Molecular mechanism of CD98 function

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Abstract

CD98 is expressed on both haematopoietic and non-haematopoietic cells and has been implicated in a variety of different functional role in cell physiology and immunobiology such as amino acid transport, oncogenic transformation and cell adhesion and fusion. Little is known about molecular mechanism of CD98 function, including interaction with other molecules and signalling pathways induced by CD98 activation.

The first study examined the functional interactions between CD98 and other adhesion molecules such as CD29 and CD147 on the surface of the promonocyte line U937, using a quantitative assay of cell aggregation and blocking monoclonal antibodies to adhesion molecules. Two antibodies to CD147, an immunoglobulin superfamily member whose function has remained unclear, were also potent inhibitors of the aggregation induced via the ligation of both CD98 and CD29. CD98 blocking mAbs also diminished CD29-induced homotypic aggregation. The results suggest that CD98, CD29 and CD147 are functionally associated within a multi-molecular unit which regulates cell aggregation.

To dissect signalling pathways induced by CD98 activation, CD98-mediated U937 homotypic aggregation was further carefully evaluated with pharmacological and biochemical approaches. CD98 ligation induced a selective membrane translocation of the novel PKCδ isoform, as an essential step in mediating U937 homotypic aggregation. CD98-induced PKCδ activation mediated activation of MAPK, ERK1/2 and p38, as
well as tyrosine phosphorylation. Collectively, CD98 activation may be selectively
linked to PKCδ activation.

The CD98-induced aggregation was negatively regulated by PMA-responsive PKC
isoforms because PKC inhibitors and activators enhanced or inhibited CD98-induced
intercellular adhesion. PMA also induced the translocation of PKC α, β, and γ,
suggesting that the PMA-responsive PKC isoforms were conventional isoforms of PKC.
Together, these data provide strong evidence that PMA-responsive conventional PKC
may play a key role in the negative regulation in CD98-induced signalling and
homotypic aggregation.
To my parents, with love
Acknowledgements

I gratefully acknowledge the contributions of many people to the completion of this project.

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Abbreviations

aa : amino acids
APC : antigen presenting cells
BCH : 2-(-)-endoamino-bicycloheptane-2-carboxylic acid
cPKC : conventional PKC
Cys : cysteine
DAG : diacylglycerol
DC : dendritic cells
DOG : dioctanolyglycerol
DONS : dominant negative suppression
DTT : dithiothreitol
ERK1/2 : extracellular signal-regulated kinases 1 and 2
FAK : focal adhesion kinase
FCS : fetal calf serum
FITC : fluorescein isothiocyanate
FRP : fusion regulatory protein
h : hour
HC : heavy chain
hCD98 : human CD98
HIV : human immunodeficiency virus
hsp70 : heat shock protein 70
ICAM-1 : intercellular adhesion molecule-1
kD : kilodalton
Chapter 1

General introduction

CD98 is a central component within a multi-molecular complex
Abstract

CD98 is a heterodimeric glycoprotein composed of a 85 kD glycosylated heavy chain (CD98HC) and a 35 kD non-glycosylated light chain (CD98LC). CD98 activation is linked to an extraordinary diversity of normal biological functions, such as transport of amino acids or Na⁺/Ca²⁺, T cell costimulation, cell aggregation and fusion, and cell differentiation, survival or death. These effects are mediated by various CD98-induced signalling pathways, which involve tyrosine and serine/threonine phosphorylation, and changes in actin cytoskeleton, upon the formation of a molecular complex including integrins and CD147. The impairment of CD98 function is associated with several diseases, including lysinuric protein intolerance and oncogenic transformation. This chapter will summarise and review publications which address the role of function of CD98 in terms of biochemical, molecular biological, immunobiological and pathological aspects.
1.1 Introduction

Human CD98 is a type II transmembrane glycoprotein that is extensively expressed on most tissues, and on both normal and tumor cells. The protein is composed of two chains (Fig. 1.1); a glycosylated heavy chain (CD98HC, 529 amino-acid (aa), 85 kD) (Hemler and strominger, 1982; Teixeira et al., 1987; Quackenbush et al., 1987) and a non-glycosylated light chain (CD98LC, 507 aa, 40 kD) (Tsurudome et al., 1999; Mastroberardino et al., 1998). The heavy chain is known both to control membrane trafficking of the light chain and to maintain the membrane topology of the heterodimer (Nakamura et al., 1999). The light chain is reported to function as an amino acid transporter (Nakamura et al., 1999; Segawa et al., 1999). These two proteins are covalently linked by structurally important disulfide bond between cysteines from the heavy and light chains on the membrane surface (Kubota et al., 1994).

To date, the physiological role of CD98 remains unclear, but CD98 has been implicated in several different cellular functions, including involvement as an amino acid or ion transporter (Michalak et al., 1986; Segawa et al., 1999; Nakamura et al., 1999; Kanai et al., 1998), controlling cell growth, survival, differentiation and oncogenic transformation (Hara et al., 1999; Hara et al., 2000; Shishido et al., 2000b; Papetti and Herman, 2001; Warren et al., 1996), functioning as an adhesion molecule to regulate cell-to-cell adhesion (Warren et al., 1996; Ohgimoto et al., 1995, 1996; Tabata et al., 1994), and induction of T cell proliferation and activation as a costimulatory molecule (Woodhead et al., 2000; Stonehouse et al., 1999; Diaz et al., 1997b; Friedman et al., 1994). Because the wide variety of cellular functions of CD98 cannot be simply explained by its role in amino acid transport, this suggests that the molecule may
Fig. 1.1 Schematic representation of heterodimers formed by LCs and CD98HC or rBAT (Verrey et al., 1999).
participate in a variety of cellular activities in many different guises as suggested previously (Helmer et al., 1982).

This chapter reviews our knowledge about CD98, including its structure and its biological functions. It specifically addresses several unresolved questions about CD98 such as how can CD98 have such an extraordinary diversity of biological functions, what is the relationship between β1 integrins (CD29) and CD98 as one of the integrin function-regulating proteins, is amino acid transport (light chain) functionally linked to the other biological activities mediated by CD98HC, how is CD98 activated and what enzymes and associated proteins are involved in CD98-induced signaling pathways?

1.2 Other names of CD98: 4F2 or FRP-1

CD98 was originally identified as an activation antigen of lymphocytes, the 4F2 antigen (Haynes et al., 1981). Recently, it has also been shown that fusion regulatory protein (FRP)-1, which is a newly defined cell surface molecule, is identical to CD98/4F2 by biochemical data such as N-terminal amino acid sequences, molecular mass and covalent modification (Ohgimoto et al., 1995). Therefore, three names (CD98/4F2/FRP-1) are now simultaneously cited in CD98-related works (in this thesis, however, CD98 will be used as a general name).

1.3 Structure of CD98

As shown in Fig. 1.1, CD98 is a heterodimeric protein of approximately 125 kD which is composed of a glycosylated heavy chain (CD98HC, 85 kD) and a non-glycosylated light chain (CD98LC, 40 kD). These two proteins are covalently linked by a disulfide
bond to maintain a stable membrane bound form. Specific monoclonal antibodies (mAbs) produced by immunisation with the stable heterodimer mostly bind to the CD98HC, suggesting that the glycosylated heavy chain could be more structurally immunogenic than the light chain.

The cloning of the genes for CD98HC and LC allowed predictions of possible structures and the membrane topology of CD98 (Kanai et al., 1998; Mastroberardino et al., 1997; Rossier et al., 1999; Markovich and Repeer, 1999; Tsurudome et al., 1999; Broer et al., 1997; Nakamura et al., 1999; Kanai et al., 1998; Segawa et al., 1999). Currently, there are no studies of the structural properties of CD98, such as a crystal structure or any data on structural regulation of CD98 function. Predictive amino acid sequences have, however, suggested sites of potential modifications such as phosphorylation or glycosylation. N-linked glycosylation plays an important role in the cyclical appearance of CD98 and its association with 45-kD light chain because tunicamycin (a glycosylation inhibitor)-treated cells do not displayed a 125 kD heterodimer but only CD98HC of 85 kD (Papetti and Herman, 2001).

The heterodimeric structure of CD98 and its characteristics are analogous to the structural regulation of the Na\(^+\),K\(^+\)-ATPase (Verry et al., 1999). This ion-exchange pump is composed of two subunits, a catalytic multitransmembrane subunit (α-chain) and a type II glycoprotein (β-subunit). Thus, CD98HC and the β-subunit of Na\(^+\),K\(^+\)-ATPase seem to be required for stabilisation of the complex, its maturation and to allow transport of CD98LC or the α subunit of the ATPase from the endoplasmic reticulum to the plasma membrane (Glitscher and Ruppel, 1991; Verry et al., 1999). A similar
situation also exists for rBAT (Fig. 1.1), a surface glycoprotein, which is able to covalently associate with b\textsuperscript{0+} type amino acid transporters (Tsurudome and Ito, 2000; Verry et al., 1999).

### 1.3.1 Heavy chain (CD98HC)

The heavy chain of human CD98 was first cloned from a genomic \(\lambda\) library by Teixeira et al (1987) and the murine form was cloned soon after (Parmacek et al., 1988). The cDNA was about 1.8 kb in length including a poly (A) tail and open reading frame, and encoding 529 amino acids (aa) with a 81-aa cytoplasmic domain and a 425-aa ectodomain, as summarised in Table 1.1.

Even though the crystal structure of CD98HC is currently unknown, a number of structural features have been predicted, based on the DNA sequences and hydropathicity analyses. These include an internal transmembrane spanning region, 4 (human) or 7 (murine) potential glycosylation sites in the C-terminal extracellular membrane domain, and a N-terminal cytoplasmic domain having about 81 amino acids (Warren et al., 1996; Parmacek et al., 1989; Lumadue et al., 1987; Quackenbush et al., 1987; Teixeira et al., 1987) The CD98HC has two structurally important cysteine residues at residues 109 and 330. Cys 109 forms a disulfide bond linking CD98HC to the light chain (Teixeira et al., 1987; Pfeiffer et al., 1998). The highly conserved nature of the Cys 109 in many different species confirms its importance for structural stability. Cys 330 is supposed to be involved in forming a homodimer between heavy chains (Ohta et al., 1994; Papetti and Herman, 2001) and particularly for stabilising the overall structural conformation (Shishido et al., 2000b).
### Table 1.1 Summary of heavy chain of CD98.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Teixeire et al., 1987</th>
<th>Quackenbush et al., 1987</th>
<th>Ludadue et al., 1987</th>
<th>Parmacek et al., 1989</th>
<th>Warren et al., 1996</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Origin</strong></td>
<td>human</td>
<td>human</td>
<td>human</td>
<td>murine</td>
<td>murine</td>
</tr>
<tr>
<td><strong>mRNA size</strong></td>
<td>2.1 kb</td>
<td>1.854 kb</td>
<td>1.8 kb</td>
<td>1.8 kb</td>
<td>1.8 kb</td>
</tr>
<tr>
<td><strong>Number of AA</strong></td>
<td>529</td>
<td>495 - 526</td>
<td>529</td>
<td>526</td>
<td>533</td>
</tr>
<tr>
<td><strong>M.W. (estimated)</strong></td>
<td>58 kD</td>
<td>58 kD</td>
<td></td>
<td></td>
<td>58.817 kD</td>
</tr>
<tr>
<td><strong>NH$_2$-terminal signal peptide</strong></td>
<td>lacks</td>
<td></td>
<td></td>
<td></td>
<td>lacks</td>
</tr>
<tr>
<td><strong>Cys for disulfide bond</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cys 109</td>
</tr>
<tr>
<td><strong>Transmembrane-spanning region</strong></td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Potential glycosylation sites</strong></td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td>7</td>
</tr>
<tr>
<td><strong>Cytoplasmic N-terminal AA size</strong></td>
<td>81</td>
<td>50-81</td>
<td></td>
<td>75</td>
<td></td>
</tr>
<tr>
<td><strong>Tissue distribution</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>high level: testis, lung, brain kidney, spleen low level: liver, cardiac muscle</td>
</tr>
<tr>
<td><strong>Remarks</strong></td>
<td></td>
<td></td>
<td></td>
<td>pi : 5.55</td>
<td></td>
</tr>
</tbody>
</table>

Interestingly, a comparison of the human and murine heavy chain cDNA sequences indicates a marked level of homology with regard to the general structure, and amino acid sequences (Parmacek et al., 1989; Warren et al., 1996). Thus, 77.5% nucleotide sequence and almost 80% amino acid sequence are identical between murine and human, suggesting that the protein has an important and conserved function in the normal physiology of cells or tissues. In particular, the highest levels of identity between two species were observed within the intracytoplasmic (86% identity at the amino acid level) and the transmembrane (100% identity at the amino acid level) regions (Parmacek et al., 1989), indicating the structural importance of these domains.

cDNA sequence analysis (Warren et al. (1996)) also identified potential modification sites of CD98. These are two potential cAMP/cGMP-kinase phosphorylation (R/T-Xaa-S/T) sites, eight potential protein kinase C (PKC) phosphorylation (S/T-Xa-R/K), and eight potential Casein kinase II phosphorylation (S/T-Xaa-D/E) sites. However, there are no consensus sites for tyrosine phosphorylation, nor are there protein tyrosine kinase (PTK) or other catalytic motifs in the murine heavy chain (mCD98HC) cDNA.

Intriguingly, the large COOH-terminal extracellular domains of the CD98HC are similar to those of glycosidases, which show a globular tertiary structure (Wells and Hediger, 1992). Catalytic activity has not been shown yet, and it remains unclear whether this function may or may not be related to amino acid transport.

CD98HC has an important role in maintaining overall structure of CD98 complex. The CD98HC transports the light chain from the Golgi apparatus to the plasma membrane.
Unlike CD98LC, which remained intracellular when expressed alone, CD98HC reaches the plasma membrane in the absence of light chain (Mastroberardino et al., 1998; Teixeira et al., 1987). However, it is still an open question as to whether the heavy chain has an impact on the amino acid transport specificity and/or kinetics of the hetero-oligomer transporters or whether it only fulfills a chaperone function (Tsurudome and Ito, 2000; Verrey et al., 1999).

The human heavy chain (hCD98HC) gene is located on the long arm of chromosome 11 (Francke et al., 1983), and is composed of nine exons and spans approximately 8 kb. The gene also includes a housekeeping promoter which is G+C rich and four potential binding sites for the ubiquitous Sp1 transcription factor in 5' end of the gene (Gottesdiener et al., 1988). The regulation of CD98 gene expression is controlled by major regulatory elements, locating on exon 1-intron 1 region, and which act through blocking transcriptional elongation (Leiden et al., 1989). An active and powerful enhancer has been reported in the first intron from some cells including malignant T cells (Karpinski et al., 1989). This enhancement is regulated by binding with two novel nuclear proteins (NF-4FA and NF-4FB), which flank a consensus binding sites for the AP-1 transcription factor (Karpinski et al., 1989).

1.3.2 Light chain (CD98LC)

Although the early biochemical studies had revealed that CD98 is a heterodimer containing two chains, there was little evidence on the nature of the light chain before 1997. One year later, however, Mastroberadino and coworkers (1998) found indirect evidence that the permease-related protein E16, functioning in amino acid transport,
was able to covalently associate with CD98HC when this chain was co-expressed with various amino acid transporters in Xenopus oocytes. Recently, biochemical approaches have directly verified that the CD98HC-associated light chain is a glycoprotein-associated amino acid transporter (Tsurudome et al. 1999). To do this, CD98LC was co-purified with CD98HC from HeLa S3 cells, and a partial N-terminal sequence was obtained. Using this sequence, a full length cDNA clone of CD98LC was cloned from a cDNA library, and shown to be identical to the amino acid transporter hLAT1/E16.

The hLAT1/E16 gene locates on the long arm of chromosome 16 (16q24.3) (Tsurudome et al., 1999). A related human LAT-2 gene was found at chromosome 14q11.2-13 (13 cR or approximately 286 kb from marker D14S1349) (Pineda M et al., 1999). The human y+LAT-1 gene localizes to chromosome 14q11.2 (17cR approximately 374 kb from D14S1350) (Torrents et al., 1998), within the lysinuric protein intolerance (LPI) locus (Lauteala, et al., 1997).

Since the first finding of CD98LC, 6 kinds of light chains (Table 1.2), called (i) CD98-lc1 (LAT1: E16, TA1, AmAT-L-lc, ASUR4) (Kanai et al., 1998; Mannion et al., 1998; Nakamura et al., 1999; Mastroberadino et al., 1998), (ii) CD98-lc2 (y+LAT1) (Torrents et al., 1998), (iii) CD98-lc3 (y+LAT2) (Pfeiffer et al., 1999), (iv) CD98-lc4 (xCT) (Sato et al., 1999), (v) CD98-lc5 (LAT2) (Pineda et al., 1999; Rossier et al., 1999; Segawa et al., 1999) and (vi) CD98-lc6 (Rajan et al., 1999) have been reported. According to Na+ dependency and substrate specificity, these light chains are divided into 4 types (Verrey et al., 1999); (i) LAT1 and LAT2 (specific for system L), (ii) y+LAT1 and y+LAT2 (specific for system y+L), (iii) xCT (specific for system Xc-) and (iv) CD98-lc6
Table 1.2 Summary of light chain of CD98 (I).

<table>
<thead>
<tr>
<th>Reference</th>
<th>Mastroberadino et al., 1998</th>
<th>Torrents et al., 1998</th>
<th>Pfeiffer et al., 1999</th>
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<tr>
<td>Name</td>
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<td>y+LAT1 (CD98-lc2)</td>
<td>y+LAT2 (CD98-lc3)</td>
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<td></td>
<td></td>
<td></td>
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<tr>
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<td>system L</td>
<td>system y+L</td>
<td>system y+L</td>
</tr>
<tr>
<td>- AA transport (Na+ dependency)</td>
<td>large,neutral (-)</td>
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<td>cationic; large neutral(+)</td>
</tr>
<tr>
<td>- Representative AA</td>
<td>leucine</td>
<td>arginine; leucine</td>
<td>arginine; leucine</td>
</tr>
<tr>
<td>Biochemical properties</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Cloned sites</td>
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<td>(human)</td>
<td></td>
</tr>
<tr>
<td>- AA size</td>
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<td>511</td>
<td>515</td>
</tr>
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</tr>
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<td>16q24.3</td>
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<td>Cys 151</td>
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<td></td>
</tr>
<tr>
<td>- Phosphorylation sites</td>
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<td>1</td>
<td></td>
</tr>
<tr>
<td>- for PKC-dependent</td>
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</tr>
<tr>
<td>- for cAMP-, cGMP-dependent PK</td>
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<tr>
<td>- for casein kinase II-dependent</td>
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</tr>
<tr>
<td>- Sensitivity to</td>
<td></td>
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</tr>
<tr>
<td>- BCH</td>
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<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>- MeAIB</td>
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<td>NO</td>
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<tr>
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<td>a candidate for lysinuric protein intolerance</td>
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**Table 1.2 Summary of light chain of CD98 (II).**

<table>
<thead>
<tr>
<th>Reference</th>
<th>Sato et al., 1999</th>
<th>Pineda et al., 1999</th>
<th>Rajan et al., 1999</th>
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</thead>
<tbody>
<tr>
<td><strong>Name</strong></td>
<td>xCT (CD98-lc4)</td>
<td>LAT2 (CD98-lc5)</td>
<td>CD98-lc6</td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
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<td>system Xc^-</td>
<td>system L</td>
<td>system b^+</td>
</tr>
<tr>
<td>- AA transport (Na^+ dependency)</td>
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<td>small, large zwitterionic(-)</td>
<td>cationic (-); neutral (-); cystine (-)</td>
</tr>
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<td>arginine; alanine</td>
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<tr>
<td><strong>Biochemical properties</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>- Tissue distribution (high level)</td>
<td>activated macrophage; brain</td>
<td>kidney; small intestine</td>
<td>kidney; small intestine</td>
</tr>
<tr>
<td>- Chromosomal location</td>
<td>14q11.2-13</td>
<td>Cys 154</td>
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</tr>
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<td>- Cys for disulfide bond</td>
<td>Cys 158</td>
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<td><strong>Pharmacological properties</strong></td>
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<tr>
<td>- Sensitivity to</td>
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<tr>
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<td>Yes</td>
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<tr>
<td><strong>Pathophysiology</strong></td>
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</tr>
<tr>
<td></td>
<td>a possible candidate for cystinuria</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(specific for b_{o}^{+}-like amino acid transport system which is specific to rBAT). A defect in the y^{+}LAT1 gene has recently been shown to be responsible for the genetic disorder lysinuric protein intolerance (Rajan et al., 1999) (see Section 1.7.1.1) (Gitomer and Pak, 1996; Pierides, 1997). Type I Cystinuria has been shown to be mediated by a mutation in the gene encoding rBAT (Calonge et al., 1994; Chairoungdua et al., 1999; Chillaron et al., 1997).

Table 1.2 shows that these light chains have several common characteristics. All are predicted on the basis of hydrophobicity plots to have 12 membrane-spanning regions (unlike the heavy chain which has only one hydrophobic region) and a highly conserved L-cysteine (164 Cys) which locates on the second extracellular loop between the putative third and fourth TM domains (Pfeiffer et al., 1998). The light chain is therefore a membrane-bound protein, and has structural similarity to other transport proteins. The conserved Cys 164 residue of CD98LC is believed to take part in disulfide bond formation with the heavy chain (Rajan et al., 1999; Mastroberardino et al., 1998; Sato et al., 1999; Kanai et al., 1998; Nakamura et al., 1999). In addition, CD98LCs possess a conserved central hydrophobic domain (418-432 amino acids without cytoplasmic tails) and variable NH\textsubscript{2}- and COOH-terminal residues, respectively (Verrey et al., 2000). By pairwise comparison, the minimal level of identity between the different CD98LCs is more than 40%. 21.1% of amino acids are identical in all CD98LC sequences and 82.9% are similar (conservative substitutions), out of the 454 residues which are always present (Verrey et al., 2000).

A prediction from cDNA sequences is that CD98-lc6 has one potential site for N-linked
glycosylation in the extracellular loop between transmembrane domains 7 and 8, three potential sites for PKC-dependent phosphorylation and one potential site for cAMP- and cGMP-dependent protein kinase phosphorylation in the intracellular domain (Rajan et al., 1999).

1.4 Regulation of CD98 expression

CD98 expression is potentiated by some mitogens such as lectin, alloantigens (Haynes et al., 1981; Yagita and Hashimoto, 1986), lipopolysaccharide (LPS) and phorbol 12-myristate 13-actate (PMA)/calcium ionophores (Tanaka et al., 1989) in T cells. A low level of CD98HC expression in resting T cells is markedly up-regulated upon activation, reaching a maximum level within 6 to 12 hours. The maximal expression of CD98HC is shown at the late G1 phase and the levels are maintained throughout the rest of cell cycle before the expression of interleukin (IL)-2 and transferrin receptor (Helmer et al., 1982; Haynes et al., 1981; Yagita et al., 1986). Mouse mRNA of CD98HC is also shown to be very low in resting mouse spleen cells. However, phorbol-12-myristate-13-acetate (PMA) strongly stimulates the transcription of the gene up to 60 fold (Teixeira and Kuhn, 1991). Furthermore, it has been reported that the expression of the fourth amino acid transporter of CD98, mouse xCT, is greatly enhanced when mouse macrophages are stimulated by LPS (Sato et al., 1999). This massive increase of CD98 mRNA by activation stimuli is mediated by a transcriptional enhancer (Karpinski et al., 1989).

1.5 Tissue distribution

The tissue distribution pattern is more or less consistent with an understanding of the
functional role of CD98. Thus, CD98HC is highly expressed in tissues related to proliferation, peptide or protein secretion, and cell fusion such as hair follicle epithelium, skeletal muscle sarcolemma, stomach surface and glandular epithelia, pancreatic islets, kidney proximal tubules, parathyroid gland, follicular epithelium, testicular seminiferous tubules and ova in the ovary (Tabata et al., 1995; Azzarone et al., 1984) (Table 1.1 and 1.2). Other cells such as peripheral blood monocytes, alveolar macrophages and some bone marrow stem cells are also reported to exhibit higher surface level of CD98HC (Azzarone et al., 1984; Woodhead et al., 1998).

The tissue distribution of murine CD98HC showed a different pattern from human. The mCD98HC is highly expressed in brain, kidney, lung, spleen and testis, but a lower level of CD98HC was found in heart, skeletal muscle, thymus, pancreas and liver. The mCD98HC is also expressed on thymocytes, splenocytes, peripheral lymphocytes and blood monocytes (Parmacek et al., 1989). Another report similarly demonstrated that mCD98HC is highly expressed in adult testis, lung, brain, kidney, and spleen, and is expressed at significantly lower levels in adult liver and cardiac and skeletal muscle (Tsumura et al., 1999).

hCD98HC expression is linked to the maturation of dendritic cells (DC) derived from 2-hr adherent peripheral blood monocytes, and cultured for 7 days with granulocyte-macrophage colony-stimulating factor and IL-4. The kinetics of monocyte to DC transition revealed a rapid activation phase within the first 24 hr, with a considerable increase in expression of CD98; this was followed by a down-regulation of CD14 and a more gradual development of the other dendritic cell features over the remaining 6 days.
Thus, the report suggests that CD98HC may regulate DC maturation or participate in DC function at an early transition stage.

Warren et al (1996) also showed that mCD98HC is expressed on haematopoietic stem cells, T- and B-cell precursors, and a subset of myeloid and erythroid-lineage cells. mCD98 is strongly detected in 70 to 90% of haematopoietic cells in murine yolk sac at day 8/8.5 gestation and 100% of mononuclear cells from fetal liver at day 12 of gestation. Furthermore, CD98 is also expressed in more than 90% of thymocytes at days 13, 14 and 15 of gestation.

The tissue distribution of human CD98LC has also been evaluated by Northern hybridization analysis. Although it is controversial whether or not normal tissues including brain, lung, liver, kidney, colon and stomach do express hCD98LC mRNA (Gaugitsch et al., 1992; Wolf et al., 1996), all colorectal carcinoma and adenocarcinomas from breast, endometrium, salivary gland and esophagus have a strong detectable level of hCD98LC mRNA (Prasad et al., 1999). A weak but easily detectable signal was found in other normal tissues, such as heart, colon, thymus, spleen, kidney, liver, lung and leukocytes (Prasad et al., 1999). There was no detectable level in retina and thymus (Prasad et al., 1999). Leucocytes or quiescent peripheral blood lymphocytes displayed lower level of CD98LC transcripts or were negative, whereas stimulation with PMA/ionomycin clearly increased mRNA expression (Gaugitsch et al., 1992).

Northern blot analysis of the distribution of rat and mouse CD98LC also suggests that
neoplastic tissues and tissues which undergo extensive proliferation possess abundant expression of mCD98LC. Some other tissues, such as brain also express mCD98LC mRNA (Kanai et al., 1998; Sang et al., 1995). However, normal liver does not express mCD98LC, whereas fetal liver evidently expresses mCD98LC (Nakamura et al., 1994).

Abnormal level of CD98 in some diseases is consistent with this idea that CD98 could contribute to the functional impairment of lymphocytes. The diseases includes Wiskott-Aldrich syndrome (Gerwin et al., 1996), ulcerative colitis (UC) (Yacyshyn, 1993), rheumatoid arthritis (Hoy et al., 1993), chronic infection with Leishmania major (Rosat et al., 1994), chronic hepatitis type B (Garcia-Monzon et al., 1992) and active systemic lupus erythematosus (Alcocer-Varela et al., 1991). In these diseases, B or T lymphocytes from peripheral blood, UC lamina propria or synovial fluid showed relatively increased or significantly decreased expression level of CD98 as well as other activation markers.

1.6 Ligand for CD98HC

One valuable approach to understand the extraordinary diversity of functional roles of CD98 is to identify possible ligands for CD98. To date, galectin-3 is regarded as the only identified CD98 ligand (Dong and Hughes 1996). Galectin-3 is a carbohydrate-binding protein of approximately 30 kD expressed on the surface of inflammatory macrophages and several macrophage cell lines. Addition of galectin-3 to Jurkat cells induced a CD98-mediated sustained influx of extracellular Ca\(^{2+}\) by a voltage-gated Ca\(^{2+}\) channel-independent manner, suggesting that galectin-3 released by accessory cells such as macrophages may bind to CD98 and participate in Ca\(^{2+}\) signalling (Dong and Hughes,
However, it is not clear yet whether galectin-3 is able to induce other CD98-mediated biological activities. Several groups are now searching for other possible ligands for CD98.

1.7 The function of CD98 in physiological and pathological processes

Presently, the function of CD98 under normal physiological conditions remain largely unknown, but its surface abundance and the high degree of cross-species conservation in some domains of the protein (Parmacek et al., 1989) are suggestive of an important role. Experimentally, CD98 has been shown to regulate amino acid transport, T cell costimulation, cell aggregation and adhesion, and cell death, survival and differentiation (Fig. 1.2), although the exact mechanisms remain unknown (see Tsurudome and Ito, 2000; Deves and Boyd, 2000; Diaz and Fox, 1998 for review).

1.7.1 Involvement in transport systems

Amino acid and metal ions play a major role in many aspects of cellular metabolism and metabolic communication, such as protein synthesis, ATP formation and activation of cell signalling, between cells and tissues. Essential to these roles is their rapid transport across the plasma membrane, which is catalysed in part by the recently identified family of CD98LC upon cooperation with CD98HC (Verrey et al., 1999). Although the understanding of the functional relationship between CD98HC and LC is still deficient, molecular cooperation between two chains seems to be necessary to carry out the functional role of CD98 in these cellular events.
Fig. 1.2 CD98-mediated regulation of cellular function.
1.7.1.1 Amino acid transport

Since it was first reported that CD98HC has structural and functional similarities to rBAT, which is a surface membrane glycoprotein bound to a cysteine transporter (Bertran et al., 1992; Wells and Hediger, 1992), a possible function of CD98 has been proposed to be amino acid transport. For example, CD98 and rBAT display 30% overall amino acid sequence identity with 52% sequence similarity, and the cysteine residue at position 109 is highly conserved in CD98HC as well as rBAT (Bertran et al., 1992; Wells and Hediger, 1992). Direct evidence was obtained by showing that CD98HC cRNA injected into Xenopus oocytes stimulated transport of dibasic amino acids and neutral amino acids in a sodium-independent manner (Bertran et al., 1992; Wells and Hediger, 1992; Kanai et al., 1992). However, it was unlikely that CD98HC itself had transporter properties, because of its structure, as compared to other known amino acid transporters. CD98HC is a simple type II membrane glycoprotein in which there is no evidence of multiple membrane-spanning regions which are necessary to carry out transport function (Verrey et al., 1999). This suggests that CD98HC regulates amino acid transport, indirectly. This prediction was later confirmed by the evidence that CD98HC undergoes molecular association with one of six amino acid transporters, possessing multiple membrane-spanning regions (Verry et al., 1999).

As mentioned above, 6 different amino acid transporters have been reported to associate with the CD98HC (Kanai et al., 1998; Mannion et al., 1998; Nakamura et al., 1999; Mastroberadino et al., 1998; Torrents, et al., 1998; Pfeiffer et al., 1999; Sato et al., 1999; Pineda et al., 1999; Rossier et al., 1999; Segawa et al., 1999; Rajan et al., 1999). Commonly, these light chains transport neutral amino acids such as leucine, but the
properties are divided by their differing specificity for substrates and by difference in their sodium dependency (Verrey et al., 1999; Verrey et al., 2000).

As indicated in Table 1.2, the first representative class of CD98LC is an L-type specific amino acid transporter which exchanges large neutral amino acids, in particular those with branched and aromatic side chains (Christensen, 1990). Two CD98HC-associated light chains (LAT1 and LAT2) have been identified to be L-type specific (Kanai et al., 1998; Mastroberardino et al., 1998; Pineda et al., 1999; Rossier et al., 1999; Segawa et al., 1999). In spite of being of the same L type, these two light chains show striking differences in tissue distribution and substrate specificity. LAT1 transports neutral amino acids (leucine, histidine, isoleucine, phenylalanine, tyrosine, tryptophan, valine, methionine and glutamine) and is predominantly localised to the testis, ovary, placenta, brain, activated lymphocytes and some tumor cells (Kanai et al., 1998; Nakamura et al., 1999; Rossier et al., 1999; Wolf et al., 1996). In contrast, LAT2 has a higher affinity for small neutral amino acids (phenylalanine, tyrosine, tryptophan) and it is more specifically found in kidney (in epithelial cells of the proximal tubules, mostly localised to the basolateral membrane) and small intestine, suggesting that this transporter contributes to the renal reabsorption of neutral amino acids by the epithelial cells. The molecular mass (total aa : 534 or 531) of LAT2 is also larger than that of LAT1 (total aa : 512) (Verrey et al., 1999).

Two y+L-type amino acid transporters (y+LAT1 and y+LAT2) have also been identified as CD98HC-associated light chains (Pfeiffer et al., 1999; Torrents et al., 1998). Like LAT2, y+LAT1 is highly expressed in small intestine and proximal kidney tubules,
whereas y+LAT2 is ubiquitously found in most tissues and cells (Pfeiffer et al., 1999; Torrents et al., 1998). These light chains transport neutral amino acids more effectively than cationic amino acids in a Na⁺-dependent manner (Deves et al., 1992). Defective y+LAT1 function due to mutation of its gene (SLC7A7) is regarded as a highly probable cause of autosomal recessive disease lysinuric protein intolerance (see below) (Borsani et al., 1999; Rajantie et al., 1981; Torrents et al., 1998, 1999). y+LAT2 shows very similar molecular characteristics to y+LAT1.

The cysteine-glutamate exchanger xCT is also a significant CD98HC-associated light chain (Sato et al., 1999). This amino acid transporter is found in activated macrophages and some cultured cells. This transporter exchanges extracellular L-cystine in its anionic form for intracellular L-glutamate, in a Na⁺-independent manner. Thus, this light chain might regulate the immune response by controlling L-cystine/L-cysteine and intracellular glutathione levels in activated cells (Sato et al., 1998).

The last member of the CD98LC family (CD98-lc6) is a Na⁺-independent amino acid transporter, b⁰⁺AT, which is also known to associate with rBAT (Well et al., 1992; Bertran et al., 1992; Tate et al., 1992). CD98-lc6 mediates apical influx of L-cysteine and cationic amino acids in epithelial cells of the kidney proximal tubule and the small intestine. Because of this, it has been suggested that mutations of CD98-lc6 might be a cause of cystinuria (Rajan et al., 1999), a genetic disease in which cystine and other dibasic amino acids fail to be reabsorbed from the tubular fluid.

Impairment of amino acid transport has been linked to some genetic diseases, including
lysinuric protein intolerance (LPI; OMIM 222700) (Mylkanen et al 2000; Torrents et al 1999; Torrents et al 1998; Gitome and Pak, 1996; Pierlides, 1997). LPI is a rare, autosomal recessive disorder characterised by defective transport of the cationic amino acids lysine, arginine and ornithine at the basolateral membrane of the polar epithelial cells in the intestine and renal tubules, and by hyperammonemia after high-protein meals (Navar, 1980). Symptoms include failure to thrive, growth retardation, muscle hypotonia and hepatosplenomegaly (Navar, 1980). LPI is caused by mutations, such as deletions, missense mutations, frameshift mutations, nonsense mutations, a splice site mutation and a tandem duplication, in the SLC7A7 (solute carrier family 7, member 7) gene encoding y(+)LAT-1 (y(+)L amino acid transporter-1) (Torrents et al., 1999). Functional genetic analysis using five mutations (L334R, G54V, 1291delCTTT, 1548delC and LPI(Fin)) also indicated that residues L334 and G54 play a crucial role in the function of the y(+)LAT-1 transporter (Mykkanen et al., 2000).

Light chain may also be implicated in abnormal modulation of T4-T3/rT3 metabolism in the hypothyroid state (Ritchie et al., 2001). Although experimental hypothyroidism (28-day propylthiouracil treatment) has no significant effect on System L-like transport of the amino acid tryptophan in adipocytes, uptake of T3 and especially T4 is substantially reduced in adipocytes from hypothyroid rats, partly due to reduction of the BCH [2-(-)-endoamino-bicycloheptane-2-carboxylic acid] -sensitive transport component (L-type amino acid transporters) in liver cells. This may be due to down-regulation or dissociation of iodothyronine receptors from the System L transporter complex.
1.7.1.2 Na⁺/Ca²⁺ transport

It has been suggested that the CD98HC participates in the regulation of intracellular Ca²⁺ concentration via activation of a Na⁺/Ca²⁺ exchanger (Michalak et al., 1986; Latarte et al., 1986; Wacholtz et al., 1992). This activity is specific to certain cell types; especially, cardiac and skeletal muscle sarcolemmal vesicles. An mAb to CD98HC, when added to rabbit skeletal muscle or bovine cardiac sarcolemmal vesicles, inhibited Na⁺-dependent Ca²⁺ uptake by up to 90%. Neither Na⁺-dependent release of accumulated Ca²⁺ nor Ca²⁺/calmodulin-dependent ATPase activity and ATP-dependent Ca²⁺ uptake by sarcolemmal vesicles were inhibited (Wacholtz et al., 1992). Cardiac and skeletal muscle sarcolemmal vesicles are also reported to express higher levels of CD98HC than skeletal muscle transverse tubule membrane and sarcoplasmic reticulum membranes (Michalak et al., 1986). The role of CD98HC in regulation of Ca²⁺ uptake was also investigated with human parathyroid adenoma cells. Incubation with the anti-CD98HC antibodies transiently increased intracellular Ca²⁺, resulting in decreasing basal level of parathyroid hormone secretion (Posillico et al., 1987). Therefore, these findings suggest that CD98 is able to regulate Na⁺/Ca²⁺ exchanger molecules in a specific manner.

1.7.2 CD98 and the control of cell survival, growth and differentiation

1.7.2.1 Regulation of cell proliferation

CD98 is reported to be abundant at the surface of endothelial cells in metaphase, anaphase, and cytokinesis but not the surrounding interphase cells (i.e.: mitotic cells are intensely stained by CD98 mAb whereas interphase cells are not) suggesting that cells
with high mitotic index such as tumor cells would possess abundant cell surface CD98. Indeed, the localisation and expression level of CD98 in tumor cells parallels that shown in mitotic primary cells (Papetti and Herman, 2001). CD98 mAb also block DNA synthesis in activated peripheral blood mononuclear cells (Diaz et al., 1997a).

CD98 is also important in the regulation of tumor cell proliferation, because some anti-CD98 mAbs inhibited cell growth in vitro (Azzarone et al., 1984; Yagita et al., 1986; 1986b; Papetti and Herman, 2001). Especially, CD98 mAbs strongly suppressed fibroblast sarcoma cells, colon carcinoma cells and prostate tumor cells, but it showed little inhibition in certain tumor cell line (HEPG2 hepatocarcinoma) and primary cells. The inhibition did not due to apoptosis. The cells arrest at a particular phase of the cell cycle, and proliferation is restored if the mAb is removed from the culture.

Early reports have demonstrated that significant amino acid transport occurs in mitosis as well as in interphase (MacMillan and Wheatley, 1981). Therefore, the mAbs might be act by inhibiting amino acid transport during interphase and possibly mitosis.

1.7.2.2 Programmed cell death (apoptosis)

Like other surface molecules such as CD43 (Pace et al., 1999) and CD99 (Sohn et al., 1998), it has been reported that CD98 might regulate cell survival and death via the induction of apoptosis. Warren et al (1996) demonstrated that CD98 ligation with a CD98 mAb, Joro 177 induces programmed cell death (apoptosis). Joro 177 increased DNA fragmentation in Lin' haematopoietic precursor (FL) cells, resulting in inhibition of differentiation and induction of apoptosis. However, conflicting observations report
that CD98 may have oncogenic potential (Hara et al., 1999; Hara et al., 2000; Shishido et al., 2000b), and that CD98 ligation did not affect cell viability of U937 cells (Cho et al., 2001). CD98-induced apoptosis may, therefore, be restricted to certain cell types or to specific differentiation steps.

1.7.2.3 Regulation of differentiation

Although the underlying mechanism is not completely understood, it is generally accepted that CD98 plays an important role in the regulation of differentiation of Lin− haematopoietic precursors and blood monocytes (Warren et al., 1996).

1.7.2.3.1 Differentiation of Lin− haematopoietic precursors

Warren et al. (1996) showed that CD98 participates in the differentiation of Lin− haematopoietic precursors. A CD98 mAb inhibited the generation of lymphoid, myeloid, and erythroid lineage cells in vitro from early Lin− haematopoietic precursors. This inhibition may be due to suppression of cell survival and proliferation, because CD98 HC ligation also induced apoptosis (see above).

1.7.2.3.2 Induction of osteoclast-like cells

Long exposure of blood monocytes to CD98HC mAbs for 7 to 14 days induced the formation of multinucleated giant cells (Higuchi et al., 1998; 1999; Tajima et al., 1999). These cells were positive for the osteoclast markers (such as tartrate-resistant acid phosphatase (TRAP), actin ring and calcitonin receptors) (Suda et al., 1992) and gained bone resorption ability (Higuchi et al., 1998; 1999; Tajima et al., 1999). In agreement with this data, CD98 is expressed on both osteoclasts isolated from human bone and the
osteoclast-like cells obtained from human giant cell tumors of bone (Higuchi et al., 1999).

1.7.2.3.2.1 Molecular mechanism of CD98-osteoclastogenesis

To define the molecular mechanisms by which CD98 induces differentiation of monocyte to osteoclast-like cells, Higuchi et al (1999) analysed messenger (mRNA) expression of osteoclast markers, during differentiation of osteoclasts from monocytes. Of selected markers (cathepsin-K, carbonic anhydrase II, vacuolar H⁺-ATPase, vitronectin receptor, TRAP, osteopontin, galectin-3, receptor activator of NF-KB and its ligand, c-src, c-fos, and c-fms), only c-src mRNA was selectively expressed from 3 h after CD98 ligation, suggesting that c-src may play an important role in the CD98-induced differentiation process. In turn, c-src induction requires tyrosine kinase activity, Ras activation, mitogen-activated protein kinase (MAPK) activation and Sp1, a ubiquitously expressed transcription factor (see Section 1.9.2.4) (Mori et al., 2001; Higuchi et al., 1999; Tajima et al., 1999). Specific pharmacological inhibitors of these classes of enzymes could block c-src expression and osteoclastogenesis induced by CD98 mAb.

1.7.2.3.2.2 Possible regulation of CD98-osteoclastogenesis

Importantly, it has been reported that some mAbs to CD98 can block osteoclast formation (Table 1.3). The CD98 mAb, HBJ 127, induced extensive cell aggregation, but not polykaryocyte formation (Tajima et al., 1999). Interestingly this mAb also blocked the ability of other anti-CD98 mAbs (6-1-13, 4-5-1, or 38-2-2) to induce osteoclast formation, and the induction of c-src message. CD98 may therefore play a
Table 1.3 Differential biological activities of anti-CD98 mAb (Tsurudome and Ito, 2000).

<table>
<thead>
<tr>
<th>mAb</th>
<th>U937-2 (U937-2/gp160)</th>
<th>NDV-infected HeLa Monocytes</th>
<th>Effects on Cd+JEM2 (Jurkat/gp160) of 4-5-1 in Cd+U2ME-7</th>
<th>Effects on biological activity of HB127 in Cd+U2ME-7</th>
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<tbody>
<tr>
<td>Aggregation event</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>4-5-1</td>
<td>++++</td>
<td>+++</td>
<td>+++</td>
<td>NT</td>
</tr>
<tr>
<td>6-1-13</td>
<td>++++/+</td>
<td>+++/-</td>
<td>+++</td>
<td>Inhibitory</td>
</tr>
<tr>
<td>4F2</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>Inhibitory</td>
</tr>
<tr>
<td>HBJ127</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>NT</td>
</tr>
<tr>
<td>H227</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>No effect</td>
</tr>
<tr>
<td>38-2-2</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>No effect</td>
</tr>
<tr>
<td>Fusion event</td>
<td></td>
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<tr>
<td>4-5-1</td>
<td>++++</td>
<td>+++</td>
<td>++</td>
<td>No effect</td>
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<td>6-1-13</td>
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<td>4F2</td>
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<td>HBJ127</td>
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<td>H227</td>
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<td>38-2-2</td>
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</table>
dual role in both inducing or inhibiting c-src expression, and hence osteoclast formation.

1.7.3 A potential role for CD98 in oncogenesis

A cancerous (transformed) state is due to abnormal control of cell proliferation. Many studies have shown that CD98 expression is strongly increased during lymphocyte proliferation, suggesting that CD98 is linked to the cell cycle. One possible role for CD98 is to increase the intracellular pool of amino acids, including several essential amino acids (notably large aliphatic ones), and hence allow increased protein synthesis. This could be one explanation why actively proliferating cancer cells, including osteosarcomas and rhabdomyosarcomas as well as simian virus-transformed cells (Haynes et al., 1981; Azzarone et al., 1984; Dixon et al., 1990; Esteban et al., 1990) display high levels of CD98.

The involvement of CD98 in tumorigenicity was firstly suggested by clinical findings that CD98 was highly expressed on various cancer cells such as thyroid cancer cells, cholesteatoma (Sudhoff et al., 1994; Holly et al., 1993), childhood acute lymphoblastic leukemia (Taskov et al., 1996), squamous cell carcinomas (Esteban et al., 1990), plasmacytic neoplasms and B-cell non-Hodgkin's lymphomas (Lewandrowski et al., 1990). The level of CD98 expression was shown to be correlated with different tumor-spreading, differentiation and metastatic behaviors in some cells (Esteban et al., 1990), including childhood acute lymphoblastic leukemia (Taskov et al 1996), brain tumors and non-Hodgkin's lymphomas (Holte et al 1989).

However, several experiments have demonstrated a more direct link between CD98 and
Hara et al., (1999; 2000) and Shishido et al., (2000b) overexpressed CD98 in BALB3T3 or NIH3T3 cells, and showed that these cells had increased tumorigenicity. The cells grew to higher saturation density, had a greater efficiency in colony formation in soft agar, and allowed development of tumors in athymic mice (Hara et al., 1999).

In contrast, ligation of CD98HC inhibited tumor cell proliferation (Yagita et al., 1986) as well as mitogen-induced DNA synthesis in T and B lymphocytes (Haynes et al., 1981). Thus, although CD98 and cell cycle are linked in some way, the physiological role of CD98 in the regulation of the cell cycle requires further investigation.

1.7.3.1 Mechanism of CD98-induced tumorigenicity

To dissect further the structural requirements for CD98-mediated tumorigenicity, the effect of introducing a number of mutations was investigated. Truncated forms of CD98 in which extracellular domains are deleted (Hara et al., 2000) or mutants (C103S, C325S and 103/325) in which Cys 103 and/or Cys 325 were replaced with serine (Shishido et al., 2000) were tested. Truncation (up to 292 aa) of the extracellular domain did not affect CD98-mediated tumorigenicity, and indeed further enhanced tumor growth (Hara et al., 2000). However, disulfide linkage between CD98HC and CD98LC was found to be essential to induce malignant transformation (Shishido et al., 2000b). These results suggest that the interaction of CD98HC with its ligand (if such exists) is not necessary for induction of cell transformation, but that the light chain(s) probably play an essential role.
1.7.3.2 Other possible functions in tumor cells

The L-phenylalanine transporter, which is one of the CD98 light chains, mediates the uptake of melphalan, an anticancer drug susceptible to development of drug-resistance (Harada et al., 2000). Down-regulation of CD98LC might be therefore responsible for drug resistance, by reducing melphalan uptake. Altered tumor associated gene-1/CD98LC (LAT1) is also hypothesized to be involved in an early event in hepatocarcinogenesis giving neoplastic cells a growth or survival advantage, particularly under conditions of limited amino acid availability (Campbell et al., 2000).

1.7.4 Role of CD98 as an adhesion molecule

In parallel to its role in transport, and regulation of cell growth/differentiation, CD98 has been implicated in the regulation of cellular adhesion and virus-induced cell fusion (Suga et al., 2001; Cho et al., 2001; Fenczik et al., 1997; Ohta et al., 1994; Ohgimoto et al., 1995).

1.7.4.1 Involvement in cell adhesion

To carry out many biological activities, cells must dynamically interact with other cells or with the extracellular matrix. These activities include both physiological and pathological processes, such as development, wound healing, homeostasis, migration, tumor cell metastasis, immune activation, inflammation, and thrombosis (Springer, 1990; Albelda and Buck, 1990). The control of cell adhesion plays an especially important role in the regulation or generation of immunological and inflammatory responses (Patarroyo, 1991). Cell adhesion is initiated and maintained by a complex interaction and association between cell surface and intracellular molecules (Springer,

### 1.7.4.1.1 Cell-to-extracellular matrix adhesion

One example of an adhesion process is integrin-mediated adhesion to matrix components. Binding of many types of cells to extracellular matrices (e.g. fibronectin, collagen etc.) through integrin transmembrane receptors initiates the assembly of an actin cytoskeletal complex at the inner surface of the membrane, which in turn drives formation of filopodia, lamellipodia, focal adhesions, and stress fibers (Giancotti and Ruslahti, 1999; Hubbard and Rothlein, 2000). Continuously, multiple intracellular signalling molecules are stimulated during this process, many of which are dependent on assembly of these cytoskeletal structures for their function.

CD98 has also been reported to regulate cell adhesion to extracellular matrix (Fenczik et al., 1997; Chandrasekaran et al., 1999). A CD98 mAb increased cell adhesion of the small cell lung-cancer line (SCLC) and breast carcinoma cell line (MDA-MB-435 cells) to laminin, collagen, fibronectin and other sulfate glycoproteins (Fenczik et al., 1997). The adhesion of SCLC was abrogated by a blocking mAb to CD29 (β1 integrin) and EDTA treatment, suggesting that CD98-induced cell adhesion is dependent on β1 integrin activation. The thrombospondin (TSP)-1-mediated adhesion of MDA-MB-435 to fibronectin and collagen is also functionally regulated by CD98, because CD98 ligation increased cell adhesion of the cell line, via activation of β1 integrins (Chandrasekaran et al., 1999).

### 1.7.4.1.2 Cell-to-cell adhesion
In the immune system, cell-to-cell adhesion (homotypic and/or heterotypic aggregation) has been observed for neutrophils (Simon et al., 1993), lymphocytes (Andrew et al., 1995), eosinophils (Teixeira et al., 1995), platelets (Gordon, 1981), dendritic cells (Delemarre et al., 2001) and macrophage/monocytes (de Smet et al., 1993) after activation. These aggregation events are believed to play important roles in many immunological processes, including antigen presentation, cell-mediated cytotoxicity, and cell extravasation into inflammation sites (Singer, 1992; O'Rourke, 1996; Gille and Swerlick, 1996). Like adhesion events, cell aggregation is accomplished through a variety of cell surface receptors, including the β2 integrin / lymphocyte function-associated molecule-1 (LFA-1, CD18/CD11) (Zapata et al., 1995), intercellular adhesion molecule-1 (ICAM-1, CD54), tetraspans (such as CD9, CD53, CD81 and CD82) (Lagaudriere-Gesbert et al., 1997), β1-integrins (CD29) (Nieswandt et al., 2001), CD43 (de Smet et al., 1993), CD54 (Smith et al., 1989), CD99 (Hahn et al., 2000), CD147 (Kasinrerk et al., 1999), CD151 (Fitter et al., 1999) and T cell activation antigen (TABS) (Andrew et al., 1995).

1.7.4.1.3 Homotypic aggregation induced by CD98 ligation

CD98 mAb-induced homotypic aggregation has been reported in many experiments with various cells such as U937 (Cho et al., 2001), FTH cells (lymphoid progenitor cells) (Warren et al., 1996), BAF-3 (pro-B cells) and BAF3/hLAF-1 (BAF3 cells transfected with human LFA-1α (αL and β2) chain cDNAs) (Suga et al., 2001), U2ME-7C (CD4+ U937 cells transfected with the human immunodeficiency virus (HIV) gp160 gene) (Ohgimoto et al., 1996), U937-2 cells (CD4+ U937 cells) (Ohta et al., 1994; Ohgimoto et al., 1995) and JME2 cells (Ohgimoto et al., 1996). Although it is unclear
what physiological purpose (if any) is served by this type of homotypic aggregation, it was found to lead to cell fusion in certain cases, such as virus-infection or osteoclastogenesis (Ohta et al., 1994; Ohgimoto et al., 1996).

The aggregation of JME2, U2ME-7C and U937-2 cells by CD98 mAb is strongly induced within 1 to 2 hours, but these aggregates become loose after approximately 6 hours (Ohta et al., 1994; Ohgimoto et al., 1996). In contrast, U937 homotypic aggregation is slowly induced, maximal at 5 to 6 hours, and clusters remain for up to 2 days. In comparison, CD29- and CD43-induced aggregation is much more rapid, and is strongly induced within 1 hour (Cho et al., 2001). Since cytochalasin, azide, deoxyglucose and low temperature (4°C) all strongly block the formation of aggregates, CD98-induced homotypic aggregation requires both cytoskeletal changes and metabolic energy ((Ohta et al., 1994; Ohgimoto et al., 1996; Tabata et al., 1997; Warren et al., 1996). In agreement with this, it has been reported that CD98 associates or co-localises with cytoskeleton proteins such as heat shock protein (hsp) 70, actomyosin, vimentin and various tropomyosin isoforms (Suga et al., 1995; Nakamura et al., 1999; Shishido et al., 2000a). As will be discussed in Section 1.9, CD98-induced homotypic aggregation requires a series of intracellular signalling steps, such as MAPKs and G-proteins (Ohta et al., 1994; Ohgimoto et al., 1996; Warren et al., 1996; Suga et al., 2001; Tabata et al., 1997).

1.7.4.2 Adhesion molecule involvement
Although the mechanism whereby CD98 ligation induces adhesion is not fully understood, the involvement of adhesion molecules is well documented. Most studies
report that CD98-induced adhesion is mediated by activation of β1 and β2 integrins. Both monocyte and BAF3/hLAF-1 aggregation is suppressed by β2 integrin (LFA-1) mAbs (Ohgimoto et al., 1995; Suga et al., 2001), providing evidence of β2 integrin involvement. Similarly, blockade of β1 integrins attenuated the aggregation of U2ME-7C and U937-2 cells (Ohta et al., 1994; Ohgimoto et al., 1996).

However, mouse CD98 HC mAb Jorol77-induced aggregation of FTH5 Pro-T cells was not blocked by several blocking mAbs against CD49d/CD29, CD49f/CD29, CD11a/CD18 and CD54, indicating that integrin-independent adhesion molecules might be involved in mCD98-mediated homotypic aggregation (Warren et al., 1996). As described in Chapter 3 (Cho et al., 2001), CD147, which is a member of immunoglobulin superfamily, also plays an important role in U937 homotypic aggregation (Cho et al., 2001).

1.7.5 Regulation of cell fusion

Normal cells do not normally undergo cell fusion, with the exception of sperm fusion with an egg, myogenesis, osteogenesis and placenta formation (White, 1990; Burger and Verkleij, 1990). However, virus infection often induces cell fusion, and formation of giant polykaryocytes and/or syncytia (White, 1990). Many enveloped viruses such as paramyxoviruses, HIV and herpes simplex virus can induce cell fusion (White, 1990). Although the underlying mechanisms remain unknown, these viruses seem to be able to overcome the inhibitory mechanisms by which cell fusion is normally prevented (Tsurudome and Ito, 2000). Virus-induced cell fusion is started by the fusion between the virus envelope and the cell membrane, and usually results in cell death.
In order to understand how virus-induced cell fusion is regulated, Ito and colleagues screened a number of mAbs for their ability to induce fusion of the U2ME-7 cell line, and identified two such mAbs (Tsurudome and Ito, 2000). One of these mAbs recognised a protein which was shown to be identical to CD98/4F2, both by N-terminal amino acid sequence analysis and other biochemical characteristics, such as molecular mass and covalent association with a non-glycosylated light chain (Ohgimoto et al., 1995).

Studies on the role of CD98 in membrane fusion have been conducted mostly in two models: 1) Virus-mediated cell fusion by Newcastle disease virus (NDV)-infected HeLa cells (Yamamoto et al., 1984; Ito et al., 1987) and 2) HIV gp160-mediated cell fusion by HIV gp160-transfected U937-2 (Cd+U2ME-7) cells or CD4+ Jurkat T-cell (JME2) line (Ohgimoto et al., 1995; Ohgimoto et al., 1996). In the first model, CD98 ligation using CD98 mAb was shown to increase HeLa cell fusion in a coculture system in which dispersed BHK cells infected with NDV were seeded on monolayers of HeLa cells (Ito et al., 1992). In the second model, CD98 mAbs initiate the formation of syncytia and polykaryocytes of HIV gp160-transfected U937-2 cells (Ito et al., 1992). The polykaryocytes begin to appear at about 10 h and increase in number for approximately 15 h (Ohta et al., 1994).

The role of CD98 was further confirmed using dominant negative mutants (Okamoto et al., 1997a). Two mutated CD98HC molecules, [CD98/HN, in which the cytoplasmic domain is replaced by human parainfluenza virus type 2 haemagglutinin neuraminidase
(HN), and CD98/330 (serine), in which Cys 330 is replaced by serine], showed little or no effect on CD98 mAb-mediated enhancement of polykaryocyte formation in parent HeLa cells induced by NDV, and rubulavirus-induced cell fusion (Okamoto et al., 1997a).

The CD98-mediated cell fusion phenomenon is further complicated, because CD98 mAbs can differentially modulate the event (Table 1.3). For example, CD98 mAb (HBJ127) displayed two opposite effects on virus-induced fusion events; this mAb enhanced cell fusion in NDV-infected HeLa cells, but delayed human parainfluenza type 2 virus (hPIV2)-induced fusion without diminishing virus growth or viral protein synthesis (Okamoto et al., 1997a).

1.7.6 T cell co-stimulation

CD98 expression is strikingly regulated during T cell differentiation and activation, but the role of CD98 in T lymphocyte responses is not yet understood. Using ligation with mAbs, several functions have been proposed. Firstly, CD98 showed comitogenic properties when T lymphocytes were treated with CD98 mAbs and soluble or immobilized CD2 and CD3 mAbs (Freidman et al., 1994; Warrant et al., 2000), because these CD98 mAb strongly up-regulated T cell proliferation even in the absence of antigen presenting cells (APC). These results suggest that CD98 might act as a costimulatory molecule. Secondly, some different CD98HC mAbs inhibited IL-2 receptor expression and progression of T cells from G1 to S phase, suggesting that CD98 might be involved in T cell activation (Diaz et al., 1997b). Finally, the effects of CD98 ligation may also be mediated via the regulation of APC function, since CD98HC
is expressed at very high levels on human peripheral blood DC (Woodhead et al., 1998) as well as monocyte and monocyte-derived lines (Stonehouse et al., 1999). Diaz et al., (1997) demonstrated that the interaction between CD98 mAb and CD98 on monocytes, acting as APC, was more important than a direct effect on the T cell in mediating regulation of T cell activation. This model used a novel xenogeneic system, which took advantage of the lack of xenoreactivity of purified human T cells against mouse splenocytes (Diaz et al., 1997b). Mouse splenocytes, therefore, acted as "accessory cells" for CD3-mediated activation of T cells. In this model, mAbs recognised mouse but not human CD98. Two further studies (Stonehouse et al., 1999; Woodhead et al., 2000) demonstrated a functional role for CD98 on APC. In these studies, CD98 mAbs inhibited T cell proliferation induced by CD3 mAb in the presence of U937 or DC. This suppressive effect was maintained, even when CD98 mAb was prepulsed on U937 cells and DC, and was washed off before the U937 cells and DC were added to the T cells. Taken together, these results implicate that CD98 is involved in a novel pathway for APC regulation of T cell activation and proliferation.

1.8 CD98-associating proteins

CD98 ligation induces activation-dependent signalling pathways, particularly tyrosine phosphorylation, despite the fact that CD98 contains no catalytic region within its cytoplasmic domain (Warren et al., 1996). This suggests that phosphorylation might result from association with membrane-associated tyrosine kinases, or with serine/threonine-specific protein kinases to activate the tyrosine phosphorylation system. More generally, CD98 function may be mediated by proteins that associate with CD98. Recent data indeed support this hypothesis. Immunohistochemical, immunological and
biochemical studies have shown that CD98 can associate with various cytoskeletal and structural proteins, such as heat shock protein 70 (hsp70), actomyosin, vimentin, ADAM3 disintegrin, tropomyosin, β1 integrins and cadherin from various type of cells (Suga et al., 1995; Nakamura et al., 1999; Shishido et al., 2000a; Takahashi et al., 2001). These results further suggest that the function of CD98 may be linked to the regulation of cell cytoskeleton and integrin function.

1.8.1 CD98 as an integrin function regulator

1.8.1.1 What are integrins?
Integrins are heterodimeric transmembrane proteins composed of α and β subunits that functionally mediate cell adhesion to extracellular matrix components or cell-to-cell interaction and serve as signalling receptors for cell migration, cell growth and differentiation, cell cycle and adhesion events (Kishimoto et al., 1989; Larson and Springer, 1990). The α and β chains each include an extracellular region, a transmembrane segment and a small cytoplasmic domain. These β chains have been identified as β1, β2, β3 and β7. The β1-integrins are mainly involved in binding of cells to extracellular matrix such as fibronectin, etc. (Wood and Shimizu, 2001), β2-integrins participate in leucocyte adhesion to endothelium and to other immune cells (Hubbard and Rothlein, 2000), and β3-integrins are involved in the interactions of platelets and neutrophils at inflammatory sites or sites of vascular damage (Seftor, 1998). Each β chain associates with one of a number of different α chains to make at least 22 different integrin receptors (Springer, 1990). The binding of the integrin complex to its ligand is accompanied by rearrangements in the actin cytoskeleton. This rearrangement is mediated by activation and nucleation of a complex of proteins inside the cell, including
the cytoplasmic tail of the β subunit of the integrin receptor and a number of other signaling proteins. The β subunit is important in determining which proteins are nucleated (to form the focal adhesion) and which signals are generated. β and α chains also bind directly to structural proteins (such as talin, actin, vinculin and α-actinin) and signaling proteins (such as focal adhesion kinase (FAK), p130CAS, Src, Csk and Crk) to regulate cytoskeleton rearrangement, formation of focal adhesion and signalling (Giancotti and Ruoslahti, 1999; Boudrea and Jones, 2000; Schlaepfer et al., 1997).

Integrin-mediated signalling pathways are very complex, but include activation of MAPK via Rho GTPases (especially Cdc42) (Boudreau and Jones, 1999; Hill and Treisman, 1995).

1.8.1.2 Regulation of integrins is of therapeutic interest

Integrins have been implicated in the pathogenesis of many diseases, such as rheumatoid arthritis, inflammatory bowel disease, asthma, cancer and coronary heart disease (Kishimoto et al., 1989; Larson and Springer, 1990; Engers and Gabbert, 2000). For this reason, it is of great interest to learn how to control and regulate the function of integrins, which are promising targets for the development of therapeutic agents. To do this, there are two main approaches at present. The first is to develop antagonists of the extracellular domain and the second is to identify new regulators of integrin extra- and intracellular domain function.

1.8.1.3 Direct inhibition of integrin function: receptor antagonism

Interest in the development of specific antagonists of integrin function has been principally driven by efforts to design more potent curative (e.g. anti-thrombotic) agents
than conventional drugs (Scarborough, 1999). One example is the development of a RGD tripeptide mimic as a functional blocker of \( \beta_3 \) integrins. Several possible clinical settings for targeting the \( \beta_3 \) integrins are currently being evaluated.

### 1.8.1.4 Indirect inhibition of integrin function

Recently a dominant and concentration-dependent suppression of integrin activation was reported, using a chimaeric integrin protein. Fenczik et al (1997) showed that adhesion, spreading or migration mediated by integrin function was strongly blocked by a chimaeric protein (Tac-\( \beta_1 \)), composed of the cytoplasmic region of the \( \beta_1 \) integrin chain and the extracellular domain of the Tac (CD25) subunit of the IL-2 receptor. The suppression was believed to be due to the binding of the free \( \beta_1 \) integrin cytoplasmic domains of the Tac-\( \beta_1 \) chimaeras to putative cellular factors. Identifying this factor, or factors, was clearly an important question (Lasky, 1997). Integrins were already known to associate physically with many cell-surface and intracellular proteins (so called integrin-associating proteins). Such proteins included CD47, glycosylphosphatidylinositol-liked receptor (CD87, CD16b and CD14), the tetraspan or transmembrane-4 superfamily (CD9, CD53, CD63, CD81, and CD82), CD98 and CD147 (Hemler, 1998; Berditchevski, 1997; Zhang et al., 2001). Any of these proteins might be involved in the regulation of integrin function. However, the clever genetic complementation strategy employed by Fenczik and collaborators (1997), specifically identified that CD98 is involved in Tac \( \beta_1 \) dominant suppression as the putative cellular factor. Blocking CD98 function may therefore be an alternative tool to suppress integrin function, and such a blockade may have potential therapeutic applications, for example anti-inflammatory or anti-metastatic (Lasky, 1997).
1.8.1.5 Biochemical evidence: molecular mechanism

The hypothesis that CD98 regulates integrin function was therefore explored further. The CD98HC gene was manipulated to make several deletion mutants and the function of these recombinant CD98 molecules was evaluated using immunoprecipitation and the capacity to reverse dominant negative suppression (DONS) of integrin activation (Fenczik et al., 1997; Zent et al., 2000; Fenczik et al., 2001). Using this approach, CD98 was shown to specifically associate with the integrin β1A cytoplasmic domains (Fenczik et al., 1997). There was no association with other domains, such as the muscle-specific splice variant, β1D, or the leukocyte-specific β7 cytoplasmic domain (Zent et al., 2000). Both the cytoplasmic and transmembrane domains of CD98HC were found to be required for its interaction with integrins, because deletion or replacement of either domain completely blocked DONS capacity of CD98, and physical association with β1 integrins (Fenczik et al., 2001).

1.8.1.6 Functional evidence that CD98 associates with integrins

Several further reports support the biochemical evidence for CD98/β1 integrin interaction. Most studies have used cell adhesion models, and immunological blockade using blocking mAbs.

The first evidence was obtained in a model system in which CD98 ligation induces adhesion to collagen (see Section 1.7.4.1.1). In this model, the β1 integrin blocking mAb P5D2 strongly diminished CD98-mediated cell adhesion to collagen, suggesting that antibody-mediated crosslinking of CD98 stimulated β1 integrin-dependent cell
adhesion (Fenczik et al., 1997). The second example uses a model in which cell adhesion is mediated by TSP-1 (Chandrasekaran et al., 1999; 2000), a matricellular protein that displays both pro- and anti-adhesive activities (Bornstein, 1995). Attachment and spreading of MDA-MB-435 (breast carcinoma) cells on immobilized TSP-1 are known to be primarily β1 integrin-dependent. Under these conditions, CD98 ligation increased the activity of β1 integrins. Conversely, serum stimulation, which leads to integrin activation, is accompanied by increased surface expression of CD98. Thus, it was suggested that the pro-adhesive activity of TSP1 for breast carcinoma cells was in part controlled by CD98 activation through regulation of the α3β1 integrin.

Evidence for β1 integrin regulation by CD98 was also shown in the case of virus-mediated cell fusion (Tabata et al., 1994; Ohta et al., 1994) (see Section 1.7.5). β1 integrin mAb completely inhibited the enhancement of NDV-mediated cell fusion by CD98 mAb, whereas mAbs to β3 and α4 integrins, did not affect the CD98-mediated potentiation of the cell fusion. Furthermore, β1 integrin mAb also blocked syncytium formation in CD98 mAb-treated Cd^+U2ME-7 cells, suggesting that β1 integrins are necessary for CD98-induced fusion.

A role for β1 integrins was also demonstrated in the co-stimulatory activity of CD98 for T cells (Warren et al., 2000) (see Section 1.7.6). As reported previously (Nakao et al., 1993), some CD98 mAbs synergised with CD3 mAb in stimulating proliferation of peripheral blood T lymphocytes. However, a blocking β1 integrin mAb suppressed the CD98-mediated costimulatory activity. Therefore, it was proposed that interaction between CD98 and integrins is necessary for the T cell costimulatory role of CD98.
Although β1 integrins have been the main CD98-associated molecule studied, other important proteins may exist. The evidence for one such molecule, CD147, is presented in Chapter 4.

1.9 Role of CD98 as a signal transduction molecule

Despite the multitude of studies addressing CD98 function, there is little information on the intracellular signalling pathways which underlie the extraordinary diversity of function. Tyrosine phosphorylation, G-protein involvement, and MAPK activation following CD98 ligation with activating mAbs have all been documented as possible signalling pathways in CD98 function (Fig. 1.3). Most of the experiments have been carried out using U937 (Cho et al., 2001), BAF/hLFA-1 cell (Suga et al., 2001), FTH5 cells (Warren et al., 1996), Cd+U2ME-7 (Tabata et al., 1997) and blood monocytes (Miyamoto et al., 2000; Tsurudome and Ito, 2000), under normal culture or virus-infected conditions. A definite consensus pathway for CD98-mediated signalling cannot yet be clearly defined, however. This may be due to the variety of cell types examined or the different outcomes evaluated with the frequent absence of quantitation. The data do suggest that different CD98 functions may be mediated by different intracellular signalling pathways.
The interaction of CD98 ligand with CD98 and the formation of molecular complex within the cell membrane initiates signal transduction. CD98 may transduce signals by association with β1 integrins, cytoskeleton proteins (CP) and other signalling molecules such as PTK (protein tyrosine kinases), and PI3-K (phosphatidylinositol 3-kinase). The activation can lead to downstream activation of MAPKs. With the activation of MAPK, transcription factor (e.g., Sp1) activation directs new protein synthesis (e.g., c-src).
1.9.1 Engagement or ligation of CD98

To stimulate activation / interaction of surface molecules with their ligands, cells are often incubated with crossing-linking antibodies, a process called engagement or ligation. To date, dissection of the signal transduction pathways induced by activated CD98 has been mostly done by means of CD98 ligation. The CD98 activation by mAb treatment leads to activation of protein tyrosine phosphorylation, serine/threonine phosphorylation, G-protein/Ras cascade, and MAPK to mediate CD98-induced biological activities (see also Chapter 4).

1.9.2 Intracellular changes upon CD98 ligation

1.9.2.1 Tyrosine phosphorylation

CD98 ligation rapidly induces or strongly potentiates tyrosine phosphorylation of many cellular proteins in U2ME-7 (U937-2 cells transfected with HIV gp160 gene), U937 and FTH5 (pro-T cell line) cells (Warren et al., 1996; Tabata et al., 1997; Miyamoto et al., 2000; Cho et al., 2001). However, in contrast to integrin-mediated signalling, none of the CD98-induced tyrosine-phosphorylated proteins have been previously identified (Warren et al., 1996; Tabata et al., 1997). Using U2ME-7 cells undergoing aggregation and cell fusion, it was demonstrated that CD98 ligation induces and enhances tyrosine phosphorylation of several cellular proteins; two proteins including pp130 were newly phosphorylated, phosphorylation of ten proteins was increased, and the phosphorylation of two polypeptides was decreased (Tabata et al., 1997). The CD98-induced phosphoprotein, pp130, was clearly different from FAK (pp125FAK), β1 integrin, and vinculin which all have similar molecular weights, suggesting that CD98-induced tyrosine phosphorylation is distinct to the integrin-mediated phosphorylation cascade.
In terms of biological function, CD98-induced tyrosine phosphorylation was shown to be an early obligatory signal to induce multinucleated cell formation (Tabata et al., 1997), c-src expression (Miyamoto et al., 2000), and murine pro-T cell homotypic aggregation (Warren et al., 1996), but not U2ME-7 homotypic aggregation (Tsurudome and Ito, 2000). Phosphorylation was strongly linked to c-src expression, an essential step in osteoclast-like cell formation (Miyamoto et al., 2000; Higuchi et al., 1999), because PTK inhibitors strongly blocked both c-src mRNA expression and osteoclastogenesis. CD98-induced tyrosine phosphorylation was also shown to be necessary for FTH5 homotypic aggregation (Warren et al., 1996), whereas CD98-induced U2ME-7 cell aggregation was not affected by PTK inhibitors (Tabata et al., 1997). Therefore, CD98-induced tyrosine phosphorylation seems to be specifically required for certain cellular roles of CD98, especially the initiation of cell fusion. Some discrepancies between murine and human CD98 in this regard remains unresolved.

Because CD98 contains no PTK catalytic region or consensus PTK substrate sequences within its cytoplasmic domain (Warren et al., 1996), CD98-induced tyrosine phosphorylation is thought to reflect its association with membrane associated tyrosine kinases or with serine/threonine-specific protein kinases, which indirectly activate tyrosine phosphorylation. Clear identification of CD98-induced tyrosine phosphorylated proteins, elucidation of possible association with PTK and the relationship between tyrosine phosphorylation and other kinase activities will be an interesting task for further investigation (see Chapter 3).
1.9.2.2 Serine/threonine phosphorylation

Serine/threonine phosphorylation is mediated by several enzymes such as protein kinase A (PKA), PKC, phosphatidylinositol 3-kinase (PI3-K) and AKT. Like tyrosine phosphorylation, these enzymes play important roles in many cellular processes such as cytokine production. The involvement of these enzymes can be pharmacologically evaluated by broad or more specific inhibitors. In several experiments, these pharmacological inhibitors have been employed to investigate whether these enzymes control CD98-induced signalling pathways.

Broad specificity serine/threonine phosphorylation inhibitors (H-7 and H-89) inhibited CD98-induced cell aggregation and fusion of human monocyte cell lines (Tabata et al., 1997), and a mouse pro-T cell line (Warren et al., 1996). However, other reports did not find any increase in either PKC or PKA activities of whole cells after CD98 ligation, and there was no evidence of membrane translocation of PKCs by CD98 ligation in U2ME-7 (Tabata et al., 1997).

1.9.2.3 G protein or G-like protein involvement

The heterodimeric guanine nucleotide-binding regulatory proteins (G-proteins) mediate signalling from a large number of diverse seven transmembrane spanning cell surface receptors to a variety of intracellular effectors (Ross and Wilkie, 2000; Sprang, 1997). These signal transduction pathways control numerous essential functions in all tissues and cells. Ras proteins also play an important role in relaying signals from receptor tyrosine kinases to the nucleus to stimulate cell proliferation and differentiation
(Kaibuchi et al., 1999). They function as signal mediators for receptor tyrosine kinases and tyrosine-associated receptors. Activation of cell surface receptors by a signalling molecule converts Ras proteins from their inactive, GDP-bound state, to active, GTP-bound state. In particular, these signalling molecules are of interest because they are specifically linked to regulation of cytoskeleton rearrangement which is necessary to mediate signal transduction pathways and changing cell shape, required for cell aggregation or adhesion.

There is some evidence that CD98 function does indeed require activation or involvement of G-protein or Ras proteins, particularly in cell cluster formation and fusion. Using murine cell lines, BAF3 and BAF3/hLFA-1, Suga et al (2001) found that cross-linking CD98 with mAb induces activation of Rap1 which is a Ras-related small GTPase. This activation was found to be linked to induction of homotypic aggregation via activation of PI3-K (Suga et al., 2001), although it was required only for fusion, and not aggregation or s-src expression (Miyamoto et al., 2000). In contrast, however, in Cd\textsuperscript{U2ME-7} and blood monocytes, the Ras pathway was apparently not involved in triggering homotypic aggregation (Miyamoto et al., 2000). A Ras inhibitor, manumycin A, displayed inhibitory effects on c-src gene expression and cell fusion (Miyamoto et al., 2000), suggesting that Ras-mediated pathways may play an important role in CD98-mediated cell fusion.

1.9.2.4 MAPK/ transcription factor activation

A few studies have evaluated the effects of CD98 engagement on MAPK signaling pathways and transcription factor activation. Ligation of CD98 on BAF3/hLFA-1 cells
activated extracellular signal-regulated kinases 1 and 2 (ERK1/2) (Suga et al., 2001). MAPK involvement was also demonstrated pharmacologically in cell fusion events, although MAPK inhibitors did not affect cell aggregation (Miyamoto et al., 2000). Specific MAPK inhibitors, PD98059 (a selective inhibitor of ERK 1/2 activator kinases) and SB20380 (an inhibitor of p38 MAPK) strongly suppressed fusion of CdU2ME-7 cells or blood monocytes (Miyamoto et al., 2000).

CD98-induced MAPK activation was also linked to activation of transcription factors. Two transcription factors, Sp1 and AP2, bind to the 5' flanking region of the c-src gene (Bonham and Fujita, 1983). PD98059 and SB203580 blocked CD98-dependent induction of Sp1 mRNA in blood monocytes (Miyamoto et al., 2000). CD98 ligation may therefore induce Sp1 transcription and hence c-src and monocyte differentiation / fusion via activation of MAPK.

1.9.3 Possible view of CD98 and signal transduction

An overall model can be proposed in which interaction of ligands (eg. galectin-3) with CD98 leads to the formation of CD98 multimers, and this in turn triggers out-side-in signal transduction (Fig. 1.2). The model incorporates the information from amino acid sequence analysis, suggesting that CD98 has no intrinsic tyrosine kinase activity, nor any consensus tyrosine phosphorylation motifs, but that CD98 could possibly be regulated by serine/threonine phosphorylation. The model also incorporates the evidence that CD98 activation is able to activate different biological functions in a specific and cell-dependent manner.
In human primary monocytes or monocytic cell lines, protein tyrosine kinases appear to be required for CD98 induction of cell fusion. Tyrosine phosphorylation is specifically required for induction of c-src expression, which is an essential step for osteoclast-like cell formation. Unidentified serine/threonine kinases also seem to be involved in both homotypic aggregation and cell fusion processes. Although the details of the signalling pathways related to G-protein or Ras proteins are still to be determined, these pathways may participate in CD98-induced signalling via PI3-K and MAPK. Clearly, the available data are not enough to define the full details of CD98-induced signalling, and further studies are needed to complete our understanding of CD98 regulation of cellular events.

1.10 Conclusion

Although CD98 was originally identified as a phenotypic marker indicating lymphocyte activation, this molecule has now been implicated in several fundamental cellular functions. These are not all mediated by amino acid transport function by CD98LC, and indeed the functions for amino acid transport and CD98HC-mediated β1 integrin regulation can be separated by molecular manipulation. Presently, CD98LC-mediated amino acid transport might be involved in regulating proliferation, but CD98HC seems to play important roles in other cellular functions such as Ca\(^{2+}\) transport, cell adhesion and fusion and T cell costimulation via association with cytoskeleton proteins and β1-integrins. The CD98 molecular complex mediates its multiple biological functions via activation of intracellular signalling pathways, including tyrosine phosphorylation and serine/threonine phosphorylation.
1.11 Aims

CD98 is important in many aspects of cellular function, such as amino acid transport, cell aggregation and adhesion, virus-induced cell fusion, T cell costimulation, and oncogenic transformation, but an understanding of its molecular mode of action, which would link together its diverse functional roles, is still lacking.

The first aim of this project is to establish a suitable model for looking at CD98-induced signalling and its function in cell aggregation and costimulation of antigen presenting cells. Previous work in this laboratory has described a role for CD98 in the interaction between antigen presenting cells (either U937 as a model cell line, or human peripheral blood dendritic cells) and T cells. The molecular analysis of antigen presenting systems, however, are made much more difficult because the experiments involve co-culture of two cell types, both of which express CD98, and both of which can be the target for pharmacological intervention. As a first step in understanding the role of CD98 on these cells, therefore, we established a quantitative model system in which CD98 induced homotypic aggregation of U937 cells. The establishment and characterisation of this system is described in the first part (Chapter 3) of this thesis. In this context, we mainly focused on understanding the aggregation mechanism, and which surface adhesion molecules cooperated in mediating CD98-induced U937 homotypic aggregation.

The second aim of this thesis is to use the model described above to dissect the major signalling pathways activated in response to CD98 ligation (Chapter 4 and 5). As mentioned above, only a few papers address CD98-induced intracellular signalling. Furthermore, the available data are not enough to define the full details of CD98-
induced signalling, and further studies were needed to understand CD98 regulation of cellular events. Using pharmacological and biochemical approaches, therefore, more quantitative dissection of CD98-induced signalling was carried out and the results will be described in this thesis (Chapter 4 and 5).
Chapter 2

Materials and Methods.
2.1 Materials

Metabolic inhibitors (Table 2.1) and broad or more specific signaling enzyme inhibitors or activators (Table 2.2) were purchased from Sigma (Poole, UK), Calbiochem (La Jolla, CA, USA) and Daewoong Pharm. (Sungnam, Korea), respectively.

Table 2.1 List of metabolic inhibitors.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Specificity</th>
<th>Solvent</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azide</td>
<td>ATP synthesis</td>
<td>Medium</td>
<td>Sigma</td>
</tr>
<tr>
<td>Colchicine</td>
<td>Microtubule formation</td>
<td>Ethanol</td>
<td>Sigma</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>Protein synthesis</td>
<td>Ethanol</td>
<td>Sigma</td>
</tr>
<tr>
<td>Cytochalasin B</td>
<td>Cytoskeleton arrangement</td>
<td>Ethanol</td>
<td>Sigma</td>
</tr>
<tr>
<td>Deoxyglucose</td>
<td>ATP synthesis</td>
<td>Medium</td>
<td>Sigma</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ca^{2+}</td>
<td>Medium</td>
<td>Sigma</td>
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</table>

Table 2.2 List of enzyme inhibitors.

<table>
<thead>
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<th>Compound</th>
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<th>Company</th>
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</thead>
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<tr>
<td>Genistein</td>
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<td>Calbiochem</td>
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<tr>
<td>Herbimycin A</td>
<td>PTK inhibitor</td>
<td>Enthanol</td>
<td>Sigma</td>
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<td>PTK inhibitor</td>
<td>Medium</td>
<td>Daewoong</td>
</tr>
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<td>Broad specific serine/threonine kinase inhibitor</td>
<td>Medium</td>
<td>Calbiochem</td>
</tr>
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<td>H-89</td>
<td>Broad specific serine/threonine kinase inhibitor</td>
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<td>KT5720</td>
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<td>DMSO</td>
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<tr>
<td>Forskolin</td>
<td>Adenylate cyclase activator</td>
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<td>Inhibitor</td>
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<td>Cyclosporin A</td>
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A panel of mAbs to surface proteins (Table 2.3), direct-labelled mAbs (Table 2.4) and
mAbs to signaling enzymes (Table 2.5) were used. The mAb concentration was determined by SDS PAGE with Coomassie Blue staining compared to standard mAb (CD44, El/2). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and all other chemicals were purchased from Sigma Chemical Co. (Poole, UK). Protein G-Sepharose was obtained from Calbiochem (La Jolla, CA, USA). Nitrocellulose membrane and ECL reagent were from Amersham Life Science (Little Chalfont, Buckinghamshire, UK). Bradford reagent was obtained from Bio-Rad (Heidemannstraße, München, Germany). Fetal calf serum (FCS) and RPMI1640 were obtained from GIBCO (Grand Island, NY, USA). U937 cells were purchased from ATCC (Rockville, MD, USA).

Table 2.3 List of unlabelled mAbs to surface molecules.

<table>
<thead>
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<th>Specificity</th>
<th>MAb name</th>
<th>Isotype</th>
<th>Species</th>
<th>Originator / Company</th>
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<td></td>
<td>BU86</td>
<td>G1</td>
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</tr>
<tr>
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<td>Mouse</td>
<td>V. Horejsi</td>
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<td></td>
<td>P5D2</td>
<td>G1</td>
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### Materials and Methods

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Table 2.4 List of direct labelled antibodies.

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<td>FITC</td>
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<td>CD54</td>
<td>FITC</td>
<td>Immunotech</td>
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<td>UM-8D6</td>
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<td>9-49</td>
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Table 2.5 List of antibodies to signaling enzymes.

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<th>Ab name or catalogue #</th>
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<td>phospho-JNK</td>
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<td>06-333</td>
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<td>Promega</td>
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<td>9211S</td>
<td>phospho-p38</td>
<td>1/1000</td>
<td>Rabbit</td>
<td>Cell signaling</td>
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Materials and Methods

P16520 PKCα 1/1000 Mouse Transduction lab
P17720 PKCβ 1/250 Mouse Transduction lab
P20420 PKCγ 1/5000 Mouse Transduction lab
P36520 PKCδ 1/500 Mouse Transduction lab
P14820 PKCe 1/1000 Mouse Transduction lab
P15120 PKCθ 1/250 Mouse Transduction lab
P22529 PKCλ 1/250 Mouse Transduction lab
C-20 PKCζ 1/2000 Rabbit Santa Cruz
T(P)505 phospho-PKCδ 1/2000 Rabbit P. J. Parker
T(P)560 phospho-PKCζ 1/2000 Rabbit P. J. Parker

2.2 Cell culture

Promonocytic U937 cells, established from malignant cells from the pleural effusion of a 37 year-old Caucasian male with diffuse histiocytic lymphoma (Sundstrom and Nilsson, 1976) were maintained in RPMI 1640 supplemented with 10% FCS. Cells were grown at 37°C and 5% CO₂ in humidified air.

2.3 Quantitative homotypic cell aggregation assay

Twenty μl of cells in RPMI1640 medium supplemented with 10% FCS at 10⁶ cells/ml were placed in round bottom wells of a 96-microwell plate. An equal volume of medium, with or without appropriate antibody, was added, and the cells were incubated at 37°C for 4-7 h. The cells were resuspended gently, so as not to break up the clusters, and the number of unaggregated and total cells were counted in a haemocytometer and percentage of cells in aggregates was determined by the following equations:

Equation 1: % of cells in aggregates = [total cells-free cells/total cells] × 100.
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**Equation 2**: \% of control = \[\% of aggregation (drug or blocking mAb-treated group / \% of aggregation (only aggregating mAb-treated group)\] \times 100.

**2.4 Cytofluorometric analysis**

Expression of CD98 on cell surface of U937 cells was determined by flow cytometry. Cells \((10^5)\) were washed with phosphate-buffered saline (PBS) staining buffer (containing 2% FCS and 0.1% sodium azide) and incubated in 50 \(\mu\)l of staining buffer containing 10% rabbit serum for 10 min on ice and then with the primary antibody for a further 45 minutes. After washing three times with staining buffer, cells were treated with 1/20 dilution of fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG secondary antibody (Dako, Dakopatts, High Wycombe, UK). Cells were then washed three times with staining buffer and analyzed on a FACScan (Becton Dickinson). Expression of CD29, CD147, CD18, CD45 and HLA-DR was analysed by direct binding of fluorescein-conjugated antibodies (Table 2.4) using flow cytometry as above.

**2.5 Subcellular fractionation for PKC translocation assay**

Four ml U937 cells \((5 \times 10^6 \text{ cells/ml})\) were plated in 6 well plates under serum-free conditions. After 3 h recuperation, pharmacological inhibitors were added to the cells as appropriate, for a further 30 min. CD98 mAb (ANH-18) was then added for 20 min, and the cells were then transferred on to ice, and washed three times with ice-cold phosphate-buffered saline. 800 \(\mu\)l of homogenisation buffer A \((20 \text{ mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA, 50 mM } \beta\text{-glycerophosphate, 1 mM dithiothreitol (DTT), 1 mM Na}_3\text{VO}_4\), each 10 \(\mu\)g/ml of leupeptin, aprotinin and pepstatin, 0.4 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzimide, 2 mM hydrogen peroxide,
0.25 M sucrose) were added and the cell lysate was sonicated for 10 s three times. The homogenate was centrifuged at 2000 × g for 5 min at 4°C. The supernatant was further centrifuged at 100,000 × g for 25 min at 4°C to obtain the cytosolic fraction. The pellet was resuspended in 300 μl of homogenisation buffer B (2% Triton X-100 in buffer A) and sonicated for 10 s twice. After incubation on ice for a further 25 min, the suspension was centrifuged at 12,000 × g for 10 min at 4°C. The supernatant was collected and used as membrane fraction. Protein concentration of each sample was determined by the Bradford method and 2 × Laemmli sample buffer was added before boiling for 5 min.

For preparing whole cell lysates, cells (5× 10^6 cells/ml) were washed three times in cold PBS containing 1 mM sodium orthovanadate, and lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM DTT, 1% Triton X-100, 10 % glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 1 mM benzimide and 2 mM hydrogen peroxide) for 30 min with rotation at 4°C. The lysates were clarified by centrifugation at 16,000 × g for 10 min at 4°C.

2.6 Western Blotting

Samples containing equal amount (40 or 80 μg/lane) of protein were loaded in each lane and separated on 10% SDS-polyacrylamide gel. The gel was transferred by electroblotting to nitrocellulose membrane. Membranes were blocked for 60 min in TTBS (3% BSA in Tris-buffered saline containing 20 mM NaF, 2 mM EDTA, 0.2% Tween 20) at room temperature. Immunoblots for phosphorylated or non-
phosphorylated proteins of eight PKC isoforms, MAPK/p38, MAPK/ERK, and MAPK/JNK, and phosphotyrosine proteins were carried out using specific antibodies against phosphorylated or nonphosphorylated forms, and secondary horseradish peroxidase-conjugated rabbit anti-mouse or swine anti-rabbit immunoglobulins. Peroxidase activity was visualised by chemiluminescence detection (ECL reagents, Amersham, Little Chalfont, Buckinghamshire, UK). Intensity of bands on film was quantified using Synoptic image analysis system and software (Synoptic, Cambridge, UK).

2.7 Immunoprecipitations

The cells were washed with ice-cold PBS and lysed in lysis buffer. After centrifugation for 5 min at 12,000 × g, the supernatant (whole cell extract (0.5 mg/ml)) were subjected to immunoprecipitation with both anti-PKCδ and anti-phospho PKCδ antibodies overnight at 4°C. Then, protein G-Sepharose was added, and the mixture was incubated for 2 h. The immunoprecipitates were washed three times with washing buffer containing 50 mM Tris-HCl, 0.5% NP-40, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM Na3VO4, 1 mM DTT, 1 mM NaF, 1 mM PMSF and each 10 μg/ml pepstatin, aprotinin and leupeptin. The immunoprecipitates were boiled in the sample buffer of SDS-PAGE. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The residual binding sites were blocked by incubating the membrane with 3% BSA or 5% non-fat milk protein in TTBS. The membranes were incubated with anti-phosphotyrosine antibody for 1 h. After washing, the membranes were incubated with anti-mouse IgG peroxidase conjugate and developed by ECL.
2.8 Purification of CD98 mAb (AHN-89)

Protein A coupled to Sepharose CL-4B (Sigma, 1 ml containing 2 mg protein) was swollen in PBS containing 0.1% sodium azide for 24 hours. One ml of gel solution was packed into the column and washed with 10 ml 0.1 M phosphate buffer (pH 8.0), 4 ml citric acid solution and 10 ml 0.1 M phosphate buffer, respectively. On the other hands, mAb culture supernatant (total 2 l, a gift from Prof. K.M. Skubitz, Minnesota University, USA) was precipitated with ammonium sulfate and dialysed with 0.1 M sodium phosphate buffer (pH 8.0, final 100 ml). Ten ml from the dialysate was applied to the Protein A column at approximately 5 ml/h. After collecting a flow-through, the column was washed with 5 ml of 0.1 M phosphate buffer. The unretarded fraction containing non-immunoglobulin components was also collected. Elution to purify IgG1 isotype was started by treatment with 15 ml 0.1 M sodium citrate buffer (pH 4.5). The fraction size was 1 ml per Eppendorf tube, containing 60 µl of 2 M Tris solution. The column was washed with 4 ml citric acid solution and then stored in 0.1 M phosphate buffer containing 0.2% sodium azide. The protein elution profile was monitored by measurement of the absorbance at 280 nm. The purity was checked by SDS-PAGE. Three fractions with the highest OD values were pooled and dialysed two times in PBS.

2.9 Preparation of F(ab’)2 and Fab fragments from purified AHN-18

CD98 mAb (AHN-18) solution dialysed against 0.1 M citrate buffer, pH 3.5 were subjected to pepsin digestion to prepare F(ab’)2 fragments. Briefly, 3 ml of mAb solution was digested with 50 µg pepsin (EC 3.4.23.1) (Sigma) for F(ab’)2 or papain for Fab. The mixture was incubated at 37°C for 10 h and then neutralized with 2 M Tris (270 µl in 3 ml mixture) to stop the enzymatic reaction. Undigested antibody was
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removed on a protein A-Sepharose column by allowing it to bind to protein A. Purity of F(ab')2 or Fab fragments (flow-through) was confirmed by SDS-PAGE.

2.10 Biotin conjugation of AHN-18

CD98 mAb (AHN-18) solution dialysed against 0.1 M sodium bicarbonate buffer was subjected to biotin conjugation to prepare labelled antibody. 5 ml of mAb solution was mixed with 150 \( \mu \)l of 2 mg/ml biotin (biotin N-hydroxysuccinimide, sigma) solution dissolved in DMSO. The mixture was incubated at room temperature for 4 h with stirring. Unreacted biotin was removed by dialysis against PBS overnight.

2.11 Protein determination (Bradford method)

Protein determination was performed by using the Bradford reagent as described by the manufacturer (Bio-Rad). Eight hundred \( \mu \)l of diluted samples was mixed with 200 \( \mu \)l Bradford reagent and incubated for 10 min at room temperature. Optical density (OD) values were determined at 595 nm. Bovine serum albumin was used as standard protein.

2.12 MTT assay (colorimetric assay) for measurement of cell viability

Cell viability was measured by standard MTT assay. 10 \( \mu \)l of MTT solution (10 mg/ml in phosphate buffered-saline) was added to each well of U937 cultures for 3 h before the end of the culture period. The cells were lysed by the addition of 15% SDS for solubilization of formazan and the OD at 570 nm (\( \text{OD}_{570-630} \)) was measured using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA, USA).
2.13 Statistical analysis

All values expressed as mean ± SEM were obtained from 3 ~ 6 observations (for one experiment). The Student's t-test for unpaired observations between control and experimental samples was carried out to evaluate statistical difference; p values of $=\langle 0.05$ were regarded as statistically significant.
Chapter 3

The functional interactions between CD98, β1-integrins, and CD147 in the induction of U937 homotypic aggregation
Abstract

CD98 is expressed on both haematopoietic and non-haematopoietic cells and has been implicated in a variety of different aspects of cell physiology and immunobiology. This study examines the functional interactions between CD98 and other adhesion molecules on the surface of the promonocyte line U937, using a quantitative assay of cell aggregation. Several of the CD98 antibodies induced homotypic aggregation of these cells, without affecting cellular viability or growth. Aggregation induced by CD98 antibodies could be distinguished from that induced by β1-integrin (CD29) ligation by lack of sensitivity to EDTA, and by increased sensitivity to deoxyglucose. Aggregation induced via CD98 and CD29 could also be distinguished by the pattern of protein tyrosine phosphorylation induced. Some CD29 antibodies partially inhibited CD98-induced aggregation, and these antibodies were neither agonistic for aggregation, nor inhibitors of β1-integrin binding to substrates. Conversely, some CD98 antibodies were potent inhibitors of CD29-induced aggregation. Antibodies to β2-integrins also partially inhibited CD98-induced aggregation. Unexpectedly, two antibodies to CD147, an immunoglobulin superfamily member whose function has remained unclear, were also potent inhibitors of both the aggregation and the protein tyrosine phosphorylation induced via CD98 ligation. The results of this study support a central role for CD98 within a multi-molecular unit which regulates cell aggregation.
3.1 Introduction

CD98 is the heavy chain (85 kD) of a cell-surface dimeric molecule found on the surface of many haematopoietic cells (Warrant et al., 1996; Lumadue et al., 1987). It is a type II integral membrane protein, with a long cytoplasmic portion of 81 amino acids. Some studies have suggested that it may act as ligand for the cell surface lectin galectin-3 (Dong and Hughes, 1997). CD98 is found covalently linked to one of several (six have been identified to date) alternative light chains, at least four of which have been identified as members of an amino acid transporter family (Verrey et al., 1999). There is also good evidence that CD98 is functionally associated with β1 integrin molecules on the cell membrane (Zent et al., 2000; Fenczik et al., 1997), although the consequences of this association remain unclear.

The most striking feature of CD98 is the extraordinary diversity of functions in which it has been implicated (Deves and Boyd, 2000). Antibodies against CD98 block the formation of cell syncitia by HIV and other viruses (Ohgimoto et al., 1996; Okamoto et al., 1997), and it appears also to play a more general role in regulation of integrin-mediated cell adhesion (Fenczik et al., 1997; Tabata et al., 1994; Chandrasekaran et al., 1999). CD98 has been implicated in haematopoietic cell differentiation, growth, transformation and apoptosis (Warrant et al., 1996; Hara et al., 1999), and recently this role has been extended to the regulation of osteoblast differentiation (Higuchi et al., 1999). CD98 also plays a role in the regulation of amino acid transport by virtue of its associated light chain, as discussed above. Most recently, the molecule has been implicated in the regulation of both antigen presenting cell function and T cell
activation (Diaz and Fox, 1998; Diaz et al., 1997). Our group's interest in CD98 arose
first because we described its presence at very high levels on the surface of human
dendritic cells Woodhead et al., 1998). Subsequently we showed that some antibodies to
this molecule could block the ability of both the U937 promonocyte line (Stonehouse et
al., 1999) and human peripheral blood derived dendritic cells (Woodhead et al., 2000) to
deliver essential co-stimulatory signals to T cells.

One central question in the biology of CD98 has been how to understand the nature of
its functional interactions with integrins and other adhesion molecules at the cell surface.
In this study we have developed and used a new quantitative assay of homotypic
aggregation of U937 cells, and we have re-examined this question in detail. Our results
confirm that there is an important interaction between CD98 and CD29 (β1-integrin),
but demonstrate that the cellular events following activation via these two molecules
can be clearly distinguished pharmacologically, biochemically and in terms of their
differential sensitivity to antibody modulation. Thus the down stream functional effects
of CD98 ligation are not mediated solely via β1-integrins. Furthermore, both the
specificity profile and kinetic data suggest that β1 integrins are involved in modulating
CD98 activity, but not in mediating the U937 homotypic adhesion. Finally, our study
identifies a new member of the CD98 functional unit, the immunoglobulin superfamily
member CD147, whose function has remained unclear hitherto. Taken together with the
previously published data on CD98, the results presented below suggest that CD98
plays a central role within a multi-molecular unit which may serve to regulate cellular
responses to changes in the tissue micro-environment.
3.2 Results.

3.2.1 Aggregation-inducing activity of CD98 antibodies is heterogeneous, and is not correlated with binding activity.

The binding of a panel of seven mAbs specific for CD98 to U937 cells is shown in Fig. 3.1A. All showed a unimodal binding profile by flow cytometry, and the level of binding was dependent on antibody concentration. The panel of mAbs was then tested for their ability to induce homotypic aggregation of U937 cells (Fig. 3.1B panels B-E, and Table 3.1). The mAb CD98-AHN-18 was the most potent inducer of aggregation; several others induced weaker aggregation (BK19.9, BU53, 4F2), while some mAbs did not induce any aggregation. An mAb known to activate signalling and homotypic aggregation through the CD29 β1-integrin chain (MEM 101A), and an mAb to CD43 (161-46), which is believed to induce aggregation via a β1-integrin independent pathway (King and Katz, 1989) were also tested in the aggregation assay. Both these mAbs did induce aggregation of the U937 cells (panels F, G), although the morphology of the clusters was different from that induced by CD98-AHN-18, as the clusters tended to be tighter and more compact. Aggregation was not induced simply by the presence of mAb on the surface of U937, since mAbs to CD44, another molecule present on the surface of U937 (Freistadt and Eberle, 1997), did not induce aggregation (panel H).

The ability of the panel of CD98 mAbs to induce aggregation did not correlate with the level of binding to the U937 cell surface. To confirm this further, flow cytometry at a range of mAb concentrations was used to select a titre that gave a mean fluorescent
Fig. 3.1A CD98 mAbs differ in their ability to bind to the surface of U937 cells, and their ability to induce homotypic aggregation. A panel of seven CD98 mAbs were tested for their ability to bind U937 cells using flow cytometry. All mAbs were tested over a wide range of concentrations (1-30 µg/ml), and the histograms shown in the figure are those obtained at saturating doses of mAb (between 1 and 30 µg/ml).
Fig. 3.1B CD98 mAbs differ in their ability to bind to the surface of U937 cells, and their ability to induce homotypic aggregation. U937 cells were incubated with mAbs (at dilutions which induced maximal aggregation in each case, between 0.3 and 1.5 μg/ml in each case) for 6 h, as described in methods. Images of cells in culture at this time point were obtained using an inverted phase contrast microscope, attached to a video camera, and captured using NIH image software. A. Medium alone. B. CD98-AHN-18 (CD98). C. BU53 (CD98). D. BU89 (CD98). E. 4F2 (CD98). F. MEM 101A (CD29). G. 161-46 (CD43). H. El/2 (CD44).
Table 3.1 Lack of correlation between cell surface binding and aggregation-inducing activity of CD98 mAbs.

<table>
<thead>
<tr>
<th>MAb</th>
<th>Cell surface binding</th>
<th>Aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHN-18.1</td>
<td>134 ± 0.6</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>AHN-18</td>
<td>159 ± 19</td>
<td>54 ± 9</td>
</tr>
<tr>
<td>BK19.9</td>
<td>152 ± 10</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>BU53</td>
<td>200 ± 4</td>
<td>32 ± 6</td>
</tr>
<tr>
<td>BU89</td>
<td>184 ± 5</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>MEM108</td>
<td>193 ± 1</td>
<td>11 ± 6</td>
</tr>
<tr>
<td>4F2</td>
<td>108 ± 2</td>
<td>20 ± 2</td>
</tr>
</tbody>
</table>

U937 cells were incubated with the CD98 mAbs shown at a wide variety of concentrations (between 0.5 and 30 µgm/ml), and tested both for binding to U937 and for the ability to induce aggregation. The data shown in the Table 3.1 were selected so as to show aggregation at a concentration that gave comparable cell surface binding, with MFI between 100-200. Binding to the U937 cells was measured by indirect immunofluorescence and flow cytometry. MFI was calculated using WIN-MDI software on a minimum of 5000 cells. Aggregation at 6 h was measured using the quantitative assay described in Methods. The results are shown as mean for triplicate cultures ± SEM.
channel number (MFI) of between 100 and 200. Aggregation at this titre was then measured using the quantitative assay. As shown in Table 3.1, aggregating activity is clearly independent of binding activity, and aggregation levels vary widely even when the concentrations of mAb used were chosen to give comparable levels of binding to the U937 cells.

The characteristics of CD98-AHN-18 induced aggregation, as well as binding, were examined in more detail (Fig. 3.2). Binding increased in a linear fashion for six hours and then reached a plateau. In contrast, aggregation induced via CD29 (MEM 101A) or CD43 (161-46) was much more rapid (Fig. 3.2A). The quantitative level of CD98-AHN-18 induced aggregation was dose dependent, but was maximal at sub-saturating doses of bound mAb. Further increase in mAb concentration above this optimal level resulted in a lesser degree of aggregation (Fig. 3.2B). F(ab')2 fractions of CD98-AHN-18 also induced aggregation (Fig. 3.2B). The presence of high levels of human or rabbit Ig did not inhibit clustering (data not shown). In contrast, Fab fragments of CD98 failed to induce any clustering.

3.2.2 CD98-induced aggregation does not lead to cell death.

Since a previous study (Warrant et al., 1996) had suggested that CD98 triggering could induce cell death, we tested whether the CD98 mAb AHN-18 also caused killing of U937. As shown in Fig. 3.3, CD98-AHN-18 did not induce decrease MTT reduction at 48 h (Fig. 3.3A). Furthermore, CD98-AHN-18 did not appear to cause growth arrest of the U937 cells, since cell numbers during 48 h in culture increased to the same amount in both treated and control groups (Fig. 3.3B).
Fig. 3.2  Time and dose dependency of aggregation induced by CD98-J3/AHN-18. A) U937 cells were incubated with CD98-AHN-18 (1 µg/ml), MEM 101A (CD29, 0.3 µg/ml) or 161-46 (CD43, 0.3 µg/ml) for various times as shown. Aggregation was measured as described in Methods. The results show mean aggregation from triplicate cultures for one representative experiment out of 2. B) U937 cells were incubated with CD98-AHN-18 or fragments of CD98-AHN-18 at different concentrations for 6 h. Aggregation and binding were measured as for Fig. 3.1. The results show mean aggregation ± SEM for triplicate cultures for one representative experiment out of 2.
**Fig. 3.3** **CD98-AHN-18** mAb does not induce apoptosis of U937 cells, or inhibit cell division. A) U937 cells \((5 \times 10^5 \text{ cells/ml})\) were incubated in the presence of different concentrations of CD98-AHN-18 mAb or isotype control in complete medium. MTT reduction was measured after 48 h. Results are expressed as % OD (mean ± SEM for triplicate cultures), relative to OD in the absence of mAb. The isotype control significantly stimulated MTT reduction at the highest concentration \((p < 0.01)\), but all other values did not differ significantly from control. B) U937 cells \((5 \times 10^5 \text{ cells/ml})\) were seeded into 96 well plates, and incubated for 24 or 48 h in the presence of CD98-AHN-18 mAb \((1.5 \mu g/ml)\) or an isotype control. Cells were harvested and counted using trypan blue staining and light microscopy. The results show mean ± SEM for triplicate cultures. No value differed significantly from control.
3.2.3 CD98-induced aggregation, but not CD29 induced aggregation is resistant to EDTA but sensitive to deoxyglucose.

Since CD98 has been reported to associate with CD29 (β1-integrin) in the cell surface (Fenczik et al., 1997), we compared the ability of a number of inhibitors of cell function to block the aggregation induced by mAbs to these two molecules, as well as aggregation induced by CD43. As shown in Fig. 3.4A, aggregation by mAbs to CD29 and CD98 share the properties of being sensitive to colchicine, cytochalasin B, and low temperature, but insensitive to cycloheximide. EDTA blocks the ability of the activating β1-integrin mAb (MEM 101A) to induce aggregation; however, aggregation induced by the CD98 mAb AHN-18 is insensitive to the presence of EDTA. Aggregation induced by CD43 mAb, in contrast, was sensitive only to low temperature, suggesting a completely different mechanism of action for this molecule.

CD98-dependent aggregation was also much more sensitive than CD29 or CD43-dependent aggregation to inhibition by the metabolic inhibitor deoxyglucose (Fig. 3.4A). However, sensitivity to deoxyglucose was lost soon after addition of aggregating m (Fig. 3.4B), suggesting that ATP-dependence was an early step in the CD98-induced signalling pathway.

3.2.4 CD98-induced aggregation and CD29-induced aggregation are associated with distinct patterns of tyrosine phosphorylation.

Previous studies have identified tyrosine phosphorylation as a down-stream event in signalling by both β1-integrins (Meng et al., 1998) and CD98 (Tabata et al., 1997). As
Fig. 3.4 Metabolic inhibitor profile for CD98-, CD29- and CD43-induced aggregation. A) Aggregation was measured under standard conditions as described in Methods, in the presence of CD98-AHN-18 (1.5 μg/ml), MEM 101A (CD29, 0.3 μg/ml) and 161-46 (CD43, 0.3 μg/ml). The inhibitors were added 1 h prior to the mAb and remained in the culture. Results are expressed as aggregation relative to control cultures in the presence of each aggregating antibody, but absence of inhibitor (mean ± SEM, triplicate cultures). All values which differ significantly from controls (p < 0.01) are indicated by an asterisk. B) As for A) but the deoxyglucose (10 mM) was added either before, simultaneously or after CD98-AHN-18. Aggregation in all cases was measured at 6 h. Results are expressed as aggregation relative to control cultures in the presence of each aggregating antibody, but absence of inhibitor (mean ± SEM, triplicate cultures).
shown in Fig. 3.5, a comparison of the pattern of phosphotyrosine proteins induced following activation of the U937 cells with CD98-AHN-18 is similar, but distinct to that induced by the activating CD29 mAb MEM 101A. In particular, CD98-AHN-18, but not MEM 101A, induces a rapid strong phosphorylation of a 72 kD band. Both mAbs induced bands at 114 and 155 kD.

3.2.5 Reciprocal cross-inhibition by antibodies to CD98 and CD29.

In order to probe further the interaction between CD29 and CD98 in the induction of U937 aggregation, the abilities of blocking mAbs to each of these two molecules to inhibit aggregation induced by the other was tested. As shown in Fig. 3.6A two non-aggregating CD98 mAbs, MEM108 and BU89 strongly inhibited CD98-AHN-18 induced aggregation. At the same concentration, CD98-MEM108 showed no inhibition of aggregation induced by CD29 agonist mAb MEM 101A, while CD98-BU89 showed a significant, but partial inhibition (panel B). In the reciprocal experiment, two inhibitory mAbs to CD29, P5D2 and MAR4 showed strong inhibition of CD29-induced (MEM 101A) aggregation (panel E). P5D2, which almost totally blocked the agonist action CD29-MEM 101A, and is known to block binding of β1-integrin dimers to fibronectin (Fenczik et al., 1997; Wayner et al., 1993) did not have any inhibitory activity against CD98-AHN-18 induced aggregation, and indeed reproducibly enhanced the aggregation observed (panel D). The other CD29 blocking mAb, MAR4 (which does not block binding of β1-integrins to fibronectin (Tanaka et al., 1997)) showed a small but reproducible inhibition of CD98-AHN-18 induced aggregation (Panel D).

None of the blocking mAbs to either CD98 or CD29 tested had any inhibitory effect on CD43-induced aggregation (Panels C and F).
Fig. 3.5 Ligation of CD98 or CD29 induces rapid and distinct patterns of tyrosine phosphorylation. U937 cells (5 x 10^6 cells/ml) were incubated in the presence of CD98-AHN-18 (1.5 μg/ml, panel A) or MEM 101A (CD29, 0.5 μg/ml, panel B) for various time periods. Cells were lysed and analysed for phosphotyrosine proteins as described in Methods. Molecular weights of the major phosphorylated species are shown on the left, and were calculated by image analysis of the Western blot, using molecular weight standards to calibrate the software.
Fig. 3.6 Cross-inhibition of U937 homotypic aggregation between CD98 and CD29 mAbs. Aggregation was measured under standard conditions in the presence of CD98-AHN-18 (1.5 μg/ml, left panels), MEM 101A (CD29, 0.3 μg/ml, middle panels) or 161-46 (CD43, 0.3 μg/ml, right panels). Blocking mAbs were added to U937 cultures 1 h prior to the aggregating mAbs. Blocking mAbs used were to CD98 (panels A, B and C) or to CD29 (panels D, E and F). Results are expressed as % aggregation relative to the aggregation in the absence of blocking mAb (column labelled 0 in each panel). Means which differ significantly from control (absence of inhibitory mAb, p < 0.05) are shown with an asterisk. Antibodies were tested at a series of dilutions, starting with the most dilute and increasing in two fold steps along the x-axis. To obtain the actual concentration of mAb at each point (in μg/ml), the x-axis value should be multiplied by 0.6 for BU89 (CD98, empty bars), 0.3 for MEM108 (CD98, solid bars), 1.25 for P5D2 (CD29, empty bars) and 0.5 for MAR4 (CD29, solid bars).
3.2.6 Inhibition of CD98-induced homotypic aggregation by antibodies to β2 integrins.

Previous studies have implicated β2, as well as β1 integrins, in mediating CD98-induced adhesion. As predicted, blocking mAbs to CD18, the β2-integrin chain, partially blocked aggregation induced via CD98 (Fig. 3.7 top panel), but not via CD29 (middle panel B) or via CD43 (bottom panel). Both antibodies, at the same concentrations, showed strong inhibition of aggregation induced by PMA (not shown). A panel of ICAM-1 and -2 and -3 (CD54, CD102, CD50,) mAbs were also tested, but none inhibited aggregation induced by CD98 (not shown).

U937 cells express relatively little β2-integrin at their cell surface, and the majority is retained within intracellular vesicles. One mechanism of action for CD98 might therefore be to cause a redistribution of CD18 to the cell surface. The cell surface levels of both β1 and β2 integrin chains were therefore measured following CD98 ligation, using directly fluoresceinated anti-CD18 or CD29 mAbs, and flow cytometry. The levels of expression of CD29 and CD18 were unchanged, however, following CD98-AHN-18 induced aggregation (Fig. 3.7B).

3.2.7 Inhibition of CD98-induced homotypic aggregation by antibodies to CD147.

Since inhibition by integrin mAbs was only partial, we tested other mAbs previously shown to be expressed on U937 cells (Stonehouse et al., 1999) for the ability to block CD98-induced aggregation. Unexpectedly, the only significant inhibition was by two mAbs to CD147 (Fig. 3.8A left panel, and Fig. 3.8B). The CD147 mAbs also significantly inhibited aggregation induced via CD29 (Fig. 3.8A middle panel). Neither
CD147 mAbs inhibited aggregation induced via CD43 (Fig. 3.8A left panel). Addition of CD147 mAb together with CD18 mAb, CD29 mAb or all three together did not result in further inhibition over the level observed using each antibody independently (data not shown).

In order to establish whether CD147 was involved in the induction (signalling) phase of CD98-induced aggregation, the influence of CD147 mAb on CD98-AHN-18 induced tyrosine phosphorylation was examined (Fig. 3.9). The presence of CD147 antibody, as well as the blocking CD98 mAb BU89, almost completely blocked phosphorylation of the 72 and 155 kD band induced by CD98-AHN-18 ligation (lanes 3 and 5). Phosphorylation of the band(s) at around 114 kD was less strongly inhibited. CD147 also partially inhibited CD29-induced tyrosine phosphorylation (lanes 4 and 6). CD147 ligation alone did not induce any changes in protein tyrosine phosphorylation (Fig. 3.9 lane 2). CD147 mAbs did not alter the level of CD98 expression when added either 4, 9 or 24 h before staining (data not shown).

Finally, to try to distinguish further between induction and effector (cell-cell binding) phases of the CD98-AHN-18 induced homotypic aggregation, the ability of the various mAbs to block aggregation was tested when added either prior to, simultaneously or after addition of the agonist antibody CD98-AHN-18 (Fig. 3.10). CD29 mAb MAR4 blocked CD98-AHN-18 induced aggregation when added 1.5 h before addition of CD98-AHN-18, but not when added simultaneously or 1.5 h afterwards. In contrast, mAbs to CD98 itself, to CD18 and to CD147 inhibited to the same extent whether added before CD98-AHN-18 or up to 1.5 h afterwards.
Fig. 3.7A Functional interactions between CD98 and β2 integrins (CD18). Aggregation was measured under standard conditions in the presence of CD98-AHN-18 (1.5 µg/ml, top panel), MEM 101A (CD29, 0.3 µg/ml, middle panel) or 161-46 (CD43, 0.3 µg/ml, bottom panel). Blocking mAbs to CD18 were added to U937 cultures 1 h prior to the aggregating mAbs. Results are expressed as % aggregation relative to the aggregation in the absence of blocking mAb (column labelled 0 in each panel). Means which differ significantly from control (absence of inhibitory mAb, p < 0.05) are shown with an asterisk. mAbs were tested at a series of dilutions, starting with the most dilute and increasing in two fold steps along the x-axis. To obtain the actual concentration of mAb at each point (in µg/ml), the x-axis value should be multiplied by 0.5 for both CLB-LFA1 (CD18, empty bars) and BU86 (CD18, filled bars).
Fig. 3.7B  CD98 ligation does not alter cell surface levels of CD18 or CD29. U937 cells were incubated in the presence of CD98-AHN-18 (1.5 μg/ml) or control for 24 (top panel) or 48 (bottom panel) hours. Levels of cell surface CD18 or CD29 were measured by flow cytometry as described in Methods. Note that the two fluorescence histograms obtained in the presence and absence of CD98 are almost coincident. Histograms marked C are the fluorescence profile of CD98-treated cells stained with isotype control.
Fig. 3.8A  CD147 mAbs block aggregation induced via CD98.

Aggregation was measured under standard conditions in the presence of CD98-AHN-18 (1.5 μg/ml, left panel), MEM 101A (CD29, 0.3 μg/ml, middle panel) or 161-46 (CD43, 0.3 μg/ml, right panel). Blocking mAbs to CD147 were added to U937 cultures 1 h prior to the aggregating mAbs. Results are expressed as % aggregation relative to the aggregation in the absence of blocking mAb (column labelled 0 in each panel). Means (mean ± SEM, triplicate cultures) which differ significantly from control (absence of inhibitory mAb, p < 0.05) are shown with an asterisk. mAbs were tested at a series of dilutions, starting with the most dilute and increasing in two fold steps along the x-axis. To obtain the actual concentration of mAb at each point (in μg/ml), the x-axis value should be multiplied by 1.2 for MEM M6/1 (CD147, empty bars) and 1.1 for H84AF (CD147, filled bars).
Fig. 3.8B CD147 mAbs block aggregation induced via CD98. U937 cells were incubated in medium alone (A), CD98-AHN-18 (1.5 μg/ml) (B), or CD98-AHN-18 and MEM M6/1 (CD147, 1.2 μg/ml) (C) for 6 h, as described in Methods. Images of cells in culture at this time point were obtained using an inverted phase contrast microscope, attached to a video camera, and captured using NIH image software.
Fig. 3.9 CD147 regulates tyrosine phosphorylation induced via CD98 or CD29 ligation. A) U937 cells (5 × 10^6 cells/ml) were incubated in the presence of CD98-AHN-18 (1.5 µg/ml), MEM 101A (CD29, 0.5 µg/ml), MEM M6/1 (CD147, 2.5 µg/ml), BU89 (CD98, 2.3 µg/ml) or combinations of these mAbs for 20 min. Cells were lysed and analysed for phosphotyrosine proteins as described in Methods. Molecular weights of the major phosphorylated species are shown on the left, and were calculated by image analysis of the Western blot, using molecular weight standards to calibrate the software. Lane 1 – isotype control; lane 2 – MEM M6/1 (CD147); lane 3 – CD98-AHN-18; lane 4 – MEM 101A (CD29); lane 5 – CD98-AHN-18 + CD147; lane 6 – MEM101A (CD29) + CD147; lane 7 – BU89 (CD98); lane 8, CD98-AHN-18 + BU89 (CD98). B) The relative intensities of the major phosphotyrosine species in bands 3, 5 and 8, calculated using gel image analysis software.
Fig. 3.10 Sensitivity of CD98 induced aggregation to blocking antibodies added at different times. U937 cells aggregation was measured under standard conditions in the presence of CD98-AHN-18 (1.5 µg/ml). mAbs to CD29 (MAR4, 0.5 µg/ml), CD98 (BU89, 1.2 µg/ml), CD147 (MEM M6/1, 1.6 µg/ml) and CD18 (CLB-LFA1, 1.5 µg/ml) were added to the culture before, together with or after the aggregating mAb as shown. Results are expressed as % aggregation relative to the aggregation in the absence of blocking mAb (column labelled 0 in each panel). Means which differ significantly from control (absence of inhibitory mAb, p < 0.05) are shown with an asterisk.
3.3 Discussion

The diversity of responses in which the 85 kD type II membrane protein, CD98, has been implicated supports the notion that the molecule probably plays a key role in cell–cell interaction and signalling, but (perhaps because of CD98 diversity and ubiquity) this role remains poorly understood. Our recent studies identified CD98 as a major component of the human dendritic cell surface (Woodhead et al., 1998), and confirmed earlier reports (Diaz et al., 1997) that it is involved in T cell co-stimulation (Stonehouse et al., 1999; Woodhead et al., 2000). During the course of these studies, however, we also confirmed previous reports (Tabata et al., 1994) that some CD98 mAbs induced homotypic aggregation of the U937 cell line, and we focused on this model system to dissect the molecular basis of CD98 function, because of the advantages of working with a uniform cell line rather than a mixed population of T cells and antigen presenting cells.

A reproducible quantitative assay of U937 homotypic aggregation was developed as a prelude to pharmacological and molecular dissection of CD98 function, and the results from this assay form the basis for the present study. Using this assay, it was clear that CD98 mAbs are highly heterogenous both in function and ability to bind to CD98 on the U937 cell surface (Fig. 3.1). It seems unlikely that this heterogeneity reflects simply concentration or affinity of the mAbs used, since each mAb was tested over a wide range of concentrations. The heterogeneity is very reminiscent of aggregation induced by anti-β1 integrin mAbs (Tanaka, 1997), and presumably reflects the presence of specific conformational epitopes on the CD98 molecule, only some of which are
involved in stimulating the down-stream signalling cascade which leads to aggregation. A cross-linking event, rather than a direct physical effect of antibody-binding to CD98 is also suggested by the requirement for divalent F(ab')2 fragments, and by the lack of correlation between mAb binding levels and aggregation. Indeed very high levels of CD98 occupancy, by either intact mAb or F(ab')2 fragments, result in lower levels of aggregation, perhaps because cross-linking by mAb becomes less efficient under conditions of mAb excess.

The next question we addressed, using the same assay, was the relationship between CD98 and other cell surface molecules thought to have a role in cell-cell interaction. The functional association between CD98 and CD29 (β1 integrin) in the cell membrane (Fenczik et al., 1997; Warren et al., 2000) is well-documented, and has led to the suggestion that CD98 signalling into the cell is in fact mediated via integrin activation. No clear biochemical data showing interaction between CD98 and CD29 in the cell membrane has been published, although a recent study has shown that CD98 can associate with isolated cytoplasmic portions of some β1 integrin isoforms (Zent et al., 2000). The results from the present study support the general hypothesis that there is a close link between β1 integrin and CD98 function, but not the suggestion that CD98 functions simply by cross-linking β1 integrins. Specifically, although aggregation by both CD29 mAb and CD98 mAb share many properties, such as a requirement for an intact cytoskeleton, and although there is some cross-inhibition by mAbs to the two molecules, significant differences between the two pathways exist. Thus β1-integrin mediated aggregation is completely abrogated by EDTA, reflecting the fact that both integrin chains require divalent cations for activity. In contrast, CD98-induced
aggregation is EDTA insensitive. An intact integrin heterodimer cannot therefore be an essential intermediate for CD98-induced signalling in these cells. Furthermore, the initial phase of signalling via CD98 appears to be much more sensitive to the metabolic inhibitor deoxyglucose, again suggesting a pathway distinct from that induced via CD29. Finally, the pattern of protein tyrosine phosphorylation induced via CD98 appears to be quite distinct from that induced via CD29, though some substrates of phosphorylation appear to be shared between the two pathways. The identity of the phosphorylated proteins remains unknown, although some likely candidates, including FAK, vinculin and CD29 itself have been ruled out (Tabata et al., 1997). CD98 itself is also not tyrosine phosphorylated ((Tabata et al., 1997) and own unpublished data), and indeed its sequence does not contain any known tyrosine phosphorylation motif.

Several features of the CD29/CD98 results presented in this paper suggest that CD29 molecules play a role in the inductive phase of the response, rather than mediating the actual cell-cell adhesion. Firstly, the CD29 mAb which inhibited aggregation induced via CD98 is not the same as that which blocks the ability of β1-integrins to bind their ligand (e.g. fibronectin) in other systems. For example, the mAb P5D2 blocks aggregation induced by the agonist CD29-directed mAb MEM 101A, and also blocks the ability of β1-integrins to bind extracellular substrates (Fenczik et al., 1997; Wayner et al., 1993), but does not influence CD98-induced aggregation at all. Conversely, MAR4 which does not block binding of β1 integrin dimers to extracellular substrates (Tanake, 1997) does inhibit CD98-induced aggregation. Secondly, MAR4, the CD29 mAb which does block CD98-induced aggregation does so when added prior to CD98, but not after CD98, even though significant aggregation does not occur until 2-3 h post-
activation. These results suggest that mAb blocking may reflect interference in CD98/CD29 interaction in the membrane, rather than reflecting a block of CD29/ligand interaction in mediating the cell-cell binding event itself.

In contrast, mAbs to β2-integrins block aggregation even when added after CD98, consistent with a role in the actual cell-cell binding step. A role for β2-integrins in mediating both homotypic (Hewison et al., 1994) and heterotypic (King and Katz, 1989) aggregation is now well-documented. However, in comparison to other systems, the role of the β2 integrins here seems rather minor, since aggregation is blocked only by approximately 20-30%. Furthermore, no significant inhibition of aggregation was observed by ICAM-1, 2, or 3 mAbs, perhaps because of significant overlap in the function of these molecules as β2 integrin ligands.

The blocking of CD98-induced aggregation by CD147 was an unexpected finding. CD147 is a member of the immunogloubulin superfamily, originally believed to be involved in blood-brain barrier function (Schlosshauer, 1993). However, CD147 knockouts did not reveal any defect in this function, but showed some behavioural and immunological abnormalities (Igakura et al., 1996). The ligand (if such a molecule exists) for CD147 has not been described, and hence the partner for its role in mediating cellular aggregation remains unknown. Most intriguingly, however, recent reports have identified some striking parallels between CD147 and CD98. CD147 associates physically with β1-integrins in the membrane (Berditchevski et al., 1997), as does CD98 with isolated cytoplasmic β1 domains (Zent et al., 2000). Antibodies to both molecules can induce aggregation of U937 cells, and the aggregation appears to be
mediated in part by β2-integrin activation (Kasirnerk et al., 1999). Levels of CD98 and CD147 correlate on T cells, with high levels in the thymus, low in resting mature T cells, and higher levels on activated mature T cells (Kirsch et al., 1997). Finally, and most intriguingly it appears that CD147, like CD98 is acting as a chaperone for multi-membrane spanning transporter molecules. In the case of CD98 these are amino acid transporters (Pfeiffer et al., 1999); in the case of CD147 they are the MCT family of proton-linked monocarboxylic acid transporters (Halestrap and Price, 1999).

In CD98-mediated U937 aggregation it is unclear whether CD147 is acting as an ancillary adhesion molecule, or, alternatively mediates the aggregation event itself. The inhibitory action of CD147 is manifest even when antibody is added 1.5 h after CD98-AHN-18, consistent with a possible role for CD147 in mediating the cell-cell binding. However, an alternative hypothesis for this data is that continuous signalling via CD98 is required to produce aggregation, and that CD147 interferes with this signalling event, or even sends negative signals that oppose CD98-induced changes. This model is consistent with the observation that CD147 ligation profoundly inhibits the tyrosine phosphorylation induced by CD98-AHN-18 ligation.

The data presented above, taken together with previous data on this pleiotropic molecule, suggest that CD98 is a central component within a multi-molecular complex which can regulate outcomes as diverse as adhesion, growth, differentiation and antigen presentation. This data prompts us to suggest a speculative model which links CD98 induced aggregation, the central role of β1 integrins in CD98 function, the involvement of CD147, and the curious structural parallels between this molecule and CD98 (Fig.
3.11). This envisages that CD98 forms one component of a “sensory complex”, containing β1 integrins, CD98 and CD147, together with all their associated transporter molecules. The complex induces signals, perhaps via the transporter molecules themselves, to regulate multiple aspects of cell physiology. Parameters which would regulate the function of such a complex may include extracellular matrix, levels of amino acids and levels of carboxylic acids (e.g. lactic acid) which are found in their environment. Work is therefore in progress to dissect how this complex might function at a molecular level.
Fig. 3.11 Model of possible functional complex containing CD98 (heavy and light chains, β1-integrins (α/β chains) and CD147 (heavy and light chains). The figure shows the 3 components of the CD98 signalling complex and their topology within the plasma membrane.
CD98 regulates mitogen activated protein kinases and adhesion via the selective activation of protein kinase Cδ
Abstract

CD98 is a protein found on the surface of many activated cell types, and is implicated in the regulation of cellular differentiation, adhesion, growth and apoptosis. Despite many studies addressing CD98 function, there is little information on the intracellular signalling pathways that mediate its activity. In this study, we examine the major signalling pathways which are activated following ligation by the CD98 antibody AHN-18, an antibody which induces U937 homotypic aggregation, and inhibits antigen presenting activity and T cell activation. Pharmacological and biochemical analysis, using a quantitative assay of U937 aggregation, demonstrate that CD98 ligation induces a selective membrane translocation of the novel PKCδ isoform, as an essential step in mediating U937 homotypic aggregation. PKCδ activation in turn mediates activation of ERK1/2 and p38, as well as tyrosine phosphorylation of multiple proteins, but only MAPK activation is essential for cellular aggregation. One of the targets of CD98 induced tyrosine phosphorylation is itself PKCδ, suggesting that this phosphorylation may act as a negative feedback to limit the overall activation of the CD98 pathway.
4.1 Introduction

CD98 is the heavy chain (85 kD) of a dimeric molecule found on the surface of many haematopoietic cells (Warren et al., 1998; Lumadue et al., 1987). It is a type II integral membrane protein, with a long cytoplasmic portion of 81 amino acids. Some studies have suggested that it may act as ligand for the cell surface lectin galectin-3 (Dong and Hughes, 1997). CD98 is found covalently linked to one of several alternative light chains. Thus far six have been identified, of which at least four are members of an amino acid transporter family (Verry et al., 1999). CD98 is functionally associated with both β1 integrin molecules (Zent et al., 2000), and the immunoglobulin superfamily member CD147 on the cell membrane (Cho et al., 2001; Fenczik et al., 1997). The most striking feature of CD98 is the extraordinary diversity of functions in which it has been implicated (Deves and Boyd, 2000). Antibodies against CD98 block the formation of cell syncitia by HIV and other viruses (Ohgimoto et al., 1996; Okamoto et al., 1978), and it appears also to play a more general role in regulation of integrin-mediated cell adhesion (Fenczik et al., 1997; Tabata et al., 1994; Chandrasekaran et al., 1999). CD98 has been implicated in haematopoietic cell differentiation, growth, transformation and apoptosis (Warren et al., 1996; Hara et al., 1999), and this role has been extended to the regulation of osteoblast differentiation (Higuchi et al., 1999). CD98 also plays a role in the regulation of amino acid transport by virtue of its associated light chain, although this function, in contrast to its association with integrins in the membrane, is mediated principally via the extracellular portion of the molecule. Most recently, the molecule has been implicated in the regulation of both antigen presenting cell function and T cell activation (Diaz et al., 1998; Diaz et al., 1997). CD98 is present at very high levels on
the surface of human dendritic cells (Woodhead et al., 1998), and our group showed that some antibodies to this molecule could block the ability of both the U937 promonocyte line (Stonehouse et al., 1999) and human peripheral blood derived dendritic cells (Woodhead et al., 2000) to deliver essential co-stimulatory signals to T cells.

Despite the multitude of studies addressing CD98 function, there is little information on the intracellular signalling pathways which underlie the extraordinary diversity of function of this molecule. A few studies have documented tyrosine protein phosphorylation following CD98 ligation (Warren et al., 1996; Tabata et al., 1997), but there is limited evidence linking this to downstream functional outcomes. In this study, we examine the major signalling pathways which are activated following ligation of CD98 by the CD98 mAb AHN-18, an antibody which we previously selected as the most potent activator of U937 homotypic aggregation (Cho et al., 2001), as well as being the most potent inhibitor of antigen presenting activity and T cell activation. Pharmacological and biochemical analysis, using a quantitative assay of U937 homotypic aggregation which we described previously (Cho et al., 2001), demonstrate that CD98 ligation induces a very selective membrane translocation of the novel PKC6 isoform, as an essential step in mediating U937 homotypic aggregation. PKC6 activation in turn mediates activation of ERK1/2 and p38, as well as tyrosine phosphorylation of multiple proteins, but only MAPK activation is essential for cellular aggregation. Interestingly, one of the major downstream targets of CD98 induced tyrosine phosphorylation is itself PKC6, suggesting that this phosphorylation may be part of a negative feedback loop which limits the overall activation of the CD98 pathway. This study suggests that selective regulation of PKC6 is a key intracellular
Target of the CD98 signalling complex.
4.2 Results.

4.2.1 Protein tyrosine phosphorylation induced by CD98 ligation is not essential for U937 homotypic aggregation.

The addition of CD98-AHN-18 mAb (or F(ab’)2 fragment, not shown) to U937 cells induced strong homotypic clustering (Fig. 4.1A) as described previously (Cho et al., 2001). The extent of clustering was dependent on the concentration of mAb, and on time, with maximum clustering observed after 3-6 h (Cho et al., 2001). In parallel, CD98-AHN-18 induced rapid tyrosine phosphorylation of multiple proteins. The addition of genistein (Fig. 4.1B) almost completely blocked tyrosine phosphorylation, in a dose dependent manner. However, neither genistein (Fig. 4.1C, left panel) nor herbimycin A (Fig. 4.1C, right panel), nor A77,1726 (not shown), all inhibitors of tyrosine phosphorylation, blocked CD98 dependent clustering. Both genistein and herbimycin A enhanced U937 clustering at lower concentrations.

4.2.2 Protein kinase C activity is necessary for CD98-induced aggregation.

Since CD98 mediated aggregation did not require protein tyrosine phosphorylation, a number of inhibitors of serine/threonine kinase activity were tested in this model. Two broad specificity serine/threonine kinase inhibitors, H-89 and H-7, both blocked aggregation at concentrations of 10 (H-89) or 50 (H-7) μM respectively (Fig. 4.2A). Two major classes of protein serine/threonine kinases are the PKA and PKC families. The selective PKA inhibitor KT5720, however, did not block CD98-induced aggregation, and indeed enhanced aggregation (Fig. 4.2B). Furthermore, forskolin, a potent inducer of cAMP, which itself activates PKA, inhibited CD98-induced
aggregation. The pharmacological data therefore suggest that PKA plays a negative, rather than positive role in CD98 signal transduction.

In contrast, the broad specificity PKC inhibitors, chelerythrine chloride and GF109203X both completely inhibited CD98-mediated aggregation (Fig. 4.2C). Chelerythrine chloride was a potent inhibitor, showing maximal inhibition at concentrations of 3 μM and above. In contrast, GF109203X showed a biphasic dose response, inducing enhanced aggregation at concentrations of 10 μM or below, but inhibiting aggregation at concentrations of 80 μM.

The involvement of PKC family members was dissected further in Fig. 4.3. Two selective inhibitors of the classical Ca\(^{2+}\) dependent PKC isoforms (α,β and γ), Go6976 and 19-27, showed no inhibitory activity. 19-27, a pseudosubstrate peptide which very specifically blocks the α and β isoforms, enhanced aggregation at concentrations of 25 μM or above. In contrast, aggregation was inhibited by low concentrations of rottlerin, an inhibitor which has been reported to show 10 fold or more selectivity in vitro for the PKCδ isoform over other PKC isoforms (although recent studies have suggested that other serine/threonine kinases in the cell may also be inhibited (Davies et al., 2000)) (Fig. 4.3B, C and D). Rottlerin blocked aggregation when added prior to, or together with CD98-AHN-18, but did not block aggregation when added 1.5 h after addition of CD98-AHN-18 (but before aggregation occurred) (Fig. 4.3C). The rottlerin-sensitive step was thus an early signalling event in the aggregation process. Rottlerin also strongly inhibited CD98-induced protein tyrosine phosphorylation (Fig. 4.3E), suggesting that the rottlerin-sensitive step was up-stream of tyrosine kinase activation.
Fig. 4.1 CD98-induced aggregation is independent of tyrosine phosphorylation. A) U937 cells were incubated for six hours in the presence of CD98-AHN-18 (1 μg/ml). Images of cells in culture at this time point were obtained using an inverted phase contrast microscope, attached to a video camera, and captured using NIH image software. A representative field is illustrated, showing strong U937 homotypic aggregation. B) U937 cells were incubated for 20 min in the presence or absence of CD98-AHN-18 (1 μg/ml), with or without the prior addition of genistein as shown. Cells were washed, lysed and analysed for protein tyrosine phosphorylation as described in Methods. C) U937 cells were incubated for six hours in the presence of CD98-AHN-18 (1 μg/ml), with or without the prior addition of genistein or herbimycin A as shown. Aggregation was measured as described in Methods. The results [aggregation % (% of control)] show mean aggregation ± SEM for triplicate cultures. * p < 0.01.
Fig. 4.2 CD98-induced U937 aggregation requires protein kinase C activity. U937 cells were incubated for 4 h in the presence of CD98-AHN-18 (1 μg/ml), with or without the prior addition of inhibitors as shown. A) Broad spectrum threonine/serine kinase inhibitors H-89 and H-7. B) An inhibitor of PKA, KT5720, and the cAMP agonist forskolin. C) Broad spectrum PKC inhibitors chelerythrine chloride, and GF109203X. Aggregation was measured as described in Methods. The results [aggregation % (% of control)] show mean aggregation ± SEM for triplicate cultures. * p < 0.05. ** p < 0.01.
Fig. 4.3 Rottlerin, a selective inhibitor of novel PKCδ, blocks CD98-induced aggregation and protein tyrosine phosphorylation. A) – C) U937 cells were incubated for 6 h in the presence of CD98-AHN-18 (1 μg/ml), with or without the prior addition of inhibitors as shown. Aggregation was measured as described in Methods. The results [aggregation % (% of control)] show mean aggregation ± SEM for triplicate cultures. * p < 0.01. A) Selective inhibitors of conventional Ca^{2+}-dependent PKC isoforms, Go6976 and peptide 19-27 (a peptide substrate analogue of PKCa/β. B) - D) Selective inhibitor of the novel (Ca^{2+}-independent) PKCδ isoform, rottlerin. In panel C), the inhibitor was added either 1.5 h before, or together with, or 1.5 h after CD98-AHN-18. Aggregation was measured after 4 h. In panel D) images of cells in culture at 4 h were obtained using an inverted phase contrast microscope, attached to a video camera, and captured using NIH image software. E) U937 cells were incubated for 20 min in the presence or absence of CD98-AHN-18 (1 μg/ml), with or without the prior addition of rottlerin as shown. Cells were washed, lysed and analysed for protein tyrosine phosphorylation as described in Methods.
Rottlerin did not block U937 aggregation induced by mAbs to CD43 (Cho et al., 2001), suggesting the inhibitory effect was not a generalised effect on U937 viability, or aggregation (data not shown), but was dependent on CD98 ligation.

4.2.3 CD98 ligation selectively induces PKCδ membrane translocation.

The activation of PKC enzymes is frequently associated with translocation of the enzyme from a soluble to a membrane bound form. The relative distribution of eight PKC isoforms between cytoplasm and membrane following CD98 ligation is shown in Fig. 4.4A. A proportion of several PKC isoforms was found in the membrane fraction even before stimulation, perhaps reflecting the transformed phenotype of the U937 cell line. However, CD98 ligation induced a very selective additional translocation of the nPKCδ isoform, while the distribution of all other isoforms tested remained unchanged. In contrast, PMA, which is a potent agonist of many cPKC and nPKC isoforms, induced translocation of PKCα, β, and γ (all the conventional isoforms) but not δ (not shown). PKCδ translocation was not a general effect of U937 aggregation, since antibodies to CD43, another cell surface molecule which induces U937 aggregation via different signalling pathways (Cho et al., 2001), does not affect PKCδ translocation (not shown). Rottlerin, which inhibited aggregation as shown in Fig. 4.3C above, also abolished the translocation of PKC δ (Fig. 4.4B), consistent with its known inhibitory activity against PKCδ.

4.2.4 CD98 induced aggregation requires downstream activation of ERK and p38 MAPkinases.

CD98 ligation induced rapid phosphorylation of both ERK1 and ERK2, and a slower
**Fig. 4.4** CD98-ligation induces a selective membrane translocation of PKCδ. **A)** U937 cells were incubated for 20 min in the presence (right hand lanes) or absence (left hand lanes) of CD98-AHN-18 (1 µg/ml). Cells were washed, lysed and fractionated into membrane and cytoplasmic fractions, as described in Methods. Whole lysate (W), membrane (M), and cytoplasmic (C) fractions were analysed for the presence of specific PKC isoforms by Western blot as described in Methods. The right hand panel shows the the relative intensities of membrane associated PKCδ bands in the presence or absence of CD98-AHN-18, calculated using Syngene gel image analysis software. The results from three separate experiments are shown. **B)** As for A) above, but rottlerin (10 µM) was added to some cultures 30 min prior to CD98-AHN-18, as shown.
Phosphorylation of p38, but no change in levels of phosphorylated JNK (Fig. 4.5A). ERK phosphorylation was blocked by rottlerin (Fig. 4.5B), confirming that MAPK/kinase activation is downstream of PKCδ activation. In addition, selective inhibitors of MEK (PD98059), which is required for ERK activation, and of p38 (SB203580) inhibited CD98-induced aggregation (Fig. 4.5C).

4.2.5 CD98 induced activation of PKCδ is independent of diacylglycerol, and leads to PKCδ phosphorylation.

PKCδ can be regulated by binding of DAG. However, a number of pieces of evidence suggested that CD98 activation (translocation) of PKCδ is independent of DAG formation. Firstly, calphostin C, a competitive inhibitor of the DAG/PKC interaction, did not block CD98-induced aggregation (Fig. 4.6). Secondly, inhibition of phosphatidyl choline-phospholipase C (D609 inhibitor), which catalyses the conversion of phosphatidyl choline into DAG, also did not inhibit CD98-induced aggregation (Fig. 4.6B). DAG can also be produced as a result of the cleavage of phosphatidylcholine by phospholipase D (PLD). Propranolol, which is often used as a selective blocker of phosphatidic acid phosphohydrolase, and hence an inhibitor of PLD-dependent DAG formation, did block CD98-induced aggregation (Fig. 4.6B). However, the activity of PLD appeared to be downstream of PKCδ activation, since propranolol did not block PKCδ translocation (Fig. 4.6C). In addition, propranolol blocked aggregation when added up to 1.5 h after CD98-AHN-18, consistent with a role in the late (effector) stages of aggregation. Alternative pathways of PKCδ activation involve phosphorylation of PKC, on either serine/threonine or on tyrosine. CD98 ligation appeared to induce a small increase in total levels of phosphorylated T505 PKCδ (Fig. 4.7A). The
Positive signalling pathway

Phosphorylated form is predominantly cytoplasmically localised in resting U937 cells (Fig. 4.7B), but is strongly translocated to the cell membrane following CD98 ligation.

CD98 induced ligation also caused selective tyrosine phosphorylation of PKCδ, as shown by immunoprecipitation of PKCδ, followed by blotting with anti-phosphotyrosine antibody (Fig. 4.7C). The tyrosine phosphorylation of PKCδ was strongly inhibited by genistein (data not shown), suggesting that this phosphorylation was not necessary for the induction of aggregation by PKCδ.
Fig. 4.5 CD98-induced aggregation requires PKCδ-dependent ERK and p38 activation. A) U937 cells were incubated for various times in the presence of CD98-AHN-18 (1 μg/ml). Cells were washed, lysed and analysed for ERK, p38 and JNK expression by Western blot as described in methods. The upper blots show gels probed with antibodies specific for phosphorylated forms of the kinases, while the lower blots show gels probed with antibodies which recognise both phosphorylated and non-phosphorylated forms of the kinases. B) U937 cells were incubated for 30 min in the presence or absence of CD98-AHN-18 (1 μg/ml), and in the presence or absence of rottlerin (10 μM), PD598059 (50 μM) or SB203580 (20 μM) as shown. Cells were washed, lysed and analysed for phospho-ERK, by Western blot as described in methods. C) U937 cells were incubated for 6 h in the presence of CD98-AHN-18 (1 μg/ml), with or without the prior addition of inhibitors as shown. Aggregation was measured as described in Methods. The results [aggregation % (% of control)] show mean aggregation ± SEM for triplicate cultures. ** p < 0.01.
Fig. 4.6 CD98-induced aggregation and PKCδ translocation is independent of diacylglycerol formation. A) and B) U937 cells were incubated for 6 h in the presence of CD98-AHN-18 (1 μg/ml), with or without the prior addition of inhibitors as shown. Aggregation was measured as described in Methods. The results [aggregation % (% of control)] show mean aggregation ± SEM for triplicate cultures. * p < 0.01. A) Calphostin C is a PKC inhibitor, which acts by binding to the regulatory subunit at the same site as diacylglycerol. B) D609 is a selective inhibitor of phosphoryl choline phospholipase C. Propranolol is a selective inhibitor of phosphatidic acid phosphohydrolase, and hence of phospholipase D-dependent DAG formation. C) U937 cells were incubated for 20 min in the presence or absence of CD98-AHN-18 (1 μg/ml), and the presence or absence of propranolol (50 μM). Cells were washed, lysed and fractionated into membrane and cytoplasmic fractions, as described in Methods. Membrane fractions were analysed for the presence of PKCδ isoform by Western blot as described in Methods.
Fig. 4.7  CD98 regulation of PKCδ phosphorylation. A) U937 cells were incubated for various times in the presence of CD98-AHN-18 (1 μg/ml). Cells were washed, lysed and analysed by Western blot, using an antibody specific for phospho PKCδ (T(P)505) and phospho PKCζ (T(P)560) as described in Methods. B) U937 cells were incubated for 20 min in the presence (right hand lanes) or absence (left hand lanes) of CD98-AHN-18 (1 μg/ml). Cells were washed, lysed and fractionated into membrane and cytoplasmic fractions, as described in Methods. Whole lysate (W), membrane (M), and cytoplasmic (C) fractions were analysed for the presence of phospho (T505)-PKCδ by Western blot as described in Methods. C) U937 cells were incubated for 20 min in the presence or absence of CD98. Cells were washed, lysed and immunoprecipitated using an antibody specific for PKCδ. The immunoprecipitate was solubilised and analysed by Western blot using an anti-phosphotyrosine antibody.
4.3 Discussion

CD98 forms part of a multimolecular complex on the cell surface of many activated, or transformed cell types, and has been implicated in the regulation of amino acid transport, cell growth, differentiation, death, adhesion, fusion and immunological function. The intracellular events which transmit the necessary signals from the CD98 cell surface complex to the effector molecules that regulate these various functions remain very poorly understood. In this study, we have used a model system in which CD98 ligation by certain antibodies leads to homotypic aggregation of the U937 cell line, mediated by β1 and β2 integrins. The functional properties of this model have been investigated in some detail both by our laboratory (Cho et al., 2001) and others (Tabata et al., 1997; Ohgimoto et al., 1995). An important advantage of the model is that it allows a detailed pharmacological and biochemical analysis of the underlying molecular changes which follow CD98 ligation.

Previous studies on CD98 signalling have focused almost entirely on protein tyrosine phosphorylation, which is a rapid event following CD98 ligation in a variety of systems (Cho et al., 2001 Tabata et al., 1997; Warren et al., 1996). Therefore we focused initially on the role of these signals in mediating aggregation of U937. Pharmacological data, however, clearly demonstrate that tyrosine protein phosphorylation is not necessary for this aspect of CD98 signalling. Neither genistein nor herbimycin A, both potent inhibitors of PTKs, block cell aggregation in this model. Indeed both inhibitors significantly enhanced CD98-dependent aggregation, suggesting that tyrosine phosphorylation may act as negative regulator of this signalling pathway (see below).
contrast U937 cell fusion, which can be induced by CD98 ligation in the presence of HIV gp160, does require protein tyrosine phosphorylation (Tabata et al., 1997).

Our attention turned, therefore, to the role of serine/threonine kinases in mediating CD98 signalling. Both the pharmacological and biochemical data from these experiments suggest that activation of the “novel” isoform PKCδ is a key central step in orchestrating CD98 function. Pharmacological data include the inhibition of CD98-induced clustering by rottlerin. This inhibitor has been shown in vitro to have at least a ten-fold or greater selectivity for the PKCδ isoform over others (Keenan et al., 1997), although may also inhibit other unrelated serine/threonine kinases (Davies et al., 2000). The inhibition by rottlerin was not complete, and some homotypic aggregation occurred even in the presence of rottlerin, suggesting that additional pathways may exist. The dose response curve of GF109203X, which has a high affinity for “conventional” PKC isoforms, but also inhibits “novel” PKC s at higher concentrations (Chen et al., 1999), is also consistent with this model. A parallel biochemical approach used the membrane translocation of PKCδ as an indication of PKC activation. Using this method, we showed that PKCδ is the only PKC isoform tested which was translocated following CD98 ligation in the U937 cells. The increase in membrane associated PKCδ was even more marked when an antibody recognising pThr505 PKCδ was used, suggesting a selective recruitment of the phosphorylated (activated) form to the membrane or selective phosphorylation of the membrane-bound form (see below). Finally, the pharmacological and biochemical data could be linked, since rottlerin inhibited not only U937 aggregation, but also PKCδ translocation. CD98 ligation is thus a highly selective way of regulating the activity of PKCδ, and this activation is likely to be critical not
only for the induction of U937 homotypic aggregation but for many of the other documented downstream effects of CD98 ligation. In contrast, activation of conventional PKC isotypes may be involved in negative regulation of this pathway, since the selective inhibitor of PKCα/β, as well as low concentrations of GF109203X enhanced aggregation, while PMA inhibited aggregation and PKCδ translocation (Chapter 5).

Having established that regulation of PKCδ is a pivotal step in CD98 signal transduction, we have examined both upstream and downstream steps in this pathway. Activation of MAPkinases, especially ERK and p38 isoforms, has been previously implicated both in the regulation of cellular adhesion/migration (Lu et al., 1998; Sano et al., 2001; Rousseau et al., 1997) and as a downstream event following PKCδ activation (ERK) via integrin activation (Miranti et al., 1999). A similar signalling connection is apparently involved in the CD98 response, since CD98 ligation induces a PKCδ-dependent activation of both ERK, and p38 kinases, and inhibition of either of these two MAPkinases by selective inhibitors blocks the CD98-induced aggregation. In contrast to integrin mediated signalling (Miranti et al., 1999), however, the pathway from PKCδ to ERK activation does not appear to involve tyrosine kinase activity, since the pathway is resistant to genistein. The specific downstream targets of ERK and p38 which mediate aggregation in this model have not yet been investigated in detail. However, a number of likely candidates, which regulate cytoskeletal rearrangements and integrin activity have previously been identified, including paxillin (Ku et al., 2000) and pleckstrin (Brumell et al., 1997). ERK-mediated PLD phosphorylation has also been implicated in the regulation of cellular adhesion (Sano et al., 2001). The relationship between PLD
and PKCδ is complex, since PLD can be both an upstream activator of PKCs, via the release of DAG from phosphoryl choline, and a downstream target of PKCδ (Lee et al., 2000; Siddigi et al., 2000). Our data is more consistent with a downstream role for PLD, since aggregation is blocked by the propranolol, but PKCδ translocation is not affected by propranolol. However, propranolol is likely to have other pharmacological effects, and further work is required to dissect the role of PLD in this system further.

The activation and regulation of PKCδ is extremely complex, and can involve binding of DAG to the regulatory subunit (Gschwendt, 1999), serine/threonine phosphorylation (Parekh et al., 2000) (although the significance of this phosphorylation has been questioned (Stempka et al., 1999)), and multiple tyrosine phosphorylation sites (Li et al., 1994). The activity of the enzyme is also probably modulated by binding to specific sets of receptors, or linker molecules within the cell, which may act as scaffolds for promoting enzyme/substrate interaction (Mochly-Rosen and Gorden, 1998). In the present study, CD98 regulation of PKCδ appears to be largely independent of DAG generation, since it is resistant to the DAG binding site inhibitor calphostin C, and also to phosphatidyl choline-PLC inhibition. Furthermore, PMA which is a potent DAG activator, and the DAG analogue dioctanoylglycerol, inhibits CD98 induced aggregation, and blocks PKCδ translocation (Chapter 5). Increased phosphorylation at Thr505 is also unlikely to be the major regulatory step in PKCδ regulation in this model, since resting U937 cells already contain high levels of this phosphorylated form (although there may be a small increase in the total levels of this phosphorylated form following CD98 ligation). Finally, it is interesting that PKCδ is itself tyrosine phosphorylated following CD98 ligation (this is the first identification of a CD98-induced...
Positive signalling pathway

phosphotyrosine species). It is likely that this phosphorylation is itself a regulating step, since phosphorylation of PKCδ has previously been shown to regulate function both positively and negatively Haleem-Smith et al., 1995; Deszo et al., 2001; Kronfeld et al., 2000). In the case of CD98-mediated aggregation, this phosphorylation may be part of a negative feed-back loop, since genistein totally abolished PKCδ tyrosine phosphorylation, and simultaneously enhanced aggregation.

The major steps in CD98 signalling identified in this study are depicted in Fig. 4.8. Ligation of CD98 (by antibody in this study, but by interaction with appropriate counter-receptor in vivo), leads to a rapid recruitment of PKCδ to the membrane. An analogous model has been proposed for the events which follow cross-linking of the IgEγ chain in mast cells (Haleen-Smith et al., 1995). Recruitment may require some post-translational modification of PKCδ itself, or alternatively modification of a PKCδ receptor associated with the CD98/integrin complex at the cell surface. Activated PKCδ then activates a signalling cascade which involves both phosphorylation/activation of ERK/p38, and downstream activation of unidentified proteins which initiate the changes in cell adhesion which result in U937 aggregation. In parallel, PKCδ activation activates, either directly or indirectly, tyrosine kinase activity (both lyn and src have previously been shown to be regulated by PKCδ (Song et al., 1998)). One target of this tyrosine kinase activity is PKCδ itself, which may negatively regulate PKCδ (Denning et al., 1993), or alter the specificity of PKCδ so as to phosphorylate different intracellular target proteins (Haleem-Smith et al., 1995; Deszo et al., 2001), and hence generate other functional outcomes. Other targets of the PKCδ-activated tyrosine kinases remain undefined, but may be downstream pathways which regulate other CD98-dependent
functions, including growth and differentiation. CD98 forms part of multi-molecular complex, which includes integrins and CD147 (Cho et al., 2001). This complex has been suggested to act as a sensory unit, which can be regulated by extracellular amino acid levels, pH, and matrix. CD98 is itself a key element in transducing signals from this complex to within the cell (Cho et al., 2001). The observation documented in this study that PKCδ is a pivotal element in this pathway provides further support for this enzyme's role as a key component of the intracellular signalling pathways which regulate a cell's interaction with its complex microenvironment.
Fig. 4.8 Model of CD98-induced signalling. Signal transduction steps which are essential for CD98-induced U937 aggregation:

PKC translocation: 

Regulatory signal pathways: 

Other functions
Chapter 5

Conventional PKC plays a critical role in negative regulation of CD98-induced homotypic aggregation
Abstract

CD98, a heterodimeric type II transmembrane protein, is involved in many different cellular events, ranging from amino acid transport to cell-cell adhesion. Little is known about the signalling pathways involved in these responses. Therefore, we examined the role of conventional PKC isoforms during CD98-induced U937 homotypic aggregation.

The CD98-induced aggregation was blocked by a protein kinase C (PKC) activator PMA, and enhanced by broad specific protein kinase inhibitors, GF109203X and staurosporin, and by specific PKCα/β peptide inhibitor 19-27. PMA inhibition was not affected by specific enzyme inhibitors of PKA, PTK, protein phosphatase (PPTase), and PI3-K. PMA inhibition was diminished by treatment with PKC inhibitors recognising the ATP-binding site in PKC (e.g. staurosporin, GF109203X and Go6983). Inhibitors which bind to DAG or Ca²⁺-binding sites of PKC (calphostin C and Go6967) had no effect. In agreement with these observations, conventional PKC (cPKC) isozymes (α, β and γ) were translocated during PMA treatment. PMA treatment did not decrease expression of CD18, CD29 and CD147 which have been implicated in CD98-induced cell adhesion, suggesting that PMA inhibition is not mediated by surface adhesion molecule. These data provide evidence that PMA-responsive conventional protein kinase C isoforms (α, β and γ) play a key role in negative regulation of CD98 signalling and homotypic aggregation.
5.1 Introduction

Cell-cell adhesion interactions are known to play an important role in many immune functions, such as cell migration, effector target recognition and activation (Martz, 1987; Belitsos et al., 1990). Examples of such cell-to-cell interactions include those between T cell and APC, follicular dendritic cells and B cells, and tumor cells and cytotoxic T cells. Cell adhesion is strictly regulated by multiple molecules with different modes of action (Springer and Anderson, 1986; Pardi et al., 1992; Petty and Todd, 1996; Hubbard and Rothlein, 2000), including integrins, selectins and intercellular adhesion molecules (ICAMs).

CD98 is a type II integral membrane glycoprotein, composed of a 80 kDa glycosylated heavy chain and a 45 kDa nonglycosylated light chain (Tsurudome and Ito, 2001). The molecule is highly expressed on both haematopoietic and non-haematopoietic cells. CD98 expression is known to be tightly regulated in the process of differentiation. It has been implicated in haematopoietic cell differentiation, growth, transformation and apoptosis, and osteoclastogenesis (Warren et al., 1996; Hara et al., 1999; Higuchi et al., 1999; Hara et al., 2000; Shishido et al., 2000). There is also tight regulation of CD98 expression during differentiation from monocytes to dendritic cells (Woodhead et al., 1999). CD98 has been reported to be a regulator of cell-cell adhesion (Warren et al., 1996; Ohgimoto et al., 1995; Ohgimoto et al., 1996; Ohgimoto et al., 1997; Fenczik et al., 1997; Chandraseran et al., 1999, Chapter 3), of $\beta$1 integrin function (Fenczik et al., 1997; Chandraseran et al., 1999), of T cell co-stimulation (Diaz et al., 1997; Stonehouse et al., 1999; Woodhead et al., 2000), and of virus-induced cell fusion (Ohgimoto et al.,...
The light chain functions as an amino acid transporter and is transported to the cell membrane by association with the heavy chain (Verrey et al. 1999).

A key question in understanding the biology of CD98 is to resolve how the molecule can participate in such an extraordinary diversity of functions. Two approaches which could be used to explain this problem are (i) identification of CD98 ligand(s) and (ii) investigation of the intracellular signalling pathways that are triggered following CD98 ligation. At present, galectin-3 is the only identified ligand of CD98 (Dong and Hughes, 1997). There is very little information, however, on the intracellular signalling pathways which may mediate CD98-induced changes in cell function. Previous studies have identified tyrosine phosphorylation (Warren et al., 1996; Tabata et al., 1997; Miyamoto et al., 2000; Chapter 3), ras involvement (Miyamoto et al., 2000; Suga et al., 2001), activation of MAPK (Miyamoto et al., 2000; Suga et al., 2001) and PI3-K (Suga et al., 2001) as possible intracellular signals mediating CD98 ligation.

In a previous study, we reported that CD98 interacts functionally with both β1 integrins and CD147 to induce homotypic aggregation of U937 cells (Chapter 3), and we suggested that CD98 plays a central role within a multi-molecular unit which could serve to regulate cellular responses to changes in the tissue micro-environment. Further studies from our laboratory have focused on the role of the novel PKC (nPKC) isoform, PKCδ, in mediating CD98-induced homotypic aggregation of U937 cells (Cho et al., manuscript submitted). This work has now been extended to look at the role of other PKC isoforms in this model system. We provide evidence that CD98-induced cell-cell
adhesion is suppressed by the activity of cPKC isoforms. The antagonism was not mediated by changes in surface adhesion molecule expression, but seemed to act downstream of the MAPK/ERK pathway. cPKCs may form part of a physiological regulatory system, which acts to limit cell-cell adhesion/aggregation mediated by ligation of CD98.
5.2 Results

5.2.1 Comparative kinetic analysis of CD98-induced homotypic aggregation.

The initial studies were based upon our observations that CD98 mAb (both intact and F(ab')2 fragments) not only abrogated U937-triggered T lymphocyte proliferation in the presence of soluble CD3 (Stonehouse et al., 1999), but also elicited homotypic aggregation of the U937 cells themselves under standard culture conditions (Chapter 3). U937 homotypic aggregation was not induced by mAbs of the same isotype directed against several other major cell-surface molecules known to be expressed on U937 cells (e.g. CD44, CD45 and CD147). However, other mAbs to adhesion molecules, such as CD29 and CD43, did cause strong homotypic aggregation of the cell line (Chapter 3).

In this study, CD98-induced aggregation was carefully compared to aggregation mediated by CD29 and CD43, because the shapes of the aggregates induced by these molecules were strikingly distinct. The kinetics of a representative assay are shown in Fig. 5.1A and B. Aggregation induced by CD29 and CD43 was more rapid than the CD98 response, reaching 30% (CD29) and 50% (CD43) at 30 min, and increasing further at one hour incubation, whereas CD98 induced cluster formation slowly, with only approximately 15% of the cells within clusters at one hour (Fig. 5.1A). The maximum level of CD98-induced aggregation was also significantly less than that seen with CD29 and CD43 (Fig. 5.1C). These data raise the possibility that CD98-induced aggregation may be negatively regulated in some way to restrict cluster formation.
Fig. 5.1 D98-induced aggregation shows a kinetically different pattern from CD29- and CD43-induced aggregation. A) U937 cells were incubated for 1 h in the presence of mAbs to CD98 (AHN-18, 1 μg/ml), CD29 (MEM101A, 1 μg/ml) and CD43 (161-46, 1 μg/ml). Images of cells in culture at this time point were obtained using an inverted phase contrast microscope, attached to a video camera, and captured using NIH image software. A representative field is illustrated, showing strong U937 homotypic aggregation. (1) Medium alone. (2) CD98 (AHN-18) (3) CD43 (161-46). (4) CD29 (MEM101A). B) U937 cells were incubated for indicated times in the presence of mAbs to CD98, CD29 and CD43 and aggregation was measured under standard conditions as described in Materials and Methods. The results show mean aggregation ± SEM for triplicate cultures for 1 representative experiment of 2. C) U937 cells were incubated for 6 h in the presence of mAbs to CD98, CD29 and CD43 and the results show mean aggregation ± SEM for triplicate cultures for 1 representative experiment of 2.
5.2.2 PKC inhibitors enhance CD98-mediated homotypic aggregation

GFX109203X is a broad-specificity PKC inhibitor, which has been shown to inhibit both classical (α, β, and γ) and novel (δ, ε, η, and θ) forms of the enzyme family (Martiny-Baron et al., 1993; Wilkinson et al., 1993). **Fig. 5.2A** shows that GFX109203X increased CD98-induced aggregation significantly. This effect was dose-dependent, between 1 and 10 μM of the inhibitor. Furthermore, GFX109203X (**Fig. 5.2B**) increased the rate of CD98-induced aggregation two or three fold, to rates comparable to that seen in the presence of CD29 and CD43. GFX109203X alone did not induce any clustering (**Fig. 5.2A and B**). The same enhancement was seen with another structurally related PKC inhibitor, staurosporin (Courage et al., 1995) (**Fig. 5.2C**) and structurally unrelated PKC inhibitors, polymixin B sulfate and D-sphingosine (data not shown). Isoform specific cPKC inhibitor, myristoylated PKC peptide inhibitor (19-27), which is a selective inhibitor of PKCα/β, also strongly up-regulated CD98-induced aggregation (**Chapter 4**). Collectively, these data suggest that CD98-induced aggregation is under negative regulation by cPKC.

5.2.3 PKC activators inhibit CD98-induced homotypic aggregation.

Because PKC inhibitors potentiated CD98-induced aggregation, the next experiments examined the effect of PKC activators. PMA (a direct activator of DAG-dependent PKC isozymes), and dioctanoylglycerol (DOG) (a DAG analog), both induced a time- and concentration-dependent inhibition of homotypic aggregation of up to 90% (**Fig. 5.3A, B, C and D**). PMA inhibited aggregation when added either before or up to 1 h after CD98 mAb (**Fig. 5.3B**), suggesting that PMA may inhibit the middle or late phase of CD98-induced aggregation. These results suggest that PMA (or DOG)-responsive PKCs
Fig. 5.2 Conventional and novel PKC inhibitors potentiate CD98-induced intercellular adhesion. 

A) and C) U937 cells were incubated for 4 h in the presence or absence of AHN-18 and aggregation was measured under standard conditions as described in Materials and Methods. GF109203X and staurosporin were added 30 min prior to the antibody and remained in the culture. 

B) U937 cells were incubated for indicated times in the presence or absence of AHN-18 and GF109203X (5 μM), and aggregation was measured under standard conditions as described in Materials and Methods. The results show mean aggregation ± SEM for triplicate cultures for 1 representative experiment of 2.
Fig. 5.3 PKC activators down-regulate CD98-mediated aggregation. A) and C) U937 cells were incubated for 4 h in the presence or absence of AHN-18 (1 µg/ml) and aggregation was measured under standard conditions as described in Materials and Methods. PMA (A and B) and dioctanolyglycerol (DOG (C)) were added 30 min prior to the antibody and remained in the culture. B) U937 cells were incubated for indicated times in the presence or absence of AHN-18 and PMA, and aggregation was measured under standard conditions as described in Materials and Methods. The results show mean aggregation ± SEM for triplicate cultures for 1 representative experiment of 2. D) U937 cells were incubated for 4 h in the presence of AHN-18 (1 µg/ml) and PMA (50 ng/ml). Images of cells in culture at this time point were obtained using an inverted phase contrast microscope, attached to a video camera, and captured using NIH image software. A representative field is illustrated, showing strong U937 homotypic aggregation. (1) AHN-18. (2) AHN-18 + PMA. (3) PMA alone.
have a negative regulatory role in CD98-mediated cell-cell adhesion.

5.2.4 PMA-mediated inhibition is abrogated by conventional PKC inhibitors

To characterise the inhibitory mechanism of PMA, further pharmacological dissection was carried out using a panel of PKC inhibitors. These included three groups, competitive inhibitors for the ATP-binding (GF109203X, staurosporin and Go6983), Ca\(^{2+}\)-binding (Go6976) and DAG-binding (calphostin C) sites of conventional and novel PKC isoforms. Fig. 5.4 shows that ATP-binding site inhibitors abrogated the PMA effect (Fig. 5.4A), but neither calphostin C (DAG-binding site inhibitor) (Kobayashi et al, 1989), nor Go6976, (Ca\(^{2+}\) binding site inhibitor) (Martiny-Baron, et al., 1993), diminished PMA inhibition (Fig. 5.4B and C).

5.2.5 PMA induces translocation of conventional PKC isoforms to the plasma membrane.

To identify which PKCs were responding to PMA, the membrane translocation of a panel of PKC isoforms was examined. U937 cells express all eight isoforms of PKC tested, as demonstrated by Western blot (Fig. 5.5A), although PKCe expression was relatively low. Only a very small proportion of the conventional isozymes was associated with the membrane fraction in unstimulated cells, but a significant proportion of PKC \(\delta\), \(\zeta\), and \(\lambda\) was membrane bound (Fig. 5.5B). CD98 ligation induced an increase in membrane associated PKC\(\delta\) (Chapter 4), but no change in membrane association of any other isotypes (Fig. 5.5B). In contrast, PMA treatment, both in the presence and absence (data not shown) of CD98, induced strong membrane translocation of PKC \(\alpha\), \(\beta\), and \(\gamma\), but not atypical forms (\(\zeta\) and \(\lambda\)), as reported
**Fig. 5.4A** Conventional and novel PKC inhibitors toward ATP-binding site abolish PMA-mediated inhibition of CD98-induced homotypic aggregation. U937 cells were incubated for 3 or 4 h in the presence or absence of AHN-18 (1 μg/ml) and aggregation was measured under standard conditions as described in Materials and Methods. All compounds were added 30 min prior to the mAb and remained in the culture. The results show mean aggregation ± SEM for triplicate cultures for 1 representative experiment of 2.
Fig. 5.4B and 5.4C Conventional and novel PKC inhibitors toward ATP-binding site abolish PMA-mediated inhibition of CD98-induced homotypic aggregation. U937 cells were incubated for 3 or 4 h in the presence or absence of AHN-18 (1 μg/ml) and aggregation was measured under standard conditions as described in Materials and Methods. All compounds were added 30 min prior to the antibody and remained in the culture. Aggregation was measured under standard conditions as described in Materials and Methods. The results show mean aggregation ± SEM for triplicate cultures for 1 representative experiment of 2.
Fig. 5.5 PMA induces translocation of conventional PKC isoforms to the plasma membrane. A) and B) U937 cells were incubated for 20 min in the presence or absence of CD98-AHN-18 (1 μg/ml) and PMA (50 ng/ml). Cells were washed, lysed and fractionated into membrane and cytoplasmic fractions, as described in Methods. Total (whole lysate (A)) and membrane (B) [lane 1: normal, 2: CD98 mAb (AHN-18), 3: CD98 mAb + PMA] were analysed for the presence of specific antibodies to PKC isoforms by Western blot as described in Methods. The results from three separate experiments are shown.
5.2.6 PMA does not inhibit CD98-induced ERK activation.

PKC activation has been linked previously to up-regulation of MAPK activity (Monick et al., 2001; Yoon et al., 2000; Chen et al., 1999), and PMA treatment increases phosphorylation of MAPK, an indicator of MAPK activation. Previous studies (manuscript submitted) showed that CD98 induced activation of MAPK/ERK and MAPK/p38, and that this activation was essential for CD98-induced homotypic aggregation. To identify possible target substrates of PMA-dependent inhibition, we first examined whether PMA inhibits CD98-induced MAPK activation. Fig. 5.6 shows that PMA enhanced CD98-induced ERK activation, but the total MAPK/ERK was not changed by PMA treatment (data not shown), suggesting that the inhibitory target of PMA-responsive PKC is downstream of MAPK. PMA-induced ERK phosphorylation, like the inhibition of aggregation, was also blocked by GF109203X, but not calphostin C or Go6983.

5.2.7 PMA does not block adhesion molecule expression during CD98-induced aggregation

CD98-mediated homotypic aggregation has been reported to be regulated by β1 and β2 integrins and CD147. The expression level of these adhesion molecules following PMA treatment was therefore determined. The time period selected (three hours) is that required for maximum inhibition. As shown in Fig. 5.7 and Table 5.1, there was no marked down-regulation of any marker by PMA treatment. However, the surface
Fig. 5.6 PMA up-regulates CD98-mediated phospho-MAPK/ERK expression. U937 cells were incubated for various times in the presence or absence of CD98-AHN-18 (1 μg/ml) and PMA (50 ng/ml). Cells were washed, lysed and analysed for phospho-MAPK/ERK expression by Western blot as described in methods. The blots show gels probed with antibody specific for phosphorylated forms of the kinase. GF109203X (10 μM), Go6976 (1 μM), and calphostin C (1 μM) were added 30 min prior to the antibody and remained in the culture. The result shows a representative data of 3 separate experiments.
Fig. 5.7A Effect of PMA on the surface level of U937 adhesion molecule. U937 cells were incubated for 4 hours in the presence or absence of PMA (50 ng/ml or indicated concentrations). Surface adhesion molecule levels were tested with a panel of U937 adhesion molecule mAbs by means of flow cytometry. The histograms only are representative of the obtained data. Histograms marked C are the fluorescence profile of CD98-treated cells stained with isotype control (Black colour : Normal, Green colour: PMA).
**Fig. 5.7B and C** PMA down-regulates the surface level of U937 CD98. U937 cells were incubated for 4 h (B) or indicated times (C) in the presence or absence of PMA (50 ng/ml or indicated concentrations). Surface adhesion molecule levels were tested with a panel of U937 adhesion molecule antibodies by means of flow cytometry. MFI values were calculated by means of WIN-MDI software on a minimum of 5000 cells. The results are shown as mean ± SEM (n=4) (*: p<0.05, **: p<0.01).
Table 5.1 Effect of PMA on surface level of U937 adhesion molecules.

<table>
<thead>
<tr>
<th>Group</th>
<th>MFI values</th>
<th>Normal</th>
<th>PMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Ab</td>
<td>10.2 ± 0.7</td>
<td>11.2 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>CD98</td>
<td>355.7 ± 12.1</td>
<td>288.6 ± 14.2</td>
<td>*</td>
</tr>
<tr>
<td>CD147</td>
<td>2181.4 ± 57.3</td>
<td>2130.9 ± 83.7</td>
<td></td>
</tr>
<tr>
<td>CD44</td>
<td>1403.4 ± 18.6</td>
<td>1549.2 ± 11.4</td>
<td>*</td>
</tr>
<tr>
<td>CD29</td>
<td>539.3 ± 7.1</td>
<td>584.8 ± 11.3</td>
<td></td>
</tr>
<tr>
<td>CD43</td>
<td>429.6 ± 67.2</td>
<td>398.2 ± 24.9</td>
<td></td>
</tr>
<tr>
<td>CD18</td>
<td>72.4 ± 1.8</td>
<td>89.0 ± 2.5</td>
<td>**</td>
</tr>
<tr>
<td>CD45</td>
<td>66.1 ± 0.4</td>
<td>72.2 ± 2.2</td>
<td></td>
</tr>
</tbody>
</table>

U937 cells were incubated with PMA (50 ng/ml) for 4 h and tested its effect on surface level of adhesion molecule. MFI values were calculated by means of WIN-MDI software on a minimum of 5000 cells. The results are shown as mean ± SEM for triplicate for 1 representative experiment of 2 (*: p<0.05 and **: p<0.01).
expression of CD98 itself was significantly decreased, and the surface levels of CD18 and CD44 were significantly increased, in agreement with previous reports (Nueda et al., 1995). No significant changes were seen in levels of CD29, CD43 and CD147. The down-regulation of CD98 occurred at inhibitory doses of PMA and began after 1 hour incubation (Fig. 5.7B and C).

5.2.8 The relationship between PMA-responsive PKC and other signalling pathways

A previous study (manuscript submitted) demonstrated that CD98-induced aggregation is strikingly potentiated by a selective PKA inhibitor, KT5720 and inhibited by PKA activator, forskolin. Protein tyrosine kinase (PTK) inhibitors also strongly augmented CD98-induced cell adhesion, indicating that both PKA and PTK might be involved as negative regulator of CD98-induced signalling pathways.

The relationship between PKC, PTK and PKA pathways was therefore explored. As reported previously, GF109203X, KT5720 and genistein potentiated CD98-induced aggregation, whereas PKC activators such as forskolin and PMA had a strong inhibitory effect (Fig. 5.8A and B). The potentiation by genistein was not seen in the presence of PMA (Fig. 5.8A), suggesting that PTK activity may lie upstream of PKC in this negative regulatory loop. In contrast, KT5720 potentiated clustering even in the presence of PMA, while GF109203X potentiated clustering in the presence of forskolin (Fig. 5.8B). The PKA inhibitory pathway is therefore parallel to PKC.
Fig. 5.8A abd B Relationship between PMA-inhibition and other enzyme activation in U937 homotypic aggregation. A) and B) U937 cells were incubated for 3 or 4 h in the presence or absence of AHN-18 (1 µg/ml) and PMA (50 ng/ml), and aggregation was measured under standard conditions as described in Materials and Methods. All compounds including genistein (50 µM), GFX (10 µM), KT5720 (1 µM) and forskolin (50 µM) were added 30 min prior to the antibody and remained in the culture. Aggregation was measured under standard conditions as described in Materials and Methods. The results show mean aggregation ± SEM for triplicate cultures for 1 representative experiment of 2.
5.2.9 The effect of other intracellular signalling enzyme inhibitors on PMA inhibition

Previously, PMA has been shown to activate a variety of intracellular signalling enzymes (PMA-inducible enzymes) such as phosphatidylinositol 3-kinase (PI3-K) and serine/threonine phosphatase (STPPase) (Tardif et al., 1998; Djerdjouri et al., 1995). Therefore, to address whether or not PMA inhibition is mediated by down-stream activation of these PMA-inducible enzymes, a panel of enzyme inhibitors were tested.

Fig. 5.8C illustrates that the inhibitory effect of PMA does not appear to be related to any of these pathways. Thus, a PI3-K inhibitor [LY294002 (LY)], STPPase inhibitors [okadaic acid (OKA) and cyclosporin (Cys)], and a Ca^{2+} chelator (EDTA) did not interfere with the PMA inhibition of CD98-induced aggregation, suggesting that these PMA-inducible enzymes were not involved in PMA inhibition.
Fig. 5.8C Relationship between PMA-inhibition and other enzyme activation in U937 homotypic aggregation. U937 cells were incubated for 3 or 4 h in the presence or absence of AHN-18 (1 μg/ml) and PMA (50 ng/ml), and aggregation was measured under standard conditions as described in Materials and Methods. All compounds including LY294002 (LY), okadaic acid (OKA), cyclosporin (Cys) and EDTA were added 30 min prior to the antibody and remained in the culture.
5.3 Discussion

CD98 is now recognised as a cell surface molecule that is important in a range of cellular processes, but very little is known about the CD98 induced signal transduction pathways, and about how these might affect the many proposed functions of the molecule. In this study, therefore, we have continued our exploration of the CD98 signalling mechanism, using a quantitative assay of CD98-induced homotypic aggregation of U937.

Initial experiments showed that the kinetics of cluster formation induced by CD98 was slower than the cluster formation seen with other adhesion molecule (CD29 and CD43)-mediated homotypic aggregation events, indicating that a more complex process than rapid receptor-ligand surface interactions is probably involved. The PKC activators PMA and DOG inhibited the aggregation in a dose- and time-dependent manner, while cPKC inhibitors (GF109203X, staurosporin, and a selective cell-permeahle, myristoylated protein kinase Ca/β inhibitor 19-27) enhanced the response. The PKC inhibitors (GF109203, staurosporin and Go6983) also reversed the PMA-induced inhibition, thus confirming that PMA itself inhibits via PKC. cPKC isoforms, and not novel or atypical PKCs, were translocated to the cell membrane during PMA-inhibition. CD98 ligation did not induce cPKC translocation. These results therefore suggest that constitutive activity of cPKC isoforms limit CD98-induced aggregation, and that this activity is amplified by pharmacological activation of these enzymes. The results extend our previous findings that PKCα is a key enzyme in initiating CD98-induced aggregation, by demonstrating that cPKCs play an opposing negative regulatory role on
this process.

PKC activity is regulated by DAG, phospholipids, Ca^{2+} and ATP, each of which bind to distinct sites on the enzyme. Aggregation was enhanced by GF109203X, staurosporin, and Go6983, all competitive inhibitors of the PKC ATP-binding site (Toullec et al., 1991; Martiny-Baron et al., 1993), whereas the other inhibitors were ineffective. Furthermore, PMA inhibition was not affected by Ca^{2+} depletion conditions (EDTA/EGTA) and by DAG-binding inhibitor (calphostin C). The binding of ATP to the N-terminal pseudosubstrate binding domain therefore seems to be the key regulatory step in mediating the negative regulation of CD98-induced U937 aggregation.

An important question raised by these studies is what are the target molecules of activated PKC which inhibit the CD98-induced aggregation? cPKC activation could result either in direct phosphorylation of CD98, or indirect regulation of other enzymes which are able to phosphorylate CD98, and this phosphorylation could negatively regulate the function of the molecule. Similar regulatory loops, including the regulation of serine/threonine kinase Akt and Btk by PKC (Zheng et al., 2000; Yao et al., 1994) and serine/threonine phosphorylation of CD43, have been reported for other signalling pathways. Furthermore, CD98 has eight predicted potential PKC phosphorylation (S/T-Xa-R/K) sites and two potential cAMP/cGMP-kinase phosphorylation (R/K-Xaa-S/T) sites (Warren et al., 1996). However, our data suggest that the target of cPKC is downstream of the initial signalling events since PMA enhanced, rather than inhibited CD98-induced MAPK/ERK phosphorylation. Furthermore, PMA inhibited aggregation even if added one hour after CD98 ligation.
An alternative possibility is that inhibition of homotypic adhesion is mediated by ERK itself. A similar negative regulation of IGF-1-induced Akt activation and insulin receptor signalling has been reported to be mediated by PMA-mediated MAPK activation (Zheng et al., 2000). However, this pathway is unlikely to operate in our model, since the selective MAPK/ERK inhibitor, PD98059 strongly blocked CD98-induced aggregation, (Chapter 3; Lu et al., 1998) and did not reverse the PMA inhibitory effect. Furthermore, many actin-binding proteins regulated by conventional PKC more usually enhance cell movement, spreading and cytoskeletal organisation, and adhesion (Ono et al., 1997; Adams et al., 1999; Rojnunckarin and Kaushansky, 2001). Identification of the targets of cPKC in this system therefore requires further investigation.

The experiments also investigated the relationship between the inhibitory cPKC activity, and that of two other families of enzyme also apparently involved in the negative regulation of CD98-induced homotypic adhesion, PKA and PTK. PMA completely inhibited the enhancement of CD98-mediated aggregation induced by genistein (a PTK inhibitor), but not by KT5720 (a PKA inhibitor). One model to explain these findings is that a PTK lies up-stream of cPKC, and hence can be by-passed by the addition of exogenous PMA (see Fig. 5.9). In an analogous pathway, we recently reported that PKCδ is one of the targets of CD98-induced PTK activation (manuscript submitted). In contrast, PKA mediated inhibition may be independent of the cPKC pathway. Further biochemical studies will be required to resolve these issues.
In conclusion, this study defines a cPKC mediated regulatory pathway which limits both the speed and the maximum extent of CD98-induced aggregation. The pathway appears to operate constitutively, but can be enhanced by PKC activators such as PMA and DOG. cPKC regulation is mediated predominantly by alterations in binding of ATP to the regulatory site on the cPKC. The target(s) of cPKC remain undefined, but lie downstream to the activation of MAPK/ERK. These results need to be seen in the context of our previous studies, which identified PKCô as a key enzyme involved in triggering of homotypic aggregation in response to CD98 ligation. The balance between cPKC activity and PKCô activity therefore determine the extent of CD98-triggered homotypic aggregation. Further studies will be required to determine whether a similar regulatory circuit operates in mediating other CD98-dependent functions.
Fig. 5.9 Model of PKC-mediated negative regulation of CD98-induced cell adhesion (CD98HC : heavy chain of CD98. CD98LC : light chain of CD98. PKC: protein kinase C. PTK: protein tyrosine kinase. cPKC : conventional PKC. PKA : protein kinase A. - : negative feedback loop).
Chapter 6

Conclusions
6.1 Role of CD98 in the regulation of integrin function

Recently molecular genetic analysis has demonstrated that CD98 is able to regulate integrin functions such as adhesion, spreading and migration. It has been suggested that the mode of action for this modulation is regulated very specifically by means of structural interaction with β1 integrins, but not with other integrin families (Fenczik et al., 1997; 2001; Zent et al., 2000). The cytoplasmic and transmembrane domains of CD98HC are absolutely required for its interaction with integrins (Fenczik et al., 2001). The significance of CD98-mediated control of integrin function is supported by a wide variety of in vivo studies, including those dealing with cell aggregation, adhesion, fusion and APC-derived T cell costimulation (Fenczik et al., 1997; Chandrasekaran et al., 1999; 2000; Tabata et al., 1994; Ohta et al., 1994; Stonehouse et al., 1999; Warren et al., 2000; Woodhead et al., 2000).

Chapter 3 of this thesis provides new support for a functional association between CD98 and integrins, since blocking antibodies (MAR4 and CLB-LFA1) to CD29 and CD18 significantly inhibited CD98-induced aggregation, although the inhibitory activities are weaker than for CD29- or PMA-induced aggregation (data not shown). An interesting observation was that P5D2, a mAb to CD29 which strongly inhibits integrin-mediated adhesion (Fenczik et al., 1997), did not inhibit CD98-induced homotypic aggregation, but rather enhanced cluster formation. This discrepancy in inhibitory action of P5D2 on aggregation and adhesion events is still not fully explained. One explanation could be that β1 integrins are not critically involved in mediating CD98-induced cell-cell adhesion, and that some other adhesion molecule is involved.
Alternatively, different integrin epitopes are involved in mediating cell-cell aggregation and cell adhesion to ECM, respectively.

On the basis of the data in Chapter 3, we favor the former hypothesis for several reasons. Firstly, all three blocking mAbs (P5D2, MAR-4 and Lia1/2 (not shown)) either enhanced or showed small inhibitory effects on CD98-induced intercellular aggregation. Secondly, the inhibitory effect of the CD29 mAb (MAR4) is abrogated if the mAb is added after CD98 ligation, suggesting that β1 integrin might be involved in the induction phase of CD98-induced homotypic aggregation, rather than mediating cell-cell adhesion. Thirdly, we did identify another molecule, CD147 which may mediate CD98-induced aggregation, because two mAbs (MEM M6/1 and H84AF) to CD147 strongly diminished CD98-mediated cluster formation. Interestingly CD29-induced aggregation was also inhibited by the mAbs to CD147, as much as by some mAbs to CD98 (BU89 and MEM108), suggesting that these three molecules may be functionally associated to make an adhesion complex. The blocking effect of CD147 mAbs was seen even when these mAbs were added subsequent to CD98 ligation, suggesting that CD147 may be directly involved in mediating cell-cell adhesion. This is further supported by confocal analysis (work in progress) showing that both CD147 and CD98 are concentrated at the sites of cell contact, suggesting that CD147 may also physically associate with CD98 at the contact area. Taken in the context of previous work showing that CD147 is a β1 integrin-associated protein (Berditchevski et al., 1997), our results suggest that the three molecules CD98, β1 integrins and CD147 can form a molecular cluster. Further studies, using biochemical (such as co-immunoprecipitation) and molecular biological (such as transfection of wild type and mutant genes) methods are
required to probe the nature of this complex.

The physiologic regulation of CD98-induced aggregation, and other CD98 functions remains unclear. However, it is intriguing that CD147, like CD98, acts as a chaperon for multimembrane spanning transporters. While CD98 associates with amino acid transporters, CD147 chaperons members of the monocarboxylate transporter family (Halestrap and Price, 1999). The multimolecular complex of CD98, CD147, their associated transporters, and the β1 integrins might act as a sensory unit, which regulates outcomes as diverse as adhesion, growth, differentiation and antigen presentation. These functions would then be regulated by multiple parameters, including ECM (integrins), levels of amino acids (CD98), and levels of carboxylic acids (eg, lactic acid) (CD147). Our preliminary results that nutrient-deprived culture conditions suppressed CD98-induced aggregation, but not CD43-induced aggregation (Table 6.1) appear to support such a hypothesis.

6.2 CD98 induces intracellular signalling

CD98 participates in an extraordinary diversity of cell functions. How this molecule can be implicated in such a wide range of activities is still not fully explained. One possible approach to this question is to study how activation of CD98 via extracellular ligation affects the intracellular signalling pathways and hence provides an ‘inside-out’ signal as well as ‘out-side-in’.
Table 6.1 Effect of nutrient-deprived culture conditions on CD98-induced aggregation.

<table>
<thead>
<tr>
<th>Recuperation time (h)</th>
<th>Aggregation (% of aggregation)</th>
<th>CD98</th>
<th>CD43</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RPMI1640</td>
<td>HBSS</td>
<td>RPMI1640</td>
</tr>
<tr>
<td>1</td>
<td>27.5 ± 5.0</td>
<td>22.9 ± 2.5</td>
<td>77.8 ± 1.7</td>
</tr>
<tr>
<td>2</td>
<td>26.5 ± 4.5</td>
<td>13.3 ± 3.7</td>
<td>48.9 ± 8.6</td>
</tr>
<tr>
<td>3</td>
<td>33.0 ± 3.3</td>
<td>9.3 ± 4.6</td>
<td>70.4 ± 1.9</td>
</tr>
</tbody>
</table>

U937 cells washed 3 times with PBS were cultured in RPMI1640 or HBSS solution with Ca\(^{2+}\)/Mg\(^{2+}\) for indicated recuperation times and were stimulated with aggregating mAb solution [CD98: AHN-18 (1 µg/ml) and CD43: 161-46 (0.5 µg/ml)] with 10% dialysed FCS for 4 h. Aggregation was measured as described in Materials and Methods. The results are expressed as mean aggregation ± SEM (n=3).
It is perhaps surprising that despite the multitude of studies that have addressed CD98 function, there is little information on these pathways. Possible signaling events, which have been suggested as transducing CD98-mediated function post-ligation, include tyrosine phosphorylation, G-protein activity, and MAPK activation. Many of these experiments have been carried out using BAF/hLFA-1 cell (Suga et al., 2001), FTH5 cells (Warren et al., 1996), Cd+U2ME-7 (Tabata et al., 1997) and blood monocytes (Miyamoto et al., 2000; Tsurudome and Ito, 2000), have also been used, sometimes in the presence of virus.

A definitive pathway for CD98-mediated signalling has not been defined, however, this may be due to the variety of different cell types examined, the different functional outcomes, and the frequent absence of quantitation. The data only suggest that CD98-induced function is mediated by a complex interaction of intracellular signalling pathways.

In this thesis, therefore, using a reproducible and quantitative aggregation assay, intracellular signalling events have been dissected, using a combination of pharmacological and biochemical approaches. One of the major findings in this thesis is that members of the PKC family may play a central role in induction of CD98-mediated homotypic aggregation, thus extending the known role of PKCs in cell migration, adhesion and aggregation via the regulation of cytoskeleton and activation of adhesion molecules (Yue et al., 1999; Masur et al., 2001; Kermorgant et al., 2001).

6.2.1 Positive signalling
Conclusions

The first part of the thesis dealing with signalling (Chapter 4) presents evidence that PKCδ is selectively activated by CD98 ligation and in turn activates MAPK (ERK and p38). Thus, the partially selective PKCδ inhibitor, rottlerin, and selective MAPK inhibitors, PD98059 (ERK) and SB20380 (p38) showed strong inhibitory effects on CD98-induced U937 aggregation. Biochemical studies confirmed both PKCδ membrane translocation, regarded as an indicator of activation of PKCδ, and a time-dependent increase in expression level of phospho-ERK and phospho-p38. Both these events were inhibited by rottlerin treatment. Both biochemical and pharmacological data suggest, therefore, that PKCδ may be involved in mediating CD98 signals. Further molecular analysis is required to confirm this model. Our results conflict with a previous report that CD98 ligation did not induce PKC activation in mediating gp120-mediated cell fusion (Tabata et al., 1997). The discrepancy may be due either to the use of a different cell line, or to using different CD98 mAbs, or because cell fusion and cell aggregation depend on different signalling pathways. There are no reports connecting PKC, MAPK activation and CD98, although it has been reported that MAPK activation accompanies CD98 ligation (Suga et al., 2001; Miyamoto et al., 2000).

A second interesting finding in Chapter 4 is that CD98 is capable of regulating tyrosine phosphorylation of PKCδ itself. Because the tyrosine phosphorylation itself appears to be a negative regulator of CD98-induced intercellular adhesion (see below), tyrosine phosphorylation of PKCδ may act as a negative feedback on PKCδ activity (Haleem-Smith et al., 1995; Deszo et al., 2001; Kronfeld et al., 2000). In summary, the data suggest a model in which CD98 selectively activates PKCδ, and MAPKs leading to induction of homotypic aggregation. This is the first report that PKCδ is a key regulator
of cell-cell aggregation.

6.2.2 Negative signalling

In the second part of the thesis (Chapter 5), a key finding is that CD98-induced aggregation is negatively regulated by cPKC isoforms (α, β, and γ). Thus, there was no spontaneous aggregation-inducing effect of the cPKC inhibitor, GF109203X, but it strongly augmented CD98-induced aggregation. In parallel, a strong PKC activator, PMA, suppressed CD98-mediated aggregation. This suppression was antagonised by conventional PKC inhibitors, suggesting that PMA-inhibition is due to activation of conventional PKC, but not nPKC. The suppression appears to occur downstream of MAPK, since PMA enhances, rather than inhibits, CD98-induced MAPK phosphorylation. This data is the first evidence that cPKC can negatively antagonise nPKC (PKCδ)-induced aggregation, stimulated by CD98 ligation. Although the exact mechanism by which cPKC interferes with PKCδ effect is still not known, cPKC presumably regulate the phosphorylation state of downstream protein substrate(s) following MAPK activation, so that this hyper- or hypo-phosphorylated condition may play an important role in the regulation of CD98-mediated homotypic aggregation.

6.3 Proposed signaling pathway in U937 cells

On the basis of the data presented in Chapter 4 and 5 and the information available in the literature (reviewed in Chapter 1), we have proposed a model for the intracellular signalling pathway which connects CD98 ligation to U937 homotypic aggregation. The major features of the model are depicted in Fig. 6.1. Ligation of CD98 (by mAb in this study, but by interaction with appropriate counter-receptor in vivo) leads to a rapid
recruitment of PKCδ to the membrane. An analogous model has been proposed for the events which follow cross-linking of the IgEγ chain in mast cells (Haleen-Smith et al., 1995). Recruitment may require some post-translational modification of PKCδ itself, or alternatively modification of a PKCδ receptor associated with the CD98/integrin/CD147 complex at the cell surface. Activated PKCδ then activates a signalling cascade which involves both phosphorylation/activation of ERK/p38, and downstream activation of unidentified proteins (including cytoskeleton and PLD) which initiate the changes in cell adhesion which result in U937 aggregation. In parallel, PKCδ activation activates, either directly or indirectly, tyrosine kinase activity [both lyn and src have previously been shown to be regulated by PKCδ (Song et al., 1998)]. One target of this tyrosine kinase activity is PKCδ itself, which may negatively regulate PKCδ (Denning et al., 1993), or alter the specificity of PKCδ so as to phosphorylate different intracellular target proteins (Haleem-Smith et al., 1995; Deszo et al., 2001), and hence generate other functional outcomes. Other targets of the PKCδ-activated tyrosine kinases remain undefined, but may be downstream pathways which regulate different CD98-dependent functions, such as growth and differentiation. Simultaneously, the molecular complex activates negative regulatory systems (including cPKC and PKA) which are able to limit cell aggregation. These enzymes may catalyse serine/threonine phosphorylation of target substrate protein(s) to allow hyper- or hypo-phosphorylation states, which regulate CD98-induced homotypic aggregation.
Ligation of CD98/integrins/CD147 complex

Membrane

CD98LC

PKC\(\delta\)

ERK/p38

Tyrosine phosphorylation

Other functions

Unidentified substrates

Hyper-phosphorylation

Hypo-phosphorylation

Negative regulators

cPKC

PKA

Cytoskeleton

Integrins

PLD

Down-regulation

Aggregation

Up-regulation

Fig. 6.1 Signal transduction steps which regulate CD98-induced U937 aggregation. Ligation of CD98 leads to a rapid recruitment of PKC\(\delta\) to the membrane. Activated PKC\(\delta\) then activates a signalling cascade which involves both phosphorylation/activation of ERK/p38, and downstream activation of unidentified proteins, including cytoskeleton and PLD (phospholipase D). Simultaneously, the molecular complex activates negative regulatory systems [including cPKC (conventional PKC) and PKA (protein kinase A)] which are able to limit cell aggregation. These enzymes may catalyse serine/threonine phosphorylation of target protein(s) to allow hyper-phosphorylation state, resulting in down-regulation of cluster formation.
6.4 CD98-induced signaling in mammalian cells

A broad view of how CD98 might regulate other signalling molecules in mammalian cells is depicted in Fig. 6.2. The interaction of CD98 ligand with CD98, and the formation of a molecular complex within the cell membrane, initiates CD98-mediated signal transduction. The complex may then incorporate further surface molecules (including β1 integrins, CD147), cytoskeletal proteins (CP), and other signalling molecules (such as PTK, PI3-K, ras protein and PKCδ). Subsequently, activation leads to downstream activation of the MAPK signalling cascade. With the activation of MAPK, there is continuous activation of either transcription factors (e.g. Sp1) to direct new protein synthesis (e.g. c-src in fusion events, or other substrates, including PLD, resulting in cell adhesion events.

6.5 Functional role of CD98

In addition to its original description as a phenotypic marker for activated lymphocytes, CD98 is now known to be a multifunctional molecule involved in many cellular events (Fig. 6.3). The function of CD98 appears to depend on making molecular complexes with other surface and intracellular molecules. The ability to form complexes with a variety of molecules may be a possible way to determine differential CD98 function for each cell or tissue. For example, CD98HC association with one of 6 light chains, with each of different specificity (Deves and Boyd, 2000; Verrey et al., 2000) may help to determine the nature of the response. At the same time as diversity, these complexes also perform one of the most basic cellular functions, i.e. transport of amino acids and Na⁺/Ca²⁺ into and out of the cytoplasm. The transport function of the CD98 complex could augment and control both protein biosynthesis and/or other intracellular signalling.
pathways. The molecular complex can be regarded as a focus for recruitment and regulation of integrand functions, once the signalling complex has been formed, and then this can participate in regulating an extraordinary diversity of cellular functions, such as proliferation, costimulation, haematopoiesis, apoptosis, cell aggregation, fusion, and osteoclastogenesis.

6.6 Future direction

On the basis of the data presented in this thesis, several future avenues of research can be proposed. These summarised below focus on understanding CD98 function in cell adhesion and T cell costimulation, the major immunobiological roles of CD98.

6.6.1 CD98 ligation and the reorganization of cell surface molecules such as integrins and CD147

Antibodies to several cell surface molecules have been shown to have a potential blocking effect on CD98-induced homotypic aggregation. As expected, these include partial block by β1 integrin, and β2 integrin antibodies. Unexpectedly, our results have suggested that aggregation is also reduced by antibodies to CD147. Since the avidity of β1 and β1 integrins and perhaps other adhesion molecules such as CD147 is known to be strongly regulated by their distribution within the membrane (Jun et al., 2001; Bleijs et al., 2001), we propose to measure changes in this parameter following CD98 ligation in two ways:
Fig. 6.2 The interaction of CD98 ligand with CD98 and the formation of molecular complex within the cell membrane initiates signal transduction. CD98 may transduce signals by association with several proteins, including β1 integrins, CD147, cytoskeleton proteins (CP) and other signalling molecules such as PTK (protein tyrosine kinases), PI3-K (phosphatidylinositol 3-kinase), PKCδ (protein kinase Cδ). The activation can lead to downstream activation of MAPKs. With the activation of MAPK, transcription factor (e.g., Sp1) activation directs new protein synthesis (e.g., c-src)
CD98 activation → Intracellular events → Cell → Outcome

CD98LC
Amino acids
Na+/Ca2+
Others (?)

CD98HC
Transport

 Integrin regulation
β1 integrins
Cytoskeleton rearrangement

CD147

Integrin regulation

Regulation of cellular function

- Tumor cells
- T lymphocytes
- Cardiac/skeletal muscle cells
- Kidney cells
- Hematopoietic progenitor cells
- Virus-infected cells
- Monocytes
- Proliferation/
- Costimulation/
- Activation
- Amino acid reabsorption
- Hematopoiesis
- Apoptosis
- Aggregation
- Fusion
- Osteoclastogenesis

Fig. 6.3 CD98-mediated regulation of cellular function.
6.6.1.1 Lateral mobility of β1 and β2 integrins and CD147 in the membrane following CD98 ligation

Cells will be treated with CD98 mAb, (or as controls, CD29 mAb or CD43 mAb, both of which induce homotypic aggregation) and then capping and patching of adhesion molecules (both processes known to be tightly controlled by cytoskeletal interactions) will be followed using fluorescently-labelled antibodies, with or without addition of a second layer to promote capping. This approach is now a well-established way of looking at changes in integrin/cytoskeleton interaction. If interesting changes are observed in the distribution of cell surface molecules using this approach, this could be followed up by more quantitative and sophisticated assay of lateral mobility using photo-bleaching, and fluorescence recovery.

6.6.1.2 Molecular complex of CD98 and adhesion molecules in lipid rafts

Recently, it has become clear that the phenomena described in section 6.6.1.1 above, have a biochemical correlation, in the re-assortments of proteins within lipid rafts within the cell membrane (Peyron et al., 2000; Claas et al., 2001). Thus U937 cells will be treated with CD98 mAb or controls, and the lipid rafts will be isolated using gradient centrifugation as described. The rafts will be then assayed for the presence of β1 and β2 integrins or CD147 (or other adhesion molecules) by Western blot.

6.6.2 Signalling pathways

Having identified the PKCs and MAPKs principally activated following CD98 ligation, the identification of the down-stream protein targets of this phosphorylation is an obvious next step. For this purpose, $^{32}$P-labeling will allow detection of the major
protein species which become phosphorylated following CD98 ligation. Molecular weights alone of these species may provide some clues as to their identity, but attempts to scale up and obtain identifying sequence data by mass spectroscopy from gels will also be explored.

6.6.3 New cellular models with which to analyse CD98 structure and functions in cell aggregation and T cell costimulation

The objective would be to set up a system where 1) mutant variants of CD98 can be introduced, and ligated independently of or in the absence of endogenous CD98 and 2) CD98 can be ligated on the surface of antigen presenting or U937 cells, without at the same time ligating CD98 on the surface of the responding T cells.

6.6.3.1 myc-tagged variants of CD98

U937 cells will be transfected with CD98 constructs with a C-terminal (i.e. extracellular) myc-tag added to the CD98-encoding sequence. CD98 ligation will be then be accomplished independently of endogenously CD98 by use of an anti-myc antibody.

6.6.3.2 CD98-negative variants of U937 cells

U937 cells will be mutagenised using UV or chemical mutagenesis, and then CD98-negative variants will be isolated by repeated rounds of cell sorting. This is potentially the most strait forward approach, and has been used previously to identify class II MHC negative variants of a B cell line (Poirier et al., 1993) and this would allow for the identification of CD98-dependent versus independent events within the multimolecular
complexes postulated above.

### 6.6.3.3 Human/mouse chimaeric model

Antibodies to human CD98 do not cross-react with murine CD98. Therefore, human CD98 cDNA will be transfected into mouse antigen presenting cells or into related cell lines, and the effects of CD98 ligation on homotypic aggregation and also on antigen presentation by the transfected cells to murine T cells will be measured. This approach would also address questions raised in sections 6.6.3.1 and 6.6.3.2 above, allowing ligation independent of both endogenous CD98 (mouse) and T cell CD98 (mouse).
Chapter 7

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