2A lineage Glioblastoma number 1.</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin.</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobase.</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilodalton.</td>
</tr>
<tr>
<td>KDR</td>
<td>Kinase insert domain-containing receptor kinase.</td>
</tr>
<tr>
<td>LIMK</td>
<td>LIM-kinase.</td>
</tr>
</tbody>
</table>
M Molar.
MAPK Mitogen activated protein kinase.
MBP Myelin basic protein.
mg Milligrams.
ml Millilitres.
mM Millimolar.
mRNA Messenger ribonucleic acid.
MS Multiple sclerosis.
MyT1 Myelin transcription factor 1.
ng Nanogram.
nM Nanomolar.
NT-3 Neurotrophin-3.
Ø Diameter.
O-2A Oligodendrocyte-type-2 astrocyte.
OAc Acetate.
OD Optical density.
PBS Phosphate buffered saline.
PCR Polymerase chain reaction.
PDGF Platelet derived growth factor.
PDGFRα Platelet derived growth factor receptor alpha.
PLL Poly-l-lysine.
RNA Ribonucleic acid.
rpm Revolutions per minute.
SDS Sodium dodecyl sulphate.
SSC Standard saline citrate.
TE Tris-EDTA.
TGF β2 Transforming Growth Factor β2.
Tris Tris (hydroxymethyl) amino methane.
Tween 20 Polyoxyethylene (20)-sorbitan monolaurate.
µg Microgram.
µl Microlitre.
µm Micrometre.
UV Ultraviolet.
VEGF Vascular endothelial growth factor.
v/v Volume/volume.
w/v Weight/volume.
INTRODUCTION

General Introduction

In order to understand the cellular and molecular mechanisms involved in diseases of the human central nervous system affecting glia, (such as gliomas and multiple sclerosis), it is probably necessary to understand the biological properties and developmental origin of glial cells, their interaction with other cells in their microenvironment and to develop suitable markers that would allow the study of these cells in vivo. Progress in these fields has moved rapidly over the last few years.

1.1. The oligodendrocyte-type-2 astrocyte lineage

1.1.1. The history of neuroglia

The mammalian central nervous system (CNS) consists of neurons, glial cells, endothelial cells and meningial cells. The neuroglia were first described by Virchow in 1846. He viewed this as a supporting matrix for the 'fibrous' nerves, (but with a cellular component), and therefore called it neuroglia, 'nerve glue' (reviewed in Somjen 1988). With the advent of electron microscopy and perfusion fixation, the CNS was demonstrated to be mainly composed of cells, and Virchow's interneuronal matrix was seen to consist of the perikarya and processes of neuroglial cells. Knowledge of the shape and
Chapter 1  Introduction

classification of neuroglia came with the metallic impregnation techniques of Ramno y Cajal (1909-11) and del Rio Hortega (1921). The glia were classified into macroglia (subdivided into astrocytes, oligodendrocytes and ependymal cells) and microglia. Although further morphological studies and definitions have taken place there is a major difficulty with the application of this approach to the study of development, lineage and cell function. All conclusions of dynamic processes are based on inference rather than on actual demonstration of proposed lineage relationships or functional interactions.

In recent years two strategies have helped to overcome these problems. Firstly, tissue culture which allows the cellular and fluid environment to be controlled and manipulated, and secondly identification of the cell types of interest by the use of cell-type specific markers.

1.1.2. Glial cell lineages in the CNS

Several different lineages can give rise to glial cells in the rodent CNS, the species in which CNS glial development has been most extensively characterized.

There are unipotent cells such as the cells of the rat optic stalk, (the embryonic anlage of the optic nerve), which give rise to only a single glial population (Small et al. 1987), called the type-1 astrocyte (Raff et al. 1983a). It is thought that the CNS contains several types of astrocytes, but the true complexity of this family of cells is not yet established. Some experiments conducted on spinal cord cultures have suggested the existence of as many as five distinct classes of astrocytes just in this single tissue (Miller and Szigeti 1991). Certainly, it is clear that although the type-1 astrocytes of the optic nerve appear to be similar to astrocytes present in cultures generated from many other regions of the CNS (e.g. Noble and Murray 1984; Raff et al.
1983a; Raff et al. 1988), their precursor cells are not the only source of this category of glia.

There are also totipotent cells which are able to give rise to both neuronal and glial lineage cells, producing a variety of neurons as well as oligodendrocytes and astrocytes (Davis and Temple 1994; Reynolds and Weiss 1992; Vescovi et al. 1993; Williams et al. 1991). Several such precursor cell populations have been described in the CNS of embryonic, perinatal and adult rats, although the relationship between the cells studied in different laboratories is not yet clear.

Of the lineages which have restricted developmental potential, the oligodendrocyte-type-2 astrocyte (O-2A) lineage has been most extensively characterized. The precursor cells of this lineage were first identified in cultures derived from perinatal rat optic nerves (Raff et al. 1983c), where they were found to give rise to two mature glial cell populations; oligodendrocytes (the cells that produce myelin in the central nervous system) and type-2 astrocytes. Oligodendrocytes are a well characterized cell type in vivo. Although it is unclear at present whether type-2 astrocytes occur in vivo (Fulton et al. 1991), implantation of O-2A progenitor cell lines into demyelinating lesions of the rat spinal cord have shown production of both oligodendrocytes and astrocytes (reviewed in Franklin and Blakemore 1995).

Oligodendrocytic differentiation of O-2A progenitor cells occurs when progenitors were grown in chemically defined medium and progress along this differentiation pathway does not require the presence of inducing factors. In contrast, astrocytic differentiation requires the presence of appropriate inducing factors, such as the still unidentified factor or factors present in foetal sera of a number of different species (Raff et al. 1983a; Raff et al. 1983b; Raff et al. 1983c).
1.1.3. Antigenic phenotype of O-2A lineage cells

Several antigenic markers have proved useful in studying O-2A lineage cells. Cells of the O-2A lineage are characterized by being monoclonal antibody A2B5 positive (A2B5 is an antibody to a cell surface ganglioside, Eisenbarth et al. 1979) and monoclonal antibody Rat neural antigen-2 (RAN-2) negative (RAN-2 is a cell surface protein antibody, Raff et al. 1984a). Perinatal O-2A progenitor cells are A2B5 positive, Galactocerebroside (GalC) negative [GalC is a cell surface marker (Ranscht et al. 1982)], and glial fibrillar acidic protein (GFAP) negative, (GFAP is an intracellular cytoskeletal protein), (Aloisi et al. 1988; Behar et al. 1988; Raff et al. 1983c; Raff et al. 1984b). In vitro type-2 astrocytes are A2B5 positive, GalC negative and GFAP positive (Levi et al. 1986; Raff et al. 1983a; Raff et al. 1984b; Williams et al. 1985), while oligodendrocytes are A2B5 negative, GalC positive and GFAP negative, (Miller et al. 1985; Raff et al. 1978).

The antigenic progression from a perinatal O-2A progenitor cell to an oligodendrocyte involves at least four stages. The perinatal O-2A progenitor cell (first stage) which is monoclonal antibody 04 negative, (04 is an antibody to cell surface sulphatide Sommer and Schachner 1981), acquires 04 expression and becomes A2B5 positive, 04 positive and GalC negative (second stage), (McMorris and Dubois Dalcq 1988; Sanes et al. 1986). Cells in this second stage are still bipotential precursor cells which can be induced in vitro to form type-2 astrocytes (Gard and Pfeiffer 1989; Gard and Pfeiffer 1990; Trotter and Schachner 1989). During the very brief third antigenic stage, cells become A2B5 positive, 04 positive and GalC positive. However once they express GalC, they very quickly lose A2B5 expression and become A2B5 negative, 04 positive and GalC positive oligodendrocytes (stage four), (Abney et al. 1983; Levi et al. 1987). The point when they begin to express GalC appears to mark the point where O-2A lineage cells are committed to
become oligodendrocytes. Another monoclonal antibody O1 (an antibody to cell surface galactocerebroside Sommer and Schachner 1981) recognises similar but more specific epitopes than anti-GalC (Bansal et al. 1989).

1.1.4. Biological studies on the O-2A progenitor

Several small molecules have been found to have biological effects on O-2A lineage cells. Purified cortical astrocytes promote O-2A progenitor division (Noble and Murray 1984) and allow their correctly timed differentiation in vitro (Raff et al. 1985). The cortical astrocytes used in these studies expressed a phenotype similar to those of optic nerve type-1 astrocytes (Miller et al. 1985). The effects of purified cortical astrocytes appear to be mediated by Platelet Derived Growth Factor (PDGF, Noble et al. 1988; Raff et al. 1988; Richardson et al. 1988). O-2A progenitors exposed to either PDGF or astrocyte-conditioned medium exhibit a bipolar morphology, migrate extensively (with an average migration rate of 21.4 ± 1.6 µm/hr) and divide with an average cell cycle length of 18 hours (Noble et al. 1988). PDGF had the same potency as type-1 astrocytes in promoting the correctly timed differentiation in vitro of embryonic O-2A progenitors into oligodendrocytes (Raff et al. 1988). PDGF antibodies blocked the mitogenic effect of type-1 astrocytes on embryonic O-2A progenitor cells, causing these cells to cease division and to differentiate prematurely even when they were growing in type-1 astrocyte conditioned medium (Noble et al. 1988; Raff et al. 1988; Richardson et al. 1988). Neurons are also a source of PDGF and promote division of O-2A progenitors in vitro (Gard and Pfeiffer 1990; Hunter and Bottenstein 1991). The results of in situ labelling of normal adult CNS sections by PDGF complementary DNA probes suggests that neurons are the major producers of this mitogen (Sashara et al. 1991; Yeh et al. 1991),
although the specific contributions of neuronal or astrocytic production of PDGF to the development of the O-2A lineage *in vivo* is not yet known.

O-2A progenitors can also be induced to divide by Basic Fibroblast Growth Factor (bFGF) but they have a longer cell cycle length, a reduction in migratory behaviour and a different morphology, from those seen in PDGF (Bögler et al. 1990). bFGF unlike PDGF is able to promote division of oligodendrocytes (Bögler et al. 1990; Eccleston and Silberberg 1985; Saneto and DeVellis 1985). bFGF also inhibits the differentiation of purified O-2A progenitors (Mayer et al. 1993; McKinnon et al. 1990), this can be overridden by a factor or factors secreted by astrocytes (Mayer et al. 1993), and also by Ciliary Neurotrophic Factor (CNTF) and leukaemia inhibitory factor (Mayer et al. 1994).

Combining PDGF and bFGF allows O-2A progenitor division to continue in the absence of oligodendrocyte differentiation (Bögler et al. 1990). Progenitors expanded in such a manner function normally and are able to repair experimentally demyelinated lesions following transplantation *in vivo*, (Groves et al. 1993). There is also a synergistic effect of PDGF and Neurotrophin-3 (NT-3) in perpetuating the self renewal of perinatal O-2A progenitors (Barres et al. 1994) and antibodies to NT-3 have been shown to lead to decreased numbers of perinatal O-2A progenitor cells and oligodendrocytes *in vivo* (Barres et al. 1994). The ability of appropriate growth factor combinations to promote extended precursor cell division may be a general phenomenon as indicated for example by the importance of growth factor co-operation in promoting the extended division *in vitro* of haematopoetic stem cells (Cross and Dexter 1991) and primordial germ cells (Matsui et al. 1992).
1.1.5. O-2A progenitors in the adult CNS

O-2A progenitors of the adult CNS have also been studied in order to gain insights into the cellular mechanisms underlying repair of demyelinated lesions in the adult animal. Adult O-2A progenitor cells of the rat optic nerve differ from their perinatal counterparts in several ways. Adult O-2A progenitors have a unipolar morphology \textit{in vitro} (Wolswijk and Noble 1989), whereas perinatal O-2A progenitors are usually bipolar (Small et al. 1987; Wolswijk and Noble 1989). Adult O-2A progenitors also have a longer average cell-cycle time \textit{in vitro} than perinatal O-2A progenitors (65 hours versus 18 hours, Noble et al. 1988; Wolswijk and Noble 1989), migrate more slowly (4 mm/hr versus 21 mm/hr, Small et al. 1987; Wolswijk and Noble 1989) and take longer to differentiate (5 days versus 2 days for 50% differentiation, Wolswijk and Noble 1989). Furthermore, adult O-2A progenitors stimulated to divide by type-1 astrocytes are vimentin negative, (vimentin is an intracellular cytoskeletal protein), and O4 positive while dividing perinatal O-2A progenitors are vimentin positive and O4 negative (Raff et al. 1984b; Wolswijk and Noble 1989). The replacement of the perinatal progenitor population by the adult one occurs gradually (Wolswijk et al. 1990). Perinatal O-2A progenitor cells give rise to either all adult O-2A progenitors or a combination of adult O-2A progenitor cells and oligodendrocytes within a small number of cell divisions (Noble et al. 1992; Wren et al. 1992). The maintenance of these cells in the adult optic nerve may be the result of asymmetric division and differentiation of adult O-2A progenitors themselves (Wren et al. 1992). The capacity of adult O-2A progenitors to undergo asymmetric division and differentiation is consistent with the hypothesis that these cells are stem cells (Wren et al. 1992). Adult O-2A progenitors also express other properties of stem cells, such as long cell-cycle times (Wolswijk and Noble 1989; Wolswijk et al. 1991b; Wren et
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Moreover, these cells seem to be capable of undergoing prolonged self-renewal, at least *in vitro* (Wren et al. 1992). Finally, these cells have the important quality of being maintained in the optic nerve throughout life (Wolswijk and Noble 1989; Wolswijk et al. 1990). Thus adult O-2A progenitors are stem cells, (in the specific sense that these cells are capable of functioning as a self-renewing population throughout life), and perinatal O-2A progenitors differentiate within a limited time-span.

1.1.6. Studies of the human O-2A lineage

The ultimate goal of many research groups is to study the human O-2A lineage thereby verifying work in the rodent and bringing fresh insights into disease processes and possibly novel treatments. Unfortunately such studies have been complicated by the difficulties of obtaining fresh human central nervous system tissue. Preliminary studies have shown A2B5 positive perinatal O-2A progenitor-like cells from human optic nerve foetal tissue obtained from saline abortions (Kennedy and Fok-Seang 1986) and O4 positive and GalC negative cells in cultures from 7 to 8 week old human embryos (Aloisi et al. 1992). The presence of transitional or bipotential glial cells has been shown in glial cultures for adult human corpus callosum obtained at autopsy (Kim 1985) and studies have also identified putative oligodendrocyte precursor cells in cultures prepared from adult temporal lobe biopsy material (Armstrong et al. 1992b; Gogate et al. 1994). These putative oligodendrocyte precursor cells did not show division with either factors secreted by cortical astrocytes, nor with PDGF or bFGF, although such cells have been shown to synthesize deoxyribonucleic acid (DNA) when grown on an astrocyte monolayer (Scolding et al. 1995), neither did they differentiate into type-2 astrocyte like cells when exposed to foetal sera (Armstrong et al. 1992b). Because of the difficulties in obtaining enough quality fresh human
material, a profitable strategy for studying human glial lineages may be to develop tumour cell lines from gliomas, analogous to previous success in studying lineages by using tumour cell lines of the haematopoetic system.

1.2. Analysis of glioma lineages

1.2.1. Glioma classification

A glioma is a neoplasm arising from the neuro-ectodermally derived neuroglial cells of the central nervous system. Gliomas, in common with neoplasias occurring elsewhere in the body, consist of a mass of disorganised tissue formed by the abnormal and inappropriate proliferation of cells. The abnormal cell proliferation is not inhibited by the mechanisms normally controlling cell division and in most cases appears to be irreversible (Wyllie 1992). As a group gliomas vary widely in their incidence, cellular composition and most importantly in the way they affect their host. In the context of this thesis the generic term glioma is applied to astrocytic, oligodendroglial, ependymal and choroid plexus papillary tumours. The incidence of gliomas is 4 to 6 people per 100,000 of the population (Barker et al. 1976; Tola et al. 1994).

There are differences in the incidence and site of gliomas between adults and children possibly reflecting differences in their origin and biological behaviour. In adults approximately 70% of gliomas are above the tentorium cerebelli, (the frontal lobes being most commonly affected), whereas in children approximately 70% are below. Glioblastoma multiforme, a highly malignant form of glioma, accounts for approximately 50% of gliomas in adults but only 10-15% in children. Primitive neuroectodermal tumours are much commoner in children than in adults (reviewed in Green et al. 1976; Leestma 1980; Russell and Rubinstein 1989).
Current treatment strategies for gliomas involve the use of surgery, radiotherapy and chemotherapy and the application of these strategies depends on the tumour classification. The first classification of CNS tumours was formulated by Bailey and Cushing in 1926 and founded on the science of embryology (Bailey and Cushing 1926). Kernohan and colleagues in 1949 then proposed the concept of tumour grading for the purposes of assessing prognosis (Kernohan et al. 1949). In 1979 The World Health Organisation (WHO) consolidated and reconciled several diverse classifications and the WHO classification is now the main one used. Classification is based on morphology seen on histological preparations, coupled with the analysis of gene products. The resemblance of the predominant tumour cell type to a developing or a mature glial cell is used to classify the type of tumour. The tumour is then graded according to how closely its cells resemble a 'normal' adult cell: tumours composed of cells resembling mature cells are low grade or benign, while those showing minimal resemblance to mature cells are high grade or malignant (reviewed in Green et al. 1976; McComb and Bigner 1984; Russell and Rubinstein 1989).

Using the above systems of classification the main types of glioma in adults are:

1) Astrocytic tumours;
   a) Low grade astrocytic tumours (approximately 20% of adult glial tumours);
   b) Anaplastic astrocytic tumours (approximately 20% of adult glial tumours);
   c) Glioblastoma multiforme (approximately 50% of adult glial tumours);
2) Oligodendroglialomas (>5% of adult glial tumours);
3) Ependymal cell tumours (>5% of adult glial tumours);
4) Mixed tumours.
Most low grade astrocytomas occur in adults during the third and fourth decades of life. They usually arise within deep structures of the cerebral hemispheres and approximately 15% can evolve into highly malignant tumours within 5 years of presentation. The mean age for patients presenting with anaplastic astrocytomas is 46 years, most are situated in the cerebral hemispheres and the average survival is 2 years. Glioblastoma multiforme was first recognised as a tumour of glial cells by Virchow more than 100 years ago. It is the most malignant primary brain tumour with a peak age of presentation between 40 and 60 years and usually arises from the deep white matter, basal ganglia or thalamus. The hallmark of glioblastoma multiforme is necrosis, extensive vascularization and extensive migration of cells outside the primary tumour site. The average survival after diagnosis is 10 months (reviewed in Heffner 1991).

Oligodendrogliomas are usually calcified and can be haemorrhagic. Assessment of the malignant potential of oligodendrogliomas has been difficult (MacKenzie et al. 1988), but the average survival is 5 years. In general, benign ependymomas infrequently evolve into more malignant forms. The average survival of patients with benign intracerebral ependymomas is 30 to 40 months, but this varies widely, and survival is impossible to predict from histological appearances (Mork and Loken 1977).

The anatomical site of a glioma obviously determines the ultimate fate of the patient, as most patients succumb because of the effects of a space occupying lesion.

Currently used classifications have their limitations as they do not always predict the behaviour of a particular tumour in a particular site and they do not address why histologically similar tumours in different sites may behave in a dissimilar manner. These difficulties may be due to morphologically based classifications failing to identify fundamental parameters which define the biological behaviour of a particular tumour.
1.2.2. Analysis of glioma lineages

Biological analysis of glioma lineages may aid our understanding of the behaviour of particular gliomas in vivo. Neoplasia tends to deregulate or disrupt pre-existing mature as well as dormant developmental cellular mechanisms leading to tumour initiation and maintenance of preferential tumour growth. Therefore, the simultaneous study of normal glial neurobiology and glioma biology may be advantageous as discoveries in one field might lead to discoveries in the other field.

In addition, recent evidence suggests that the genetic abnormalities which are thought to be causal in the generation of gliomas seem to be grouped and these groups may identify particular families of tumours (e.g., Kraus et al. 1995; von Deimling et al. 1994; von Deimling et al. 1993). These families may arise because the ability of a genetic abnormality to alter the normal control of differentiation is dependent upon the cellular lineage in which the abnormality arises. The effects of oncogene or suppressor genes might therefore be most usefully analysed in the context of the particular cell lineage from which the tumour arose.

Biological analysis of tumour lineage might aid in the selection of appropriate treatments for various glioma subgroups, a strategy which has proved successful in haematopoietic system tumours. Consistent with such a possibility is the finding that patients with oligodendrogliomas appear to derive more benefit from chemotherapy than do patients with tumours thought to be of astrocyte lineages (Cairncross and MacDonald 1988; MacDonald et al. 1990). A better understanding of the cellular and molecular biology of gliomas and their lineages might also lead to new 'molecular targets' and more selective strategies for treating patients with gliomas.
1.2.3. The mesenchymal paradox of glioma biology

It is found that a very large proportion, (up to 90%), of human glioma specimens grown in standard tissue culture conditions give rise within several passages to populations that have no glial characteristics and instead resemble mesenchymal cells (Bigner et al. 1981; Kennedy et al. 1987; Westphal et al. 1990). These mesenchyme like cells can be derived from tumours of every category and every grade of malignancy. To make matters even more confusing, it is also clear that it is possible to derive such fibronectin positive and GFAP negative populations simply by cloning of GFAP positive parental glioma lines (Westphal et al. 1988) and that such populations can also be derived from rat astrocytes immortalized with Simian Virus 40 Large T antigen (Geller and Dubois-Dalcq 1988).

The present state of knowledge about the mesenchymal like derivatives of gliomas is limited. Some of the mesenchyme like cells that arise from growth of gliomas in standard tissue culture conditions may be derived from meningeal cells rather than from the glioma cells themselves, but it is clear that in other cases these are bona fide tumour cells displaying the molecular abnormalities characteristic of gliomas (Bigner et al. 1981; Davenport and McKeever 1987a; Davenport and McKeever 1987b). Mesenchymal like cells can also contribute to the tumour mass in the patient (Kennedy et al. 1987).

There are many questions that remain unanswered about the mesenchymal like cells which can be derived from gliomas. Firstly, what is the molecular basis for the transition from glial phenotype to mesenchymal phenotype and is this associated with an alteration in the biological properties of the resultant cells. It is uncertain whether this transition represents an aberrant pathway that becomes accessible to neural tumours or reflects instead the developmental potential of the cell of origin of the tumour. Finally how often does this transition occur in vivo and does it have any
relevance to therapy responsiveness. If experiments in tissue culture provide a valid insight into what may occur *in vivo*, then this transition may affect the patient's prognosis and treatment. The two resulting populations may differ in their growth and survival requirements, migratory behaviour, and sensitivity to radiation and chemotherapy.

1.2.4. **Mesenchymal derivatives of gliomas impede lineage based analysis**

Whether the mesenchymal derivatives of gliomas are of profound or trivial importance, the frequency with which these cell populations arise has greatly impeded the development of a lineage based analysis of gliomas that relies on cellular biology rather than histopathology. In order to attempt to derive more useful glioma derived populations *in vitro* traditional tissue culture techniques based on sera may need to be abandoned.

It might be possible to grow glioma cells in the micro-environment in which they would find themselves *in vivo*. Astrocytes are primarily responsible for maintaining extracellular fluid homeostasis in the CNS and serum proteins are not normally present in CNS extracellular fluid. Using such techniques (i.e. astrocyte conditioned medium), a human glioma cell line of the O-2A lineage was derived (see Chapter 3).

1.2.5. **The role of growth factors and their receptors in gliomas**

Growth factors provide a key link between cellular proliferation, cellular differentiation, cell survival and oncogenesis. The persistent and uncontrolled proliferation of malignant cells could result from several mechanisms; the abnormal autoproduction of a growth factor to which the cell normally responds, overproduction of a growth factor to which the cell normally responds, overproduction of a growth factor receptor, a production of an
uncontrollable growth factor receptor or a combination of these possibilities. In addition, many other oncogenes have been found to encode for proteins that are part of the growth factor intracellular second messenger pathway from the cell surface receptor to nuclear transcription.

Several roles for growth factors and their receptors have already been found in human glioma biology, but of course no subdivision on a lineage basis has yet proved possible. With the establishment of serum free culture conditions it has been found that human gliomas respond mitogenically to many growth factors with known roles during normal gliogenesis (for example, Epidermal Growth Factor (EGF), PDGF, IGF-1, Nerve Growth Factor (NGF), Acidic Fibroblast Growth Factor (aFGF) and bFGF, (Engenbraaten et al. 1993; Pollack et al. 1991). Human gliomas have also been shown to actively express growth factors (for example EGF, bFGF, IGF-1, Insulin-like Growth Factor 2 (IGF-2), NGF, Brain Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3), Transforming Growth Factor β (TGF β), Transforming Growth Factor α (TGF α) and Vascular Endothelial Growth Factor (VEGF), (Ekstrand et al. 1991; Hamel et al. 1993; Melino et al. 1992; Morrison et al. 1993; Plate et al. 1992; Sandberg-Norqvist et al. 1993; Tada et al. 1993; Torp et al. 1991) and several normal and rearranged growth factor receptors such as EGF and PDGF receptors, (Ekstrand et al. 1991; Fleming et al. 1992; Hurtt et al. 1992), see also Chapter 6. While the effects of individual growth factors have been studied, little is known of the potential role of co-operative combinations of growth factors in glioma biology (as opposed to normal glial biology, section 1.1.4).
1.3. The O-2A lineage in neuro-embryology

1.3.1. The O-2A lineage in vivo

The ultimate purpose of investigating the O-2A lineage in vitro is to apply these findings to the study of this lineage during development and myelination (the present section) and also during repair and remyelination (section 1.4).

1.3.2. General neuro-embryology

Formation of the mammalian CNS begins with the notochord derived from the mesoderm inducing the overlying ectoderm to form neuroectoderm. These cells respond by forming taller columnar epithelium and involuting along a longitudinal axis to form the neural tube. This 'neurulation' begins at two points, the cervical-hindbrain and the midbrain-forebrain junction, and continues in rostral and caudal directions. The dorsal edges of the neurulating tube break away and form the neural crest, which ultimately gives rise to the peripheral nervous system (autonomic and somatic nervous system), the enteric nervous system, several cranial nerves, portions of the branchial arches and melanocytes. It is at the point of closure of the neural tube that neuroectodermal cells begin to differentiate from one another.

In the part of the neural tube destined to become the spinal cord, the lumen gradually shrinks until in the adult it becomes the central canal. In the part of the neural tube destined to become the brain, the lumen dilates to form three segmentally arranged vesicles (the prosencephalon or forebrain, the mesencephalon or midbrain and the rhombencephalon or hindbrain) separated by two annular constrictions. In the brain the neural tube lumen
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persists as the ventricular system. The adult brain develops from the
differential growth of the three primary vesicles, the emergence of four
flexures (the cervical flexure - concave ventrally, the mesencephalic flexure -
concave ventrally, the pontine flexure - concave dorsally and the
telencephalic flexure - concave dorsally) and a series of complex three
dimensional foldings.

The cellular organisation of the tissue between the internal and external
limiting membranes in both the rudimentary spinal cord and brain remains
remarkably similar right up to the point of active glio- and neurogenesis. As
the tall columnar epithelium transforms into pseudostratified columnar
epithelium, the lateral walls of the neural tube thicken, while the dorsal and
ventral surfaces thin to form respectively the roof and floor plates. Early in
development all cells in the neural tube divide through a process of
interkinetic nuclear migration where the nucleus travels to-and-fro within the
cell cytoplasm and where the nucleus position is determined by its stage in
the mitotic cycle (actual cell division occurring near the internal limiting
membrane), (Sauer 1935).

Initially the pseudostratified columnar epithelium forms three zones
(Boulder Committee 1970). The layer nearest the lumen is the ventricular
zone, defined as the space to which the to-and-fro nuclear movement during
interkinetic nuclear migration is confined. The layer nearest the external
limiting membrane is the relatively acellular marginal zone. The layer
between the other two zones is the intermediate zone made up of the first
cells which have ceased interkinetic nuclear migration. Slightly later a fourth
layer, the subventricular zone, is formed, which is located between the
ventricular and intermediate zones. The subventricular zone is very cell
dense, composed of a morphologically homogeneous population of small
round cells with a high proliferative rate. Unlike ventricular cells, they
remain stable in position and are not attached to the internal limiting

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membrane. The last fifth layer is the cortical plate which arises between the intermediate and marginal zones and consists of cell bodies that eventually form the cortical grey matter.

Although a morphological description and categorization of mature glial phenotypes was possible, (due in a large part to the efforts of del Rio Hortega and Ramno y Cajal), glial precursors eluded definition. Glia appeared to arise from the very homogeneous subventricular and ventricular zones but could not be reliably identified by morphology or standard histochemical stains.

1.3.3. Markers for the O-2A lineage during embryogenesis

By culturing cells from portions of the developing CNS and using markers to study cells of interest it has been found that the cells of the ventricular and subventricular zones are likely to be a heterogeneous population of multipotential neuro-glial precursors, neuronal precursors and perinatal O-2A progenitor cells. Ventricular and subventricular zone heterogeneity exists both at a cellular and region level within both zones. For instance the subventricular zone has highly localised regions that serve as sources of neuronal precursor cells (Luskin 1993). In addition, perinatal O-2A progenitor cells only arise from very specific regions of the ventricular (spinal cord) and subventricular zones (brain). It was first noticed that cells isolated from the dorsal half of embryonic day 14 rats failed to produce oligodendrocytes while those in the ventral half were able to do so (Warf et al. 1991). The perinatal O-2A precursor cells of the spinal cord originate from the ventral half of the ventricular zone and migrate into the dorsal half as well as out to the periphery of the spinal cord. This agrees with the findings from in situ hybridisation for PDGF receptor α (PDGFRα) messenger RNA (mRNA), which is expressed in perinatal O-2A progenitors, (Pringle and Richardson 1993) and also DM-20 mRNA (a splice variant of
proteolipid protein mRNA, which seems to be expressed by perinatal O-2A progenitors (Lubetzki et al. 1991; Timsit et al. 1992). The *in situ* hybridisation studies also suggest that perinatal O-2A progenitors arise from distinct regions of the subventricular zone of the brain, the ventral, caudal rhombencephalon\-rostral spinal cord and the lateral basal plate of the diencephalon, (Pringle and Richardson 1993; Timsit et al. 1995).

In the optic nerve perinatal O-2A progenitors can be identified from primary cultures at embryonic day 16 in rats, (Miller et al. 1985; Small et al. 1987), they migrate from the brain end of the optic nerve to the orbit end by embryonic day 21 (Small et al. 1987). A similar migration of cells positive for PDGFRα has also been seen *in vivo* (Pringle and Richardson 1993).

Myelin Transcription factor 1 (MyT1) a transcription factor named for its ability to recognise the proteolipid protein gene also seems to recognise mainly O-2A progenitors in the developing rodent central nervous system (Armstrong et al. 1995).

It has been suggested that perinatal O-2A progenitor cells are GD3 positive *in vivo*, (GD3 is a cell surface ganglioside antibody), (see for example LeVine and Goldman 1988; Reynolds and Wilkin 1988), but recent studies have failed to confirm this finding (Wolswijk 1995).

Identification of O-2A lineage cells using a single marker may not be entirely reliable and therefore a combination of markers may prove more useful. There is still a need for further markers of the O-2A lineage. The findings of new molecular markers may also provide new insights into the functional changes that occur within these cells *in vivo*. 
1.4. The O-2A lineage in repair

1.4.1. The O-2A lineage and multiple sclerosis

An important reason for studying the O-2A lineage in the adult CNS is to understand the failure of remyelination in the relatively common neurological disease multiple sclerosis (MS).

MS is a human disease first fully identified by Charcot in his lecture on *la sclérose en plaque disseminées* in 1868. The clinical features of MS are manifestations of the pathological lesions seen in the CNS, namely repeated demyelination with, in general, preservation of axons. Only in a minority of acute lesions in MS is early remyelination found, (Prineas et al. 1993; Raine et al. 1981). It is therefore possible that strategies to promote myelin repair might influence the course of MS. The oligodendrocyte is the myelinating cell of the CNS and in order to study the origin of remyelinating cells in MS it would be useful to have *in vivo* markers for cells of the O-2A lineage.

1.4.2. Regeneration of the oligodendrocyte population following demyelinating damage

Regeneration of the oligodendrocyte population has been observed in many experimental models of CNS demyelination (reviewed in Ludwin 1981). Two observations suggest that rodent adult O-2A progenitors may play an important role in replenishment of the oligodendrocyte population following demyelinating damage. Firstly cells with the antigenic characteristics of adult O-2A progenitor cells grown *in vitro* have been shown to synthesize DNA and/or increase in number during the recovery process from demyelinating damage (Carroll et al. 1990; Godfraind et al. 20
1989). Secondly the oligodendrocyte lineage cells that proliferate in response to experimentally-induced demyelination (Arenella and Herndon 1984; Carroll et al. 1990; Ludwin 1979; Ludwin 1984; Rodriguez et al. 1991) and those that are present at the edges of demyelinating lesions in patients with multiple sclerosis (Raine et al. 1981) are ultrastructurally similar to adult O-2A progenitor cells grown in vitro (Wolswijk et al. 1991a).

If adult O-2A progenitor cells are involved in damage repair, substances involved in stimulating the division of adult O-2A progenitor cells must be expressed or released following injury. There are several candidate molecules, including cytokines secreted by reactive astrocytes, inflammatory cells or microglia, myelin breakdown products and components present on demyelinated axons. Due to the complexity of the in vivo situation, in vitro studies can also be helpful in understanding possible mechanisms.

One factor that both appears to be expressed at increased levels following injury to the adult CNS (Eckenstein et al. 1991; Finklestein et al. 1988; Gómez-Pinilla et al. 1992; Logan 1988; Logan 1990; Logan et al. 1992; Nieto-Sampedro et al. 1988) and is capable of increasing the rate of division of adult O-2A progenitor cells grown in vitro is bFGF (Wolswijk and Noble 1992).

Adult O-2A progenitor cells exposed to bFGF can be induced to divide even more rapidly if they are also exposed to PDGF (Wolswijk and Noble 1992); this response would allow a rapid increase in the number of adult O-2A progenitor cells [as observed, for example, in lesions of animals recovering from experimentally-induced demyelination, (Carroll et al. 1990; Godfraind et al. 1989)]. Consistent with the hypothesis that PDGF as well as bFGF may be involved in the increased generation of adult O-2A progenitor cells in vivo are the findings that mechanically-induced injury to the adult CNS is associated with transient increases in the levels of bFGF, PDGF and both their mRNA's (Eckenstein et al. 1991; Finklestein et al. 1988; Gómez-
Pinilla et al. 1992; Logan 1988; Logan 1990; Logan et al. 1992; Lotan and Schwartz 1992; Nieto-Sampedro et al. 1988). However, it is not yet known whether demyelinating damage is associated with increases in the levels of these two growth factors, either in experimental models of demyelinating diseases or in patients with MS. In addition to PDGF and bFGF other cytokines, such as those secreted by the inflammatory cells which are often present in demyelinating lesions (Ludwin 1981), may play key roles in regenerative responses in the O-2A lineage. The identification of such cytokines in vivo and the analysis of their effect on adult O-2A progenitor cells grown in vitro may be crucial in furthering the understanding of the process of myelin repair.

Although oligodendrocyte proliferation has been seen following a variety of insults to the adult CNS (Arenella and Herndon 1984; Ludwin 1984; Ludwin and Bakker 1988), it only occurs in a small proportion of cells suggesting that primary oligodendrocyte proliferation makes only a small contribution to the generation of new oligodendrocytes. In vitro, oligodendrocyte division can be promoted by bFGF (Bögler et al. 1990; Eccleston and Silberberg 1985; Saneto and DeVellis 1985; Wolswijk and Noble 1992) and also when co-cultured with dorsal root ganglion cells (Wood and Bunge 1986; Wood and Bunge 1991), exposed to axolemmal enriched fractions (Chen and DeVries 1989) or grown on an extra-cellular matrix (Ovadia et al. 1984).

1.4.3. Factors limiting repair of demyelinated lesions

Whilst there is some evidence for myelin repair at the edges of MS lesions (Prineas and Connell 1979; Prineas et al. 1989; Raine et al. 1981), the centres of old lesions or 'plaques' are often virtually completely devoid of oligodendrocytes, and bare axons are surrounded instead by astrocytic
processes. At present very little is known about why remyelination is usually unsuccessful in MS patients or about the cause of the initial destruction of oligodendrocytes. *In vitro* it has been shown that rodent adult O-2A progenitor cells and oligodendrocytes activate complement in the absence of antibody, resulting in their destruction (Wren and Noble 1989). It is unknown whether complement mediated destruction plays a role in the formation of chronically demyelinated lesions, but complement does appear to be activated in areas of myelin breakdown (Compston et al. 1991). It has also been shown that X-ray irradiation depletes the adult O-2A progenitor population *in vivo* (Van der Maazen et al. 1992), followed by subsequent demyelination: such a progenitor depletion could account for the demyelination which is often seen following X-ray irradiation (Van der Kogel 1991).

Additionally, there is a reduction in the number of adult O-2A progenitor cells and their progeny seen *in vitro* with age (Van der Maazen et al. 1992; Wolswijk and Noble 1989; Wolswijk and Noble 1992). This is mirrored by a reduction in the potential for recovery from optic neuritis, a human demyelinating disease of the optic nerve, with age (Kriss et al. 1988; McDonald 1983) and by less successful repair of ethidium bromide-induced demyelinated lesions in rodent spinal cord with increasing age of the animal (Gilson and Blakemore 1993).

Myelin repair could be hampered by the differentiation of adult O-2A progenitor cells into type-2 astrocytes instead of oligodendrocytes. Cells with the antigenic phenotype of type-2 astrocytes have been identified in mouse hepatitis virus-induced demyelinated lesions as well as in cultures prepared from this tissue (Armstrong et al. 1990; Godfraind et al. 1989). This suggests that demyelinated lesions may contain factors which induce type-2 astrocytic differentiation in adult O-2A progenitor cells. Therefore the astrocytic scars often found in experimentally-induced demyelinated and MS lesions (Ludwin
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1981) could be due to the generation and proliferation of type-2 astrocytes, rather than proliferation of astrocytes from a different glial lineage as has previously been suggested (Miller et al. 1986; Raff et al. 1987).

Impaired oligodendrocyte survival could also hamper remyelination. Appropriate amounts of critical growth factors would be needed for oligodendrocyte survival in *vivo*. Factors that have been found to promote long term survival of oligodendrocytes *in vitro* include IGF-1, IGF-2, insulin (but only at doses that secondarily saturate IGF-1 receptors), CNTF, leukaemia inhibitory factor, NT-3 and interleukin-6 at very high doses, (Barres et al. 1992; Barres et al. 1993; Louis et al. 1993; Mayer et al. 1994). PDGF also promotes early oligodendrocyte survival, but this is short lived, (Barres et al. 1992), as the PDGF receptors are then lost (McKinnon et al. 1990). Combinations of these growth factors may be more effective in long term oligodendrocyte survival (Barres et al. 1993).

1.4.4. *In vivo* markers of the adult O-2A progenitor cell

In order to study the role of O-2A progenitor cells in the repair of demyelinated lesions *in vivo*, they must be identified in sections of adult CNS tissue, using specific antigenic and/or molecular markers. Several potential markers for rodent adult O-2A progenitor cells *in vivo* have been identified. For example, PDGFRα in the adult optic nerve (Pringle et al. 1992), glutamate receptor linked cation channels of the quisqualate/kainate type in the adult CNS (Fulton et al. 1992), and NG2 chondroitin-sulphate proteoglycan in the adult cerebellum (Levine et al. 1993), have all been suggested to mark O-2A progenitors *in vivo*. Although the expression of these markers in demyelinated lesions has not yet been examined, NG2 chondroitin sulphate proteoglycan positive cells after mechanically brain injury, transiently express higher levels of NG2 and become proliferatively
active (Levine 1994). However the evidence that adult O-2A progenitor cells express PDGFRα and NG2 in both the normal and damaged adult CNS is indirect and it may be that other cell types also express these molecules in vivo.

In the normal adult mouse spinal cord it has been found that cells binding O4, but not antibodies to the oligodendrocyte-specific enzyme CNP (2', 3' cyclic-nucleotide 3' phosphohydrolase) are rare, but they increase in number following experimentally-induced demyelination (Godfraind et al. 1989). As CNP is expressed by oligodendrocytes following the expression of galactocerebrosides (GalC) (Pfeiffer et al. 1993), the population of O4 positive/CNP-negative cells includes both adult O-2A progenitor cells and immature oligodendrocytes and therefore the relative contribution to the regeneration by each of these two cell types by increases in cell number is not known from this study.

As previously noted, the identification of a cell as an O-2A progenitor cell (or any other cell type) on the basis of the expression of a single marker in vivo may not be reliable and several markers should be used. Therefore, there is still a need to identify further markers and verify the ones currently available in human tissue.

1.5. Protein kinases

1.5.1. The O-2A lineage and protein kinases

Growth factors and their receptors provide a key link between cellular proliferation, differentiation and survival in cell systems, including the O-2A lineage. They are also important in oncogenesis. In addition one growth factor receptor, PDGFRα, is an O-2A progenitor marker in vivo (Pringle et al. 1992; Pringle and Richardson 1993). Growth factor receptors are protein
kinases involved in signalling cascades transmitting signals to the nucleus, where they can bring about changes in gene expression. The cell context in which a signal is processed is determined by the molecules present in the cell, and is a function of the developmental history of the cell. However, the cell response not only depends on qualitative differences in signal transducing molecules but also on the quantitative differences of the signal (Marshall 1995; Traverse et al. 1992).

1.5.2. Protein kinases are key players in signal transduction

In 1955 Krebs and Fischer discovered that glycogen phosphorylase is activated through the reversible addition of an activated phosphate group, (Fischer and Krebs 1955). We now recognise that protein phosphorylation is a fundamental mechanism for regulating numerous cellular processes, including signal transduction. The enzymes catalysing the transfer of the γ-phosphate group of magnesium adenosine triphosphate (MgATP) to a protein substrate are known as protein kinases (Hunter 1991).

Protein kinases are integral components of several signalling pathways and modulate the interaction of signalling molecules in two main ways. Firstly, they can alter the enzymatic activity of downstream proteins, either causing activation as in the case of the insulin receptor kinase following tyrosine phosphorylation of the β unit (Yu and Czech 1984) or inhibition as in the case of the epidermal growth factor receptor following phosphorylation of a threonine residue (Countaway et al. 1990). Secondly, protein kinases can regulate signalling pathways by controlling the formation of complexes between signalling and effector molecules. This is exemplified by the formation of high affinity binding sites for src homology 2 domain (SH2) containing molecules through tyrosine phosphorylation of substrates (reviewed in Pawson 1995). In the case of the platelet derived growth factor
receptor, autophosphorylation promotes the binding of other signalling molecules to activated SH2-sites in the intracellular domain of the receptor. Interaction with these molecules plays an important role in transmitting the receptor generated signal into the cytoplasm (Kazlauskas and Cooper 1989; Schlessinger and Ullrich 1992).

1.5.3. Protein kinases contain a catalytic domain with conserved features

The superfamily of protein kinases is enormous, with an estimated 2000 protein kinases present in the human genome (Hunter 1994). All protein kinases have a catalytic kinase domain of approximately 270 amino acids, which has been subdivided on the basis of sequence comparisons into subdomains I-XI. Within these lie at least twelve highly conserved amino acid residues required for enzyme activity, complexing MgATP and mediating the phosphotransfer reaction (figure 1.1., Hanks et al. 1988).

Crystal structure analysis of the cyclic adenosine monophosphate-dependent protein kinase revealed that many of these conserved residues are located in loops which come together at the active site (Knighton et al. 1991).

The protein kinase family can be further subdivided into two main groups, the serine/threonine and tyrosine protein kinases, depending on the conservation of other amino acids in each subgroup. Although the protein serine/threonine kinase subfamily is more diverse than the protein tyrosine kinases, short sequence motifs in subdomains VI and VIII have been used to distinguish between protein tyrosine and serine/threonine kinases on the merit of amino acid sequence alone (figure 1.1., Hanks et al. 1988). The consensus sequences $IHRDL_{A}^{R}A_{R}^{A}A_{R}^{A}N$ in subdomain VI and $P_{V}^{I}K_{R}^{K}W_{R}^{X}APE$ in subdomain VIII are predominantly found in tyrosine kinases and the sequence motifs $HRLDK_{S}^{P}E_{N}$ and $G_{S}^{T}x_{T}^{x}Y_{T}^{x}APE$ are
Figure 1.1. All protein kinases contain a catalytic kinase domain.

Subdomains

I II III IV VIa VIb VII VIII IX XI

Subdomains I-XI of the catalytic core are depicted according to Hanks et al., 1988. Amino acid residues are shown in single letter code. Dash represents variable amino acids. Certain highly conserved amino acid residues are required for complexing MgATP and mediating the phosphotransfer reaction (bold). Other amino acids are conserved in either protein tyrosine kinases (PTK) or protein serine/threonine kinases (PSK) and may be involved in determining hydroxy-amino acid specificity. The enumeration of amino acids follows the amino acid sequence of cyclic adenosine monophosphate-dependent kinase α.
frequently found in subdomains VI and VIII of serine/threonine kinases.

However, several protein kinases have been reported that are not closely related in sequence to either of the above subfamilies. Some of these can phosphorylate both serine/threonine and tyrosine residues, and have been labelled 'dual-specificity' kinases (Lindberg et al. 1992). The catalytic domains of these kinases show no significant homology beyond the highly conserved residues described above. The best known members of this third subfamily of protein kinases are the mitogen activated protein kinase kinases (MAPKK1 and 2, also known as MEK1 and 2, Ashworth et al. 1992; Crews et al. 1992; Kosako et al. 1992). Activated mitogen activated protein kinase kinases phosphorylates mitogen activated protein kinase (MAPK) (reviewed in Marshall 1994). MAPK is a serine/threonine kinase.

The classification of protein kinase subfamilies relies only in part on the hydroxy-amino acid specificity. Other criteria include the regulators of kinase activity, such as the cyclic adenosine monophosphate-dependent kinases, or the growth factor receptor kinases. This is often supported by additional sequence homologies in- and outside the kinase domain (Hanks et al. 1988; Hunter 1991). In addition, related members of a subfamily may also share other properties such as cellular localization and additional, conserved functional domains.

In light of the tremendous importance of protein kinases as key regulatory molecules, the highly conserved amino acid residues of the catalytic domain have also been exploited to isolate numerous new members of this important protein super family. This has lead to an exponential increase in the number of known protein kinases over the past fifteen years (Hunter 1991). An often employed strategy involves the use of degenerate oligonucleotide primers, directed against the conserved regions of the kinase domain, in a polymerase chain-reaction (PCR) based protocol to screen for
novel protein kinases (examples and variations of this method have been reported by Hanks 1987; Levin et al. 1987; Reith et al. 1990; Wilks 1989).

1.5.4. Protein tyrosine kinases

The protein tyrosine kinases can be divided into two subfamilies on the basis of structure and function, that is receptor and non-receptor tyrosine kinases. The non-receptor cytosolic kinases are localized to the cell interior, although they may be associated with the cell membrane or nucleus. The structural features of these kinases vary, but most contain at least one non-catalytic regulatory domain such as a SH2 or a src homology 3 domain (SH3) or a regulatory terminus in addition to the kinase domain. The protein tyrosine kinase receptors are discussed below.

1.5.5. Protein tyrosine kinase receptors

Many of the known receptors for growth factors have tyrosine kinase activity, for example the receptors for PDGF, EGF and IGF-1 (reviewed in Ullrich and Schlessinger 1990). These protein tyrosine kinase growth factor receptors are characterized by three major functional domains: firstly, an intracellular domain which may vary in length, but which always contains the catalytic kinase domain; secondly, a single hydrophobic membrane spanning domain and thirdly, a glycosylated extracellular domain whose structure determines ligand binding specificity or may also point to other functions such as interactions with extracellular matrix and the surface of adjacent cells (van der Geer et al. 1994). According to these different extracellular domains, the family of RTKs can be divided into several distinct classes in which each member of a particular class will share certain structural features. These structural domains are discernible in the primary amino acid sequence.
Therefore, the characterization of such features can be used to help predict properties of novel family members from the polypeptide sequence (Hanks et al. 1988). The six main classes of protein tyrosine kinase growth factor receptors are listed below, (there are several other receptors that do not fall into these main groupings):

**Class 1:** The EGF receptor family, (Ullrich et al. 1984), characterized by two cysteine rich domains in the extracellular region.

**Class 2:** The insulin receptor family (Ebina et al. 1985), each receptor is characterized by two subunits each having a cysteine rich domain in the extracellular region.

**Class 3:** The PDGF receptor family (Claesson-Welsh et al. 1989; Yarden et al. 1986), most of which have five immunoglobulin-like domains in their extracellular region, (although there is a subgroup which has seven immunoglobulin-like domains), they also have an insert region within the intracellular kinase domain.

**Class 4:** The FGF receptor family (Lee et al. 1989; Reid et al. 1990), characterized by three immunoglobulin-like domains in their extracellular region and an insert region within the intracellular kinase domain.

**Class 5:** The EPH receptor family, (EPH was initially cloned from an erythropoietin-producing human hepatocellular carcinoma cell line, Hirai et al. 1987), characterized by an immunoglobulin-like domain, a cysteine rich region and two fibronectin-type III repeats in the extracellular domain.

**Class 6:** The *trk* (tropomyosin receptor kinase) receptor family (Martin-Zanca et al. 1986), characterized by a leucine rich region and two immunoglobulin-like domains in the extracellular region and an insert within the intracellular kinase domain.

In each case signal transduction is initiated by the binding of growth factors to the extracellular domain of their cognate receptors. Ligand binding
facilitates dimerization of the receptor which can then induce receptor autophosphorylation, (Ullrich and Schlessinger 1990; Yarden and Schlessinger 1987). Both soluble and membrane associated protein ligands have been shown to function in this manner. The activated receptor binds and in some cases phosphorylates proteins containing one or more SH2 domains (Koch et al. 1991). This elicits a cascade of cellular events which are not yet fully elucidated but involve for example Ras-guanine triphosphate activating protein, Growth-factor Receptor Bound protein 2 (GRB2), phospholipase Cγ, and cytoplasmic kinases like MAPK.

1.6. The scope of this thesis

In this introduction I have outlined the main areas of interest relevant to the work of this thesis. The rodent O-2A lineage, the possible relevance of these findings to the human, the need for a lineage based classification of gliomas, the requirement for further O-2A lineage markers in vivo to investigate development and repair and the relevance of protein kinases to all these fields.

The following chapters present; the derivation and characterization of a cell line of the O-2A lineage from a human glioblastoma multiforme (Chapter 3), the finding of a novel effect of a growth factor on oligodendrocyte maturation within this cell line which is also applicable to the rodent O-2A lineage (Chapter 4), the cloning and initial characterization of a protein kinase found in both the O-2A lineage and gliomas (Chapter 5) and finally the finding of a recently described receptor tyrosine kinase in cells of the O-2A lineage and during human development (Chapter 6).
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2 MATERIALS & METHODS

2.1. Materials

2.1.1. Tissue culture supplies

a) Culture Media and Animal Sera
Dubeccco's modification of Eagle's medium (DMEM), Leibovitz L-15 Medium, animal sera, glutamine and gentamycin were obtained from Gibco-BRL. Tissue culture grade water was supplied by Imperial Laboratories.

| DMEM plus 10% foetal calf serum (DMEM+10%FCS) |
| DMEM supplemented with the addition of 10% (v/v) foetal calf serum (FCS), heat inactivated, (56°C for 45 mins), 25μg/ml gentamycin and 2mM glutamine. |

Chemically defined medium (DMEM-BS) was modified from that previously described by Bottenstein and Sato (Bottenstein and Sato 1979; Wolswijk and Noble 1992).

| DMEM-BS |
| DMEM supplemented with, 25 μg/ml gentamicin, 2 mM glutamine, 1μg/ml bovine pancreas insulin (Sigma), 100 μg/ml human transferrin (Sigma), 0.026% (v/v) bovine serum albumin Path-O-Cyte* 4 (ICN Biochemicals), 180 nM progesterone (Sigma), 90 nM putrescine (Sigma), 200 nM sodium selenite (Sigma), 450 nM 3,3',5-triiodo-L-thyronine (T3; Sigma), 400 nM L-thyroxine (Sigma) |

b) Other stock reagents
These were filter sterilised if they were not known to be sterile initially.

| Collagenase |
| A 2000U/ml solution of collagenase (Worthington Biochemical) was made up in Leibovitz L-15 medium. |

| Cytosine arabinoside |
| Solutions of 1mM cytosine arabinoside (Sigma) in DMEM (Gibco) were stored in 1ml aliquots at -20°C, and used at a final concentration of 20μM. |

| EDTA-medium |
| Sodium ethylenediamine tetraacetic acid (EDTA; Sigma) was dissolved in DMEM-Calcium and Magnesium free medium (kindly supplied by the Imperial Cancer Research Fund) to a final concentration of 0.25mM. |
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**Fibronectin**
Fresh fibronectin (Sigma) was made up with sterile water to a final concentration of 30µg/ml and kept at 4°C and reused for up to a month.

**Laminin**
Laminin (basement membrane-Engelbreth Holm Swarm mouse sarcoma; Sigma) was made up with sterile water to give a final concentration of 1-4µg/ml.

**Poly-L-lysine**
4 mg/ml poly-l-lysine (PLL, 175,000 MW; Sigma) was made up with sterile water and used at a final concentration of 20 µg/ml.

**Soybean trypsin inhibitor (SBTI-DNAse)**
This solution consists of 0.52 mg/ml soybean trypsin inhibitor, 0.04 mg/ml bovine pancreatic DNAse and 3mg/ml BSA Fraction V (all from Sigma) made up in DMEM.

**Trypan blue**
0.5% (v/v) trypan blue in 0.85% (w/v) sodium chloride (Gibco).

**Trypsin**
A 3mg/ml (30,000 units/ml) solution of bovine pancreatic trypsin (Sigma) in DMEM-Calcium and Magnesium free medium (kindly supplied by the Imperial Cancer Research Fund) was made up.

c) Disposable Plastic Ware
Tissue culture flasks, Petri dishes, multiwell trays, cell scrapers, syringes, centrifugation tubes and freezing vials were supplied by Falcon or Nunclon Delta (Gibco). Microscope slides and coverslips were supplied by BDH/Merck.

Tissue culture surfaces were coated with either PLL, fibronectin or laminin by placing the solution on the surface for >30min. at 37°C, the solution was then removed and the surface air dried in the tissue culture hood.

2.1.2. Animals

Adult and embryonic rats were obtained from the Biological Services Unit, University College London.

2.1.3. Molecular biology supplies

a) Growth Media and Solutions
Where buffers and solutions have not been listed with the appropriate method in section 2.3., they were prepared as previously described by (Sambrook et
al. 1989). Reagents were always molecular biology grade and were tested by the manufacturer for the presence of contaminating nucleases.

Water used was always deionized and filtered using a Millipore water-purification system (to 15-18 megaOhm resistance) and subsequently autoclaved (ddH₂O). For use in experiments which involve intact RNA, water was di-ethyl-pyro-carbonate (DEPC) treated before being autoclaved.

Growth media (Luria Broth (LB); NZYCM (NZamine Yeast Casamino acids and Magnesium) Media for lambda phage growth), antibiotics, agarose and phosphate buffered saline (PBS) were purchased from Gibco-BRL.

Radioactive agents were obtained from Amersham.

b) Other Materials
Plastic labware was washed, autoclaved and dried, while glassware was washed and baked at 180°C for 2-4 hours. Pipettes, micropipette tips and microcentrifuge tubes were supplied by different manufacturers (Costar, Treff, Multi, Gilson).

Auto radiography film was supplied by Kodak (X-OMAT, XAR-5) and Fuji (RX). Images were also presented using computer supported graphics (ImageQuant Phosphoimager, Apple Macintosh, Microsoft Adobe Photoshop).

2.2. Cell biology methods

2.2.1. Preparation of monolayers of purified cortical astrocytes and harvesting astrocyte conditioned medium

Monolayers of purified cortical astrocytes were prepared by modifications of previously described methods (McCarthy and deVellis 1980; Noble et al. 1984; Noble and Murray 1984). Cerebral cortices were dissected out of one day old Sprague-Dawley rat pups and the tissue placed into sterile petri dishes containing Leibovitz L-15 medium and 25μg/ml gentamycin. The hippocampus, caudate and meninges were removed from the cerebral cortices and the remaining tissue was finely chopped with a fresh scalpel (Swann-Morton) and transferred to a universal containing 0.25ml collagenase, 2.5ml trypsin and 5ml EDTA-medium and incubated for 37°C.

The reaction was stopped by adding 5ml SBTI-DNAse, the tissue was triturred through a 5ml pipette and then centrifuged for 2 min at 4000 rpm. The supernatant was discarded and the pellet resuspended in 5ml DMEM+10%FCS and dissociated into a single cell suspension by gently trituratung tissue through a 5ml pipette followed serially by a 25-gauge and then a 27-gauge needle (Sherwood Medical) and syringe. Cells were plated into PLL-coated 80cm² flasks (2 brains per flask) in DMEM+10%FCS (final
volume, 10ml per flask). The flasks were incubated at 37°C with 5.5% CO₂; 94.5% air and the medium changed after 2 hours, on day 1, on day 3 and on day 6. After 7 days in vitro, the top cells were removed by shaking the cultures over night on a rotary shaker (100 rpm; Luckman Ltd.). The medium was changed and the following day treatment with 20μM cytosine arabinoside for two 48 hour periods was given, changing the medium in between, to eliminate the faster dividing non-astrocytes. The cells were grown for a further 2 days in DMEM+10%FCS. They were then rinsed in DMEM-BS and grown in DMEM-BS for two 48 hour periods, at a ratio of 1ml of medium for every 8 cm² of astrocyte monolayer, after each period the medium was harvested and the cells were then allowed to recover for 3 days in DMEM+10%FCS before further growth in DMEM-BS, then the cycle of harvesting in DMEM-BS and recovery in DMEM+10%FCS was repeated. Harvested astrocyte conditioned medium was either, filtered using a 0.22μm filter (Gellman Science) and used fresh or stored at -20°C and filtered on thawing. Astrocyte monolayers provided conditioned medium for up to 10 weeks and were then discarded.

2.2.2. Maintenance of Hu-O-2A/Gb1 cells

Hu-O-2A/Gb1 cells were maintained in culture (7.5% CO₂; 92.5% air) in tissue culture flasks coated with poly-L-lysine (PLL) using medium termed ACM which was half DMEM-BS and half astrocyte conditioned medium. The medium was changed every second or third day. When confluent cells were passaged to a larger surface area.

2.2.3. Passaging

The medium was removed from the cells which were then washed in EDTA-medium, a 1:10 dilution of stock trypsin/EDTA-medium was added, enough to cover the surface area, and the cells were allowed to detach (approximately 5min.). SBTI-DNase was added (1ml per 1.5ml trypsin/EDTA-medium). This was made up to 10ml with appropriate medium in a sterile universal tube and centrifuged at 1000 rpm for 5 min. The medium was then removed and the cell pellet triturated, resuspended in the required volume of medium and plated out.

2.2.4. Freezing of cultures

The passaging protocol was followed until after centrifugation when the medium was removed and replaced by 10ml of fresh medium and the cell
thoroughly resuspended a small aliquot was then counted using a
haemocytometer, the remaining sample was centrifuged at 1000 rpm for 5
min. The cells were resuspended at 1 million/ml in medium + 10% dimethyl
sulphoxide (DMSO; Merck), 1ml of the cell suspension was placed in each
labelled freezing vial. The vials were placed in a cell cryo-container
(Nalgene) containing isopropanol at -70°C overnight and then placed in
liquid nitrogen for long term storage.

2.2.5. Thawing of cells

The vial of cells was removed from liquid nitrogen to dry ice and
thawed quickly in a 37°C water bath. The outside of the vial was very
thoroughly washed with 70% (v/v) alcohol and the 1ml cell suspension was
added to 9ml of medium in a universal container and centrifuged at 1000 rpm
for 5 min. The medium was removed and the cell pellet resuspended in 10ml
of medium and plated out.

2.2.6. Disaggregation protocol for glioma samples

The glioma sample was minced finely with a sterile scalpel blade,
suspended in 2ml of DMEM+10%FCS and transferred to a vial containing 1
ml of collagenase and incubated for 1 hr at 37°C. 7 mls of DMEM+10%FCS
was added and the tissue was dissociated by trituration through a 10ml, 5ml
and 1 ml blow-out pipette and 22-gauge and 25-gauge hypodermic needles
attached to a syringe. The sample was centrifuged at 1000 rpm for 5 min., the
supernatant discarded and the tissue resuspended and triturated in 10ml of
fresh medium and plated out.

2.2.7. Growth factors

The following human recombinant growth factors were used: Vascular
Endothelial Growth Factor (VEGF) 165 homodimer, a kind gift from Dr.
H.A.Weich, (Dept. of Gene Expression/ZWE Genexpression, Braunshweig,
FRG) and latterly from Pepro Tech EC Ltd, Basic Fibroblast Growth Factor
(bFGF; Precision), acidic FGF (Boehringer Mannheim), Epidermal Growth
Factor (EGF; Calbiochem-Novabiochem Corp.), Neurotrophin-3 (NT-3;
Pepro Tech EC Ltd), Platelet Derived Growth Factor-AA (PDGF-AA)
(British Biotechnology Products Ltd.), Transforming Growth Factor β2
(TGFβ2; Genzyme) and Glial Growth Factor 2 [GGF 2; clone HBS5
expressed in Sf9 insect cells (Cambridge Neuroscience) purified by Dr.
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P. Stroobant (Ludwig Institute for Cancer Research) was used at a final concentration causing conversion of 50% of Schwann cell precursors into myelin forming cells. Rodent recombinant Ciliary Neurotrophic Factor (CNTF; Precision) was used.

Lyophilized growth factors were dissolved in Lebovitz L-15 medium supplemented with 1% bovine serum albumin PATH-O-CYTE*4 at the desired concentration (allowing a ≥1:100 dilution for final use). The solution was aliquoted and stored at -20°C.

### 2.2.8. Chemical agents

Details of preparation of these reagents refer to there initial dilution and storage, final dilution in each case was in DMEM-BS. All-trans-retinoic acid (Sigma) was diluted in DMSO to 100mM and stored at -20°C away from light, further dilution to 1mM was in 100% ethanol (Merck). Phorbol 12,13 dibutyrate (Sigma) was diluted in ethanol to a concentration of 10mM and stored at -20°C. N6,2'-O-Dibutyryladenosine 3',5' cyclic monophosphate (Sigma) was diluted in water to a stock concentration of 100 mM and stored at -20°C. N,N-Dimethylformamide and Phenylacetate were obtained from Sigma.

### 2.2.9. Immunocytochemistry

Cells were plated out in 25μl drops of medium onto round 13mm diameter (Ω) coated glass coverslips and allowed to attach for 30 min., the volume of the medium was adjusted to 500μl. The cells were grown in the required conditions. For experiments longer than 3 days the medium was changed every 2 days.

The cells were usually fixed in 4% paraformaldehyde (Merck) dissolved in Phosphate buffered Saline (PBS; Gibco) for 15 min., except when immunolabelling involved 5-bromodeoxyuridine (BrdU) in which case the cultures were immunolabelled without prior fixation.

All antibodies were diluted in Hanks' staining medium prior to use. Cells were first immunolabelled with both primary and secondary antibodies to surface antigens (A2B5, O4, O1, GalC and fibronectin (FN)), followed by methanol permeabilisation (-20°C for 10 min.), and immunolabelling with both primary and secondary antibodies to internal antigens using anti-BrdU, anti-GFAP and anti-myelin basic protein (MBP) antibodies. Cultures were incubated in 25μl of the primary antibody solution for 30 min. at room temperature, rinsed several times in Hanks' staining medium and then incubated for 30 min. at room temperature with the secondary antibodies.
The binding of the biotin-conjugated antibodies was visualised with
coumarin-labelled streptavidin (diluted 1:50; Molecular Probes Inc., Eugene,
Or, USA). In several experiments cells were immunolabelled with three
different antibodies and the binding of these antibodies was visualised using
three different fluorochromes. After staining, coverslips were washed in
distilled water and mounted cell side down in anti-fade (Johnson et al, 1982)
and sealed with clear nail varnish.

Cultures were examined using an infinity corrected x20 or a x40
objective mounted on a Zeiss Axiophot microscope employing transmission
phase-contrast and epi-illuminated fluorescence optics, the latter with filters
optimised for distinguishing between fluorescein (FITC), rhodamine (RITC)
and coumarin emission. Immunolabelled cells were photographed using Fuji
Ektachrome or Ilford XP2 400 films.

### Hanks' balanced salt medium
Hanks' balanced salt solution, (Imperial Laboratories) containing 0.05% (w/v)
sodium azide (Sigma), 5% heat-inactivated new born calf serum (Gibco) and
buffered to pH7.4 with 40mM Hepes (Hydroxyethylpiperazine ethanesulphonic
acid; Sigma)

<table>
<thead>
<tr>
<th>Primary antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antibody (source)</strong></td>
</tr>
<tr>
<td>A2B5 (mouse monoclonal)</td>
</tr>
<tr>
<td>O4 (mouse monoclonal)</td>
</tr>
<tr>
<td>O1(mouse monoclonal)</td>
</tr>
<tr>
<td>Anti-GalC (mouse monoclonal)</td>
</tr>
<tr>
<td>Anti-FN (goat polyclonal)</td>
</tr>
<tr>
<td>Anti-BrdU (mouse monoclonal)</td>
</tr>
<tr>
<td>Anti-GFAP (rabbit polyclonal)</td>
</tr>
<tr>
<td>Anti-MBP (mouse monoclonal)</td>
</tr>
<tr>
<td>Anti-MBP (rabbit polyclonal)</td>
</tr>
</tbody>
</table>
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### Secondary antibodies

The second layer conjugated antibodies were obtained from Southern Biotechnology Associates Inc., Birmingham, AL, USA and diluted 1:100.

- Goat anti-mouse IgM-FITC
- Goat anti-mouse IgM-RITC
- Goat anti-mouse IgM-biotin
- Goat anti-mouse IgG1-FITC
- Goat anti-mouse IgG1-RITC
- Goat anti-mouse IgG3-FITC
- Goat anti-mouse IgG3-RITC
- Goat anti-mouse IgG3-biotin
- Goat anti-rabbit Ig-FITC
- Goat anti-rabbit Ig-RITC
- Goat anti-rabbit Ig-biotin
- Rabbit anti-goat Ig-RITC

### Anti-fade

Glycerol containing 2.5% (w/v) 1,4-diazobicyclo[2,2,2] octane (Sigma)

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2.2.10. 5-bromodeoxyuridine (BrdU) incorporation assay

DNA cell synthesis was measured by immunofluorescent analysis of the incorporation of the thymidine analogue 5-bromodeoxyuridine (BrdU) into the cell nuclei (Gratzner 1982). Cell cultures were incubated with 10 μM BrdU (Sigma) for a period of 24 hours. The cells were permeabilised with methanol (10 min at -20°C), then incubated in a solution of 0.2% paraformaldehyde (dissolved in Hanks' staining medium) for 1 min at room temperature followed by a solution of 0.07M NaOH for 7 min. (Gratzner 1982). The anti-BrdU and secondary antibodies were added as described above.

### 2.3. Molecular biology methods

2.3.1. Small scale DNA isolation using alkaline lysis

**a) Bacteria Culture:**

A single colony was picked from an agar plate and transferred to a replica plate. The same colony was used to inoculate 1.5ml Super Broth Media. The culture was then incubated overnight in a shaker at 37°C.
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**Super Broth**
10g NaCl (Merck), 20g Bacto-Tryptone (Gibco), 35g Yeast extract (Gibco), 10g Sodium Morpholinopropansulphate (MOPS-Na; Merck) completed to 1 litre with ddH2O

b) DNA Isolation:
The bacterial suspension was centrifuged at 13000 rpm for 40 sec. and the supernatant was aspirated. The pellet was mixed with 100μl Solution I and incubated for 5 min. at room temperature. 200μl of Solution II was added and mixed by inverting gently until the solution appeared clear. This mixture was incubated for 5 min. on ice before 150μl of cold Solution III were added to the suspension. The mixture was spun at 13000 rpm for 10 min. at 4°C and the supernatant was transferred to a fresh tube.

The supernatant was mixed with 0.6 volume isopropanol (Merck) and centrifuged at 8000 rpm for 10 min. at room temperature. The upper phase was transferred into a fresh microcentrifuge tube, mixed with 700μl 2M ammonium acetate (NH4OAc) and spun at 13000 rpm for 5 min. at 4°C. The supernatant was transferred to a fresh tube and extracted with 400μl isopropanol. After spinning at 13000 rpm for 5 min. at room temperature, the precipitate was washed with 75% (v/v) ethanol, precipitated again and the DNA pellet was dissolved in 100μl TE (Tris EDTA) buffer.

<table>
<thead>
<tr>
<th><strong>Solution I</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>50mM Glucose (Sigma), 10mM EDTA (Sigma), 25mM Tris, HCl pH 8.0 (Merck).</td>
</tr>
<tr>
<td>Add powdered lysozyme (Sigma) to 4mg/ml just before use.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Solution II (fresh)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2M NaOH (Merck); 1% (w/v) sodium dodecyl sulphate (SDS; Merck)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Solution III</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>3M KAcetate (Merck), 2M Glacial Acetic Acid (Merck)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>TE Buffer</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>10mM Tris HCl, pH7.5 (Merck), 1mM EDTA, pH8 (Sigma)</td>
</tr>
</tbody>
</table>

c) RNase Treatment:
For use as a template in sequencing reactions, the RNA was removed by digestion with RNase A. The DNA was resuspended in 100μl TE buffer before adding 1μl 20mg/ml RNase A and incubating the mixture at 37°C for 30 min. The sample was then mixed with 60μl 20% (w/v) polyethylene glycol (PEG6000)/2.5M NaCl (Merck) and incubated for 1 hr on ice. After precipitating the DNA at 13000 rpm for 15 min. at 4°C the pellet was dissolved in 100μl TE buffer and twice extracted with Chloroform/Isooamyl alcohol (24:1 v/v). The final precipitation was carried out in 10μl 3M sodium acetate (NaOAc) and 60μl isopropanol. After spinning for 10 min. at 13000 rpm the DNA pellet was washed in 75% (v/v) ethanol and dissolved in 100μl TE buffer.
2.3.2. Large scale DNA isolation using CsCl-gradient centrifugation

a) Bacteria Culture
500ml of Luria Broth (LB) Medium (Gibco) containing antibiotic were inoculated with 25ml late log phase bacterial culture. The bacterial culture was amplified to an optical density at 600nm (OD600) of 0.4 (ca. 2.5 hours) and chloramphenicol was added to a final concentration of 150μg/ml. The bacteria suspension was pelleted at 6000 rpm for 10 min. at 4°C in a Sorvall GSA Rotor, the pellet was dissolved in 100ml ice cold STE buffer and again pelleted at 6000 rpm for 10min. at 4°C. The supernatant was discarded and the pellet well drained.

b) Lysis by Alkali
The bacterial pellet was resuspended in 40ml Solution I, mixed well and lysed with 5mg/ml lysozyme for 10 min. at room temperature. To denature the lysate 80ml Solution II were added for 10 min. at room temperature. 40ml Solution III were mixed well into the solution and incubated for 10 min. to 1 hr on ice. The denatured bacterial lysate was pelleted at 10000 rpm for 20 min. at 4°C and the supernatant was filtered through one layer of gauze, or Whatmann No.1 filter paper into fresh GSA centrifugation cups. The DNA was mixed with 0.6 volumes isopropanol and immediately spun at 9000 rpm for 10 min. at room temperature. The pellet was rinsed carefully with 100% ethanol and resuspended in 7 ml TE-50 buffer.

| STE buffer: | 0.1M NaCl (Merck), 10mM Tris, HCl pH 8 (Merck), 1mM EDTA, pH 8 (Sigma) |
| Solution I: (cold) | 50mM Glucose (Sigma), 10mM EDTA 25mM (Sigma), Tris, HCl pH 8.0 (Merck). Add powdered lysozyme to 4mg/ml just before use. |
| Solution II: (fresh) | 0.2M NaOH (Merck), 1% (w/v) SDS (Merck) |
| Solution III | 3M Potassium Acetate (Merck), 2M Glacial Acetic Acid (Merck) |
| TE-50 buffer: | 50 mM Tris-HCl pH 8 (Merck), 50mM EDTA-Na pH 8 (Sigma) |

c) CsCl/Ethidium Bromide Gradient Centrifugation
In a 50ml tube, 9g CsCl (Merck) were mixed with 8.63g DNA solution (in TE-50) to obtain a refraction index of 1.39. Ethidium bromide (EtBr; Sigma) was added to a final concentration of 9g/17.1g DNA solution. The solution was spun at 4000 rpm for 20 min. at room temperature and the supernatant was transferred into Ti70.1 tubes suitable for a fixed angle rotor. The tubes were topped up with a solution containing 1.58 g/ml CsCl in TE-50 and spun at 55000 rpm for 20 hours at 20°C. The fixed angle provided a shallow gradient with an upper band showing chromosomal DNA, a lower band...
containing plasmid DNA and a pellet representing RNA/ethidium bromide complexes. The plasmid band was removed and transferred into VTi.65 tubes suitable for a vertical rotor, filled to the top with 1.58 g/ml CsCl in TE-50 Solution and centrifuged at 65000 rpm for 4 hours at 20°C or at 30000 rpm overnight. The plasmid band was removed and the ethidium bromide was washed out by repeatedly adding 2 volumes of 1-Butanol/H$_2$O until the solution appeared colourless. The plasmid DNA was mixed with 3 volumes TE and 0.6 volumes Isopropanol (Merck), incubated for 2 hours at -20°C and precipitated at 12000 rpm for 20 min. The DNA pellet was washed with 70% (v/v) ethanol and resuspended in 200µl TE buffer. This purification procedure yielded on average 3-5 mg plasmid DNA/500 ml bacteria culture. The DNA was stored at 4°C to avoid frequent freeze/thaw cycles.

1-Butanol/ H$_2$O:
Add 1 volume 1-Butanol (Merck) to 1 volume H$_2$O, let phases separate.

TE Buffer
10mM Tris HCl, pH7.5 (Merck), 1mM EDTA, pH8 (Sigma)

### 2.3.3. Digestion of DNA with restriction endonucleases

DNA was restricted according to the manufacturers' specifications. The three most frequently occurring reaction conditions are listed below. Wherever possible restriction buffer supplied by the enzyme manufacturer was used. Restriction enzymes were obtained from: New England Biolabs, Boehringer Mannheim and Promega.

<table>
<thead>
<tr>
<th></th>
<th>Low salt</th>
<th>Medium salt</th>
<th>High salt</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>0</td>
<td>50 mM</td>
<td>100 mM</td>
</tr>
<tr>
<td>Tris-HCl [pH 7.4]</td>
<td>10 mM</td>
<td>10 mM</td>
<td>50 mM</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>10 mM</td>
<td>10 mM</td>
<td>10 mM</td>
</tr>
<tr>
<td>DTT</td>
<td>1 mM</td>
<td>1 mM</td>
<td>1 mM</td>
</tr>
</tbody>
</table>

### 2.3.4. Electrophoresis of DNA fragments

DNA molecules were fractionated according to size by electrophoresis through an agarose gel. 1% (w/v) gels were generally used, although 2% (w/v) gels were employed for analysis of DNA fragments less than 500 base pairs (bp) in size, and 0.7% (w/v) gels for DNA larger than 10 kilobases (Kb). The appropriate volume of 1% (w/v) agarose (Gibco) in 1x (Tris Acetate EDTA) TAE Buffer was made and heated to dissolve the agarose. The agarose solution was cooled to 60°C and ethidium bromide added to a concentration of 1µg/ml and poured into the gel cast. 1/10 volume Loading
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Buffer was added to samples and horizontal electrophoresis performed in 1xTAE Buffer at 5V/cm against standard molecular weight markers (Gibco-BRL).

\[
\text{50x TAE Buffer} \\
0.2M \text{Tris-Acetate (Merck), 50mM EDTA (Sigma)}
\]

\[
\text{5x Loading Buffer} \\
0.25\% \text{ (w/v) xylene cyanol, 0.25\% (w/v) bromophenol blue, 15\% (w/v) Ficoll type 400 (Pharmacia) in ddH}_2\text{O}
\]

2.3.5. DNA elution using glass bead suspension

Purification of DNA was performed using the Gene Clean/Bio 101 Inc. kit and reagents. Briefly, after separating the DNA on an agarose gel, the desired band was excised under long wavelength UV light (365nm) and cut into small pieces. The running buffer for the gel was TAE and 3 volumes of NaI Stock Solution (provided by manufacturer) were added to the gel slices. The agarose was dissolved at 55°C. The agarose/DNA suspension was mixed with Glassmilk Suspension using 5µl/5µg total DNA. The suspension was incubated for 5 min. on ice and gently mixed every 1-2 min. The sample was twice centrifuged for 5 seconds at 13000 rpm and each time the supernatant was discarded carefully. The pellet was washed with 200µl NaI and incubated for 2 min. at 55°C to remove undissolved agarose. After spinning for 5 seconds at 13000 rpm and removing the remaining supernatant, the pellet was washed twice with 10-50 volumes NEW Wash buffer and resuspended in 1 volume TE Buffer. The DNA was incubated for 3 min. at 55°C, spun down and the supernatant was recovered, which typically contained 80% of the DNA. This procedure was repeated once, recovering an additional 15% of DNA.

\[
\text{NEW Wash Buffer:} \\
14 \text{ml NEW Concentrate, 280 ml H}_2\text{O, 310 ml ethanol (100\%)}
\]

\[
\text{TE Buffer} \\
10mM \text{Tris HCl, pH7.5 (Merck), 1mM EDTA, pH8 (Sigma)}
\]

2.3.6. DNA ligation

T4 DNA ligase was used for all ligation reactions. This enzyme is suitable for sticky and blunt end ligations. The enzyme is unstable at temperatures higher than 30°C and is inhibited by high salt concentrations (>150mM).
The relative concentrations of vector DNA and fragment (insert) DNA used in the ligation reaction were calculated using the following formula: Fragment [μg/μl] = (molecular weight (MW) Fragment/ MW Vector) x Vector [μg/μl]. To the appropriate amounts of Insert and Vector DNA 1μl 10x Ligase buffer, 1μl T4 Ligase [400 U/ml] (Promega) and 10μl H2O were added. Reactions containing small DNA fragments were incubated for 3-4 hours at room temperature. Ligation of large inserts required incubation overnight at 15°C to allow annealing of sticky ends. Blunt end ligations were carried out in the presence of polyethylene glycol (PEG 8000). The ligation reaction was stopped by incubation of the reaction mixture for 5 min. at 70°C.

10x Ligase Buffer for sticky end Ligation:
0.5M Tris-HCl, pH 7.5 (Merck), 0.1M MgCl2 (Merck), 0.2M dithiothreitol (DTT) (Sigma), 10mM adenosine triphosphate (ATP; Promega)

5x Ligase buffer for blunt end Ligation:
25mM Tris-HCl, pH 7.5, (Merck) 50mM MgCl2 (Merck), 25% (w/v) PEG 8000 (Merck) 5mM ATP (Promega), 5mM DTT (Sigma)

2.3.7. Phosphatase treatment of DNA termini

To prevent self ligation of digested vector in a ligation reaction, the vector was first dephosphorylated. Briefly, the vector was digested with the appropriate restriction enzyme, extracted with chloroform, ethanol precipitated and resuspended in medium salt restriction enzyme buffer (see 2.3.3.). To this was added 0.2 units of calf intestinal alkaline phosphatase (Boehringer), SDS to a final concentration of 0.2% (w/v) and the sample incubated at 37°C for 30 min. The reaction was then terminated by the addition of ethylene glycol tetra-acetic acid (EGTA; Sigma) to a final concentration of 20 mM to chelate all Mg2+ ions and heat inactivation of enzyme at 68°C for 15 min. The DNA was then phenol (Fisons)/chloroform (Merck) extracted twice and ethanol precipitated.

2.3.8. Preparation of competent bacteria

A single bacterial colony was picked from a non-selective agar plate and inoculated in 5ml Luria Broth. The culture was shaken at 37°C overnight. To 5ml overnight-culture 100 ml fresh Luria Broth was added and incubated in a 500 ml flask at 37°C to an OD550 of 0.48. The mixture was cooled rapidly on ice and centrifuged at 2500 rpm for 5 min. at 4°C. The bacterial pellet was resuspended in 30ml ice cold TFB1. After incubation on ice for 15min. the suspension was pelleted at 2500 rpm for 5 min. at 4°C. The pellet was gently
resuspended in 4ml ice cold TFB2 and incubated for 15 min. on ice. After spinning at 2500 rpm for 5 min. at 4°C the bacteria pellet was resuspended and aliquoted into microcentrifuge tubes at a volume of 200μl. The aliquots were stored at -70°C. This procedure produced competent bacteria capable of achieving an average of more than $5 \times 10^8$ transformant colonies/μg of transformed plasmid DNA.

<table>
<thead>
<tr>
<th>TFB1</th>
<th>100mM RbCl (Sigma), 50mM Mn-II-Cl₂ (Merck), 30mM KOAc (Sigma), 10mM CaCl₂ (Sigma), 15% (v/v) Glycerol (Merck)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFB2</td>
<td>10mM RbCl (Sigma), 10mM MOPS, pH7 (20.9g free acid/100ml, add ca.3ml M NaOH) (Sigma), 75mM CaCl₂ (Sigma), 15% (v/v) Glycerol (Merck).</td>
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</tbody>
</table>

2.3.9. Transformation of E. coli

The DNA was added to competent cells, mixed by gentle pipetting and incubated on ice for 30 min. to allow the DNA to be absorbed onto the cells. The cells were then induced to take up the DNA by heat-shock at 42°C for 90 seconds, after which 0.5 ml of Luria Broth was added and the cells incubated at 37°C with shaking for 30 min. The cells were then gently spread onto a pre-dried agar plate containing the relevant antibiotic selection and incubated at 37°C overnight.

2.3.10. Polymerase chain reaction complementary DNA (cDNA) cloning

a) First Strand cDNA Synthesis (Reverse Transcription)
First strand cDNA was generated using the PTKII oligonucleotide (Wilks et al. 1989) and 10μg total RNA. The subsequent PCR reaction was performed with Cetus Taq polymerase, using the provided buffers and oligonucleotides PTKI and PTKII (1 μg each) (Wilks et al. 1989). Specifically, 2μl reverse transcriptase (RT) hybridisation buffer were mixed with 150 ng 3' oligonucleotide and were added together with 20μg ethanol-precipitated total RNA. The mixture was incubated for 2 hours at 40°C to allow annealing. The resulting RNA/oligonucleotide complex was mixed with 5μl 0.5M Tris, pH 8.3, 5μl 0.1M MgCl₂, 1μl RNasin, 1μl 1M NaPyrophosphate(Merck; optional), 2μl 1M KCl (Merck), 1μl 0.1M DTT (Sigma), 10μl 2mM dNTP's (Promega), 1μl Avian Myeloblastosis Virus Reverse Transcriptase (20u, Boehringer Mannheim) and 1μl Moloney Murine Leukaemia Virus Reverse Transcriptase (20u, Pharmacia). The reaction mix was completed to a total volume of 50μl with DEPC-H₂O, incubated for 2 hours at 40°C and stored at -20°C.
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RT hybridisation buffer
2M NaCl (Merck), 0.2M PIPES (Piperazine diethane sulphonylic acid) pH6.5 (Sigma), 5mM EDTA (Sigma)

2M PIPES: (100ml)
60.86 PIPES (very acidic) (Sigma), 80ml DEPC H2O, 10ml 10M NaOH (Merck), complete to 100ml with DEPC H2O

0.5M EDTA: (100ml)
18.61g Na2EDTA 2H2O (Sigma), 70ml DEPC H2O, 5ml 10M NaOH (Merck), complete to 100ml with DEPC H2O

0.5M Tris pH8.3: (500ml)
30.28g Tris base (Merck), 400ml DEPC H2O, <15ml conc HCl (Merck), allow to cool before adjusting pH, complete with DEPC H2O to 500ml

0.1M MgCl2: (100ml)
2.03g MgCl2 6 H2O (Merck), 80ml DEPC H2O, complete with DEPC H2O

RNasin Ribonuclease Inhibitor
20U/μl Recombinant RNasin (Promega)

dNTP’s: (2mM and 10mM)
100mM dATP, dCTP, dGTP, dTTP 2mM: 1:50 DEPC H2O (Cat.No. U1240, pH7, ddH2O), 10mM: 1:10 DEPC H2O, (Promega)

Avian Myeloblastosis Virus Reverse transcriptase
10U/μl Boehringer Mannheim, RNA-dep. DNA polymerase

Moloney Murine Leukaemia Virus Reverse transcriptase
1000U Pharmacia, cloned, fast performance liquid chromatography pure

b) Polymerase Chain Reaction (PCR) Amplification
To hydrolyse the RNA, 5μl first strand cDNA from a) was incubated with 2μl 1M NaOH for 10 min. at 50°C. The reaction was then neutralised with 2μl 1M HCl. The neutralized cDNA was mixed with the following components: 10μl 10x Taq Buffer, 10μl 10mM dNTP’s, 2μl 10mg/ml Gelatin, 1μl 5’ oligonucleotide (0.5-1μg), 1μl 3’ oligonucleotide (0.5-1μg), 0.5μl Taq polymerase (2.5U Cetus, Perkin-Elmer), 66μl ddH2O. The reaction was overlayed with 100μl mineral oil (Sigma).

Oligonucleotide Purification:
Oligonucleotides were supplied in NH4Cl solution (ca. 3ml), (20-mer/6600 kD: 0.2μM= ca.1.32mg, 40mM= ca. 264μg optimal) and distributed in 6 microcentrifuge tubes (a 400μl), 1ml 100% ethanol and 50μl 3M NaOAc, pH5.2 were added and oligonucleotides were precipitate at -20°C overnight. Nucleotides were pelleted at 15000 rpm for 15 sec at 4°C and resuspended in 100μl DEPC water. The concentration was measured: 1 OD260 =20 μg/ml

10x Taq Buffer
500mM KCl (Merck), 100mM Tris-HCl, pH8 (Merck), 100mM NaCl (Merck), 0.1mM EDTA (Sigma), 1% (v/v) Triton X100 (Sigma)

c) PCR Cycle (30 cycles)
To amplify the cDNA the following series of cycles were run: 1.5 min. at 93°C, 2 min. at 45°C and 4 min. at 63°C for 30 cycles obtaining 210bp
fragments. The PCR products were cloned into pBluescript (Stratagene) and sequenced.

2.3.11. λ Phage library screening

a) Plating λ Phage Clones/Libraries:
A single colony of *E. coli* bacteria ("SURE" strain, Stratagene) was used to inoculate a small volume of Luria broth containing 10mM MgSO₄ and 0.1% (w/v) maltose. This culture was incubated overnight at 30°C. NZYCM (NZamine, Yeast, Casamino acids and Magnesium) agarose plates were prewarmed to 37°C and λ Phage eluents (from λ Phage library stocks or from λ Phage plugs; see below) were diluted to a final concentration of 1:1000 in SM buffer. 600μl bacteria culture were mixed gently at 37°C for 15 min. with 1μl of λ Phage diluent to allow the phage to adhere to the bacterial cell surface. 6.5 ml Top Agar (liquified and cooled to 37°C) were added and the mixture was plated on a 15cm diameter petri dish. After the overlay has solidified, the plates were incubated overnight at 37°C and then transferred to 4°C for storage.

<table>
<thead>
<tr>
<th>NZYCM Broth (1000ml)</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>85mM NaCl (Merck), 8mM MgSO₄·7H₂O (MW 246.38, Merck), 5g Yeast Extract (Gibco), 10g NZ Amine/Casein Hydrolysate (Gibco), adjust to pH 7.5 with NaOH</td>
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<table>
<thead>
<tr>
<th>NZYCM Plates</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>15g Difco Agar (Gibco) per liter NZY Broth (1.5%), (Gibco). Approximately 50-80ml per 15cm diameter (Ø ) dish.</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Top Agar</th>
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<tbody>
<tr>
<td>3.5g Difco Agar per 500ml NZY Broth (0.7%). Approx. 7ml per 15cm Ø dish.</td>
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<table>
<thead>
<tr>
<th>SM Buffer</th>
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<tbody>
<tr>
<td>100mM NaCl (Merck), 16.6mM MgSO₄ (Merck), 50mM Tris-HCl, pH 7.5 (Merck), 0.01% (w/v) Gelatin (Sigma)</td>
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<table>
<thead>
<tr>
<th>LB Broth (1000ml):</th>
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</thead>
<tbody>
<tr>
<td>10g NaCl, 10g Bacto-Tryptone, 5g Yeast Extract, H₂O to 1000ml final volume</td>
<td></td>
</tr>
</tbody>
</table>

Note: To harvest an amplified library from a 15cm Ø dish, 10ml of SM Buffer are spread on one plate containing ca. 50-80ml of NZYCM Agarose. This is allowed to diffuse overnight. The magnesium ion concentration of the eluent (library) is 8-16mM.

b) Replica Filter Lifts:
Membranes/filters (Hybond N, Amersham) were labelled with a pencil (A: first, B: second lift). Three flat dishes were prepared containing: a) Denaturing Solution: 1.5M NaCl/ 0.5M NaOH, b) Renaturing Solution: 1.5M NaCl/ 0.5M Tris pH 8 and c) 2x SSC.
For the first lift the membrane was gently lowered onto the plate and the position marked by piercing through the filter into the agar with a needle dipped into ink. The filters were allowed to adsorb for 2 min. before they were transferred to the Denaturing Solution (Plaque/DNA side down) and incubated for 2 min. Filters were then transferred to the Renaturing Solution for 5 min. followed by a washing step for 1 min. in 2xSSC. Filters were placed on Whatmann paper, "DNA/Plaque"-side up and allowed to dry.

20xSSC
3M NaCl (Merck), 0.3M sodium citrate (Merck), adjusted to pH7

c) Second Lift:
Membranes were placed on all plates at once and the membrane marked as described in (a). Filters were allowed to adsorb for 10 min. and transferred to the Denaturing Solution, following the same Renaturing and washing procedure as described for the first lift. Filters were stacked with one interleaf, put between Whatmann paper and baked at 80°C in a vacuum for 2 hours.

d) Hybridisation:
20ml of Hybridisation Solution (prewarmed to 42°C) were filled into a 15cm Ø dish, before the filters were added one by one; each filter was submerged thoroughly. The prehybridisation was carried out at 42°C for 2-3 hours in a shaker. The prehybridisation buffer was drained and the filters were arranged as a rosette on the lid. The prehybridisation buffer was replaced by 15ml Hybridisation Solution containing the radiolabelled boiled DNA probe. Filters were added one by one and submerged thoroughly. The hybridisation was carried out at 42°C in 30% (v/v) formamide for 12-18 hours in a shaker. The Hybridisation buffer was drained and filters were washed at room temperature in a series of buffers (10 min. per step): 2xSSC/0.1% (w/v) SDS, 1xSSC/0.1% (w/v) SDS, and 0.1xSSC/0.1% (w/v) SDS. Followed by further washes of 0.1xSSC/0.1% SDS for 10 min. at 37, 42 and if necessary 65°C. Filters were exposed to an auto radiography film and developed 12 to 18 hours later.

Hybridisation Solution
30% (v/v) Formamide (Fluka), 6xSSPE, 5x Denhardt’s Solution, 300μg/ml Calf Thymus DNA (autoclaved and boiled; Sigma), 0.3% (v/v) SDS (Merck)

50x Denhardt’s Solution
1% (w/v) Ficoll (Pharmacia), 1% (w/v) polyvinylpyrrolidone (Pharmacia) and 1% (w/v) bovine serum albumin (Sigma).

20x SSPE
3M NaCl (Merck), 0.2M NaH₂PO₄, H₂O (Merck), 25 mM EDTA (Merck), adjusted to pH7.4 with NaOH.
e) Isolating Plaques of Interest:
Signals on the auto radiogram were checked against the following criteria; that the signal was present on both replicas and that it did not align to an air bubble on the agar plate. Plaques of interest were cored with the wide end of a pasteur pipette and the plug was transferred into a microcentrifuge tube which contained 500μl SM Buffer and 20μl chloroform. The phage were eluted for 1-2 hours at room temperature or at 4°C overnight. Such a λ phage stock can be stored for up to one year at 4°C.

f) In Vivo Excision of Phage Inserts Using λ ZAP System:
200μl of an overnight culture of XL1-Blue *E.coli* (Stratagene) were mixed with 200μl Phage eluent from a plug and 1μl of R408 helper phage with a titer >10^6 plaque forming units/ml. The mixture was incubated at 37°C for 15 min. followed by the addition of 3ml of 2x Yeast Tryptone Media. This mixture was incubated at 37°C for 2-2.5 hours in a shaker. The tubes were heated at 70°C for 20 min. and the suspension was spun at 2000 rpm (4000g) for 5 min. The supernatant was decanted into a sterile tube. This supernatant contains pBluescript phagemid packaged as filamentous phage particles and can be stored at 4°C for 1-2 months.

50μl of this pBluescript phagemid stock was mixed with 200μl XL1-Blue overnight culture in a 1.5ml test tube. The same amount of XL1-Blue overnight culture was also mixed with 10μl 1:10^2 pBluescript phagemid stock dilution and incubated at 37°C for 15 min. 100μl of each mixture were plated individually on 10cm Ø Luria Broth/Ampicillin (50μg/ml) plates. The plates were pre-coated with 100μl isopropyl-β-D-galactosidase (IPTG) [100mM] and 40μl (5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-Gal) [2%] for blue/white selection. Plates were incubated overnight at 37°C. Plasmid DNA isolated from white colonies was purified and further analysed.

2.3.12. DNA sequencing

Double-stranded DNA sequencing was performed using either the Sequenase 2.0 DNA Sequencing Kit (United States Biochemical) or the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems).

a) Sequenase 2.0 DNA Sequencing:
2-4μg of RNA-free plasmid DNA resuspended in 18μl H2O were added to 2μl of 2M NaOH, 2mM EDTA and incubated for 5 min. at 37°C. The DNA was precipitated by adding 8μl 5M NH4OAc, pH5 and 100μl 100% ethanol on dry ice for 5 min., pelleted at 4°C at 13000 rpm for 20 min., washed with 70% (v/v) ethanol, dried and resuspended in 7μl ddH2O.

The DNA was mixed with 2μl Annealing Buffer and 1μl oligonucleotide primer (molar stoichiometry of primer to template= 1:1). The mix was incubated at 65°C for 2 min and allowed to cool slowly to 35°C.
sequencing reaction was carried out by mixing the annealed DNA sample with 1μl 0.1M dithiothreitol (DTT), 2μl 1x Labelling Mix, 1μl 35S-dATP, and 2μl diluted Sequenase Polymerase (prediluted 1:8 in Enzyme Dilution Buffer). The reaction was incubated for 5 min. at room temperature.

From the labelling reaction, 3.5μl were added to each of the termination tubes containing 2.5μl of either of the four different ddNTPs: ddGTP, ddATP, ddTTP or ddCTP. After incubating for 5 min. at 37°C, the reaction was stopped by adding 4 μl Stop Solution. 2-3μl of each termination reaction were heat denatured and resolved on a 6% polyacrylamide/urea gel. The gel was pre-run for 30 min. at 60W with 1xTBE as running buffer. DNA samples were run for approximately 4 hrs at 60W. The gel was fixed with 5% (v/v) methanol/5% (v/v) acetic acid and then in 5% (v/v) methanol for 15 min. and transferred on to filter paper. The gel was dried in a vacuum for 1 hr at 70°C and exposed to an auto radiogram overnight.

<table>
<thead>
<tr>
<th>5x Annealing Buffer</th>
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<tbody>
<tr>
<td>200mM Tris-HCl, pH7.5, 100mM MgCl2, 250mM NaCl</td>
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<tr>
<th>Enzyme Dilution Buffer</th>
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<tbody>
<tr>
<td>10mM Tris-HCl, pH7.5, 5mM DTT, 0.5mg/ml Bovine Serum Albumin</td>
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<table>
<thead>
<tr>
<th>Labelling Mix:</th>
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<tbody>
<tr>
<td>7.5μM dGTP, 7.5μM dCTP, 7.5μM dTTP</td>
</tr>
</tbody>
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<tr>
<th>Stop solution</th>
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<tbody>
<tr>
<td>95% Formamide, 20mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol FF</td>
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<thead>
<tr>
<th>Acrylamide/Urea gel (6%,):</th>
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<tbody>
<tr>
<td>8.3M Urea (Sigma), 20% (w/v) Acrylamide (Bio-Rad), 2.8 ml TBE (20%), 0.6 ml Ammonium persulphate (10%) (Bio-Rad), 35μl N,N,N',N'-tetramethylethylenediamine (TEMED) (Bio-Rad)</td>
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<tr>
<th>10x TBE buffer</th>
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<tbody>
<tr>
<td>0.89 Tris HCl (Merck), 0.89M Boric Acid (Sigma), 2mM EDTA (Sigma)</td>
</tr>
</tbody>
</table>

b) Taq DyeDeoxy Terminator Cycle Sequencing:
Double-stranded DNA templates do not need to be denatured. 1μg of plasmid DNA in ddH2O was mixed with: 3.2pmol oligonucleotide primer, 4μl 5x TACS Buffer, 1μl dNTP Mix, 1μl DyeDeoxy A Terminator, 1μl DyeDeoxy T Terminator, 1μl DyeDeoxy G Terminator, 1μl DyeDeoxy C Terminator and 0.5μl AmpliTaq DNA Polymerase in a total volume of 20μl. The reaction was overlaid with mineral oil (Sigma) and incubated for 25 cycles as follows: at 96°C for 30sec., at 50°C for 15sec. and at 60°C for 4min. Samples were brought up to 80μl volume with ddH2O and the aqueous phase transferred to a fresh microcentrifuge tube, followed by two extractions with 100μl of phenol:H2O:chloroform (68:18:14). Extension products were precipitated by adding 15μl of 2M sodium acetate, pH4.5 and 300μl 100% ethanol and centrifuging for 15min. at 13000 rpm at room temperature. Samples were washed once with 70% (v/v) ethanol, dried and resuspended in
4μl Formamide Buffer. Prior to loading onto Applied Biosystems 373A DNA sequencer, samples were heated at 90°C for two min.

<table>
<thead>
<tr>
<th>5x TACS Buffer</th>
<th>400mM Tris HCl, 10mM MgCl₂, 100mM (NH₄)₂SO₄, pH9</th>
</tr>
</thead>
<tbody>
<tr>
<td>dNTP Mix</td>
<td>150μM dGTP, 150μM dATP, 150μM dTTP, 150μM dCTP</td>
</tr>
<tr>
<td>Formamide Buffer</td>
<td>80% Formamide, 10mM EDTA, pH8</td>
</tr>
</tbody>
</table>

2.3.13. Southern blot analysis

a) DNA transfer:
Purified DNA was digested using appropriate restriction enzymes and fractionated by electrophoresis in an agarose gel containing TAE buffer at 3 to 4V/cm. The gel was stained with ethidium bromide (5μg/ml) and photographed under longwave UV light. The DNA was then transferred to reinforced nitro-cellulose (Schleicher & Schuell) by a modification of the Southern blot protocol (Sambrook et al. 1989). The agarose gel was incubated twice for 30min. in Solution A and then twice for 30min. in Solution B. The DNA was transferred to nitro-cellulose in Solution B over a period of 16-24 hours. The nitro-cellulose filter was then allowed to dry and was baked in a vacuum at 80°C for 2 hours.

<table>
<thead>
<tr>
<th>TAE Buffer</th>
<th>0.04M Tris-acetate (Merck), 2mM EDTA (Sigma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution A</td>
<td>1.5M NaCl (Merck), 0.5M NaOH</td>
</tr>
<tr>
<td>Solution B</td>
<td>1M Ammonium Acetate (Sigma), 5mM NaOH</td>
</tr>
</tbody>
</table>

b) DNA detection
DNA was detected by hybridisation of a radiolabelled, complementary DNA probe. To denature purified DNA fragments, the samples were heated to 95°C for 5 min. and cooled on ice. For radiolabelling a labelling kit designed for random priming was used (Boehringer Mannheim) to incorporate [α³²P]dCTP (74MBq/ml, Amersham International) into DNA by adding: dATP, dTTP, dGTP (0.5nmol each), [α³²P]dCTP (0.37MBq), 2U Klenow DNA polymerase and 10% v/v of a solution containing a random mixture of hexanucleotides to the denatured DNA sample. The reaction was incubated for 30 min. at 37°C. The labelled DNA was purified from unincorporated nucleotides by using a chromatography column (Stratagene NucTrap). The activity of the probe was counted in 1μl fraction of purified DNA in a scintillation counter (Beckman Instruments).
The blot was pre-hybridised in a rotary hybridisation oven in Southern Hybridisation Buffer at 42°C overnight. Filters were washed once in 2xSSC/0.1%SDS at room temperature for 30 min, once in 1xSSC/0.1%SDS at room temperature for 30 min, and finally once in 0.1xSSC/0.1%SDS for 30 min at 60°C. The filters were then exposed to auto radiography film at -70°C for up to 7 days using intensifier screens, or to a phosphoimager storage plate up to 48 hours. Images were presented using computer supported graphics (Apple Macintosh, Microsoft Adobe Photoshop).

### Southern Hybridisation Solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% Formamide (Fluka)</td>
<td>6xSSPE</td>
</tr>
<tr>
<td>5x Denhardt's Solution</td>
<td>300µg/ml Calf Thymus DNA (autoclaved and boiled; Sigma)</td>
</tr>
<tr>
<td>0.3% SDS (Merck)</td>
<td></td>
</tr>
</tbody>
</table>

### 20xSSPE

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>3M NaCl (Merck)</td>
<td>0.2M NaH2PO4. H2O(Merck)</td>
</tr>
<tr>
<td>25 mM EDTA (Merck)</td>
<td>adjusted to pH7.4 with NaOH</td>
</tr>
</tbody>
</table>

### 50x Denhardt's Solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% (w/v) Ficoll (Pharmacia)</td>
<td>1% (w/v) polyvinylpyrrolidone (Pharmacia)</td>
</tr>
<tr>
<td>1% (w/v) bovine serum albumin. (Sigma)</td>
<td></td>
</tr>
</tbody>
</table>

### 20xSSC

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>3M NaCl (Merck)</td>
<td>0.3M sodium citrate (Merck)</td>
</tr>
</tbody>
</table>

### 2.3.14. Total RNA isolation

#### a) Tissue culture:

Cells were lysed directly in the culture dish with 1.8ml Guanidinium Thiocyanate (GT) Solution/100mm dish (ca 60cm²) or 100µl GT Solution/10⁶ cells of suspension culture.

#### b) Dissected tissue samples:

Tissue was transferred into ice cold Leibovitz Medium (L-15), washed with 1xPBS and centrifuged at 1200 rpm for 5 min.. The cell pellet was resuspended in 1.8 ml GT Solution and cells were triturated through a pipette.

#### c) RNA Purification:

The cell/ GT Solution mixture was transferred into a 4ml snap-cap tube and mixed thoroughly after adding 180 µl 2M NaOAc, pH4, 1.8 ml phenol (H2O saturated only for use with RNA) and 360µl chloroform:isoamyl alcohol (24:1 v/v (CIA) Merck). The suspension was shaken well and cooled on ice for 15 min, before spinning at 10000 rpm for 20 min. at 4°C. The aqueous phase was transferred to a fresh tube and mixed with 1.8ml isopropanol (Merck) per 1.8ml RNA/GT Solution and left at -20°C overnight. The RNA was precipitated at 10000 rpm for 20 min. at 4°C and resuspended in 300µl GT Solution. 600µl 100% ethanol were added and the mixture was again incubated at -20°C overnight. The RNA was precipitated at 15000 rpm for
10 min. at 4°C, washed with 80% (v/v) ethanol, dried and resuspended in 50µl 0.2% (w/v) DEPC-SDS. This RNA preparation is ready to use for total RNA Northern blot or poly-A selection.

**Guanidinium Thiocyanate (GT) Solution**
250g Guanidinium Thiocyanate (Fluka), 293ml ddH2O, 17.6ml 0.75M NaCitrate (Merck), pH7, 26.4ml 10% (w/v) Sarcosyl (Fluka). Compounds were dissolved at 65°C and prior to use 0.36ml 2-Mercaptoethanol were added per 50ml GT-Stock solution.

2.3.15. Northern blot analysis
15µg of total RNA were heat denatured, size fractionated on a 1% (w/v) agarose/0.7% (v/v) formaldehyde gel in 1x MOPS Running Buffer. The fractionated RNA was then blotted onto Hybond-N membranes (Amersham) using 10xSSC transfer buffer. Membranes were probed at high stringency as described (Sambrook et al. 1989), using random primed ^32P-γ-dCTP labelled cDNA probes (Feinberg and Vogelstein 1983), and see section 2.3.13b. Auto radiography was performed using either a phosphoimager and final images presented by computer based graphics (Macintosh, Adobe Photoshop) or Kodak film using intensifying screens at -70°C.

**10x MOPS**
0.4M Na-Morpholinopropansulphate (MOPS, Sigma), 0.1M NaOAc (Sigma), 10mM EDTA (Sigma), adjust pH to 7.0 with NaOH (ca. 15ml/L)

2.3.16. Protein isolation

a) **Tissue samples**
Tissues samples (0.5 cm cubes) were put into approximately 500 µl of ice cold lysis buffer on ice and cut into fine pieces with a scalpel blade, vortexed three times and placed at 4°C with agitation for 40 min. Samples were then centrifuged at 13 000 rpm for 10 min at 4°C and the supernatant used. The lysis buffer for myelin basic protein (MBP) is shown below, other lysis buffers are found in the appropriate section.

b) **Cells**
The cells were washed once in ice cold phosphate buffered saline (PBS) and lysed on ice for 5-10 min. in ice-cold lysis buffer. The lysates were vortexed and centrifuged at 13 000 rpm for 10 min at 4°C and the pellet discarded. The lysis buffer for myelin basic protein is shown below, other lysis buffers are found in the appropriate section.
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**Myelin basic protein (MBP) lysis buffer**

20 mM Tris-HCl pH 7.5 (Merck), 150 mM NaCl (Merck), 0.1% SDS (Merck), 1% Nonidet P-40 (NP-40; Sigma), 0.5% sodium deoxycholate (Sigma), 1 mM EDTA (Sigma), 2 mM phenylmethylsulphonyl fluoride (Sigma), 20 μg/ml leupeptin (Sigma), 15 nM pepstatin (Sigma), 0.5 mM Nα-p-tosyl-L-lysine chloromethyl ketone (Sigma) and 20 μg/ml aprotinin (Sigma)

2.3.17. Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis

Gel equipment was assembled according to the manufacturers instructions, (plates were cleaned with detergent, then dH₂O and finally 100% ethanol). The resolving gel was prepared and filled to approximately twice the length of the comb teeth, from the top edge of the gel plate. Depending on the size of the protein to be analysed, different concentrations of polyacrylamide were used (8% (w/v) for ca. 160kD and 15% (w/v) for ca.40kD and under. The gel was immediately overlaid with H₂O-saturated butanol (Merck) and allowed to set. The butanol was rinsed away with dH₂O and excess water removed with 3MM Whatman paper, before the stacking gel was poured on top of the resolving gel, 5% (w/v) stacking gels were used. The comb was inserted deep enough into the gel to exclude air and the gel was allowed to set. The comb was then removed and the gel was inserted into the gel tank (Gibco) and secured. Upper and lower chambers were filled with 1xSDS Running Buffer and the wells rinsed with buffer. The protein samples were boiled for 5min. and put on ice. Samples in Sample buffer were pipetted into the wells and the gel was run usually overnight (16 hr) at 12 mA. When gels were stained, this was with Coomassie Blue for 10 min., destaining was with 40% (v/v) methanol, 10% (v/v) acetic acid for 45min. to overnight and these gels were dried on 2 layers of 3MM Whatman paper in a vacuum at 65°C for 1 hr.

**Resolving Gel (8%)**

For a total of 40 ml gel volume : 16.6 ml H₂O, 8 ml Acrylamide (40%, 29:1) (Merck), 15 ml Tris-HCl pH 8.8, 200 μl SDS (20%), 200 μl Ammonium persulphate (20%), 20μl N,N,N′,N′-tetramethylethylenediamine (TEMED) (Bio-Rad).

**Resolving Gel (15%)**

For a total of 40 ml gel volume : 9.59 ml H₂O, 15 ml Acrylamide (40%, 29:1) (Merck), 15 ml Tris-HCl pH 8.8, 200 μl SDS (20%), 200 μl Ammonium persulphate (20%), 20μl TEMED (Bio-Rad).

**Stacking Gel (5%)**

For a total of 20 ml gel volume: 15.4 ml H₂O, 1.88 ml Acrylamide (40%, 29:1) (Merck), 2.5 ml Tris-HCl pH 6.8, 100 μl SDS (20%), 100μl Ammonium persulphate (20%), 20μl TEMED (Bio-Rad).
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5x SDS Running Buffer (1000ml)
30 g Tris Base (Merck), 144g Glycine (Merck), 5g SDS (Merck)

5x Sample Buffer
100mM Tris, pH 8 (Merck), 4% (w/v) SDS (Merck), 50% (v/v) Glycerol (Merck),
50mM β-Mercaptoethanol (Sigma), 0.5% (w/v) Bromophenol Blue (Bio-Rad)

Coomassie Blue:
10% (v/v) Acetic Acid (Merck), 0.3% (w/v) Coomassie R250 (Bio-Rad), 50% (v/v)
Methanol

Fast Destain
10% (v/v) Acetic Acid, 40% (v/v) Methanol

Prestained MW-Markers (Sigma)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein +Dye</th>
</tr>
</thead>
<tbody>
<tr>
<td>180kD</td>
<td>a2-Macroglobulin</td>
</tr>
<tr>
<td>116kD</td>
<td>b-Galactosidase (E.coli)</td>
</tr>
<tr>
<td>84kD</td>
<td>Fructose-6-Phosphate Kinase</td>
</tr>
<tr>
<td>58kD</td>
<td>Pyruvate Kinase</td>
</tr>
<tr>
<td>48kD</td>
<td>Fumarase</td>
</tr>
<tr>
<td>36.5kD</td>
<td>Lactic Dehydrogenase</td>
</tr>
<tr>
<td>26.6kD</td>
<td>Triosephosphate Isomerase</td>
</tr>
</tbody>
</table>

Preparation: 0.24g Urea, 500μl dH2O, 200μl 5x Sample Buffer (Laemmli) were added to the marker vials, boiled and completed to 1ml, 20μl aliquots were made and stored at -20°C.

2.3.18. Western blot analysis

Proteins separated by an SDS-polyacrylamide gel can be selectively identified using specific antisera by Western blotting. Specifically, the gel was immersed in Transfer Buffer for approximately 5 min. The gel was then placed on top of nitro-cellulose paper (Schleicher & Schuell) presoaked in transfer buffer. The gel/nitro-cellulose was sandwiched between several layers of presoaked Whatman 3 MM and placed in the transfer apparatus (Biorad) with the nitro-cellulose between the anode and the gel. The proteins were transferred from the gel onto the nitro-cellulose by applying a current, which was dependent on the dimensions of the gel (mA=gel area cm² x 0.8). After 6 hrs the gel was then Coomassie stained to analyse whether transfer was complete.

The nitro-cellulose membranes were blocked overnight in Blocking Buffer at 4°C with agitation. Membranes were then washed in TBST, (three 10 min. washes), and incubated with the primary antibody, diluted in Blocking Buffer, for 2 hours at room temperature. After three washes (10 min. each) in TBST, membranes were treated in one of two ways; either incubated with 125I-Protein A (Amersham) in blocking buffer (dilution 1:1000) for 40 min. at room temperature, and then washed 3 times in TBST (10 min. each) and autoradiographed with Fuji RX film using intensifying screens at -70°C; or incubated with anti-horse radish peroxidase conjugate
(Amersham; diluted 1:8000) in blocking buffer for 1 hr at room temperature followed by 3 washes in TBST (10 min. each) and development using the Amersham Enhanced Chemiluminescence (ECL) System, according to the manufacturers instructions and Fuji RX film.

**Transfer Buffer**
192 mM glycine, 25 mM Tris-HCl, pH 7.5, 0.1% (w/v) SDS, 20% (v/v) methanol

**Tris-buffered saline and Tween (TBST) buffer**
Tris-buffered saline (20 mM Tris-HCl, pH 7.5 (Merck), 150 mM NaCl (Merck)) with 0.1% (v/v) Tween-20 (Sigma)

**Blocking Buffer (5% Marvel in TBST)**
Tris-buffered saline with 5% (w/v) Marvel milk powder and 0.1% (v/v) Tween-20 (Sigma)

**Primary Antibodies**
- anti-Myelin Basic Protein (MBP) rabbit polyclonal 1:2500 (Pep7 a kind gift from Dr. P.J. Brophy, Stirling)

**Secondary Antibodies**
- 125I-Protein A 1:1000 (Amersham)
- Anti-rabbit horse radish peroxidase conjugate 1:8000 (Amersham)

### 2.3.19. Mitogen Activated Protein Kinase (MAPK) Assay

The cells were stimulated by the addition of growth factors [such as VEGF, EGF or PDGF-AA (1 μg/ml)] which were added to the medium for 10 min. The cells were then washed once in ice cold phosphate buffered saline and lysed on ice for 10 min. in MAPK lysis buffer.

The lysates were vortexed and centrifuged at 13 000 rpm for 10 min. at 4°C. The pellet was discarded, 3μl of supernatant was taken for protein concentration measurement by the Bradford-assay (BioRad) at 595nm. Lysates containing equal amounts of protein (adjusted with MAPK lysis buffer to a final volume of 250μl) were incubated with the 122 MAPK rabbit antiserum (1:100 dilution); (Leevers and Marshall 1992); a kind gift from Dr. S. Leeveres) for 1 hr at 4°C with agitation. The antibody complexes were immunoprecipitated with 100 μl of 10% protein-A-Sepharose (Sigma; which had been washed three times in MAPK lysis buffer) for 2 hrs at 4°C with agitation. They were then washed twice in MAPK lysis buffer and once in kinase buffer.

The immunoprecipitates were incubated in 30μl of Kinase buffer containing myelin basic protein (250 μg/ml; Sigma) and [γ³²P]ATP (33 μCi/ml) for 30 min. at 30°C with occasional agitation. The immunoprecipitates were boiled after the addition of 8μl of 5x Sample buffer and analysed on a 15% SDS-polyacrylamide gel. The gel was subsequently western blotted onto nitro-cellulose paper (Schleicher & Schuell) and
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Radiolabelled myelin basic protein was detected with Fuji RX film using intensifying screens at -70°C.

\[
\text{MAPK lysis buffer} \\
20 \text{ mM Tris-HCl pH 8.0 (Merck), 40 mM sodium}\, \textit{tetra} \text{ pyrophosphate (Merck), 50 mM NaF (Merck), 5 mM MgCl}_2 \text{ (Merck), 100 } \mu\text{M sodium orthovanadate (Sigma), 10 } \mu\text{M EGTA (Sigma), 1}\% \text{ TritonX-100 (Sigma), 0.5}\% \text{ sodium deoxycholate (Sigma), 0.1}\% \text{ SDS (Merck), 20 } \mu\text{g/ml leupeptin (Sigma), 20 } \mu\text{g/ml aprotinin (Sigma), 2 mM phenylmethylsulphonyl fluoride (Sigma)}}
\]

\[
\text{Kinase buffer} \\
30 \text{ mM Tris-HCl, pH 8.0 (Merck), 20 mM MgCl}_2 \text{ (Merck), 2 mM MnCl}_2 \text{ (Merck), 10 } \mu\text{M ATP (Sigma)}}
\]

\[
\text{5x Sample Buffer:} \\
100\text{mM Tris pH 8 (Merck), 4}\% \text{ (w/v) SDS (Merck), 50} \% \text{ (v/v) Glycerol (Merck), 50mM } \beta\text{-Mercaptoethanol (Sigma), 0.5}\% \text{ (w/v) Bromophenol Blue (Bio-Rad)}}
\]

2.3.20. \textit{In vitro} kinase assay for Human Embryo Kinase 2 (HEK2)

The cells were grown to 70\% confluency, washed once in ice cold phosphate buffered saline and lysed on ice for 5 minutes in ice-cold HEK2 lysis buffer.

The lysates were vortexed and centrifuged at 13000 rpm for 10 min. at 4°C and the pellet discarded. The protein concentration was measured by the Bradford-assay. Lysates containing equal amounts of protein (adjusted with HEK2 lysis buffer to a final volume of 500 \mu l) were incubated with specific rabbit immune serum to HEK2 (a kind gift from Dr. B. Böhme at a dilution of 1:100) for 1 hr at 4°C with agitation. The antibody complexes were immunoprecipitated with 100 \mu l of 10\% protein-A-Sepharose (which had been washed three times in HEK2 lysis buffer) for 2 hrs at 4°C with agitation. The immunoprecipitates were washed twice in ice-cold HEK2 lysis buffer and once in kinase buffer.

The immunoprecipitates were incubated in 50\mu l of kinase buffer containing \[^{32}\text{P}]\text{ATP} (200 \mu\text{Ci/ml}) for 30 minutes at 30°C. The kinase reactions were washed once in HEK2 lysis buffer and boiled in 30\mu l of 2.5x Sample buffer and centrifuged at 13000 rpm for 5 minutes and the Sepharose pellet discarded. The immunoprecipitates were then size fractionated by SDS-polyacrylamide gel electrophoresis on an 8\% (w/v) gel and the dye front allowed to run off the edge of the gel. The gel was fixed for 30 min. in 10\% (v/v) acetic acid and 30\% (v/v) methanol with gentle shaking, then incubated in 1M KOH at room temperature for 10min. and afterwards incubated in fresh 1M KOH for 90 min. at 55°C with occasional shaking. The gel was refixed in 10\% (v/v) acetic acid and 30\% (v/v) methanol for three times 30min. and dried onto Whatmann paper on a gel drier at 70°C for 2hrs. Radiolabelled proteins were detected with Fuji RX film using intensifying screens at -70°C.
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<table>
<thead>
<tr>
<th>HEK2 lysis buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM Tris-HCl, pH 7.5 (Merck), 150 mM NaCl (Merck), 0.1% SDS (Merck), 1% Nonidet P-40 (NP-40; Sigma), 0.5% sodium deoxycholate (Sigma), 1 mM EDTA (Sigma), 1 mM phenylmethylsulphonyl fluoride (Sigma), 10 ( \mu )g/ml aprotinin (Sigma), 10 ( \mu )g/ml leupeptin (Sigma) and 10 ( \mu )M sodium orthovanadate (Sigma)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Kinase buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 mM Hydroxyethylpiperazine ethanesulphonic acid (HEPES) pH 7.4 (Sigma), 10 mM MnCl(_2) (Merck), 1mM EDTA (Sigma) and 0.1% TritonX-100 (Sigma)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>5x Sample Buffer:</th>
</tr>
</thead>
<tbody>
<tr>
<td>100mM Tris pH 8 (Merck), 4% (w/v) SDS (Merck), 50% (v/v) Glycerol (Merck), 50mM ( \beta )-Mercaptoethanol (Sigma), 0.5% (w/v) Bromophenol Blue (Bio-Rad)</td>
</tr>
</tbody>
</table>

### 2.3.21. In situ hybridisation

The tissue specific expression of LIMK, HEK2 and PDGFR\(\alpha\) were studied using human wax embedded sections. The in situ hybridisation protocol applied here is based on the detection of digoxigenin (DIG) labelled RNA by alkaline phosphatase conjugated anti-DIG Fab-fragments (Boehringer Mannheim) as described (Birren et al. 1993; Wilkinson 1992).

**a) Microscope Slide Preparation:**

Microscope slides were either soaked overnight in Dichrol in a fume hood or for 1 hr in surface cleaning detergent (Decon 90). The slides were rinsed in ddH\(_2\)O and dried for 20min. at 150°C. Slides were then dipped for 20-60 seconds in a solution of 2\% (w/v) 3-triethoxysilyl-propylamine (TESPA; Sigma) in acetone, followed by two washing steps in acetone and three washing steps in DEPC-treated water. After drying the slides overnight, they were stored dry, at room temperature. All glass ware was baked at 180°C for twelve hours and then treated as RNAse-free. Double-distilled water (or water of equivalent quality) was made RNase-free by treating one litre of water with 1ml Di-Ethyl-Pyrocarbonate (DEPC, Sigma) for 12 hours at room temperature, followed by autoclaving at 120°C for 20 min. to remove remaining traces of DEPC.

**b) Section Preparation:**

Human tissues were placed overnight in 4\% (w/v) paraformaldehyde in DEPC-PBS at 4°C, in a flat bottomed glass vial. The solution was replaced with DEPC-PBS for 30-60min. (depending on size of tissue >0.5cm cube for the longer time mentioned) at 4°C, and then replaced with 0.85\% (w/v) sodium chloride at 4°C for 30-60 min. twice. The solution was then changed to half 0.85\% (w/v) sodium chloride to which an equal volume of 100\% ethanol was added, the mixture was swirled and left at room temperature for 15-30 min. All following incubations were done at room temperature. The solution was successively replaced with 50\% (v/v) ethanol made up with
0.85% (w/v) sodium chloride and followed by two incubations in 70% (v/v) ethanol made up with DEPC-H$_2$O, each incubation was for 15-30 min. The tissue was left in 70% (v/v) ethanol overnight at 4°C. The solution was replaced with 85%, 95%, 100% and 100% (v/v) ethanol each for 30-60 min. and then two changes with histoclear (National Diagnostics) each for 30-60 min. In a 60°C oven the histoclear was replaced with paraffin wax (Raymond Lamb), three changes were made, each lasting 20-40 min. The tissue was orientated in a plastic mould containing wax at 60°C on a heating block. The sample was allowed to set and stored at 4°C.

The wax block was removed from the mould, trimmed to a square block and clamped in the microtome (Reichert-Jung). The blade was positioned to approach the surface by 50μm cuts. Once the surface was reached sections were cut at a thickness of 6μm into ribbons which were lowered onto DEPC-H$_2$O at 50°C in a waterbath and left for 2 min. to unfold. The ribbons were transferred onto slides drained vertically and dried on a 50°C slide drier. Sections were stored at 4°C.

**Phosphate Buffered Saline (PBS)**

140mM NaCl (Merck), 3mM KCl (Merck), 1mM K$_2$HPO$_4$ (Merck), 7mM Na$_2$HPO$_4$ · 2H$_2$O (Merck), 0.5mM MgCl$_2$ · 6H$_2$O (Merck)

4% (w/v) Paraformaldehyde in 1x PBS

8g EM-grade Paraformaldehyde (Sigma) was dissolved in 200ml DEPC-H$_2$O and 200μl 5M NaOH by stirring for 30min. at 60°C. Two PBS tablets (Sigma P-4417) were added and allowed to dissolve. The solution was cooled to room temperature, filtered and the pH was adjusted to pH7 with 1M HCL.

c) **Template Preparation:**

To generate digoxigenin-labelled probes templated DNA was linearised to allow run-off transcription using the RNA polymerase promoter present upstream of the cloned insert (probe sequence). Specifically 20-40μg plasmid DNA were digested with appropriate restriction enzymes and precipitated in 1/9 volume RNAse-free 3M NaOAc, pH 4.8 and 2 volumes 100% ethanol at -70°C for 30 min. The DNA was resuspended in 20μl DEPC-H$_2$O and diluted to a final concentration of 0.5μg/μl.

d) **Preparation of Digoxigenin (DIG) Labelled RNA Probes:**

In a final volume of 50μl the following reagents were added: 2.5μg template DNA, 10μl 5x Transcription Buffer (supplied by manufacturer), 5μl 100mM DTT, 20μl 2.5x rNTP/DIG-UTP Mix, 1μl RNasin (10U/μl) and RNA polymerase (T3 or T7; Boehringer Mannheim). The mixture was incubated for 2hrs at 37°C and a small aliquot (2μl) was retained. The rest of the sample was then incubated with 10μl RQ-DNase [1U/μl] (Boehringer Mannheim) at 37°C for 30 min.

After the incubation another 2μl aliquot was removed. This and the previous aliquot were then analysed side-by-side on an agarose gel to test for complete degradation of the DNA template. To the remaining sample (56μl)
1 μl of 0.5M EDTA, pH 8 was added to stop the reaction and the sample was purified using ammonium acetate precipitation by adding the following reagents, 50 μl DEPC-H2O, 72 μl of 5M NH4OAc (Sigma) and 170 μl of 100% ethanol (the 2M final concentration of NH4OAc, excluding the ethanol, does not precipitate nucleotides) precipitation was on dry ice for >1 hr, followed by centrifugation 13000 rpm for 30 min. at 4°C. The RNA pellet was washed with 95% (v/v) ethanol and dissolved in 50 μl 40 mM NaHCO3/60 mM Na2CO3. One microlitre of the sample was used in a UV-spectrophotometer to determine the amount of single-stranded RNA probe generated (OD260 nm 1 = 40 μg/μl). To hydrolyse the RNA strands into smaller fragments which can diffuse into the tissue more readily, the sample was incubated at 60°C for various amounts of time (t) according to the following formula:

\[
\frac{L_0 \text{ [kb]} - L_f \text{ [kb]}}{t} = \frac{0.11 \times L_0 \text{ [kb]} \times L_f \text{[kb]}}{L_0 \text{[kb]}}
\]

L0: original length of transcripts (= length of template DNA) in kb
Lf: desired length of probe fragments in kb (ideally 0.2-0.4 kb)

To stop hydrolysis, the sample was precipitated with 5.5 μl 1M sodium acetate, pH 4.8 and 100 μl 100% ethanol at -70°C for 30 min. The sample was washed with 95% (v/v) ethanol and suspended in 200 μl Hybridisation Buffer to a final concentration of 40 μg/ml. Probes were then stored at -70°C. The incorporation of DIG-rUTP into the RNA probes was assayed by dot-blot analysis: a Hybond-N nylon membrane (Amersham) was subdivided into small squares using a soft pencil. 1-5 μl of probe were spotted on to the membrane and allowed to air dry. The RNA was then cross-linked to the membrane using UV light (Stratagene UV-Crosslinker, 1200 μJoules). All subsequent treatments were carried out on a shaking platform and at room temperature: 1 min. in Buffer 1, 30 min. in Buffer 2 (blocking), 1 min. in Buffer 1, 30 min. To detect the DIG-groups incorporated into the probe, the membrane was incubated in a 1:5000 dilution of anti-DIG antibody coupled to alkaline phosphatase (Boehringer Mannheim) in Buffer 1 for 30 min. The membrane was then washed twice for 15 min. in Buffer 1 and for 2 min. in Buffer 3. The colour reaction was carried out for 5-20 min. in Colour Substrate Buffer. The reaction was stopped with Buffer 4 and the membrane stored moist, wrapped in cling film.

**2.5x rNTP/DIG-UTP Mix**
- 2.5 mM rATP, 2.5 mM rCTP, 2.5 mM rUTP, 2.5 mM rOTP (Promega), 0.87 mM DIG-rUTP (Boehringer Mannheim)

**Hybridisation Buffer**
- 50% (v/v) Formamide (Fluka), 1 mg/ml Yeast tRNA (Sigma), 100 μg/ml Heparin, 5xSSC, 1x Denhardt's Solution, 0.1% (v/v) Tween 20 (Sigma), 0.1% (w/v) CHAPS (Sigma), 0.5 mM EDTA pH 8 (Sigma)
e) Fixation and Hybridisation of Tissue Sections

The slides were warmed to room temperature. The microscope slides were then placed in suitable slide racks. All following treatments were carried out in baked, glass troughs at room temperature unless otherwise indicated.

The sections were de-waxed in histoclear for 10 min. twice and then placed through an alcohol rehydration series, 100% ethanol for 2 min. to remove most of the histoclear and then quick changes of 100%, 100%, 95%, 85%, 70%, 50% and 30% (v/v) ethanol. The slides were then placed in 0.85% (w/v) sodium chloride followed by DEPC-PBS each for 5 min., fixed for 20 min. in 4% (w/v) paraformaldehyde, pH7 (Sigma) and then washed twice for 5 min. in DEPC-PBS.

To allow better penetration of the probe, the samples were permeabilised in 20µg/ml Proteinase K (Boehringer Mannheim) in Protease Buffer for 6 min. and then placed in DEPC-PBS for 5 min. The sections were refixed for 20 min. in 4% (w/v) paraformaldehyde, pH7 (Sigma), and rinsed in DEPC-H2O.

To reduce background signal, the tissue sections were acetylated. This was done by placing them in 200 ml 0.1M Triethanolamine-HCl, pH8 and slowly adding 420 µl acetic anhydride whilst stirring, by a stir bar to aid dispersal. The samples were allowed to stand for 10 min. and placed for 5 min. in DEPC-PBS followed by 5 min. in 0.85% (w/v) sodium chloride. The tissue sections were then ready for hybridisation. All hybridisations were carried out at 60°C. The microscope slides were covered with 25x55 mm siliconised coverslips and kept in a moist chamber. For the prehybridisation (3-4hrs) 250 µl Hybridisation Buffer per microscope slide were used. The probe was used at a final concentration range of 1-4 µg/ml.
Chapter 2  Materials & Methods

\[
\begin{align*}
Protease Buffer & \quad 50\text{mM Tris-}\text{HCl, pH7.5 (Merck), 5mM EDTA, pH8 (Sigma)} \\
Hybridisation Buffer & \quad 50\% (v/v) \text{ Formamide (Fluka), 1mg/ml Yeast tRNA (Sigma), 100}\mu\text{g/ml Heparin (Sigma), 5xSSC, 1x Denhardt's Solution (Sigma), 0.1\% (v/v) Tween-20 (Sigma), 0.1\% (w/v) 3-[3-Cholamidopropyl]dimethylammonio]-1-propane sulphonate (CHAPS; Sigma), 0.5mM EDTA pH8 (Sigma).}
\end{align*}
\]

\textbf{f) Washing Steps after Hybridisation:}

After hybridisation it was no longer necessary to use RNase-free solutions, but it was important not to let the sections dry out. The hybridisation buffer containing the probe could be collected and reused several times. The slides were washed as followed: 10min./ 60°C in Buffer A, 10min./ 60°C in Buffer B, and twice for 20min./ 37°C in Buffer C. This was followed by an RNase treatment to further reduce unspecific background signal by incubating the slides for 30min./ 37°C in Buffer C containing 20\mu g/ml RNase A and 10U/ml RNase T1. Slides were washed for 10min. at room temperature in Buffer C, for 30min./ 60°C in Buffer D, two times for 10min./ 60°C in Buffer E, for 10min./ room temperature in PTw and finally 15min. in PBT.

\[
\begin{align*}
\text{Buffer A} & \quad 1\times\text{SSC, 0.3\% (w/v) 3-[3-Cholamidopropyl]dimethylammonio]-1-propane sulphonate (CHAPS; Sigma)} \\
\text{Buffer B} & \quad 1.5\times\text{SSC, 0.3\% (w/v) CHAPS (Sigma)} \\
\text{Buffer C} & \quad 2\times\text{SSC, 0.3\% (w/v) CHAPS (Sigma)} \\
\text{Buffer D} & \quad 0.2\times\text{SSC, 0.3\% (w/v) CHAPS (Sigma)} \\
\text{Buffer E} & \quad 0.3\% (w/v) \text{ CHAPS (Sigma), 0.1\% (v/v) Tween-20 in PBS} \\
\text{PTw} & \quad 0.1\% (v/v) \text{ Tween-20 (Sigma) in PBS} \\
\text{PBT} & \quad 2\text{mg/ml Bovine Serum Albumin (Fraction V, Sigma), 0.1\% (v/v) Triton X-100 (Sigma) in PBS}
\end{align*}
\]

\textbf{g) Anti DIG Antibody Detection:}

Slides were treated for 1 hr with PBT + 20\% (v/v) heat inactivated newborn sheep serum. This treatment was followed by an overnight incubation at 4°C in 1/1000 preabsorbed Anti-DIG in PBT + 20\% (v/v) newborn sheep serum, 250\mu l of solution was used per slide. The slides were covered with siliconized coverslips. Slides were washed three times in PBT at room temperature for 30 min. following one wash in alkaline phosphatase buffer for 5 min. and one wash in alkaline phosphatase buffer + 5mM Levamisole (Sigma) for 5 min. at room temperature. To develop the colour reaction, slides were incubated with fresh Colour Substrate Solution, using 250\mu l solution/slide. Slides were not covered with coverslips. After 2-20hrs (depending on the abundance of mRNA) slides were washed for 5 min. in PBS at room temperature. The slides were incubated for 15min. to 2hrs in MEMFA and either mounted in glycerol or for permanent mounting the following procedure was followed. After a rinse in dH\textsubscript{2}O the slides were put through an alcohol dehydration series 30, 50, 70, 80, 90, 100, 100\% (v/v)
ethanol each for 2 min. and then two 10 min. incubations in histoclear followed by mounting in DPX mountant (Merck).

**Preabsorption of Anti-DIG Antibody**
The generation of 1 ml of 1:200 Anti-DIG antibody required 8-10 rat embryos aged 12.5 days. Embryos were fixed for 2 hrs at room temperature in 4% (w/v) paraformaldehyde, washed for 5 min. in DEPC-PBS and stored at -20°C in Methanol. To rehydrate, the embryos were washed for 5 min. at room temperature in a descending Methanol series (75%, 50%, 25% (v/v) in PTw). After a final wash for 5 min. in PTw embryos were transferred to 10% NSS in PTw. Embryos were then dissociated using a 5 ml pipette, followed by a pasteur pipette. The tissue was incubated for 1 hr at room temperature on a rotating wheel and centrifuged at 3000 rpm for 5 min. An equal volume of an anti-DIG antibody diluted 1:200 in 1% NSS/PTw was added. This mixture was incubated for 3 hrs at room temperature on a rotating wheel, before spinning at 3000 rpm for 5 min. The pellet was discarded and the antibody-containing supernatant brought to a final dilution of 1:1000 in 20% NSS/PBT.

**Alkaline Phosphatase Buffer**
100 mM Tris-HCl, pH 9.5 (Merck), 50 mM MgCl₂ (Merck), 100 mM NaCl (Merck), 0.1% (v/v) Tween 20 (Sigma)

**5x MEMfa Salts**
0.1 M MOPS (free acid, Sigma), 2 mM EGTA (Sigma), 1 mM MgSO₄ (Sigma), adjust pH to 7.5 with NaOH

**MEMFA**
1x MEMfa Salts, 3.7% (v/v) Formaldehyde (Fluka). Made up fresh each time.

**Colour Substrate Buffer**
Alkaline Phosphatase Buffer + 5 mM Levamisole (Sigma) + 0.45% v/v Nitrobluetetrazolium (in 70% dimethyl formamide) and 0.35% v/v 5-bromo-4-chloro-indoyl phosphate (in 100% dimethyl formamide), (both from Boehringer Mannheim)
3

BIOLOGICAL CHARACTERIZATION OF Hu-O-2A/Gb1 CELLS

3.1. Introduction

There are difficulties in obtaining enough quality fresh normal human material even for preliminary studies of the human O-2A lineage (see section 1.1.6.). A profitable strategy for studying the human O-2A lineage might be to develop tumour cell lines from gliomas, analogous to previous successes in the haematopoietic system. The study of such cell lines might allow a lineage based classification of gliomas to be developed, and might aid in the selection of appropriate treatments for various glioma subgroups as has proved the case for tumours of the haematopoietic system. Unfortunately, a lineage based analysis of gliomas has been impeded by the mesenchymal derivatives grown using standard tissue culture techniques (see section 1.2.3.).

An attempt was therefore made to re-create the micro-environment in which precursor cells would find themselves in vivo, by using techniques previously developed to study rodent O-2A progenitor cells in vitro and applying them to human glioma samples, rather than using traditional tissue culture techniques.

Glioma samples were cultured in chemically defined medium that had first been conditioned by purified rat cortical astrocytes, as had been described a decade ago for the growth of rat O-2A progenitor cells (Noble and Murray 1984). Just before my arrival Mrs. K. Bevan used this technique
to isolate a human cell line from a glioblastoma multiforme. This cell line has subsequently been named Hu-O-2A/Gb1 (for Human O-2A lineage Glioblastoma number 1).

Hu-O-2A/Gb1 cells were derived from a 59 year old man who underwent a left temporal lobectomy for debulking of a malignant glioma at the Gough-Cooper Department of Neurological Surgery, The National Hospital, Queen Square, London. The pathological report confirmed a glioblastoma multiforme. Biopsy samples from the tumour specimen were dissociated. Cells grown in traditional conditions (DMEM+10%FCS) expressed abundant fibronectin on their surfaces but no detectable glial specific antigens. Those grown in a combination of 50% DMEM-BS and 50% rodent cortical astrocyte conditioned medium (see section 1.1.4.), a condition which was termed ACM, were very different from those in DMEM+10%FCS. These Hu-O-2A/Gb-1 cells expressed antigens characteristic of the O-2A lineage of the rat, cells were positive for A2B5 and GD3 (ganglioside markers), 01 and GaIC (cerebroside markers), as well as GFAP. The term cell line is applied to the Hu-O-2A/Gb-1 cell population because all the cells have the same cytogenetic abnormalities (see section 3.3.3.), although they are not clonal in the true sense.

I characterized the response of Hu-O-2A/Gb-1 cells to biological stimuli, (aided by my findings from growth factor receptor tyrosine kinase cloning), and their consequent changes in antigenic phenotype. This work is presented in the results section of this chapter. These results and related findings, which define the Hu-O-2A/Gb-1 cell line as being unambiguously a glioblastoma multiforme derived cell line of the O-2A lineage, are then discussed.
3.2. Results

3.2.1. Cloning of tyrosine kinase fragments from Hu-O-2A/Gb1 cells

In order to screen for growth factors of biological relevance to the Hu-O-2A/Gb1 cell line, a strategy was employed of cloning 210 base pair complementary DNA (cDNA) fragments of growth factor receptor tyrosine kinases from passage 13 Hu-O-2A/Gb1 total RNA. This was achieved by using a modified reverse transcriptase-polymerase chain reaction (PCR) based approach and degenerate oligonucleotide primers PTK1 (5'-CGGATCCAC/GNGAC/TCT/T-3') and PTK2 (5'-GGAATTCCA/GAGGA/CTCCA/TTAGGA CCA/G/ACG/A/TC-3') which were based on the conserved motifs VI and IX found in the catalytic domain of protein kinases (see sections 1.5.3. and 2.3.10).

Twenty-one clones containing cDNA inserts from the PCR were sequenced using oligonucleotide primers T3 and T7 (Stratagene). Sequence analysis was performed using the University of Wisconsin GCG software package (Devereux et al. 1984). Nineteen of the clones had a single cDNA insert of which eleven were protein tyrosine kinase sequences and eight did not contain protein kinase sequences. Two of the clones contained triple cDNA inserts of which four were independent protein tyrosine kinase sequences and the other two did not contain protein kinase sequences. Therefore, in total there were fifteen independent cDNA insert fragments containing protein tyrosine kinase sequences, these are shown in table 3.1.

Five cDNA inserts contained sequences for receptor tyrosine kinases whose growth factor ligands were known to produce effects when applied to rodent O-2A cells. One clone contained a Platelet Derived Growth Factor Receptor alpha (PDGFRα) cDNA fragment (Claesson-Welsh et al. 1989;
Matsui et al. (1988), the receptor through which PDGF-AA mediates its response (Claesson-Welsh et al. 1989; Matsui et al. 1988). This receptor maps to Chromosome 4q11-12 (Gronwald et al. 1990) and in the rodent central nervous system it is a marker for O-2A progenitors (Pringle et al. 1992).

### Table 3.1. Protein tyrosine kinases found in Hu-O-2A/Gb1 cells by reverse transcriptase-PCR.

A modified reverse transcriptase-PCR based approach was used to clone 210bp cDNA fragments encoding part of the catalytic kinase domain of protein kinases from Hu-O-2A/Gb1 total RNA passage 13. The cDNA inserts of twenty-one clones were sequenced; 2 contained triple ligations. In total there were 15 cDNA fragments containing protein tyrosine kinase sequences. Chromosomal locations of the various genes are also given, the short arm of a chromosome is designated 'p' and the long arm 'q'.

<table>
<thead>
<tr>
<th>Protein tyrosine kinases</th>
<th>Number of cDNA fragments</th>
<th>Chromosomal location</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell surface receptors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDGFRα</td>
<td>1</td>
<td>4q11-12</td>
</tr>
<tr>
<td>FGFR3</td>
<td>1</td>
<td>4p16.3</td>
</tr>
<tr>
<td>FGFR4</td>
<td>3</td>
<td>5q33</td>
</tr>
<tr>
<td>FLT4</td>
<td>2</td>
<td>5q33</td>
</tr>
<tr>
<td>HEK2 (tyro 6)</td>
<td>2</td>
<td>3p21</td>
</tr>
<tr>
<td><strong>Cytoplasmic kinases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JAK1</td>
<td>2</td>
<td>1p22 or 1p31.3</td>
</tr>
<tr>
<td>tyk2</td>
<td>4</td>
<td>19p13.2</td>
</tr>
</tbody>
</table>

Another clone contained a Fibroblast Growth Factor Receptor 3 (FGFR3) cDNA fragment (Keegan et al. 1991), a receptor for bFGF and also acidic fibroblast growth factor (aFGF), different relative affinities for these growth factors are reported, (Keegan et al. 1991; Ornitz and Leder 1992), this receptor maps to Chromosome 4p16.3 (Keegan et al. 1993). Both PDGF-AA
Chapter 3

The Hu-O-2A/Gb1 cell line

and bFGF are known to produce biological responses in rodent O-2A lineage cells (Bögler et al. 1990; Noble et al. 1988; Raff et al. 1988). Three clones contained Fibroblast Growth Factor Receptor 4 (FGFR4) cDNA fragments, FGFR4 has a higher affinity for aFGF than bFGF (Partenen et al. 1991; Ron et al. 1993) and maps to Chromosome 5q33 (Armstrong et al. 1992a).

Unexpectedly, two cDNA inserts contained sequence for the receptor tyrosine kinase fms-like tyrosine kinase 4 (FLT4), which maps to Chromosome 5q33 (Aprelikova et al. 1992; Galland et al. 1992; Galland et al. 1993; Pajusola et al. 1992); the significance of this finding is discussed in chapter 4. The sequence of two further cDNA inserts gave the same predicted amino acid sequence as rodent tyro-6 (Lai and Lemke 1991), cloning of a longer portion of this gene was attempted from a cDNA library of Hu-O-2A/Gb1 cells but without success. The full length sequence of the human homologue of tyro 6 was then published as human embryo kinase 2 (HEK2, Böhme et al. 1993) and mapped to 3q21. The possible relevance of HEK2 to gliomas and the O-2A lineage is the subject of chapter 6.

Six cDNA fragments contained sequence for cytoplasmic tyrosine kinases of the Janus kinase family. This family includes JAnus Kinase 1 (JAK1) (Wilks et al. 1991) which maps to Chromosome 1p22 or 1p 31.3 (Howard et al. 1992; Pritchard et al. 1992) and also tyrosine kinase 2 (tyk2 Firmbach Kraft et al. 1990; Krolewski et al. 1990) which maps to Chromosome 19q13.2. These kinases are characterised by a functional C-terminal kinase domain, a second kinase-related domain and by the absence of src homology 2 and 3 domains (Wilks et al. 1991). In spite of their structural similarities and their broad distribution, particularly in haematopoietic cells, each member of the JAK family is preferentially involved in the signalling pathway of different cytokine receptors. JAK1 and tyk2 associate with the receptor for interferons α- and β- (Muller et al. 1993;
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Velazquez et al. 1992) and also recently have been found to associate with the receptors for the CNTF family of cytokines (Stahl et al. 1994).

The chromosomal locations of the protein kinase genes found did not show significant clustering on any particular chromosome. The frequency of a particular sequence found using the reverse transcriptase-PCR does not reflect the relative abundance in the original messenger RNA because of the possibility of bias in the PCR.

3.2.2. Application of biological stimuli to Hu-O-2A/Gb1 cells

The tyrosine kinase fragments that were found suggested that a number of the conditions that were applicable to analysing the rodent O-2A lineage might also apply to Hu-O-2A/Gb1 cells.

The division and differentiation characteristics of Hu-O-2A/Gb1 cells in various conditions were investigated by using immunocytochemistry. The conditions applied produced striking differences after 3 days. The following conditions were used; chemically defined medium alone (DMEM-BS), PDGF, bFGF, PDGF and bFGF and DMEM+10% FCS. aFGF was also studied in light of the finding of FGFR4 in the Hu-O-2A/Gb1 cell line.

Hu-O-2A/Gb1 cells were maintained in ACM routinely on Poly-l-lysine (PLL) coated plastic (PLL is the standard coating for studying rodent O-2A lineage cells in the laboratory). ACM promoted both cell division and generation of oligodendrocytes in Hu-O-A2/Gb1 cultures. For experiments, the cells were plated out onto PLL coated coverslips at a density of 5000 cells/coverslip and grown in the particular condition for 3 days and stained. The results from passage 13 Hu-O-2A/Gb1 cells are presented in the following sections, similar results were found for passages up to 20, although there was a relative reduction in the percentage of cells having an oligodendrocytic phenotype in any given condition at the higher passage
numbers 17 to 20. Growth factors were added each day at a concentration of 10ng/ml to DMEM-BS. It was found that Hu-O-2A/Gb1 cells could not be maintained in growth factors long-term (i.e., >3 weeks) because of the formation of a non-infectious coating on the PLL.

Characteristic Hu-O-2A/Gb1 cells are shown in figure 3.1. They have a process bearing morphology, and express antigens associated with rodent O-2A lineage. The oligodendrocyte-like cells do have branching processes, but not to the extent of rodent oligodendrocytes and neither do they form molecular layers in any of the conditions applied.

3.2.3. Stimulation of DNA synthesis

Cell DNA synthesis was measured by BrdU incorporation, cells were maintained in the condition of interest for 3 days and over the last 24 hours the medium was supplemented by BrdU: the results are shown in figure 3.2. Although some Hu-O-2A/Gb1 cells continued to incorporate BrdU when grown in chemically defined medium lacking exogenous mitogens [DMEM-BS; mean±standard error of the mean (s.e.m.) 25±2%], the proportion of such cells was reduced as compared with cultures exposed to mitogens. In the rodent O-2A lineage progenitor cells grown in DMEM-BS cease division and differentiate into oligodendrocytes. Division of Hu-O-2A/Gb1 cells was stimulated not only by ACM (46±2%; p= 0.0001 versus DMEM-BS), but also by PDGF (49±3%), which is secreted by purified rodent cortical astrocytes, and by bFGF (46±1%), these are known mitogens for rodent O-2A progenitors (Bögler et al. 1990; Noble et al. 1988; Raff et al. 1988; Richardson et al. 1988). Simultaneous exposure to PDGF and bFGF increased the division rate further (59±2%, p=0.003 versus PDGF), another characteristic shared with rodent O-2A progenitors (Bögler et al. 1990; McKinnon et al. 1991). Hu-O-2A/Gb1 cells grown in the presence of aFGF
Figure 3.1. Hu-O-2A/Gb1 cells.

Pictures of Hu-O-2A/Gb1 cells cultured in ACM on PLL are shown. Scale bar is 10μm. A, B and C are the same field of view. D, E and F are the same field of view. A and D are phase contrast pictures. B is O1 rhodamine staining and E is A2B5 rhodamine staining. C and F are GFAP fluorescein staining.
Figure 3.2. PDGF and bFGF are mitogens for Hu-O-2A/Gb1 cells.

Passage 12 Hu-O-2A/Gb1 cells cultured in ACM were plated out onto PLL coated coverslips at a density of 5000 cells/coverslip. Cells were then grown for 3 days in the conditions shown. Growth factors were added each day at a concentration of 10ng/ml to cells in DMEM-BS. The mitogenic activity of the various conditions for Hu-O-2A/Gb1 cells is compared to DMEM-BS alone (see text). BrdU uptake is a measure of DNA synthesis. PDGF and bFGF are mitogens and there is a synergistic effect between them. aFGF produces a mitogenic response but not to the same extent as bFGF. The results shown are for a single experiment. The % mean ± standard error of mean (s.e.m.) for 3 separate coverslips is shown, >400 cells were counted/coverslip. The experiment was repeated 3 times with similar results.
without the addition of heparin showed an increase in division rate (39±3%) in comparison to that seen in DMEM-BS (p=0.002), but not to the same extent as that seen for bFGF. Cells grown in DMEM+10%FCS had a similar division rate to those in DMEM-BS. The increases in BrdU uptake recorded in the above conditions were confirmed to lead to an actual increase in cell proliferation by size measurements of Hu-O-2A/Gb1 cell spheroids grown in each condition; these were made by Dr. M. Linskey.

3.2.4. Antigenic phenotype changes

Several oligodendrocytic cell surface markers were used, O4 (a sulphatide marker) labels both immature oligodendrocytes and also more mature oligodendrocytes, whereas O1 (a galactocerebroside marker) labels only more mature oligodendrocytes, therefore all cells that are O1 positive are also O4 positive. Similar results to those obtained with O1 were seen with anti-GalC. Anti-GalC is an antibody for galactocerebroside recognising this lipid, but which is less restricted in its specificity than the O1 antibody. Slightly more cells were GalC positive than O1 positive in any given condition, (approximately 5% more in DMEM-BS, ACM and PDGF).

The variation in the number of cells having an oligodendrocytic antigenic phenotype that were GFAP negative in the various conditions is shown in figure 3.3. In agreement with previous studies on rodent O-2A lineage cells, Hu-O-2A/Gb1 cultures contained more cells expressing oligodendrocyte specific antigens if grown in DMEM-BS, ACM or PDGF (O4, 35-65%; O1, 20-30%), than if they were grown in bFGF or PDGF+bFGF (O4, <15%; O1, <2%). Hu-O-2A/Gb1 cells cultured in the presence of aFGF but without exogenous heparin, also showed a suppression of the oligodendrocyte pathway similar to that seen for bFGF. Only a small percentage of Hu-O-2A/Gb1 cells that were O1 positive divide,
Passage 12 Hu-O-2A/Gb1 cells cultured in ACM were plated out onto PLL coated coverslips at a density of 5000 cells/cover slip. Cells were then grown for 3 days in the conditions shown. Growth factors were added each day at a concentration of 10ng/ml to cells in DMEM-BS. The graphs show the variation in the number of cells having an oligodendrocyte antigenic phenotype in the various conditions (increased in DMEM-BS, ACM and PDGF; similar to that seen in the rodent O-2A lineage). O4 is a marker for early and more mature oligodendrocytes (above), whereas O1 is a specific marker for oligodendrocytes (below). All cells that are O1+ are also O4+, GFAP is an astrocyte marker. FCS refers to DMEM+10%FCS. The results are the % mean ± s.e.m. for 3 separate coverslips, >400 cells were counted/cover slip. The experiment was repeated 3 times with similar results.
Passage 12 Hu-O-2A/Gb1 cells cultured in ACM were plated out onto PLL coated coverslips at a density of 5000 cells/coverslip. Cells were then grown for 3 days in the conditions shown. Growth factors were added each day at a concentration of 10ng/ml to cells in DMEM-BS. GFAP is an astrocyte marker. FCS refers to DMEM+10%FCS. The top graph shows a large increase in GFAP expression in Hu-O-2A/Gb1 cells exposed to FCS, similar to that seen for rodent O-2A progenitors exposed to FCS. Otherwise, cells having an A2B5 and GFAP positive phenotype (early type-2 astrocyte phenotype) were small in number in all conditions, lower graph. The results shown are the % mean ± s.e.m. for 3 separate coverslips, >400 cells/coverslip counted. This experiment was repeated 3 times with similar results.
Chapter 3 The Hu-O-2A/Gb1 cell line

(O1+BrdU+cells <3% in any condition), an observation similar to the finding of rodent O-2A progenitors falling out of division when they become oligodendrocytes (Noble and Murray 1984; Noble et al. 1988; Raff et al. 1988; Raff et al. 1983c; Raff et al. 1984b).

The behaviour of Hu-O-2A/Gb1 cells also resembled that of rodent O-2A progenitors in that cultures grown in DMEM+10%FCS for 3 days showed suppression of oligodendrocyte generation (figure 3.3) and greatly increased astrocyte differentiation, (>75% GFAP positive; figure 3.4.). Only a small percentage of cells in any condition, however, showed the early rodent type-2 astrocyte antigenic phenotype (A2B5+GFAP+; as rodent type-2 astrocytes mature they become A2B5-GFAP+), <7% of total cell number (figure 3.4., lower graph). Also in contrast to the rodent O-2A lineage in all the conditions analysed a proportion of cells remained GFAP positive >34%. This expression of GFAP seemed to be due to an astrocyte-inducing agent produced by these cells, (further experiments concerning this finding are discussed in Chapter 4).

Triple staining showed that some cells expressed a perinatal progenitor-like phenotype (i.e., A2B5+GalC-GFAP-): this percentage varied from 4±2% in ACM to 18±2% in bFGF. In addition, a proportion of cells failed to stain with either O1, O4, A2B5, GalC or GFAP in the conditions examined: this group varied from 15% in ACM to 45% in PDGF+bFGF.

3.2.5. Exogenous heparin did not potentiate the effects of the FGF's

Heparin is required for fibroblast growth factor (FGF) stimulation of biological responses, it induces FGF oligomerization and is responsible for FGF receptor dimerization and activation (Spivak-Kroizman et al. 1994). In an attempt to potentiate the effects of the fibroblast growth factors, in particular aFGF, Hu-O-2A/Gb1 cells were grown in the presence of heparin.
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(Sigma) in DMEM-BS. Previous studies in the rodent system had suggested that 2.5 units/ml of heparin might give maximum potentiation (Engele and Bohn 1992). But even at low heparin concentrations (0.05 units/ml) Hu-O-2A/Gb1 cells failed to remain attached to the PLL coating, although they were viable as assessed by trypan blue testing and reattachment to fresh PLL. PLL is known to bind to heparin (Dauchel et al. 1989), and therefore it is possible that the exogenous heparin displaced the cells by competing for PLL binding sites. Hu-O-2A/Gb1 cells were therefore plated out on fibronectin in DMEM-BS, growth factors were added daily (10 ng/ml/day, either bFGF or aFGF) and varying heparin concentrations were used (up to 5 units/ml). Under these conditions neither the antigenic phenotype nor BrdU uptake varied significantly from that seen in DMEM-BS with the same growth factor on PLL.

3.2.6. β-interferon was not a stimulus for Hu-O-2A/Gb1 cells

The two members of the Janus family of tyrosine kinases, (JAK1 and tyk2), found in the Hu-O-2A/Gb1 cell line (section 3.2.1.) were known to be part of the signalling pathway for the α- and β- interferons (Muller et al. 1993; Velazquez et al. 1992). β-interferon is an agent of possible benefit in multiple sclerosis (Paty et al. 1993; The IFNB Multiple Sclerosis Study Group 1993), where there is repeated damage to O-2A lineage cells. It might therefore have biological effects in the Hu-O-2A/Gb1 cell line.

Hu-O-2A/Gb1 cultures in DMEM-BS were supplemented by various concentrations of β-interferon (Sigma). It was found that Hu-O-2A/Gb1 cells were unable to tolerate concentrations ≥ 25U/ml/day and died. At a concentration of 12.5U/ml/day for 3 days, β-interferon did not influence either the antigenic expression (as assessed by the markers A2B5, O4, O1 or GFAP) or DNA synthesis as assessed by BrdU, compared to cultures...
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maintained in DMEM-BS alone. The β-interferon receptor is located on Chromosome 21 (Langer et al. 1990), this seemed normal on cytogenetic analysis of the Hu-O-2A/Gb1 cell line (Dr. X. Mao, personal communication). It was later shown that β-interferon did not produce any biological affect on rodent O-2A lineage cells (Dr. M. Mayer, personal communication).

Recently the combination of JAK1 and tyk2 has been found in the signalling pathway for the receptors of the CNTF family of cytokines (Stahl et al. 1994). This is an alternative explanation for the presence of JAK1 and tyk2 in Hu-O-2A/Gb1 cells and is supported by the finding that CNTF induces astrocytic differentiation in >70% of Hu-O-2A/Gb1 cells (see section 4.2.2.). CNTF is also known to have several effects on rodent O-2A lineage cells (Barres et al. 1993; Mayer et al. 1994).

3.3. Discussion

3.3.1. The biological behaviour of Hu-O-2A/Gb1 cells, similarities to rodent O-2A lineage cells

The results show that Hu-O-2A/Gb-1 cells express antigens characteristic of the rodent O-2A lineage and generate both more progenitor-like cells and also O4+O1+ oligodendrocytes when grown in chemically defined medium conditioned by purified rat cortical astrocytes. Plating these cells in DMEM+10%FCS leads to suppression of the oligodendrocyte pathway and enhancement of differentiation along the astrocyte pathway, as is the case for rodent O-2A progenitor cells (Raff et al. 1983a; Raff et al. 1983b; Raff et al. 1983c). Hu-O-2A/Gb-1 cells are stimulated to divide by both PDGF and bFGF, as are rodent O-2A progenitors (Bögler et al. 1990;
Noble et al. 1988; Raff et al. 1988; Richardson et al. 1988). In addition, bFGF suppresses the oligodendrocyte pathway as is found for purified O-2A progenitors (Mayer et al. 1993; McKinnon et al. 1990). Simultaneous exposure to both PDGF and bFGF suppresses differentiation along the oligodendrocyte pathway and increases division rate, another characteristic shared with rodent O-2A progenitors (Bögler et al. 1990; McKinnon et al. 1991). All of these properties can be maintained through at least 20 passages of these cells in vitro. These findings along with three other groups of results confirm that Hu-O-2A/Gb1 cells are a glioblastoma multiforme derived cell line of the O-2A lineage. These other results are discussed in the next three sections.

3.3.2. Proton nuclear magnetic resonance (1H-NMR) spectra of Hu-O-2A/Gb1 cells

All of the major cell types of the CNS can be unambiguously distinguished from each other by their 1H-NMR spectra profiles of free amino acids and other small metabolites (Urenjak et al. 1992; Urenjak et al. 1993). Experiments conducted in the laboratory of Dr. S. Williams by Dr. J. Urenjak and Dr. K. Bhakoo showed that Hu-O-2A/Gb1 cells express a 1H-NMR spectrum which is essentially identical to that otherwise uniquely expressed by perinatal O-2A progenitors derived from rat tissues (figure 3.5). In particular, high levels of N-acetyl aspartate and hypotaurine were seen in these cells. The 1H-NMR spectra obtained from 9 other glioblastoma cell populations, did not possess these characteristic N-acetyl aspartate and hypotaurine peaks and did not appear on biological grounds (antigenic phenotype) to be of the O-2A lineage.
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Figure 3.5. Proton nuclear magnetic resonance ($^1$H-NMR) spectra of Hu-O-2A/Gbl cells.

$^1$H-NMR spectra obtained from (A) Hu-O-2A/Gbl cells grown in ACM; (B) rodent O-2A progenitors; (C) a representative glioblastoma cell population. Note the similarity between the peaks in A and B indicated by a star. $^1$H-NMR spectroscopic analysis was performed at pH 8.9 with 512 scans recorded at 500MHz. Spectra were referenced, and displayed between 0.5 and 4.0 parts per million (ppm). The amplitude of each peak is proportional to the number of hydrogen atoms resonating at that frequency. Identified signals from right to left, Valine (Val), Leucine (Leu), Isoleucine (Ile), Alanine (Ala), Acetate (Ace), N-acetyl aspartate (NAA), Glutamate (Glu), Glutamine (Gln), Hypotaurine (H-Tau), Creatinine (Cr) and Choline containing compounds (Cho).
3.3.3. Cytogenetic analysis of Hu-O-2A/Gbl cells

Cytogenetic analysis of the Hu-O-2A/Gbl cell line was conducted in the laboratory of Dr. D. Sheer by Miss T. Jones and Dr. X. Mao, using cells which I provided. Hu-O-2A/Gbl cells show reproducible cytogenetic abnormalities consistent with them being derived from a glioblastoma multiforme. There was both trisomy 7 and monosomy 10, (common findings in malignant gliomas Bigner et al. 1988), loss of material from the remaining short arm of chromosome 10 and deletions of regions affecting chromosome 16 involving both the short arm and long arms. Loss of heterozygosity on chromosome 10 appears to occur exclusively with glioblastoma multiforme as opposed to other glioma types, (Fults et al. 1992; Rasheed et al. 1992; Venter and Thomas 1991; Watanabe et al. 1990). It is noteworthy that chromosomes 13, 17 and 22, where abnormalities are seen in glioblastomas (James et al. 1988), were normal even on fluorescent in situ hybridisation.

3.3.4. In vivo tumour formation by Hu-O-2A/Gbl cells

Hu-O-2A/Gbl cells maintain the ability to produce tumours in vivo. Experiments conducted by Dr. M. O'Leary in the laboratory of Dr. W.F. Blakemore have shown that Hu-O-2A/Gbl cells injected into nude rats, as either a cell suspension into the spinal cord or as spheroids subventricularly in the brain, produce tumours with evidence of invasion of the central nervous system. These tumour cells stain with markers specific to human chromosomes and show a morphology similar to the original tumour with many extracellular spaces.
3.3.5. A human glioblastoma multiforme derived cell line of the O-2A lineage

The biological properties of Hu-O-2A/Gb1 cells, taken together with their $^1$H-NMR spectra, cytogenetic analysis and ability to form tumours \textit{in vivo} confirm that this is a glioblastoma multiforme derived cell line of the O-2A lineage.

These findings may be of interest to understanding development and neoplasia in the human O-2A lineage. It suggests that studies of the rodent O-2A lineage may have considerable potential utility for understanding the human O-2A lineage. It is important to note though that the behaviour of Hu-O-2A/Gb1 cells in different conditions was not completely identical to that of the rodent O-2A lineage. Rodent O-2A progenitors all differentiate into oligodendrocytes when grown in DMEM-BS and cease dividing (Raff et al. 1983c). In contrast, although BrdU incorporation by Hu-O-2A/Gb1 cells was reduced in DMEM-BS, it did not cease completely nor did all the cells turn into oligodendrocytes. Expression of myelin basic protein a marker of oligodendrocyte maturation was not seen in Hu-O-2A/Gb1 cells during the set of experiments reported in this chapter, (see Chapter 4). In addition no condition has been found in which Hu-O-2A/Gb1 cultures are completely GFAP negative, this is probably a result of Hu-O-2A/Gb1 cells producing an astrocyte inducing agent (see Chapter 4).

Re-creating the micro-environment in which precursor cells would find themselves \textit{in vivo} was critical in allowing the isolation of Hu-O-2A/Gb-1 cells. If the original tumour biopsy specimen had been grown in medium containing foetal sera using the tissue culture techniques utilized in most laboratories, oligodendrocytes would not have been found. In addition Hu-O-2A/Gb1 cells grown in the presence of foetal serum loose their N-acetyl aspartate and hypotaurine peaks, (K. Bhakoo, personal communication).
Therefore there would have been no indication that this was a tumour of the O-2A lineage.

Using analytical techniques such as those described in this chapter, it may be possible to create a biologically-based lineage analysis of human gliomas based upon cellular biology rather than morphology. Such a classification system might aid in the recognition of appropriate treatments for various glioma subgroups, as is the case for tumours of the haematopoietic system. A necessary step would be to define markers of O-2A lineage gliomas, allowing all of them to be identified and also their frequency could then be determined. The defining of this family of gliomas will then make it easier to identify the next biological family of gliomas.

The $^1$H-NMR spectroscopic analysis is consistent with the existence of a minimum of two separate lineage contributions to human glioblastoma multiforme. Analyses of rodent meningeal cells and human meningioma cells (Florian et al. 1995) also show a surprising consistency between the small metabolite profiles expressed by primary cells of the rodent central nervous system and tumours of the human central nervous system. In future, $^1$H-NMR spectroscopy of living patients may be useful in detecting tumours of the O-2A lineage, possibly by showing an N-acetyl-aspartate peak.

As there are difficulties in obtaining enough quality fresh material even for preliminary studies of the human O-2A lineage, Hu-O-2A/Gbl cells may help in the cellular and molecular characterization of the human O-2A lineage, analogous to previous success in using tumour cell lines to study lineages in the haematopoietic system.
Chapter 4

A NOVEL ROLE FOR VASCULAR ENDOTHELIAL GROWTH FACTOR

4.1. Introduction

An important conclusion that could be drawn from the studies in Chapter 3 was that analysis of the rodent O-2A lineage could in several instances correctly predict characteristics of the Hu-O-2A/Gb1 cell line. This showed that analysis of cells from the rodent central nervous system appear to have considerable utility for attempts to study human cells.

Here two important findings are described. A new factor, Vascular Endothelial Growth Factor (VEGF), was found to have biological activity in the Hu-O-2A/Gb1 cell line and also on the rodent O-2A lineage. Thus, the results of using VEGF on the Hu-O-2A/Gb1 cell line could be applied to normal O-2A lineage cells.

The cloning of receptor tyrosine kinase fragments from Hu-O-2A/Gb1 cells had lead to the suggestion that VEGF could have an effect on Hu-O-2A/Gb1 cells (section 3.2.1.). Two fragments had unexpectedly contained sequence for the receptor tyrosine kinase FLT4 (fms-like tyrosine kinase 4) (Aprelikova et al. 1992; Galland et al. 1992; Galland et al. 1993; Pajusola et al. 1992). FLT4 is in the same receptor tyrosine kinase subclass as two high affinity VEGF receptors [fms-like tyrosine kinase 1 (FLT1) and kinase insert domain-containing receptor tyrosine kinase/foetal liver kinase 1 (KDR/FLK1) (de Vries et al. 1992; Millauer et al. 1993; Quinn et al. 1993; Terman et al. 1992)]. At the time of the finding FLT4 was a candidate
receptor for VEGF, although this has not yet been confirmed (Pajusola et al. 1994).

It was subsequently found that VEGF enhanced oligodendrocyte maturation in the Hu-O-2A/Gb1 cell line. By studying the effect of VEGF on rodent optic nerve oligodendrocytes it was discovered that VEGF acted as a survival factor for oligodendrocytes in vitro, in addition to enhancing oligodendrocyte maturation.

4.2. Results

4.2.1. VEGF enhances oligodendrocyte maturation in the Hu-O-2A/Gb1 cell line

To ascertain if VEGF was biologically relevant to Hu-O-2A/Gb1 cells the effect of this growth factor was assessed on the mitogenic and antigenic phenotype of these cells. It was found that the only effect of VEGF (5 or 10 ng/ml/day for 8 days) was to enhance Hu-O-2A/Gb1 oligodendrocyte maturation as assessed by myelin basic protein (MBP) expression, (figure 4.1.).

At a plating density of 1500 cells/cover slip even in DMEM-BS, cells having an O1 and MBP positive phenotype resembling mature oligodendrocytes were seen. The number of cells showing DNA synthesis as measured by BrdU uptake did not change significantly if the cells were treated with VEGF. For example, the percentage of O1-BrdU+ cells on PLL in DMEM-BS was 19±3% (mean ± s.e.m.) and in VEGF 5ng/ml/day 15±1%; p=0.12, (the number of O1+BrdU+ cells in all conditions was <2%). At this plating density (1500 cells/cover slip) in DMEM-BS a similar total percentage of O1 positive (data not shown) and also O1 and MBP positive cells were seen whether the cover slips were coated with PLL (32±5%) or fibronectin
Figure 4.1. Enhanced MBP expression in Hu-O-2A/Gb1 oligodendrocytes in the presence of VEGF.

Enhanced MBP expression in Hu-O-2A/Gb1 oligodendrocytes in the presence of VEGF. Hu-O-2A/Gb1 cells passage 15 were plated at a density of 1500 cells/coverslip onto either fibronectin (FN) or PLL and cultured in the presence of DMEM-BS with or without VEGF at concentrations of 5 or 10 ng/ml/day for 8 days. Cells were stained on the eighth day and >400 cells were counted/coverslip. The values are the % mean ± standard error of mean (s.e.m.) for 3 separate coverslips. The percentage of cells that were both O1 and MBP positive in each condition are shown. Similar results were obtained from 2 further experiments.
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(30μg/ml; Sigma), (29±4%; p=0.4, figure 4.1.), (although at a higher cell density, 5000 cells/cover slip, fibronectin did enhance O1 expression; see below). Compared to cells grown in DMEM-BS, there was a significant enhancement of MBP expression in O1 positive oligodendrocytes when VEGF was added at a dose of 5ng/ml/day (71±8% on PLL and 57±5% on fibronectin; p<0.002) and also 10ng/ml/day (67±5% on PLL and 60±9% on fibronectin). The morphology of O1 and MBP positive Hu-O-2A/Gb1 oligodendrocytes did not differ upon VEGF supplementation, but was influenced by the nature of the extracellular matrix, such that finer less branching processes were produced on fibronectin as opposed to PLL (figure 4.2.).

The finding of enhanced MBP expression in Hu-O-2A/Gb1 oligodendrocytes exposed to VEGF was confirmed by western blot analysis using a different MBP antibody from that used for immunofluorescence, (this was a rabbit anti-sera, known to recognise all isoforms of MBP, a kind gift from Dr. P.J. Brophy, Stirling University), (figure 4.3.). Low levels of MBP isoforms were seen in cells cultured in ACM, as well as those maintained in DMEM-BS for 8 days. There was however a marked increase in MBP expression in cells exposed to VEGF (10ng/ml/day) for 8 days. By 15 days cells cultured in DMEM-BS achieved a similar level of MBP expression to those receiving VEGF supplementation. These results are comparable to immunohistochemical studies showing enhancement of rodent oligodendrocyte maturation by CNTF, although eventually oligodendrocytes in DMEM-BS also reach full maturation, (Mayer et al. 1994). The main isoform seen at approximately 16 kDa is consistent with an embryonic-neonatal form of MBP previously described (Mathisen et al. 1993) and may be attributed to the cell line being derived from a glioblastoma multiforme, and therefore not able to form fully mature myelin.
Confocal pictures of Hu-O-2A/Gb1 oligodendrocytes at 8 days after plating, showing double positive (O1 and MBP) oligodendrocytes, (scale bar is 10μm). These pictures were taken with the help of Dr. A. Entwistle as has been previously described (Bögler et al. 1993; Entwistle and Noble 1994). A and C are fluorescein MBP staining, and B and D are rhodamine O1 staining. A and B are cells grown on PLL in DMEM-BS supplemented with VEGF 10ng/ml/day and C and D are cells grown on fibronectin in DMEM-BS alone.
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Figure 4.3. Western blot analysis confirming enhanced MBP expression in Hu-O-2A/Gb1 oligodendrocytes exposed to VEGF for 8 days.

Equal amounts of protein were loaded in each lane. 2.5x10^5 Hu-O-2A/Gb1 cells passage 15 were grown on 6cm diameter PLL coated dishes (Nunc), A431 cells, an epidermoid carcinoma cell line, (Stoscheck and Carpenter 1983) were grown to 70% confluence on uncoated dishes. Lanes 1 to 5, Hu-O-2A/Gb1 cells; in DMEM-BS supplemented with VEGF 10 ng/ml/day for 15 days (lane 1), DMEM-BS alone for 15 days (lane 2), DMEM-BS supplemented with VEGF 10ng/ml/day for 8 days (lane 3), DMEM-BS alone for 8 days (lane 4) and ACM (lane 5). Lane 6, A431 cells, a negative control; lane 7 purified human MBP a positive control, (a kind gift from Professor N. Groome, Brookes University, Oxford, UK re-suspended in lysis buffer); lane 8, a positive control, white matter from the brain of an adult rat (see section 2.3.16.).

A low level of a MBP isoform at approximately 16 kDa is seen, (and also a weaker band at approximately 11 kDa and a faint band at approximately 14 kDa), in cells grown in ACM and DMEM-BS for 8 days (lanes 4 and 5), but there was a marked increase particularly in the approximately 16 kDa MBP band in cells exposed to VEGF 10ng/ml/day for 8 days, lane 3. By 15 days cells grown in DMEM-BS have a similar level of MBP expression to those exposed to VEGF. The isoforms of MBP found in Hu-O-2A/Gb1 cells are of a smaller molecular weight than those normally seen in humans (for review see Campagnoni 1988). The main isoform seen at approximately 16 kDa is consistent with an embryonic-neonatal form of MBP previously described (Mathisen et al. 1993), and may be attributed to the cell line being derived from a glioblastoma multiforme, and therefore not being able to produce fully mature myelin.

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10μg of total RNA were loaded in each lane. 7x10^5 Hu-O-2A/Gb1 cells passage 15 were grown on 80cm^2 PLL coated flasks (Nunc) for 8 days and the RNA extracted. They were grown in the following conditions either ACM lane 2, VEGF 10ng/ml/day in DMEM-BS lane 3 or 10% FCS+DMEM lane 4. The A431 cell line (lane 1) was a negative control. The blot was probed firstly with a DM20 probe which showed a 3.2kb band corresponding to the PLP mRNA (top) and then with glyceraldehyde phosphate dehydrogenase (GAPDH; below) which confirmed equal loading of RNA in all lanes. An up regulation of PLP mRNA is seen in lane 3 compared with lane 2, consistent with oligodendrocyte maturation in the presence of VEGF and a down regulation is seen in lane 4, as cells grown in 10% FCS have only an astrocytic phenotype (GFAP positive cells).
Further evidence supporting Hu-O-2A/Gb1 oligodendrocyte maturation in the presence of VEGF came from the finding on northern blot analysis of increased myelin proteolipid protein (PLP) mRNA expression in this condition compared to that seen in ACM after 8 days, (a mouse probe for the DM20 portion of PLP mRNA was used, a kind gift from Dr. B. Zalc, INSERM U., Paris, France), (figure 4.4.). PLP is the most abundant myelin protein in the CNS. The presence of PLP mRNA in the ACM lane is consistent with these progenitor- and early oligodendrocyte-like cells expressing a low level of expression of the PLP locus, similar to findings previously reported for progenitor cells, (Timsit et al. 1995). A down regulation of PLP mRNA was also confirmed in cells cultured in DMEM+10% FCS for 8 days when only an astrocytic phenotype is expressed (>75% of all cells are GFAP positive).

4.2.2. Other molecules did not enhance Hu-O-2A/Gb1 oligodendrocyte maturation

Apart from VEGF, other conditions which were applied to the Hu-O-2A/Gb1 cell line failed to enhance oligodendrocyte maturation. Previously it has been shown that Hu-O-2A/Gb1 cells plated at a density of 5000 cells/PLL coated coverslip and maintained in ACM or cultured for 3 days in either DMEM-BS alone or supplemented with PDGF-AA produced immature oligodendrocytes that were BrdU negative and O1 positive but did not express MBP (Chapter 3). Prolonging culture of these cells from passages 14 to 16 in DMEM-BS alone, repeatedly failed to allow MBP expression and the cells would die after 8 days. The addition of 1% FCS to DMEM-BS did not influence cell survival, although it did show the sensitivity of Hu-O-2A/Gb1 cells to the effects of serum as after 3 days the cells showed a similar
antigenic phenotype to those grown in DMEM supplemented with 10% FCS (>70% of cells were GFAP positive and O1 staining was absent).

The addition of either Ciliary Neurotrophic Factor CNTF (4ng/ml/day) or Glial Growth Factor 2 (GGF 2; added daily at a concentration which caused 50% of Schwann cell precursors to convert into myelin forming cells; Dr. P. Stroobant, personal communication) to DMEM-BS favoured the astrocytic pathway increasing the number of GFAP positive cells to >70%, with a complete loss of oligodendrocyte markers. Transforming Growth Factor β2 (TGFβ2; 10ng/ml/day) did not influence antigen expression of Hu-O-2A/Gb1 cells cultured in DMEM-BS.

Culturing with Hu-O-2A/Gb1 conditioned media (DMEM-BS which had been incubated on a layer of 75-90% confluent Hu-O-2A/Gb1 cells for 2 days) diluted 1:1 with DMEM-BS again favoured an astrocytic phenotype for Hu-O-2A/Gb1 cells (figure 4.5.). After 3 days there was a significant increase in the number of GFAP positive cells (61±3% versus ACM 36±2%; p<0.01) and also an increase in DNA synthesis as measured by BrdU uptake (56±3% versus ACM 44±3%; p<0.01), O4 and O1 positive cells accounted for <2% of the total.

Chemical agents at doses which had proved successful in permitting differentiation in other cell types were applied individually, as single doses, to Hu-O-2A/Gb1 cells on PLL coated coverslips (5000 cells/coverlip) in DMEM-BS. A range of final concentrations for each agent were applied; all-trans-retinoic acid (1nM, 10nM or100nM), N,N-dimethylformamide (6mM, 60mM, 600mM), phorbol 12,13 dibutyrate (0.016nM, 0.16nM, 1.6nM or 16nM), phenylacetate (0.4mM, 4mM, or 40mM), and dimethyl sulphoxide (0.125%, 1.25% or 12.5%). After 3 days at the higher concentrations of each agent >90% of cells were dead while at the lower concentrations GFAP positive cells accounted for >50% of live cells and there were no O1 positive cells. Dibutyryladenosine 3',5' cyclic monophosphate was also tried as single
Figure 4.5. Hu-O-2A/Gb1 cells condition their own medium.

Five thousand Hu-O-2A/Gb1 cells passage 15 were plated onto PLL coated coverslips and cultured in the presence of either DMEM-BS or ACM or Hu-O-2A/Gb1 conditioned medium (50% DMEM-BS which had been incubated on a layer of 75-90% confluent Hu-O-2A/Gb1 cells for 2 days and 50% DMEM-BS). The values are the % mean ± s.e.m. for 3 separate coverslips, >400 cells counted/coverslip. Hu-O-2A/Gb1 conditioned medium contains mitogens for Hu-O-2A/Gb1 cells as shown by an increase in the % of BrdU positive cells (p<0.01 versus ACM, see text) and also induces an astrocytic phenotype as shown by an increase in the % of GFAP positive cells (p<0.01 versus ACM, see text) and a reduction in O4 positive cells. Similar results were obtained from 2 further experiments.
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doses providing final concentrations of either 0.01mM, 0.1mM or 1mM. At a concentration of 1mM after 3 days >90% of cells were dead, whilst at the lower concentrations although a few O1 positive cells remained (<10%) these failed to express MBP and most live cells (>70%) were GFAP positive.

Changing the extracellular matrix from PLL to either laminin or fibronectin did not enhance oligodendrocyte MBP expression. Hu-O-2A/Gb1 cells failed to attach to coverslips coated with laminin (1 to 4μg/ml; basement membrane-Engelbreth Holm Swarm mouse sarcoma; Sigma) on 3 separate occasions, although cell viability was maintained as assessed by trypan blue staining (0.4%; Sigma) and by the ability of the cells to reattach to PLL coated coverslips. Although Hu-O-2A/Gb1 cells plated at a density of 5000 cells onto fibronectin (30μg/ml; Sigma) coated coverslips and cultured in DMEM-BS did show a small but significant increase in the number of O4 positive cells (58±3%) after 3 days compared to those grown on PLL (48±3%; p<0.01) and also a much larger increase in O1 positive cells (50±12%) compared to those on PLL (21±2%; p<0.015) (figure 4.6.), MBP expression was not seen despite continuing culture for 10 days. This enhancement in the number of O4 and O1 positive Hu-O-2A/Gb1 cells when they were attached to fibronectin over those on PLL was not seen at the lower plating density of 1500 cells/coverslip (see above).

4.2.3. VEGF also enhances rodent oligodendrocyte maturation

Hu-O-2A/Gb1 cells had been isolated and maintained in culture by adopting methods from the rodent O-2A lineage. The unexpected finding that VEGF enhanced oligodendrocyte maturation in the Hu-O-2A/Gb1 cell line might be applicable only to this malignant cell line, as it has not been examined in the rat O-2A lineage. It was therefore interesting to investigate whether the VEGF response was specific for this human malignant cell line.
At a higher Hu-O-2A/Gb1 cell density, plating on fibronectin (FN) increases the number of oligodendrocytes. 5000 Hu-O-2A/Gb1 cells passage 15 were plated out on coverslips coated with either PLL or fibronectin (FN) and cultured in DMEM-BS and stained for either O1 or O4 after 3 days, >400 cells were counted/coverslip. The values are the % mean ± s.e.m. for 3 separate coverslips. At this higher cell density fibronectin increases the expression of these oligodendrocyte markers (see text), but does not allow MBP expression. Similar results were obtained from 2 further experiments.
or reflected a property of rat O-2A lineage cells. This was investigated by examining the effect of VEGF on O-2A lineage cells from rodent optic nerves. This system was chosen because it is the most characterised part of the O-2A lineage in the rodent central nervous system.

Panned purified rodent neonatal (postnatal day 7) O-2A progenitors were provided by Dr. M. Mayer using a previously described protocol (Mayer et al. 1994). Progenitor expansion, when required, was in DMEM-BS without thyroid hormones, supplemented with 10ng/ml/day PDGF and 5ng/ml/day Neurotrophin-3 (NT-3). This has been found to be the optimal combination of factors for expansion, (Dr. M. Mayer, personal communication), NT-3 promotes the mitogenic activity of PDGF and blocks premature differentiation of progenitor cells into oligodendrocytes.

The effect of VEGF on the maturation of oligodendrocytes was examined in two different assay systems. In the first assay VEGF was added to the cells when they still expressed the progenitor cell phenotype immediately after purification. In this assay cells were cultured in DMEM-BS with or without supplementation by VEGF 10 or 20 ng/ml/day. The O-2A progenitors differentiate quickly once grown in DMEM-BS, most becoming mature by 3 days, and therefore cells were stained after 2 days to allow a change in the rate of oligodendrocyte MBP expression to be seen (figure 4.7.a). There was a significant enhancement of oligodendrocyte maturation in the presence of VEGF as shown by an increase in double stained O1 and MBP oligodendrocytes in medium supplemented by VEGF 10 or 20ng/ml/day (28±5% and 33±1% respectively) versus DMEM-BS alone (11±3%; p<0.0001).

In the second assay progenitor cells were purified and plated at a density of 2000 cells/coverslip in DMEM-BS. After two days, when 100% of the cells were A2B5-GalC+, oligodendrocytes were exposed to various concentrations of VEGF. After 24 hours the cells were stained for GalC and
Figure 4.7. VEGF enhances MBP expression in rodent optic nerve oligodendrocytes.

Enhanced maturation of rodent oligodendrocytes in the presence of VEGF.

A) Effect of VEGF starting from progenitor cells. Panned purified O-2A progenitors from P7 rat optic nerves were plated out at 4000 cells/PLL coated coverslip and cultured in either DMEM-BS or DMEM-BS supplemented with VEGF at either 10 or 20ng/ml/day for two days. The values are the % mean ± s.e.m. of O1 and MBP positive cells for 4 separate coverslips, all cells on each coverslip were counted, there were equivalent numbers of cells on coverslips. Similar results were obtained on 3 other occasions.

B) Effect of VEGF starting from premature oligodendrocytes. Panned purified O-2A progenitors from P7 rat optic nerves were plated out at 2000 cells/PLL coated coverslip and cultured in DMEM-BS for 2 days (100% of cells A2B5-GalC+). Coverslips were then changed to new wells containing DMEM alone or supplemented with VEGF at either 10 or 20ng/ml. Cells were cultured for one additional day and then stained with GalC and MBP, (the GalC antibody recognises a similar epitope to O1). The values are the % mean ± s.e.m. for 4 separate coverslips, all cells on each coverslip were counted.
MBP, (the GalC antibody recognises a similar epitope to O1). In cultures, where the medium was supplemented with VEGF at 10 or 20ng/ml, 31±8% or 54±2% respectively expressed MBP, compared to only 28±1% in cultures without VEGF (figure 4.7.b). There was no further enhancement of MBP expression with concentrations of VEGF above 20ng/ml (data not shown).

Therefore, VEGF enhances the maturation of oligodendrocytes regardless of the developmental stage of the O-2A cells at the time of exposure. The effect is seen when cells are either A2B5+/GalC- progenitor cells or A2B5-/GalC+ premature oligodendrocytes at the time of exposure.

Increased rodent oligodendrocyte MBP expression in the presence of VEGF was confirmed by western blot analysis. In order to obtain enough protein, panned purified postnatal day 7 rodent optic nerve O-2A progenitors were expanded for two weeks in DMEM-BS without thyroid hormones but supplemented with PDGF-AA 10ng/ml/day and NT-3 5ng/ml/day. Under these conditions it was found necessary to allow oligodendrocytes to remain in culture for 4 days before mature MBP expression could be visualised by western blot. Only oligodendrocytes cultured in DMEM-BS supplemented by either VEGF 10ng/ml/day or CNTF 4ng/ml/day expressed mature MBP (figure 4.8.). Oligodendrocytes exposed to CNTF were used as a positive control as this condition is known to enhance oligodendrocyte maturation, (Mayer et al. 1994).

4.2.4. VEGF is a survival factor for rodent oligodendrocytes but does not effect O-2A progenitors

CNTF not only enhances rodent oligodendrocyte maturation but also acts as a survival and differentiation factor (Mayer et al. 1994) and therefore the effect of VEGF on rodent oligodendrocyte survival was assessed.
Panned purified P7 rodent optic nerve O-2A progenitors were expanded for two weeks in DMEM-BS without thyroid hormones, supplemented by PDGF-AA 10ng/ml/day and NT-3 5ng/ml/day. Progenitors were grown to a density of 5x10^5 cells on 3.5cm diameter PLL coated dishes (Nunc) and cultured in either DMEM-BS supplemented with CNTF 4ng/ml/day (lane 1), DMEM-BS supplemented with VEGF 20ng/ml/day (lane 2), DMEM-BS (lane 3) or maintained as progenitors (lane 4) for 4 days, white matter from the brain of an adult rat was included as a positive control (lane 5). Equal amounts of protein were loaded in each lane. Mature MBP isoforms of 18.5 and 17 kDa were only seen in lanes 1 (a positive control for growth factor enhanced rodent oligodendrocyte maturation) and 2. A less mature isoform of MBP, the embryonic-neonatal form previously described (Mathisen et al. 1993), was seen running at approximately 16 kDa in lanes 1, 2, 3 and 4.
Dr. M. Mayer performed the survival experiments using MTT (3-[4,5-dimethythiaziazol-2yl]-2,5-diphenyl tetrazolium bromide; Sigma). The MTT assay distinguishes between live and dead cells, by assaying the mitochondrial activity of the cells. Only live cells are able to cleave the tetrazolium ring of MTT into a visible dark blue formazan reaction product. The assay was performed as previously described (Mossman 1983). MTT was dissolved in phosphate buffered saline at 5mg/ml, the stock solution was filtered through a 0.22 μm filter and added to the culture medium at a dilution 1:10. Plates were incubated at 37°C for 2 hours. Live cells appeared with dark blue cytoplasm in bright-field phase microscopy.

In order to establish whether VEGF is also a survival factor, panned purified postnatal day 7 rodent optic nerve progenitors were plated out onto PLL coated coverslips (2000 cells/coverstrip) and cultured in DMEM-BS for 3 days. Coverslips which now contained pure oligodendrocyte populations were washed in DMEM and transferred to new wells containing DMEM±VEGF for 3 days. These coverslips were then stained with MTT and live cells were counted. Oligodendrocyte survival was significantly enhanced by 100ng/ml/day (figure 4.9.).

The actions of VEGF, like CNTF, were limited to rodent oligodendrocytes. Their precursors, the O-2A progenitors, were not affected in terms of their survival (figure 4.10.) and division as assessed by staining dividing cells with BrdU (data not shown).

To determine whether VEGF could promote differentiation of O-2A progenitors towards oligodendrocytes the following strategy was used, (this had previously been used to show that CNTF promotes differentiation, Mayer et al. 1994). As division of O-2A progenitor cells is required to prevent their premature differentiation into oligodendrocytes (Noble and Murray 1984; Noble et al. 1988; Raff et al. 1988; Raff et al. 1983c; Raff et al. 1984b), the effects of VEGF were examined on cells grown in the presence of
Figure 4.9. Survival of oligodendrocytes in the presence of VEGF.

Enhanced survival of rodent oligodendrocytes in the presence of VEGF. Panned purified O-2A progenitors from P7 rat optic nerves were plated out onto PLL coated coverslips (2000 cells/coverslip) and cultured in DMEM-BS for 3 days. Control coverslips were stained with GalC to confirm that all cells differentiated into oligodendrocytes. Cells were then switched to DMEM+VEGF and cultured for another 3 days. Cells were stained with MTT, live cells are MTT positive with intact cell bodies and well defined processes. Dead cells were identified as 'ghosts' with no nuclei and no visible MTT reaction. The control was oligodendrocytes remaining in DMEM-BS [100% = cells in DMEM-BS (99±10)], all cells on each coverslip were counted. The values are the % mean ± s.e.m. for 6 separate coverslips. Similar results were obtained from 2 further experiments.
Panned purified O-2A progenitors from P7 rat optic nerves were plated onto PLL coated coverslips (2000 cells/coverslip) and cultured in DMEM for 3 days with either a suboptimal dose of PDGF 0.01ng/ml/day alone (PDGF at a higher dose would act as a survival factor itself, Barres et al. 1993) or in addition to VEGF 10ng/ml/day. Cells were stained for A2B5 (for progenitors) and GalC (for oligodendrocytes); because of the low concentration of PDGF all cells differentiated into oligodendrocytes during the 3 day incubation. The number of total cells on each coverslip was similar for all conditions, regardless of whether the cells were exposed to VEGF when they were still progenitors. The values are the mean ± s.e.m. for 6 separate coverslips. Similar results were obtained from 2 further experiments.
bFGF. In these conditions the differentiation of O-2A progenitors is inhibited so long as they are first purified away from other cells of the optic nerve (Mayer et al. 1993; McKinnon et al. 1990). Moreover, the ability of bFGF to promote oligodendrocyte division means that the generation of oligodendrocytes in the presence of bFGF is not necessarily associated with a cessation of cell division. Purified progenitor cells grown in the presence of bFGF [10ng/ml] ± VEGF [1,10 or 50 ng/ml] showed no increase in the appearance of oligodendrocytes as examined by staining cells for GalC (data not shown). VEGF seemed not to be able to override the effect of bFGF on the block of differentiation. VEGF therefore differs from CNTF (Mayer et al. 1994) which promotes O-2A progenitor differentiation, (and VEGF also differs from PDGF as in combination with bFGF, PDGF increases progenitor division rate (McKinnon et al. 1991).

In addition, when cells were grown in the presence of PDGF [10ng/ml] ± VEGF [10 or 100ng/ml] the proportion of oligodendrocytes and progenitor cells was not altered, suggesting, that the presence of VEGF did not influence the timed differentiation of progenitor cells into oligodendrocytes established by PDGF.

4.2.5. VEGF leads to MAPK activation in both Hu-O-2A/Gb1 and rodent oligodendrocytes

Growth factors such as Epidermal Growth Factor (EGF) and PDGF are known to act through intracellular signalling pathways that include mitogen activated protein kinase (MAPK) activation. In order to confirm that VEGF was having a biochemical intracellular effect, MAPK activation was assayed in both Hu-O-2A/Gb1 cells and rodent oligodendrocytes exposed to VEGF. MAPK activation was determined by the ability of MAPK to phosphorylate myelin basic protein (Leevers and Marshall 1992). Stimulation by 1μg/ml of
Biochemical confirmation of MAPK activation by VEGF in both Hu-O-2A/Gb1 cells and rodent oligodendrocytes was obtained by the ability of MAPK to phosphorylate myelin basic protein in vitro. Hu-O-2A/Gb1 cells passage 15 were plated at a density of 2.5x10^5 cells onto 6cm diameter PLL coated dishes (Nunc) in DMEM-BS for 24 hours and then either maintained in DMEM-BS alone (lane 1), or VEGF was added at a concentration of 1μg/ml for 10 min. (lane 2; which shows an up regulation of MAPK activation) or EGF was added at a concentration of 1μg/ml for 10 min. as a positive control (lane 3; EGF is a known mitogen for Hu-O-2A/Gb1 cells). Expanded panned purified P7 rodent optic nerve O-2A progenitors were grown to a density of 5x10^5 cells on 3.5cm diameter PLL coated dishes (Nunc) and placed into DMEM-BS for 24 hours, lanes 4 and 5, or maintained as progenitors lane 6. Rodent oligodendrocytes (lane 5) were stimulated by the addition of VEGF 1μg/ml for 10 min. and the enhanced level of MAPK activation was similar to that seen for the addition of the mitogen PDGF-AA (1μg/ml for 10 min.) to progenitors.
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VEGF for 10 minutes produced a similar level of MAPK activation to that seen for 1µg/ml of EGF a known mitogen for Hu-O-2A/Gb1 cells (EGF produces a similar mitogenic response as a combination of PDGF and bFGF in Hu-O-2A/Gb1 cells) or 1µg/ml of PDGF-AA a known mitogen for rodent O-2A progenitors, (figure 4.11.).

4.3. Discussion

4.3.1. A new role for VEGF

The findings for VEGF were new and unexpected. VEGF enhanced oligodendrocyte maturation in the Hu-O-2A/Gb1 cell line. In addition, VEGF was tested in the normal rodent O-2A lineage and was shown to enhance oligodendrocyte maturation and survival in vitro. The suggestion that the VEGF family of growth factors may have biological relevance for Hu-O-2A/Gb1 cells came during the cloning of tyrosine kinase fragments from Hu-O-2A/Gb1 cells. Unexpectedly, the tyrosine kinase receptor FLT4 (Aprilikova et al. 1992; Galland et al. 1992; Galland et al. 1993; Pajusola et al. 1992) was found to be expressed by these cells (section 3.2.1.). FLT4 forms a subclass of the class III receptor-type tyrosine kinases along with two high affinity VEGF receptors FLT1 and mouse FLKI and its human homologue KDR (de Vries et al. 1992; Millauer et al. 1993; Quinn et al. 1993; Terman et al. 1992). All three receptors have an extracellular domain comprising seven immunoglobulin-like loops and a highly homologous tyrosine kinase domain. Recent evidence suggests, however, that FLT 4 is not a VEGF receptor (Pajusola et al. 1994).

VEGF enhanced Hu-O-2A/Gb1 oligodendrocyte maturation as reflected by MBP protein expression and PLP mRNA levels. As is the case for other
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non-endothelial cells VEGF did not act as a mitogen for Hu-O-2A/Gb1 cells (see section 4.3.4.).

4.3.2. Other agents did not enhance Hu-O-2A/Gb1 oligodendrocyte maturation

The role of VEGF in enhancing Hu-O-2A/Gb1 oligodendrocyte maturation seems specific, as judged by the effects observed for other differentiation and maturation agents assessed. The growth factors CNTF, (which is known to have a role in the oligodendrocytic as well as a type-2 astrocyte differentiation Mayer et al. 1994), TGFβ (McKinnon et al. 1993) and GGF2 (GGF belongs to the same family as acetylcholine receptor-inducing activity protein, Vartanian et al. 1994), were all good maturation candidates from rodent O-2A lineage studies but failed to promote Hu-O-2A/Gb1 oligodendrocyte differentiation and/or maturation, possibly because of an astrocytic inducing agent made by the cell line. Changing the extracellular matrix to laminin, which is known to permit differentiation of many cells including melanoma cells (Kleinmann et al. 1985), did not permit Hu-O-2A/Gb1 cell attachment. Fibronectin increased the number of cells expressing the oligodendrocyte antigens O4 and O1 compared to those maintained on PLL, but only at a higher Hu-O-2A/Gb1 plating density of 5000 cells/coverslip; it did not influence MBP expression. In contrast, occupancy of fibronectin binding sites has been shown to allow rodent oligodendrocytes to increase synthesis of sulpholipid and myelin basic protein (Cardwell and Rome 1988). Several chemical agents at concentrations known to induce differentiation in other cell systems, promoted the astrocytic and suppressed the oligodendrocytic pathway. These were: retinoic acid, DMSO, dimethyl formamide, phorbol dibutyrate (as used for instance in the HL-60 promyelocytic leukaemic cell line, reviewed in
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Collins 1987) and phenylacetate (as used in gliomas, Ram et al. 1994; Samid et al. 1994). Dibutyryladenosine cyclic monophosphate has been shown to induce differentiation of both gliomas (Edstrom et al. 1974) and O-2A progenitors (Raible and McMorris 1989), but failed to enhance the oligodendrocyte pathway or allow myelin basic protein expression in the Hu-O-2A/Gb1 cell line. These findings suggest that the favoured pathway for differentiation in Hu-O-2A/Gb1 cells is astrocytic. Support for the suggestion that Hu-O-2A/Gb1 cells favour astrocytic differentiation comes from the extreme sensitivity of the cells to low concentrations of foetal calf serum and the finding of astrocytic induction of Hu-O-2A/Gb1 cells by Hu-O-2A/Gb1 conditioned medium. This may account for part of the cell density effect on oligodendrocyte differentiation. In addition, the Hu-O-2A/Gb1 conditioned medium also induces type-2 astrocyte generation in rodent cultures.

Due to the long period (> 7 days) that Hu-O-2A/Gb1 cells could survive in even the most basic culture medium (DMEM alone), meaningful survival experiments could not be conducted.

4.3.3. The effects of VEGF on rodent O-2A lineage cells

The finding that rodent optic nerve oligodendrocyte maturation was also enhanced by VEGF shows that studies on the Hu-O-2A/Gb1 cell line can be relevant to normal O-2A lineage cells. VEGF is also a survival factor for oligodendrocytes in vitro, another similarity to CNTF, although it does not seem to influence O-2A progenitor differentiation down either the type-2 astrocytic or oligodendrocytic pathway unlike CNTF (Mayer et al. 1994). The studies on rodent cells were undertaken in cultures growing in chemically defined medium (either DMEM or DMEM-BS) conditions in which no division occurs (Noble and Murray 1984; Raff et al. 1983c) indicating that the effects of VEGF were unlikely to be secondary to a simple
inhibition of cell division. The actions of VEGF seem limited to rodent oligodendrocytes. Their precursors, the O-2A progenitors, did not seem to be affected in terms of their survival, differentiation or division.

VEGF was found to lead to MAPK activation in both Hu-O-2A/Gb1 cells and rodent oligodendrocytes. This has also recently been found to be the case for endothelial cells (Seetharam et al. 1995). VEGF has been shown to influence cytoskeletal organisation resulting in morphological changes in a cell line of endothelial origin by inducing phosphorylation of cellular proteins (Enomoto et al. 1994). It is possible that VEGF could act in a similar manner in oligodendrocytes allowing an increased accumulation of major myelin proteins.

4.3.4. VEGF

VEGF is a homodimeric glycoprotein structurally related to PDGF. Its actions as an endothelial cell mitogen and on vascular permeability lead to its discovery (Connolly et al. 1989; Ferrara and Henzel 1989; Gospodarowicz et al. 1989). VEGF mRNA undergoes alternative splicing events that generate four different homodimeric isoforms, VEGF 121, VEGF 165, VEGF 189 and VEGF 206 (Houck et al. 1991; Tischer et al. 1991). All have equal potency for endothelial mitogenesis (Fiebich et al. 1993), although they have different binding affinities for heparin sulphate proteoglycans which behave as low affinity receptors (Gitay Goren et al. 1992). VEGF 121 is a freely diffusible nonheparin binding acidic protein. The longer forms, VEGF 189 and VEGF 206, are highly basic proteins tightly bound to extracellular heparin containing proteoglycans (Houck et al. 1991; Houck et al. 1992; Peretz et al. 1992). VEGF 165 is the most abundant and best characterised form (Ferrara et al. 1991). This isoform has intermediate properties and was the isoform used in the experiments reported here.
The expression pattern of VEGF and the locality of its binding sites has suggested involvement in the development and maintenance of normal vasculature and also in tumour angiogenesis (Berkman et al. 1993; Berse et al. 1992; Brown et al. 1993; Jakeman et al. 1992; Plate et al. 1992; Shweiki et al. 1992). This view is supported by the finding of expression of two high affinity VEGF receptors, FLT1 and KDR/FLK1, in developing vascular and glioblastoma multiforme endothelia (Millauer et al. 1993; Plate et al. 1992; Shweiki et al. 1992; Yamaguchi et al. 1993).

In the central nervous system, high levels of VEGF mRNA have been reported throughout the foetal rat brain (day 14) with diffuse low levels in adult brain (Ferrara et al. 1991), this contrasts with findings in the normal adult human brain (frontal lobe) where the mRNA has been found in cells scattered in the cortex but is not found in the underlying white matter (Plate et al. 1992). Interestingly at 17-20 weeks in the human foetus VEGF mRNA was not detectable in the brain (Kaipainen et al. 1993). This is a stage before significant myelination has started, (Brody et al. 1987; Yakovlev and Lecours 1967). VEGF protein has been detected within neurones of the normal human cortex whose axons in the white matter are ensheathed by myelin membranes from oligodendrocytes, a localization possibly consistent with our finding of VEGF influencing oligodendrocyte maturation. Only in specific regions (such as the hippocampus) do normal human astrocytes express VEGF, although it is found in reactive and neoplastic astrocytes (Alvarez et al. 1992).

It is possible that VEGF may also have an effect in the repair of demyelinated lesions. VEGF is likely to be released in the demyelinating lesions of multiple sclerosis where vascular permeability is one of the first findings (Katz et al. 1993). This would be similar to recent findings of VEGF in the synovial fluid of patients in the active phase of another chronic inflammatory condition, rheumatoid arthritis, where there is also vascular
permeability (Fava et al. 1994). The released VEGF theoretically might enhance oligodendrocyte maturation and therefore help in remyelination.

The potent mitogenic effects of VEGF seem to be restricted to endothelial cells (Conn et al. 1990; Connolly et al. 1989; Ferrara and Henzel 1989; Gospodarowicz et al. 1989; Leung et al. 1989; Yamane et al. 1994). VEGF is known, however, to have other effects on cells originating outside the endothelium. It induces foetal bovine osteoblast differentiation (Midy and Plouet 1994), foetal rat islet β-cell maturation (probably through FLK1, Oberg et al. 1994) and monocyte migration and chemotaxis (Clauss et al. 1990). VEGF receptors have been found on monocytes (Shen et al. 1993) and several human melanoma cell lines, (but not normal human melanocytes, Gitay Goren et al. 1993), where they are mainly 150 and 130 kDa in size rather than the larger receptors such as FLT1 or KDR. Similar receptor types are also found on HeLa, 3T3 and bovine granulosa cells (Gitay Goren et al. 1992). VEGF receptor binding is regulated not only by the specific receptor type but also by the amount and composition of heparin like molecules present on the cell surface (Tessler et al. 1994). For both the Hu-O-2A/Gb1 cell line and rodent oligodendrocytes the cell surface receptor mediating the VEGF response has not yet been identified, using available antibodies to KDR/FLK1 and FLT1, although recent evidence suggests it is not FLT4 (Pajusola et al. 1994).

4.3.5. The difficulty of in vivo studies

In vivo studies looking at the role of VEGF in oligodendrocyte maturation and survival would be problematical because the effects on angiogenesis and vascular permeability would lead to many secondary effects. In many other situations though, in vitro data in the O-2A lineage seems to mirror the situation in vivo for example, the timed differentiation of

4.3.6. Conclusion

In conclusion, VEGF was the only agent found to enhance oligodendrocyte maturation in a human glioblastoma derived cell line of the O-2A lineage. Interestingly this finding also applies to the rodent O-2A lineage, where VEGF is also a survival factor for oligodendrocytes in vitro. VEGF has been shown to lead to the intracellular activation of MAPK in both Hu-O-2A/Gbl cells and rodent oligodendrocytes. VEGF is known to be present during development of the rodent (Ferrara et al. 1991) and murine (Breier et al. 1992) central nervous system indicating that it may be available to oligodendrocytes in vivo to aid maturation and survival. Using the Hu-O-2A/Gbl cell line as a research resource it may be possible to conduct further molecular studies which may have relevance to understanding of not only the rodent but also the human O-2A lineage.
5

THE CLONING & EXPRESSION OF LIM-KINASE

5.1. Introduction

It has already been observed that protein kinases are key elements in every aspect of cell metabolism. This includes the regulation of cell survival, proliferation and differentiation. Protein kinases are not only required during embryogenesis and in the maintenance of the adult organism, but also contribute significantly to tumour formation and growth. The O-2A lineage is no exception to these observations.

A novel murine protein kinase gene (murine LIM-kinase1; mLimkl) was discovered by Dr. C. Pröschel and Dr. M.-J. Blouin in the laboratory whilst I was characterizing the Hu-O-2A/Gb1 cell line. This gene was a member of a completely novel family of protein kinases. In addition to having a putative catalytic kinase domain at the carboxy-terminal end (which has since been shown to be active in vitro, Pröschel et al. 1995), it also had two LIM domains at the amino terminus. The LIM-domain proteins (reviewed in Sanchez-Garcia and Rabbitts 1994) are another newly emerging family of regulatory proteins. These proteins carry a structural motif of approximately fifty amino acids first described in three transcription factors: lin-11 (Freyd et al. 1990), isl1 (Karlsson et al. 1990) and mec-3 (Way and Chalfie 1988), from which the acronym LIM was derived. The consensus sequence for the LIM-domain exhibits conservatively spaced cysteine and histidine residues,
arranged in a manner reminiscent of metal-binding domains found in zinc-finger proteins.

It was found that \textit{mLimk1} was highly expressed in the murine central nervous system. I investigated the possibility that there might be a human gene similar to \textit{mLimk1} expressed in the Hu-O-2A/Gb1 cell line. Such a finding would prove interesting because firstly it would be a new human gene, and secondly it might prove relevant to the study of gliomas and possibly also the O-2A lineage.

\textit{mLimk1} was initially found using a modified reverse transcriptase-polymerase chain reaction (PCR) based approach and degenerate oligonucleotide primers PTK1 (5'- CGGATCCAC\textsubscript{A}/CGNGAC\textsubscript{T}/T-T-3') and PTK2 (5'-GGAATTCCA\textsubscript{A}/TAGGACCA\textsubscript{G}/TAC\textsubscript{A}TC-3'), (section 2.3.10) to screen for protein kinases of biological relevance to murine liver cells. I had used a similar strategy in studying the Hu-O-2A/Gb1 cell line (section 3.2.1). The complementary DNA (cDNA) fragment obtained was slightly longer than that normally found for protein kinases [240 base pairs (bp) versus 210bp], but its predicted amino acid sequence contained characteristic motifs for protein kinases. Longer-length cDNA clones were obtained by Dr. C. Pröschel from a mouse pancreas cDNA library using the 240bp cDNA fragment as a probe. One of the isolated clones was C5. This encoded 2.5kilobases (kb) of cDNA, including the kinase domain and lacked the polyadenosine [poly(A)\textsuperscript{+}] tail.

5.2. Results

5.2.1. Northern blot analysis using \textit{mLimk1} as a probe

In order to ascertain if a human gene homologous to \textit{mLimk1} was expressed in Hu-O-2A/Gb1 cells, clone C5 was used as a probe on a northern
Figure 5.1. Northern blot analysis suggesting the presence of LIMK or a family member in the Hu-O-2A/Gb1 cell line.

Northern blot analysis using total RNA (10μg/lane), showing hybridisation of the C5 mLimkl probe to a 3.3kb band in lane 2, RNA from the Hu-O-2A/Gb1 cell line and also weakly in lane 5, RNA from the A431 cell line (a cell line derived from an human epidermoid carcinoma). There is also non-specific hybridisation to ribosomal RNA bands 28S and 18S because of the low stringency used to pick up a positive signal across species. Lanes 1 and 4 contain RNA from other glioma cell populations (see table 5.1. later: lane 1 is RNA from G4 and lane 4 is from G8). Lane 3 contains RNA from HeLa cells, a human epitheloid carcinoma of the cervix derived cell line. Lane 6 contains RNA from line 157, an oligodendroglioma cell line contaminated by rhabdomyosarcoma (Venter et al. 1992).
blot containing total RNA from a variety of human cell lines including the Hu-O-2A/Gb1 cell line (figure 5.1.). At the low stringency washes used to see hybridisation across species, there was cross hybridisation to the ribosomal RNA bands, but a specific 3.3kb band was seen in RNA from the Hu-O-2A/Gb1 cell line. This suggested that the Hu-O-2A/Gb1 cell line did contain either the human homologue of \textit{mLimkl} or a family member of this novel class of protein kinases. Therefore the cloning of a human gene related to \textit{mLimkl} might be possible from the Hu-O-2A/Gb1 cell line. This human gene is referred to as LIMK.

\section*{5.2.2. Cloning of LIMK}

An unamplified Hu-O-2A/Gb1 cDNA library was made from passage 4 Hu-O-2A/Gb1 cells by Mr. R.Ludwig. I screened this library using the murine C5 probe and reduced stringency hybridisation conditions (30\% instead of 50\% formamide (v/v) in both the pre-hybridisation and hybridisation solutions, see section 2.3.11.).

Using this strategy, after primary screening of $2 \times 10^6$ plaques 13 positives were picked. On secondary screening of these 13 plaques, 8 remained positive. \textit{In vivo} excision of phage inserts into pBluescript (Stratagene) was undertaken for the 8 positive plaques and the DNA was isolated. The 8 positive plasmids at this stage were designated 1, 2, 3, 4, 5, 6, 7 and 8.

In order to ascertain which of the plasmids might contain LIMK sequence, a double restriction endonuclease digest of the plasmids was obtained using the enzymes \textit{Kpn}1 and \textit{EcoR}1. The products of the \textit{Kpn}1/\textit{EcoR}1 digest were fractionated by electrophoresis on a 1\% agarose gel and Southern blotted. The membrane was probed using the murine C5 probe.
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(figure 5.2.). This showed strong positive hybridisation to 2, 3, 4 and 5. The other 4 plasmids (1, 6, 7 and 8) were therefore discarded.

Linearization of the plasmids by a NotI restriction endonuclease digest, which was known to cut in the vector, was also undertaken. Fractionation of these fragments by electrophoresis showed three bands for 5 (suggesting the possibility of three separate plasmids in 5), figure 5.2c. Therefore individual plasmids were obtained from 5 by streaking the glycerol stock of 5 onto fresh LB/ampicillin (50μg/ml) plates and picking individual clones, plasmid DNA was isolated from 9 of these clones. The three plasmids containing the largest insert, (3kb as determined by a NotI digest allowing for a vector size of 3kb), were used for sequencing the others were not sequenced as any LIMK insert would have been smaller than the remaining three plasmids (2, 3 and 4).

Sequence was obtained from both ends of each cDNA insert using the oligonucleotide primers T7 and T3 (Stratagene). Sequence analysis was performed using the University of Wisconsin GCG software package (Devereux et al. 1984). Sequencing of plasmids 2, 3 and 4 revealed the same sequence at one end of each cDNA insert starting with a polyadenosine tail. This sequence was found at the T3 primer end of 2 and 4 and the T7 primer end of 3 and confirmed that they all represented cDNA's derived from the same gene, which had no significant homology to sequences on the data base. In addition, sequence from the T7 primer end of 4 contained sequence for the mRNA of a protein phosphatase type 1 catalytic subunit (Durfee et al. 1993) starting from the polyadenosine tail. This indicated that plasmid 4 had two separate cDNA inserts due to an artefact of cloning. The full length of the protein phosphatase type 1 catalytic subunit cDNA was known to be 1.4kb. Therefore assuming plasmid 4 contained its whole length, the remaining length of the candidate LIMK cDNA insert was approximately 3.3kb, allowing for a known total insert length in plasmid 4 of 4.7kb. This candidate LIMK cDNA insert was longer than that for plasmid 2 which was

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Further selection from plasmids 1, 2, 3, 4, 5, 6, 7 and 8 was necessary to ascertain which of these plasmids contained possible LIMK inserts. (A) photograph of a 1% agarose gel, lanes show the products of a double restriction endonuclease digest with enzymes *KpnI* and *EcoR1* for each plasmid. (B) Southern blot of the gel in (A) probed with C5 *mlimk* showing positive hybridisation to bands from the digested plasmids 2 (at 1.9kb), 3 (at 5.5kb), 4 (at 2.0kb) and 5 (at 1.9kb). There is also a weak band in the 6 lane at approximately 2.0kb suggesting possibly the presence of a gene related to LIMK, although sequencing showed that this cDNA had 85.2% homology to a rodent S5 ribosomal sequence (see text). (C) photograph of a 1% agarose gel showing the products of a *Not1* restriction endonuclease digest for each plasmid, this cuts in the vector at a single site allowing plasmid linearization and suggests the possibility of three separate plasmids in 5.
2.7kb and plasmid 3 which was 2.55kb. The three purified plasmids from 5 had the same sequence, that of human mitochondrial DNA, and were therefore discarded.

The other plasmids discarded after the Southern blot were also sequenced. The cDNA insert of plasmid 1 had 84% homology to rodent Brain Derived Neurotrophic Factor, that of plasmid 6 had 85.2% homology to a rodent S5 ribosomal sequence, that of plasmid 7 was a human mitochondrial messenger RNA and that of plasmid 8 was a human translationally controlled tumour protein.

5.2.3. Full length cDNA sequencing of LIMK

Complete sequence of the cDNA's for 2, 3 and 4 was determined using two strategies (figure 5.3.). Firstly fragments were subcloned following restriction enzyme digests of the plasmids (the enzymes BamH1, BstX1, EcoR1, Kpn1, Pst1, Sac1 and Xho1 were used), the subcloned fragments were then sequenced using the primers T3, T7 and also those shown in figure 5.4. Secondly, to derive additional sequence information for oligonucleotide primer design the degenerate primers PTK1 and PTK2 were used in a PCR with each of the plasmids 2, 3 and 4 to amplify a 240bp fragment encoding part of the catalytic kinase domain (bases 1523 to 1763). This fragment was subcloned and sequenced. Primer oligonucleotides were then made from the sequence obtained from the PCR fragment (see figure 5.4). These primers were used for sequencing from the original 2, 3 and 4 cDNA's to allow complete sequencing in a region where there were no convenient restriction enzyme sites to enable sequencing from directly subcloned fragments.

The composite cDNA sequence of 3290bp for LIMK is shown in figure 5.4. Only plasmid 4 contained the most 5' region of 595bp. As estimated by northern blot analysis this approximated well with the full length cDNA
Figure 5.3. Schematic representation of human LIMK, restriction enzyme sites, positive clones and subcloned fragments.

A) schematic representation of LIMK cDNA, the large open box indicates the open reading frame (95 to 2036 bases), within which the two black boxes are the LIM-domains and the shaded box is the kinase domain. The full length cDNA sequence was 3290 bases. The key to the restriction endonuclease enzyme sites that were used are: B-BamHI (707), Bs-BsrXI (370, 2380, 3005), E-EcoRI (737), K-KpnI (2716), P-PstI (257, 983, 2857, 2990), S-Sacl (914) and X-Xhol (855).

B) shows the alignment of LIMK cDNA clones used to construct the composite cDNA sequence and fragments which were subcloned to aid sequencing. Subcloned fragment (b) containing 625 bp of 3' untranslated sequence was used for most northern blot and in situ hybridisation analysis, although similar results were obtained by using the subcloned fragment (a) which contained the kinase domain and was 1970 bp long.

The degenerate primers PTK1 and PTK2 were used for PCR to isolate a 240 bp fragment encoding part of the catalytic kinase domain (bases 1523 to 1763). This was subcloned and sequenced allowing primer oligonucleotides to be made to complete the sequencing in a region where there were no convenient restriction enzyme sites to permit sequencing from directly subcloned fragments (see figure 5.4).
This sequence is identical to that which has since been published by Mizuno et al., 1994 as LIMK. Regions from which important primer oligonucleotides were made to allow sequencing are shown underlined, (mainly covering the kinase domain). Those extending 3' are shown, those which were made to extend in a 5' direction using the anti-sense strand sequence are not shown. The position of two overlapping polyadenylation signals is shown in bold lettering and boxed.
Figure 5.5. Predicted amino acid sequence of human LIMK, using the standard single letter abbreviations for each amino acid.

```
35   G Q Y L Q A L N A D W E A D C F R G C D G S A S L S H O Y Y E K D G
69   Q L F C K K D Y W A R Y G E S G H G C S E Q I T K G L V M V A G E L
137  G Y Y Q T V V T P V I E Q I L P D S P G S H L P H T V T V L V S I P A
171  S S H G K R G L S V S I D P P H G P G C G T E H S T V R V Q G V
205  D P G C M S P D V K N S I H V G D R I L E I N G T P I R N V P L D E
239  I D L L I Q E T S R L L Q L T L E H D P H D T L G H G L G P E T S P
307  S L G S P A S Q R K D L G R S E S L R V V C R P H R I F R P S D L I
409  L N F I T E Y I K G G T L R G I K S M D S Q Y P W S Q R V S F A K
443  D I A S G M A Y L H S M N I I H R D L N S H N C L V R E N K N V V
511  G N P Y W M A P E M I N G R S Y D E K V D V E S F G I V L C E I I G
579  F F P I T V R C C D L D P E K R P S F V K L E H W L E T L R M H L A
613  G H L P L G P Q L E Q L D R G F W E T Y R R G E S G L P A H P E V P
647  D
```

The two putative initiation methionines are shown in bold type and underlined. The two LIM-domains are singly underlined and conserved cysteine, histidine and aspartate residues (believed to be involved in zinc ion binding), within these domains are in outlined lettering. Two putative MAPK phosphorylation sites (PDSP, amino acids 152 to 155 and SLGSPA, amino acids 307 to 312) and a putative casein kinase I phosphorylation site (SPLSS, amino acids 271 to 275) are shown in bold large lettering. The kinase domain is double underlined, the putative nuclear localisation signal insert has a dotted line beneath it.
(figures 5.1. and 5.7.). This data was confirmed to be correct by others (Mizuno et al. 1994).

In order to be absolutely certain that there was no further 5' untranslated sequence primer extension with RNase protection would be necessary. The deduced amino acid sequence and protein domains for LIMK are shown in figure 5.5.

5.2.4. Structural characteristics of LIMK

Depending on which of two potential ATG initiation codons is used, the open reading frame of LIMK encodes a protein of 647 or 633 amino acid residues, with a predicted molecular weight of approximately 73 kilodaltons (kDa). No Kozak consensus sequence (which modulates translation) is present at either ATG (Kozak 1986). The LIMK cDNA has a 95bp 5' untranslated leader sequence and the open reading frame is followed by a 1254bp 3' untranslated region which contains two overlapping polyadenylation signals and a polyadenosine tail (Proudfoot and Brownlee 1976).

The predicted amino acid sequence of LIMK showed two important characteristics. Firstly a pair of LIM domains was arranged in tandem at the amino-terminus of LIMK, (amino acid residues 25-75 and 84-137 Karlsson et al. 1990), and secondly a catalytic protein kinase domain was located at the carboxy-terminal end, amino acids 346-604 (Hanks and Quinn 1991; Hanks et al. 1988). Some of the serine residues between the LIM domains and the catalytic protein kinase domain lie within sequence motifs which represent recognition sites for serine/threonine kinases (Kemp and Pearson 1990). Most prominent are putative phosphorylation sites for casein kinase I (SPLSS, amino acids 271 to 275) (Gonzalez et al. 1991; Tuazon and Traugh 1991) and
mitogen activated protein kinase (PDSP, amino acids 152 to 155 and SLGSPA, amino acids 307 to 312) (Gonzalez et al. 1991).

Other protein sequence motifs, which have previously been found in conjunction with either protein kinases, such as src homology domains 2 and 3 (Koch et al. 1991) or LIM-proteins, such as the homeodomain (Freyd et al. 1990), were absent. No hydrophobic signal peptide or transmembrane sequences were detected. This suggested that LIMK was an intracellular protein. Furthermore, a short and highly basic insert (RSLKKPDRKKR, amino acid residues 496 to 506) was present within the kinase domain. This sequence might act as a putative nuclear localisation signal (Chelsky et al. 1989). Uncharacteristically, though, this short insert sequence lies between subdomains VII and VIII of the catalytic protein kinase domain. In contrast, most kinase domain inserts are located between subdomains V and VIa.

Amino acid homology between the full length \( mLimk1 \) and human LIMK varied between 97.3% in the kinase domain, 94% in the LIM domains and 92% in the intervening sequence. Overall homology on the DNA level was 78%, while the 5' and 3' untranslated ends show a higher degree of divergence (10% and 65% homology, respectively). The high amino acid sequence homology, the fact that no other protein kinases were found on screening the Hu-O-2A/Gb1 cDNA library even under low stringency conditions and the finding that the \( mLimk1 \) probe crossed to human on northern blot analysis suggested that human LIMK was the homologue of \( mLimk1 \), (see also section 5.3.3.).

The sequence alignment of catalytic kinase domains between LIMK and other known protein kinases highlighted the unusual nature of the LIMK kinase domain (figure 5.6.). The alignment was based on the previously described subdomain structure of catalytic domains found in protein kinases (Hanks and Quinn 1991; Hanks et al. 1988; Lindberg et al. 1992). Alongside
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Figure 5.6. The alignment of the amino acid sequence of human LIMK to the conserved kinase subdomains of protein tyrosine kinases (PTK) and protein serine/threonine kinases (PSK).

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>VIb</th>
<th>VII</th>
<th>VIII</th>
<th>IX</th>
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</thead>
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<td>IHRDL^AA~N</td>
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<td>-pIKw-APE</td>
<td>SDWw~Fw</td>
</tr>
</tbody>
</table>

A dash represents variable residues. LIMK contains all conserved amino acids which have been shown to be necessary for kinase activity. However LIMK differs from both the PTK and PSK consensus sequences, (residues marked by dots), in regions diagnostic of either PSK or PTK's.
of LIMK, the consensus sequences of protein serine/threonine kinases (PSK) and protein tyrosine kinases (PTK) in six of the eleven defined subdomains are shown. While LIMK contains 15 of the 15 highly conserved amino acid residues defined throughout the entire kinase domain, some differences can be seen in subdomains VIb and VIII. These domains are part of the catalytic core and have been previously used to predict the hydroxy-amino acid specificity of kinases (Hanks et al. 1988). The LIMK sequence in subdomain VIb (HRDLNSHN) and VIII (GNYWMAPE), aligns only partially with either the PSK or PTK consensus motifs. The region by the highly conserved lysine (K) in subdomain II also fails to align to either the serine/threonine or tyrosine kinase consensus (figure 5.6). In addition, these regions in LIMK failed to show any significant homology to putative dual specificity kinases (Lindberg et al. 1992), placing LIMK into a new class of protein kinase.

**5.2.5. LIMK mRNA is predominantly expressed in the developing and adult human nervous system**

LIMK mRNA expression was investigated mainly using a probe containing 625bp of the 3' untranslated sequence of LIMK (figure 5.3.), although similar results were obtained by using a probe which was 1970bp long and contained the kinase domain as well as some 3' untranslated sequence.

Northern blot analysis showed that LIMK is predominantly found in both the developing and adult human nervous system (figure 5.7.) and is almost equally expressed in various deep structures within the adult brain (figure 5.7.).

*In situ* hybridisation of human embryo sections from a 50 day postovulatory embryo (O'Rahilly and Muller 1994) confirmed LIMK expression in the developing nervous system, figure 5.8; these sections were
Figure 5.7. Northern blot analysis of LIMK expression in normal human tissues.

Human foetal, adult, and adult brain northern blots are shown. Abbreviations; M.-muscle, N.-nucleus, Ni.-Nigra and Callos.-Callosum. The blots were obtained from Clontech, approximately 2μg of poly A+ RNA was loaded in each lane. The foetal blot contained RNA from 19 to 23 week foetus. The blots were probed firstly (above) for LIMK showing a 3.3kb band, highest expression is seen in the brain and secondly (below) with a full length murine GAPDH probe, to indicate the amount of RNA.
Figure 5.8. *In situ* hybridisation analysis of LIMK expression in the nervous system of a 50 postovulatory day human embryo.

The LIMK 625bp cRNA probe (see figure 5.3.) was labelled with DIG-UTP and visualised using anti-DIG alkaline phosphatase antibody. (A) a section through rhombencephalon/medulla, (see figure 5.9 for orientation), fourth ventricle is above. LIMK expression is seen in sub-ventricular zone of the fourth ventricle and a lower level of expression extends out into the sub-pial layer. The arrow indicates expression in the medial accessory olivary nucleus either side of the midline, this area is shown in greater detail in C. (B) a similar section to (A) hybridised with the sense-strand RNA probe, a negative control. (C) a close-up of the medial accessory olivary nuclei shown in (A). (D) a section through the cerebellum (c), (see figure 5.9 for orientation), the fourth ventricle is on the right, there is a high level of sub-ventricular expression, the ectoderm is on the left, there is also some expression seen in the mesenchyme adjacent to the ectoderm. The arrow shows expression in the dentate nucleus. (E) a section through the spinal cord (see figure 5.9 for orientation), dorsal is above. There was some generalised expression in the dorsal part of the spinal cord and single cell staining more ventrally. There is also expression in the dorsal root ganglia (d). (F) a section through the fifth nerve ganglion, (high expression, in centre of picture) (see figure 5.9 for orientation). The ectoderm is on the left. Scale bar represents either 100μm (C) or 250μm (in A, B, D, E and F).
Figure 5.9. A diagram of a 50 postovulatory day human embryo showing areas of LIMK expression in the nervous system above the thoracic spinal cord.

LIMK expression is in red, the open blue rectangles give a guide to the sections from which the photographs in Figure 5.8. were obtained. Section 1 is through the rhombencephalon/medulla (photographs A, B and C in figure 5.8), section 2 is through the cerebellum (photograph D), section 3 is through the spinal cord (photograph E) and section 4 is through the V nerve ganglion (photograph F). In situ hybridisation for LIMK was not undertaken on sections lower than the cervical spinal cord due to the scarcity of human material and therefore expression is not shown below this level.
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obtained through a collaboration with Professor P. Thorogood's laboratory in the Institute of Child Health. At this stage during development in the central nervous system high levels of mRNA expression were seen in the sub-ventricular zone of the fourth ventricle, (a lower level of expression extended out into the sub-pial layer), the brainstem nuclei and also there was some generalised expression in the dorsal part of the spinal cord and single cell staining more ventrally. In the peripheral nervous system high levels were seen in cranial nerve ganglia and dorsal root ganglia (figure 5.8.). Expression was also seen in the ectoderm and mesenchyme and around the developing eye (data not shown). The expression pattern of LIMK is represented in figure 5.9.

5.2.6. LIMK is expressed in glioblastoma multiforme tumours as well as their derived cell populations

Northern blot analysis of total RNA from 11 out of 19 low passage human malignant glioma derived primary cell populations (from grade 3 and 4 tumours; see table 5.1.), showed high LIMK expression, (as exemplified by figure 5.10. for G1, G3, G7, G10, G12, G13, G14 and Hu-O-A/Gb1 cells; and also data not shown). High LIMK expression was seen in cell populations expressing either predominantly GFAP, (6 cell populations, including the Hu-O-2A/Gb1 cell line) or fibronectin (FN; 5 cell populations). Two out of three human meningioma derived cell populations also examined by northern blot analysis showed high LIMK expression (data not shown). The high expression of LIMK seen in at least three of the malignant glioma derived cell populations was not the result of changes occurring during tissue culture, as high levels of LIMK expression were seen by in situ hybridisation of sections taken from the original glioblastoma multiforme tumours, (the cell populations derived from these three tumours all had a glial phenotype;
Table 5.1. Low passage primary cell populations derived from malignant gliomas; biological characteristics and patient details.

The other cell population derived from a malignant glioma and used for northern blot analysis, but not shown in this table, is the Hu-O-2A/Gb1 cell line; therefore making 19 cell populations in total. Cell populations were grown in either DMEM+10%FCS (FCS) or ACM as shown. The antigenic phenotype for each cell population is shown, all cell populations were stained for both GFAP and FN, where one is not mentioned no staining was seen, (mean±s.e.m. is given; 3 coverslips counted). The pathological diagnosis in each case was from the sample taken at the time of operation and reported by the Department of Neuropathology, The National Hospital, Queen Square, London. New abbreviations used: M - male; F - female; R - right; L - left; F - frontal lobe; T - temporal lobe; P - parietal lobe; O - occipital lobe; GBM - glioblastoma multiforme; GS 4 - gliosarcoma grade 4; O/A 4 - oligo-astrocytoma grade 4; O/A 3 - oligo-astrocytoma grade 3; A 3 - astrocytoma grade 3.

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Figure 5.10. Northern blot analysis of LIMK expression in cell populations derived from malignant human glioma samples.

15μg of total RNA from each cell population was loaded in each lane, several other northern blots were also probed for LIMK (data not shown). Malignant glioma cell populations having a mesenchymal antigenic phenotype are fibronectin (FN) positive, those having a glial antigenic phenotype are glial fibrillary acidic protein (GFAP) positive and those having a mixed antigenic phenotype are labelled FN/GFAP or GFAP/FN (the most abundant antigen is placed first). Lanes 1 and 12 contain RNA from the cell line A431 (an epidermoid carcinoma cell line), a positive control. RNA from Hu-0-2A/Gb1 cells grown in ACM passage 13 are shown in lanes 8 and 11. Details of the cell populations are show in table 5.1. Lane 2 is G13, lane 3 is G3, lane 4 is G8, lane 5 is G2, lane 6 is G16, lane 7 is G14, lane 9 is G1 (a very high level of LIMK), lane 10 is G4, lane 13 is G10, lane 14 is G7, lane 15 is G15, lane 16 is line G18 and lane 17 is G12. The blots were probed firstly (above) with LIMK showing a 3.3kb band (7 day exposure) and and then (below) with a full length murine GAPDH probe to indicate the amount of RNA.
Figure 5.11. *In situ* hybridisation analysis of LIMK expression in glioblastoma multiforme tumours.

The LIMK 625bp cRNA probe (see figure 5.3.) was labelled with DIG-UTP and visualised using anti-DIG alkaline phosphatase antibody. (A) is a section through the glioblastoma multiforme tumour from which the Hu-O-2A/Gb1 cell line was derived. (B) a similar section to (A) hybridised with the sense-strand RNA probe, a negative control. (C) is a section through the G1 glioblastoma multiforme tumour, see table 5.1. (D) is a section through the G3 glioblastoma multiforme tumour, see table 5.1. LIMK positive cells are seen in each tumour sample. Scale bar represents 100μm.
one was the Hu-O-2A/Gb1 cell line, figure 5.11.

Hu-O-2A/Gb1 cells did not change their relative expression of LIMK mRNA when either a more astrocytic phenotype or a more mature oligodendrocytic phenotype was expressed. This was shown by northern blot analysis, total RNA from Hu-O-2A/Gb1 cells maintained in ACM was compared to that from cells grown in either DMEM+10%FCS or DMEM-BS supplemented by 10ng/ml/day of VEGF for 8 days (data not shown).

### 5.3. Discussion

#### 5.3.1. LIM proteins can be divided into four different structural classes

The cDNA sequences of approximately twenty-four different LIM-domain proteins have been published. Based on the arrangement of LIM-domains and the presence or absence of additional protein motifs, the family of LIM-domain proteins has been be subdivided into several classes (Sanchez-Garcia and Rabbitts 1994).

The first class of LIM-proteins to be identified were the LIM-homeodomain proteins. All members of this class contain two LIM-domains arranged in tandem and a carboxy-terminal homeodomain; for example lin-11 (Freyd et al. 1990), isl1 (Karlsson et al. 1990) and mec-3 (Way and Chalfie 1988). The presence of homeodomains suggests that members of this class of LIM proteins may act as regulators of transcription. There is evidence that LIM-homeodomain genes are involved in differentiation and cell determination from the study of the nematode genes lin-11 and mec-3. Null mutants leading to the loss of asymmetric cell division have been reported in both lin-11 mutants in secondary vulval precursor cells (Freyd et al. 1990) and in mec-3 mutants in the mechano-sensory neuron lineage (Way and Chalfie 1988; Way et al. 1992).
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The second class of LIM-proteins contains one or two LIM-domains, but no other structural motifs with a known function. The majority of proteins in this class are associated with cytoskeletal components (Crawford et al. 1994; Sadler et al. 1992), they include cysteine rich protein (Liebhaber et al. 1990). This group of LIM-only proteins also includes the rhombotins (RBTN's) such as RBTN-1 or T cell translocation gene-1 (Ttg-1) (Boehm et al. 1990b; McGuire et al. 1989) and RBTN-2 or Ttg-2 (Boehm et al. 1991; Royer-Pokora et al. 1991). Unlike the cysteine rich proteins, the rhombotins are located in the nucleus and have been proposed to act as regulators of transcription (Boehm et al. 1990a; McGuire et al. 1991; Rabbitts and Boehm 1990). At present RBTN-1 and RBTN-2 are the only known LIM domain containing oncogenes. This ability of RBTN-1 and -2 to induce tumours was confirmed in transgenic mice by expressing either protein from a T-cell specific promoter (Fisch et al. 1992; Larson et al. 1994).

A third class of LIM-domain proteins comprises proteins with three or more LIM-domains. Two members of this class, zyxin (Crawford and Beckerle 1991) and paxillin (Turner and Miller 1994), are cytoskeletal proteins and contain additional protein domains, while the Enigma protein consists almost entirely of five LIM domains (Wu and Gill 1994).

The final class of LIM proteins consists of \( mLimk1 \) (Cheng and Robertson 1995; Pröschel et al. 1995) and its human homologue LIMK described here, (which has also been independently cloned and published by another group, Mizuno et al. 1994). The suggestion that LIMK is the homologue of \( mLimk1 \) is supported by the high amino acid sequence homology between \( mLimk1 \) and LIMK, and the fact that no other protein kinases were found on screening the Hu-O-2A/Gbl cDNA library with a \( mLimk1 \) probe even under low stringency conditions. Chromosomal mapping supports the suggestion that LIMK is the human homologue of \( mLimk1 \), (see section 5.3.3.).
The predicted amino acid sequence of LIMK shows a catalytic kinase domain at the carboxy-terminal end and two LIM domains at the amino terminus. *In vitro* studies reveal that *mLimk1* does have kinase activity and phosphorylates on serine, threonine and tyrosine residues (Pröschel et al. 1995). This suggests a possible dual specificity for LIMK/*mLimk1*, both serine/threonine and also tyrosine kinase activity (Lindberg et al. 1992).

### 5.3.2. LIM domain functions

The conserved arrangement of cysteine, histidine and aspartate residues in the LIM domain, allows a complex to form between one LIM domain and two Zn$^{2+}$ ions giving a symmetrical double zinc finger structure (Archer et al. 1994; Kosa et al. 1994; Li et al. 1991; Michelsen et al. 1993; Michelsen et al. 1994). Because of the similarities between zinc-fingers that mediate DNA binding in nuclear transcription factors, and the double zinc-finger/LIM motif, it was suggested that LIM domains may be involved in DNA binding (Freyd et al. 1990; Karlsson et al. 1990). However no evidence has been published to support a function for LIM-domains in DNA binding. Instead, several lines of research have pointed towards a possible role for LIM domains in mediating protein-protein binding (Rabbitts and Boehm 1990; Sadler et al. 1992).

The effect of the LIM domains on the kinase activity of LIMK is unknown, but by analogy to non-catalytic domains of other protein kinases, the LIM domains may be capable of modulating either LIMK kinase activity directly or indirectly by determining subcellular localization or substrate availability.
5.3.3. LIMK Chromosomal localization

Additional information regarding the *in vivo* function of a novel gene might be obtained by mapping it to a region of a chromosome where an abnormality can be found which is known to produce a phenotype. Using interspecific backcross analysis *mLimk1* was found to be a single copy gene mapping to the distal end of mouse chromosome 5 (Cheng and Robertson 1995; Pröschel et al. 1995). It was therefore predicted that if LIMK was the human homologue of *mLimk1* it would lie on human chromosome 7q (the long arm of 7). This was confirmed by Dr. X. Mao in the laboratory of Dr. D. Sheer who screened a human chromosome 7 specific cosmid library using the probe which contained the 625bp of the 3' untranslated sequence of LIMK. Fluorescent *in situ* hybridisation using this and other LIMK cDNA probes on chromosome preparations had previously proved unsuccessful. The gene for Williams Syndrome ("elfin" facies, mental retardation, growth deficiency, "cocktail party patter" personality, a variety of cardiac lesions and intermittent hypercalcaemia) maps to the same region as LIMK on chromosome 7, the significance of this finding is currently being studied (Dr. X. Mao, personal communication).

5.3.4. Possible locations where LIMK may act within the cell

In the absence of a LIMK specific antibody the subcellular localization of this protein is unknown. The presence of a putative nuclear localization signal in the kinase domain suggests a possible nuclear role. Alternatively LIMK might interact with other LIM-proteins in the cytoskeleton, in a fashion similar to the interaction between cysteine rich protein and zyxin (Crawford et al. 1992; Sadler et al. 1992), these possibilities are shown in figure 5.12.
Figure 5.12. Possible locations where LIMK may act within the cell.

A diagrammatic representation of the possible locations where LIMK may act. LIMK might act in the nucleus with Rhombotin, in the cytoskeleton possibly in focal adhesion complexes with Zyxin or it might also be phosphorylated by kinases involved in signal transduction such as MAPK, (here shown as part of the EGF receptor pathway as an example).
Chapter 5 LIMK a new protein kinase

Besides the cellular localization, an additional level of control of LIMK activity might be exerted through phosphorylation by other kinases involved in signal transduction, such as casein kinase I and mitogen activated protein kinase, for which putative recognition sites are found in LIMK (Marshall 1994), (figures 5.5. and 5.12.).

5.3.5. A possible role for LIMK as an oncogene and during human development

The high levels of LIMK expression seen in human glioblastoma multiforme samples and also in cell populations derived from human malignant gliomas raises the possibility that LIMK is involved in the initiation, growth or invasion characteristics of a subset of these tumours, (and possibly also meningiomas), but further investigation is needed.

The expression of the mRNA of LIMK in the developing human embryo also requires further investigation, more stages of human development need to be studied. Although, even the 50 day postovulatory stage that has been studied suggests that LIMK expression is not limited to cells of the O-2A lineage in the central nervous system. LIMK mRNA was found in developing brain stem nuclei, in a fairly widespread distribution at the single cell level in the hind brain and also in peripheral nerve ganglia. These are regions where it is unlikely O-2A lineage cells would be found at this developmental stage, but it must be remembered that one can only use developmental studies of the O-2A lineage in the rat and mouse for comparison (see for example, Pringle and Richardson 1993; Timsit et al. 1995). High levels of LIMK expression were seen in the subventricular zone of the fourth ventricle, where O-2A lineage cells could arise. Such a finding of widespread nervous system expression for LIMK are supported by the findings of high levels of LIMK mRNA expression in glioblastoma cell populations expressing either
astrocytic or mesenchymal phenotypes as well as those having an O-2A lineages phenotype. In addition meningioma cell populations expressing high levels of LIMK mRNA were found.

It therefore seems from the data that is available, that although LIMK is found in O-2A lineage cells, (the Hu-O-2A/Gb1 cell line, and also Limkl mRNA is found in rodent O-2A precursors and oligodendrocytes, Dr. C.Pröschel personal communication), its expression is not restricted to cells of this lineage in the nervous system.

5.3.6. The future

The interesting questions related to the role of LIMK in gliomas and during development could be addressed in future by using a combination of methods. Firstly the generation of LIMK specific antibodies is essential. This would allow the intracellular localization of the LIMK protein and its in vitro association with other proteins and substrates. A detailed analysis of LIMK mRNA and protein expression during human development would then be possible. Overexpression of mutant and wild-type forms of LIMK could be tested for their effects in various cell systems including the Hu-O-2A/Gb1 cell line. The production of mLimkl transgenic animals and the isolation of purified cell types (such as O-2A progenitors) and their analysis in terms of cell division, differentiation and survival would also be possible.
HUMAN EMBRYO KINASE 2, A POSSIBLE MARKER OF THE O-2A LINEAGE

6.1. Introduction

Cloning of tyrosine kinase fragments from Hu-O-2A/Gb1 cells had shown that out of 21 clones, two contained sequence which gave the same predicted amino acid sequence as rodent tyro-6 (Lai and Lemke 1991), (section 3.2.1.). An unsuccessful attempt to clone a longer portion of the human gene was made from a complementary DNA library derived from Hu-O-2A/Gb1 cells. The full length sequence of the human homologue of tyro-6 was then published as Human Embryo Kinase 2 (HEK2 Böhme et al. 1993). HEK2 is a member of the EPH family of receptor tyrosine kinases. This is the largest family of tyrosine kinase receptors, with 13 currently known members. The brain is the one tissue in which virtually all of these receptors have been reported to be expressed. A number of features serve to define newly discovered kinases as members of the EPH class. These include in the extracellular region an amino terminal immunoglobulin-like domain followed by a cysteine-rich stretch (the positions of most cysteines are conserved), and sequences corresponding to two fibronectin type III repeats (Skorstengaard et al. 1986) in close proximity to the transmembrane domain. In the intracellular region there is a catalytic tyrosine kinase domain without an insert.

HEK2 homologues have been described in a number of species; chicken (Chicken embryo kinase 10 (Cek10), Sajjadi and Pasquale 1993), mouse
(Segmentally expressed kinase 4 (Sek4), Becker et al. 1994) and rat (tyro-6 Lai and Lemke 1991). At the time of discovering HEK2 in the Hu-O-2A/Gb1 cell line, no ligands for the EPH family were known, but over the past year their has been an explosion of knowledge in this field (see section 6.3.4.).

HEK2 in common with other tyrosine kinases might have a role in oncogenesis, during development or as a marker of the O-2A lineage. HEK2 expression was therefore investigated in gliomas and human embryonic sections and findings were compared to PDGFRα a known marker of the O-2A lineage in rats (Pringle et al. 1992; Pringle and Richardson 1993).and mice (Yeh et al. 1993).

6.2. Results

6.2.1. Specific probes for HEK2 and PDGFRα.

The cDNA for HEK2 was obtained from Dr. B. Böhme (Böhme et al. 1993). The cDNA for PDGFRα was obtained from Professor C.-H. Heldin, Ludwig Institute for Cancer Research, Uppsala, it contained sequence from base pair position 251 to 3464 (Claesson-Welsh et al. 1989). Candidate probes were made by using restriction endonucleases to cut each cDNA. These candidate probe fragments were used as probes on northern blots of Hu-O-2A/Gb1 cell total RNA to test their specificity in providing a single band of the correct size, (4.6kb for HEK2 and 6.4kb for PDGFRα). The best probes were sequenced to confirm that they contained the correct tyrosine kinase receptor. It was found that for HEK2 a fragment from an Acc1 site at position 339 to an Xho1 site at position 1095 provided the ideal probe. This fragment was entirely within the extracellular domain of HEK2. For PDGFRα a fragment from an EcoR1 site at position 251 to an EcoRV site at
position 1166 was optimal. Again, this fragment was entirely within the extracellular domain of PDGFRα.

6.2.2. Selective high expression of HEK2 in glioblastoma multiforme derived cell populations having a glial phenotype

Northern blot analysis of total RNA showed high HEK2 mRNA expression in only 3 out of 19 low passage human malignant glioma derived primary cell populations (figure 6.1; see also table 5.1.). One of the three cell populations was the Hu-O-2A/Gb1 cell line. Greater than 90% of cells from the other two cell populations were GFAP positive, cells from these populations (both also derived from glioblastoma multiforme tumours; G1 and G3), had a process bearing morphology and differed from the other 16 cell populations studied (figure 6.2.). In addition, one of three human meningioma cell populations examined by total RNA northern blot analysis had a detectable level of HEK2 expression (data not shown).

High PDGFRα expression did not follow the same pattern as that seen for HEK2 (figure 6.1. and data not shown). Of the 19 malignant glioma cell populations analysed by northern blot, 5 showed strong PDGFRα expression. Two of these had a predominance of GFAP expression, (one was the Hu-O-2A/Gb1 cell line, the other G12), and 3 expressed fibronectin only (G6, G7 and G17). All three of the human meningioma cell populations tested showed expression of PDGFRα mRNA (data not shown).

In situ hybridisation showed that the high expression of HEK2 seen in the three glioblastoma multiforme derived cell populations of glial phenotype was not the result of changes occurring during tissue culture. Sections from the three original glioblastoma multiforme tumours showed cells expressing a high level of the mRNA of HEK2 (figure 6.3.). High PDGFRα mRNA expression was also confirmed in the original glioblastoma multiforme
Figure 6.1. Northern blot analysis of HEK2 and also PDGFRα expression in cell populations derived from malignant human glioma samples.

15μg of total RNA from each cell population was loaded in each lane, several other northern blots were also probed for HEK2 and PDGFRα (data not shown). Malignant glioma cell populations having a mesenchymal antigenic phenotype are fibronectin (FN) positive, those having a glial antigenic phenotype are glial fibrillary acidic protein (GFAP) positive and those having a mixed antigenic phenotype are labelled FN/GFAP or GFAP/FN (the most abundant antigen is placed first). Lane 1 is the RNA from the cell line A431 (an epidermoid carcinoma cell line). RNA from Hu-0-2A/Gb1 cells grown in ACM passage 13 is shown in lane 8. Details of the cell populations are show in table 5.1. Lane 2 is G13, lane 3 is G3, lane 4 is G8, lane 5 is G2, lane 6 is G16, lane 7 is G14 and lane 9 is G1. The blot was probed firstly for HEK2 showing a 4.6kb band in lanes 3, 8 and 9 (middle panel; 7 day exposure), and then for PDGFRα showing a 6.4kb band only in lane 8 (upper panel). Probing with full length murine GAPDH probe (lower panel), indicates the amount of RNA in each lane.
Figure 6.2. Different morphology / antigenic expression of cells derived from malignant gliomas.

Pictures of malignant glioma cells, all cells were cultured on PLL. Scale bar is 10μm. A, C and E are phase contrast pictures. B and D are GFAP fluorescein staining. F is FN fluorescein staining. A and B are the same field of view and are cells from the G1 cell population, C and D are the same field of view and are cells from the G3 cell population, E and F are the same field of view and are cells from the G15 cell population; see table 5.1. Cell populations G1 and G3 have a process bearing morphology, >90% of cells were GFAP positive and they differed from the other 16 cell populations studied. The G15 cell population is representative of the 16 other cell populations.
Figure 6.3. *In situ* hybridisation analysis of HEK2 expression in glioblastoma multiforme tumours.

The HEK2 756bp cRNA probe (see section 6.2.1.) was labelled with DIG-UTP and visualised using anti-DIG alkaline phosphatase antibody. (A) is a section through the G1 glioblastoma multiforme tumour, see table 5.1. (B) a similar section to (A) hybridised with the sense-strand RNA probe, a negative control. (C) is a section through the glioblastoma multiforme tumour from which the Hu-O-2A/Gb1 cell line was derived. (D) is a section through the G3 glioblastoma multiforme tumour, see table 5.1. HEK2 positive cells are seen in each tumour sample. Scale bar represents 100μm.
specimen from which the Hu-O-2A/Gb1 cell line was derived (data not shown).

6.2.3. Differential expression of HEK2 in the Hu-O-2A/Gb1 cell line depending on the antigenic phenotype

The expression of HEK2 was found to vary in the Hu-O-2A/Gb1 cell line according to whether the cells became more astrocyte-like or more oligodendrocyte-like (figure 6.4.). For these experiments Hu-O-2A/Gb1 cells maintained in ACM (progenitor-like condition), were compared to those grown in DMEM+10%FCS (astrocytes) or DMEM-BS supplemented by 10ng/ml/day of VEGF for 8 days (more mature oligodendrocytes). It was shown that at both the mRNA and protein levels, (HEK2 antiserum was kindly provided by Dr. B. Böhme), there was HEK2 down regulation when the astrocyte pathway was favoured and up regulation when the oligodendrocyte pathway was favoured, compared to cells maintained in ACM. By immunoprecipitating the HEK2 receptor from cells maintained in each condition, (thereby concentrating the receptor and allowing receptor phosphorylation to occur in the presence of radioactive adenosine triphosphate, section 2.3.20.), a functional reduction of in vitro HEK2 kinase activity was also shown for cells maintained in DMEM+10%FCS (when the astrocytic pathway of differentiation was favoured).

6.2.4. HEK2 expression in the developing and adult human nervous system

It had previously been reported that by northern blot analysis HEK2 is predominantly expressed in adult human lung, pancreas, placenta, brain and kidney, (Böhme et al. 1993) with lower levels in the heart, skeletal muscle
Hu-O-2A/Gb1 passage 15 cells attached to PLL were cultured in either DMEM-BS supplemented with VEGF 10ng/ml/day or DMEM+10%FCS or maintained in ACM for 8 days. A) Northern blot analysis for HEK2. 7x10^5 cells were cultured in each condition in 80 cm^2 flasks. 15μg of total RNA was loaded in each lane. The blot was probed for HEK2 (above) and GAPDH (below). More HEK2 (4.6kb) message is seen in the condition where more cells have oligodendrocyte markers (cells treated with VEGF) than when the oligodendrocyte pathway is suppressed and astrocytic differentiation is favoured (cells cultured in the presence of FCS). B) Western blot analysis confirming the northern findings. 2.5x10^5 cells in each condition were grown on 6 cm diameter PLL coated dishes for 8 days, equal amounts of protein were loaded in each lane. A HEK2 doublet (130kDa) is seen in all Hu-O-2A/Gb1 lanes, (this is also seen in A431 cells, data not shown), but is not seen in human adult epileptic brain (Ep. Brain). C) An in vitro kinase assay for HEK2 activity using cells prepared as in (B) (see section 2.3.20.), initially equal amounts of cell protein were used. It shows a reduction in HEK2 activity in cells grown in DMEM+10% FCS.
and liver. Further human tissue northern blot analysis showed high levels of HEK2 mRNA in foetal brain, lung and kidney and also in deep structures of the adult brain, at a relatively constant level (figure 6.5.).

*In situ* hybridisation of human embryo sections from a 50 day postovulatory embryo (figure 6.6.), confirmed HEK2 expression in the developing nervous system, (these sections were obtained from Professor P. Thorogood's laboratory in the Institute of Child Health). At this stage during nervous system development high levels of HEK2 mRNA were seen in the subventricular zone of the fourth ventricle, the spinal cord at the midline and also in two lateral columns, in the choroid plexus and also particularly in the eighth nerve ganglia. No expression was seen in the optic nerve or diencephalon, although there were some HEK2 positive cells in the pituitary region. PDGFRα mRNA expression was also seen at this stage in the subventricular zone of the fourth ventricle and in the spinal cord at the midline mainly dorsally (figure 6.6.E). There was no PDGFRα expression in the optic nerve or diencephalon. Therefore there were many similarities between HEK2 and PDGFRα expression, but the pattern was not identical.

6.2.5. HEK2 is expressed predominantly in progenitors of the rodent O-2A lineage

Western blot analysis using HEK2 antiserum showed that the rodent protein homologue of HEK2 was expressed not only in adult rodent brain but more specifically in panned purified rodent optic nerve O-2A progenitors (supplied by Dr. M. Mayer) rather than oligodendrocytes, type-2 astrocytes or purified cortical astrocytes (figure 6.7.). This result differed from that seen in the Hu-O-2A/Gb1 cell line where the highest levels of HEK2 expression were seen in cells treated with VEGF showing a more mature oligodendrocyte phenotype. The explanation for this difference in HEK2
Figure 6.5. Northern blot analysis of HEK2 expression in normal human tissues.

Human foetal, adult brain and adult northern blots are shown. Abbreviations; M.-muscle, N.-nucleus, Ni.-Nigra and Callos.-Callosum. The blots were obtained from Clontech, approximately 2μg of poly A+ RNA was loaded in each lane. The foetal blot contained RNA from 19 to 23 week foetus'. The blots were probed firstly (above) for HEK2 showing a 4.6kb band and secondly (below) with a full length murine GAPDH probe to indicate the amount of RNA. Northern blot analysis showed high levels of HEK2 mRNA in foetal brain, lung and kidney and also a relatively constant level in deep structures of the adult brain. Böhme et al. 1993, had previously reported that HEK2 is predominantly expressed in adult human lung, pancreas, placenta, brain and kidney, with lower levels in the heart, skeletal muscle and liver and this adult blot (right) is shown here only for comparison.
The HEK2 756bp cRNA probe or the PDGFRα 915bp probe (see section 6.2.1.) were labelled with DIG-UTP and visualised using anti-DIG alkaline phosphatase antibody. For orientation of sections see figure 5.9. (A), (C) and (D) were hybridised with the anti-sense HEK2 probe. (E) was hybridised with the anti-sense PDGFRα probe. (B) and (F) were hybridised with the sense-strand RNA probes, HEK2 and PDGFRα respectively, negative controls. (A) a section through rhombencephalon, fourth ventricle is above. HEK2 expression is seen in the sub-ventricular zone of the fourth ventricle and the choroid plexus (c). (B) is a similar section to (A) hybridised with the sense-strand HEK2 RNA probe, a negative control. (C) is a section through the spinal cord, dorsal is above. There was some generalised expression of HEK2 in the midline and the dorsal part of the spinal cord and also in two lateral columns. Expression is seen the dorsal root ganglia. (D) a section through the eighth nerve ganglion shown with an arrow, high HEK2 expression is seen. The ectoderm is on the left. (E) is a section through the spinal cord, dorsal is above. There is high expression of PDGFRα in the dorsal part of the spinal cord at the midline. (F) is a similar section to (E) hybridised with the sense-strand PDGFRα RNA probe, a negative control. Scale bar represents 250μm.
Panned purified postnatal day 7 rodent optic nerve O-2A progenitors were expanded for two weeks in DMEM-BS without triiodothyronine, supplemented by PDGF-AA 10ng/ml/day and NT-3 5ng/ml/day. Progenitors were grown to a density of 5x10^5 cells on 3.5cm diameter PLL coated dishes and then either maintained as progenitors or placed into DMEM-BS for 3 days (oligodendrocytes) or into DMEM+10%FCS for 3 days (type-2 astrocytes). Cortical astrocytes were prepared as in methods section 2.2.1. White matter from the brain of an adult rat was taken as a positive control. Lysis of all cells and tissue was in HEK2 lysis buffer (see sections 2.3.16. and 2.3.20.). Equal amounts of protein were loaded in each lane. HEK2 (a 130 kDa band) is seen in O-2A progenitors and there are very low levels in fully mature oligodendrocytes and type-2 astrocytes but not in cortical astrocytes.
expression between rodent oligodendrocytes and Hu-O-2A/Gb1 cells treated with VEGF may be because Hu-O-2A/Gb1 oligodendrocytes do not reach full maturity, (they express the embryonic form of myelin basic protein and do not form molecular layers, see Chapter 4). This suggestion is supported by the finding that after 1 day in DMEM-BS immature rodent oligodendrocytes still expressed high levels of the rodent homologue of HEK2 (data not shown), unlike fully mature rodent oligodendrocytes which were grown in DMEM-BS for 3 days.

6.3. Discussion

6.3.1. Glioblastoma multiforme tumours and high HEK2 expression

High HEK2 mRNA expression seemed limited to human glioblastoma multiforme derived primary cell populations having strong glial characteristics (those that were GFAP positive, fibronectin negative, process bearing; this included the Hu-O-2A/Gb1 cell line). HEK2 mRNA expression was also found in the three original tumour samples from which the positive cell populations were derived, this might indicate that HEK2 could have an oncogenic role in a subset of gliomas. In addition, in light of the other data presented in this chapter it is possible that HEK2 could be a marker of glioblastomas arising from O-2A lineage cells, but it should be noted that excluding the Hu-O-2A/Gb1 cell line, GFAP was the only glial antigenic marker of these cell populations. Interestingly though, one of the strongly positive HEK2 cell populations was maintained in conventional DMEM+10%FCS and therefore could have lost other antigenic O-2A lineage markers (section 3.3.5.), although the other cell population had always been maintained in ACM. Derivation of further definite O-2A lineage cell
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populations from glioblastoma multiforme samples would help to address the relevance of high HEK2 expression to O-2A lineage glioblastomas.

6.3.2. Neural expression of HEK2 homologues and their relationship to glia

_in situ_ hybridisation studies of various homologues of HEK2 have been performed at certain developmental stages. In the mouse expression between 7.5 and 10.5 developmental days has been examined (Becker et al. 1994). Sek4 is the murine homologue of HEK2 and studies were undertaken using a probe that contained most of the highly conserved catalytic kinase domain and also the 3' untranslated region, although similar results were obtained with a shorter and more specific probe containing mainly the 3' untranslated region. At 7.5 days Sek4 expression was seen in the anterior half of the neuroectoderm. By 8.5 days (4-8 somites) the expression in the neural tube had become more restricted to rhombomeres 2 and 3 in the hindbrain (although the boundaries were not well defined), the ventral midbrain and the ventro-posterior forebrain. By the 10-12 somites stage Sek4 expression was restricted to rhombomeres 3 and 5. There was then down regulation of the expression firstly rhombomere 3 and then rhombomere 5. Between 10.25 and 10.75 developmental days Sek4 expression was not seen in the murine central nervous system.

The Sek4 expression seen in embryonic 7.5-10.5 day mice corresponds in humans to approximately embryonic days 9 to 32 (Kaufman 1992). This is a long time before the stage of human development I studied (day 50) and it would be before glial cells arise, therefore the expression seen could be a marker of neuronal subgroups and would not be helpful in the study of glia. At day 50 it is possible to isolate oligodendrocyte precursors from the embryonic human spinal cord (Aloisi et al. 1992).
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Studies of tyro-6, the rodent homologue of HEK2, were undertaken using a 210bp probe for the highly conserved catalytic kinase domain sequence and therefore cross hybridisation to other family members could not be excluded (Lai and Lemke 1991). Northern analysis in rats using this probe showed abundant expression of tyro-6 in embryonic day 12 brain. This level of expression fell gradually to postnatal day 10 and was relatively constant thereafter even in the adult brain. This is in contrast to findings for PDGFRα where the percentage of positive cells increases to rodent postnatal day 10 and then declines to a lower adult level (Pringle et al. 1992). In situ analysis of tyro-6 in 33 day old and adult male rats showed a restricted expression pattern: a subset of Purkinje cells was positive in the cerebellum and weak signals were seen in the hippocampus. The expression pattern seen for tyro-6 was very similar to that for the highly related tyro-5 gene and therefore cross hybridisation by the tyro-6 probe might have accounted for some of the expression seen, especially as it would be a strong RNA-RNA bond.

In conclusion it does seem likely from the reported studies of HEK2 homologues in different species that HEK2 expression is not limited to the O-2A lineage, but occurs in neuronal subgroups. The findings reported here in a 50 day human embryo of HEK2 expression in the lateral columns of the spinal cord and the eighth nerve nucleus are likely to be a reflection of neuronal expression. Indeed it should also be noted that PDGFRα (an O-2A lineage marker) is expressed in the rodent spinal cord at embryonic days 12 to 14 in presumed interneurones in the dorsal alar plate (Pringle and Richardson 1993). PDGFRα is therefore not uniquely expressed by O-2A lineage cells in the CNS, underlining the difficulty of using a single marker for a particular cell type. Interestingly the finding of a mainly dorsal distribution of PDGFRα in the rodent spinal cord at embryonic days 12 to 14 is similar to the expression seen in the 50 day human embryo studied here (figure 6.6.).
In order to investigate the possible use of HEK2 as a marker for O-2A lineage cells in humans, embryo's older than 50 days need to be examined by \textit{in situ} hybridisation and compared to findings with PDGFR\(\alpha\) and also other possible markers of the O-2A lineage \textit{in vivo}.

\subsection*{6.3.3. HEK2 is found in O-2A progenitors}

The variation of HEK2 expression seen at the mRNA and protein levels and also the variation in HEK2 kinase activity seen in Hu-O-2A/Gb1 cells according to their antigenic phenotype, (down regulation when the astrocytic pathway was favoured), suggested two further possibilities. Firstly, HEK2 may be a differential marker within the O-2A lineage, and secondly this receptor tyrosine kinase may have functional relevance for the O-2A lineage.

In addition it was shown that the protein of the rodent HEK2 homologue was expressed predominantly in O-2A progenitors rather than other glia such as oligodendrocytes, type-2 astrocytes or purified cortical astrocytes. It was also noted that one day old rodent oligodendrocytes maintained high HEK2 expression, thereby resolving the discrepancy of HEK2 expression between the Hu-O-2A/Gb1 oligodendrocytes grown in VEGF and fully mature rodent oligodendrocytes. The very high expression of HEK2 seen in Hu-O-2A/Gb1 cells grown in VEGF having a more mature but not fully mature oligodendrocyte phenotype, would therefore more closely resemble the expression seen in one day old rodent oligodendrocytes rather than 3 day old fully mature oligodendrocytes.

Interestingly the variation of expression for HEK2/tyro-6 in the rodent O-2A system \textit{in vitro} is similar to that previously shown for PDGFR\(\alpha\). PDGFR\(\alpha\)'s are found on O-2A progenitor cells and are initially maintained when these cells differentiate into either oligodendrocytes or type-2 astrocytes (Hart et al. 1989). It is known that an acquired unresponsiveness to
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PDGF determines when a perinatal O-2A progenitor will differentiate rather than decreased availability of PDGF (Hart et al. 1989; Raff et al. 1985). Excess PDGF will not alter the timing of oligodendrocyte differentiation once there is unresponsiveness (Raff et al. 1988). It does not appear that a loss of PDGF receptors causes the unresponsiveness as these receptors are still found on oligodendrocytes and type-2 astrocytes (Hart et al. 1989). Therefore it should be noted with reference to HEK2 that the presence of this receptor does not necessarily mean that it is functional, a point that could be addressed once the physiological ligand for HEK2 is known.

6.3.4. Ligands for the EPH family

Recently the first ligands for the EPH family of receptors have been cloned and shown to be structurally related to a previously identified factor, called B61 (Holzman et al. 1990). B61 binds to the epithelial cell kinase (Eck) receptor (Bartley et al. 1994). Other members of this family of ligands have now been identified (Beckmann et al. 1994; Cheng and Flanagan 1994; Davis et al. 1994; Shao et al. 1994; Shao et al. 1995) they all have 4 conserved cysteine residues. The study of these ligands has proved difficult. To reach half-maximal tyrosine phosphorylation of Eck several hundred ng/ml of B61 were required (Bartley et al. 1994). It was speculated B61 might be more active as a membrane bound form; this has proven to be the case (Davis et al. 1994). The EPH ligands (of which seven are now known, Tessier-Lavigne 1995) appear to fall into two subclasses, those that are transmembrane and have a cytoplasmic domain such as LERK2 (ligand for eph-related kinase 2) (Beckmann et al. 1994), and those that are attached to the cell membrane because of their hydrophobic tail by a glycophosphatidylinositol (GPI) anchor (Holzman et al. 1990). It has been difficult to elicit measurable biological responses from cell lines expressing
an EPH family receptor which were exposed to the appropriate ligand. Recently, however, co-transfection of LERK2 and chimeric receptors containing an Eph-related ectodomain into NIH 3T3 cells induced their transformation (Brambilla et al. 1995). In addition it has been shown that B61, through the Eck receptor, has a role in tumour necrosis factor-α induced angiogenesis of human vein endothelial cells (Pandey et al. 1995).

The data available at present suggests that LERK2, (a transmembrane protein), is the ligand for HEK2 (Brambilla et al. 1995), such investigations though are still in their infancy. Once the physiological ligand for HEK2 is known, not only could investigations of its possible functional relevance for the O-2A lineage *in vitro* be conducted, but also a comparison between HEK2 and its ligand in the developing human nervous system *in vivo* would be possible.
By applying growth conditions developed for studying normal rodent O-2A progenitor cells, (a combination of chemically defined medium and cortical astrocyte conditioned medium), to the primary culture of malignant gliomas, the Hu-O-2A/Gb1 cell line was isolated. This cell line has promising characteristics for the investigation of glioma biology and also possibly the human O-2A lineage.

Hu-O-2A/Gb1 cells, unlike most glioma cell populations established using conventional serum containing medium, do not have a mesenchymal phenotype and fail to express fibronectin. They express antigens characteristically found in rodent O-2A lineage cells and can be manipulated in culture similarly to rodent O-2A lineage progenitors towards either a more astrocytic or a more oligodendrocytic phenotype. Hu-O-2A/Gb1 cells have similar biological responses to PDGF, bFGF or a combination of the two, compared to that seen for rodent O-2A lineage cells. Also like rodent O-2A lineage cells they do not respond to β-interferon, but are sensitive to the presence of CNTF and VEGF.

The finding of a human glioblastoma multiforme which can unambiguously be identified as being of the O-2A lineage may be an important early step in developing a biological lineage based classification of gliomas. This might have important implications for glioma diagnosis (which may be aided by the use of new molecular markers, for example possibly HEK2, see later), prognosis and therapy. Initially, though it is necessary to identify more human gliomas that arise from the O-2A lineage, this would
not only give some indication of their frequency but also allow investigation of their natural history and ascertain if their response to therapy is different from other gliomas. Further identification of O-2A lineage gliomas could be achieved in one of two ways. Firstly, by routinely culturing glioma samples in ACM or secondly, by using $^1$H NMR spectroscopy, (adult gliomas containing the characteristic N-acetyl-aspartate metabolite peak are likely to be of the O-2A lineage).

Despite many similarities to the rodent O-2A lineage, it must be remembered that Hu-O-2A/Gbl cells are transformed and therefore different from normal rodent O-2A lineage cells. Hu-O-2A/Gbl cells are derived from a glioblastoma multiforme and maintain their ability to invade CNS tissue, have cytogenetic abnormalities consist with being from a glioblastoma multiforme and continue to divide, (albeit at a reduced rate), in chemically defined medium without the presence of mitogens. Hu-O-2A/Gbl cells also produce a diffusible astrocytic inducing agent, therefore astrocytic differentiation rather than the oligodendrocytic pathway is favoured in this cell system. This astrocytic inducing agent accounts for the surprising finding that conditions used to aid oligodendrocyte differentiation or maturation, (except VEGF), tended to produce an astrocytic rather than an oligodendrocytic phenotype.

It is noteworthy that cell transformation as seen in the Hu-O-2A/Gbl cell line may have certain advantages or disadvantages for the study of cell biology depending on the context. Firstly, the observation that the immortalising oncogene simian virus 40 large T antigen can lower the requirement of cells for growth factors (Ridley et al. 1988), suggests that a complete repertoire of growth factors may be unnecessary to see response to a particular growth factor. Perhaps the absence of a complete repertoire of growth factors could account for the inability to show division of normal human adult oligodendrocyte precursors by PDGF and bFGF (Armstrong et
al. 1992b; Gogate et al. 1994; Scolding et al. 1995). It could be that a
different combination of growth factors from those used to study rodent O-
2A precursors is necessary for normal human adult oligodendrocyte
precursor division. Secondly, certain precursor cells have been shown to give
rise to other undifferentiated cell types. For example, there is some evidence
to suggest that O-2A progenitor-like cells in the forebrain are derived from
precursor cells that are responsive to PDGF, but which do not label with
markers for the O-2A lineage (Grinspan et al. 1990; Hardy and Reynolds
1991). It is possible that the presence of such undifferentiated precursor-like
cells may account for the finding that some of the Hu-O-2A/Gb1 cells
respond by division to the presence of growth factors (such as PDGF), but
fail to show antigenic markers of the O-2A lineage (Chapter 3). Also it
should be noted that in the rodent O-2A lineage perinatal O-2A progenitors
can give rise to adult O-2A progenitors (Wren et al. 1992) and it may
therefore be possible to allow transition between several different cell types
without terminal differentiation in the presence of immortalising oncogenes.

The relatively easy maintenance of the Hu-O-2A/Gb1 cell line and the
scarcity of quality normal tissue to study the human O-2A lineage means that
this cell line may prove a powerful tool in the study of the molecular
mechanisms involved in the human O-2A lineage allowing the isolation of
potential molecular markers, and there is also the advantage that changes in
antigenic phenotype can be readily recognised. This potential can be seen
from the results of chapters 4, 5 and 6.

The new role discovered for VEGF in oligodendrocyte maturation and
survival in vitro resulted from a molecular strategy to find growth factors of
potential biological relevance to the O-2A lineage by the cloning of growth
factor receptor tyrosine kinases. Confirming the in vivo significance of this
new role for VEGF is complicated by the potent effects of VEGF on vascular
permeability and angiogenesis which would cause secondary effects on a
variety of cell types in the CNS. Clarification of the relevance of this new role for VEGF may come with the isolation of the VEGF receptor on oligodendrocytes, thereby allowing co-localization of antigenic markers for oligodendrocytes and the VEGF receptor mediating the maturation effects in developmental myelinating and also remyelinating states in vivo.

LIMK, a new gene for a protein kinase (chapter 5), was cloned from a cDNA library made from Hu-O-2A/Gbl cells. LIMK is also expressed in rodent O-2A lineage cells (Dr. C. Pröschel, personal communication). The spatial distribution of LIMK from in situ hybridisation studies suggests that it is not specific for the O-2A lineage in the CNS, but expression of this protein could still be functionally relevant to this lineage. Furthermore the high expression levels of LIMK in primary human malignant glioma cell populations and original glioblastoma multiforme tumour samples suggests it may have relevance for gliomas, (and possibly other CNS neoplasms, as it was also found in meningioma cell populations). Further in vivo and in vitro investigations of LIMK expression in different grades of glioma may provide useful information. An important step will be studies of LIMK function, hopefully leading to the discovery of new mechanisms which are active in the O-2A lineage as well as gliomas.

HEK2, (chapter 6), was a receptor tyrosine kinase found during the initial cloning of receptor tyrosine kinase fragments from Hu-O-2A/Gb1 cells. It seems to have a more restricted expression pattern than LIMK. High HEK2 expression was only found in human malignant glioma cell populations which were strongly GFAP positive and had a process bearing morphology. Could high HEK2 expression be limited to glioblastomas arising from O-2A lineage cells? Apart from Hu-O-2A/Gb1 cells, the 2 cell populations that showed high HEK2 expression might not have had O-2A antigenic markers because, either they arose from cells in the astrocytic pathway of the O-2A lineage, or the culture conditions were not optimal to
allow full expression of an O-2A phenotype, (one cell population was cultured in the traditional DMEM+10%FCS medium). The possibility that high HEK2 expression might be limited to glioblastoma multiforme tumours arising from O-2A lineage cells remains speculative at present, until further gliomas of the O-2A lineage can be unambiguously identified and their HEK2 expression assessed.

The discrepancy seen between the relative levels of HEK2 expression of Hu-O-2A/Gbl cells and rodent O-2A lineage cells manipulated down the oligodendrocytic pathway as compared to cells maintained as progenitors, agrees with other differences between of Hu-O-2A/Gbl oligodendrocytes and fully mature rodent oligodendrocytes. Although more mature Hu-O-2A/Gbl oligodendrocytes, (high HEK2 expression), produce myelin basic protein this is not a mature form, neither do they produce a molecular layer (Chapter 4), nor do they lose the N-acetyl-aspartate peak on 1H NMR spectroscopy (Dr. K. Bhakoo, personal communication), all of which are features of fully mature normal rodent oligodendrocytes, (low HEK2 expression). This suggests that as the Hu-O-2A/Gbl cell line is transformed, full maturation of Hu-O-2A/Gbl oligodendrocytes is inhibited, and therefore high expression of HEK2 remains, as seen in immature rodent oligodendrocytes.

Once the physiological ligand for HEK2 is discovered, the biological relevance of the presence of the HEK2 receptor on O-2A lineage cells and gliomas could be addressed.

The finding of high levels of HEK2 expression in rodent O-2A progenitors rather than their fully differentiated progeny or cortical astrocytes may also apply to normal human O-2A lineage cells. This might allow HEK2 to be used as a marker of O-2A progenitors, but probably only in combination with other markers such as PDGFRα, DM20 or MyT1 because in situ hybridisation studies suggest that HEK2 expression is also seen in selected neuronal populations. Knowledge of human O-2A progenitor-like
cells is restricted at present. Cells expressing PDGFRα have been found on human CNS sections by *in situ* hybridisation using a rodent PDGFRα probe (Gogate et al. 1994), but surprisingly no biological response to PDGF has been demonstrated *in vitro*. This could indicate a difference from rodent O-2A progenitors or perhaps these human cells are not 'true' O-2A progenitors and have already differentiated so far along the oligodendrocyte pathway that they no longer respond to PDGF but still maintain the PDGFRα, as observed in the rodent system (Hart et al. 1989; Raff et al. 1985). Although MyT1, (another O-2A progenitor marker), was found to label similar cells to those labelling with PDGFRα in CNS sections of human tissue (Gogate et al. 1994). It should be remembered though that MyT1 like all other O-2A progenitor markers is not completely specific, it is also expressed in neuronal subgroups (Armstrong et al. 1995). This underlines the difficulty of using a single marker for O-2A progenitor cells *in vivo*, a point demonstrated further by the controversy surrounding GD3 positive cells *in vivo* (Wolswijk 1995), which appear to label both macroglial precursors and microglia. Therefore several markers of human O-2A progenitor-like cells, (or other O-2A lineage cell types), are needed to identify them unambiguously *in vivo*. The expression of such markers during the stages of division, differentiation and maturation in the O-2A lineage need to be investigated by using *in vitro* techniques. It should also be recognised that although there may be many similarities to the rodent O-2A lineage, due to evolution, the mechanisms governing the human O-2A lineage are likely to be more complex.

Because of the complex nature of the human CNS and the multiple growth factors and cytokines and extracellular matrices that are available to cells during development, maintenance and in disease, there remains a role for *in vitro* studies in the identification of responses of well characterized cell types to defined molecules under controlled conditions.
The importance of the Hu-O-2A/Gb1 cell line as a research resource for studies of the human O-2A lineage lies primarily in the identification of new molecules of potential relevance to the O-2A lineage. The large quantities of Hu-O-2A/Gb1 cells that are needed for molecular studies can be cultured with relative ease and also the biological properties of Hu-O-2A/Gb1 cells can be analysed on the basis of many known similarities to the rodent O-2A lineage. It is important to note though that these *in vitro* experiments may demonstrate growth factor requirements, susceptibility, developmental, regenerative and oncogenic capacities, that are not active or utilized in the *in vivo* cellular environment. It will only be by a combination of both *in vivo* and *in vitro* approaches, however, that the full role of the human O-2A lineage in development, maintenance, repair and oncogenesis of the human CNS will be discovered.

In conclusion, the properties of the Hu-O-2A/Gb1 cell line suggest many exciting avenues for further research in the human O-2A lineage and in the study of gliomas.
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