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FOREWORD

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Introduction

The process of metastasis and invasion of tumor cells requires that the cells regulate their ability to adhere to the surrounding extracellular matrix (2). For example, loss of cell adhesion occurs when cells detach from the primary tumor. Changes in cell adhesion are also required for cells to migrate through tissues. These alterations in the adhesive properties of tumor cells involve changes in the function of the integrin family of cell adhesion receptors. Integrins consist of an α and a β subunit, each containing large extracellular domains, transmembrane domains and short cytoplasmic domains, typically 20-60 amino acids long (21). Interference with the binding of tumor cells to extracellular matrix proteins through integrins can block tumor metastasis or invasion, showing the role of integrins in the progression of cancer (4, 12, 19, 20, 38).

Integrins are known to modulate their affinity for their extracellular ligands in response to intracellular signals (inside-out signaling), thereby regulating cell adhesion events (41). The dynamic regulation of integrin adhesion by inside-out signaling is a crucial aspect of integrin function that is important in hemostasis, leukocyte extravasation, cell migration and fibronectin matrix assembly. Inside-out signaling is energy dependent, cell-type specific and requires the cytoplasmic domains of both the α and β subunits. The proteins that mediate these cytoplasmic signals have not been fully defined.

One approach in identifying mediators of inside-out signaling is to find proteins that directly interact with the integrin cytoplasmic domains. This has been done successfully using both biochemical approaches and yeast two-hybrid screens. By both of these methods several proteins have been shown to interact with integrin cytoplasmic domains including talin (17), α -actinin(32), filamin(34), β 3-endonexin(43) and Rack1(24). However, the functional relevance of these interactions in regulating inside-out integrin signaling remains unclear.

Genetic analysis has been a successful method for mapping intracellular signal transduction pathways *in vivo*. In general, these approaches have involved the isolation and characterization of mutations in signaling cascades that perturb defined cellular functions. Recently, methods have been developed to identify proteins that complement overexpressed signaling mutants in cultured cells. For example, mammalian proteins that modulate Ras activity have been identified by using expression cloning in yeast that express an activated Ras variant (13). We have used concepts from these approaches to develop an expression cloning strategy designed to identify potential integrin regulatory proteins in a cell culture system. This screen relies on suppression of integrin activation by overexpression of isolated integrin β 1 cytoplasmic domains in the form of chimeras with the transmembrane and extracellular domains of the IL-2 receptor α subunit (Tac- β 1). This inhibition of integrin activation is structurally specific as chimeric molecules with either integrin α cytoplasmic domains or certain β cytoplasmic domain point mutants lack inhibitory activity. Suppression of integrin activation by Tac- β 1 is also cell autonomous and concentration dependent. Hence, free β 1 tails act as competitive inhibitors of integrin activation by potentially titrating out proteins necessary for integrin signaling (see Figure 1 in attached paper). We reasoned, therefore, that if we overexpressed these titrated proteins we would overcome Tac- β 1 suppression.

This strategy utilized a chinese hamster ovary (CHO) cell line ($\alpha\beta\text{py}$) stably expressing a chimeric integrin ($\alpha_{\text{IIb}}\alpha_6\beta_3\beta_1$), that contains the extracellular and transmembrane domains of $\alpha_{\text{IIb}}\beta_3$ fused to cytoplasmic domains of $\alpha_6\beta_1$ (3). This chimeric integrin has the ligand binding properties of $\alpha_{\text{IIb}}\beta_3$ and is regulated via the $\alpha_6\beta_1$ cytoplasmic domains. In $\alpha\beta\text{py}$ cells this chimeric integrin is constitutively active, as measured by the binding of a ligand mimetic monoclonal antibody, PAC1, that only recognizes the activated form of the chimeric integrin(28). Our cloning strategy was to co-express Tac- β_1 and a cDNA library in $\alpha\beta\text{Py}$ cells and isolate cDNAs that overcome Tac- β_1 suppression.

We have isolated a cDNA from this screen, 5F8, that is able to rescue Tac- β_1 suppression. We previously showed that the rescue of 5F8 was dependent upon the integrin cytoplasmic domain rather than the II-2 receptor portion of the chimeric molecule. To test the specificity of the rescue we also transfected cDNAs encoding proteins reported to interact with integrin cytoplasmic domains and assayed their ability to rescue Tac- β_1 suppression. None of the proteins tested rescued the dominant negative effects of Tac- β_1 (α -actinin, vinculin, paxillin, integrin-linked kinase, and β_3 -endonexin).

5F8 may be increasing the activation of the chimeric integrin itself rather than directly interfering with Tac- β_1 . To test whether or not the 5F8 increase in integrin activation occurs only in cells that have been suppressed by Tac- β_1 , we co-transfected 5F8 and Tac- α_5 into the CHO cell line, that expresses the chimeric integrin. The level of integrin activation in cells expressing 5F8 and Tac- α_5 was identical to the activation level in control cells showing that the increase of integrin activation that is seen when clone 5F8 is overexpressed is a recovery from Tac- β_1 suppression. In this report, we describe the identification of clone 5F8 as the hamster homologue of CD98 and discuss the possible mechanisms by which it affects integrin function.

Body

Materials and Methods

Antibodies and Reagents

The isolation and characterization of the anti- β_3 monoclonal antibody, anti-LIBS6, has been previously described (11). The activation specific anti- $\alpha_{\text{IIb}}\beta_3$ monoclonal antibody PAC1 (42) 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 $\alpha_{\text{IIb}}\beta_3$ -specific peptidomimetic inhibitor Ro43-5054 (1) 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 characterised by SDS-PAGE and exhibited characteristic mobilities.

cDNA constructs, Cell Lines and Transfection.

The Tac- β_1 and Tac- α_5 chimeras in the CMV-IL2 vector were the gift of Drs. S. LaFlamme and K. Yamada (23). pSG5 MKP-1, was a gift from Dr. N. Tonks (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) (44). pDCR-H-Ras (G12V), was gift from Dr. M. H. Wigler (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) (46). 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. The pcDNA3 construct encoding CD9 has been described (Indig et al., submitted). 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).

Chinese Hamster Ovary (CHO-K1) cells were obtained from American Type Culture Collection, (Rockville, Md.). The generation of the $\alpha\beta\gamma$ cell line has been described (3). 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 $\alpha_{IIb}\alpha_{6a}$ and $\beta_3\beta_1$. 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 μ g/ml streptomycin.

Cells were transfected using a lipofectamine procedure. For each 10 cm tissue culture dish with 40-60% confluent cells, 20 μ l of lipofectamine reagent (GibCo BRL) and 10 μ g of plasmid DNA were mixed in 200 μ l 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(9). 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 μ M 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.

Expression cloning.

A cDNA library, made from polyA(+) mRNA from CHO-K1 cells, directionally cloned into the Not I site of the mammalian expression vector, pcDNA1 was purchased from Invitrogen (San Diego, CA). The library is

reported to contain 1.8×10^7 primary recombinants and has been amplified once. Plasmid DNA (4 $\mu\text{g}/\text{plate}$) was transfected into the $\alpha\beta\text{py}$ cell line, along with the Tac- β_1 chimera (4 $\mu\text{g}/\text{plate}$) using lipofection as described above. The transfection efficiency ranged from of 30-50%, as judged by 7G7B6 binding.

After a 48 hr incubation, cells were stained for PAC1 and 7G7B6 binding. Cells positive for both PAC1 and 7G7B6 were isolated by fluorescence activated cell sorting (FACSTAR, Becton Dickinson). Gates were set by comparing cells transfected with Tac- α_5 with those transfected with Tac- β_1 only. Plasmid DNA was extracted (16) and digested with Dpn I to remove plasmids that were not replicated in transfected cells and then transformed into the host E. Coli MC1061/P3. Individual colonies were grown, and the bacteria were then pooled into groups of 16 for plasmid purification (Quiagen, Chatsworth, CA). Groups of cDNAs were transfected into the $\alpha\beta\text{py}$ cell line along with Tac- β_1 and the transfectants were screened by flow cytometry as described above. Pools containing cDNAs that altered PAC1 binding were further screened in smaller pools of four, and then the positive pools were screened as individual colonies.

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, 2×10^5 cells were transfected using as described above with 2 μg of pCMV5 HA-ERK2 (HA; hemagglutinin tagged). The cells were also transfected with 2 μg of the test plasmid e.g. pDCR H-Ras(G12V). In some experiments 4-6 μg of a second plasmid e.g. MKP-1 were co-transfected and the total amount of DNA was adjusted to a total of 10 μg , 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 (22, 36) using the anti-HA antibody 12CA5 (Boehringer Mannheim). ERK2 expression was monitored by running 25 μg 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

passed into serum free medium RPMI 1640 containing SITA (sigma). 3-5 days post passage $1-2 \times 10^6$ 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°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 (15).

Recombinant Cytoplasmic Domain Synthesis

The cytoplasmic domains of various integrin tails were synthesized using previously described methods (34). 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 $\alpha\beta$ py cells were washed twice in phosphate-buffered saline (PBS) and biotinylated 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 (1mM Na_3VO_4 , 50mM NaF, 40 mM sodium pyrophosphate, 10 mM Pipes, 50 mM NA Cl, 150mM sucrose, pH 6.8) containing 1% Triton X-100, 0.5% sodium deoxycholate, 1mM EDTA, 20 ug/ml aprotinin, 5 ug/ml leupeptin and 1mM PMSF. Lyates were sonicated and spun at 14 000 RPM for 30 minutes in a microcentrifuge.

Affinity Chromatography

500 ug of purified recombinant integrin tail protein was dissolved in a mixture of 5 ml of 20mM Pipes, 50mM NaCl, pH 6.8 (PN) and 1ml of 0.1 M sodium acetate, pH 3.5 and bound overnight to 80 ul of nickel saturated His-bind resin. The resin was washed twice with PN. Cell lysates were diluted 10 X with buffer X, 0.05% Triton-X and 3mM MgCl_2 and incubated overnight at 4°C with 50 ul of the resin for affinity chromatography experiments and 100 ul for immunoprecipitation experiments. The following day the resins were washed with this buffer and heated in reducing sample buffer for the affinity chromatography experiments. Samples were separated on 4-20% SDS polyacrylamide gels and transferred to nitrocellulose. The nitrocellulose was blocked with 5% nonfat milk in TBS and stained with streptavidin peroxidase. Bound peroxidase was detected with an enhanced chemiluminescence kit.

For the immunoprecipitation experiments the protein bound to the resin was eluted in an elution buffer (800mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9). This eluent was dissolved in 1ml of lysis buffer (20 mM Tris HCl, 150 mM Na Cl, 10 mM Benzimidine HCl, 1% Triton-X100). The lysis buffer and eluent was precleared with protein A sepharose and then immunoprecipitated with an anti-CD98 antibody bound to protein A sepharose. The protein A sepharose was washed with lysis buffer after 4 hours and then heated in reducing sample buffer. The samples were run on SDS gels and developed in a similar way to the affinity chromatography experiments.

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 its 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 (33) and human (35). 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- β_1 into $\alpha\beta$ py cells. Overexpression of human CD98 also reversed dominant suppression and we therefore conclude that we have isolated its hamster homologue (Figure 1).

To confirm the orientation of CD98 in the membrane, we expressed either amino-or carboxy-terminally HA-epitope tagged CD98 in $\alpha\beta$ py cells and then followed the expression using both flow cytometry and Western blot analysis. The predicted orientation by Tmpred analysis of CD98 being a Type II transmembrane protein is confirmed because the C-terminal HA tag is the only one recognized by flow cytometry, whereas both N-and C-terminus HA-tags are detected in Western blot analysis (Figure 2).

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 (5), CD47(IAP,(8) , and uPAR(45). Expression of these proteins in $\alpha\beta$ py cells failed to reverse suppression of integrin activation by Tac- β_1 (Figure 1). Protein expression was confirmed by flow cytometry in each case (data not shown). Thus, the rescue of Tac- β_1 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 (18). 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 (Figure 3A). In contrast, expression of MAP kinase phosphatase (MKP-1) blocked Ras suppression. CD98 was functional, since in simultaneous assays, it complemented Tac- β_1 suppression (Fig 3B). Thus, CD98 does not complement all inhibitors of integrin activation.

We also tested whether the Tac- β_1 suppressive effect was dependent on the Ras-initiated MAP kinase pathway. Overexpression of Tac- β_1 did not activate ERK-2, while ERK-2 was activated by the expression of an activated H-Ras (Figure 3C). Furthermore, co-transfection of MKP-1 failed to reverse the suppressive effect of Tac- β_1 , whereas co-expression of CD98 completely overcame this effect. Thus, the mechanisms of suppression of integrin activation by Ras or by overexpression of isolated β_1 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(10). 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 (Figure 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 (8) 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 (Figure 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 β_1A cytoplasmic domains.

In our original model we hypothesized that Tac- β_1 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 β_1A ? 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 (34) showed the utility of this system by demonstrating the direct binding of filamin/ABP-280 and talin to integrin cytoplasmic domains. Endogenously expressed CD98 from

Jurkat cells binds to the β 1A tail but not to the α IIb tail or to beads alone (Figure 5). Similar results were obtained when using cell lysates of CHO cells transiently transfected with human CD98.

Since it has been determined that CD98 does bind to the β 1A tail, we are in the process of mapping the binding site of the β 1A tail. Recombinant tail proteins were constructed which expressed several short carboxy-terminal deletions of the β 1A tail (Figure 6A). None of the mutant β 1A recombinant proteins was able to bind to CD98 (Figure 6B). It appears that all or part of the terminal NPXYEGK sequence is required for CD98 binding. Further mutations are now being constructed to test exactly which of the last seven amino acids of the β 1A tail are required for CD98 binding.

A point mutation of the β 1A tail was also tested for CD98 binding, β 1A,Y788A. Tyrosine 788 has been shown to be important in inside-out signaling (29) and affects cytoskeletal associations (37). This mutation in the recombinant β 1A cytoplasmic tail protein disrupts binding of both filamin and talin (34). As seen in figure 7B, CD98 was still capable of binding to β 1AY788A. To test whether the mechanism of Tac- β 1 suppression is titration of CD98, we expressed Tac- β 1Y788A in $\alpha\beta$ py cells. Since we know that this mutation is still capable of binding to CD98, overexpression of free β 1Y788A tails in the form of a Tac chimera should result in suppression of integrin activation if this simple model is true. The expression of Tac- β 1Y788A had no effect on the activation state of the chimeric integrin (Figure 7B) ruling out our initial hypothesis.

Binding of CD98 in integrin b tails correlates with the ability to rescue suppression.

CD98 is not specific for the β 1A tail, as CD98 expression also rescues suppression by Tac- β 3. To further examine the specificity of the interaction between CD98 and the β cytoplasmic tail, we looked at both the ability of CD98 to bind to other β integrin tails and the ability of CD98 to rescue suppression by other free β tails. CD98 does not bind to either β 1D or to β 7, the only two tails tested thus far (Figure 7C). Interestingly, both β 1D and β 7 lack the three terminal amino acids of β 1A, EGK,(Figure 7A) which we have shown may be important in binding of CD98 to β 1A tails. Chimeras between β 7 or β 1D and β 1A are also being constructed to further elucidate the CD98 binding site.

The expression of either Tac- β 7 or Tac- β 1D caused suppression of integrin activation in $\alpha\beta$ py cells (Figure 7B). CD98 was unable to rescue Tac- β 7 suppression which correlates with the inability of CD98 to bind to β 7 cytoplasmic domains. However, there is a slight, but consistent recovery of Tac- β 1D suppression by expression of CD98 even though we see no binding of CD98 to β 1D cytoplasmic domains. It could be that CD98 is able to weakly bind to the β 1D cytoplasmic domains, such that there is a weak rescue, but this binding is below the detection levels of affinity chromatography.

CD98 cytoplasmic tail is required but apparently not sufficient for rescue of suppression.

We next wanted to determine which regions of CD98 were required for its affect on integrin function. A mutant cDNA (d4F2) was constructed which caused the deletion of almost the entire cytoplasmic domain of CD98. The initiator codon was left intact as well the stop transfer sequence (VRTR), so that the mutant would be

correctly oriented in the membrane. Expression of $\Delta 4F2$ in $\alpha\beta$ py cells failed to rescue Tac- $\beta 1$ suppression (Figure 8A). Expression levels of $\Delta 4F2$ were confirmed by flow cytometry. $\Delta 4F2$ was unable to bind to $\beta 1A$ cytoplasmic tails (Figure 8B) excluding the possibility that the extracellular domain of CD98 may be interacting in a non-specific manner with the $\beta 1A$ cytoplasmic tail. The inability of $\Delta 4F2$ to bind to $\beta 1A$ cytoplasmic domains correlates with the inability to rescue integrin suppression.

To test whether the cytoplasmic domain alone is sufficient to affect integrin function, we expressed a chimera containing the N-terminal cytoplasmic domain of CD98 and the extracellular and transmembrane domain of CD69 (4F2/69) with Tac- $\beta 1$. CD69 was chosen as it has the same membrane topology as CD98 and because expression of CD69 does not alter integrin affinity in our system. Another reason for selecting CD69 is because it is known to form homodimers *in vivo* (39). Because in the SCLC system crosslinking of CD98 had an effect on integrin function, we reasoned that homodimer formation of the CD98/CD69 chimera may increase its ability to rescue integrin suppression. However, expression of 4F2/69 failed to rescue Tac- $\beta 1$ suppression and did not bind to the $\beta 1A$ cytoplasmic tail (Figure 9). The failure of the 4F2/69 construct to function in either of our assays may be because the cytoplasmic domain of CD98 is not held in the proper orientation. It could be that the transmembrane or the extracellular domains of CD98 are responsible for maintaining a certain orientation of the cytoplasmic domain. Another possibility is that when the CD69 extracellular domains forms homodimers the cytoplasmic domain of CD98 is no longer held in the correct position to bind to the $\beta 1$ tail. In order to address these concerns, we are in the process of testing additional CD98/CD69 chimeras (Figure 11). An additional chimera will be constructed which contains the CD98 cytoplasmic domain fused to the transferrin receptor (also a Type II transmembrane protein) transmembrane and extracellular domains to rule out any interference that the dimerization of the CD69 construct may have had.

The formation of the CD98 heterodimer is not required for the rescue of integrin suppression or binding to the $\beta 1$ integrin tail.

CD98 is present in cells as a 120 kD heterodimer, the heavy chain is approximately 90 kD and the light chain is 40 kD (14). The heavy chain contains two cysteines that are required for heterodimer formation. To assess the role of the heterodimer in the regulation of integrin function, we tested the ability of a mutant CD98 cDNA (Cys-less) which has both the cysteines mutated to serines to function in our assays. The Cys-less mutant was still able to rescue integrin suppression as well as bind to the $\beta 1A$ cytoplasmic domain (Figure 10) demonstrating that heterodimer formation is not required for CD98 to function as an integrin regulator. Non-reduced and reduced gels of immunoprecipitates of lysates containing CD98 and the Cys-less mutant were run to verify the formation of the heterodimer (figure 10C).

Discussion

Expression of the Tac- β_1 chimera disrupts inside-out integrin signalling, suppressing integrin activation (9). Expression of 5F8 cDNA restored integrin activation without reducing Tac- β_1 expression. The sequence of the protein encoded by 5F8 is 72% identical to human CD98. Further, cDNA for human CD98 also complements dominant negative suppression. Clone 5F8 is likely to encode hamster CD98, thus, expression of CD98 rescues the suppressive effect of overexpression of isolated integrin β cytoplasmic domains on integrin activation.

CD98 was originally identified as an early T-cell activation antigen. The heavy chain cDNA has been isolated from human (35), mouse (33) and now hamster. Antibodies against FRP-1 (fusion regulatory protein), which has been identified as CD98, enhance virus-induced cell fusion (30). Anti- β_1 integrin antibodies inhibit the anti-FRP-1 induced cell fusion (31), 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 cellular proliferation. Antibodies to this protein inhibit sodium/calcium exchange in membrane vesicles of heart and skeletal muscle (27). Microinjection of CD98 cRNA into *Xenopus* oocytes stimulates the uptake of dibasic and neutral amino acids (6). 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. Isolated β tails could titrate intracellular factors needed for integrin activation as suggested by the cell autonomous and concentration-dependent manner in which Tac- β_1 suppresses. The β_1 Y788A integrin tail mutant is able to bind to CD98. However, expression of this mutant in the form of a Tac chimera does not suppress integrin activation. This indicates that CD98 is not likely the factor that is being directly titrated out by the free β tails. A more likely explanation is that CD98 may prevent the interaction of factors with free integrin β tails. We have shown that in vitro, CD98 is able to bind to the cytoplasmic tail of β_1 integrins. This binding also correlates with the ability of CD98 to rescue Tac- β_1 suppression. Crosslinking CD98 with 4F2 antibody stimulated β_1 dependent adhesion. The rescue of dominant suppression by overexpression of CD98 in $\alpha\beta$ py cells may work by favoring clustering of CD98, which in turn may lead to the clustering of integrins through interactions of the cytoplasmic tails of each protein.

Alternatively, Blystone et al (7) have found that Tac- β_3 suppression of integrin-dependent phagocytosis may be mediated by kinases in K562 cells. However, in the CHO cells we employed, the same inhibitors utilized by Blystone *et al.*, were without effect on dominant negative suppression (Fenczik, Ramos and Ginsberg, unpublished observations). Thus, integrin suppression may differ in mechanism between cell types. Hughes *et al.*, found that activated H-Ras suppressed integrin activation. Free integrin β cytoplasmic tails can result in phosphorylation of pp125FAK (25), implying the potential to activate Ras (40). Nevertheless, CD98 did not influence Ras suppression, nor did MKP-1, reverse Tac- β_1 suppression. Thus, CD98 is unlikely to work through the Ras initiated suppression pathway. The capacity of CD98 to reverse integrin suppression seems relatively

unique. Furthermore, studies of virus induced cell fusion provide independent confirmation that this protein regulates integrin function. Thus, CD98 appears to be a novel element in integrin signaling pathways.

With respect to statement of work.

We have completed the sequence of clone 5F8, which has led to the identification of CD98 as a regulator of integrin function. Antibodies to this protein were commercially available obviating any need to complete Task 1b (production of antibodies). Because a large body of literature exists that characterizes CD98, we chose to spend the first year of this grant characterizing the specificity of 5F8 rescue (1d) as well as the interaction between 5F8 and integrins. This work will be continued as we will further narrow down the binding site of CD98 on β 1-integrin tails. The biological relevance of this binding will also be assessed by creating Tac chimeras with β 1A-tail mutants that are no longer able to bind to CD98. These chimeras will be tested for their ability to suppress integrin activation. If they suppress activation, expression of CD98 should no longer be able to rescue. These experiments will also address our hypothesis that CD98 is rescuing integrin suppression by preventing the interaction of required cytoplasmic factors with free integrin β tails by binding to either the β tails or to the necessary factors themselves. If we find a mutant β 1A tail that fails to bind CD98 and is still capable of causing suppression in the form of a Tac chimera, this could lead to the expression cloning of the actual factor that is being titrated out.

The cytoplasmic domain of CD98 is required for its ability to rescue integrin suppression as well as to bind to the β 1A cytoplasmic domain. Deletion mutagenesis of the cytoplasmic domain has just been completed (Figure 11B). These constructs are now being tested in our assays. Additional chimeras between CD98 and CD69 have also been generated to test which regions of CD98 are required for its functions as an integrin regulator (Figure 11A). Chimeras with the cytoplasmic tail of CD98 and the transmembrane and extracellular domain of the transferrin receptor, another type II transmembrane protein is currently being constructed to test whether the cytoplasmic domain is sufficient in affecting integrin function.

As well as testing which domains of CD98 are required for its function as an integrin regulator, the relationship of the light chain to integrin function will be tested in the second year of the grant. We have shown that the covalent association between the light and heavy chain is not required for integrin function. The light chain has been recently identified (26). We have received cDNA clones of this protein and are currently assessing its role in integrin activation.

Our attempts to show that CD98 affects integrin function in another system by disrupting gene expression using antisense constructs was unsuccessful. Expression of antisense constructs did not reduce the levels of CD98 on the surface of cells. We have decided to take a more direct approach and create a knock-out mouse. A P1 clone containing the full length mouse gene has been obtained. The knock-out construct is currently under construction. The production of this mouse should answer whether CD98 has an effect on integrin function in a whole animal system.

Conclusions

We describe the involvement of CD98 in integrin function by use of a novel genetic strategy. The most important findings of this paper are: 1) CD98 complements dominant negative suppression of integrin activation by isolated integrin β cytoplasmic domains. 2) CD98 does not complement the integrin suppressive pathways initiated by the small GTP-binding protein H-Ras. Activated H-Ras and isolated β_1 tails thus appear to suppress integrin activation by independent mechanisms. 3) Cross-linking of CD98 stimulated β_1 -integrin-dependent cell adhesion of SCLC cells. 4) CD98 binds β_1 -integrin cytoplasmic domain, which correlates with the ability to rescue integrin suppression. 5) The cytoplasmic domain of CD98 is required for both rescue of integrin suppression and binding to β_1 -integrins. 6) The covalent formation of the CD98 heterodimer is not required for its affect on integrin function.

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Figure legends

Figure 1 Human CD98 reverses trans-dominant suppression whereas several other membrane proteins do not. $\alpha\beta$ py cells were transfected with 4 μ g of Tac- β_1 . They were simultaneously transfected with cDNAs encoding either human CD98, CD9, CD47, or uPAR. After 48 hrs, cells were harvested and analyzed for PAC1 binding as described in Materials and Methods. To obtain quantitative estimates of integrin activation we calculated a numerical activation index defined as $100 (F_o - F_R)/(F_o\text{LIBS6} - F_R)$, where F_o is median fluorescence intensity of PAC1 binding; F_R , the median fluorescence intensity of PAC1 binding in the presence of the competitive inhibitor (1 μ M Ro43-5054) and $F_o\text{LIBS6}$, the median fluorescence intensity of PAC1 binding in the presence of 2 μ M anti-LIBS6. Depicted are the means \pm S.E. of three independent experiments for each membrane protein. CD98 rescued cells from trans-dominant suppression, whereas none of the other membrane proteins had an effect. Specificity of CD98 complementation. Percent inhibition was defined as $100(AI_o - AI)/AI_o$, where AI_o is the activation index in Tac- α_5 transfected cells. AI is the activation index in Tac- β_1 transfected cells. The co-transfected membrane protein is indicated below each column.

Figure 2. The COOH-terminus of CD98 is expressed on the surface of the cell, whereas, the NH2-terminus is intracellular. $\alpha\beta$ py cells were transfected with either 4 μ g HA-NH2 (left side) or HA-COOH (right side). *A*, Surface expression of the HA-epitope was examined by flow cytometry with the anti-HA antibody 12CA5 (top panels). Flow cytometry was also performed with the anti-CD98 antibody 4F2 to confirm the presence of CD98 at the cell surface (bottom panels). *B*, CD98 was immunoprecipitated from cell lysates of transfected cells with 4F2 antibodies. The presence of the HA-tag was confirmed by Western blot analysis of the immunoprecipitates. Lysates were also immunoprecipitated with IgG isotype control antibodies as a negative control. In addition to the CD98 band, the 60 kD band seen is the antibody heavy chain.

Figure 3. Suppression of integrin activation either H-Ras or Tac- β_1 occur through different mechanisms. *A*, H-Ras (G12V) suppression is rescued by expression of MKP-1, but not by 5F8. $\alpha\beta$ py cells were transfected with 4 μ g of H-Ras (G12V) and 4 μ g of either MKP-1 or 5F8. Bars in which no error bars are present the S.E. was less than .01. *B*, 5F8 rescues trans-dominant suppression caused by Tac- β_1 , whereas MKP-1 expression has no effect on integrin activation in these cells. *C*, MAP kinase activity. $\alpha\beta$ py cells were transfected as above, except 2 μ g 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 immuno-blots with the anti-HA ERK2 in all transfections. There was a comparable expression of HA-tagged ERK-2 in all transfection. MKP-1 was able to rescue H-Ras (G12V) suppression only, whereas 5F8 expression could only rescue Tac- β_1 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 $\mu\text{g/ml}$ 4F2 monoclonal antibody. **Right:** Addition of 4F2-Fab (100 $\mu\text{g/ml}$) to SCLC cells does not increase adhesion to plates coated with 3 $\mu\text{g/ml}$ laminin (A) or 10 $\mu\text{g/ml}$ fibronectin (B), while addition of 4F2-Fab blocks the increase of adhesion in the presence of the intact antibody. Addition of P5D2 (a β 1-integrin function blocking antibody) or 2mM EDTA also block the increase of adhesion of SCLC cells to plates coated with either 3 $\mu\text{g/ml}$ laminin (A) or 10 $\mu\text{g/ml}$ 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 \pm SEM.

Figure 5. CD98 binds to the β 1A integrin cytoplasmic domain. Ni^{2+} resin was loaded with homodimers of either G4- β 1A, G4- α IIb or left unloaded (beads). **A,** Bound proteins from biotinylated Jurkat cell lysates were separated on 4-20% polyacrylamide gels under reducing conditions, transferred to nitrocellulose membranes and biotinylated proteins detected by streptavidin peroxidase and chemilumescence. Total proteins from the lysate are also represented in the final lane. **B,** Immunoprecipitation of CD98 from column eluates reveals that CD98 was bound only to beads loaded with β 1A cytoplasmic domains. Eluates from each column were immunoprecipitated with either 4F2 antibodies (IP) or as a negative control with IgG isotype control antibodies (IgG).

Figure 6 CD98 does not bind to deletion mutants of the β 1A tail. **A,** Panel A depicts the amino acid sequence of the complete cytoplasmic domain of the human β 1A integrin, as well as the sequences of the deletion mutants of the tail. **B,** Ni^{2+} resin was loaded with homodimers of either G4- β 1A, G4- α IIb, G4- Δ 27, G4- Δ 35, or G4- Δ 41. G4- Δ 45 is work in progress. Bound proteins from biotinylated Jurkat cell lysates were eluted and subjected to immunoprecipitation with 4F2 antibodies. Immunoprecipitates were separated on 4-20% polyacrylamide gels under reducing conditions, transferred to nitrocellulose membranes and biotinylated CD98 was detected by streptavidin peroxidase and chemilumescence.

Figure 7. Binding of CD98 to β 1A cytoplasmic domains is not the only factor involved in Tac- β 1 suppression. **A,** The amino acid sequence of the cytoplasmic domains of β 1A, β 1D and β 7 are depicted. Note there is not sequence similarity after the terminal NPXY. **B,** $\alpha\beta$ py cells were transfected with either 4 μg of Tac- β 1., Tac- β 1Y788A, Tac- β 1D, or Tac- β 7. One set of plates transfected with each Tac chimera was also co-transfected with 5F8 (the hamster homologue of CD98). After 48 hrs, cells were harvested and analyzed for PAC1 binding as described in Materials and Methods. Transfected of $\alpha\beta$ py cells with Tac- β 1aY788A did not result in a decrease in PAC1 binding, whereas expression of both Tac- β 1D and Tac- β 7 resulted in integrin suppression. While CD98 is able to rescue to suppression caused by Tac- β 1A, it's expression had no effect on

Tac- β 7 suppression and only a slight effect on Tac- β 1D suppression. *C*, Ni²⁺ resin was loaded with homodimers of either G4- β 1A, G4- α IIb, G- β 1AY788A, G4- β 1D, or G4- β 7. Bound proteins from biotinylated Jurkat cell lysates were eluted and subjected to immunoprecipitation with 4F2 antibodies. Immunoprecipitates were separated on 4-20% polyacrylamide gels under reducing conditions, transferred to nitrocellulose membranes and biotinylated CD98 was detected by streptavidin peroxidase and chemiluminescence. Immunoprecipitation of the total lysate was included as a positive control (lysate). CD98 is capable of binding to both the β 1A cytoplasmic domain and to the β 1AY788A mutant but not to the β 1D or β 7 cytoplasmic domains.

Figure 8. The cytoplasmic domain of CD98 is required for both rescue of integrin suppression and for binding to β 1A cytoplasmic domains. *A*, $\alpha\beta$ py cells were transfected with Tac- α 5 or Tac- β 1A. Cells transfected with Tac- β 1A were co-transfected with either wild type CD98 (4F2), a mutant of CD98 lacking most of the cytoplasmic domain (Δ 4F2) or pcDNA1 (vector DNA). 48 hours after transfection cells were harvested and analyzed for PAC1 binding. *B*, $\alpha\beta$ py were transfected with either wild type CD98 or with the mutant CD98 (Δ CD98). 48 hrs after transfection, cells were biotinylated and lysed. The cell lysates were bound to Ni²⁺ beads that had been loaded with either G4- β 1A or G4- α IIb. Eluates were immunoprecipitated with 4F2 antibodies. To confirm expression of the transfected cDNA total protein lysates were also immunoprecipitated with 4F2 antibodies.

Figure 9. It appears that the cytoplasmic domain of CD98 is not sufficient for rescue of integrin suppression or binding to the β 1A cytoplasmic domain. *A*, $\alpha\beta$ py cells were transfected with Tac- α 5 or Tac- β 1A. Cells transfected with Tac- β 1A were co-transfected with either wild type CD98 (4F2), a chimera containing the cytoplasmic domain of CD98 and the extracellular and transmembrane domains of CD69 (4F2/69), CD69, or pcDNA1 (vector DNA). PAC1 binding was assayed and the activation index was determined as described in figure 1. *B*, Cell lysates from cells transfected with the 4F2/69 chimera were incubated with Ni²⁺ beads that had been loaded with either G4- β 1A or G4- α IIb. Bound proteins were separated on a 4-20% SDS-polyacrylamide gels under non-reducing conditions, transferred to nitrocellulose, and stained with antibodies specific for CD69 (our antibodies were only capable of recognizing the non-reduced protein). Total proteins (lysate) were also included on the gel to confirm expression of the chimera. The size of specific band seen upon immunoblotting corresponds to the size of a homodimer or the chimera.

Figure 10. Heterodimer formation of CD98 is not required for rescue of integrin suppression or binding to the β 1A cytoplasmic domain. *A*, $\alpha\beta$ py cells were transfected with Tac- α 5 or Tac- β 1A. Cells transfected with Tac- β 1A were co-transfected with either wild type CD98 (4F2), a CD98 mutant that has both cysteines changed to serines (Cys-less), or pcDNA1 (vector DNA). PAC1 binding was assayed and the activation index was determined as described in figure 1. *B*, Cell lysates from cells transfected with either wild type CD98 (CD98), the mutant CD98 with no cysteins (Cless CD98) or pcDNA1 (mock) were incubated with Ni²⁺ beads that

had been loaded with either G4- β 1A or G4- α IIb. Eluates from each set of beads were immunoprecipitated with 4F2 antibodies. Total proteins were also immunoprecipitated with either 4F2 antibodies as a positive control or IgG isotype control antibodies as a negative control. *C*, To assess the ability of both the wild type human CD98 and mutant CD98 to form heterodimers with the hamster light chain, immunoprecipitates of transfected cells were run in both reduced and non-reduced conditions. Cells transfected with either wild type human CD98 (CD98) or with the Cys-less (Cless) mutant were immunoprecipitated with 4F2 antibodies. As seen in *C*, in the non-reduced gel (left) while most of the expressed CD98 is in the form of free heavy chain, a hamster light chain and human heavy chain heterodimer is formed. As expected, only the free heavy chain is seen in the reduced gel (right). While in the case of the Cys-less mutant, there is no heterodimer formation.

Figure 11. CD98/CD69 chimeras and CD98 cytoplasmic domain mutants. *A*, On the top of panel A a model depicts the full length CD98 protein. The next two constructs, Δ 4F2 and 4F2/CD69 have been described in this report. 4F2TM which consists of the cytoplasmic and transmembrane domains of CD98 fused to the extracellular domain and CD69 TM which consists of the extracellular and cytoplasmic domains of CD98 and the transmembrane domain of CD69 have been completed and are currently being tested for their ability to both rescue integrin suppression and to bind to b1A cytoplasmic domains. *B*, The lower panel depicts a series of mutations in the cytoplasmic domain of CD98 which have been constructed and are currently being tested in our integrin function assays.

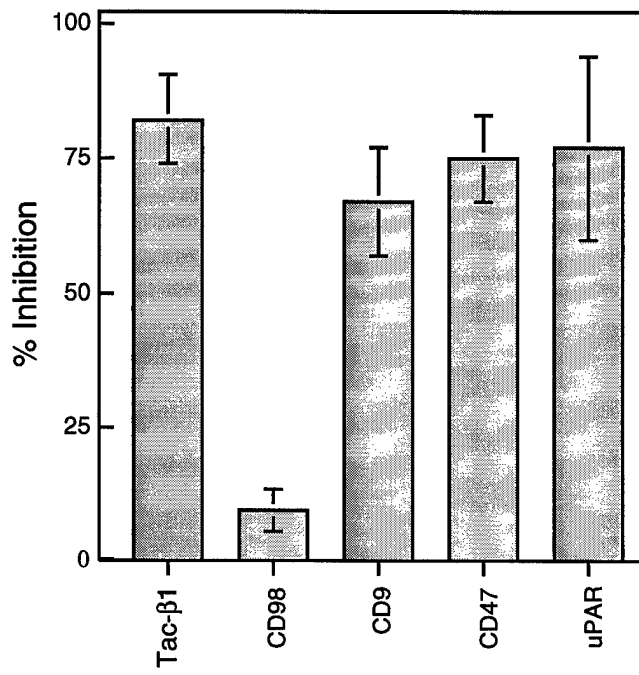


Figure 1

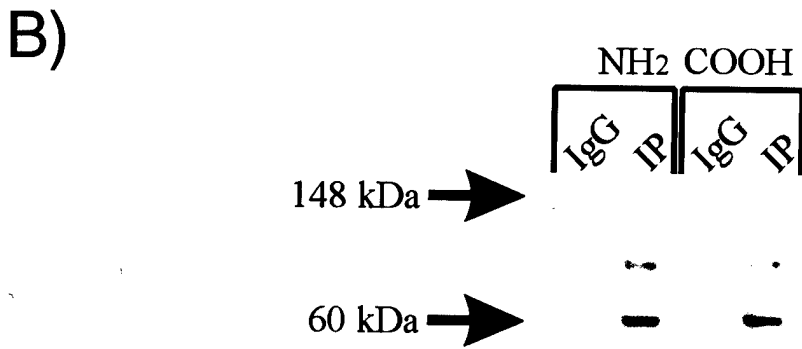
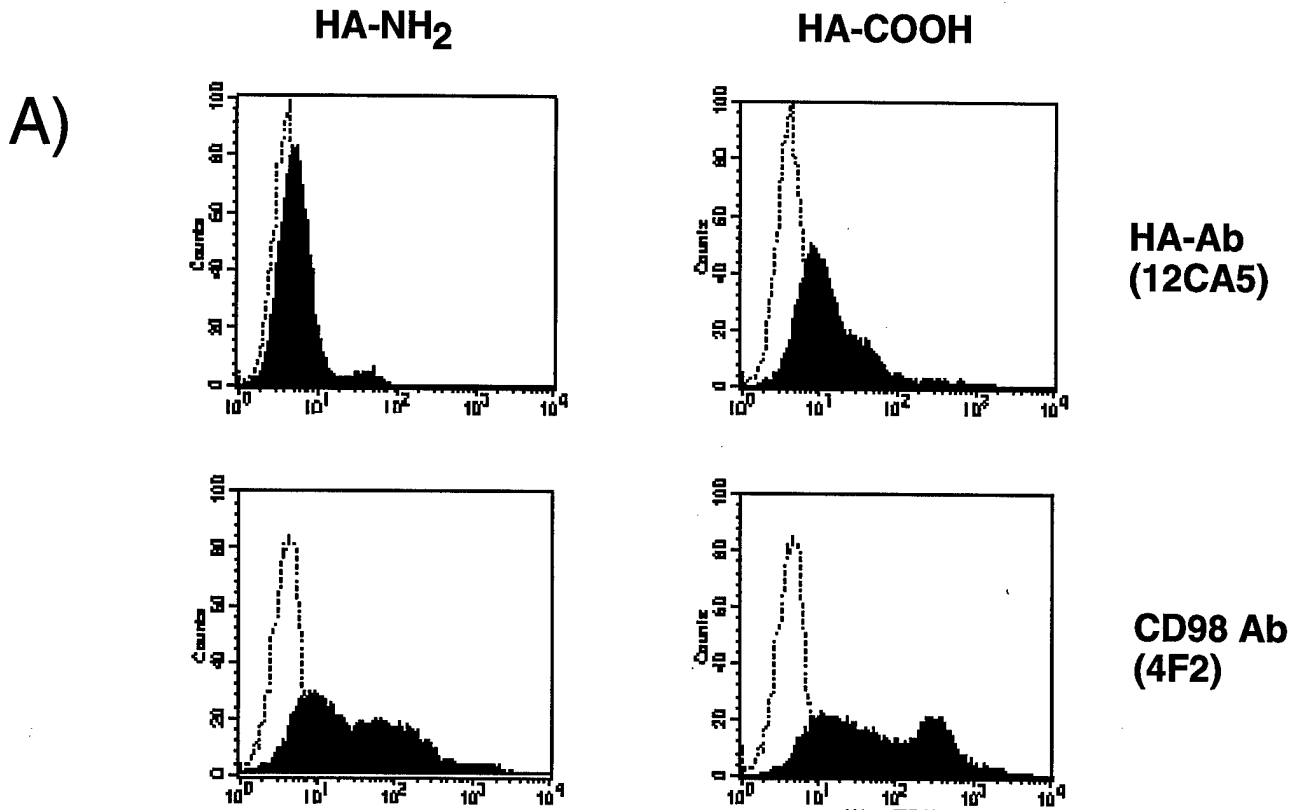


Figure 2

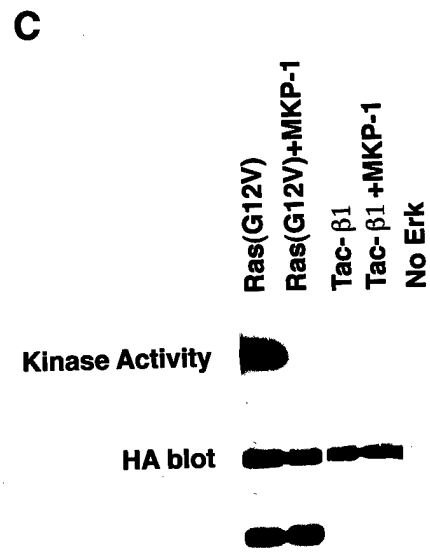
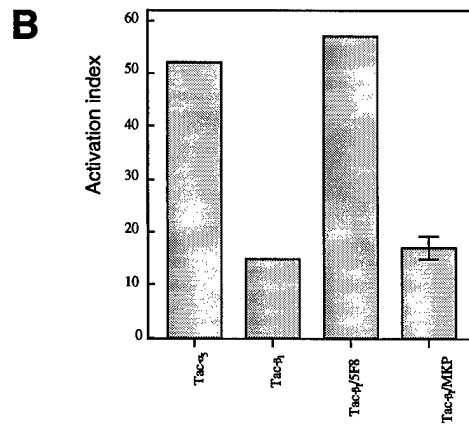
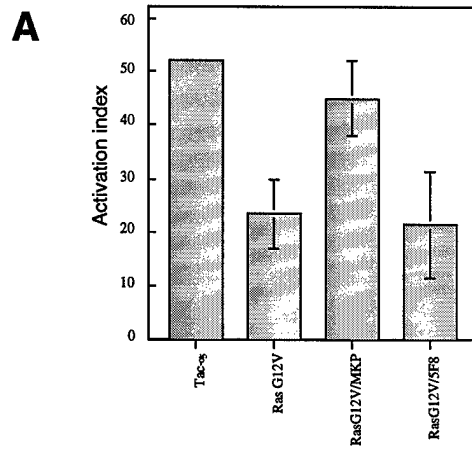


Figure 3

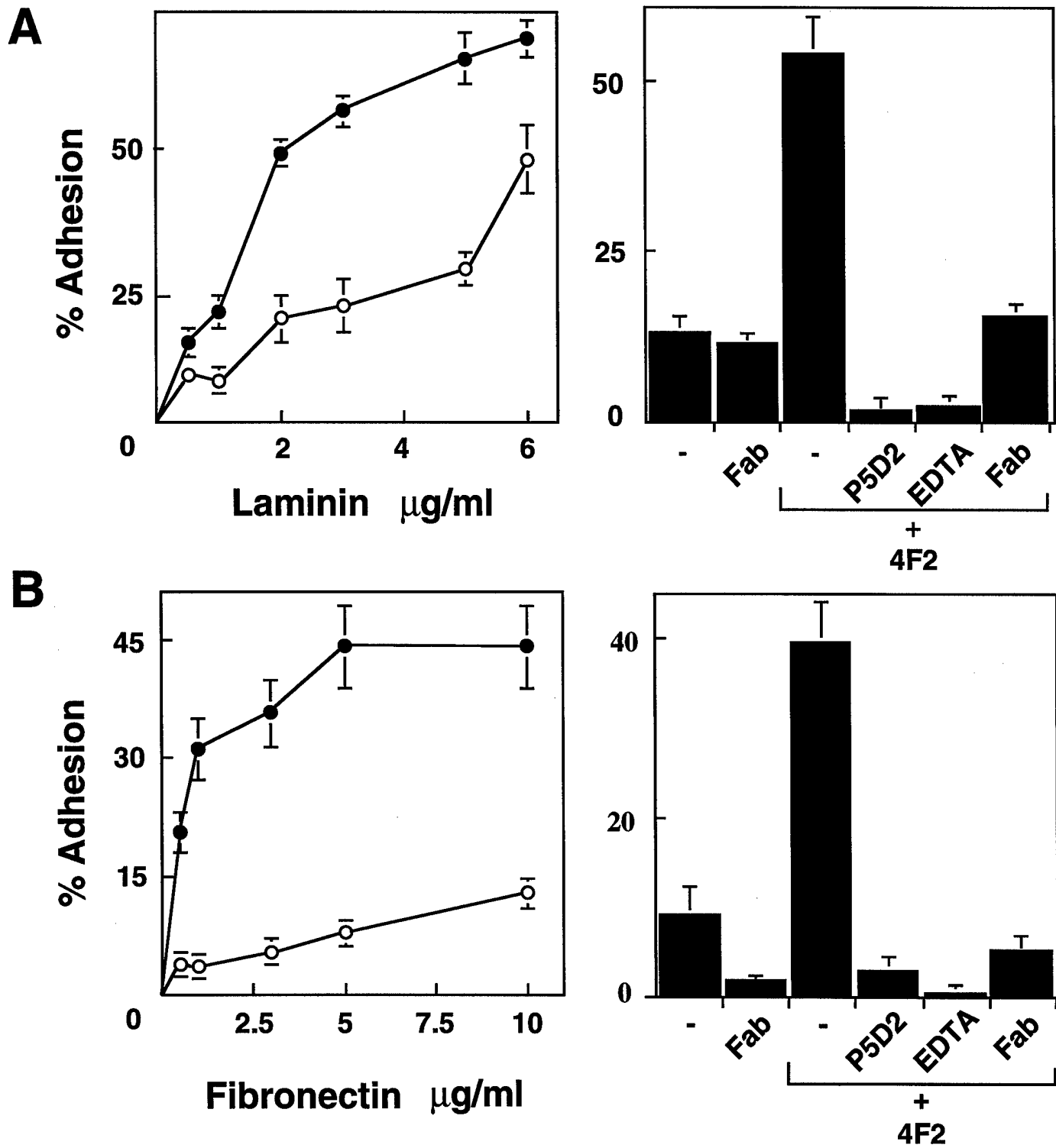


Figure 4

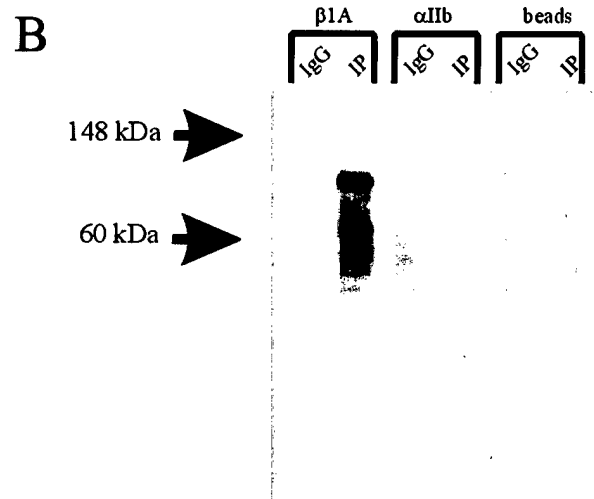
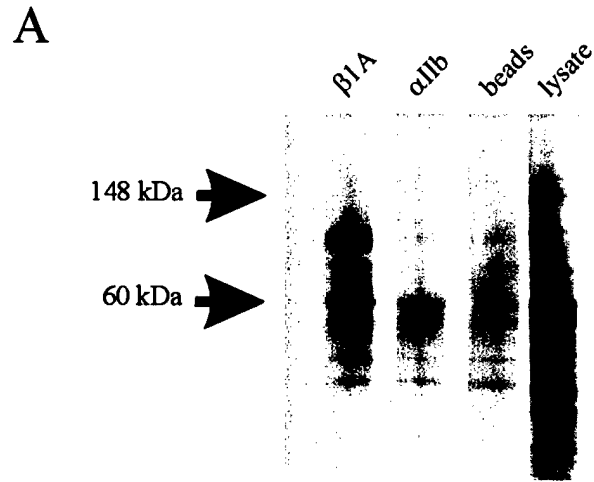


Figure 5

A) BETA 1A CYTOPLASMIC DOMAIN

KLLMIIHDRR	EFAKFEKEKM	NAKWDGTGENP	IYKSAVTTVV	NPKYEGK	Full length
KLLMIIHDRR	EFAKFEKEKM	NAKWDGTGENP	IYKSAVTTVV	NPKY	Δ45
KLLMIIHDRR	EFAKFEKEKM	NAKWDGTGENP	IYKSAVTTVV		Δ41
KLLMIIHDRR	EFAKFEKEKM	NAKWDGTGENP	IYKS		Δ35
KLLMIIHDRR	EFAKFEKEKM	NAKWDT			Δ27
KLLMIIHDRR	EFAKFEKEKM	NA			Δ23

B)

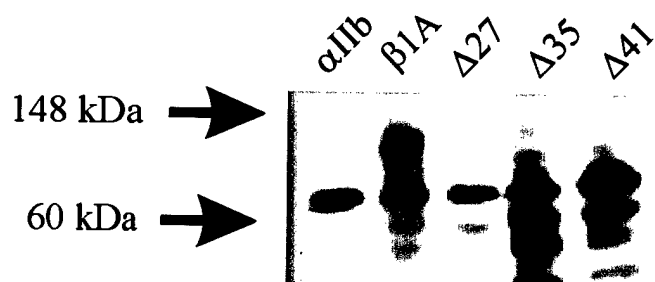
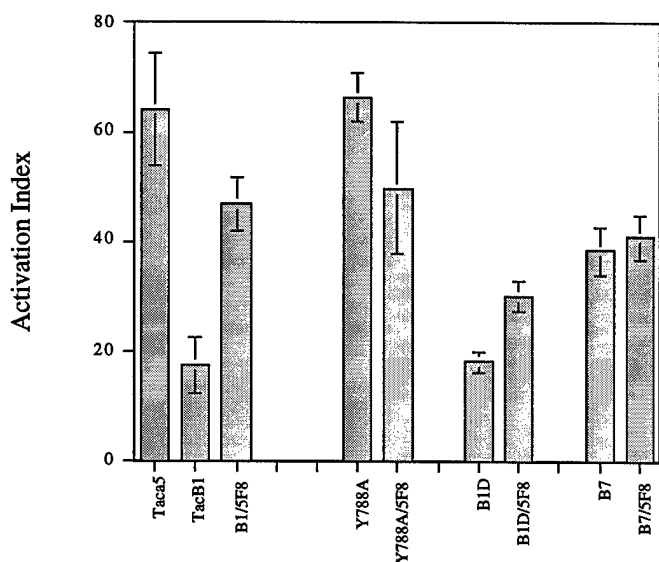


Figure 6

A)

b1A KLLMIIHRRR EFAKFEKEKM NAKWDTGENP IYKSAVTTVV NPKYEKG
b1D KLLMIIHRRR EFAKFEKEKM NAKWDYQENP IYKSPINNFK NPNYGRKAGL
b7 KLSVEIYDRR EYSRFEKEQQ QLNWKQDSNP LYKSAITTTI NPRFQEADSP TL

B)



C)

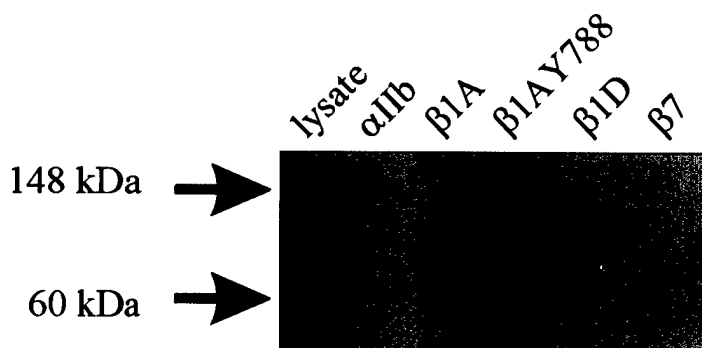
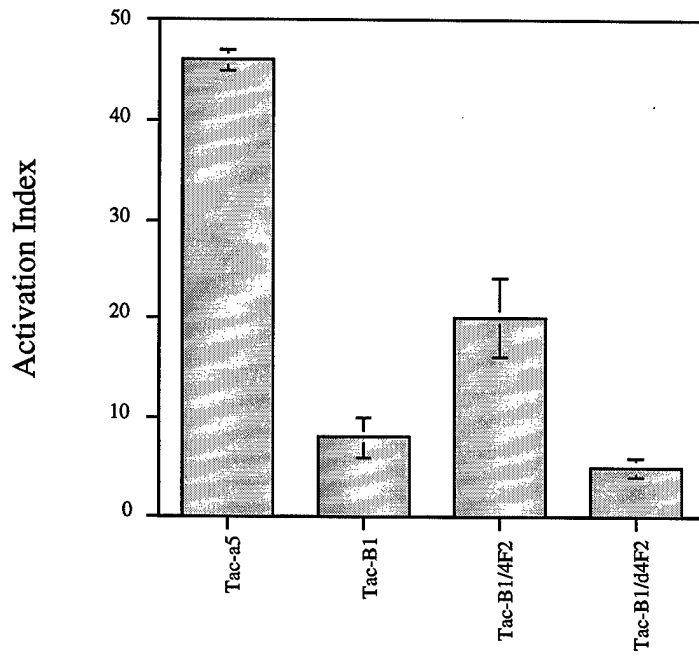


Figure 7

A)



B)

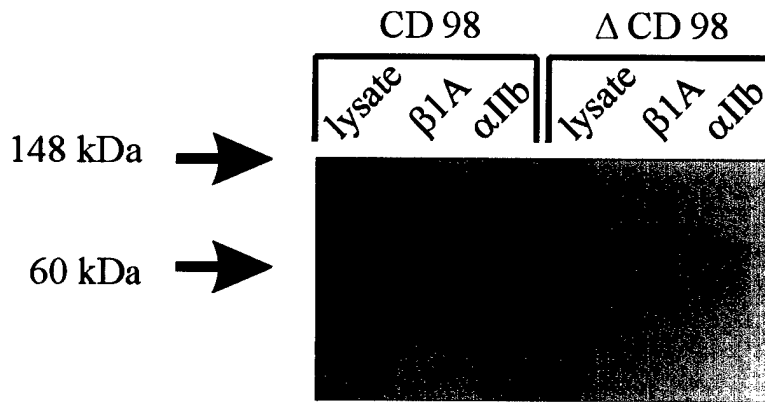
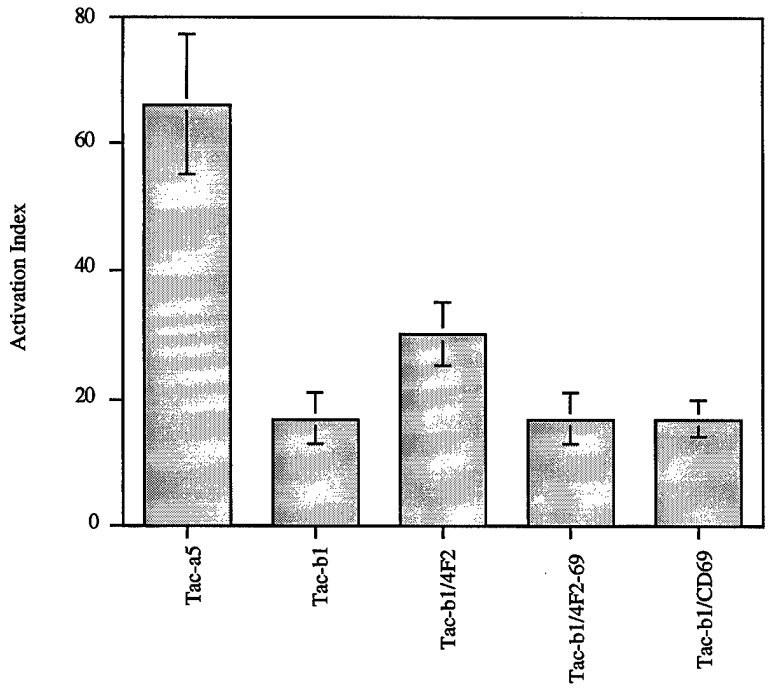


Figure 8

A)



B)

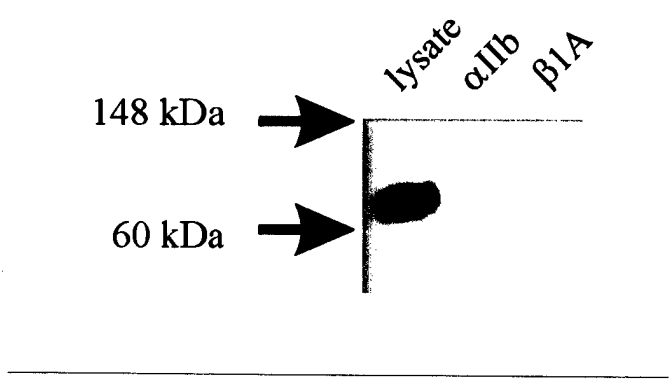
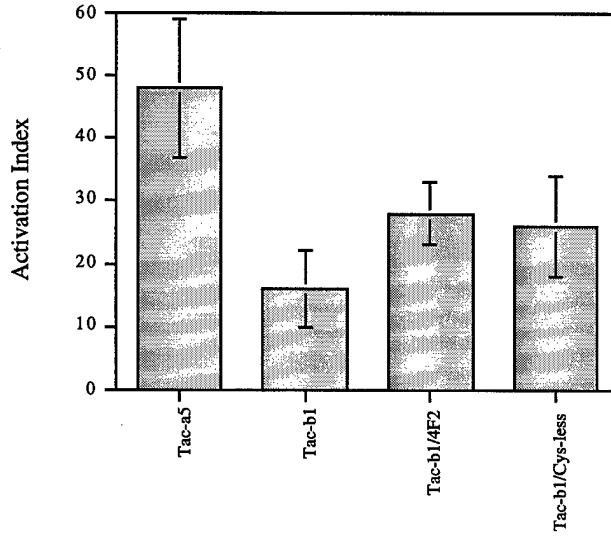
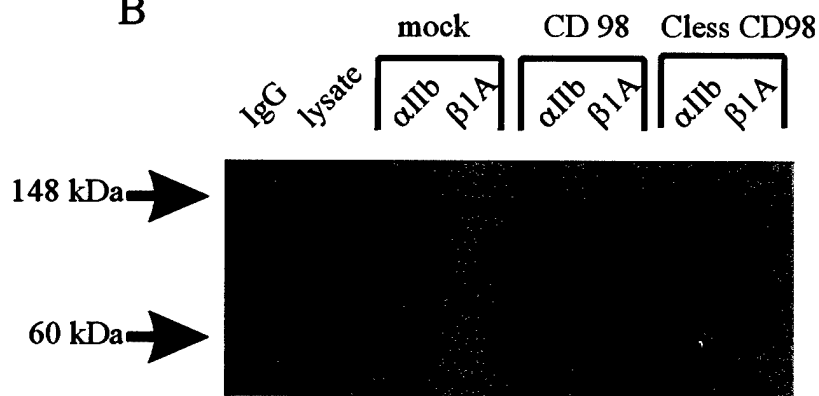


Figure 9

A



B



C

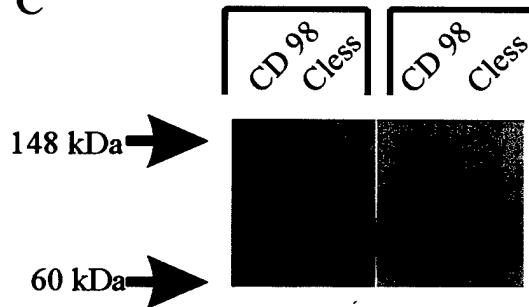
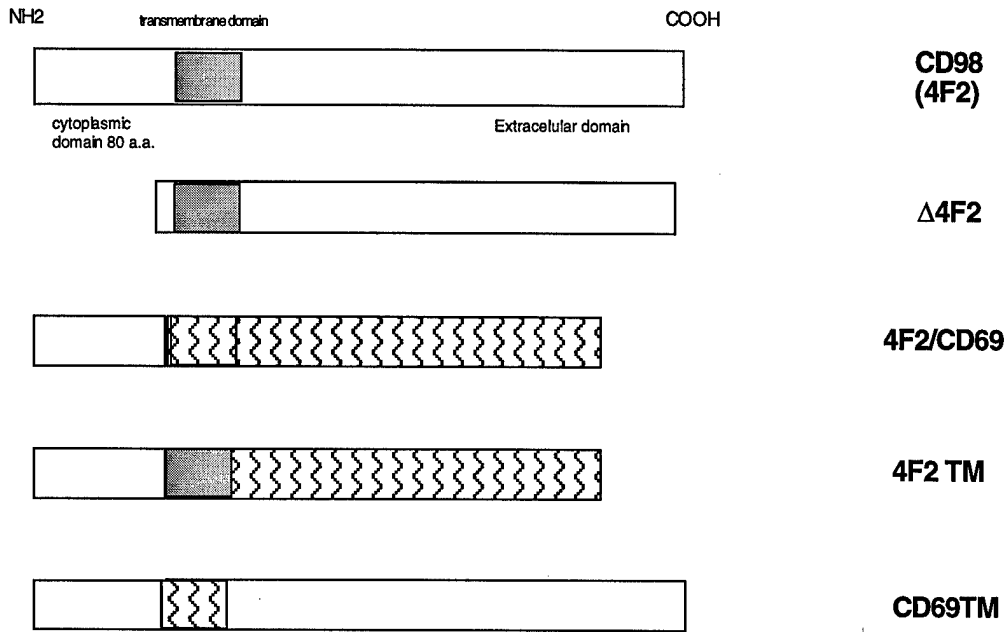


Figure 10

A) CD98/CD69 chimeras



B) CD98 cytoplasmic domain mutants

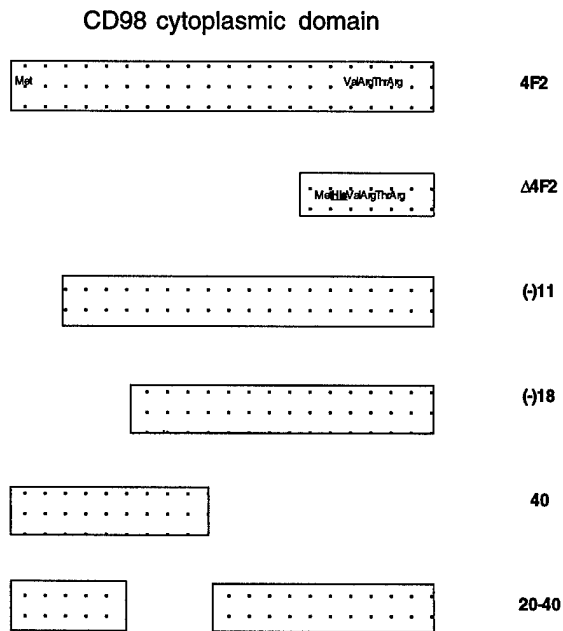


Figure 11

Complementation of dominant suppression implicates CD98 in integrin activation

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The integrin family of adhesion receptors are involved in cell growth, migration and tumour metastasis¹. Integrins are heterodimeric proteins composed of an α and a β subunit, each with a large extracellular, a single transmembrane, and a short cytoplasmic domain. The dynamic regulation of integrin affinity for ligands in response to cellular signals is central to integrin function². This process is energy dependent and is mediated through integrin cytoplasmic domains³. However, the cellular machinery regulating integrin affinity remains poorly understood. Here we describe a genetic strategy to disentangle integrin signalling pathways. Dominant suppression occurs when overexpression of isolated integrin β_1 cytoplasmic domains blocks integrin activation. Proteins involved in integrin signalling were identified by their capacity to complement dominant suppression in an expression cloning scheme. CD98, an early T-cell activation antigen that associates with functional integrins⁴, was found to regulate integrin activation. Furthermore, antibody-mediated crosslinking of CD98 stimulated β_1 integrin-dependent cell adhesion. These data indicate that CD98 is involved in regulating integrin affinity, and validate an unbiased genetic approach to analysing integrin signalling pathways.

Strategies based on genetic selection have proven to be powerful in the dissection of a number of different signal-transduction pathways. We therefore designed a new genetic cloning strategy, based on changes in integrin affinity, to identify potential modulators of integrin activation. This strategy used a Chinese hamster ovary (CHO) cell line ($\alpha\beta\gamma$) stably expressing a chimaeric integrin ($\alpha_{IIb}\alpha_{6A}\beta_3\beta_1$) that contains the extracellular and transmembrane domains of $\alpha_{IIb}\beta_3$ fused to cytoplasmic domains of $\alpha_{6A}\beta_1$ (ref. 5). This chimaeric integrin has the ligand-binding properties of $\alpha_{IIb}\beta_3$ and is activated through the $\alpha_{6A}\beta_1$ cytoplasmic domains. In $\alpha\beta\gamma$ cells this chimaeric integrin is constitutively active, as measured by

the binding of a ligand mimetic monoclonal antibody, PAC1, which recognizes only the activated form of the chimaeric integrin⁶.

Overexpression of isolated integrin β_1 cytoplasmic domains, in the form of a Tac- β_1 chimaera, results in cell-autonomous, concentration-dependent suppression of integrin signalling^{7,8}. Expression of Tac- β_1 in fibroblast cell lines interferes with cell spreading, migration, fibronectin matrix assembly, and integrin activation^{7,8}. This dominant suppression is structurally specific as neither chimaeric molecules with integrin α -cytoplasmic domains nor certain β -cytoplasmic domain variants have inhibitory activity⁷. Excess free integrin β_1 cytoplasmic domains may cause dominant suppression by titration of essential intracellular proteins that are responsible for

integrin activation (Fig. 1a). We hoped that our strategy allowed us to rescue the dominant suppression caused by Tac- β_1 expression by overexpressing these essential intracellular proteins (Fig. 1a).

To identify proteins that complement Tac- β_1 suppression, $\alpha\beta$ Py cells were co-transfected with a CHO-cell cDNA expression library and Tac- β_1 . Fluorescence-activated cell sorting (FACS) was used to isolate cells in which the chimaeric integrin was in an activated form despite the presence of Tac- β_1 (Fig. 1b). Transfected cDNAs, enriched in this way for complementing activity, were then recovered from the sorted cell populations. Six separate FACS screens resulted in the isolation of 1,200 cDNAs, which were then grouped into 75 pools each of 16 cDNAs. Each pool of cDNAs was screened

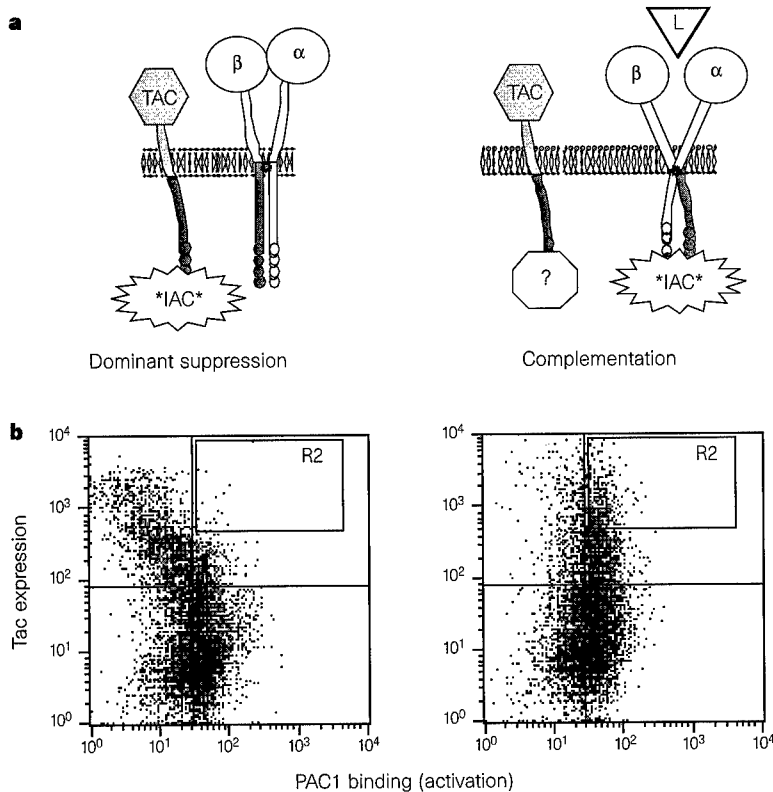


Figure 1 Complementation of dominant suppression and its effect on ligand binding. **a**, Complementation of dominant suppression. Schematic representation of the rationale behind the expression cloning strategy. Intracellular proteins that regulate integrin affinity, shown here as the integrin activation complex (IAC), interact with the cytoplasmic domains of integrins resulting in an increase in ligand binding affinity. Dominant suppression of integrin affinity by Tac- β_1 may be caused by the titration of proteins within the IAC (left). We hypothesized that overexpression of components of IAC, or proteins that block binding to the β cytoplasmic domains of the chimaeric molecule, result in an increase in integrin affinity in the presence of Tac- β_1 (right). **b**, Initial enrichment. $\alpha\beta$ Py cells were transiently transfected with Tac- β_1 and a cDNA expression library. After 48 h, cells were collected and stained for Tac expression (ordinate) and PAC1 binding (abscissa)⁷. Fluorescence-activated cell sorting (FACS) was used to isolate those cells in which the chimaeric integrin is able to bind to PAC1 in the presence of high levels of Tac- β_1 (R2). Shown here are representative dot plots obtained with cells that have been transfected with either Tac- α_5 or Tac- β_1 and analysed by two-colour flow cytometry⁶. A cell in which complementation of dominant suppression has occurred will appear in the upper right-hand quadrant (R2) in the Tac- β_1 transfected cells.

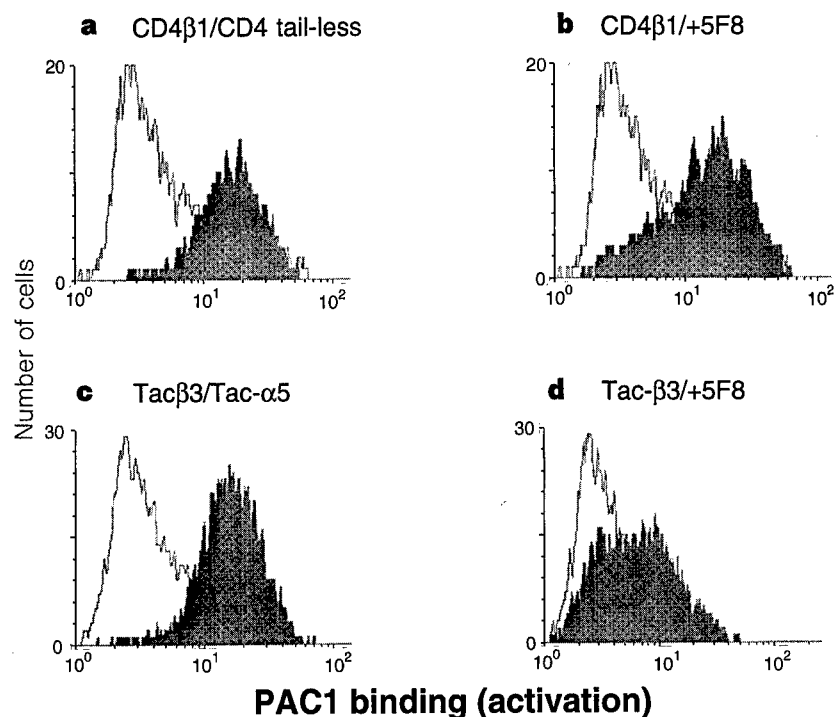


Figure 2 Cytoplasmic domain specificity of complementation of dominant suppression. Data represent PAC1 binding to the CD4- β_1 (a, b) or Tac- β_3 (c, d) positive subset of transfected $\alpha\beta$ Py cells. **a**, PAC1 binding to $\alpha\beta$ Py cells transfected with CD4- β_1 (ref. 21) (open graph) or with a CD4 construct lacking a cytoplasmic domain (shaded). **b**, Co-transfection with cDNA clone 5F8 and CD4- β_1 is indicated by the shaded graph. PAC1 binding is restored in CD4- β_1 -positive cells co-transfected with 5F8 (shaded). Transfection with 5F8 did not reduce CD4 expression (data not shown). **c**, **d**, PAC1 binding to $\alpha\beta$ Py cells transfected with Tac chimaeras. Tac- β_3 transfection is indicated by the open graphs. **c**, The shaded graph indicates binding to cells transfected with a non-inhibitory chimaera, Tac- α_5 . **d**, Co-transfection of clone 5F8 with Tac- β_3 restores PAC1 binding (shaded).

for complementing activity by transfecting the pooled cDNAs into $\alpha\beta\text{Py}$ cells along the Tac- β_1 . cDNAs whose expression caused an increase in PAC1 binding even in the presence of high levels of Tac- β_1 were identified as positives. Tac- β_1 expression levels were monitored in all experiments by staining with an anti-Tac antibody (7G7B6) to eliminate those cDNAs with complementation of dominant suppression that resulted from reduced expression of Tac- β_1 . One out of 75 pools contained cDNA that complemented dominant suppression. This pool was divided into four groups of four cDNAs and rescreened as described above, and only one pool

contained a positive cDNA. In this smaller pool, only a single cDNA, 5F8, was able to reverse Tac- β_1 suppression.

The CHO-cell cDNA library was rescreened in four additional experiments that resulted in the enrichment of 1,059 more cDNA clones. Three additional cDNAs identical to 5F8 were isolated from these clones. Consequently, in this cDNA library, 5F8 was unique in complementing suppression. This library has a reported minimal complexity of 1.8×10^7 independent clones. Thus the effect of 5F8 appears to be highly specific. Transfection of cDNAs reported to interact with or regulate integrins, including α -actinin, vinculin, paxillin, integrin-linked kinase, and β_3 -endonexin, had no effect on Tac- β_1 suppression of integrin activation (data not shown). This result further supports the idea that 5F8 complementation of dominant suppression is very specific.

To determine if the rescue by 5F8 is dependent on the cytoplasmic or extracellular domain of Tac- β_1 , we assessed the ability of 5F8 to complement suppression initiated by a β_1 tail and transmembrane domain joined to the extracellular domain of CD4 (Fig. 2). As with Tac- β_1 , co-transfection of 5F8 with CD4- β_1 resulted in a reversal of dominant suppression, indicating that the rescue was independent of the extracellular domain of the suppressive chimaera. The isolated β_3 cytoplasmic domain is similar to the β_1 cytoplasmic domain and can also initiate dominant suppression^{7,8}. We therefore investigated whether rescue by 5F8 is specific to the β_1 cytoplasmic domain. We found that 5F8 also reversed Tac- β_3 suppression (Fig. 2). Thus 5F8 can complement suppression initiated by either β_1 or β_3 cytoplasmic domains.

The 5F8 sequence has been deposited in Genbank (accession no. U93712) and contains 1,902 base pairs, with an open reading frame encoding a polypeptide of 533 amino acids. In addition, it contains a poly(A) tract and polyadenylation signal. Analysis of the predicted topology of the encoded protein using Tmpred⁹ suggested that it possesses at least one transmembrane domain with the amino terminus inside the cell (type II transmembrane protein). Furthermore, a BLAST¹⁰ database search showed that the amino-acid sequence encoded by 5F8 is 72% identical to that of the heavy chain of the 4F2 antigen, CD98 (ref. 11). Overexpression of human CD98 also reversed dominant suppression, and so we conclude that we have isolated its hamster homologue (Fig. 3a).

CD98 was originally identified as an early T-cell activation antigen. It is part of a heterodimer of relative molecular mass 120,000 (M_r 120K), consisting of a heavy chain of approximately 80K that bears CD98 epitopes, and a light chain of 40K (ref. 12). It is a membrane protein that specifically associates with β_1 integrins⁴. Indeed, it has been shown that CD98 co-precipitated with functional $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$ integrins⁴. Furthermore, co-precipitation with $\alpha_4\beta_1$ was only observed when $\alpha_4\beta_1$ was activated in the presence of 2 mM Mn^{2+} . The light chain is poorly characterized, and its cDNA has yet to be cloned. Thus we cannot assess the role of the light chain in the effect of CD98 on integrin activation.

To determine whether the rescue of dominant suppression is a function of other integrin-associated membrane proteins, we overexpressed membrane proteins previously implicated in integrin function: CD9 (ref. 13), CD47 (integrin-associated protein)¹⁴, and urokinase plasminogen activator receptor (uPAR)¹⁵; in each case, protein expression was confirmed by flow cytometry (data not shown). Expression of these proteins failed to reverse dominant suppression by Tac- β_1 (Fig. 3a). Furthermore, overexpression of CD98 or the other membrane-associated proteins in the absence of Tac- β_1 did not increase the affinity of already activated integrins (Fig. 3b). Thus the rescue of Tac- β_1 suppression by CD98 is not an artefact of overexpression of membrane proteins.

The $\alpha_3\beta_1$ integrin-mediated adhesion of the small cell lung-cancer cell line (SCLC) H345 to laminin and fibronectin can be regulated dynamically¹⁶. We therefore tested whether CD98 might be involved in the regulation of integrins in SCLC adhesion. An anti-CD98 monoclonal antibody (4F2) markedly enhanced single-

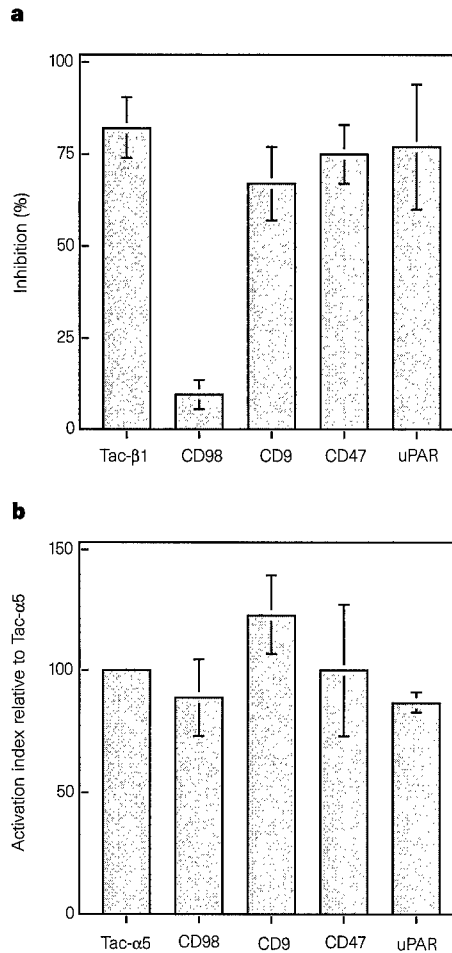


Figure 3 Human CD98 complements dominant suppression, whereas several other membrane proteins do not. $\alpha\beta\text{Py}$ cells were transfected with 2 μg of either Tac- β_1 (a) or Tac- α_5 (b). They were simultaneously transfected with 4 μg of cDNAs encoding either human CD98 (ref. 11), CD9 (ref. 22), CD47 (ref. 14) or uPAR. After 48 h, cells were collected 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 $100(F_o - F_R)/(F_{\text{LIBS6}} - F_R)$, as previously described²⁵, where F_o is 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 Ro33-5054), and F_{LIBS6} is the maximal fluorescence intensity in the presence of 2 μM anti-LIBS6, an activating monoclonal antibody. The mean \pm s.d. of three independent experiments for each membrane protein is shown. **a**, Specificity of CD98 complementation. Percentage inhibition was defined as $100(AI_0 - AI)/AI_0$, where AI_0 is the activation index in Tac- α_5 transfected cells. AI is the activation index in Tac- β_1 transfected cells. The co-transfected membrane protein is indicated below each column. **b**, Effect of membrane proteins in the absence of dominant suppression. Cells were transfected with Tac- α_5 alone or the indicated membrane protein. Data are expressed as relative activation = $100(AI_{\text{MP}}/AI_{\alpha_5})$, where AI_{MP} is the activation index in the presence of the membrane protein and AI_{α_5} is the activation index with Tac- α_5 alone. The activation index with Tac- α_5 alone was 60%.

cell adhesion of H345 cells to both laminin and fibronectin (Fig. 4). Addition of the function blocking anti- β_1 antibody (P5D2) or 2 mM 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 (ref. 17), 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, clustering of CD98 is required for 4F2-induced upregulation of β_1 integrin function.

Our genetic complementation strategy has led to the identification of CD98 as a protein involved in integrin activation. Indeed, CD98 specifically associates with β_1 integrins, and that association may be activation dependent, as CD98 is preferentially immunoprecipitated with activated integrins⁴. In addition, CD98 lacking a cytoplasmic domain fails to complement dominant suppression (C.A.F., B. Lewis & M.H.G., unpublished results). This result indicates that the association between integrins and CD98 may be mediated by the cytoplasmic domains of both proteins. It is likely that endogenous hamster CD98 is expressed in CHO cells, as 5F8 was isolated from a cDNA library derived from CHO-cell mRNA and CD98 is expressed in many tissue-culture cell lines¹¹. Cross-linking CD98 with 4F2 antibody stimulated β_1 -dependent adhe-

sion. Thus the rescue of dominant suppression by overexpression of CD98 in $\alpha\beta$ py cells may work because overexpression of CD98 may favour clustering. Further work will be required to establish relationships between CD98 clustering, its association with integrins, and the identity of CD98 ligands that might cluster CD98.

Our finding that CD98 is involved in integrin function explains the observation that CD98-mediated membrane fusion of cells transfected with HIV gp160 is integrin dependent^{18,19}. Further, when T cells are activated, CD98 expression increases before an increase in β_1 integrin-mediated adhesion²⁰. Therefore changes in integrin function in these two cell types could depend upon CD98 regulation. Collectively, these data indicate that CD98 may be an important regulator of integrin function in many contexts. □

Methods

cDNA constructs and antibodies. Expression plasmids encoding the extracellular domain of murine CD4 fused to the human β_1 transmembrane and cytoplasmic domains and CD4 tailless, which lacks the β_1 cytoplasmic domain, were a gift from M. Lukashev²¹. Human 4F2 antigen (CD98) cDNA was provided by J. M. Leiden¹¹, and was subcloned into pcDNA1 as an *EcoRI* fragment. The pCDM8 construct encoding CD9 has been described²². The pR/RV plasmid, pIAP45, encoding CD47, and B6H12, the anti-CD47 antibody, were gifts from F. Lindberg and E. Brown¹⁴. cDNA encoding uPAR, in pcDNA1, pcuPAR1, was provided by L. Miles. The hybridoma cell line 4F2(C13) was purchased from American Type Culture Collection (ATCC). Ascites were produced in pristane-primed BALB/c mice. Fab fragments were prepared by papain digestion of purified 4F2 IgG (2 mg ml⁻¹) 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 mobilities.

Expression cloning. A cDNA library, made from poly(A)⁺ mRNA from CHO-K1 cells, directionally cloned into the *NotI* site of the mammalian expression vector, pcDNA1, was purchased from Invitrogen (San Diego, CA). The library is reported to contain 1.8×10^7 primary recombinants. A CHO cell line that expresses the polyoma large T antigen and an activated recombinant chimaeric $\alpha_{IIb}\beta_3$ integrin ($\alpha\beta$ Py cells) was constructed as described⁵. $\alpha\beta$ Py cells were transfected with the Tac- β_1 (Tac- β_1 , Tac- β_3 and the non-inhibitory Tac- α_5 chimaeras were gifts from S. LaFlamme and K. Yamada⁸; 4 μ g per plate) using lipofectamine (Gibco, BRL), according to the manufacturer's instructions. The transfection efficiency ranged from 30% to 50%, as determined by the binding of the anti-Tac antibody (7G7B6)⁷. After 48 h incubation, cells were collected and stained as described⁶, and cells positive for both PAC1 and Tac were isolated by fluorescence-activated cell sorting (FACSTAR, Becton Dickinson). Plasmid DNA was extracted²³ and digested with *DpnI* to remove plasmids that were not replicated in transfected cells, and used to transform *Escherichia coli* MC1061/P3. Individual colonies were grown, and the bacteria were then pooled into groups of 16 for plasmid purification (Qiagen, Chatsworth, CA). Groups of cDNAs were transfected into the $\alpha\beta$ Py cell line along with Tac- β_1 and the transfectants were screened by flow cytometry as described above. Pools containing cDNAs that altered PAC1 binding were further screened in smaller pools of four, and then the positive pools were screened as individual colonies.

Adhesion assay. Laminin and fibronectin (Calbiochem) were used as substrates in serum-free adhesion assays. Cells of the 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). Cells ($1-2 \times 10^6$ per ml) were washed twice in RPMI 3.5 d post-passage and disaggregated into single cells. Cells in RPMI (50 μ l) were added to 96-well tissue-culture plates (Costar) coated with extracellular matrix proteins blocked with 1 mg ml⁻¹ BSA. Cells were allowed to attach for 45 min at 37 °C. Cell attachment was determined by crystal violet staining. The attachment of H345 cells to wells coated with 25 mg ml⁻¹ poly-L-lysine and fixed with 20% glutaraldehyde before aspiration was defined as 100% adhesion²⁴.

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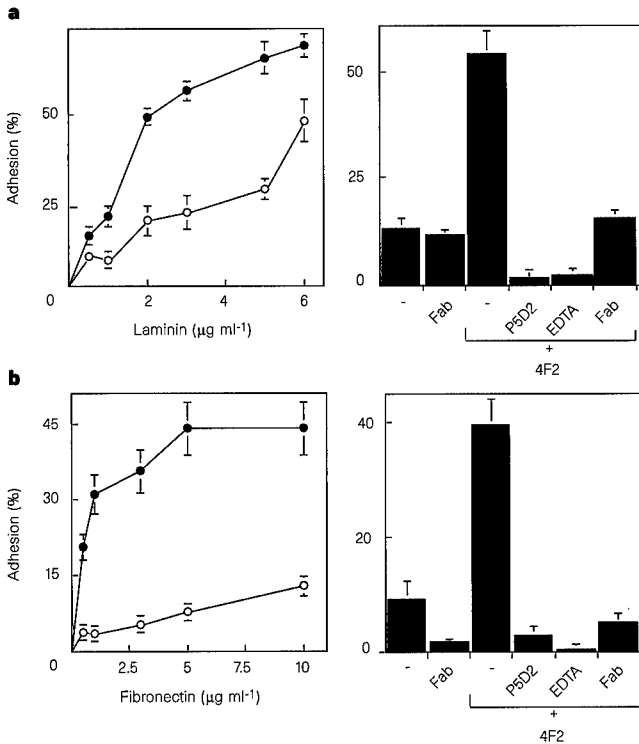


Figure 4 The effect of a 4F2 monoclonal antibody on adhesion of the small cell lung-cancer cell line (SCLC) H345 to extracellular matrix. Left, attachment of SCLC cells to 96-well tissue-culture plates coated with increasing concentrations of laminin (a) or fibronectin (b) in the presence (filled circles) or absence (open circles) of 20 μ g ml⁻¹ 4F2 monoclonal antibody. Right, addition of 4F2-Fab (100 μ g ml⁻¹) to SCLC cells does not increase adhesion to plates coated with 3 μ g ml⁻¹ laminin (a) or 10 μ g ml⁻¹ fibronectin (b); addition of P5D2 (a β_1 -integrin function-blocking antibody) or 2 mM EDTA also block the increase of adhesion of SCLC cells to plates coated with either 3 μ g ml⁻¹ laminin (a) or 10 μ g ml⁻¹ fibronectin (b). Results show the mean percentage adhesion above background (which was consistently <5%) compared with poly-L-lysine (taken as 100%) of 4-6 independent experiments in duplicate/triplicate - s.e.m.

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