CD98 modulates integrin β1 function in polarized epithelial cells

Songmin Cai[1], Nada Bulus[1], Priscila M. Fonseca-Siesser[2], Dong Chen[1], Steven K. Hanks[2], Ambra Pozzi[1,3,4] and Roy Zent[1,3,4,*]

[1]Division of Nephrology, Departments of Medicine, [2]Cell and Developmental Biology and [3]Cancer Biology, Vanderbilt University Medical Center, 1161 21st Avenue South, Nashville, TN 37232, USA
[4]Department of Research Medicine, Veterans Affairs Hospital, 1310 24th Avenue South, Nashville, TN 37232, USA
*Author for correspondence (e-mail: roy.zent@vanderbilt.edu)

Accepted 6 December 2004
Journal of Cell Science 118, 889-899 Published by The Company of Biologists 2005
doi:10.1242/jcs.01674

Summary

The type II transmembrane protein CD98, best known as the heavy chain of the heterodimeric amino acid transporters (HAT), is required for the surface expression and basolateral localization of this transporter complex in polarized epithelial cells. CD98 also interacts with β1 integrins resulting in an increase in their affinity for ligand. In this study we explored the role of the transmembrane and cytoplasmic domains of CD98 on integrin-dependent cell adhesion and migration in polarized renal epithelial cells. We demonstrate that the transmembrane domain of CD98 was sufficient, whereas the five N-terminal amino acids of this domain were required for CD98 interactions with β1 integrins. Overexpression of either full-length CD98 or CD98 lacking its cytoplasmic tail increased cell adhesion and migration, whereas deletion of the five N-terminal amino acids of the transmembrane domain of CD98 abrogated this effect. CD98 and mutants that interacted with β1 integrins increased both focal adhesion formation and FAK and AKT phosphorylation. CD98-induced cell adhesion and migration was inhibited by addition of phosphoinositol 3-OH kinase (PI3-K) inhibitors suggesting these cell functions are PI3-K-dependent. Finally, CD98 and mutants that interacted with β1, induced marked changes in polarized renal epithelial cell branching morphogenesis in collagen gels. Thus, in polarized renal epithelial cells, CD98 might be viewed as a scaffolding protein that interacts with basolaterally expressed amino acid transporters and β1 integrins and can alter diverse cellular functions such as amino acid transport as well as cell adhesion, migration and branching morphogenesis.

Key words: Integrin β1, CD98, 4F2, Epithelial cell, Adhesion, Migration

Introduction

CD98 (also known as 4F2), a type II transmembrane protein, is a heavy chain of the family of heterodimeric amino acid transporters (HAT). In polarized epithelial cells, HATs consist of a complex of a heavy chain and light chain expressed on either the basolateral or apical surface, where they induce amino acid transport (Chillaron et al., 2001; Verrey et al., 1999; Verrey et al., 2000). The light chain is the transporter and the heavy chain is required for the functional cell surface expression and localization of the HAT complex (Nakamura et al., 1999). CD98 is expressed in all cell types with the exception of platelets and its highest levels of expression are in the tubules of the kidney and the gastrointestinal tract (Quackenbush et al., 1987; Verrey et al., 2000), where it plays a critical role in the vectorial transport of amino acids across a polarized epithelium. In polarized cells, CD98 targets the HAT basolaterally, whereas rBAT, the other heavy chain found in HAT complexes, localizes the transporter to the apical compartment (Verrey et al., 2000). CD98 associates with its light chain by disulphide bonds found on the extracellular domain and these associations are required for the amino acid transport function of the HATs (Estevez et al., 1998).

In addition to its role as a component of HAT complexes, there is strong evidence supporting a role for CD98 in stimulating integrin function. The first data came from studies that showed that CD98 enhanced integrin-dependent cell aggregation (Ohgimoto et al., 1995; Ohgimoto et al., 1996; Ohta et al., 1994; Okamoto et al., 1997a; Okamoto et al., 1997b; Suga et al., 1997; Tabata et al., 1994). CD98 was also independently identified in a screen for proteins that stimulated integrin affinity (Fenczik et al., 1997). In addition, crosslinking CD98 with antibodies was shown to stimulate integrin α3β1-dependent adhesion of small cell lung and breast cancer cells (Chandrasekaran et al., 1999; Fenczik et al., 1997) and induce phosphoinositol 3-OH kinase (PI3-K) activity in a β1 integrin- and focal adhesion kinase (FAK)-dependent manner (Rintoul et al., 2002).

We previously utilized two non-physiological approaches to gain insight into the mechanism by which CD98 alters integrin function. In the first of these systems, Chinese Hamster Ovary (CHO) cells expressing constitutively active integrin chimeras consisting of α6β1 intracellular domains and αIIbβ3 transmembrane and extracellular domains (αβPy cells) were utilized (Fenczik et al., 2001; Zent et al., 2000). Wild-type CD98 and chimeric CD98 proteins, where the transmembrane, cytoplasmic and extracellular domains were replaced with another type II transmembrane protein, CD69, were assessed for their ability to complement free β1 tail-induced (Tac-β1)

dominant suppression of these 'activated' integrins. From these studies both the cytoplasmic and transmembrane domains of CD98 were found to be required for CD98 to restore affinity of the chimeric integrin. In the second system either endogenous CD98 from Jurkat cells or CD98 variants expressed in CHO cells were evaluated for their ability to bind to an affinity matrix consisting of recombinant integrin β1 cytoplasmic domains (Fenczik et al., 2001; Zent et al., 2000). Again, the transmembrane and cytoplasmic domains of CD98 were implicated in mediating the interaction with β1 cytoplasmic tails and this association was independent of CD98 interactions with the light chain. Others have also shown that CD98 coimmunoprecipitates with β1 integrins in a number of different cell lines (Kolesnikova et al., 2001; Merlin et al., 2001; Rintoul et al., 2002) and overexpression of CD98 in Madin Darby Canine Kidney (MDCK) cells alters epithelial cell cytoskeletal morphology (Merlin et al., 2001). CD98 mutants that lacked the five N-terminal amino acids of the transmembrane domain had no effect on the cytoskeletal transformation (Merlin et al., 2001), whereas mutants truncated after the first five amino acids of the cytoplasmic tail did. Thus regions within the transmembrane domain and juxtamembrane portion of the cytoplasmic tail of CD98 appear to play a critical role in CD98 association with β1 integrins and the resultant increase in integrin affinity promotes changes in cell behavior.

The present study explores the relevance of the past mechanistic findings of CD98 modulation of integrin affinity in a more physiological epithelial cell system. Utilizing chimeric and truncation mutants of CD98 expressed in mouse inner medullary collecting duct (IMCD) cells we report that only the transmembrane domain of CD98 is necessary for CD98 to interact with β1 integrins. In addition, CD98 and mutants that interact with β1 integrins were found to induce PI3-K-dependent cell adhesion and migration as well as modulate IMCD cell branching morphogenesis in three-dimensional collagen gels. Thus, although only the transmembrane domain of CD98 is required for CD98 to interact with β1 integrins, both the transmembrane domain and the proximal part of the cytoplasmic tail play an important role in modulating integrin-dependent cell adhesion and migration as well as branching morphogenesis of polarized renal epithelial cells.

Materials and Methods

Materials

The hybridoma cell line 4F2 (C13) (anti-CD98) was purchased from American Type Culture Collection (ATCC, Manassas, VA) and the anti-CD98 (4F2) antibody was purified from conditioned medium from the hybridoma cell lines by protein A affinity chromatography. A rabbit polyclonal antibody (1952) to the β1 integrin was obtained from Chemicon (Temecula, CA). The monoclonal antibody to human CD69 (FN50) was obtained from Pharmingen (La Jolla, CA). A goat polyclonal antibody to human CD98 (C20) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-mouse FAK and phospho-FAK [pY^{397}] antibodies were purchased from Biosource International (Camarillo, CA). Rabbit anti-mouse AKT, phospho-AKT (pY 379), ERK, phospho-ERK and phosphopaxillin antibodies were from Cell Signaling Technology Company (Beverly, MA). FITC-conjugated anti-rabbit IgG was purchased from Jackson Immunoresearch Laboratories Westgrove, PA). The PI3-K inhibitors, Wortmannin and LY294002, were purchased from Chemicon.

Generation of CD98 mutants

All numbering uses the amino acid sequence reported in entry 4F2-human (entry P08195) of the Swiss-Prot database (May 2004). The cytoplasmic truncation mutants CD98-77, CD98-82 and CD98-87 were made by PCR utilizing the human full-length CD98 cDNA subcloned into pcDNA3 (Invitrogen, La Jolla, CA). To generate the CD98-77 mutant (deletion of nucleotides 1-337) the primer 5′-TCGGATCCGCCGCCATGTGGGTACGCACCCGCTGGGCACTG-3′ was used, and the CD98-87 deletion mutant (nucleotides 1-367) was generated by using the sense primer 5′-TCGGATCCGCCGCCATG-CTCTTCTGGCTCGGCTGGCTCGGC-3′. The anti-sense primer for both deletion mutants was 5′-AGTCTAGACTATCAGGCCGCGTAG-GGGAAGCG-3′. The generation of CD98-82 and the chimeric constructs (CD98/CD69 constructs) have been previously described (Fenczik et al., 2001). CD98 as well as the different mutant constructs were subcloned into the eukaryotic expression vector pcDNA3.

Generation of cell populations expressing the different CD98 constructs

Immortalized mouse inner medullary collecting duct (IMCD) cells were transfected with vector only (pcDNA3), the full-length human CD98, or the different CD98 constructs described above using Lipofectin transfection reagent (Invitrogen) according to the manufacturer's instructions. Selection was started 48 hours after transfection by adding Geneticin (G418 sulfate) (Invitrogen) at 1 mg/ml to the medium. Stable cell populations of IMCD cells expressing the different CD98 constructs were established utilizing flow cytometry under sterile conditions. Briefly, a suspension of IMCD cells was incubated with monoclonal antibodies directed against human CD98 or CD69 (1:50 dilution), followed by incubation with the appropriate FITC- or PE-conjugated secondary antibodies (1:100 dilution). Flow cytometry was performed with a FACScan instrument (Becton Dickinson, Franklin Lakes, NJ) and cell sorting was performed with a BD FACSAria cell-sorting system.

Cell adhesion

Ninety-six-well plates were coated with collagen I at the indicated concentrations in PBS for 1 hour at 37°C. Plates were then incubated with 0.1% BSA in PBS for 60 minutes to block non-specific adhesion. 100 μl of single-cell suspensions (1×10^6 cells/ml) in serum-free DMEM containing 0.1% BSA were added to 96-well plates and incubated for 60 minutes at 37°C. In some experiments, cells were preincubated with Wortmannin (100 nM) and LY294002 (5 μM) for 4 hours prior to the assay. Non-adherent cells were removed by washing the wells with PBS. Cells were then fixed with 4% paraformaldehyde, stained with 1% crystal violet, solubilized in 2% SDS, and the O.D. of the cell lysates was read at 570 nm. Cells bound to 100% fetal calf serum-coated wells were used to indicate 100% adhesion and cells bound to 1% BSA-coated wells were used to evaluate background. Background values were subtracted from those obtained on serum or collagen I. Four independent experiments were performed in triplicate.

Cell migration

Cell migration was assayed in transwells consisting of polyvinylpyrolidone-free polycarbonate filters with 8-μm pores. The bottom of the filters were coated with collagen I (1 μg/ml) in PBS overnight at 4°C and subsequently incubated with 1% BSA in PBS for 1 hour at 37°C to inhibit non-specific migration. 100 μl of a cell suspension (1×10^6 cells/ml) in serum-free medium containing 0.1% BSA were added to the upper wells and cells were allowed to migrate into the bottom wells for 4 hours at 37°C. In some experiments, cells were preincubated with the PI3-K inhibitors at the concentrations indicated above. Cells on the top of the filter were removed by wiping

and the filter was fixed in 4% formaldehyde in PBS. Migrating cells were stained with 1% crystal violet, and nine randomly chosen fields counted at 200× magnification. Four independent experiments were performed in triplicate.

Immunoblotting

The different IMCD cell populations were serum starved for 12 hours and detached from the plates with trypsin. The trypsin was inactivated by the addition of 1 mg chicken egg white trypsin inhibitor/ml (Sigma). Cells were then centrifuged and resuspended in serum-free medium. One half of the cells was kept in suspension for 30 minutes at 37°C, while the other half was replated on collagen I (10 μg/ml) for 10 and 30 minutes at 37°C, as previously described (Hanks et al., 1992). Cells were then washed twice with PBS and lysed using RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, protease inhibitor cocktail, 1 mM Na_3VO_4, 1 mM NaF) for 20 minutes. Lysates were clarified by centrifugation at 12,000 ***g*** for 10 minutes at 4°C. 20 μg total protein was run onto an SDS gel and subsequently transferred to nitrocellulose membranes. Membranes were blocked in 5% milk/TBS Tween and then incubated with the different primary antibodies followed by the appropriate HRP-conjugated secondary antibodies. Immunoreactive bands were identified using enhanced chemiluminescence according to the manufacturer's instructions.

Immunoprecipitation

The different IMCD cell populations were lysed in a lysis buffer consisting of 20 mM HEPES, 150 mM NaCl, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1% (v/v) Brij-35 and protease inhibitor cocktail. The lysates were centrifuged at 12,000 ***g*** for 10 minutes and the supernatants were utilized for immunoprecipitation. 200 μg total cell lysates were incubated with 4 μg of the appropriate primary antibody and 20 μl packed protein-G Sepharose (Amersham Biosciences, Piscataway, NJ) and incubated overnight at 4°C. Beads were washed three times in lysis buffer and bound proteins were eluted by boiling the beads in SDS-PAGE sample buffer under reducing conditions. Samples were separated on an SDS gel and processed as indicated above.

Immunofluorescence

Glass coverslips were coated with 20 μg/ml collagen I overnight at 4°C and blocked with 1% bovine serum albumin (BSA) in PBS for 1 hour at room temperature. Cells were plated onto collagen I-coated coverslips in serum-free medium for 1 hour at 37°C. Cells were fixed in 4% paraformaldehyde in PBS for 15 minutes and permeabilized in 0.4% Triton X-100 in PBS for 10 minutes. Non-specific binding sites were blocked with 1% BSA in PBS for 1 hour and cells were incubated for 14 hours with anti-phosphopaxillin antibody (1:100 in 1% BSA/PBS). Cells were then incubated with a FITC-conjugated anti-rabbit secondary antibody together with 3.3 nM Alexa 594-conjugated phalloidin (Molecular Probes) for 45 minutes at room temperature. Coverslips were mounted on glass slides with ProLong Anti-fade kit (Molecular Probes, Eugene, OR) and imaged on a Zeiss Axiophot microscope and a RT Slider Spot digital camera.

Three-dimensional cell culture

Tubulogenesis of IMCD cells was performed in 3-D collagen gels composed of 0.1 mg/ml collagen I in Dulbecco's minimal essential media containing 20 mM HEPES (pH 7.2) as previously described (Chen et al., 2004). An equal volume of DMEM/F12 medium and 10% FBS was added to the gels. The cells were allowed to grow for 7-9 days at which time phase-contrast photomicrographs were taken using a Nikon Diaphot TMD inverted microscope.

Statistical analysis

The Student's *t*-test was used for comparisons between two groups, and analysis of variance using Sigma-Stat software was used for statistical differences between multiple groups. $P \leq 0.05$ was considered statistically significant.

Results

The transmembrane domain and juxtamembrane portion of the cytoplasmic tail of CD98 are highly conserved

Sequence analysis revealed that the juxtamembrane portion of the cytoplasmic tail and the transmembrane domains are 100% identical in all known mammalian CD98 orthologs (Fig. 1A). Notably, these domains are also the most

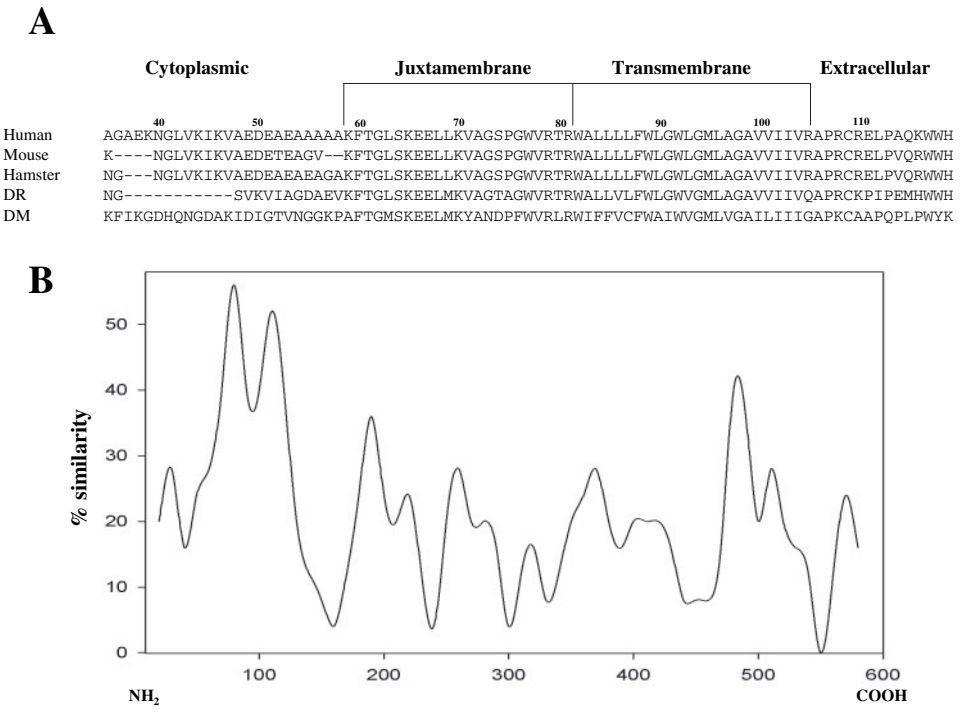

Fig. 1. The transmembrane and juxtamembrane domains of CD98 are highly conserved. (A) Amino acid sequences of the cytoplasmic, transmembrane and proximal extracellular domains of the human, mouse, hamster, zebrafish (DR) and *Drosophila* (DM) CD98 protein or CD98 homologs. Zebrafish and human CD98 had 44 identical amino acids out of 47 (94%) in the transmembrane and juxtamembrane domains but only 32% identity for the rest of the protein (data not shown). (B) The sequence of the *Drosophila* homolog accession number AY070626 was compared to the human CD98 sequence at 10-amino acid intervals utilizing alignment software MultAlin (Corpet, 1988). The percentage of sequence similarity was plotted on the *y* axis and the amino acid number on the *x* axis.

highly conserved part of the *Drosophila* and zebrafish CD98 homologs (Fig. 1A,B). By contrast, the amino acid sequence of rBAT, the closest mammalian paralog to CD98, shows only a 25% similarity in amino acid sequence in the transmembrane and juxtamembrane cytoplasmic domains. The high conservation of the transmembrane domain and juxtamembrane portion of CD98 suggest they play a critical role in mediating specific cellular functions.

The transmembrane domain of CD98 interacts with β1 integrins and induces integrin-dependent cell adhesion and migration

IMCD cells are polarized mouse renal tubular epithelial cells that undergo tubulogenesis in three-dimensional collagen I gels and whose integrin profile has been previously defined (Chen et al., 2004). These cells adhere and migrate on collagen I in an integrin α1β1- and α2β1-dependent manner. In addition, IMCD cells express endogenous CD98 but not CD69 (data not shown).

To test the importance of the transmembrane domain and the cytoplasmic tail of CD98 on integrin-dependent epithelial cell adhesion and migration, we generated IMCD cell populations expressing full-length human CD98 as well as chimeras of CD98 with CD69 (Fig. 2). IMCD cell populations stably expressing the different CD98 mutants were obtained by sorting the cells by flow cytometry utilizing monoclonal antibodies directed against either the extracellular domain of human CD98 or human CD69. In cell populations expressing CD98 or chimeras in which the extracellular domain was CD98, cells with similar levels of surface expression were obtained (Fig. 2). Similarly, in all the cell populations expressing chimeras in which the extracellular domain was CD69, cell sorting achieved populations with similar levels of expression. Overexpression of CD98 or the chimeras did not alter the expression levels of β1 integrins in IMCD cells (data not shown).

To determine the effects of overexpression of full-length CD98 or its chimeras on cell adhesion and migration, the cells were allowed to adhere to collagen I at different concentrations for an hour in serum-free conditions, or migrate onto collagen I for 4 hours. IMCD cells overexpressing the full-length CD98 adhered to and migrated on collagen I significantly better than IMCD cells transfected with vector only (Fig. 3A,B). Similar to our previous studies, IMCD cell populations expressing the cytoplasmic and transmembrane domains of CD98 and extracellular domain of CD69 (C98T98E69) adhered and migrated significantly better than the vector-transfected IMCD cell population, but significantly less than IMCD cells expressing full-length CD98 (Fig. 3A,B). Surprisingly, IMCD cell populations expressing the transmembrane and extracellular domain of CD98 and the cytoplasmic domain of CD69 (C69T98E98) migrated significantly better than cells expressing the full-length CD98. However, populations expressing only the transmembrane (C69T98E69) or the cytoplasmic (C98T69E69) domains of CD98 adhered and migrated in a similar manner as vector controls (Fig. 3A,B). Thus the transmembrane domain of CD98 is required, but is not sufficient, for increased cell adhesion and migration. As deletion of the cytoplasmic tail enhanced cell adhesion and migration relative to full-length CD98, it appears that the cytoplasmic tail acts as a negative regulator of the CD98 transmembrane domain.

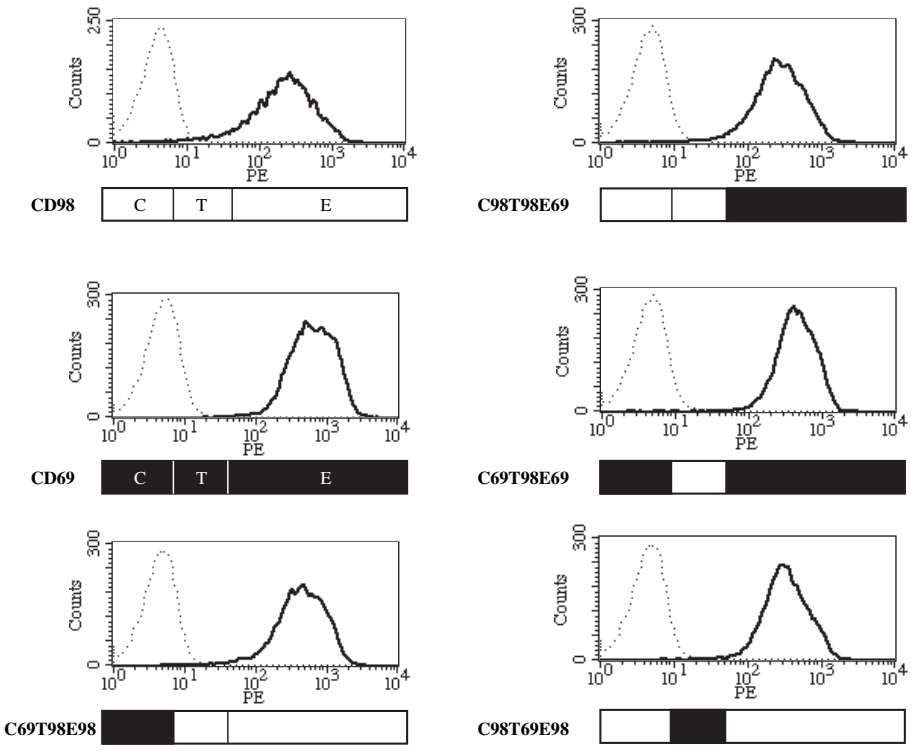

Fig. 2. Generation of stable IMCD cell populations expressing CD98 or CD98/CD69 chimeras. IMCD cells were transfected with the constructs illustrated. Transfected cell populations were sorted by flow cytometry utilizing antibodies to either the human CD98 or the human CD69 extracellular domain. The expression levels of the cell populations (solid lines) are shown relative to IMCD cells transfected with pcDNA3 vector only (dotted lines). C, cytoplasmic domain; T, transmembrane domain; E, extracellular domain.

The transmembrane domain of CD98 is sufficient for its interaction with β1 integrins

To determine whether the increased adhesion and migration induced by CD98 and the CD98/69 chimeras was due to an interaction with β1 integrins, we performed coimmunoprecipitation experiments. In cells overexpressing full-length CD98, β1 integrin and CD98 interactions were demonstrated utilizing either β1 or CD98 antibodies in the precipitation step (Fig. 4A). Similarly β1 integrin coimmunoprecipitated with the chimeric construct C98T98E69 (Fig. 4B), however this was only demonstrated using an anti-CD98 antibody in the precipitation step, as there are no antibodies that recognize the extracellular domain of human

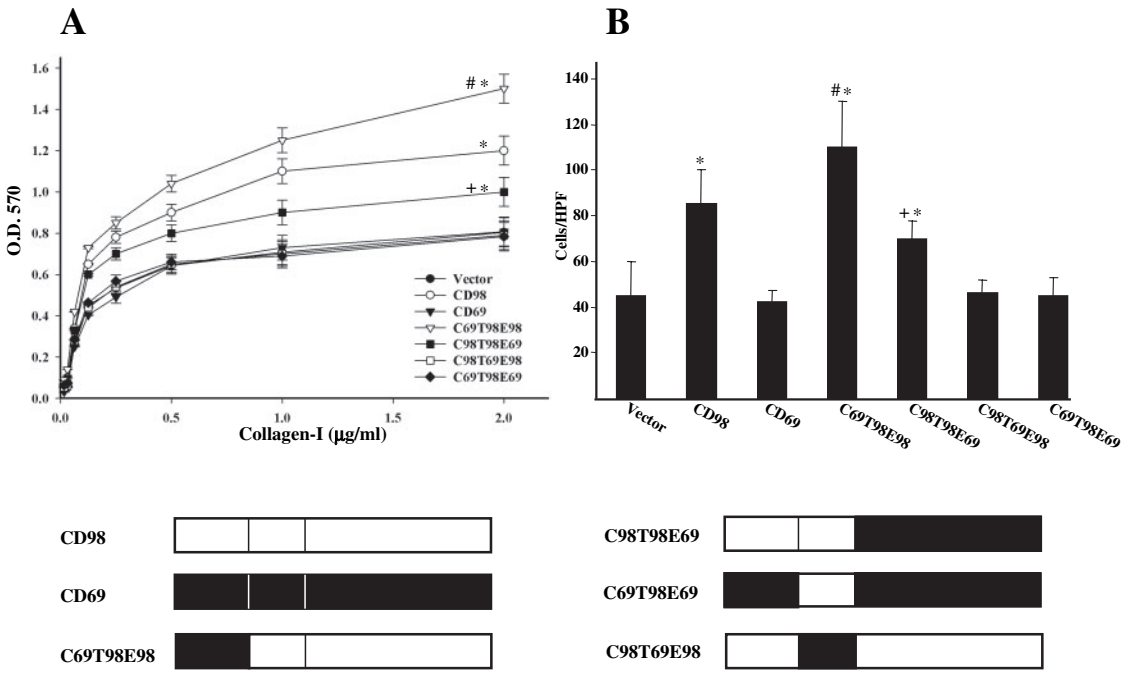

Fig. 3. The transmembrane domain of CD98 is required to enhance cell adhesion and migration. (A) The IMCD cell populations transfected with the constructs indicated were plated onto 96-well plates coated with collagen I at the concentrations indicated for 1 hour in serum-free medium. Adherent cells were then stained with crystal violet, lysed and the OD measured. Data represent the mean±s.d. of quadruplicate samples/cell population. (B) IMCD cell populations were plated in transwell dishes coated with 1 μg/ml collagen I and migration was evaluated 4 hours after plating. The values indicate the mean±s.d. of three independent experiments. Differences in cell adhesion and cell migration between CD98 or CD98 chimeras and vector control cells (*), or between C69T98E98 and CD98 (#), or CD98 and C98T98E98 (+) were significant with $P<0.05$.

CD69 for use in immunoblotting. These results support our previous affinity chromatography observations that the transmembrane and cytoplasmic domains of CD98 are necessary for its interaction with β1 integrin (Fenczik et al., 2001). However, in contrast to our past results, but in agreement with the adhesion and migration results, we observed that the cytoplasmic domain of CD98 was not required for the interaction as the C69T98E98 chimera coimmunoprecipitated with the β1 integrin (Fig. 4A). Interestingly, expression of only the transmembrane domain of CD98 (C69T98E69) was sufficient for CD98 binding with the β1 integrin (Fig. 4B), even though the amount of β1 coimmunoprecipitating with this chimera was significantly less than that detected with the C69T98E98 chimera (Fig. 4A). The chimera with the transmembrane domain of CD98 replaced with CD69 (C98T69E98) was unable to bind β1 integrins (Fig. 4A). These results suggest

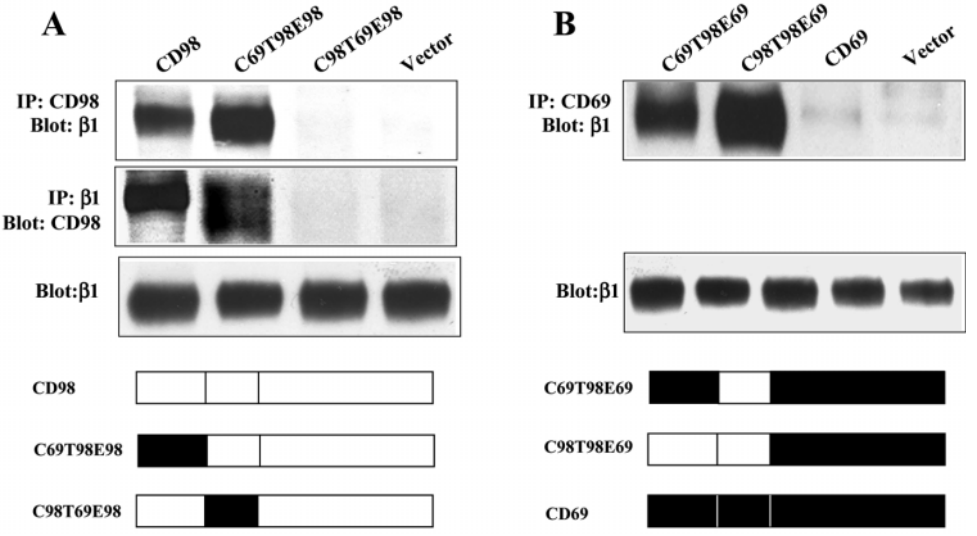

Fig. 4. The transmembrane domain of CD98 associates with β1 integrin. (A) Equal amount of cell lysates (500 μg) from cell populations transfected with either control vector (Vector) or the constructs illustrated were immunoprecipitated with an anti-human CD98 (upper panel) or an anti-mouse β1 integrin (middle panel) antibody. The immunoprecipitates were separated by 7% SDS-PAGE and transferred to nitrocellulose. Membranes were subsequently immunoblotted with an anti-mouse integrin β1 (upper panel) or an anti-human CD98 (middle panel) antibody. The lower panel represents an immunoblot of total cell lysates for mouse β1 integrin to confirm equal loading. (B) Cell lysates from cell populations prepared as described in A were immunoprecipitated with an anti-human CD69 (upper panel) and immunoblotted with an anti-mouse integrin β1. The lower panel is an immunoblot of the cell lysates for mouse integrin β1 to demonstrate equal loading.

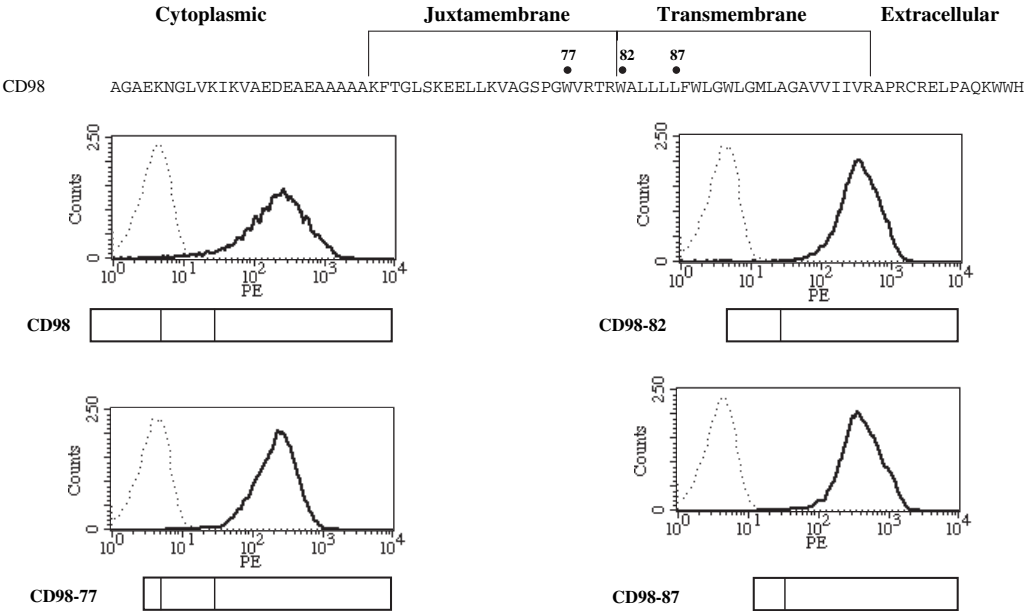

Fig. 5. Generation of stable IMCD cell populations expressing CD98 or CD98 deletion mutants. IMCD cells were transfected with the full-length or deletion CD98 constructs illustrated. The cell populations were sorted by flow cytometry utilizing an antibody to the human CD98 extracellular domain. The expression levels of the cell populations (solid lines) are shown relative to IMCD cells transfected with vector only (dotted line)

that in polarized IMCD cells the transmembrane domain of CD98 is sufficient for the interaction with the β1 integrin. However the binding to this integrin is highly enhanced when the transmembrane domain of CD98 is expressed together with either its cytoplasmic or extracellular domains.

The WALLL sequence in the transmembrane domain of CD98 is required for the increased cell adhesion

Although the transmembrane domain of CD98 appeared to be sufficient for interactions with β1 integrins (Fig. 4), the extracellular domain of CD98 was required to increase cell adhesion and migration mediated by the CD98 transmembrane domain (Fig. 3A,B). To identify the critical regions of the transmembrane domain and/or juxtamembrane region required for integrin-dependent functions, a series of truncation mutants of CD98 were analyzed (Fig. 5). The CD98-87 deletion mutant had the N-terminal five amino acids (WALLL) of the transmembrane domain deleted. The CD98-82 mutant was truncated before the tryptophan residue of the WALLL sequence, which is the last amino acid of the putative transmembrane domain. The CD98-77 mutant retained the first five amino acids (WVRTR) of the cytoplasmic tail. IMCD cell populations expressing equal amount of these three mutants were generated (Fig. 5). There was no difference in β1 integrin expression between the cell populations (data not shown).

Similar to the C69T98E98 chimera, expression of the CD98-82 mutant significantly enhanced IMCD cell adhesion and migration when compared to cells transfected with vector only, or overexpressing full-length CD98 (Fig. 6A,B). The CD98-87 mutant did not increase the adhesion or migration of the cells when compared to the wild-type controls and the CD98-77 deletion mutant adhered and migrated on collagen to a similar degree as the CD98-expressing cells. These results indicate that the WALLL sequence in the transmembrane domain is required for the increased cell adhesion and migration. Furthermore, the

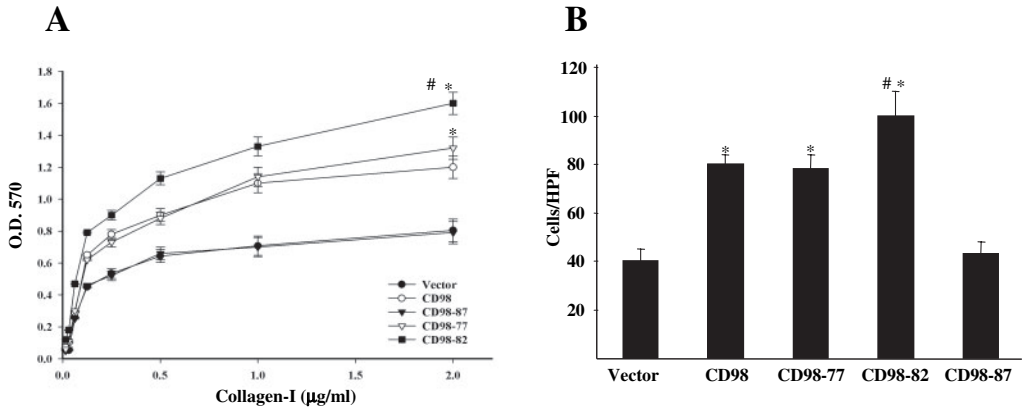

Fig. 6. The five N-terminal amino acids of the transmembrane domain of CD98 are required for increased cell adhesion and migration. (A) The different IMCD cell populations transfected with the CD98 constructs indicated were plated on collagen I at the concentrations indicated as described in Fig. 3. The values represent the mean±s.e. of quadruplicate samples/cell population. (B) Migration assays were performed as described in Fig. 3. Bars and errors represent the mean±s.e. of three independent experiments. Differences in cell adhesion and migration between CD98 or CD98 truncations and vector control cells (*) or between CD98-82 and CD98 (#) were significant with $P<0.05$.

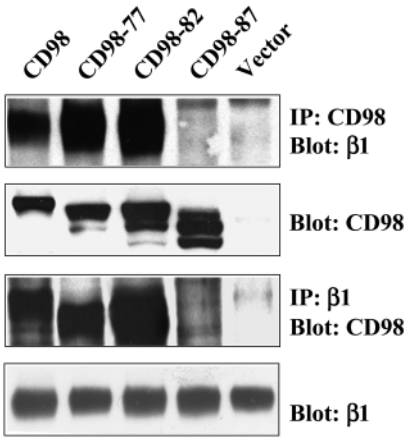

Fig. 7. The five N-terminal amino acids of the transmembrane domain of CD98 are required for CD98 association with β1 integrin. (A) Equal amount of cell lysates (500 μg) from cell populations transfected with either control vector or the CD98 constructs indicated were immunoprecipitated with an anti-human CD98 (upper panel) or an anti-mouse β1 integrin (third panel). The immunoprecipitates were separated by SDS-PAGE as indicated in Fig. 4 and membranes were incubated with an anti-mouse integrin β1 (upper panel) or an anti-human CD98 antibody (third panel). Equal amounts of cell lysate were immunoblotted with an anti-human CD98 (second panel) or an anti-mouse integrin β1 antibody (fourth panel) to demonstrate equal amounts of CD98 and β1 integrin in the different cell populations.

WVRTR sequence of the juxtamembrane domain appears to downregulate the effect of the transmembrane domain.

To confirm that these alterations in cell adhesion and migration are related to an interaction with β1 integrins, coimmunoprecipitation experiments were performed. β1 integrin was detected in immunoprecipitates of the CD98-77 and CD98-82 deletion mutants, but not the CD98-87 deletion mutant (Fig. 7).

CD98 expression induces FAK phosphorylation and PI3-K activation

Crosslinking CD98 has been demonstrated to activate PI3-K in an integrin β1- and FAK-dependent fashion (Rintoul et al., 2002). To determine the effects of CD98 and the truncation mutants on integrin-dependent activation of FAK and PI3-K, as well as the ERK pathway, levels of phosphorylated FAK, AKT and ERK were measured following IMCD cell adhesion to collagen I. Cells overexpressing full-length CD98, CD98-77 and CD98-82, but not CD98-87, induced FAK phosphorylation in cells either kept in suspension or following plating on collagen I when compared to vector controls (Fig. 8, upper panel). Levels of phosphorylated AKT were also significantly increased following cell plating on collagen I in CD98, CD98-77 and CD98-82, but not CD98-87 transfected cells (Fig. 8, middle panel). No difference in ERK activation was seen between the different cell populations (Fig. 8, lower panel). Thus overexpression of CD98 and truncation mutants able to interact with β1 integrins result in increased FAK and AKT phosphorylation.

The role of PI3-K activation in increasing cell adhesion and migration of CD98, CD98-77 or CD98-82 was investigated further. Cells were starved of serum for 24 hours and incubated with the PI3-K inhibitors Wortmannin (100 nM) or LY294002 (5 μM) for 4 hours following which cell adhesion and migration assays were performed. Both inhibitors significantly decreased cell adhesion and migration of CD98, CD98-77 and CD98-82 expressing cells when compared to vector controls and CD98-87 expressing cells (Fig. 9). Phosphorylation of AKT was completely inhibited 10 minutes after plating onto collagen following addition of the PI3-K inhibitors (Fig. 9). Thus increased adhesion and migration of IMCD cells overexpressing full-length CD98, CD98-77 and CD98-82 mutants was mediated by PI3-K activation.

CD98 expression increases focal adhesion formation

The increased FAK phosphorylation observed in CD98, CD98-77 and CD98-82 cells suggested an increase in integrin clustering that should result in increased focal adhesion formation. We therefore examined the effects of CD98 and the mutants on the localization and formation of focal adhesions as well as the actin cytoskeleton. To do this, cells were allowed to adhere on collagen I for 1 hour and subsequently incubated with an anti-phosphopaxillin antibody (a component of the activated focal adhesions) and fluorescently labeled phalloidin

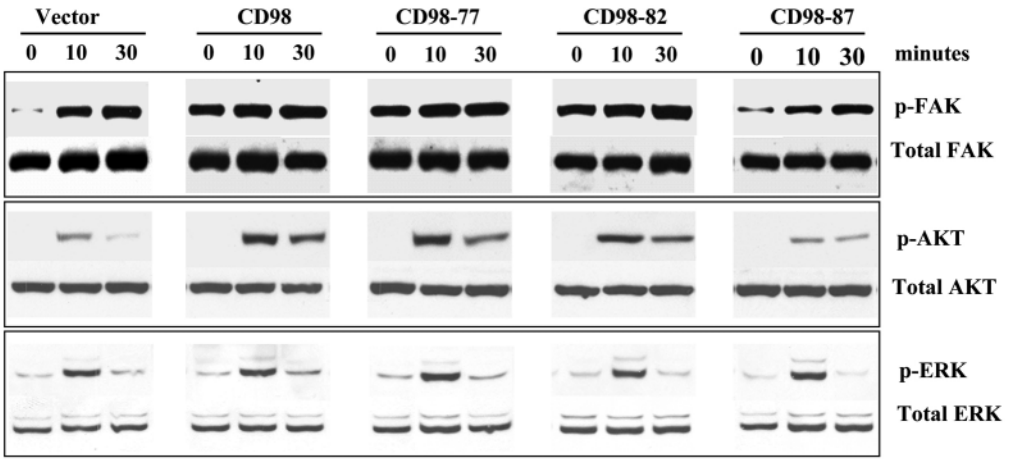

Fig. 8. CD98 association with integrin β1 induces FAK and AKT phosphorylation. The different cell populations transfected with the CD98 constructs indicated were serum starved for 12 hours, trypsinized and left in suspension or replated on 10 μg/ml collagen I for 10 or 30 minutes. Equal amounts of cell lysate were separated by 10% SDS-PAGE and transferred to nitrocellulose. The membranes were immunoblotted with antibodies to phospho-FAK (p-FAK), total FAK, phospho-AKT (p-AKT), total AKT, phospho-ERK (p-ERK) and ERK.

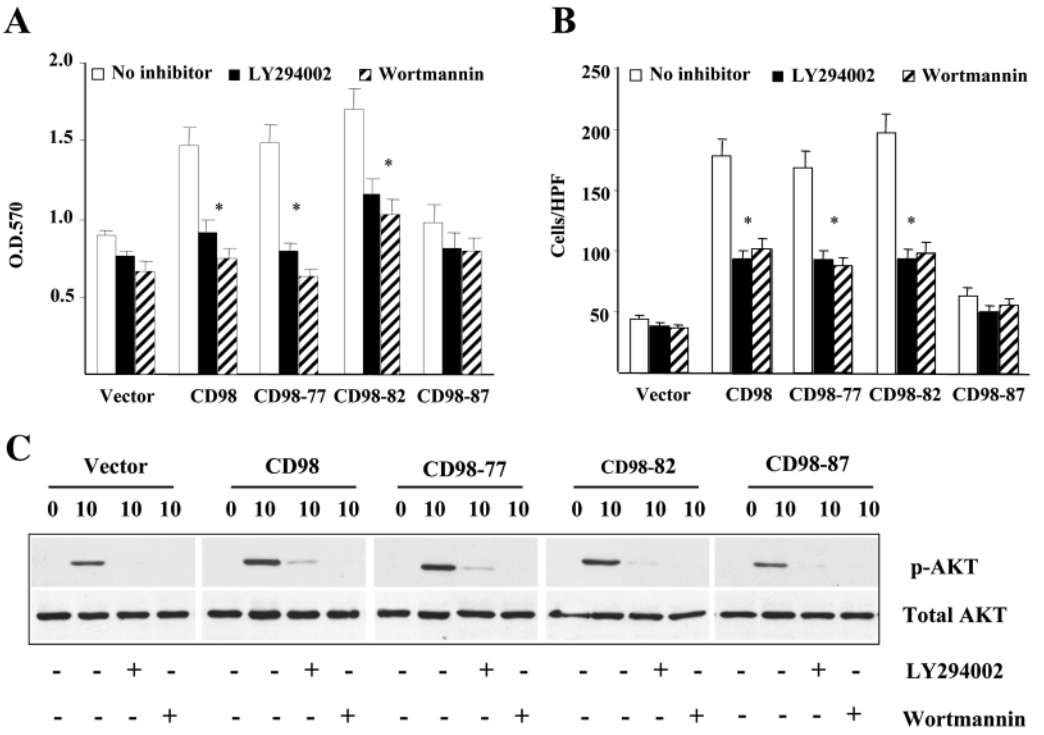

Fig. 9. CD98-induced cell adhesion and migration is mediated by PI 3-kinase. The cell populations transfected with the CD98 constructs indicated were serum starved for 12 hours and then kept untreated or treated with Wortmannin (100 nM) or LY294002 (5 μM) for 4 hours. The cells were trypsinized and their adhesion (A) and migration (B) analyzed as described in Fig. 3. To verify that Wortmannin and LY294002 inhibited PI3-K activity, cells cultured with or without the inhibitors were lysed 10 minutes after plating on collagen I and immunoblotted for phospho-AKT and total AKT (40 μg lane). Differences in adhesion and migration between untreated and Wortmannin- or LY294002-treated cells (*) were significant with $P<0.05$.

(to visualize the cytoskeleton). IMCD cells overexpressing full-length CD98, as well as the CD98-77 and CD98-82 mutants had defined rings of cortical F-actin around their periphery and had an increased number of activated focal adhesions when compared to vector controls and the CD98-87 mutant (Fig. 10). Thus, CD98 and mutants that interact with the β1 integrins result in increased cortical actin formation and the establishment of focal adhesions.

CD98 alters IMCD branching morphogenesis in three-dimensional collagen gels

As 3D-culture assays are a well-described model system used to recapitulate the cellular events of branching morphogenesis of polarized epithelial cells, we performed these assays on CD98, CD98-77, CD98-82 and CD98-87 expressing IMCD cells in collagen I gels. Although CD98 transfected cells proliferated well and formed multi-branched structures in the 3D collagen gels, the cells had a mesenchymal phenotype and there was no evidence of tube formation (Fig. 11). The CD98-77 mutants also formed highly branched non tube-like structures; however they were shorter than the CD98-transfected cells. The CD98-82 mutants formed cysts with little evidence of branching, whereas the CD98-87 cells underwent branching morphogenesis similar to that seen in the vector-transfected control cells. These results indicate that the WALLL sequence in the transmembrane domain, the WVRTR sequence of the juxtamembrane domain and the cytoplasmic tail of CD98 distal to the WVRTR sequence induce differential effects on cell biological functions required for branching morphogenesis.

Discussion

The cytoplasmic and transmembrane domains of CD98 were previously shown to be required for interactions with β1 integrins in affinity chromatography assays (Fenczik et al., 2001). The same domains were required to restore β1 integrin affinity in a non-physiological system where a constitutively active integrin chimera was suppressed by overexpression of free β1 integrin tails in CHO cells (Fenczik et al., 2001). We now show in a physiologically relevant polarized renal tubular epithelial cell system that: (1) the transmembrane domain of CD98 is required, but not sufficient, for the enhancement of integrin-dependent adhesion and migration; (2) the WALLL sequence within the transmembrane domain of CD98 is required for CD98-β1 integrin interactions; (3) CD98-β1 integrin interactions increase focal adhesion formation and FAK phosphorylation; (4) CD98-induced cell adhesion and migration is dependent on PI3-K activation; and (5) CD98 and its mutants that interact with β1 integrins impair IMCD cell branching morphogenesis in collagen gels. Thus, in addition to its other functions, CD98 can modulate β1 integrin-dependent cell adhesion and migration as well as branching morphogenesis of polarized epithelial cells.

The observation that IMCD cell adhesion and migration was enhanced by overexpression of CD98 and CD98 coimmunoprecipitated with β1 integrins, strongly suggests that CD98-β1 integrin interactions are required for CD98 to increase integrin-dependent cell functions. This result is consistent with the observations that CD98 reverses Tac-β1 dominant suppression (Fenczik et al., 2001); crosslinking of CD98 with antibodies increases integrin-dependent cell adhesion (Fenczik et al., 1997) and crosslinking of CD98 requires integrin β1 and FAK to induce PI3-K activation and cell proliferation (Rintoul et al., 2002).

Utilizing the CD98/CD69 chimeras we demonstrated that only the transmembrane domain of CD98 was required, but not sufficient, for the induction of increased IMCD cell adhesion

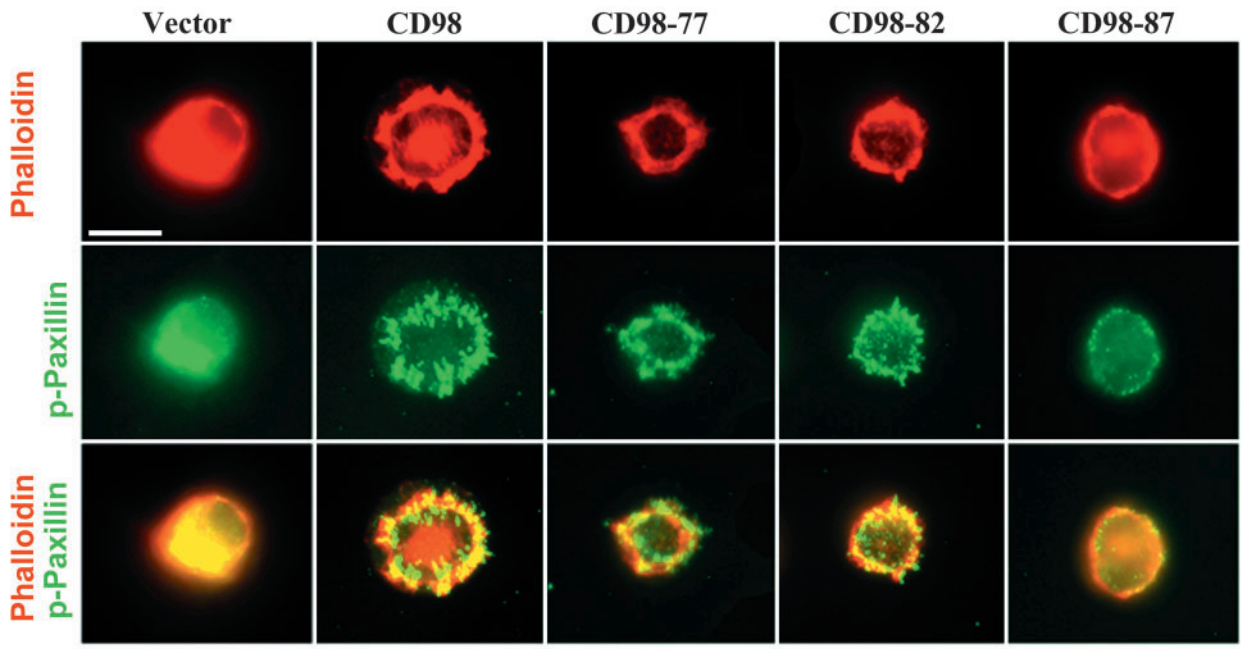

Fig. 10. CD98 association with integrin β1 induces focal adhesion formation. IMCD populations expressing the different constructs indicated were plated onto collagen I-coated coverslips for 1 hour and cells were subsequently stained with Alexa 594-conjugated phalloidin to visualize the cytoskeleton (upper panel) and anti-phosphopaxillin antibody to identify activated focal adhesions (middle panel). The lower panels show merged images. Bar, 50 μm.

and migration. In contrast to our previous findings that both the cytoplasmic and transmembrane domains of CD98 were required for reversal of Tac-β1 suppression in CHO cells (Fenczik et al., 2001), we found that changing the cytoplasmic tail of CD98 to CD69 enhanced cell adhesion and migration compared to either full-length CD98 or the chimera where only the extracellular domain of CD69 was expressed. The reason for this discrepancy may be due to the different model system utilized. In the more physiological system used in this study, the transmembrane domain of CD98 interacts, either directly or indirectly, with endogenously expressed β1 whereas in our previous studies on CHO cells, CD98 altered integrin affinity by reversing transdominant suppression induced by free integrin β1 tails (Fenczik et al., 2001). Similarly, differences in experimental systems could explain why the transmembrane domain of CD98 is necessary and sufficient for interactions with β1 integrin in polarized epithelial cells, whereas both the transmembrane and the cytoplasmic domains of CD98 were required for CD98-β1 interactions in our previous study (Fenczik et al., 2001). In the present study coimmunoprecipitation of CD98 and endogenous β1 integrins was performed, whereas affinity chromatography with recombinant β1 cytoplasmic tails was used previously (Fenczik et al., 2001; Zent et al., 2000).

Utilizing the truncation mutants we confirmed that the transmembrane domain, specifically the WALLL sequence within this region of CD98 was required for the increased cell adhesion, migration as well as the interaction with β1 integrins. As cells expressing the CD98-77 mutant behaved in a similar fashion to cells expressing the full-length CD98, we believe that the WVRTR sequence of the proximal cytoplasmic tail conferred some regulatory functions of CD98.

Although we do not provide direct mechanistic evidence, we hypothesize that the CD98 enhanced integrin-mediated cell adhesion and migration may be mediated by increasing integrin affinity for ligand as previously shown in CHO cells (Fenczik et al., 2001; Zent et al., 2000). This speculation is based on the fact that substitution of bulky amino acids within the transmembrane helix of the β3 integrin subunit induces constitutive activation of integrin αIIbβ3, thus promoting

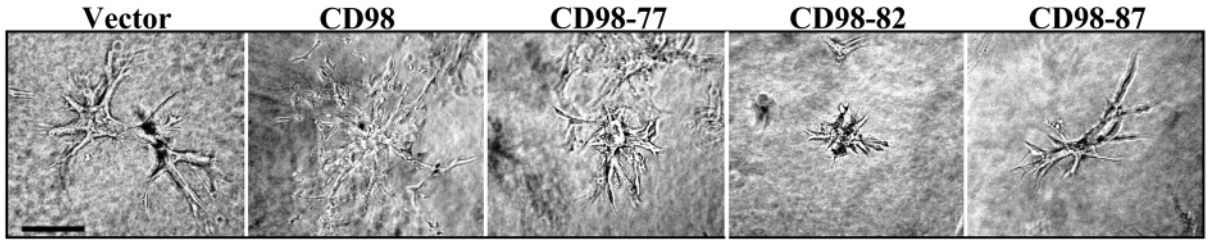

Fig. 11. CD98 association with integrin β1 alters IMCD cell branching morphogenesis. IMCD populations expressing the different constructs indicated were grown in 3-D collagen I gels for 9 days. Phase-contrast images of typical examples of branching structures are shown. Bar, 50 μm.

association and oligomerization of integrin αIIbβ3 and increased clustering on the cell membrane (Li et al., 2003). As the transmembrane domain of CD98 (WALLLLFWLGW) contains three highly conserved bulky tryptophan residues, it is possible that overexpressed CD98, via its interaction with β1 integrins, promotes homo-oligomerization of the β1 transmembrane domains and increased αβ1 clustering on the cell surface. The mechanism whereby the WVRTR sequence within the cytoplasmic tail of CD98 decreased the cell adhesion and migration is also unknown, however the juxtamembrane region of the β1 integrin tail (Armulik et al., 2004) has been shown to be critical for induction of integrin-dependent migration. Thus, it is possible that the WVRTR polypeptide of CD98 interacts with the β1 integrin tail altering integrin activation.

The fact that overexpression of full-length CD98, as well as deletion mutants that bind β1 integrins, enhanced phosphorylation of both FAK and paxillin, as well as focal adhesion formation supports the idea that CD98 induces integrin clustering. Our finding that CD98-induced cell adhesion and migration is PI3-K dependent is consistent with the data that crosslinking CD98 activates PI3-K in an integrin β1- and FAK-dependent fashion (Rintoul et al., 2002). Although it is possible that CD98 induces its effects on cell adhesion and migration by inducing FAK-dependent PI3-K activation (Chen and Guan, 1994), PI3-K may be regulated independently of FAK as only FAK and not AKT is constitutively activated by CD98. In fact PI3-K activation by CD98 might be the mechanism for increased integrin activation resulting in increased cell adhesion and migration.

For IMCD cells to undergo branching morphogenesis in collagen gels, they need to be able to polarize as well as undergo β1 integrin-dependent cell adhesion and migration (Chen et al., 2004). Although IMCD cells expressing CD98 or the mutants that interact with β1 integrins adhere and migrate well on collagen I, they were unable to form tubes. This suggests that CD98 and the mutants induced alterations in the cytoskeleton, similar to those seen in MDCK cells overexpressing CD98 (Merlin et al., 2001), which resulted in the IMCD cells being unable to polarize. We cannot explain the differences in phenotype between the CD98, the CD98-77 and the CD98-82 cells, but it is possible that the CD98-82 mutant forms cyst-like structures because they have the most profound cytoskeletal changes. The phenotypical difference between the CD98 and the CD98-77 cells is more subtle and may be accounted for by the increased proliferation of the CD98 cells in collagen I when compared to the CD98-77 mutants (N.B. and R.Z., unpublished data).

In summary we demonstrate that CD98 modulates integrin-dependent cell adhesion and migration, as well as branching morphogenesis in polarized renal epithelial cells. CD98 is also required for the surface expression of the HAT transporter complex to the basolateral aspect of polarized epithelial cells (Nakamura et al., 1999). This CD98/LAT (HAT) complex was recently demonstrated to interact with ICAM-1 and crosslinking either CD98 or ICAM-1 can affect the intrinsic transporter activity of the HAT (Liu et al., 2003). Thus CD98 can be viewed as a scaffolding protein that associates with various basolaterally expressed proteins in polarized epithelial cells where it coordinates diverse cellular functions such as amino acid transport, cell adhesion, migration and perhaps polarization.

We would like to thank Catherine Allen at the VA flow cytometry core for technical help and Mark Ginsberg for supplying some constructs. This work was supported by the Veterans Administration Advanced Career Development and Merit Award, the American Heart Association Grant in Aid Award, RO1 DK069921-01 and a Clinician Scientist award from NKF (R.Z.), by National Institutes of Health Grant NCI RO1 CA94849-01 (A.P.), National Institutes of Health Grant RO1 GM49882 (S.K.H.) and by the Vanderbilt George M. O'Brien Center for Kidney and Urologic Research Grant DK39261 (A.P. and R.Z.).

References

Armulik, A., Velling, T. and Johansson, S. (2004). The integrin beta1 subunit transmembrane domain regulates phosphatidylinositol 3-kinase-dependent tyrosine phosphorylation of Crk-associated substrate. *Mol. Biol. Cell* **15**, 2558-2567.

Chandrasekaran, S., Guo, N. H., Rodrigues, R. G., Kaiser, J. and Roberts, D. D. (1999). Pro-adhesive and chemotactic activities of thrombospondin-1 for breast carcinoma cells are mediated by alpha3beta1 integrin and regulated by insulin-like growth factor-1 and CD98. *J. Biol. Chem.* **274**, 11408-11416.

Chen, D., Roberts, R., Pohl, M., Nigam, S., Kreidberg, J., Wang, Z., Heino, J., Ivaska, J., Coffa, S., Harris, R. C. et al. (2004). Differential expression of collagen- and laminin-binding integrins mediates ureteric bud and inner medullary collecting duct cell tubulogenesis. *Am. J. Physiol. Renal Physiol.* **287**, F602-611.

Chen, H.-C. and Guan, J.-L. (1994). Association of focal adhesion kinase with its potential substrate phosphatidylinositol 3-kinase. *Proc. Natl. Acad. Sci. USA* **91**, 10148-10152.

Chillaron, J., Roca, R., Valencia, A., Zorzano, A. and Palacin, M. (2001). Heteromeric amino acid transporters: biochemistry, genetics, and physiology. *Am. J. Physiol. Renal Physiol.* **281**, F995-1018.

Corpet, F. (1988). Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res.* **16**, 10881-10890.

Estevez, R., Camps, M., Rojas, A. M., Tesrar, X., Deves, R., Hediger, M. A., Zorzano, A. and Palacin, M. (1998). The amino acid transport system y+L/4F2hc is a heteromultimeric complex. *FASEB J.* **12**, 1319-1329.

Fenczik, C. A., Sethi, T., Ramos, J. W., Hughes, P. E. and Ginsberg, M. H. (1997). Complementation of dominant suppression implicates CD98 in integrin activation. *Nature* **370**, 81-85.

Fenczik, C. A., Zent, R., Dellos, M., Calderwood, D. A., Satriano, J., Kelly, C. and Ginsberg, M. H. (2001). Distinct domains of CD98hc regulate integrins and amino acid transport. *J. Biol. Chem.* **276**, 8746-8752.

Hanks, S. K., Calalb, M. B., Harper, M. C. and Patel, S. K. (1992). Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronectin. *Proc. Natl. Acad. Sci. USA* **89**, 8489-8491.

Kolesnikova, T. V., Mannion, B. A., Berditchevski, F. and Hemler, M. E. (2001). beta1 integrins show specific association with CD98 protein in low density membranes. *BMC Biochem.* **2**, 10.

Li, R., Mitra, N., Gratkowski, H., Vilaire, G., Litvinov, R., Nagasami, C., Weisel, J. W., Lear, J. D., DeGrado, W. F. and Bennett, J. S. (2003). Activation of integrin alphaIIbbeta3 by modulation of transmembrane helix associations. *Science* **300**, 795-798.

Liu, X., Charrier, L., Gewirtz, A., Sitaraman, S. and Merlin, D. (2003). CD98 and intracellular adhesion molecule I regulate the activity of amino acid transporter LAT-2 in polarized intestinal epithelia. *J. Biol. Chem.* **278**, 23672-23677.

Merlin, D., Sitaraman, S., Liu, X., Eastburn, K., Sun, J., Kucharzik, T., Lewis, B. and Madara, J. L. (2001). CD98-mediated links between amino acid transport and b1 integrin distribution in polarized columnar epithelia. *J. Biol. Chem.* **15**, 15.

Nakamura, E., Sato, M., Yang, H., Miyagawa, F., Harasaki, M., Tomita, K., Matsuoka, S., Noma, A., Iwai, K. and Minato, M. (1999). 4F2 (CD98) heavy chain is associated covalently with an amino acid transporter and controls intracellular trafficking and membrane topology of 4F2 heterodimer. *J. Biol. Chem.* **274**, 3009-3016.

Ohgimoto, S., Tabata, N., Suga, S., Nishio, M., Ohta, H., Tsurudome, M., Komada, H., Kawano, M., Watanabe, N. and Ito, Y. (1995). Molecular characterization of fusion regulatory protein-1 (FRP-1) that induces multinucleated giant cell formation of monocytes and HIV gp160-mediated cell fusion. *J. Immunol.* **155**, 3585-3592.

Ohgimoto, S., Tabata, N., Suga, S., Tsurudome, M., Kawano, M., Nishio, M., Okamoto, K., Komada, H., Watanabe, N. and Ito, Y. (1996). Regulation of human immunodeficiency virus gp160-mediated cell fusion by antibodies against fusion regulatory protein 1. *J. Gen. Virol.* **77**, 2747-2756.

Ohta, H., Tsurudome, M., Matsumura, H., Koga, Y., Morikawa, S., Kawano, M., Kusugawa, S., Komada, H., Nishio, M. and Ito, Y. (1994). Molecular and biological characterization of fusion regulatory proteins (FRPs): anti-FRP mAbs induced HIV-mediated cell fusion via an integrin system. *EMBO J.* **13**, 2044-2055.

Okamoto, K., Ohgimoto, S., Nishio, M., Tsurudome, M., Kawano, M., Komada, H., Ito, M., Sakakura, Y. and Ito, Y. (1997a). Paramyxovirus-induced syncytium cell formation is suppressed by a dominant negative fusion regulatory protein-1 (FRP-1)/CD98 mutated construct: an important role of FRP-1 in virus-induced cell fusion. *J. Gen. Virol.* **78**, 775-783.

Okamoto, K., Tsurudome, M., Ohgimoto, S., Kawano, M., Nishio, M., Komada, H., Ito, M., Sakakura, Y. and Ito, Y. (1997b). An anti-fusion regulatory protein-1 monoclonal antibody suppresses human parainfluenza virus type 2-induced cell fusion. *J. Gen. Virol.* **78**, 83-89.

Quackenbush, E., Clabby, M., Gottesdiener, K. M., Barbosa, J., Jones, N. H., Strominger, J. L., Speck, S. and Leiden, J. M. (1987). Molecular cloning of complementary DNAs encoding the heavy chain of the human 4F2 cell-surface antigen: a type II membrane glycoprotein involved in normal and neoplastic cell growth *Proc. Natl. Acad. Sci. USA* **84**, 6526-6530. (erratum in *Proc. Natl. Acad. Sci. USA* **84**, 8618).

Rintoul, R. C., Buttery, R. C., Mackinnon, A. C., Wong, W. S., Mosher, D., Haslett, C. and Sethi, T. (2002). Cross-Linking CD98 Promotes Integrin-like Signaling and Anchorage-independent Growth. *Mol. Biol. Cell* **13**, 2841-2852.

Suga, S., Tsurudome, M., Ito, M., Ohgimoto, S., Tabata, N., Nishio, M., Kawano, M., Komada, H., Sakurai, M. and Ito, Y. (1997). Human immunodeficiency virus type-1 envelope glycoprotein gp120 induces expression of fusion regulatory protein (FRP)-1/CD98 on CD4+ T cells: a possible regulatory mechanism of HIV-induced syncytium formation. *Med. Microbiol. Immunol. (Berl.)* **185**, 237-243.

Tabata, N., Ito, M., Shimokata, K., Suga, S., Ohgimoto, S., Tsurudome, M., Kawano, M., Matsumura, H., Komada, H., Nishio, M. et al. (1994). Expression of fusion regulatory proteins (FRPs) on human peripheral blood monocytes. *J. Immunol.* **153**, 3256-3266.

Verrey, F., Jack, D. L., Paulsen, I. T., Saier, M. H., Jr and Pfeiffer, R. (1999). New glycoprotein-associated amino acid transporters. *J. Membr. Biol.* **172**, 181-192.

Verrey, F., Meier, C., Rossier, G. and Kuhn, L. C. (2000). Glycoprotein-associated amino acid exchangers: broadening the range of transport specificity. *Pflugers Arch.* **440**, 503-512.

Zent, R., Fenczik, C. A., Calderwood, D. A., Liu, S., Dellos, M. and Ginsberg, M. H. (2000). Class- and splice variant-specific association of CD98 with integrin beta cytoplasmic domains. *J. Biol. Chem.* **275**, 5059-5064.