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PRINCIPAL INVESTIGATOR: Csilla A. Fenczik, Ph.D.

CONTRACTING ORGANIZATION: The Scripps Research Institute
La Jolla, California 92037

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6. AUTHOR(S)
Csilla A. Fenczik, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Scripps Research Institute
La Jolla, California 92037

E-MAIL:
csilla@scripps.edu

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The process of metastasis and invasion of tumor cells requires that the cells regulate their ability to adhere to the surrounding extracellular matrix. Integrins, the family of cell adhesion receptors that mediate the adhesion of cells to the matrix are able to modulate their affinity for ligand. We have identified CD98 as a regulator of integrin affinity using an expression cloning strategy that utilizes the overexpression of free integrin beta 1 cytoplasmic domains. Cells expressing high levels of free beta 1 tails show reduced integrin affinity which results in an inhibition of cell adhesion, cell migration and fibronectin matrix assembly. Proteins involved in integrin affinity regulation were identified by their capacity to complement integrin suppression caused by overexrression of free beta 1 tails. In this report we investigate the mechanisms by which Cd98 may affect integrin function.

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INTRODUCTION

The development and function of multicellular animals requires integrin adhesion receptors. Integrin-dependent cell adhesion is regulated, in part, by ligand binding affinity ("activation") changes controlled by cellular signaling cascades. Regulation of integrin affinity is important in cell migration, extracellular matrix assembly and morphogenesis. Integrin activation is energy-dependent, and is mediated by cell type specific signals operating through integrin cytoplasmic domains.

Complementation of Dominant Suppression (CODS) is an expression cloning scheme used to identify proteins that modulate integrin affinity. CODS depends on the ability of an isolated integrin β1A cytoplasmic domain, in the form of a chimera with the α subunit of the IL-2 receptor, to block integrin activation (dominant suppression). Proteins involved in integrin activation are isolated by their ability to complement dominant suppression. CD98 heavy chain (hc), a type II transmembrane protein first discovered as a T-cell activation antigen, was identified utilizing CODS. CD98hc, although widely expressed on proliferating cells, is generally down regulated in quiescent cells. CD98hc forms disulfide-bonded heterodimers with several light chains that strongly resemble permeases. CD98hc regulates the transport of neutral and positively charge amino acids through these light chains. Thus, CODS has identified an unexpected connection between cell adhesion and certain amino acid transporters. During the tenure of this fellowship I aimed to address three specific aims:
1) To characterize a protein which when expressed reverses the inhibitory effects of Tac-β1A.
2) To understand the mechanism by which this protein reverses the inhibitory effects of Tac-β1A
3) To identify other proteins that rescue Tac-β1A inhibition using the same screen.

BODY

Materials and Methods

Antibodies and Reagents

The isolation and characterization of the anti-β3 monoclonal antibody, anti-LIBS6, has been previously described (1). The activation specific anti-αIIbβ3 monoclonal antibody PAC1 (2) was a gift from Dr. S. Shattil (Scripps Research Institute, La Jolla, CA). The anti-Tac antibody, 7G7B6 was obtained from the American Type Culture Collection (Rockville, MD, USA). The anti-Tac antibody was biotinylated with biotin-N-hydroxy-succinimide (Sigma) according to manufacturer's directions. The αIIbβ3-specific peptidomimetic inhibitor Ro43-5054 (3) was a gift from Dr. Beat Steiner (F. Hoffman,

La Roch, Basel, Switzerland). The hybridoma cell line 4F2(C13) was purchased from American Type Culture Collection (ATCC). Ascites was produced in pristane-primed BALB/c mice. Fab fragments were prepared by papain digestion of purified 4F2 IgG (2 mg/ml) for 5 h at 37°C. Digestion was terminated by the addition of iodoacetamide. Fab fragments were purified on Protein-A sepharose columns. Fab fragments were characterized by SDS-PAGE and exhibited characteristic mobility. The mouse hybridoma cell line, 12CA5 (anti-HA), was purchased from ATCC. Anti-CD69 antibody (C1, a rabbit polyclonal antibody) was a generous gift from Drs. F. Diaz-Gonzalez and F. Sanchez-Madrid (University of Madrid, Spain).

cDNA constructs

The Tac-β1 and Tac-α5 chimeras in the CMV-IL2 vector were the gift of Drs. S. LaFlamme and K. Yamada (4). pSG5 MKP-1, was a gift from Dr. N. Tonks (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) (5). pDCR-H-Ras (G12V), was a gift from Dr. M. H. Wigler (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) (6). The human 4F2 antigen (CD98) cDNA was kindly provided by Dr. J.M. Leiden (Univ. of Chicago, IL) and was subcloned into pcDNA1 as an EcoR1 fragment. Cless (C109S; C330S), C1 (C109S) and C2(C330S) were gifts from Dr. M. Palacin (Universitat de Barcelona). E-16 was a gift from Dr. F. Verrey (University of Zurich). The pcDNA3 construct encoding CD9 has been described(7). The pRc/RSV plasmid, pIAP45, encoding CD47, was a gift from Drs. F. Lindberg and E. Brown (Washington Univ. at St. Louis, MO). uPAR, in pcDNA1, pcuPAR1, was provided by Dr. L Miles (Scripps Research Institute, La Jolla, CA).

Recombinant proteins: cDNA encoding the expressed integrin cytoplasmic domains joined to 4 heptad repeats were cloned into the modified pET-15 vector as previously described(8). Point mutations in β1D and β7 were performed utilizing the Quickchange kit (Stratagene). Recombinant expression in BL21 (DE3) pLysS cells (Novagen) and purification of the recombinant model proteins were made in accordance with the manufacturers instructions (Novagen), with an additional final purification step on a reverse phase C18 high performance liquid chromatography column (Vydac). Polypeptide masses were confirmed by electrospray ionization mass spectrometry on an API-III quadrupole spectrometer (Sciex; Toronto, Ontario, Canada) and varied by less than 4 Daltons from those predicted by the desired sequence.

Construction of CD98hc chimeras: HA-NH2 was constructed by PCR with primers that included a nine amino acid Influenza Hemaglutinin (HA)-tagfollowed by a three Gly linker that was placed directly after the initiator Met. The primers used to create HA-COOH added the HA-tag to the C-terminal portion of CD98hc and was preceded by a Gly linker. All of the CD98hc chimeras were made by overlap PCR. ΔCD98 deletes amino acids 2–77 which removes the entire cytoplasmic domain of CD98, maintaining the intiator methionine as well as the presumptive stop transfer sequence ValArgThrArg. $C_{69}T_{98}E_{98}$ contains amino acids 1-38 of CD69 (Swissprot:Q07108) and amino acids 82-529 of CD98(Swissprot:P08195). $C_{98}T_{69}E_{98}$ contains amino acids amino acids 1-81 and 105-529 of CD98 and amino acids 39-64 of CD69. $C_{98}T_{98}E_{69}$ contains amino acids 1-104

of CD98 and amino acids 65-199 of CD69. $C_{98}T_{69}E_{69}$ contains amino acids 1-81 of CD98 and amino acids 39-199 of CD69.

Cell Lines and Transfection

Chinese Hamster Ovary (CHO-K1) cells were obtained from American Type Culture Collection, (Rockville, Md.). The generation of the αβpy cell line has been described (9). Briefly, CHO-K1 cells were stably transfected with pPSVE-PyE (a gift of D. M. Fukuda, Burnham Institute, La Jolla, Ca.), which encodes the polyoma large T antigen. pPSVE-PvE was co-transfected with replication deficient CDM8 expression constructs encoding αIIbα6A and β3β1A. All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker, Walkersville, MD) containing 10% fetal bovine serum, 1% non-essential amino acids, 2 mM glutamine (Sigma) and 100 units/ml penicillin and 100 μg/ml streptomycin.

Cells were transfected using a lipofectamine procedure. For each 10 cm tissue culture dish with 40-60% confluent cells, 20 μl of lipofectamine reagent (GibCo BRL) and 10 μg of plasmid DNA were mixed in 200 μl of DMEM. After a 10 minute incubation, the DNA-lipofectamine mixture was diluted 1:20 and added to the cells. The cells were incubated for 6 hours and then washed with complete medium. Cells were incubated for 48 hours at 37°C.

Flow cytometry.

PAC1 binding was analyzed by two-color flow cytometry as described(10). Transfected cells were detached with cell dissociation buffer (GibCo BRL, Gaithersburg, MD), for 5 min. at room temperature. The detached cells were pooled, centrifuged, and resuspended in complete medium containing 0.1% of PAC1 ascites. Control cells are also incubated with either 1 μM of the competitive inhibitor, Ro-43-5054 as a negative control, or with anti-LIBS6, an activating antibody, as a positive control. After a 30 minute incubation at room temperature, the cells were washed and then resuspended in complete medium containing a 1:25 dilution of the biotinylated anti-Tac antibody, 7G7B6. After 30 min on ice, the cells were washed and incubated with 10% FITC-conjugated goat anti-mouse IgM (TAGO) and 4% phycoerythrin-streptavidin (Molecular Probes, Inc.). Thirty min later cells were resuspended in phosphate buffered saline (PBS) for flow cytometric analysis. PAC1 binding was assessed in a subset of transiently transfected αβpy cells (cells positive for co-transfected Tac-α5 as measured by 7G7B6 binding). Integrin activation was quantified as an activation index (AI) defined as (F-Fo)/(FLIBS6-Fo) in which F is the median fluorescence intensity of PAC1 binding; Fo is the median fluorescence intensity of PAC1 binding in the presence of saturating concentration of a competitive inhibitor (Ro43-5054, 1 μM); and FLIBS6 is the maximal median fluorescence intensity in the presence of the integrin activating antibody, anti-LIBS6 (2 μM). Percent reversal is calculated as $(AI_{(\beta x\ +\ CD98)} - AI_{\beta x})/(AI_{\alpha 5} - AI_{\beta x})$. $AI_{\beta x}$ is the activation index of cells transfected with Tac-βx chimeras, $AI_{(\beta x\ +\ CD98)}$ is the AI of cells co-transfected with CD98 and Tac βx chimeras, and $AI_{\alpha 5}$ is the AI of cells transfected with Tac-α5. The x of βx can have values of 1A, 1D and 7 for the Tac-β1A, Tac-β1D and Tac-β7 chimeras respectively.

DNA Sequencing and Sequence Analysis.
Nucleotide sequences were determined with an ABI automated sequencer by using oligonucleotides synthesized according to the flanking sequences and obtained sequences within the insert. Sequences were aligned using the program Sequencher®. The entire length of the insert was sequenced in both directions. The sequence was analyzed with the UWGCG software package. Nucleotide and protein database searches were conducted using BLAST.

Measurement of ERK2 activity.
For ERK2 assays, 2 x 10^5 cells were transfected using as described above with 2μg of pCMV5 HA-ERK2 (HA; hemagglutanin tagged). The cells were also transfected with 2μg of the test plasmid e.g. pDCR H-Ras(G12V). In some experiments 4-6μg of a second plasmid e.g. MKP-1 were co-transfected and the total amount of DNA was adjusted to a total of 10μg, by the addition of pCDNA1, in each transfection. Transfections were done in duplicate to allow parallel analysis of both ERK2 kinase activity and PAC1 binding by flow cytometry, as described above. Forty-eight hrs post transfection, cells were harvested and lysed in 0.5% NP40 buffer containing phosphatase inhibitors (20mM NaPyrophosphate and 1mM Na_3VO_4) in addition to protease inhibitors. The activity of the HA-ERK2 was measured by the immune complex in-gel kinase assay method(11;12) using the anti-HA antibody 12CA5 (Boehringer Mannheim). ERK2 expression was monitored by running 25μg of whole cell lysate in SDS sample buffer on 12.5% SDS-polyacrylamide gels, transferring to Immobilon (Millipore, Bedford, Ma.) and immunoblotting with anti-HA antibody.

Adhesion assay
Laminin and fibronectin (Calbiochem) were used as substrates in serum-free adhesion assays. Small cell lung cancer cell line H345 (ATCC) were maintained in RPMI and 10% serum and for experimental purposes were passaged into serum free medium RPMI 1640 containing SITA (sigma). 3-5 days post passage 1-2X10^6 cells/ml were washed twice in RPMI and disaggregated into single cells. 50ml of cells in RPMI were added to 96 well tissue culture plates (Costar) coated with extracellular matrix proteins blocked with 1mg/ml BSA. Cells were allowed to attach for 45 min at 37^{0}C. Cell attachment was determined by crystal violet staining. The attachment of H345 cells to wells coated with 25mg/ml poly-L lysine and fixed with 20% gluteraldehyde prior to aspiration was defined as 100% adhesion(13).

Recombinant Cytoplasmic Domain Synthesis
The cytoplasmic domains of various integrin tails were synthesized using previously described methods(8). In brief, PCR was used to create a cDNA encoding a four-heptad repeat protein sequence, which formed a coiled-coil structure. A cDNA encoding four glycine residues was joined to the C terminus of this structure. This cDNA was ligated into a modified pET15b vector (Novagen) in such a manner that cDNAs for different integrin cytoplasmic tails could be ligated into the vector. The proteins were recombinantly expressed in BL21(De3)pLysS cells and purified according to the pET system manual (Novagen). The proteins were purified on a reverse phase C18 high

performance liquid chromatography column (Vydac) and analyzed by electrospray ionization mass spectroscopy.

Cells and Cell lysates

Human Jurkat cells and αβpy cells were washed twice in phosphate-buffered saline (PBS) and biotynylated with 1mM N-sulfohydroxysuccinimidobiotin-biotin (Pierce) in PBS for 30 minutes at room temperature. After two additional washes with Tris buffered saline (TBS) cells were lysed on ice with buffer X (1 mM $Na_3 VO_4$, 50 mM NaF, 40 mM sodium pyrophosphate, 10 mM Pipes, 50 mM NA Cl, 150 mM sucrose, pH 6.8) containing 1% Triton X-100, 0.5% sodium deoxycholate, 1mM EDTA, 20 ug/ml aprotonin, 5 ug/ml leupeptin and 1mM PMSF. Lyates were sonicated and spun at 14 000 RPM for 30 minutes in a microcentrifuge.

Affinity Chromatography

Recombinant proteins were expressed inBL21(DE3)pLysS cells (Novagen) and bound to His-bind resin (Novagen) through their N-terminal His-tag in a ratio of 1 ml of beads per liter of culture. Coated beads were washed with PN (20 mM Pipes, 50 mM NaCl, pH 6.8) and stored at 4 °C in an equal volume of PN containing 0.1% NaN_3. Beads were added to cell lysates diluted in buffer A, (0.05% Triton X-100, 3 mM MgCl2, and protease inhibitors) and incubated overnight at 4°C and then washed five times with buffer A. 100 ul of SDS-sample buffer was added to the beads and the mixture was heated at 100°C for 5 minutes. After 10000 r.p.m. centrifugation for 30 min in a microfuge (5417C Eppendorf), the supernatant was fractionated by SDS-PAGE and analyzed by Western blotting. Chimeras with the extracellular domain of CD69 were analyzed in the absence of DTT because the anti-CD69 antibody employed only detects the nonreduced form of CD69. In some experiments, proteins were eluted off the beads with 100 ul of elution buffer (1 M imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9) and 1 ml of immunoprecipitation buffer (20 mM Tris-HCl, 150 mM NaCl, 10 mM Benzamidine HCl, 1% Triton X-100, 0.05% Tween 20 and protease inhibitors) was then added. The eluted proteins were immunoprecipitated overnight at 4°C with an anti-CD98 antibody pre-bound to protein A Sepharose beads (Pharmacia). The following day, the beads were washed three times with the immunoprecipitation buffer and heated in sample buffer for SDS-PAGE containing 1 mM Dithiothreitol. Samples were separated on 4-20% SDS-polyacrylamide gels (NOVEX) and transferred to nitrocellulose membranes. Membranes were blocked with Tris-buffered saline, 5% nonfat milk powder and stained with streptavidin peroxidase or with specific antibodies and appropriate peroxidase conjugates. Bound peroxidase was detected with an enhanced chemiluminescence kit (Amersham). Equal loading of Ni^{2+} beads with recombinant proteins were verified by Coomassie Blue staining of SDS-PAGE profiles of SDS eluted proteins.

Amino Acid Transport

3H -labeled Ile (77 Ci/mM) was purchased from Amersham. αβPy cells were transfected with cDNAs encoding CD98hc or each of the chimeric cDNAs in the presence or absence of cDNA encoding E-16 using Lipofectamine 2000 (Life Sciences Technologies). Transport studies were performed on cells that were transfected with 80-90% efficiency, as judged by staining for CD98 in flow cytometry. Before the transport assays, cells

were rinsed with warm Na+-free Hanks buffered salt solution (HBSS) (136.6 mM ChCl, 5.4 mM KCl, 4.2 mM NaHCO3, 2.7 mM Na2HPO4, 1 mM CaCl2 , 0.5 mM MgCl2, 0.44 mM KH2PO4, 0.41 mM MgSO4, pH 7.8)), in which the sodium containing salts were iso-osmotically replaced with choline, to remove extracellular Na+ and amino acids. Cells were incubated in warm choline substituted HBSS for 10 min to deplete intracellular amino acids. The uptake of radiolabeled amino acids (2 μCi of ^{3}H-Ile/ml) at 100 μmol/liter in 1ml of choline substituted HBSS was measured for 20 s at 37° C. Preliminary experiments indicated that uptake of ^{3}H-Ile was linearly dependent on incubation time up to at least 3 min. Uptake was terminated by washing the cells rapidly four times with 1ml/well of ice-cold HBSS. The cells were incubated overnight with 1 ml 2 M NaOH to release intracellular radioactivity. A 0.95-ml aliquot from each well was mixed with scintillation fluid and radioactivity was quantified in a Beckman LS 6000SC liquid scintillation counter. The remaining 0.1 ml was analyzed for protein content using the BCA protein assay reagent (Pierce). Transport velocities were calculated using radioactivity, specific activities of uptake mixes, and protein absorbance values and expressed as cpm X 100/20sec/mg protein.

Results

Clone 5F8 encodes the hamster homologue of the human 4F2 antigen heavy chain, CD98. The 5F8 insert contained 1902 base pairs, containing an open reading frame encoding a 533 amino acid polypeptide. In addition, it contains a polyA tract and polyadenylation signal and it's sequence has been deposited in Genbank (Accession#U93712). Analysis of the predicted topology of the encoded protein using Tmpred suggested that it possesses a single transmembrane domain with the N terminus inside the cell (Type II transmembrane protein). A BLAST database search showed that 5F8 is related to the heavy chain of the 4F2 antigen, CD98, from both mouse and human(14). The predicted hamster protein sequence is 72% identical to the human protein and thus appears to be the hamster homologue of CD98. To test whether human CD98 could substitute for the hamster protein in reversing dominant negative suppression, the cDNA for human CD98 was co-transfected with Tac-β1 into αβpy cells. Overexpression of human CD98 also reversed dominant suppression and we therefore conclude that we have isolated its hamster homologue (Fig. 1).

To further characterize the specificity of CD98 rescue of Tac-β1 suppression I have shown that CD98 rescue was not dependent upon the alpha cytoplasmic tail. To test this, I used cells that express integrin chimeras that contain the cytoplasmic domains of α2, α5 and α6b that are all constitutively active when expressed with the β1A tail. The activation state of all of these chimeras was suppressed by Tac-β1A and the suppression was recovered by CD98 expression (data not shown).

I also have shown that the wild-type α5β1 receptor present in CHO cells is also rescued from dominant suppression by CD98 expression (data not shown). The activation state of this integrin was reported by using GST-fusion proteins containing fibronectin repeats 9-11, which have been shown to require an active integrin to bind.

As discussed in the last annual report we have decided that the most direct approach to understanding how CD98 affects integrin function would be to create a CD98 knockout mouse. The knockout construct has been completed and was sent to the Scripps Core Facility to generate a mouse that contains a deletion of the CD98 gene. Chimeric mice have been generated and are currently being bred. ES cells that contain the knockout construct were transfected with a similar construct that contained a different drug resistance gene. Cells were chosen and selected for absence of CD98 at the cell surface. Two cell lines were derived that do not express CD98. These cell lines are currently being studying for effects on both integrin function as well as amino acid transport.

Possible mechanism of CD98 affect on integrin affinity

Since CD98 is a membrane protein it was important to test whether other membrane-associated proteins would reverse dominant negative suppression. We overexpressed, membrane proteins previously implicated in integrin function: CD9(7), CD47(IAP)(15), and uPAR(16). Expression of these proteins in $\alpha\beta$py cells failed to reverse suppression of integrin activation by Tac-β1 (Fig. 1). Protein expression was confirmed by flow cytometry in each case (data not shown). Thus, the rescue of Tac-β1 suppression by overexpression of CD98 is not a non-specific effect of overexpression of membrane proteins.

CD98 does not complement suppression of integrin activation by activated Ras

A Ras-initiated MAP kinase pathway suppresses integrin activation(17). We sought to determine if CD98 could complement this effect. Transfection of an activated Ras (G12V) inhibited PAC1 binding as expected. The degree of inhibition was not affected by expression of CD98 (Fig. 2A). In contrast, expression of MAP kinase phosphatase (MKP-1) blocked Ras suppression. CD98 was functional, since in simultaneous assays, it complemented Tac-β1 suppression (Fig. 2B). Thus, CD98 does not complement all inhibitors of integrin activation.

We also tested whether the Tac-β1 suppressive effect was dependent on the Ras-initiated MAP kinase pathway. Overexpression of Tac-β1 did not activate ERK-2, while ERK-2 was activated by the expression of an activated H-Ras (Fig. 3). Furthermore, co-transfection of MKP-1 failed to reverse the suppressive effect of Tac-β1, whereas co-expression of CD98 completely overcame this effect. Thus, the mechanisms of suppression of integrin activation by Ras or by overexpression of isolated β1 tails differ in CHO cells.

Clustering of CD98 is required for affect on integrin function.

$\alpha_3\beta_1$ integrin-mediated adhesion of the small cell lung cancer cell line (SCLC) H345 to laminin and fibronectin can be dynamically regulated(18). We therefore tested whether CD98 might have a role in regulation of integrins in SCLC adhesion. An anti-CD98 monoclonal antibody (4F2) markedly enhanced single cell adhesion of H345 cells to both laminin and fibronectin (Fig. 4). Addition of the function blocking anti-β_1 antibody (P5D2) or 2mM EDTA abrogated this effect. Control antibodies for 4F2 (D57, anti-$\alpha_{IIb}\beta_3$) and for P5D2 (P41, anti-β_3) had no effect. In addition, B6H12, a monoclonal

antibody against CD47(19) did not enhance adhesion to fibronectin or laminin (results not shown). To assess the role of antibody-mediated CD98 crosslinking in enhancing integrin function, we examined the effects of monovalent Fab fragments of 4F2 (Fab-4F2) on SCLC cell adhesion. Fab-4F2 did not enhance SCLC cell adhesion to laminin or fibronectin (Fig. 4.). The binding of the Fab fragments was confirmed by their ability to block the enhanced adhesion caused by the intact antibody. Consequently, crosslinking of CD98 is required for 4F2 induced upregulation of β_1 integrin function .

CD98 directly associates with β1A cytoplasmic domains.
In our original model we hypothesized that Tac-β1 may be titrating out important factors that are required for integrin activation. To test whether this was happening with CD98 we first asked the question, is CD98 capable of binding to the cytoplasmic domain of β1A? These studies were performed by affinity chromatography from cell lysates using recombinant integrin cytoplasmic domains. This model system involves bacterial expression of integrin β subunit cytoplasmic domains, N-terminally fused to a coiled-coil motif expressing a poly histidine tag. Inclusion of the coiled-coil domain facilitates dimerization of the recombinant protein and acts as a spacer from the affinity matrix. The dimerization of the recombinant tails is used to mimic the cytoplasmic face of an occupied and clustered integrin. Pfaff *et. al.* (8) showed the utility of this system by demonstrating the direct binding of filamin/ABP-280 and talin to integrin cytoplasmic domains.

When a Jurkat cell lysate was exposed to such an affinity matrix, a cell surface polypeptide of 88 kDa bound to the β1A but not to the αIIb tail (Fig. 5A). This polypeptide was immunoprecipitated by the anti-CD98 antibody, 4F2 (Fig. 5B). Based on its mass and reactivity with anti-CD98 antibody, the β1A tail binding polypeptide was identified as CD98.

To assess the specificity of CD98 binding to β integrin tails, affinity chromatography was performed with β1D, β3 and β7 cytoplasmic domains. CD98 did not bind to β7 and binding to β1D was weak and variable (Fig. 6). In contrast, talin and filamin (Fig. 6) bound strongly to β1D and β7 tails respectively as reported(8). CD98 binding to β3 was detectable but weaker than to β1A. Thus CD98 binding to integrin tails is integrin class and splice-variant-specific.

Differential CD98 binding to β integrin tails is independent of filamin and talin binding. CD98 binds well to the β1A integrin cytoplasmic domain but not to those of β1D or β7. The binding assays were performed using talin and filamin-1 containing cell extracts. Thus, these CD98 binding differences could be due to competition for CD98 binding by filamin-1 or talin, which bind preferentially to β7 or β1D respectively(8). To test this possibility, we used filamin-1 deficient human melanoma cells (M2) and reconstituted cells (A7)(20) to examine the role of filamin-1 in CD98 binding. CD98 bound to the β1A tail, but not β7, when lysates of M2 cells were used (Fig. 7), showing that filamin-1 is not required for CD98 binding to β1A. CD98 binding

to β7 was not observed in the filamin-1 null (M2) cells. Consequently, competition with filamin-1 does not account for the failure of β7 to bind CD98.

To examine the role of talin, we used cell membrane preparations with a greatly reduced talin content (Fig. 8A). CD98 extracted from these membranes bound β1A but not β1D cytoplasmic domains (Fig. 8B). Thus talin does not prevent CD98 binding to β1D, nor is it required for CD98 binding to β1A.

The Y788A mutation of β1A disrupts filamin (Fig. 7B) and talin (Fig. 8C) binding(8). Similar Y to A mutations in β7 and β1D tails, corresponding to the Y788A mutation in β1A, also disrupted filamin (Fig. 7B) and talin (Fig. 8C) binding. CD98 binding to β integrin tails was not affected by Y to A mutations (Figs. 7B and 8C). The Y to A mutation introduced into β1D or β7 did not increase CD98 binding nor was CD98 binding reduced in the β1A(Y788A) mutant. These results confirm that talin or filamin competition does not account for the lack of CD98 binding to β1D and β7 and that talin or filamin binding is not required for CD98 binding to the β1A cytoplasmic domain.

CD98 binding to integrin cytoplasmic domains correlates with complementation of dominant suppression. Overexpression of isolated integrin β1A cytoplasmic domains, in the form of a Tac-β1A chimera, results in suppression of integrin activation. Dominant suppression is reversed by overexpression of CD98. Tac-β1A, Tac-β1D and Tac-β7 induced dominant suppression of integrin activation (Fig. 9A). As noted above (Fig. 6), CD98 bound poorly to β1D and β7 tails, showing that CD98 binding is not required for dominant suppression. However, CD98 was much less effective at reversing the suppression induced by Tac-β1D and Tac-β7 (Fig. 9B). Thus the capacity of CD98 to rescue suppression correlates with its binding to the suppressive β cytoplasmic domain.

CD98 binding is not sufficient to induce dominant suppression. As noted above, β1A tails suppress integrin activation and bind CD98. To assess whether CD98 binding alone is sufficient to induce dominant suppression, we first examined CD98 binding to a series of β1A-truncation mutants (Fig. 10B). CD98 binding was lost when the C-terminal seven residues were deleted (β1A(797X)) but not when the last three amino acids were eliminated (β1A(801X)) (Fig. 10A). In spite of maintaining its capacity to bind to CD98, the Tac-β1A(801X) mutant was a poor suppressor of integrin activation (Fig. 11). Furthermore, the β1A(Y788A) mutant, which also bound CD98 (Fig. 7 and 8), failed to suppress integrin activation (Fig. 11). Consequently, integrin β cytoplasmic domain binding to CD98 is not sufficient to induce dominant suppression.

CD98 light-heavy chain association is not required for the interaction of CD98 with integrins. CD98 is a heterodimer formed by a common heavy chain (CD98hc) disulfide bonded to one of a number of light chains that mediate amino acid transport. CD98hc has two extracellular cysteines. To examine the role of CD98 heavy-light chain association on its interactions with integrins, we first investigated the effect of mutation of these Cysteines (C109 and C330). The C109S (C1) mutation alone or in combination

with the C330S (Cless) mutation is reported to reduce the amino acid transport activity of CD98(21-23). However, mutation of both or either Cysteine did not impair the capacity of CD98hc to complement dominant suppression (Fig. 12A). We confirmed that the mutant lacking Cysteines (Cless) failed to form stable disulfide-bonded heterodimers with CD98 light chains (Fig.12C) and found that free CD98 heavy chain bound to the integrin β1A cytoplasmic domain (Fig. 12B). Thus, covalent CD98 heterodimer formation is dispensable for the interaction of CD98hc with the integrin β1A cytoplasmic domain and for CODS.

As an alternative approach to evaluate the role of the CD98 heavy-light chain association, we examined the effect of increased expression of a CD98 light chain (E-16) on integrin interactions. When CHO cells were transfected with CD98 heavy chain, there was a substantial quantity of free heavy chain (Fig. 13B). Transfection of increasing quantities of a light chain, E16, resulted in an increasing proportion of CD98 heterodimers (Fig. 13B). As expected, formation of increased CD98 heterodimers led to a marked increase in amino acid transport (Fig. 13A). However, increased heterodimer formation did not detectably alter the ability of CD98hc to bind to β1A cytoplasmic tails. Thus, the formation of CD98 heterodimers is important for the stimulation of amino acid transport. However, CD98hc alone is sufficient for binding to the integrin β1A tail and for CODS.

Membrane Topography of CD98. In the foregoing experiments, we found that the interactions of CD98 with integrins could be ascribed to its heavy chain. Consequently, we wished to analyze the structural determinants in the heavy chain responsible for these interactions. CD98hc is predicted to be a type II transmembrane protein, with the C-terminus outside and the N-terminus inside. To document the membrane topography of CD98, the amino and carboxyl terminus of CD98 were separately tagged using a using a short peptide sequence from influenza hemagglutinin (HA tag). When cells were transfected with the carboxyl terminal tag, the epitope tag was readily detected on the cell surface (Fig. 14A). In contrast, the amino terminal tag was not detected on the cell surface. The presence of the epitope tag on the N or C terminus did not effect the overall surface expression of CD98hc as measured with an anti CD98 antibody (Fig. 14A). Furthermore, both the N and C-terminal tags were present on the expressed protein (Fig. 14B). Consequently we conclude that the N-terminus of CD98 is intracellular and the C-terminus is extracellular.

The cytoplasmic and transmembrane domains of CD98 are required for its interaction with integrins. Having confirmed the membrane topography of CD98hc, we wished to examine the role of its cytoplasmic and extracellular domain in the integrin interaction. We first deleted the cytoplasmic domain of CD98hc (Fig. 15A). This deletion abolished its ability to complement dominant suppression (Fig. 15B) although it did not block surface expression (data not shown, but see Fig. 17). Deletion of the CD98hc cytoplasmic domain completely abolished its ability to bind to the β1A cytoplasmic domain (Fig. 15C). As an alternative approach, we exchanged the cytoplasmic domain of CD98 with another type II transmembrane protein (CD69). That chimera ($C_{69}T_{98}E_{98}$) failed to complement dominant suppression (Fig. 15B) and failed to

bind to the β1A cytoplasmic domain (Fig. 15C). Thus, the cytoplasmic domain of CD98 is required for its capacity to interact with integrins.

To assess whether the CD98hc cytoplasmic domain was sufficient for this interaction, additional chimeric exchanges were made. A construct containing the extracellular and transmembrane domains of CD69 ($C_{98}T_{69}E_{69}$) joined to the cytoplasmic domain of CD98hc failed to bind to the β1A cytoplasmic domain (Fig. 16). However, addition of the transmembrane domain of CD98 ($C_{98}T_{98}E_{69}$) resulted in binding that was comparable to that observed with full length CD98. As further evidence for the requirement for the transmembrane domain of CD98, a construct was made in which only transmembrane domain was replaced with that of CD69 and the extracellular and cytoplasmic domains were retained ($C_{98}T_{69}E_{98}$). That construct also failed to bind to the β1A cytoplasmic domain (Fig. 16). Thus, binding of CD98hc to the β1A cytoplasmic domain requires both its cytoplasmic and transmembrane domain.

Amino acid transport activity and CODS require structurally distinct regions of CD98. The foregoing studies established that both the cytoplasmic and transmembrane domains of CD98hc were required for its capacity to bind to the β1A cytoplasmic domain. To investigate whether the functional effects of CD98 correlated with its binding to the β1A cytoplasmic domain, each of these chimeras was analyzed for its capacity to mediate CODS and to promote Ile transport. In these experiments, the expression of each chimera was verified by flow cytometry and equivalent expression was observed for each one (data not shown). As previously noted, the substitution of the cytoplasmic domain of CD69 for that of CD98hc abolished CODS ($C_{69}T_{98}E_{98}$). However, that substitution stimulated Ile transport to comparable levels to wild-type CD98hc (Fig. 16C). Similarly, substitution of the transmembrane domain of CD98 ($C_{98}T_{69}E_{98}$) markedly suppressed effects on integrin function but had little effect on the ability to stimulate Ile transport. Thus, the amino acid transport activity of CD98hc is not sufficient for CODS. Conversely, the substitution of the extracellular domain of CD69 for that of CD98 ($C_{98}T_{98}E_{69}$) preserved effects on integrin function but abolished amino acid transport activity (Fig. 16C). Thus, the extracellular domain of CD98 is necessary and sufficient (in the context of another type II transmembrane protein) for its ability to stimulate amino acid transport. Conversely, the transmembrane and cytoplasmic domains of CD98 are necessary and sufficient for binding to the β1A tail and for augmentation of integrin function.

Discussion

We describe the involvement of CD98hc in integrin function by use of a novel genetic strategy. CD98hc complements dominant negative suppression of integrin activation by isolated integrin cytoplasmic domains. Complementation of dominant negative suppression by CD98hc is specific as transfection with several other cytoplasmic and membrane proteins implicated in integrin function lack this effect. CD98 complementation is independent of the extracellular domain of the suppressive chimera, and can complement suppression initiated by either the β1A or β3 tail but not β7 or β1D. CD98 interacts with β cytoplasmic tails in a class and splice variant specific manner,

which is independent of the capacity of the tails to bind the cytoskeletal proteins, talin and filamin. CD98's capacity to bind to integrin tails correlates with its ability to overcome dominant suppression of integrin activation. CD98 binding to integrin tails is neither necessary nor sufficient for dominant suppression of integrin activation. The physical interaction of CD98 with integrin cytoplasmic domains may regulate the function and localization of these membrane proteins

CD98hc combines with several different light chains to form a series of heterodimers that are involved in amino acid transport. We have compared the structural requirements of CD98 for interaction with integrins with those involved in regulation of amino acid transport. Mutation of cysteines that disrupt CD98-light chain association and reduce amino acid transport do not disrupt the binding to β1A or its effect on integrin activation. The cytoplasmic and transmembrane domains of CD98 fused to other type II transmembrane proteins are both necessary and sufficient for binding to the integrin β1A tail and for effects on integrin activation. These chimeras failed to stimulate amino acid transport. Replacement of the cytoplasmic or transmembrane domains of CD98 with those of CD69 blocked the capacity of CD98 to bind to β1A and regulate integrin activation. These exchanges had minimal effects on the amino acid transport function of CD98. The amino acid transport function of CD98 is not required for its effects on integrin function.

CD98 was originally identified as an early T-cell activation antigen. It is present in cells as a 120 kD heterodimer, the heavy chain is approximately 90 kD and the light chain is 40 kD(24). The heavy chain cDNA has been isolated from human, mouse, rat and now hamster. Antibodies against FRP-1 (fusion regulatory protein), which has been identified as CD98, enhance virus-induced cell fusion(25). Anti-β1 integrin antibodies inhibit the anti-FRP-1 induced cell fusion, thus suggesting a connection between CD98 and integrins. CD98 has been found in early embryonic cells and actively dividing cells, and antibodies to CD98 can affect proliferation suggesting a role in cell growth and proliferation(26;27). Antibodies to this protein inhibit sodium/calcium exchange in membrane vesicles of heart and skeletal muscle(28). Microinjection of CD98 cRNA into Xenopus oocytes stimulates the uptake of dibasic and neutral amino acids(29). The relationship of these earlier findings to the capacity of CD98 to complement dominant negative suppression remains to be established.

Several mechanisms could account for complementation of dominant negative suppression by CD98. Hughes *et al.* (17) found that activated H-Ras suppressed integrin activation. Free integrin β cytoplasmic tails can result in phosphorylation of pp125FAK(30), implying the potential to activate Ras(31). Nevertheless, CD98 did not influence Ras suppression, nor did MKP-1, reverse Tac-β1 suppression. Thus, CD98 is unlikely to work through the Ras initiated suppression pathway. The capacity of CD98 to reverse dominant negative suppression seems relatively unique. Furthermore, studies of virus induced cell fusion provide an independent indicator that this protein regulates integrin function. Thus, it appears to be a novel element in integrin signaling pathways.

CD98 binds to integrin β cytoplasmic domains with unique splice variant and class specificity. CD98 bound well to the β1A tail and the β3 tail. Binding to the β1D and β7 tails was negligible. The specificity of CD98 binding differs markedly from the specificity of talin and filamin binding, since talin binds preferentially to the β1D tail and filamin to the β7 tail(8). Moreover, the binding of both cytoskeletal proteins is sensitive to the Tyr substitution with Ala in the first "NPXY" (8) in β1A and, as shown here, in β7 and β1D. Strikingly, CD98 binding was insensitive to this mutation. Finally, although the last three residues of β1A were dispensable, the last seven residues were required for binding. Thus, the features of the β tail defined here for CD98 binding identifies a novel structural specificity for integrin β tail function.

CD98 binding to β tails correlates with its capacity to complement dominant suppression. CD98 was implicated in integrin activation by its capacity to reverse the suppression of integrin activation caused by an isolated β1A cytoplasmic domain. We found that CD98 binds to the β1A cytoplasmic domain, but fails to bind well to the β7 or B1D cytoplasmic domain. Strikingly, CD98 failed to complement dominant suppression initiated by either β7 or β1D cytoplasmic domains. Consequently, the mechanism of CODS appears to involve CD98 binding to the suppressive β tail. Furthermore, cross-linking of CD98 stimulates integrin α3β1-dependent adhesion in small cell lung cancer cells and in certain breast cancer cell lines (32) and β1 integrin-dependent cell fusion events(25;33-39). Thus, our finding that CD98-β1 cytoplasmic domain interactions correlate with effects on integrin function effects is relevant to integrin-dependent events involved in mulinucleate giant cell formation, viral induced cell fusion, and regulation of cell adhesion.

The physical interaction of CD98 with integrin cytoplasmic domains may be involved in modulating amino acid transport regulation. CD98 is known to regulate amino acid transport of the y+L and L type(40). This regulation is probably due to disulfide bonded heterodimer formation with a variety of light chains, that resemble permease amino acid transporters(40). In fact, mutations in one of these light chains(23) are a likely cause of lysinuric protein intolerance(41). CD98 may function to regulate both the expression and localization of its light chains(21). In certain cells CD98 has a basolateral localization(42). β1A integrins also manifest basolateral polarization in many cells(43;44), probably due to interactions with underlying matrix components(45) or recruitment to lateral cell contacts(46). It is noteworthy that β7 integrins are primarily involved in lymphocyte homing and β1D integrins primarily form mechanical linkages in striated and cardiac muscle(47;48). Thus, the failure of these cytoplasmic domains to bind to CD98 correlates well with their lack of a role in establishing polarity in epithelial or mesenchymal cells. Consequently, the physical association of CD98 with β1A integrin cytoplasmic domains may participate in the polarization and regulation of amino acid transporters and to modulate the function of certain integrins.

The formation of a covalent CD98 heterodimer is not required for its effects on integrin function. CD98hc has two extracellular cysteines C^{109} and C^{330}. C^{109} is near the transmembrane domain of CD98hc and results in a disulfide bridge with a cysteine in an extracellular loop in the light chain between transmembrane domains 3 and 4(21).

Mutation of C^{109} and C^{330} disrupted the covalent association with the light chain but did not impair interactions with or effects on integrins. While the covalent association was lost, it is possible that there was still a non-covalent interaction. Indeed, Pfeiffer(21) reported that the C109S mutant can still support the surface expression of the light chain. The C109S mutation still displays the same transport characteristics as the disulfide bound heterodimers, albeit at a reduced rate. Moreover, we also found that over expressed free heavy chains could also bind to the β1A tail. Furthermore, co-transfection of the E16 light chain increased formation of heterodimers and amino acid transport but did not augment integrin interactions or effects. Consequently, our results indicate that the covalent association of CD98hc with a light chain is not required for its effects on integrin function.

The cytoplasmic and transmembrane domains of CD98 are both necessary and sufficient for binding to the integrin β1A tail and for the effects on integrin function. When either of these domains was removed from CD98, integrin effects were lost. Conversely, effects on integrins could be conveyed to CD69 by addition of these two domains. What is the role of the CD98hc transmembrane domain in binding to the β1A cytoplasmic tail? It is possible that the CD98 transmembrane domain influences the conformation of the cytoplasmic domain to promote binding to integrin cytoplasmic domains. Alternatively, our integrin cytoplasmic domain model protein was based on that predicted from the sequence (ITB1_human) in the "Swissprot" database (P05556). Glycosylation mapping studies have suggested that β1A (K^{752}-I^{757}) of the predicted "cytoplasmic" domain may reside in the membrane(49). Consequently, the CD98hc transmembrane domain may directly interact with a transmembrane portion of our model protein "tail." Furthermore, other integrin binding proteins, such as cytohesin, Rack1 and skelemin also bind this region(50). Thus, the membrane localization of this region in an intact integrin heterodimer may specify preferential binding of integrin-associated proteins. Finally, the CD98hc solubilized from membranes could be associated with other proteins via the transmembrane domains. These "adapters" might contribute to the CD98hc-β1A tail interaction. In any case our studies provide the first delineation of a specific functional role for the cytoplasmic and transmembrane domains of CD98hc interaction with and regulation of β1A integrin function.

The capacity of CD98hc to regulate amino acid transport and integrins are distinct and separable functions of the polypeptide. Chimeras in which the cytoplasmic or transmembrane domains of CD98 were replaced with those of CD69 lost the capacity to bind to β1A and regulate integrin activation. In contrast, these replacements had little effect on the amino acid transport function of CD98. Conversely, the exchange of the extracellular domain of CD98 with that of CD69 resulted in a protein that was still capable of affecting integrin function but could not stimulate amino acid transport. Thus, the amino acid transport activity of CD98 is not required for its effect on integrin function.

CD98hc functions as a chaperone to bring the associated light chains (LAT1, LAT2, y^+LAT1, y^+LAT2, xCT, and $b^{0,+}$AT) to the plasma membrane, and recent evidence suggests that CD98hc can also influence their affinity for amino acids(51). We found

that the interaction of CD98hc with integrins and amino acid transporters are ascribable to distinct domains of the protein and are not mutually exclusive. Integrins mediate the adhesion of cells and contribute to cell polarity. Consequently, the integrin-CD98 interaction may serve to polarize amino acid transport. Conversely, CD98hc can influence multiple integrin dependent functions including virus-induced cell fusion, T-cell co-stimulation, and cell adhesion(25;52;53). Thus, the CD98hc-integrin association can promote integrin-mediated cell adhesion that in turn could serve to localize the activities of CD98-linked amino acid transporters.

KEY RESEARCH ACCOMPLISHMENTS

- We have devised an expression cloning strategy that isolates proteins that interact with integrin cytoplasmic domains in a cell-based assay.
- We have identified a protein, CD98 that complements integrin suppression in a cytoplasmic domain specific manner.
- We have identified clustering as a possible mechanism of CD98's effect on integrins.
- We have shown that CD98 physically associates with integrin cytoplasmic domains.
- We have shown that the amino acid transport function of CD98 is not required for its effects on integrin function, and amino acid transport can occur in the absence of CD98-integrin association.
- We have generated chimeric mice, which will lead to a mouse with the CD98 gene deleted.
- We have generated ES cells in which the CD98 gene has been "knocked-out".

REPORTABLE OUTCOMES

Manuscripts

Fenczik, C.A. and Zent, R. * M. Dellos, D.A. Calderwood, and M.H. Ginsberg (in preparation) Structure function analysis of CD98 function. * These authors contributed equally to this work.

Zent R.*, **Fenczik, C.A***., D.A. Calderwood, S. Liu, M. Dellos, and M.H. Ginsberg: (2000) Class and splice variant specific interactions of CD98 with integrin _ cytoplasmic domains. * These authors contributed equally to this work. *J. of Biol. Chem* . 275: 5059-5064.

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Abstracts

Fenczik, C.A., R. Zent, and M. Ginsberg: (2000) Isolation of an Integrin Regulatory Protein: Role of CD98 in Integrin Affinity Modulation. Era of Hope Meeting DoD Breast Cancer Research Program June 8-12, 2000, Atlanta, Georgia.

Fenczik, C.A., R. Zent, D. Calderwood, S. Liu, M. Dellos and M. Ginsberg: (1999) Class and Splice Variant Specific Interactions of CD98 with Integrin β Cytoplasmic Domains. Annual Meeting of the American Society of Cell Biology, December, 1999, San Francisco, California.

Presentations

Fenczik, C.A. : (1998) Genetic Analysis of Integrins. The International Symposium on Vascular Protection: From Basic Sciences to the Clinic. December, 6-9, 1998, Los Angeles, California

Cell lines

ES cell lines that contain either one or both of the alleles of CD98 replaced with a drug resistance gene (pKO4F2 #74, pKO4F2 double ko P5, pKO4F2 double ko P6).

CONCLUSIONS

We describe the involvement of CD98 in integrin function by use of a novel genetic strategy. CD98 complements dominant negative suppression of integrin activation by isolated integrin β cytoplasmic domains. CD98 clustering may lead to the activation of integrin function. The clustering of CD98 is likely to occur through the cytoplasmic domains of integrins and the cytoplasmic and transmembrane domains of CD98 through a physical association. The physiological consequences of CD98-integrin interactions are currently being investigated by studying cells that have the CD98 gene deleted. We have shown that the amino acid transport function of the CD98 heterodimer is not required for its effects on integrin function. Also, absence of the CD98-integrin association does not have a negative effect on amino acid transport in CHO cells. It will be interesting to see if this is also true in polarized cells, where integrins may be acting as a chaperone to

CD98, localizing it the the basolateral surface of cells. The physiological association between integrins and CD98 may play a role in breast cancer as Chandrasekaran *et.al.* (32) have shown that antibodies against CD98 have an effect on cell adhesion events in certain breast cancer cell lines.

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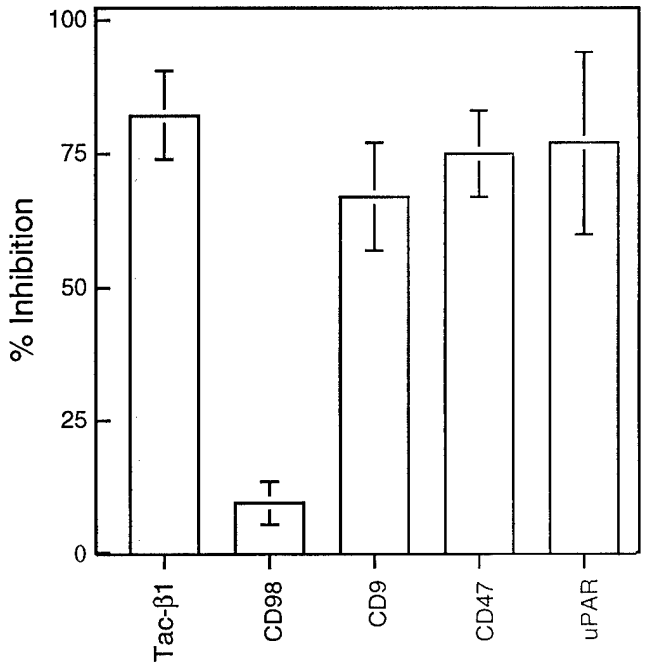


Figure 1. Human CD98 complements dominant suppression whereas several other membrane proteins do not. αβPy cells were transfected with 2 μg of Tac-β1. They were simultaneously transfected with 4 μg of cDNAs encoding either human CD9811, CD922, CD4714, or uPAR. After 48 hr, cells were harvested and analysed for PAC1 binding to the Tac positive subset of cells. To obtain quantitative estimates of integrin activation we calculated a numerical activation index defined as 100 (Fo - FR)/(FLIBS6 - FR), as previously described, where Fo is median fluorescence intensity of PAC1 binding; FR is the background fluorescence intensity of PAC1 binding in the presence of a competitive inhibitor (1 μM Ro43-5054) and FLIBS6 is the maximal fluorescence intensity in the presence of 2μM anti-LIBS6, an activating monoclonal antibody. Depicted are the mean±SD of three independent experiments for each membrane protein. Specificity of CD98 complementation. Percent inhibition was defined as 100(AI0 - AI)/AI0, where AI0 is the activation index in Tac-α5 transfected cells. AI is the activation index in Tac-β1 transfected cells. The co-transfected membrane protein is indicated below each column.

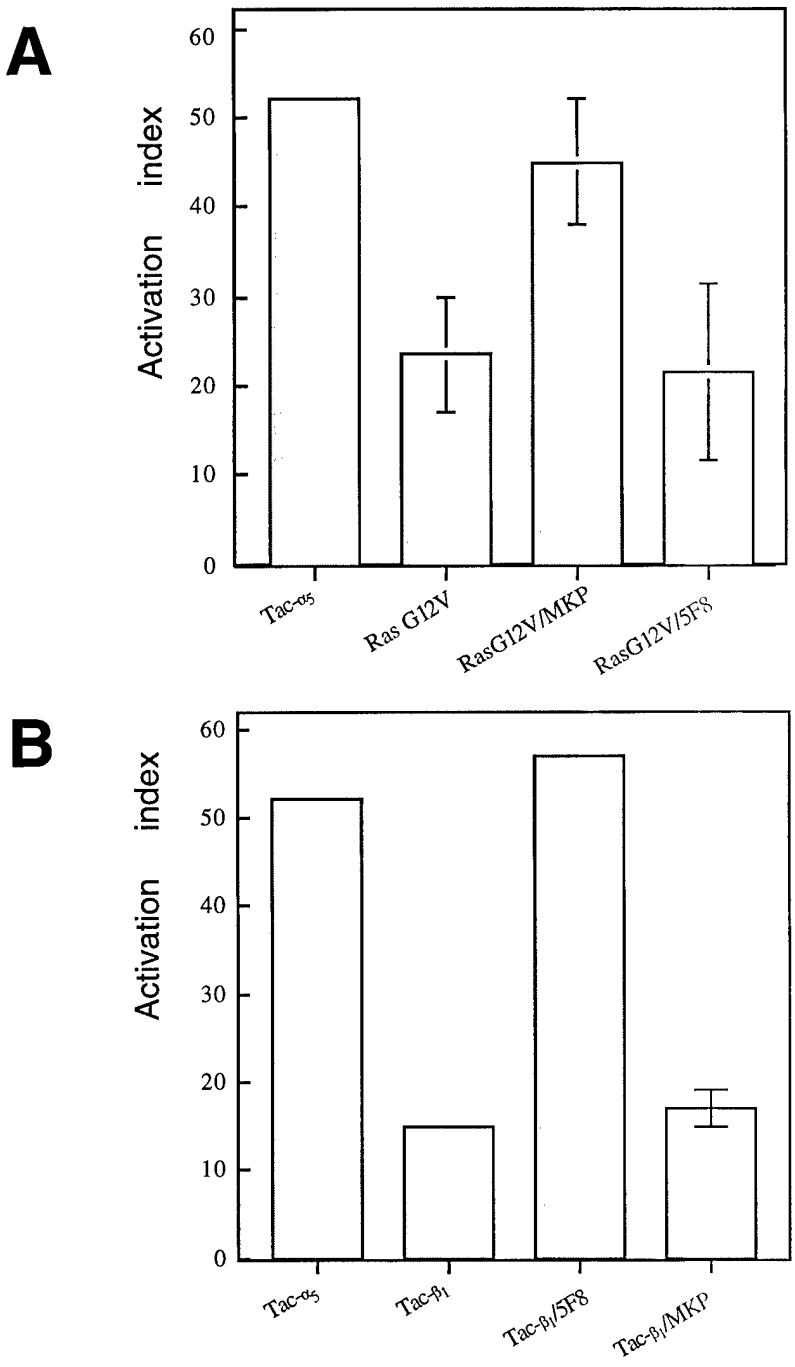


Figure 2. **Suppression of integrin activation either H-Ras or Tac-β1 occur through different mechanisms.**
A, H-Ras (G12V) suppression is rescued by expression of MKP-1, but not by 5F8. αβ-Py cells were transfected with 4 μg of H-Ras (G12V) and 4 μg of either MKP-1 or 5F8. Bars in which no error bars are present the S.E. was less than 0.1. *B*, 5F8 rescues trans-dominant suppression caused by Tac-β1, whereas MKP-1 expression has no effect on integrin activation in these cells.

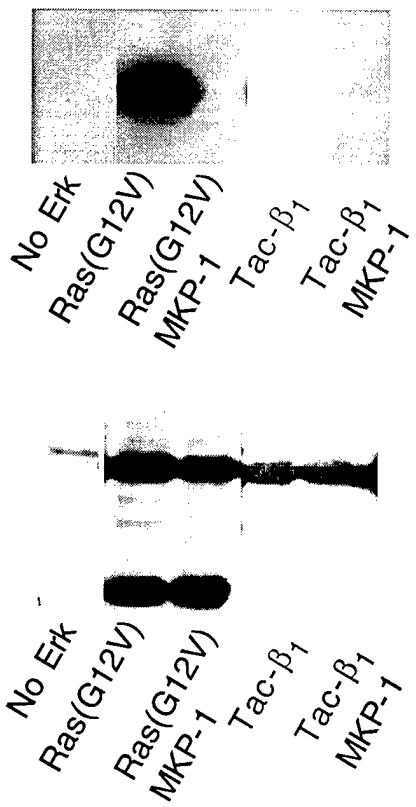


Figure 3. **CD98 does not affect ERK activation**. MAP kinase activity. αβ-Py cells were transfected as above, except 2 μg of expression vector encoding HA-tagged ERK2 was also added. The transfected kinases were immunoprecipitated with anti-HA antibody, 12CA5. ERK-2 activity was measured by phosphorylation of myelin basic protein by an in-gel kinase assay. The upper panel depicts the relative ERK kinase activity. The lower panel show immunoblots with the anti-HA ERK2 in all transfections. There was a comparable expression of HA-tagged ERK-2 in all transfections. MKP-1 was able to rescue H-Ras (G12V) suppression only, whereas 5F8 expression could only rescue Tac-β1 suppression.

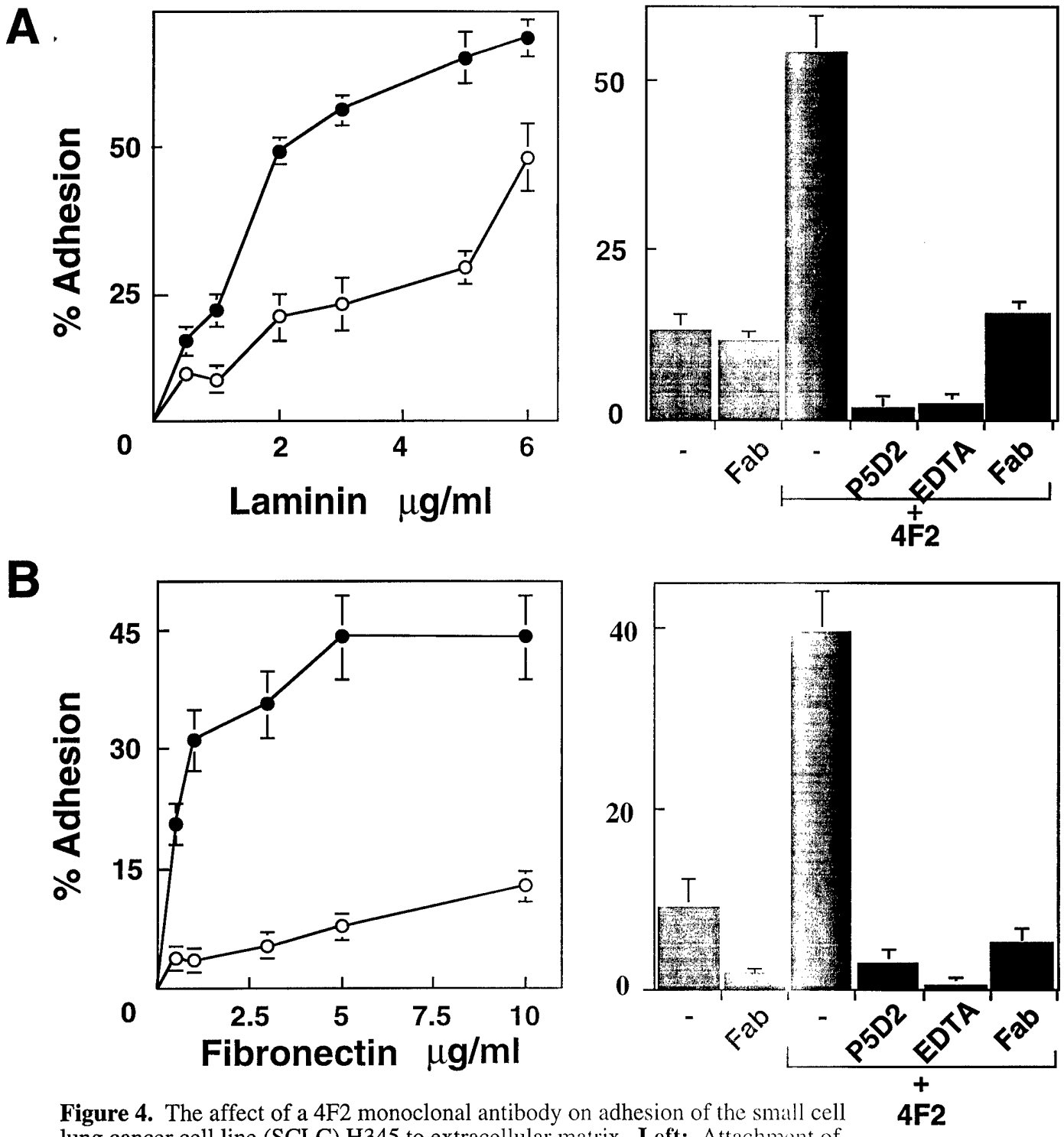


Figure 4. The affect of a 4F2 monoclonal antibody on adhesion of the small cell lung cancer cell line (SCLC) H345 to extracellular matrix. **Left:** Attachment of SCLC cells to 96 well tissue culture plates coated with increasing concentrations of laminin (**A**) or fibronectin (**B**) in the presence (closed circles) or absence of 20 µg/ml 4F2 monoclonal antibody. **Right:** Addition of 4F2-Fab (100µg/ml) to SCLC cells does not increase adhesion to plates coated with 3µg/ml laminin (**A**) or 10µg/ml fibronectin (**B**), while addition of 4F2-Fab blocks the increase of adhesion in the presence of the intact antibody. Addition of P5D2 (a β1-integrin function blocking antibody) or 2mM EDTA also block the increase of adhesion of SCLC cells to plates coated with either 3µg/ml laminin (**A**) or 10µg/ml fibronectin (**B**). Results show the mean % adhesion above background (which was consistently <5%) compared to poly-L lysine (taken as 100%) of 4-6 independent experiments in duplicate/triplicate ± SEM.

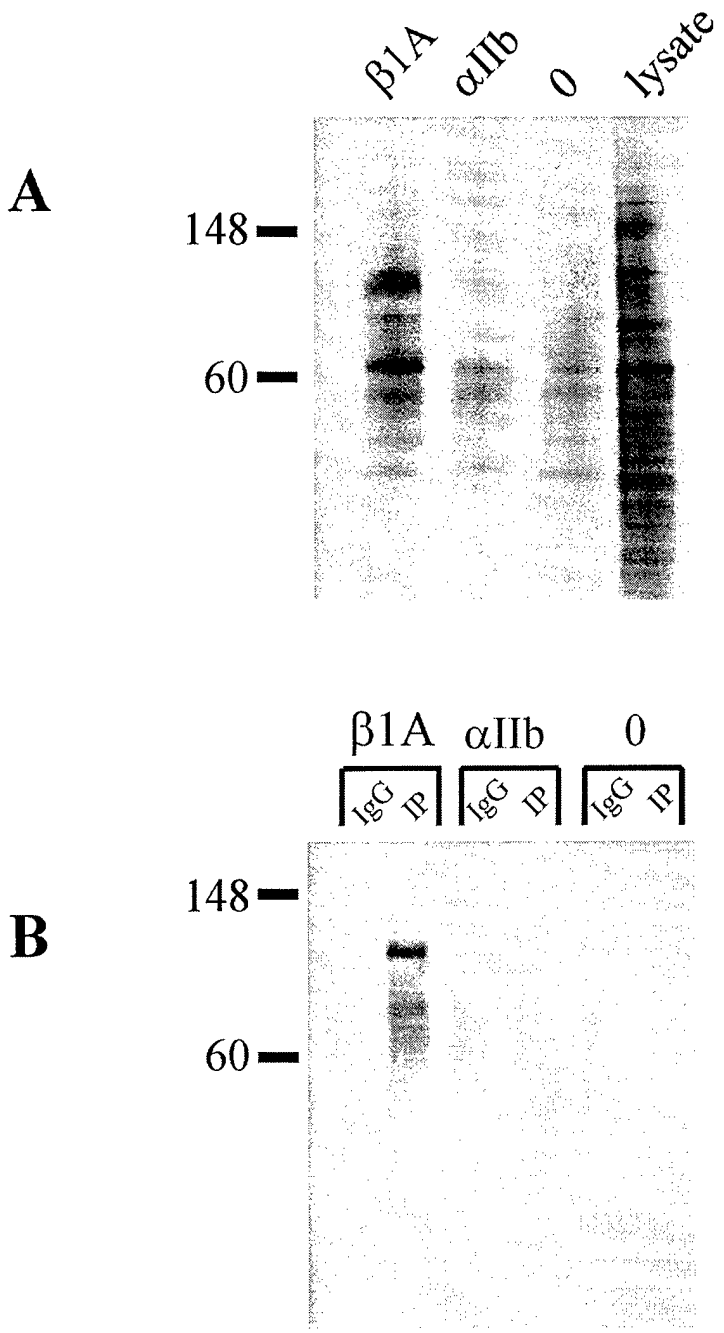


***Figure 5*. CD98 binds to β1A integrin cytoplasmic tails.** Jurkat human T cells were surface-labeled with Sulfo -Biotin N -hydroxy succinimide and the cells were lysed in buffer A (1 mM Na_3VO_4, 50 mM NaF, 40 mM sodium pyrophosphate, 10 mM Pipes, 50 mM NaCl, 150 mM sucrose, pH 6.8 containing 1% Triton 100, 0.5% sodium deoxycholate, 1mM EDTA and protease inhibitors). Panel A depicts a reduced SDS -PAGE analysis of the biotinylated proteins that bound to Ni^{2+} beads, coated with model proteins containing β1A (β1A) or αIIb (αIIb) cytoplasmic tails. Adjacent lanes show the surface proteins prese nt in the lysate (lysate) or the ones that bound to uncoated Ni^{2+} beads (0). In panel B, the biotinylated surface proteins that bound to the β1A (β1A) or αIIb (αIIb) tails or uncoated beads (0) were immunoprecipitated with CD98 antibody (IP) or a control IgG (IgG). The immunoprecipitates were fractionated by reduced SDS -PAGE and biotinylated proteins were detected by streptavidin-peroxidase-generated chemiluminescence.

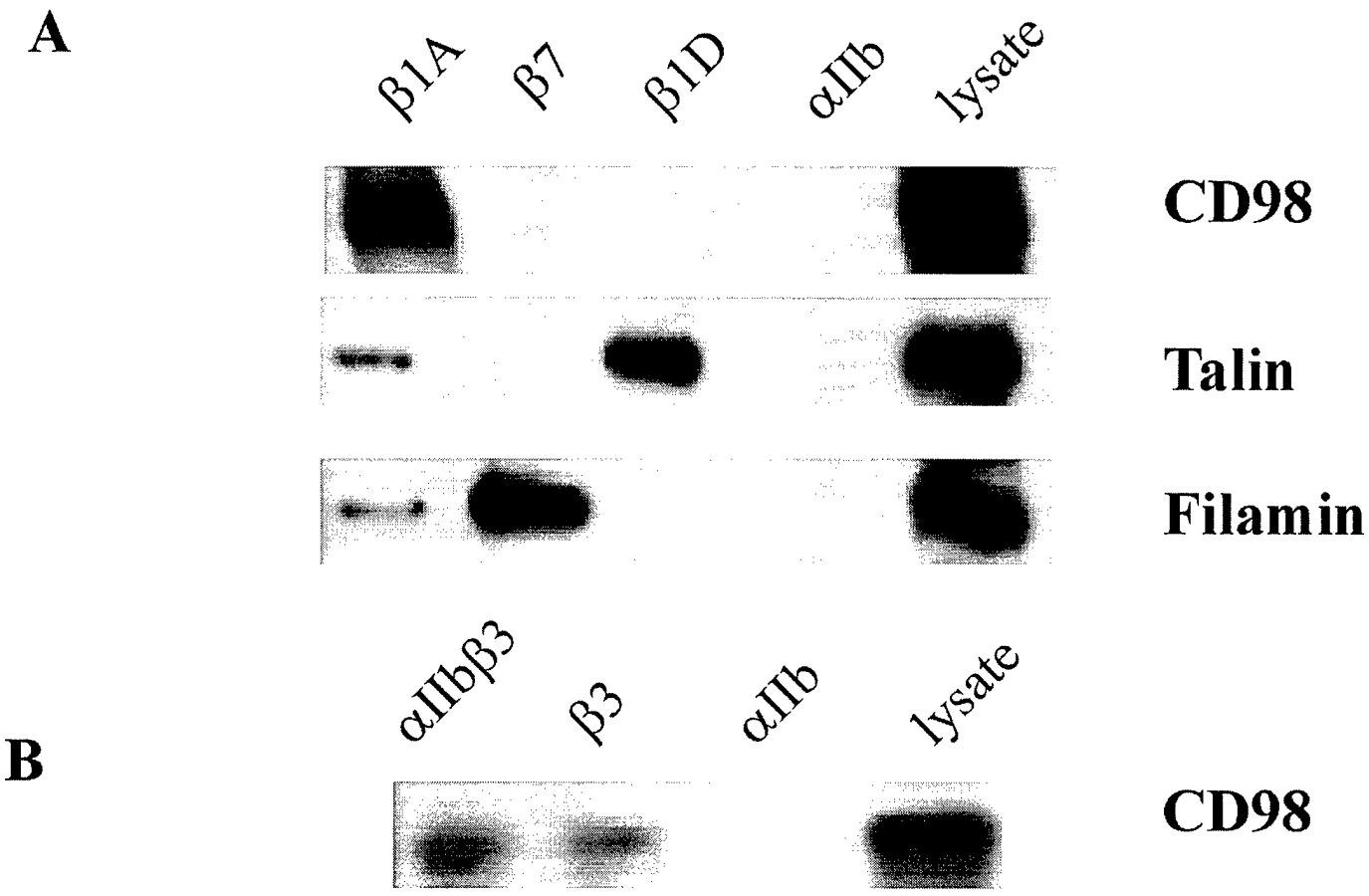


Figure 6. In the upper panel surface biotinylated Jurkat cell lysates were allowed to bind to model proteins containing the β1A, β7, β1D or αIIb integrin tails. The bound fractions were immunoprecipitated with CD98 antibo dy and analyzed by SDS -PAGE. In the lower two panels, human platelet lysates were incubated with the same tail constructs, bound proteins were fractionated by reduced SDS -PAGE and immunoblotted with antibodies to talin or to filamin. The loading of each tail was verified by Coomassie blue staining of the model proteins eluted from the beads and fractionated by SDS -PAGE (data not shown). CD98 did not bind to β7 and binding to β1D was weak and variable. In contrast, talin and filamin bound strongly to β1D and β7 tails respectively as reported previously. B) The surface -labeled Jurkat T cell lysate used in panel A, was allowed to bind to model proteins contain ing a heterodimer of the αIIb and β3 tails, or to model proteins containing only the individual tails. Bound fractions were immunoprecipitated with CD98 antibody and analyzed by SDS -PAGE. CD98 binding was not altered by the presence of the αIIb cytoplasmic domain.

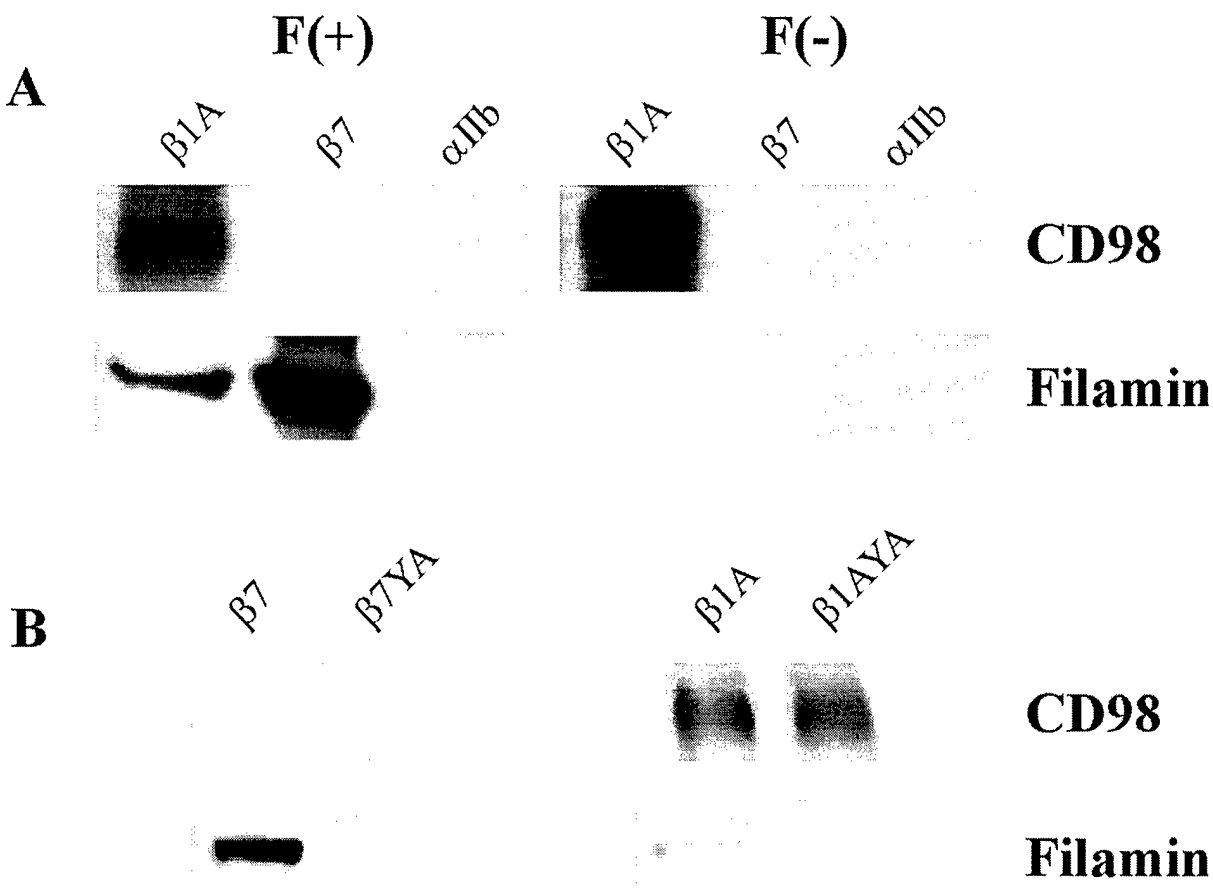


Figure 7. The inability of CD98 to bind to β1D and β7 may be due to competition for binding sites by talin and filamin respectively. To test this possibility, we used filamin -1 deficient human melanoma cells (M2) and reconstituted cells (A7) to examine the role of filamin-1 in CD98 binding. Affinity chromatography was performed using surface biotinylated M2 (F -) or A7 (F+) cell lysates and various cytoplasmic tails (aIIb, β1A, β7). Bound proteins were immunoprecipitated with anti -CD98 antibody, fractionated by reduced SDS -PAGE and the biotinylated polypeptides were detected by streptavidin - peroxidase chemiluminescence (Panel A, CD98). Lysates of A7 and M2 cells were incubated with the indicated integrin cytoplasmic tails and bound proteins were fractionated by SDS -PAGE and immunoblotted with MAB1680 anti-filamin mononclonal antibody (Panel A, filamin). In panel B, surface biotinylated Jurkat cell lysates were incubated with β1A and β7 tails and their corresponding YA (β1YA, β7YA) mutants. CD98 and filamin binding was assessed as described in panel A. L oading of integrin tails was equal as verified by Coomassie blue staining (data not shown). CD98 still binds to β1A in the absence of filamin showing that filamin -1 is not required for CD98 binding. Failure of CD98 to bind to β7 is not due to competition for binding sites with filamin, as CD98 from M2 cells [F(-)] did not bind to β7 tails. Furthermore, the Y788A mutation which disrupts filamin binding to β1A and β7 cytoplasmic tails does not allow binding of CD98 to β7.

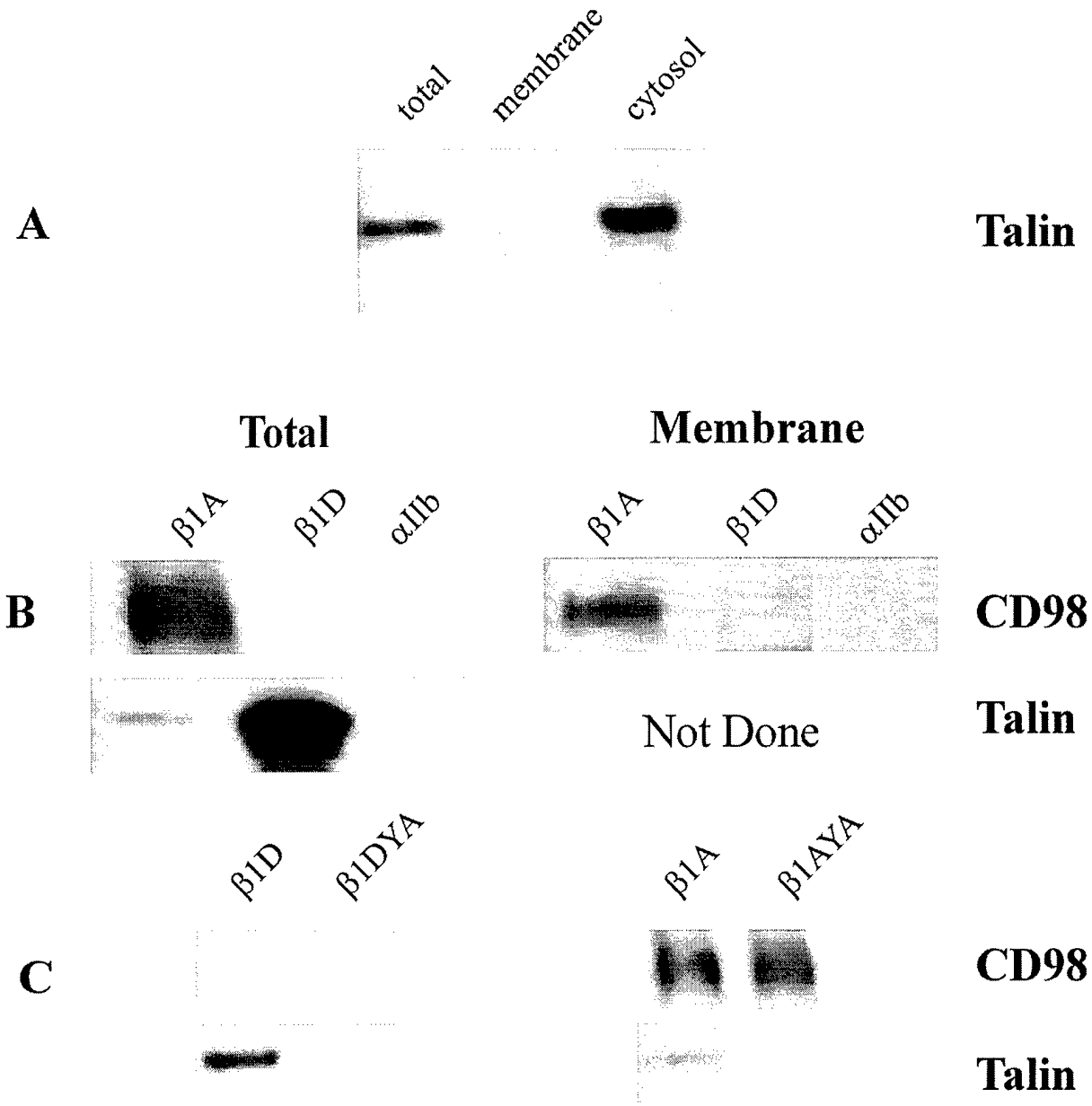


Figure 8. To assess the role of talin in CD98 binding, we used cell membrane preparations with a greatly reduced talin content. Jurkat cells were surface-labeled with biotin, lysed in bufferA and fractionated into membrane and cytosolic fractions. Whole cell lysate (Total), membrane (Membrane) and cytosolic fractions (Cytsosol) were fractionated by SDS-PAGE and immunoblotted with an anti-talin antibody (Panel A). The membrane fraction and whole cell lysate were incubated with αIIb, β1A or β1D integrin tails and bound CD98 was detected by immunoprecipitation as described in the Methods (panel B). In panel C, lysates of Jurkat cells (upper) and platelets (lower) were analyzed for binding of CD98 and talin to β1A and β1D tails, and their corresponding YA (β1YA, β1DYA) mutants as described in figure 4. Loading of integrin tails was equal as verified by Coomassie blue staining (data not shown). CD98 extracted from membranes bound β1A but not β1D cytoplasmic tails. Thus talin does not prevent CD98 binding to β1D, nor is it required for CD98 binding to β1A.

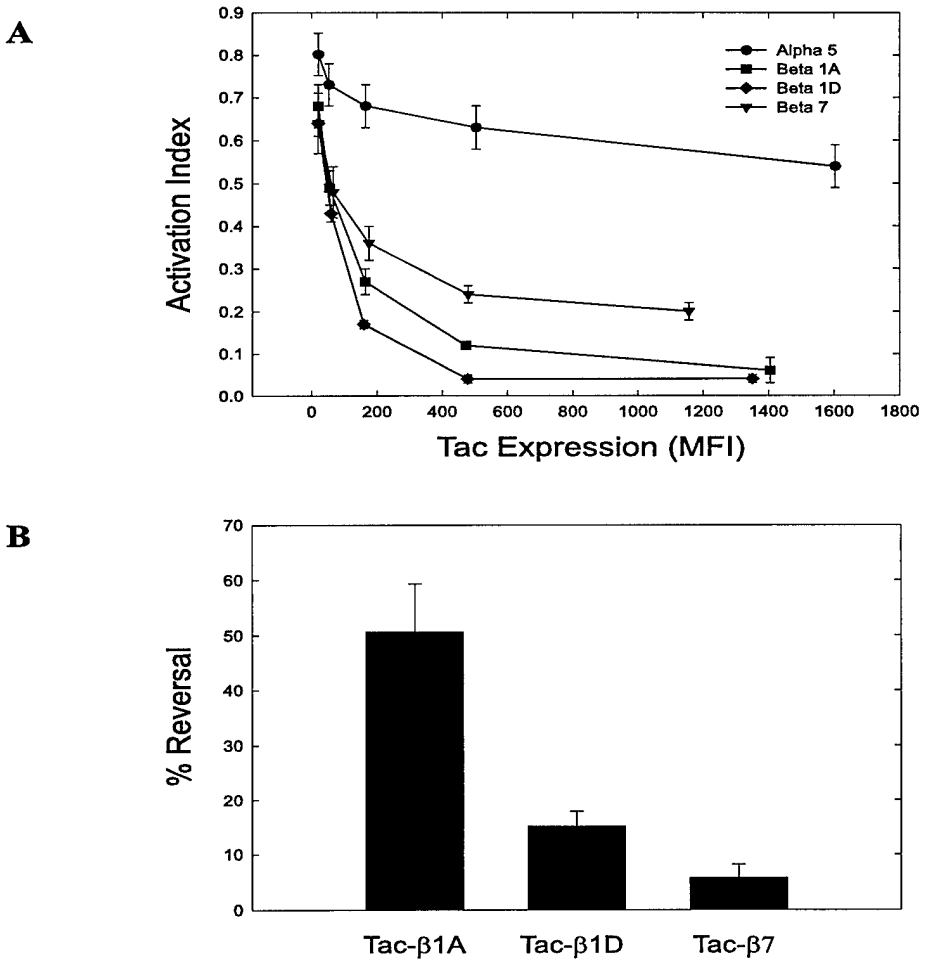
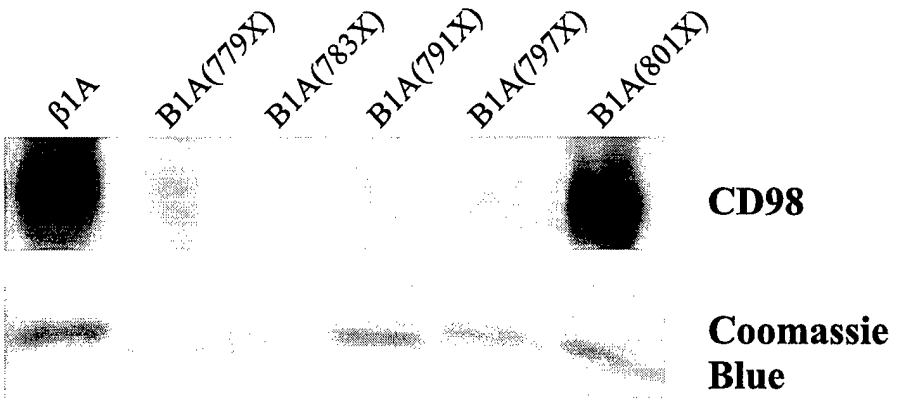


Figure 9. A) β *tails induce varying amounts of integrin suppression.* αβpy cells were transfected with Tac-β1A μg), Tac-β1D (1.0 μg), Tac-β7 (3.0 μg) or Tac-α5 (1.0 μg). After 24 h, cells were collected and analyzed for PAC1 binding to the Tac-positive subset of cells. The activation index was calculated for cells expressing different amounts of each Tac chimera. Activation index is defined as 100(F_o-F_R)/(F_{LIBS6}-F_R), where F_o is the median fluorescence intensity of PAC1 binding; F_R is the background fluorescence intensity of PAC1 binding in the presence of a competitive inhibitor (1 μM Ro43-5054), and F_{LIBS6} is the maximal fluorescence intensity in the presence of 2 anti-LIBS6, an activating monoclonal antibody. The mean ± S.D. of at least five independent experiments for each Tac chimera is shown.

B) CD98 binding to β *tails correlates with its ability to reverse dominant suppression* . αβpy cells were transfected with each of the Tac chimeras in the presence or absence of 4 μg of cDNA encoding full length CD98. 24 h after transfection, cells were collected and the Tac positive subset of cells were analyzed for the ability to bind to the PAC1 antibody. Data are expressed as percentage reversal which is calculated as ($AI_{\beta x + CD98}$ - $AI_{\beta x}$)/($AI_{\alpha 5}$ - AI is the activation index, $AI_{\beta x}$ is the AI of cells transfected with Tac β chimeras, $AI_{\beta x + CD98}$ the AI of cells transfected with CD98 and Tac βx chimeras and AI_{a5} is the AI of cells transfected with the Tac- α5. The x of βx can have values of 1A, 1D and 7 for the Tac-β1A, Tac-β1D and Tac-β7 chimeras respectively.

A



B

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   760          770          780          790          800
    |            |            |            |            |
KLLM IIHDRREFAK FEKEKMNAKW DTGENPIYKS AVTTVVNPKY EGK          β1A
KLLM IIHDRREFAK FEKEKMNAKW DTQENPIYK_ PINNFKNPNY GRKAGL      β1D
KLSV EIYDRREYSR FEKEQQQLNW KQDSNPLYKS AITTTINPRF QEADSPTL    β7
KLLM IIHDRREFAK FEKEKMNA                                     β1A779X
KLLM IIHDRREFAK FEKEKMNAKWD T                                β1A783X
KLLM IIHDRREFAK FEKEKMNAKWD TGENPIYKS                        β1A791X
KLLM IIHDRREFAK FEKEKMNAKWD TGENPIYKS AVTTVV                 β1A797X
KLLM IIHDRREFAK FEKEKMNAKWD TGENPIYKS AVTTVVNPKY             β1A801X
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Figure 10. Truncation mutants of β1A cytoplasmic tails were const ructed using PCR mutagenesis (panel B). Lysates of surface biotinylated Jurkat cells were incubated overnight with affinity matrices containing β1A, β1A(779X), β1A(783X), β1A(791X), β1A(797X) or β1A(801X) integrin tails and the bound fractions were immunoprecipitated with CD98 antibody and analyzed by SDS -PAGE (panel A). Biotinylated polypeptides were detected by streptavidin -peroxidase chemiluminescence (CD98). Loading of the affinity matrix with each tail was verified by Coomassie blue staining of model proteins eluted from the resin and fractionated by SDS -PAGE (Coomassie Blue). CD98 binding was lost when the C -terminal seven residues were deleted (β1A797X) but not when the last three amino acids were eliminated (β1A801X).

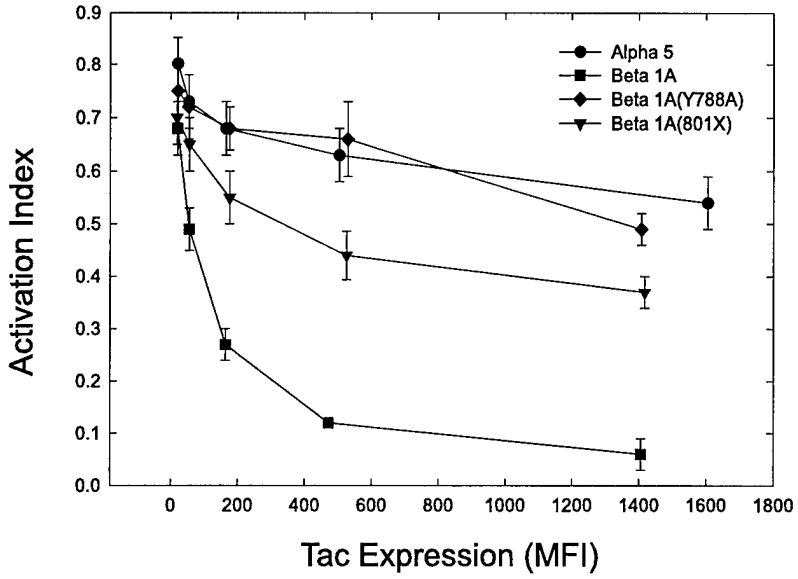


Figure 11. αβpy cells were transfected with Tac -β1 (0.5 μg), Tac-β1A(801X) (1.0 μg), Tac-β1A(Y788A) (1.0 μg) or Tac -α5 (1.0 μg). After 24 h, cells were detached and analyzed for PAC1 binding to the Tac -positive subset of cells by flow cytometry as described previously. The activation index was calculated for cells expressing different amounts of each Tac chimera as described in Figure 6. In spite of maintaining its capacity to bind to CD98, the Tac -β1A(801X) mutant was a poor suppressor of integrin acitvation and this was not due to a quantitative reduction in the association of CD98 with β1A(801X) (data not shown). Furthermore, the β1A(Y788A) mutant, which also bound CD98 (Figure 4 and 5), failed to suppress integrin activation. Consequently, integrin β cytoplasmic domain binding to CD98 is not sufficient to induce dominant suppression.

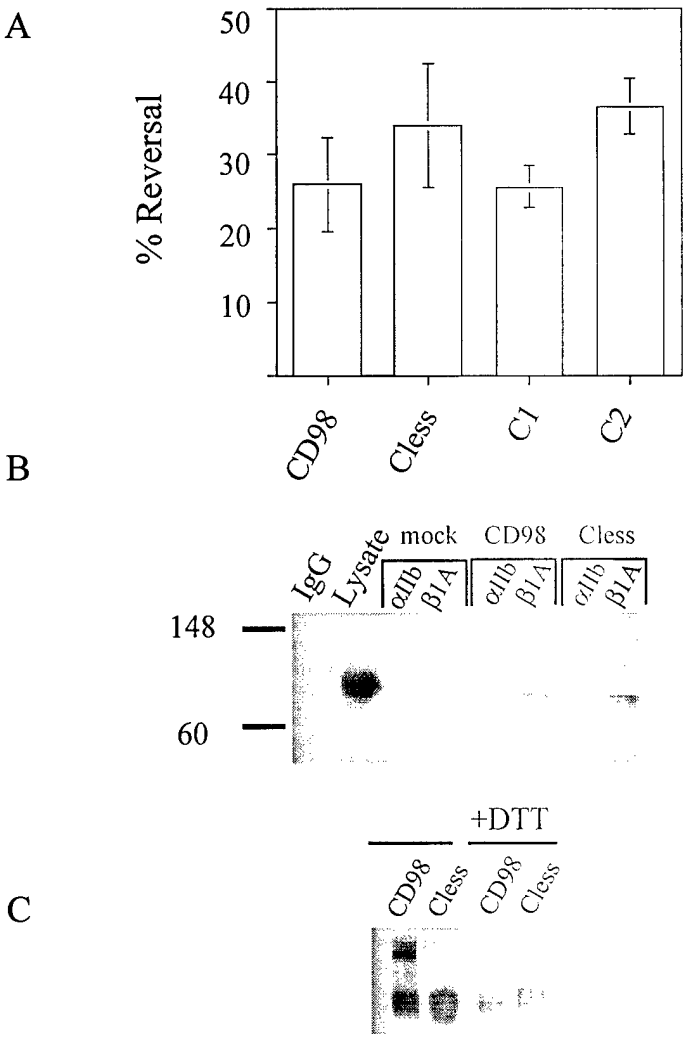
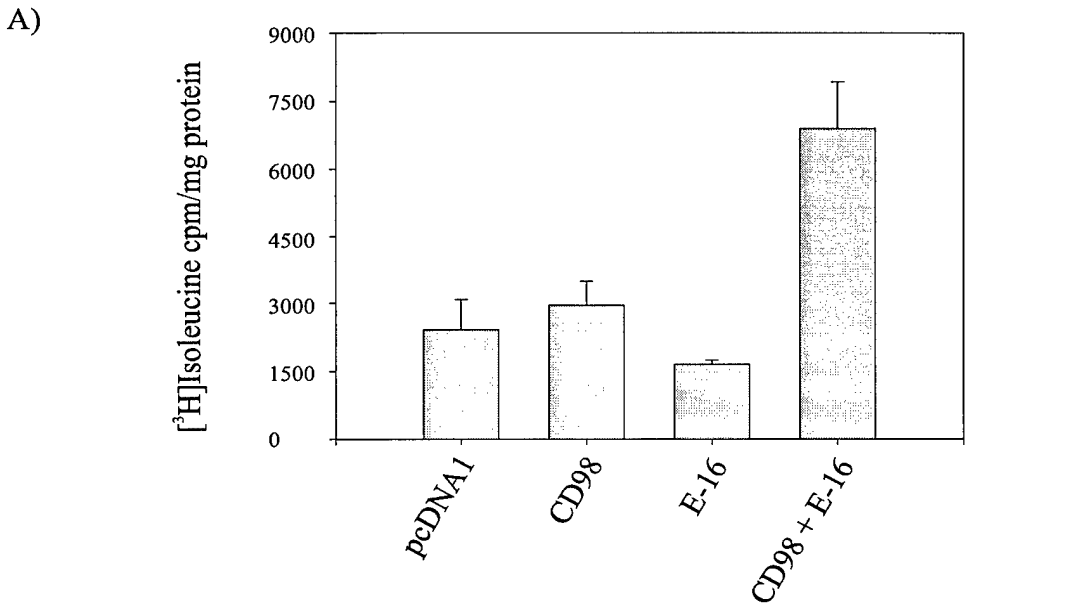
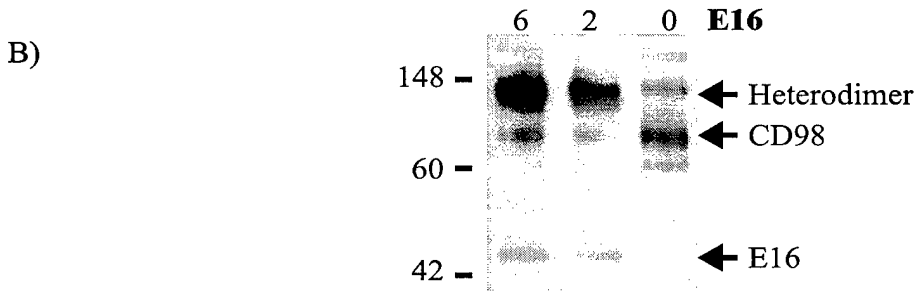


Figure 12. **Stable formation of the CD98 heterodimer is not required for its effects on integrin function.** A, αβPy cells were transfected with Tac-β1 and either wild-type CD98 or CD98 mutants with cysteines required for heterodimer formation mutated to serines. 24 h after transfection, cells were collected and the Tac-positive subset of cells were analyzed for the ability to bind to the PAC1 antibody. Data are expressed as percentage reversal, which is calculated as (AITac-β1 + CD98 - AITac-β1)/(AITac-α5 - AITac-β1). AI is the activation index of cells transfected with constructs listed in the subscript. B, αβPy cells were transfected with either CD98, the Cless mutant, or vector DNA. After 24 h surface proteins were labeled with Sulfo-Biotin N-hydroxysuccinimide, and the cells were lysed in buffer A (see "Experimental Procedures"). Cell lysates were allowed to bind to Ni2+ beads, coated with model proteins containing β1A or αIIb cytoplasmic tails. CD98 was detected by eluting bound proteins and immunoprecipitating with anti-CD98 antibody. The starting lystate was also immunoprecipitated with either anti-CD98 antibodies (Lysate) or with a control IgG (IgG). Proteins were fractionated by reduced SDS-PAGE, and biotinylated proteins were detected by strptavidin-peroxidase-generated chemiluminescence. C, Biotinylated lysates containing either CD98 or Cless CD98 were immunoprecipitated with anti-CD98 antibodies and then run on either reduced (+DTT) or non-reduced SDS gels.





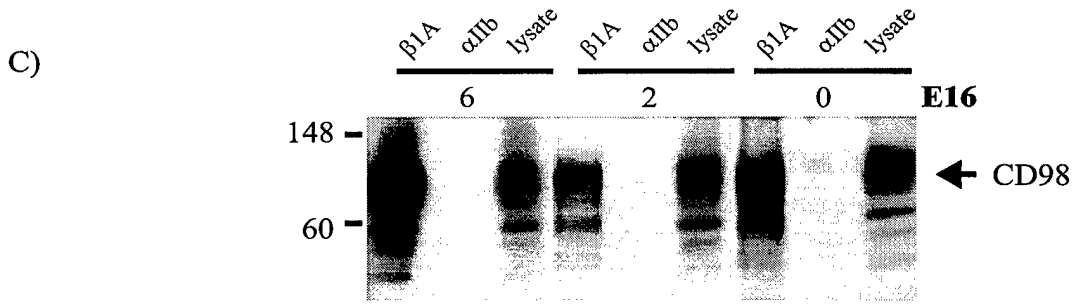


Figure 13. **Effect of Co-expression of the E-16 light chain on amino acid transport, heterodimer formation, and the binding of CD98 to the β1A cytoplasmic domain**. A. **Amino acid transport** :αβPy cells were transfected with cDNAs encoding CD98hc, E-16, CD98hc plus E-16 or vector DNA and assayed for the uptake of [^{3}H]isoleucine after 24 h. Ile uptake was measured in a Na^+ -free solution, and the values are expressed as cpm/mg protein. B. **Heterodimer formation** : CHO cells were transfected with cDNA encoding CD98hc (4 μg) plus increasing amounts of E-16 light chain (0, 2, 6 μg). Cell surface proteins were biotinylated and CD98 was immunoprecipitated from cell lysates with an anti-CD98 antibody. Heterodimers were visualized by running non-reduced SDS-PAGE as described in Fig. 1C. C. **Binding to the β1A cytoplasmic domain**: CHO cells were transfected with cDNA encoding CD98hc and increasing amounts of E-16. Twenty four h later cells were surface biotinylated and lysed. Lysates were mixed with beads coated with β1A or αIIb tails. Beads were washed, bound and eluted proteins were immunoprecipitated with anti-CD98 antibody and fractionated by reduced SDS-PAGE. The biotinylated polypeptides were detected by streptavidin-peroxidase chemiluminescence.

Figure 3

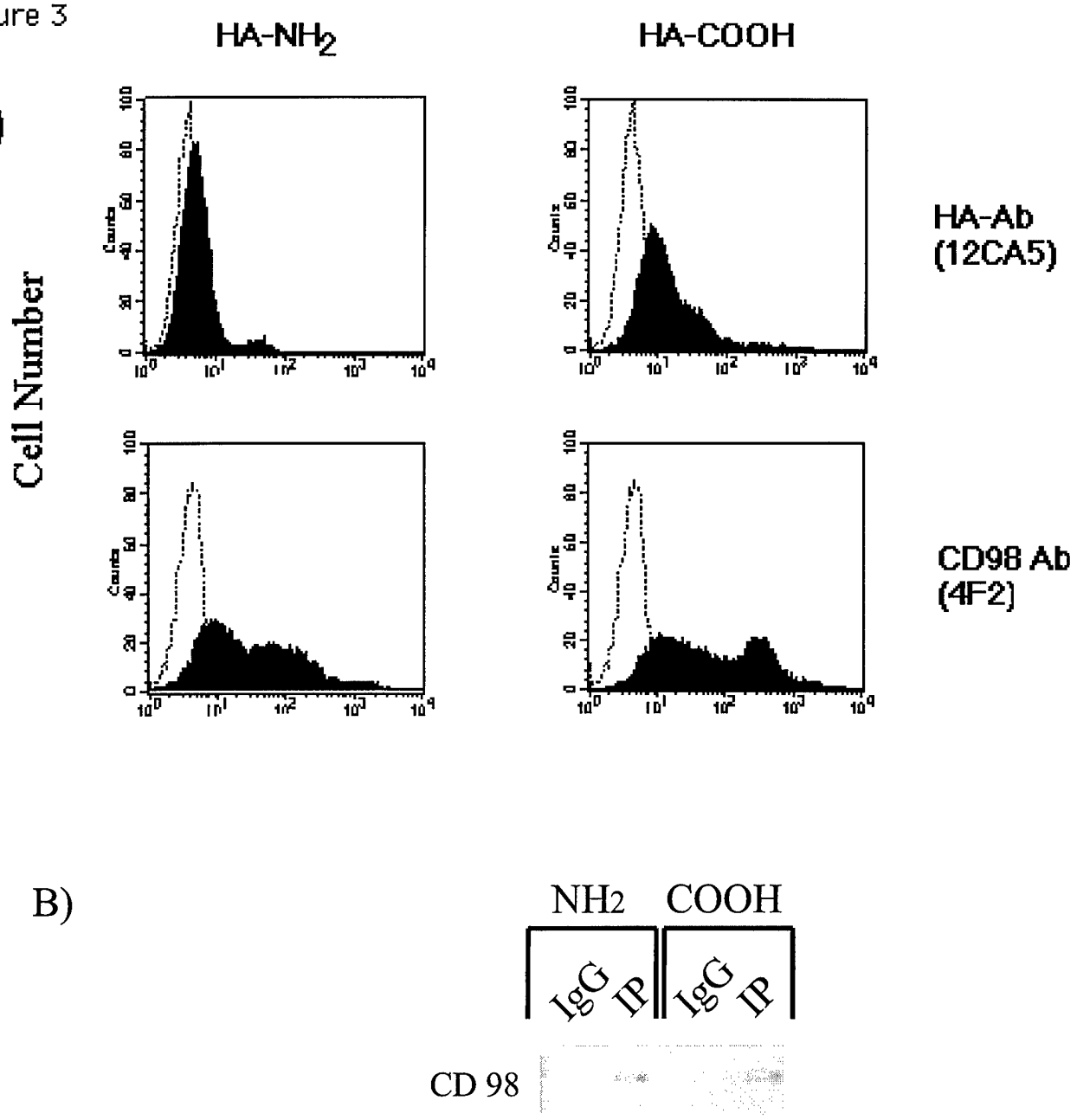


Figure 14. **The N-terminal domain of CD98 is cytoplasmic.** CHO cells were transfected with CD98 HA-tagged either at the N-terminus (HA-NH_2) or at the C-terminus (HA-COOH). A, Twenty four h after transfection cells were with stained with either an anti-HA antibody (top two panels) or with anti-CD98 antibody, 4F2 (bottom panels), and analyzed by flow cytometry. B, Expression of HA tags: CHO cells transfected as in A were lysed and immunoprecipitated with an anti-CD98 antibody (IP) or Normal Mouse IgG (IgG). The immunoprecipitates were fractionated by reduced SDS-PAGE. The immunoblots were stained with an anti-HA antibody and developed by peroxidase-mediated chemiluminescence.

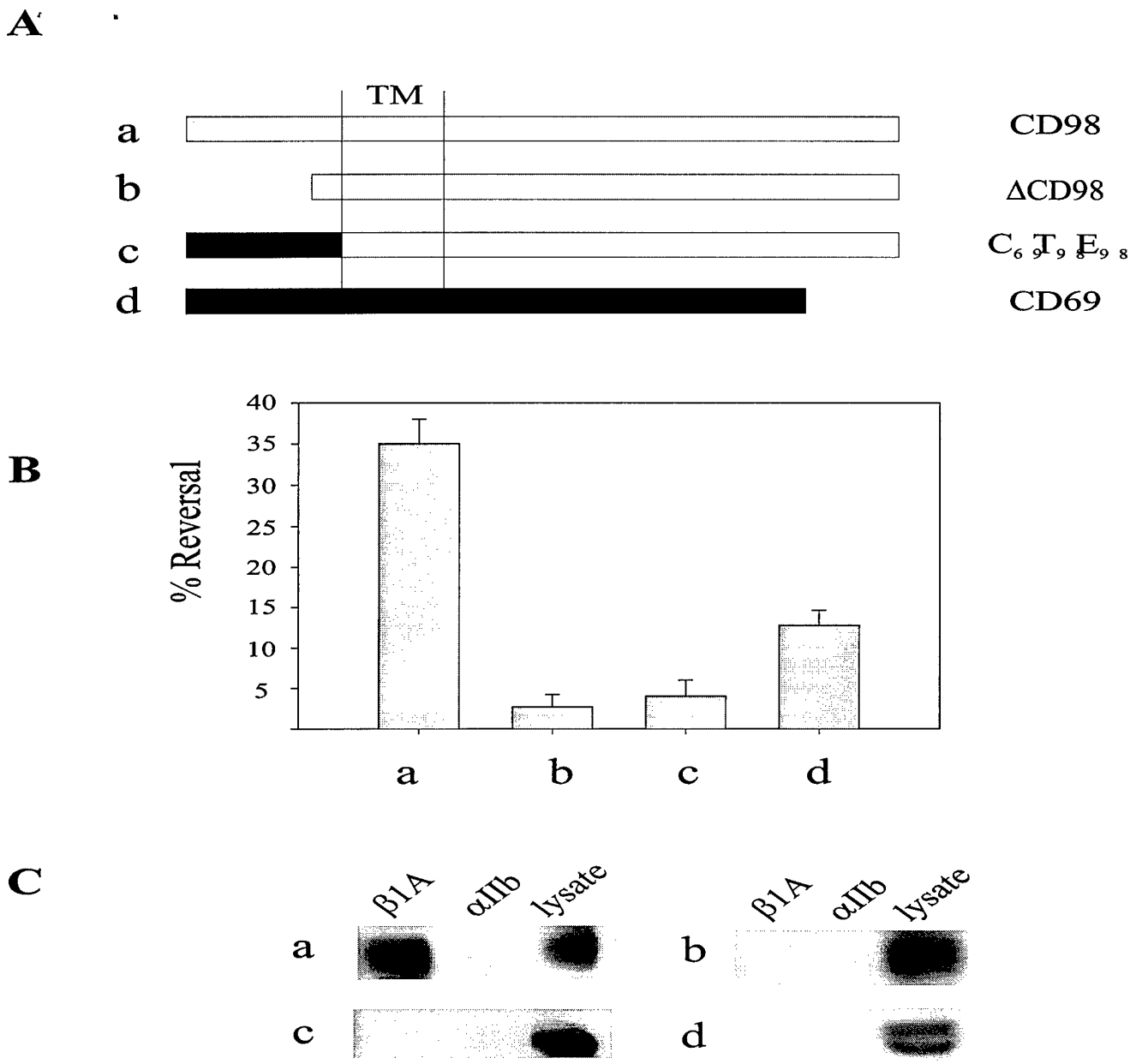


Figure 15. **The cytoplasmic domain of CD98 is required for integrin interactions.** A. Model of CD98/CD69 chimeras. B. Complementation of Dominant Suppression (CODS): αβPy cells were transfected with Tac-β1 and the CD98hc constructs depicted in panel A. Twenty four h after transfection, cells were collected and the Tac-positive subset of cells were analyzed for the ability to bind to the PAC1 antibody. Data are expressed as percentage reversal of Tac-β1A suppression, as described in Figure 1. C. Binding to β1A tail: Affinity chromatography with β1A or αIIb tails was performed using lysates of surface-biotinylated CHO cells that had been transfected with the constructs described in panel A. Bound proteins that contain the extracellular domain of CD98hc (a, b, c) were eluted from the beads, immunoprecipitated with anti-CD98 antibody, fractionated by SDS-PAGE, and the biotinylated polypeptides were detected by streptavidin-peroxidase chemiluminescence. Bound proteins that contain the extracellular domain of CD69 (d) were fractionated by SDS-PAGE and CD69 was detected by immunoblot with anti-CD69 antibodies.

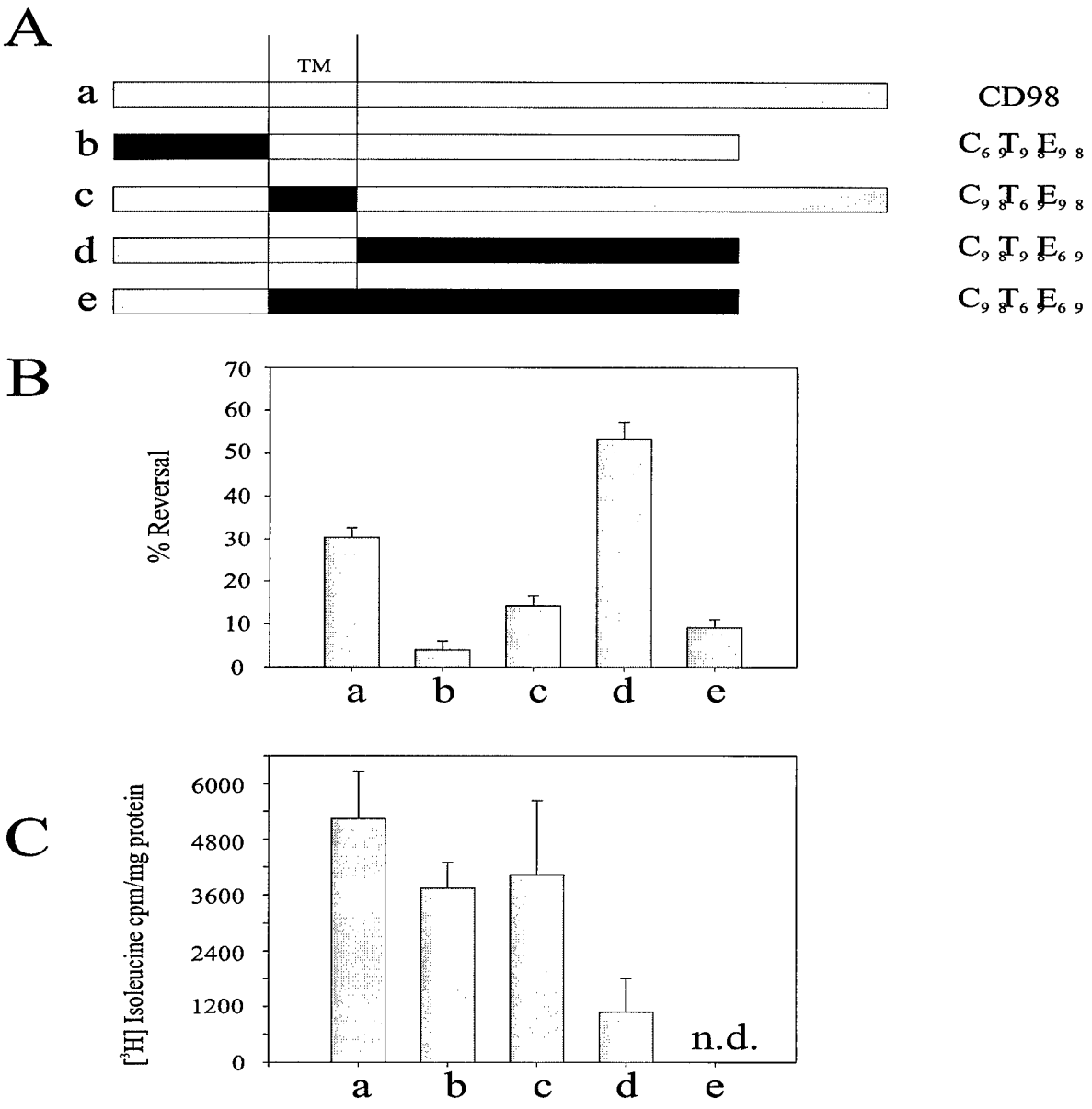


Figure 16. **The cytoplasmic and transmembrane domains of CD98 are necessary and sufficient for its effect on integrin function, but not amino acid transport**. A. Model of CD98/CD69 chimeras. B. CODS: αβPy cells were transfected with Tac-β1 and the CD98hc chimeras depicted in panel A. Twenty four h after transfection, cells were collected and the Tac-positive subset of cells were analyzed for the ability to bind to the PAC1 antibody. Data are expressed as percentage reversal of Tac-β1suppression, as described in Figure 1. C. Amino acid transport: αβPy cells were transfected with cDNAs encoding the E-16 light chain and the CD98hc chimeras depicted in panel A. The uptake of [^{3}H]isoleucine was measured 24 h after transfection as described in experimental procedures. The uptake was measured in a Na^+ -free solution, and the values are expressed as cpm/mg protein, where the baseline uptake in cells transfected with E-16 alone has been subtracted.

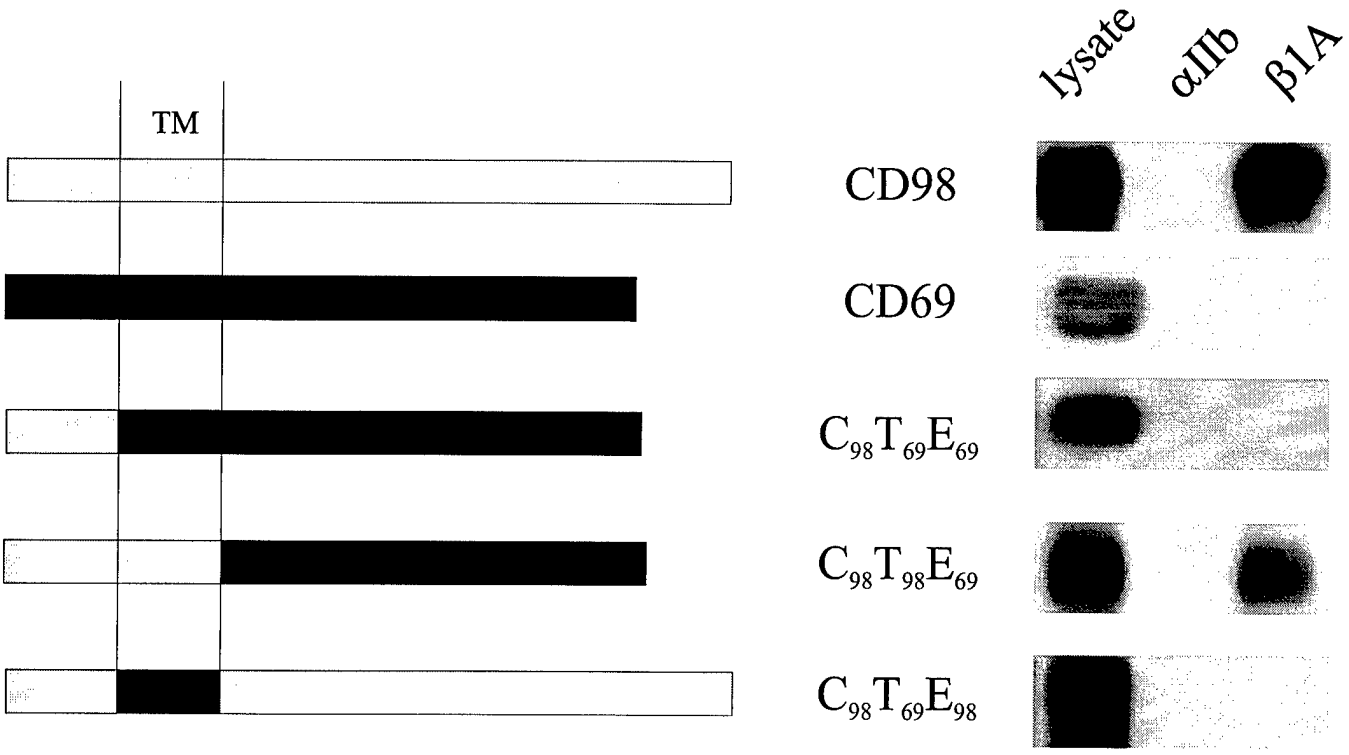


Figure 17. **The cytoplasmic and transmembrane domains of CD98 are necessary and sufficient for binding to β1A cytoplasmic domains.** CHO cells were transfected with each of the chimeric cDNAs depicted on the left-hand side of the figure. Twenty four h later, surface proteins were labeled with Sulfo-Biotin N-hydroxysuccinimide, and the cells were lysed. Cell lysates were incubated with beads coated with model proteins containing β1A or αIIb cytoplasmic tails. Bound and eluted proteins that contain the extracellular domain of CD98 (CD98, $C_{98}T_{69}E_{98}$) were immunoprecipitated with anti-CD98 antibody and fractionated by reduced SDS-PAGE and the biotinylated polypeptides were detected by streptavidin-peroxidase chemiluminescence. Bound proteins that contain the extracellular domain of CD69 (CD69, $C_{98}T_{69}E_{69}$, $C_{98}T_{98}E_{69}$) were detected by Western blot with the anti-CD69 antibody.

Communication

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The Death Effector Domain of PEA-15 Is Involved in Its Regulation of Integrin Activation*

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Joe W. Ramos‡, Thomas K. Kojima, Paul E. Hughes‡, Csilla A. Fenczik§, and Mark H. Ginsberg¶

From the Department of Vascular Biology, The Scripps Research Institute, La Jolla, California 92037

Increased integrin ligand binding affinity (activation) is triggered by intracellular signaling events. A Ras-initiated mitogen-activated protein kinase pathway suppresses integrin activation in fibroblasts. We used expression cloning to isolate cDNAs that prevent Ras suppression of integrin activation. Here, we report that PEA-15, a small death effector domain (DED)-containing protein, blocks Ras suppression. PEA-15 does not block the capacity of Ras to activate the ERK mitogen-activated protein kinase pathway. Instead, it inhibits suppression via a pathway blocked by a dominant-negative form of the distinct small GTPase, R-Ras. Heretofore, all known DEDs functioned in the regulation of apoptosis. In contrast, the DED of PEA-15 is essential for its capacity to reverse suppression of integrin activation. Thus, certain DED-containing proteins can regulate integrin activation as opposed to apoptotic protease cascades.

Integrins are transmembrane heterodimers that mediate cell-cell and cell-extracellular matrix adhesion (1). The affinity of some integrins for ligand is regulated by "inside-out" cell signaling cascades (2, 3). Regulation of integrin affinity for ligand (activation) is important in cell migration (4), fibronectin matrix assembly (5), platelet aggregation in hemostasis and thrombosis (6), and morphogenesis (7, 8). This cellular regulation of integrin activation is energy-dependent, cell type-specific, and is mediated through integrin cytoplasmic domains (9).

In fibroblastic cells, activation of the small GTP-binding protein Ha-Ras or its effector kinase, c-Raf-1, initiates a signaling pathway that blocks integrin activation (10). This suppressor activity correlates with the activation of the ERK MAP[1] kinase pathway and does not require mRNA transcription or protein synthesis. The downstream effectors or regulators of this integrin suppression pathway remain to be identified.

Integrin activation is readily measured by the binding of activation-dependent ligands, which can be used as a selective marker in expression cloning schemes. One such scheme (10, 11) uses a Chinese hamster ovary cell line ($\alpha\beta$py cells) stably expressing a chimeric integrin ($\alpha_{IIb}\alpha_{6A}\beta_3\beta_1$) that contains the extracellular and transmembrane domains of $\alpha_{IIb}\beta_3$ fused to the cytoplasmic domains of $\alpha_{6A}\beta_1$. This chimeric integrin has the ligand binding properties of $\alpha_{IIb}\beta_3$, and its activation state is regulated through the $\alpha_{6A}\beta_1$ cytoplasmic domains. Consequently, flow cytometry (FACS) can be used to assess the activation state of the chimeric integrin by measuring the binding of fibrinogen or the ligand-mimetic monoclonal antibody, PAC1. To elucidate Ras-induced integrin suppression, we modified this scheme to identify proteins that prevent Ras suppression. Specifically, we used Ras to suppress integrin activation in $\alpha\beta$py cells and isolated co-transfected cDNAs that blocked this suppression. Here we report that PEA-15 (phosphoprotein enriched in astrocytes), a small death effector domain (DED)-containing protein, blocks Ras suppression downstream of MAP kinase via a pathway blocked by a dominant interfering mutant of a distinct small GTPase, R-Ras.

EXPERIMENTAL PROCEDURES

Cell Culture—$\alpha\beta$py cells are a CHO cell line that expresses the polyoma large T antigen and a constitutively active recombinant chimeric integrin ($\alpha_{IIb}\alpha_{6A}\beta_3\beta_1$) (12). $\alpha\beta$py cells were maintained in Dulbecco's modified Eagle's medium (BioWhitaker, Walkersville, MD) supplemented with 10% fetal calf serum (BioWhitaker), 1% non-essential amino acids (Life Technologies, Inc.), 1% glutamine (Sigma), 1% penicillin and streptomycin (Sigma), and 700 μg/ml G418 (Life Technologies, Inc.).

Antibodies, Reagents, and cDNA Constructs—The activation-specific anti-$\alpha_{IIb}\beta_3$ monoclonal antibody PAC1 (13) was generously provided by Dr. S. Shattil (Scripps Research Institute). The anti-$\alpha_{IIb}\beta_3$ monoclonal antibody anti-LIBS6 has been described previously (14). The anti-Tac antibody, 7G7B6, was obtained from the American Tissue Culture Collection (Rockville, MD). 7G7B6 was biotinylated with biotin-*N*-hydroxysuccinimide (Sigma) according to the manufacturer's instructions. The mouse monoclonal anti-HA antibody (12CA5) was produced in our laboratory (15). The $\alpha_{IIb}\beta_3$–specific peptide inhibitor Ro43-5054 (16) was a generous gift from B. Steiner (Hoffmann-La Roche, Basel). The CHO-K1 oligo(dT)-primed library is directionally cloned into pcDNA1 and was obtained from Invitrogen (San Diego, CA). The library is reported to contain 1.8×10^7 primary recombinants. pDCR-Ha-RasG12V was a gift from Dr. M. H. Wigler (Cold Spring Harbor Laboratory). Tac-α5 (17) was generously provided by Drs. S. LaFlamme and K. Yamada (National Institutes of Health). HA-Erk2 was described previously (18). Dr. G. Bokoch kindly provided pCMV5-Ha-RasT17N. pcDNA3-R-RasG38V and pcDNA3-R-RasT43N (19) were gifts from Dr. E. Ruoslahti (The Burnham Institute, La Jolla, CA) with permission from Dr. A. Hall (University of London). pCHA-MEK2 222/226D was provided by Dr. M. Weber (University of Virginia).

Expression Cloning—Expression cloning was done using $\alpha\beta$py cells. $\alpha\beta$py cells were divided into 18 subconfluent 100-mm plates and co-transfected with Tac-α_5 (2 μg/plate), Ha-RasG12V (3 μg/plate) and a CHO-K1 library (4 μg/plate) using LipofectAmine™ (Life Technologies, Inc.). 48 h after transfection, cells were collected and stained for FACs analysis with antibodies PAC1 and 7G7B6 as described previously (9). Cells that bound high levels of both PAC1 and 7G7B6 were collected by fluorescence-activated cell sorting (FACSTAR, Becton Dickinson). Plasmid DNA was extracted from collected cells by Hirt Supernatant (20).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF080001.

‡ Fellows of the Leukemia Society of America.

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¶ To whom correspondence should be addressed: The Scripps Research Institute, VB-2, 10550 N. Torrey Pines Rd., La Jolla, CA 92037. E-mail: ginsberg@scripps.edu.

[1] The abbreviations used are: MAP, mitogen-activated protein; DED, death effector domain; PCR, polymerase chain reaction; 3′-UTR, 3′-untranslated region; AI, activation index; DD, death domain; FACS, fluorescence-activated cell sorter; CHO, Chinese hamster ovary; HA, hemagglutinin.

This plasmid DNA was used to transform *Escherichia coli* MC1061/P3 cells. Bacterial colonies were grown, stored, and pooled into groups of 16 for plasmid purification (Qiagen, Chatsworth, CA) and analysis. To isolate single cDNAs that reverse Ras suppression of PAC1 binding, groups of cDNAs were transfected into αβpy cells along with Ha-RasG12V and Tac-α5. Transfectants were screened by two-color flow cytometry (FACScalibur, Becton Dickinson) as described above. A group containing cDNAs that reverse Ras suppression was identified and divided into groups of four for further screening. Positive groups were finally screened as single cDNAs.

Construction of PEA-15 Mutants—An HA-tagged PEA-15 lacking the 3′-UTR was created by PCR of CHO PEA-15-pcDNA1 clone using pfu polymerase (New England Biolabs). The amplified product was subcloned into the *Bam*HI/*Eco*RI sites of pcDNA3. HA-tagged DED and C-terminal domains of PEA-15 were similarly constructed by PCR. PEA-15(D74A) was constructed using the Quickchange kit (Stratagene). DD-PEA-15 was constructed by splice-overlap PCR with pcDNA3-FADD and CHO PEA-15-pcDNA1 as templates. The insert was subcloned into the *Bam*HI/*Eco*RI sites of pcDNA3. Mutations were verified by sequencing.

Flow Cytometry—Analytical two-color flow cytometry was done as described (9). In transiently transfected αβpy cells, PAC1 binding was determined for transfected cells (cells positive for the co-transfected Tac-α_5 as measured by 7G7B6 binding). Integrin activation was quantitated as an activation index (AI) defined as $100 \times (F - F_r)/(F_{LIBS6} - F_r)$ in which F is the median fluorescence intensity of PAC1 binding; F_r is the median fluorescence intensity of PAC1 binding in the presence of competitive inhibitor (Ro43-5054, 1 μM); and F_{LIBS6} is the median fluorescence intensity in the presence of anti-LIBS6 (2 μM). From this we calculated the percent inhibition as $100 \times (AI - AI_S)/AI$, in which AI is the activation index of control cells and AI_S is the activation index in the presence of a transfected suppressing cDNA.

Measurement of ERK Activity—For ERK kinase assays, αβpy cells were transfected with HA-ERK2 (2 μg) along with test cDNA such as pcDNA3-PEA15 (3 μg) using LipofectAmine™ (20 μl/plate, Life Technologies, Inc.). In instances where more than one test plasmid is used, the amount of DNA transfected was standardized by addition of pcDNA1 control vector. Cells were lysed 48 h after transfection in ice-cold M2 buffer (0.5% Nonidet P-40, 20 mM Tris, pH 7.6, 250 mM NaCl, 5 mM EDTA, 3 mM EGTA, 20 mM sodium phosphate, 20 mM sodium pyrophosphate, 3 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, and 10 μg/ml each of leupeptin and aprotinin). ERK2 activity was measured by an immune-complex assay (from 100 μg of cell lysate protein) using myelin basic protein as a substrate (18). ERK2 activity was determined by autoradiography.

RESULTS AND DISCUSSION

Expression Cloning of PEA-15 by Prevention of Ha-Ras Suppression of Integrin Activation—To elucidate the mechanism of Ras-mediated suppression of integrin activation, we used an expression cloning strategy to identify proteins that prevent Ras suppression (Fig. 1*A*). αβpy cells were co-transfected with activated Ha-Ras and a CHO cell cDNA library. We used flow cytometry to isolate cells that still bound the activation-specific anti-$\alpha_{IIb}\beta_3$ antibody, PAC-1, despite transfection with activated Ha-Ras (Fig. 1*B*, *left panel*, *box*). Seventy-nine cDNAs were recovered from isolated cells. One of these cDNAs, R36, restored PAC1 binding in cells transfected with activated Ras (Fig. 1*B*, *middle panel*). Indeed, R36 transfection resulted in FACS profiles similar to those observed in the absence of Ras suppression (Fig. 1*B*, *right panel*). Ras expression levels in the PEA-15 transfected cells remained comparable to cells transfected with Ras alone (Fig. 1*C*). Thus, the reversal of Ras suppression was not due to a loss of Ras expression.

R36 contained 1,700 base pairs encoding an open reading frame of 130 amino acids (GenBank™ accession number AF080001). A BLAST data base search indicated that the 130-amino acid sequence is 99% identical to mouse phosphoprotein enriched in astrocytes (PEA-15, Fig. 2*A*) (21). The first 80 amino acids of PEA-15 correspond to the canonical DED sequence found in proteins that regulate apoptotic signaling pathways (22–24) (Fig. 2, *A* and *B*). In fact, the PEA-15 DED is more similar to that of FADD than that of the viral DED-containing protein MC159 (Fig. 2*B*). The remaining 50 amino acids contain a serine (Ser-104) that is phosphorylated by protein kinase C (25) and a serine (Ser-116) phosphorylated by calcium calmodulin kinase II (26). No function has yet been ascribed to PEA-15.

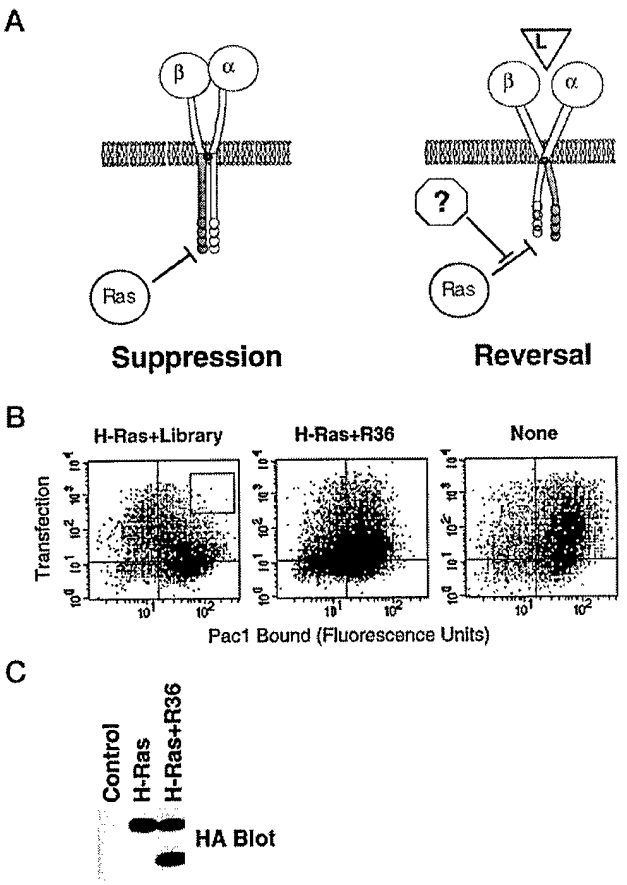

FIG. 1. **PEA-15 reverses Ras suppression of integrin activation.** *A*, depicted are the integrin α and β subunits. Ras initiates a MAP kinase pathway that inhibits integrin ligand binding (*left panel*). We used an expression cloning strategy to isolate cDNAs that encode proteins (?) that prevent Ras suppression of integrin ligand binding (*right panel*). Ligand (*L*) is shown as a *triangle*. These proteins might work by blocking a Ras-initiated pathway or activating a competing pathway. In our cloning strategy the ligand was the activation-dependent antibody, PAC1. *B*, αβpy cells were co-transfected with cDNA encoding Tac-α5 (2 μg) alone (*None*) or in combination with Ha-RasG12V (3 μg) and pcDNA1-CHO library (4 μg) or Ha-RasG12V (3 μg) and clone R36 (4 μg) as indicated. After 48 h, the cells were stained for Tac expression (*ordinate*) and PAC1 binding (*abscissa*). *C*, immunoblot with 12CA5 (anti-HA) antibody. Cells were co-transfected as in *B*.

The sequence of R36 also contained a predicted 1190-base pair 3′-UTR containing a polyadenylation signal and poly(A) tract. The final 1050 bases of this region are 70% identical to MAT1, a transforming cDNA isolated from a lithium-induced mouse mammary tumor (27). To determine whether the 3′-UTR is necessary for the reversal of Ha-Ras integrin suppression, we tested a construct without this sequence and found that it functioned like the full-length cDNA (data not shown).

PEA-15 Reversal of Ras Suppression Requires the DED—More than half of the PEA-15 protein consists of a conserved DED (Fig. 2, *A* and *B*). This domain, to date, is associated with proteins involved in apoptosis (28–31). To determine if the DED of PEA-15 is necessary or sufficient for PEA-15 reversal of Ras suppression, we overexpressed mutant forms of PEA-15 in

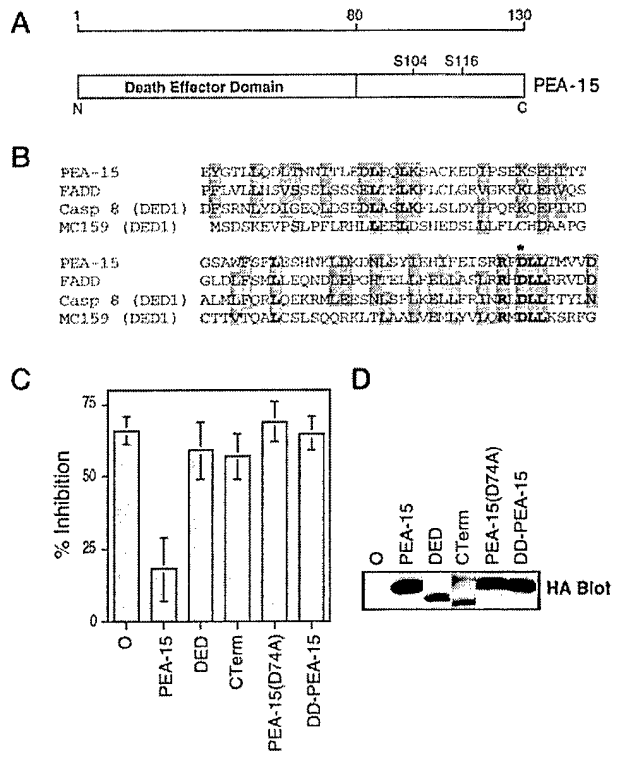

FIG. 2. **PEA-15 reversal of Ras suppression requires the DED.** *A*, the structure of PEA-15 is depicted. CHO PEA-15 contains 130 amino acids. Amino acids 1 to 80 constitute the death effector domain. Serine 104 is a protein kinase C site phosphorylated in astrocytes (25). Serine 116 is a calcium calmodulin kinase II site phosphorylated in astrocytes (43). *B*, amino acid sequence alignment of the DED of PEA-15 with DEDs of FADD, caspase 8, and MC159. Similar residues found at the same position in three or more of the DEDs are *shaded*. Residues conserved in all four DEDs are in **bold type**. The aspartate found in the highly conserved RxDLL motif is marked with an *asterisk*. Note that the DED of PEA-15 is 46% similar to that of FADD. *C*, $\alpha\beta$py cells were co-transfected with expression vectors encoding Ha-RasG12V (3 μg) in combination with PEA-15 (4 μg), DED (8 μg), C-Term (8 μg), PEA-15(D74A) (4 μg), DD-PEA-15 (4 μg), or vector lacking insert (8 μg). After 48 h, integrin activation was assayed by PAC1 binding. Shown is the mean percentage inhibition ± S.D. of at least three independent experiments. *D*, immunoblot using anti-HA antibody 12CA5. Expression levels of PEA-15 and its mutants from the experiments in *C* are similar.

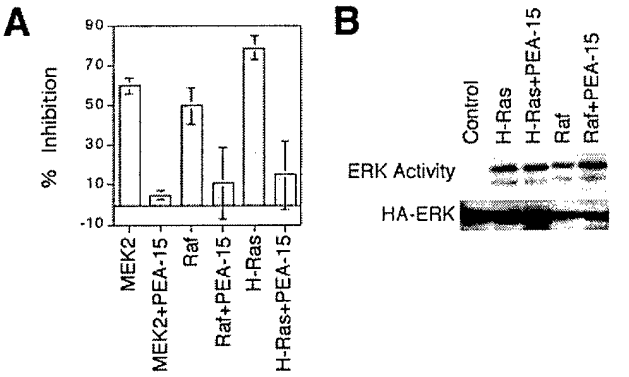

FIG. 3. **PEA-15 rescues Ras, Raf, and MEK2 suppression but does not interfere with ERK2 activation.** *A*, $\alpha\beta$py cells were co-transfected with expression vectors encoding 3 μg of MEK(222/226D), RafCAAX, or Ha-RasG12V in combination with PEA-15 (4 μg) or vector lacking an insert (4 μg). After 48 h, integrin activity was assayed by PAC1 binding. Shown is the mean percentage inhibition ± S.D. of at least three independent experiments. *B*, $\alpha\beta$py cells were co-transfected with HA-ERK2 (2 μg), and expression vectors encoding 3 μg of Ha-RasG12V or RafCAAX in combination with PEA-15 (3 μg) or vector lacking an insert (3 μg). As a control, cells transfected with only vector lacking an insert were also assayed. The transfected ERK2 was immunoprecipitated and incubated with myelin basic protein to determine activity. *Top*, relative activity of ERK2. *Bottom*, immunoblots using anti-HA antibody, 12CA5. The amount of ERK2 expressed in the experiments is comparable.

$\alpha\beta$py cells in this assay. Overexpression of only the DED of PEA-15 did not reverse Ras suppression (Fig. 2*C*). It is therefore not sufficient for this function. Mutants of PEA-15 lacking the DED (C-Term) were also unable to reverse Ras suppression (Fig. 2*C*). Furthermore, a conserved aspartate is present in a RxDLL sequence in all DEDs (32) (*asterisk* in Fig. 2*B*). Mutation of this aspartate (D74A) prevented PEA-15 reversal of Ras suppression (Fig. 2*C*). The structure of the DED is similar to that of the death domain (DD) of FADD (33). Substitution of the DD of FADD for the DED of PEA-15 yielded a chimeric molecule incapable of reversing Ras suppression (Fig. 2*C*). In all cases, mutant PEA-15 constructs were expressed (Fig. 2*D*). The DED of PEA-15 is therefore necessary, but not sufficient, for reversal of Ras suppression. Additionally, substitution of the DED of PEA-15 with the DED of FADD resulted in a chimeric protein that induced apoptosis (data not shown). This indicates that the DED of PEA-15 is functionally distinct from that of FADD and contains primary sequence information required for PEA-15 function. Thus, our studies define a new function for DEDs.

PEA-15 Does Not Block Ras Activation of ERK—Ras suppresses integrin activation by activating a MAP kinase pathway (10). When activated Ras (RasG12V) and PEA-15 were co-expressed, the integrins were not suppressed although the activated variant of Ras was present. Consequently, we assessed the effect of PEA-15 on other activated components of the MAP kinase pathway. Exchange factor mediated GTP loading, and activation of Ras initiates the MAP kinase pathway (34, 35). Active Ras recruits and thus activates Raf kinase. We found that PEA-15 could reverse suppression initiated by an activated Raf (RafCAAX, Fig. 3*A*), indicating that its site of action is distal to Raf activation. Raf phosphorylates and activates MEK, which in turn activates ERK. PEA-15 also rescued suppression mediated by activated MEK (MEK2 222/226D, Fig. 3*A*) and did not block ERK activation (Fig. 3*B*). This suggests that PEA-15 function is distal to ERK.

PEA-15 Reverses Suppression by a Mechanism Sensitive to Dominant-Negative R-Ras—PEA-15 might reverse Ras suppression by activating effectors that oppose Ras signaling to integrins. R-Ras is a Ras-related GTP-binding protein that activates integrins (19) and reverses Ha-Ras suppression of integrin activation.[2] Like PEA-15, R-Ras does not affect Ha-Ras activation of Erk. Consequently, we asked if the capacity of PEA-15 to reverse suppression depends on R-Ras. A dominant-negative R-Ras (R-RasT43N) blocked the ability of PEA-15 to reverse suppression by activated Ha-Ras (Fig. 4*A*). Cells expressing the dominant-negative R-RasT43N had moderately reduced expression of PEA-15 and Ha-Ras (Fig. 4*B*); however Ha-Ras expression levels remained sufficient to suppress integrin activity (Fig. 4*A*). Thus, the reduced expression levels of Ha-Ras do not account for the effects of dominant-negative R-Ras. Furthermore, activated R-Ras still reversed suppression when co-expressed with the dominant-negative R-RasT43N (Fig. 4*A*). Therefore, the effect of R-RasT43N was upstream of R-Ras. These results indicate that PEA-15 inhibition of Ha-Ras suppression is impaired by expression of a dominant-negative R-Ras construct.

[2] T. Sethi, M. Ginsberg, J. Downward, and P. Hughes, submitted for publication.

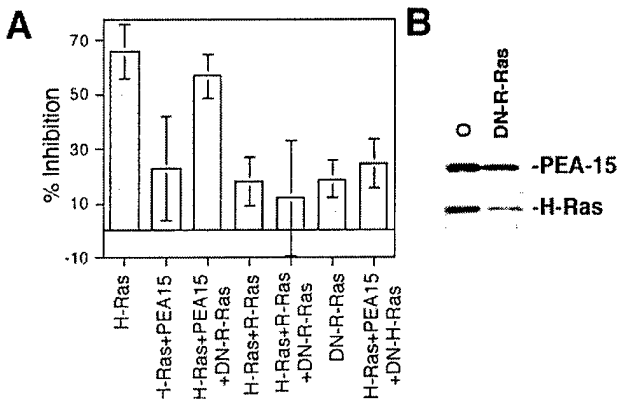

FIG. 4. **PEA-15 rescues Ras suppression by an R-Ras-dependent mechanism.** *A*, αβpy cells were co-transfected with Tac-α5 (2 μg), and the indicated combinations of Ha-RasG12V (3 μg), PEA-15 (3 μg), R-RasT43N (*DN-R-Ras*, 3 μg), R-RasVM8 (*R-Ras*, 2 μg), and Ha-RasT17N (*DN-H-Ras*, 2 μg). Total amounts of transfected plasmid were adjusted to 11 μg by addition of appropriate amounts of control vector lacking an insert. After 48 h, integrin activation was determined by PAC1 binding. Depicted is the mean percentage inhibition ± S.D. of four independent experiments. *B*, immunoblot using anti-HA antibody 12CA5. Expression levels of PEA-15 from the experiments in *A* containing Ha-Ras + PEA-15 (0, *top*) and Ha-Ras + PEA-15 + DN-R-Ras (*DN-R-Ras*, *top*) are similar, as are the levels of Ha-Ras (*bottom*).

Because R-Ras and Ha-Ras are similar (36), they may share some of the same guanine-nucleotide exchange factors (37), but R-Ras is also regulated by effectors and activators distinct from those that control Ha-Ras (38). The dominant-negative form of R-Ras we used probably sequesters GEFs (39). Consistent with this site of action, DN-R-Ras failed to affect rescue mediated by an activated variant of R-Ras (G38V). Furthermore, a DN-Ha-Ras construct did not affect PEA-15 reversal of suppression. Hence, the DN-R-Ras acts by blocking events specific for R-Ras and not ones common to both Ha-Ras and R-Ras. Similarly, dominant-negative constructs of the small GTP-binding proteins Cdc42, Rac, and Rho did not prevent PEA-15 reversal of Ras suppression (data not shown), further suggesting that the effect is R-Ras-specific. R-Ras activates integrins (19) and reverses Ha-Ras suppression of integrin activation (Fig. 4*A*). Therefore, PEA-15 may reverse Ras suppression via an R-Ras-dependent mechanism. The proteins involved in R-Ras regulation remain unclear (40). However, our data suggest that PEA-15 could be a novel upstream regulator of R-Ras activity. Alternatively, the effects of the dominant-negative R-Ras construct may be due to interference with the closely related protein TC21/R-Ras2 (41, 42). It will be interesting to analyze the potential interplay between PEA-15 and R-Ras.

In summary, we used an expression cloning scheme to identify proteins that prevent Ha-Ras suppression of integrin activation. We report that the DED-containing protein, PEA-15, blocks Ras suppression. PEA-15 does not inhibit Ras activation of the ERK MAP kinase pathway, but rather blocks Ras suppression via a pathway inhibited by a dominant-negative form of R-Ras. Finally, the DED of PEA-15 is necessary, but not sufficient, for the reversal of Ras suppression. Hence, these data provide evidence that DED-containing proteins can regulate integrin activation as well as apoptosis. Moreover, we have identified PEA-15 as a novel regulator of inside-out integrin signaling pathways.

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Class- and Splice Variant-specific Association of CD98 with Integrin β Cytoplasmic Domains*

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Roy Zent‡§, Csilla A. Fenczik§¶, David A. Calderwood‖, Shouchun Liu, Melissa Dellos, and Mark H. Ginsberg‡‡**

From the Department of Vascular Biology, Scripps Research Institute, La Jolla, California 92037

CD98 is a type II transmembrane protein involved in neutral and basic amino acid transport and in cell fusion events. CD98 was implicated in the function of integrin adhesion receptors by its capacity to reverse suppression of integrin activation by isolated integrin β_{1A} domains. Here we report that CD98 associates with integrin β cytoplasmic domains with a unique integrin class and splice variant specificity. In particular, CD98 interacted with the ubiquitous β_{1A} but not the muscle-specific splice variant, β_{1D}, or leukocyte-specific β_7 cytoplasmic domains. The ability of CD98 to associate with integrin cytoplasmic domains correlated with its capacity to reverse suppression of integrin activation. The association of CD98 with integrin β_{1A} cytoplasmic domains may regulate the function and localization of these membrane proteins.

The development and function of multicellular animals requires integrin adhesion receptors (1). Integrin-dependent cell adhesion is regulated, in part, by ligand binding affinity ("activation") changes controlled by cellular signaling cascades (1–3). Regulation of integrin affinity is important in cell migration (4–6), extracellular matrix assembly (7), and morphogenesis (8). Integrin activation is energy-dependent and is mediated by cell type specific signals operating through integrin cytoplasmic domains (9).

Complementation of dominant suppression (CODS)[1] is an expression cloning scheme used to identify proteins that modulate integrin affinity (10). CODS depends on the ability of an isolated integrin β_{1A} cytoplasmic domain, in the form of a chimera with the α subunit of the interleukin-2 receptor, to block integrin activation (dominant suppression). Proteins involved in integrin activation are isolated by their ability to complement dominant suppression. CD98, a type II transmembrane protein first discovered as a T-cell activation antigen (11), was identified utilizing CODS. CD98, although widely expressed on proliferating cells, is generally down-regulated in quiescent cells (12). CD98 forms disulfide-bonded heterodimers with several light chains that strongly resemble permeases (13–20). CD98 regulates the transport of neutral and positively charge amino acids through these light chains (14, 15, 17, 18). Thus, CODS has identified an unexpected connection between cell adhesion and certain amino acid transporters.

The mechanism by which CD98 influences integrin function is not yet clear. CODS was predicated on the idea that it would identify integrin β cytoplasmic domain binding proteins (10). Many β cytoplasmic domains manifest overall sequence similarity (1, 2); however, the cytoskeletal protein, talin, binds to the muscle-specific splice variant, β_{1D}, more tightly than to β_{1A}. In addition, the leukocyte-specific β_7 cytoplasmic domain binds to filamin more tightly than to β_{1A} (21). We have now examined interactions between CD98 and recombinant parallel-dimerized integrin β_{1A}, β_{1D}, and β_7 cytoplasmic domains by affinity chromatography (21). Here we report that CD98 interacts with the β_{1A} but not β_{1D} or β_7 integrin cytoplasmic domains. Furthermore, the CD98 interaction is insensitive to β cytoplasmic domain mutations that abolish the binding of talin and filamin. The capacity of CD98 to complement dominant suppression correlates with its capacity to bind to the suppressive β cytoplasmic domains. The interaction of the integrin β_{1A} cytoplasmic domain with CD98 may thus serve to regulate the localization and the function of these membrane proteins.

EXPERIMENTAL PROCEDURES

Antibodies—The hybridoma cell line 4F2(C13) (anti-CD98) was purchased from American Type Culture Collection (ATCC). The CD98 antibody was purified from ascites produced in pristane-primed BALB/c mice by protein A affinity chromatography. Filamin antibody (monoclonal antibody 1680) was purchased from Chemicon and talin antibody (clone 8d4) from Sigma. Dr. S. Shattil (Scripps Research Institute) generously provided the activation-specific anti-$\alpha_{IIb}\beta_3$ monoclonal antibody, PAC1 (22). The anti-$\alpha_{IIb}\beta_3$ activating monoclonal antibody, anti-LIBS6, has been described previously (23). The anti-Tac antibody, 7G7B6, was obtained from the American Tissue Culture Collection (Rockville, MD) and was biotinylated with biotin-*N*-hydroxysuccinimide (Sigma) according to manufacturer's instructions. The $\alpha_{IIb}\beta_3$-specific peptide inhibitor, Ro43-5054 (24), was a generous gift from B. Steiner (Hoffmann-La Roche, Basel, Switzerland).

DNA Constructs and Recombinant Proteins—cDNA encoding the expressed integrin cytoplasmic domains joined to 4 heptad repeats (Fig. 1) were cloned into the modified pET-15 vector as described previously (21). Point mutations in β_{1D} and β_7 (Fig. 1) were performed utilizing the Quickchange kit (Stratagene). Recombinant expression in BL21 (DE3)pLysS cells (Novagen) and purification of the recombinant products were made in accordance with the manufacturers instructions (Novagen), with an additional final purification step on a reverse phase C18 high performance liquid chromatography column (Vydac). Polypeptide masses were confirmed by electrospray ionization mass spectrometry on an API-III quadrupole spectrometer (Sciex, Toronto, Ontario,

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‡ Fellow of the National Kidney Foundation.

§ These authors contributed equally to this work.

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‡‡ To whom correspondence should be addressed: Dept. of Vascular Biology, Scripps Research Inst., 10550 N. Torrey Pines Rd., La Jolla, CA 92037. Tel.: 619-784-7143; Fax: 619-784-7343; E-mail: ginsberg@scripps.edu.

[1] The abbreviations used are: CODS, complementation of dominant suppression; PAGE, polyacrylamide gel electrophoresis; Pipes, 1,4-piperazinediethanesulfonic acid.

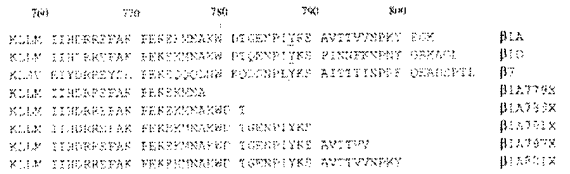

FIG. 1. **Amino acid sequences of integrin β cytoplasmic domains.** Depicted is an alignment of the integrin cytoplasmic domains used in this study. The *underlined* tyrosine (Y) was mutated to an alanine (A) to form the YA mutants. All integrin sequences with the exception of β_7 correspond to those human sequences published in the Swiss-Protein data base as of May 15, 1999. In β_7, the amino-terminal Arg was changed to Lys in order to introduce a *Hin*dIII restriction site.

Canada) and varied by less than 4 daltons from those predicted by the desired sequence.

Tac-α5 and Tac-β_{1A} DNA in modified CMV-IL2R expression vectors (25) were generously provided by Drs. S. LaFlamme and K. Yamada (National Institutes of Health, Bethesda, MD). Inserts encoding Tac-β_{1D}, Tac-β_7, Tac-β_{1A}(Y788A), and Tac-β_{1A}(801X) were subcloned into the modified CMV-IL2R expression vector as *Hin*dIII-*Xho*I fragments.

Cell Culture—αβpy cells, a Chinese hamster ovary cell line expressing the polyoma large T antigen and a constitutively active recombinant chimeric integrin, $\alpha_{IIb}\alpha_{6A}\beta_3\beta_1$ (26), were maintained in Dulbecco's modified Eagle's medium (BioWhitaker); supplemented with 10% fetal calf serum (BioWhitaker), 1% non-essential amino acids (Life Technologies, Inc.), 1% glutamine (Sigma), 1% penicillin and streptomycin (Sigma), and 700 μg/ml G418 (Life Technologies, Inc.). Human Jurkat T cell lines were obtained from ATCC and maintained in RPMI1680 (BioWhitaker) supplemented with 10% fetal calf serum, 1% nonessential amino acids, 1% glutamine, and 1% penicillin and streptomycin. The filamin-1-deficient human melanoma cell line M2 and a reconstituted line A7 (27) (kindly donated by T. P Stossel) were cultured in Eagle's medium (BioWhitaker), supplemented with 10% fetal calf serum, 1% nonessential amino acids, 1% glutamine, and 1% penicillin and streptomycin.

Cell Lysates—Jurkat cells were washed twice in phosphate-buffered saline and surface-biotinylated using Sulfo-Biotin *N*-hydroxysuccinimide in phosphate-buffered saline according to the manufacturer's instructions (Pierce). They were then washed twice with Tris-buffered saline and lysed by sonication on ice in buffer A (1 mM Na_3VO_4, 50 mM NaF, 40 mM sodium pyrophosphate, 10 mM Pipes, 50 mM NaCl, 150 mM sucrose, pH 6.8) containing 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM EDTA, and protease inhibitors (aprotinin, 5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Platelet lysates were prepared as described previously (21).

Subcellular fractionation of Jurkat cells was performed after surface biotinylation. The cells were washed three times in Hepes-saline (200 mM Hepes, 12 mM $CaCl_2{\cdot}2H_2O$, 16 mM $MgSO_4$, pH 7.3–7.4), suspended in 20 mM Hepes, and homogenized with a Dounce homogenizer. An equal quantity of buffer B (20 mM Hepes, 0.5 M sucrose, 10 mM $MgCl_2$, 0.1 M KCl, 2 mM $CaCl_2{\cdot}H_2O$ with protease inhibitors) was added to the homogenate, and the mixture was centrifuged at 500 × *g* at 4 °C for 15 min. The supernatant was collected and centrifuged at 100 000 × *g* for 30 min in a Beckman model L7-65 centrifuge. The cytoplasmic fraction (supernatant) was removed and the membrane fraction (pellet) washed in a 1:1 mixture of 20 mM Hepes and buffer B. The membrane fraction was resuspended in buffer A, 1 mM EDTA, and protease inhibitors and centrifuged at 30,000 × *g* for 20 min.

Affinity Chromatography Experiments—Recombinant proteins were expressed in BL21(DE3)pLysS cells (Novagen) and bound to His-bind resin (Novagen) through their N-terminal His tag in a ratio of 1 ml of beads/liter of culture. Coated beads were washed with PN (20 mM Pipes, 50 mM NaCl, pH 6.8) and stored at 4 °C in an equal volume of PN containing 0.1% NaN_3. Beads were added to cell lysates diluted in buffer A, (0.05% Triton X-100, 3 mM $MgCl_2$, and protease inhibitors) and incubated overnight at 4 °C and then washed five times with buffer A. 100 μl of SDS-sample buffer was added to the beads and the mixture was heated at 100 °C for 5 min. After 10,000 rpm centrifugation in a microcentrifuge, the supernatant was fractionated by SDS-PAGE and analyzed by Western blotting. In some experiments, proteins were eluted off the beads with 100 μl of elution buffer (1 M imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9) and 1 ml of immunoprecipitation buffer (20 mM Tris-HCl, 150 mM NaCl, 10 mM benzamidine HCl, 1% Triton X-100, 0.05% Tween 20, and protease inhibitors) was then added. The eluted proteins were immunoprecipitated overnight at 4 °C with an 4F2 antibody pre-bound to protein A-Sepharose beads (Amersham Pharmacia Biotech). The following day, the beads were washed three times with the immunoprecipitation buffer and heated in reducing sample buffer for SDS-PAGE under reducing conditions. Samples were separated on 4–20% SDS-polyacrylamide gels (Novex) and transferred to nitrocellulose membranes. Membranes were blocked with Tris-buffered saline, 5% nonfat milk powder and stained with streptavidin-peroxidase or with specific antibodies and appropriate peroxidase conjugates. Bound peroxidase was detected with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech). Equal loading of Ni^{2+} beads with recombinant proteins were verified by Coomassie Blue staining of SDS-PAGE profiles of SDS eluted proteins.

Flow Cytometry—Analytical two-color flow cytometry was performed as described previously (9). PAC1 binding was assessed in a subset of transiently transfected αβpy cells (cells positive for co-transfected Tac-α5 as measured by 7G7B6 binding). Integrin activation was quantified as an activation index (*AI*) defined as $(F - F_o)/(F_{LIBS6} - F_o)$, in which F is the median fluorescence intensity of PAC1 binding, F_o is the median fluorescence intensity of PAC1 binding in the presence of competitive inhibitor (Ro43-5054, 1 μM), and F_{LIBS6} is the maximal median fluorescence intensity of PAC1 binding in the presence of the integrin activating antibody anti-LIBS6 (2 μM). Percentage of reversal is calculated as $(AI_{(\beta x\,+\,CD98)} - AI_{\beta x})/(AI_{\alpha 5} - AI_{\beta x})$. $AI_{\beta x}$ is the activation index of cells transfected with Tac-β_x chimeras, $AI_{(\beta x\,+\,CD98)}$ is the *AI* of cells co-transfected with CD98 and Tac β_x chimeras, and $AI_{\alpha 5}$ is the *AI* of cells transfected with Tac-α5. The x of β_x can have values of 1A, 1D, and 7 for the Tac-β_{1A}, Tac-β_{1D}, and Tac-β_7 chimeras, respectively.

RESULTS

CD98 Binds to the β_{1A} Integrin Cytoplasmic Domain—CD98 can block reduced integrin affinity caused by overexpression of free β_{1A} cytoplasmic domains, suggesting a physical interaction between β_{1A} and CD98 (10). To assess this potential interaction, we examined the binding of solubilized membrane proteins to the β_{1A} cytoplasmic domain. For affinity matrices, we used model proteins in which the integrin cytoplasmic domain was joined to four heptad repeats (21). The repeats form parallel coiled-coil dimers so that the tails are dimerized and parallel. When a Jurkat cell lysate was exposed to such an affinity matrix, a cell surface polypeptide of 88 kDa bound to the β_{1A} but not to the α_{IIb} tail (Fig. 2*A*). This polypeptide was immunoprecipitated by the anti-CD98 antibody, 4F2 (Fig. 2*B*). Based on its mass and reactivity with anti-CD98 antibody, the β_{1A} tail binding polypeptide was identified as CD98.

To assess the specificity of CD98 binding to β integrin tails, affinity chromatography was performed with β_{1D}, β_3, and β_7 cytoplasmic domains. CD98 did not bind to β_7 and binding to β_{1D} was weak and variable (Fig. 3*A*). In contrast, talin and filamin (Fig. 3*A*) bound strongly to β_{1D} and β_7 tails, respectively, as reported (21). CD98 also bound to β_3, and binding was not altered by the presence of the α_{IIb} cytoplasmic domain (Fig. 3*B*). Thus, CD98 binding to integrin tails is integrin class- and splice-variant-specific.

Differential CD98 Binding to β Integrin Tails Is Independent of Filamin and Talin Binding—CD98 binds well to the β_{1A} integrin cytoplasmic domain but not to those of β_{1D} or β_7. The binding assays were performed using talin- and filamin-1-containing cell extracts. Thus, these CD98 binding differences could be due to competition for CD98 binding by filamin-1 or talin, which bind preferentially to β_7 or β_{1D}, respectively (21). To test this possibility, we used filamin-1-deficient human melanoma cells (M2) and reconstituted cells (A7) (27) to examine the role of filamin-1 in CD98 binding. CD98 bound to the β_{1A} tail, but not β_7, when lysates of M2 cells were used (Fig. 4*A*), showing that filamin-1 is not required for CD98 binding to β_{1A}. CD98 binding to β_7 was not observed in the filamin-1 null (M2) cells. Consequently, competition with filamin-1 does not account for the failure of β_7 to bind CD98.

To examine the role of talin, we used cell membrane preparations with a greatly reduced talin content (Fig. 5*A*). CD98 extracted from these membranes bound β_{1A} but not β_{1D} cytoplasmic domains (Fig. 5*B*). Thus, talin does not prevent CD98

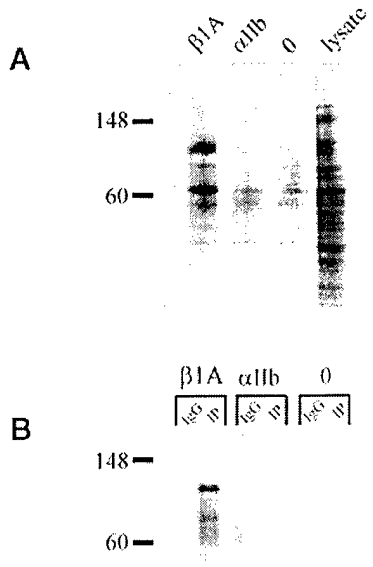

FIG. 2. **β_{1A} cytoplasmic domains bind CD98.** Jurkat human T cells were surface-labeled with Sulfo-Biotin *N*-hydroxysuccinimide, and the cells were lysed in buffer A (see "Experimental Procedures"). *Panel A* depicts a reduced SDS-PAGE analysis of the biotinylated proteins that bound to Ni^{2+} beads, coated with model proteins containing β_{1A} (*β1A*) or α_{IIb} (*αIIb*) cytoplasmic tails. Adjacent *lanes* show the surface proteins present in the lysate (*lysate*) or the ones that bound to uncoated Ni^{2+} beads (*0*). In *panel B*, the biotinylated surface proteins that bound to the β_{1A} (*β1A*) or α_{IIb} (*αIIb*) tails or uncoated beads (*0*) were immunoprecipitated with CD98 antibody (*IP*) or a control IgG (*IgG*). The immunoprecipitates were fractionated by reduced SDS-PAGE, and biotinylated proteins were detected by streptavidin-peroxidase-generated chemiluminescence.

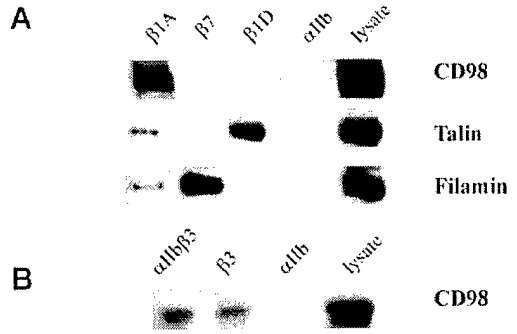

FIG. 3. **CD98 binds to the β_{1A} and β_3 cytoplasmic domain rather than that of β_{1D} or β_7.** *A*, in the *upper panel*, surface-biotinylated Jurkat cell lysates were allowed to bind to model proteins containing the β_{1A}, β_7, β_{1D}, or α_{IIb} integrin tails. The bound fractions were immunoprecipitated with CD98 antibody and analyzed by SDS-PAGE, as described under "Experimental Procedures." In the *lower two panels*, human platelet lysates were incubated with the same tail constructs, and bound proteins were fractionated by reduced SDS-PAGE and immunoblotted with antibodies to talin or to filamin. The loading of each tail was verified by Coomassie Blue staining of the model proteins eluted from the beads and fractionated by SDS-PAGE (data not shown). *B*, the surface-labeled Jurkat T cell lysate used in *panel A* was allowed to bind to model proteins containing a heterodimer of the α_{IIb} and β_3 tails, or to model proteins containing only the individual tails. Bound fractions were immunoprecipitated with CD98 antibody and analyzed by SDS-PAGE, as described under "Experimental Procedures."

binding to β_{1D}, nor is it required for CD98 binding to β_{1A}.

The Y788A mutation of β_{1A} (Fig. 1) disrupts filamin (Fig. 4*B*) and talin (Fig. 5*C*) binding (21). Similar Tyr to Ala mutations in

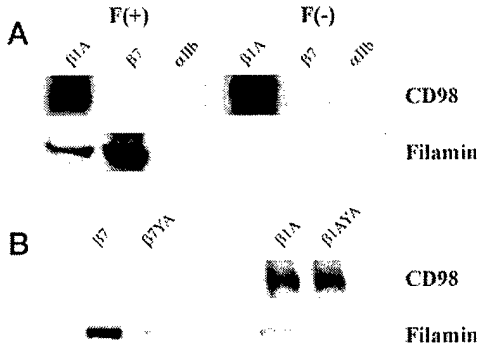

FIG. 4. **Differences in CD98 binding to β_{1A} and β_7 cytoplasmic domains are independent of the presence of filamin.** Affinity chromatography was performed using surface-biotinylated M2 (*F−*) or A7 (*F+*) cell lysates and various cytoplasmic tails (*αIIb*, *β1A*, *β7*). Bound proteins were immunoprecipitated with anti-CD98 antibody and fractionated by reduced SDS-PAGE, and the biotinylated polypeptides were detected by streptavidin-peroxidase chemiluminescence (*panel A*, *CD98*). Lysates of A7 and M2 cells were incubated with the indicated integrin cytoplasmic tails, and bound proteins were fractionated by SDS-PAGE and immunoblotted with anti-filamin monoclonal antibody 1680 (*panel A*, *filamin*). In *panel B*, surface-biotinylated Jurkat cell lysates were incubated with β_{1A} and β_7 tails and their corresponding YA (β1YA, β7YA) mutants. CD98 and filamin binding was assessed as described in *panel A*. Loading of integrin tails was equal as verified by Coomassie Blue staining (data not shown).

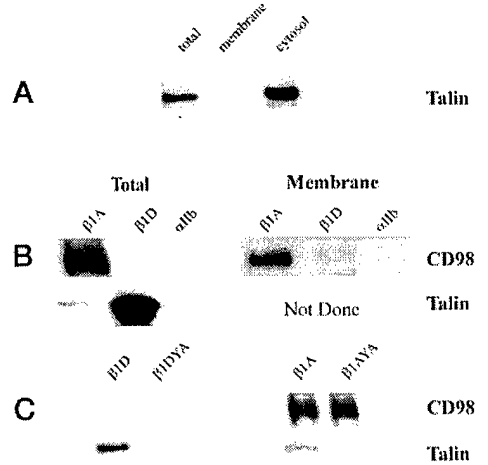

FIG. 5. **Differing CD98 binding to β_{1A} and β_{1D} cytoplasmic domains is independent of talin.** Jurkat cells were surface-labeled with biotin, lysed in buffer A, and fractionated into membrane and cytosolic fractions. Whole cell lysate (*Total*), membrane (*Membrane*), and cytosolic fractions (*Cytsosol*) were fractionated by SDS-PAGE and immunoblotted with an anti-talin antibody (*panel A*). The membrane fraction and whole cell lysate were incubated with α_{IIb}, β_{1A}, or β_{1D} integrin tails and bound CD98 was detected by immunoprecipitation as described under "Experimental Procedures" (*panel B*). In *panel C*, lysates of Jurkat cells (*upper*) and platelets (*lower*) were analyzed for binding of CD98 and talin to β_{1A} and β_{1D} tails, and their corresponding YA (β_1YA, β_{1D}YA) mutants as described in Fig. 4. Loading of integrin tails was equal as verified by Coomassie Blue staining (data not shown).

β_7 and β_{1D} tails, corresponding to the Y788A mutation in β_{1A} (Fig. 1), also disrupted filamin (Fig. 4*B*) and talin (Fig. 5*C*) binding. CD98 binding to β integrin tails was not affected by Tyr to Ala mutations (Figs. 4*B* and 5*C*). The Tyr to Ala mutation introduced into β_{1D} or β_7 did not increase CD98 binding, nor was CD98 binding reduced in the β_{1A}(Y788A) mutant. These results confirm that talin or filamin competition does not account for the lack of CD98 binding to β_{1D} and β_7 and that talin or filamin binding is not required for CD98 binding to the β_{1A} cytoplasmic domain.

CD98 Binding to Integrin Cytoplasmic Domains Correlates

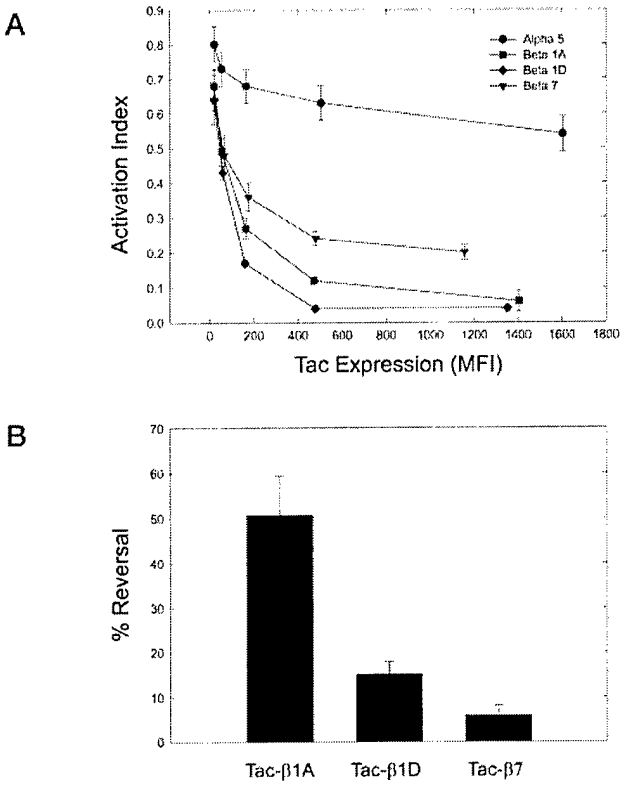

FIG. 6. *A*, β tails induce varying amounts of integrin suppression. αβpy cells were transfected with Tac-β_1 (0.5 μg), Tac-β_{1D} (1.0 μg), Tac-β_7 (3.0 μg), or Tac-α_5 (1.0 μg). After 24 h, cells were collected and analyzed for PAC1 binding to the Tac-positive subset of cells. The activation index was calculated for cells expressing different amounts of each Tac chimera. Activation index is defined as $100(F_o - F_R)/(F_{LIBS6} - F_R)$, where F_o is the median fluorescence intensity of PAC1 binding; F_R is the background fluorescence intensity of PAC1 binding in the presence of a competitive inhibitor (1 μM Ro43-5054), and F_{LIBS6} is the maximal fluorescence intensity in the presence of 2 μM anti-LIBS6, an activating monoclonal antibody. The mean ± S.D. of at least five independent experiments for each Tac chimera is shown. *B*, CD98 binding to β tails correlates with its ability to reverse dominant suppression. αβpy cells were transfected with each of the Tac chimeras in the presence or absence of 4 μg of cDNA encoding full-length CD98. 24 h after transfection, cells were collected and the Tac-positive subset of cells were analyzed for the ability to bind to the PAC1 antibody. Data are expressed as percentage reversal, which is calculated as $(AI_{\beta x + CD98} - AI_{\beta x})/(AI_{\alpha 5} - AI_{\beta x})$. *AI* is the activation index, $AI_{\beta x}$ is the *AI* of cells transfected with Tac β chimeras, $AI_{\beta x + CD98}$ the *AI* of cells transfected with CD98 and Tac β_x chimeras, and $AI_{\alpha 5}$ is the *AI* of cells transfected with the Tac-α5. The *x* of β_x can have values of 1A, 1D, and 7 for the Tac-β_{1A}, Tac-β_{1D}, and Tac-β_7 chimeras, respectively. The expression of the Tac-β_{1A} and Tac-β_{1D} chimeras were similar (mean fluorescence intensity = 340 ± 20 and 370 ± 50 units, respectively), while Tac-β_7 was better expressed (mean fluorescence intensity = 530 ± 90 units). In the absence of CD98, Tac-β_7 (55 ± 6% suppression) inhibited activation less than Tac-β_{1A} or Tac-β_{1D} (77 ± 4% and 82 ± 7% suppression, respectively).

with Complementation of Dominant Suppression—Overexpression of isolated integrin β_{1A} cytoplasmic domains, in the form of a Tac-β_{1A} chimera, results in suppression of integrin activation. Dominant suppression is reversed by overexpression of CD98 (10). Tac-β_{1A}, Tac-β_{1D}, and Tac-β_7 induced dominant suppression of integrin activation (Fig. 6*A*). As noted above (Fig. 3), CD98 bound poorly to β_{1D} and β_7 tails, showing that CD98 binding is not required for dominant suppression. However, CD98 was much less effective at reversing the suppression induced by Tac-β_{1D} and Tac-β_7 (Fig. 6*B*). Thus the capacity of CD98 to rescue suppression correlates with its binding to the suppressive β cytoplasmic domain.

CD98 Binding Is Not Sufficient to Induce Dominant Suppression—As noted above, β_{1A} tails suppress integrin activation and bind CD98. To assess whether CD98 binding alone is sufficient to induce dominant suppression, we first examined CD98 binding to a series of β_{1A} truncation mutants (Fig. 1). CD98 binding was lost when the C-terminal seven residues were deleted (β_{1A}C797X)) but not when the last three amino acids were eliminated (β_{1A}(801X)) (Fig. 7*A*). Despite maintaining its capacity to bind to CD98, the Tac-β_{1A}(801X) mutant was a poor suppressor of integrin activation (Fig. 7*B*), and this was not due to a quantitative reduction in the association of CD98 with β_{1A}(801X) (Fig. 7*C*). Furthermore, the β_{1A}(Y788A) mutant, which also bound CD98 (Figs. 4 and 5), failed to suppress integrin activation (Fig. 7*B*). Consequently, integrin β cytoplasmic domain binding to CD98 is not sufficient to induce dominant suppression.

DISCUSSION

CD98 is implicated in several cellular functions, including amino acid transport, cell fusion events, and integrin activation (12). We previously found that CD98 reverses dominant suppression of integrin function (10). We now report that: 1) CD98 associates with the β_{1A} integrin cytoplasmic domain; 2) CD98 interacts differentially with β cytoplasmic tails in a class- and splice variant-specific manner, which is independent of the capacity of the tails to bind the cytoskeletal proteins talin and filamin; 3) CD98's capacity to associate with integrin tails correlates with its ability to overcome dominant suppression of integrin activation; 4) CD98 association with integrin tails is neither necessary nor sufficient for dominant suppression of integrin activation. Thus, the association of CD98 with integrin cytoplasmic domains may regulate the function and localization of these membrane proteins.

CD98 physically associates with β_{1A} integrin cytoplasmic domains. This association was observed utilizing model protein mimics of dimerized integrin cytoplasmic tails, and it may account for the physical association of certain β_1 integrins with CD98.[2] The specificity of the interaction was confirmed by the lack of binding to mimics containing cytoplasmic domains from α_{IIb} or several other β subunits. CD98 was added to the tails in the presence of other cellular proteins, so it remains possible that an intermediary protein is required for this interaction. However, CD98 was the only surface protein observed binding to the β_{1A} tail (Fig. 2). Moreover, we observed CD98 binding in the absence of two known integrin binding proteins, talin and filamin (Figs. 3 and 4). CD98 failed to bind to β_{1D} and β_7 cytoplasmic domains, even though these tails bind many of the same polypeptides as β_{1A} (21). Thus, we conclude that CD98 associates with the β_{1A} tail and that the interaction is potentially direct.

CD98 binds to integrin β cytoplasmic domains with unique splice variant and class specificity. CD98 bound well to the β_{1A} tail and the β_3 tail. Binding to the β_{1D} and β_7 tails was negligible. The specificity of CD98 binding differs markedly from the specificity of talin and filamin binding, since talin binds preferentially to the β_{1D} tail and filamin to the β_7 tail (21). Moreover, the binding of both cytoskeletal proteins is sensitive to the Tyr substitution with Ala in the first "NPXY" (21) in β_{1A} and, as shown here, in β_7 and β_{1D}. Strikingly, CD98 binding was insensitive to this mutation. Finally, although the last three residues of β_{1A} were dispensable, the last seven residues were required for binding. Thus, the features of the β tail defined here for CD98 binding identifies a novel structural specificity for integrin β tail function.

CD98 binding to β tails correlates with its capacity to com-

[2] M. Hemler, personal communication.

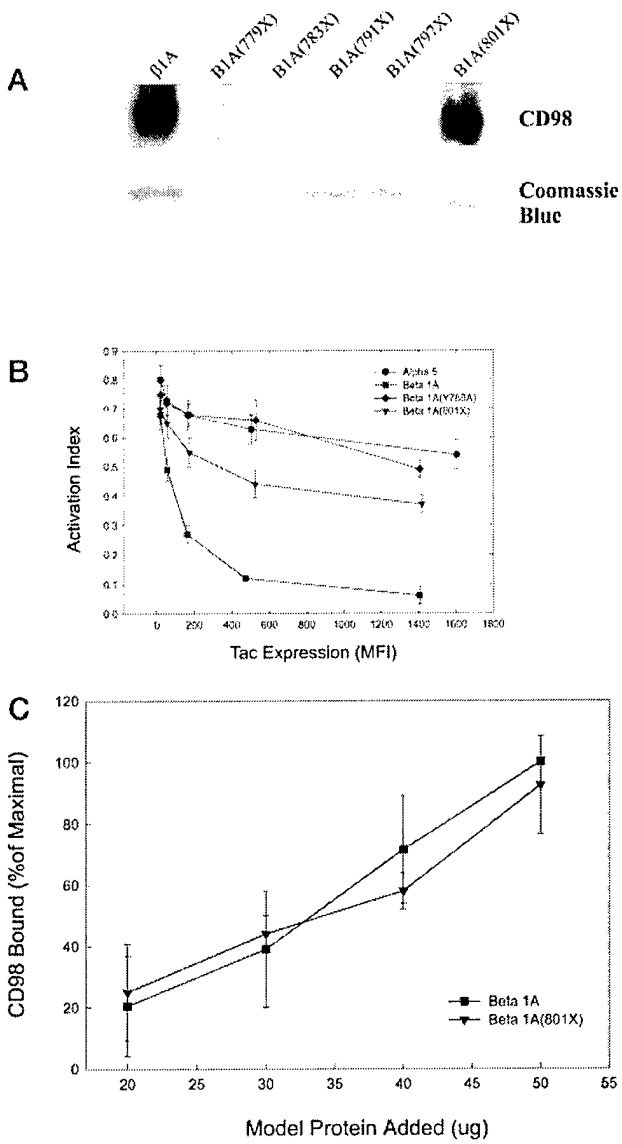

FIG. 7. *A*, binding of CD98 to truncated β_{1A} cytoplasmic domains. Truncation mutants of β_{1A} cytoplasmic tails were made as described under "Experimental Procedures." Lysates of surface-biotinylated Jurkat cells were incubated overnight with affinity matrices containing β_{1A}, β_{1A}(779X), β_{1A}(783X), β_{1A}(791X), β_{1A}(797X), or β_{1A}(801X) integrin tails, and the bound fractions were immunoprecipitated with CD98 antibody and analyzed by SDS-PAGE (*panel A*). Biotinylated polypeptides were detected by streptavidin-peroxidase chemiluminescence (CD98). Loading of the affinity matrix with each tail was verified by Coomassie Blue staining of model proteins eluted from the resin and fractionated by SDS-PAGE (*Coomassie Blue*). *B*, CD98 binding is not sufficient to induce dominant suppression. αβpy cells were transfected with Tac-β_1 (0.5 μg), Tac-β_{1A}(801X) (1.0 μg), Tac-β_{1A}(Y788A) (1.0 μg), or Tac-α_5 (1.0 μg). After 24 h, cells were detached and analyzed for PAC1 binding to the Tac-positive subset of cells by flow cytometry as described under "Experimental Procedures." The activation index was calculated for cells expressing different amounts of each Tac chimera as described in Fig. 6. Note that the β_{1A}(801X) and β_{1A}(Y788A) tails induced little suppression, even though they bound CD98. *C*, similar association of β_{1A}(801X) and β_{1A} tails with CD98. Lysates of surface-biotinylated Jurkat cells were incubated overnight with affinity matrices containing the indicated quantities of β_{1A} or β_{1A}(801X) integrin tails, and the bound fractions were immunoprecipitated with CD98 antibody and fractionated by SDS-PAGE. Biotinylated polypeptides were detected by streptavidin-peroxidase-dependent chemiluminescence and quantified by scanning densitometry. Data are reported as percentage of binding relative to the maximal binding at 50 μg of β_{1A} model protein.

plement dominant suppression. CD98 was implicated in integrin activation by its capacity to reverse the suppression of integrin activation caused by an isolated β_{1A} cytoplasmic domain (10). In the present work, we found that CD98 binds to the β_{1A} cytoplasmic domain, but fails to bind well to the β_7 or β_{1D} cytoplasmic domain. Strikingly, CD98 failed to complement dominant suppression initiated by either β_7 or β_{1D} cytoplasmic domains. Consequently, the mechanism of CODS appears to involve CD98 binding to the suppressive β tail. Furthermore, cross-linking of CD98 stimulates integrin $\alpha_3\beta_1$-dependent adhesion in small cell lung cancer cells (10) and in certain breast cancer cell lines (28) and β_1 integrin-dependent cell fusion events (29–36). Thus, our finding that CD98-β_1 cytoplasmic domain interactions correlate with effects on integrin function is relevant to integrin-dependent events involved in mulinucleate giant cell formation, virally induced cell fusion, and regulation of cell adhesion.

The physical interaction of CD98 with integrin cytoplasmic domains may be involved in modulating amino acid transport regulation. CD98 is known to regulate y+L and L type amino acid transport (14, 15, 17, 18). This regulation is probably due to disulfide-bonded heterodimer formation with a variety of light chains, that resemble permease amino acid transporters (13–20). In fact, mutations in one of these light chains (15) are a likely cause of lysinuric protein intolerance (37). CD98 may function to regulate both the expression and localization of its light chains (18). In certain cells CD98 has a basolateral localization (38). β_{1A} integrins also manifest basolateral polarization in many cells (39, 40), probably due to interactions with underlying matrix components (41) or recruitment to lateral cell contacts (42). It is noteworthy that β_7 integrins are primarily involved in lymphocyte homing and β_{1D} integrins primarily form mechanical linkages in striated and cardiac muscle (43, 44). Thus, the failure of these cytoplasmic domains to bind to CD98 correlates well with their lack of a role in establishing polarity in epithelial or mesenchymal cells. Consequently, the physical association of CD98 with β_{1A} integrin cytoplasmic domains may participate in the polarization and regulation of amino acid transporters and to modulate the function of certain integrins.

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