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FOREWORD

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Annual Report September 1999 GRANT NUMBER DAMD17-97-1-7056

TITLE: Identification and Characterization of Proteins Involved in Integrin Signaling

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

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, a type II transmembrane protein first discovered as a T-cell activation antigen, was identified utilizing CODS. CD98, although widely expressed on proliferating cells, is generally down regulated in quiescent cells. CD98 forms disulfide-bonded heterodimers with several light chains that strongly resemble permeases. CD98 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.

Progress on Specific Aim 1.

A) Complete sequence of clone 5F8
B) Production of antibodies
C) Tissue distribution and subcellular localization

Parts A-C of Specific Aim 1 were described in last years progress report.

D) Further characterize the specificity of 5F8 rescue

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 which 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. I also have shown that the wild-type α5β1 receptor present in CHO cells is also rescued from dominant suppression by CD98 expression. 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.

E) Characterize the interaction between 5F8 protein and integrins

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 will be sent to the Scripps Core Facility to generate a mouse which contains a deletion of the CD98 gene.

Progress on Specific Aim 2
Mechanisms of Rescue

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. As described in last years annual report, we have examined interactions between CD98 and recombinant parallel-dimerized integrin β1A, β1D, and β7 cytoplasmic domains by affinity chromatography. CD98 interacts with the β1A and β3 but not β1D or β7 integrin cytoplasmic domains.

To further characterize the interaction between CD98 and integrin cytoplasmic tails we tested whether other known integrin binding proteins were involved. We looked at both talin and filamin binding as they bind tightly to β1D and β7 respectively. The previous binding assays were performed using talin and filamin-1 containing cell extracts. Thus, CD98 may not be able to bind to β1D or β7 because of competition for binding sites on the integrin tails with 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 to β1A cytoplasmic tails. CD98 bound to the β1A tail, but not β7, when lysates of M2 cells were used, 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. CD98 extracted from these membranes bound β1A but not β1D cytoplasmic domains. 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 and talin binding. Similar Y to A mutations in β7 and β1D tails, corresponding to the Y788A mutation in β1A, also disrupted filamin and talin binding. CD98 binding to β integrin tails was not affected by Y to A mutations. 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, but do not rule out involvement of other cytoplasmic proteins. Furthermore, the CD98 interaction is insensitive to β cytoplasmic domain mutations that abolish the binding of talin and filamin.

To further eulcidate the mechanism of CD98 rescue we have also been performing immunofluorescence experiments. Our initial experiments attempted to show colocalization of integrins and CD98 in focal adhesions. These experiments were unsuccessful because CD98 is expressed at high levels throughout the cell surface and it is technically difficult to see focal adhesion staining above the surface staining. Rather than taking a direct approach of colocalization, we are trying to assess whether or not CD98 expression will cause a redistribution of Tac-β1A from focal adhesions. We will be using Tac-β1D as a negative control.

We are also continuing the experiments outlined in last years annual report to determine which regions of CD98 are required for it's effect on integrin function. Previously, we determined that the cytoplasmic domain was necessary but not sufficient for rescue. We have gone on to show that the transmembrane domain is also required for rescue. We have constructed a series of CD98/CD69 constructs (that were outlined in last years report) that have been tested both for their ability to rescue dominant suppression and for their ability to bind to β1A cytoplasmic domains. We have seen a strict correlation with the ability of the different chimeras to bind to the β1A cytoplasmic domain and their ability to rescue dominant suppression.