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ICAM-1 transmembrane signaling: involvement of TAXREB107 in ICAM-1-mediated signal transduction

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Abstract

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The role of membrane ICAM-1 in intercellular adhesion, through interaction with the leukocyte β2 integrin receptors LFA-1 and Mac-1, is well established. However, until recently, ICAM-1 was widely regarded as being simply an "anchor" molecule, promoting, for example, firm adhesion of lymphocytes to vascular endothelial cells. The demonstration that ICAM-1 is connected to the actin-based cytoskeleton through interactions with subcortical actin-binding proteins, such as α-actinin, enhanced this concept. However, ICAM-1 has been increasingly shown to have signaling properties of its own. Engagement of ICAM-1 extracellularly, either through its natural counter-receptors, LFA-1/Mac-1, or through antibody mediated clustering, can produce numerous changes within the cell. Recent evidence now confirms that ICAM-1 crosslinking induces multiple activation responses including: 1) phosphorylation of several cytoplasmic proteins, including cortactin, cdc2 kinase and lyn kinase; (2) activation of the Erk 1/2 MAP kinases; and (3) induction of various functional cellular responses such as oxidative burst from mononuclear leukocytes, neutrophil activation,

and rho activation in endothelial cells. Although we know of several downstream targets of ICAM-1 engagement, very little is known about upstream events, i.e., the most proximal components of the signaling cascade involving direct protein interactions with the ICAM-1 cytodomain, or through indirect interactions in a multimeric unit.

The specific aim of this project is to study the proximal aspects of ICAM-1 signaling. The hypothesis is that ICAM-1-mediated signal transduction requires interactions between currently unidentified intracellular proteins and the cytoplasmic domain of ICAM-1. In these studies, TAXREB107, a recently discovered protein, was specifically associated with the ICAM-1 cytodomain in GST-pulldown experiments using human endothelial cell extracts. This association was biochemically confirmed by co-immunoprecipitations of recombinantly expressed TAXREB107 and the ICAM-1 cytodomain, as well as endogenous TAXREB107 and ICAM-1. Recombinant TAXREB107, expressed in a wide spectrum of mammalian cell types, greatly augmented the ERK 1/2 MAPK activation induced by activating antibody or natural ligand (fibrinogen) engagement of ICAM-1. This amplifying effect was abolished by co-expression of the ICAM-1, but not VCAM-1 cytodomain. Furthermore, immunofluorescence studies demonstrated that TAXREB107, which possesses DNA-binding capability, is predominantly located in the cytoplasm in non-activated CHO or human endothelial cells, and rapidly translocates to the nucleus upon ICAM-1 engagement. These data demonstrate an interaction between the ICAM-1 cytoplasmic tail and TAXREB107, and support that this interaction can lead to a productive amplification of proximal ICAM-1-mediated signaling. This may consequently stimulate

gene expression of TAXREB-responsive genes or promote a spectrum of ERK-mediated cellular responses, also including gene activation.

ICAM-1 Transmembrane Signaling: Involvement of TAXREB107 in ICAM-1-mediated Signal Transduction

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Chapter 1 Introduction

1.1 ICAM-1 -- Classification and Early Studies

Adhesion molecules play an indispensable role in the development and homeostasis of most higher organisms. By serving to mediate cell-cell interactions, they play a role in such diverse functions as immunity, hematopoiesis, and embryonic development. Because of their ubiquitous expression and involvement in so many biological processes, this class of molecules has been extensively studied. This discussion will focus on the role of adhesion molecules in immunity and disease looking in particular at the specific activities of intercellular adhesion molecule-1 within this framework. In terms of immunity, three major groups of adhesion molecules exist (van de Stolpe et al., 1996):

A) Immunoglobulin-like cell adhesion molecules -- Single chain transmembrane molecules containing multiple in-series domains resembling those found in immunoglobulin heavy and light chains. Examples include intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and platelet/endothelial cell adhesion molecule-1 (PECAM-1).

B) Integrins -- Heterodimeric transmembrane proteins consisting of an α chain and a β chain, often capable of functioning as receptors for one or more Ig-like adhesion molecules. Integrin families are classified based on their particular β chain. For example, members of the $\beta 2$ integrin subfamily include LFA-1 (CD11a/CD18), and Mac-1

(CD11b/CD18). In their resting configuration, integrins usually have very low affinity for their ligand, e.g., LFA-1/ICAM-1 interactions have a K_d of only 10^{-5} or 10^{-6} M in the inactive state (Lollo et al., 1993). Once LFA-1 or Mac-1 are activated, affinity for ICAM-1 increases dramatically (Dustin et al., 1989). The β2 integrin-ICAM-1 interaction is also dependent upon divalent cations (Marlin et al., 1987).

C) Selectins -- Monomeric transmembrane molecules related to the sugar-binding lectins. They are involved in the early stages of leukocyte localization, by causing leukocytes in the bloodstream to "slow roll" along the vascular endothelium adjacent to sites of inflammation. These molecules bind to either mucin-like glycoproteins (GlyCAM-1 and CD34) or sialylated forms of oligosaccharides (sialylated Lewis X). Members of this family include L-selectin, P-selectin and E-selectin.

In early studies of leukocyte-leukocyte and leukocyte-endothelial interactions, lymphocyte function-associated molecules (LFA-1, -2, -3) were discovered to be essential mediators (Sanchez-Madrid et al., 1982). Monoclonal antibodies developed against the LFA's, and LFA-1 in particular, were shown to be effective in blocking adhesion between almost all known combinations of leukocyte-leukocyte and leukocyte-target cell interactions (Sanchez-Madrid et al., 1982). The hypothesis at that time was that the cell-cell binding was caused by homotypic interaction between LFA molecules on both cells. However, this was quickly disproven in studies using LAD1 cells which were deficient in β2 integrins such as LFA-1 and Mac-1 (Rothlein et al., 1986). These studies demonstrated that it is possible for phorbol ester stimulated LFA-1^+ cells to bind LFA-1^-/ Mac-1^- cells, indicating that there was an unidentified molecule serving as an LFA-1

ligand. By laboriously screening a large panel of monoclonal antibodies (mAb's) that did not bind to LFA-1, yet inhibited LFA-1 mediated adhesion, Rothlein et al. were able to eventually narrow down to one monoclonal antibody, RR1 (Rothlein et al., 1986). Building on this information, the cloning of ICAM-1 was not far behind (Simmons et al., 1988).

Since LFA-1 binds to all known ICAM's (ICAM-1, -2, -3), it is evident in retrospect why RR1 was not able to block all LFA-1-mediated interactions. Within four years after ICAM-1 was cloned, ICAM-2 and ICAM-3 had also been cloned (Staunton et al., 1989; de Fougerolles et al., 1994). At this stage, there was no known cell line whose LFA-1 dependent adhesion could not be blocked by a cocktail of anti-ICAM-1, -2, and -3 monoclonal antibodies.

1.2 Gene and Protein Structure

ICAM-1 is a 505 amino acid protein composed of five Ig-like domains in its extracellular portion (453 mostly hydrophilic residues) , a transmembrane region (24 residues), and finally, a cytoplasmic domain (28 residues) (van de Stolpe et al., 1996). The gene structure is relatively simple with seven total exons (separated by six introns), one for each of the Ig-like domains, one for the transmembrane domain, and the last exon for the cytoplasmic tail. Comparisons between various mammalian ICAM-1 sequences have been made, showing a relatively nonconserved gene across species. There appears to be only 55-65% homology between human and other mammalian ICAM-1 sequences (Siu

et al., 1989; Kita et al., 1992; Manning et al., 1995). This is surprising considering that the LFA-1/ICAM-1 interaction is very specific in its binding. Only the first Ig-like domain (the domain furthest away from the membrane) on ICAM-1 can bind to LFA-1, even though the other domains are similar in structure and sterically available (Mac-1, another β2 integrin which has similar structure to LFA-1, binds to the more inner Ig-like domain 3). Interestingly, ICAM-1 gene knockout is nonlethal in mice, although several immunological deficits are apparent (Sligh et al., 1993). Since ICAM-2 and -3 are also known to be ligands for LFA-1 (Simmons, 1995), perhaps evolutionary development of ICAM-1 need not maintain strict conservation across generations. In fact, ICAM-2 appears quite different from ICAM-1 and -3 in that it only has two Ig-like domains. Besides this, the observation that the ICAM-2 gene maps to chromosome 17q23-25 whereas ICAM-1 and -3 closely map on chromosome 19p13.2-13.3 seems to indicate that the divergence between the two sets of genes occurred relatively early on (Simmons, 1995).

Glycosylation patterns vary widely among cell types so that the molecular weight of human ICAM-1 is in the range of 80-114 kDa (van de Stolpe et al., 1996). ICAM-1 is heavily N-glycosylated, such that approximately half of its mass is generated by oligosaccharides. The projected size of a fully deglycosylated ICAM-1 is approximately 56 kDa. Why such heavy glycosylations exist remains unclear, but available evidence suggests that it may serve a regulatory purpose. When the N-linked oligosaccharides in Ig-like domain 3 of ICAM-1 were removed, Mac-1 was found to have much higher affinity (Diamond et al., 1991). ICAM-3, which is very closely related to ICAM-1, also

shows a similar effect at Ig-like domain 3 (Simmons, 1995). Presence of glycans in domain 1 of ICAM-1 do not appear to affect binding to LFA-1 or Mac-1.

Each Ig-like domain forms a β-sheet structure, which is stabilized by highly conserved cysteine residues in all but the fourth Ig-like domain, which has its own set of conserved residues, presumably necessary to stabilize its β-sheet structure in the absence of disulfide bridges (Staunton et al., 1990). ICAM-1 is presumed to form a linear chain of the five Ig-like domains based on two separate lines of work (Giranda et al., 1990; Staunton et al., 1990).

An interesting peculiarity about the extracellular domain of the ICAM molecules is that they do not possess the RGD domains usually seen in integrin ligands (Staunton et al., 1990). This is consistent with the lack of Ca^{2+} requirement in ICAM/β2 integrin binding, although Mg^{2+} and/or Mn^{2+} play important roles. In addition to interactions with β2 integrins, ICAM-1 has been reported to interact with a number of other molecules, including fibrinogen, hyaluronan, and CD43 (Rosenstein et al., 1991; Languino et al., 1993; McCourt et al., 1994).

The cytoplasmic tails of the ICAM's are the most nonhomologous regions among the different ICAM molecules, which has led to speculation that this may provide a basis for outside-in signaling specificity (Simmons, 1995). All three ICAM's have short cytoplasmic tails without any apparent enzymatically active domains. However, several potential phosphorylation sites exist within each. While potential signaling functions have been shown for ICAM-1 and ICAM-3, none has yet been shown for ICAM-2.

1.3 ICAM-1 signaling

There have been multiple demonstrations of intracellular events occurring upon membrane ICAM-1 engagement by activating antibodies. Among the earliest reports, Carpen et al. provided biochemical evidence (in a nonfunctional setting) of a direct association between α-actinin and the ICAM-1 cytodomain (CD) by demonstrating binding of purified α-actinin to a synthetic ICAM-1 cytodomain peptide, as well as to immunoaffinity purified ICAM-1 (Carpen et al., 1992). Later, brain microvessel endothelial cells that were stimulated with either anti-ICAM-1 antibodies or syngeneic encephalitogenic T cells were shown to undergo p60 src tyrosine phosphorylation, which led to phosphorylation of a src substrate, cortactin (Durieu-Trautmann et al., 1994). Since a previous report demonstrated that p60src-mediated tyrosine phosphorylation led to disassembly of adherens junctions (Volberg et al., 1991; Tsukita et al., 1992), the link between ICAM-1 and cytoskeletal elements was further strengthened. Recently, Adamson et al. also showed that antibody-mediated ICAM-1 crosslinking resulted in a reorganization of the endothelial actin cytoskeleton to form stress fibers and activation of the small guanosine triphosphate (GTP)-binding protein Rho, an effect blockable by addition of C3 transferase, a specific inhibitor of Rho (Adamson et al., 1999). Occludin, an integral membrane protein localizing at tight junctions and linked to the actin cytoskeleton, is phosphorylated after ICAM-1 crosslinking (Hirase et al., 2000). This event correlates with an increase in tight junction permeability in endothelial cell monolayers. This, and the occludin phosphorylation, are blocked by addition of a Rho

kinase inhibitor, Y-27632, or adenoviral transduction of the endothelial cells with a dominant-negative Rho A mutant.

Other pathways not immediately linked with the cytoskeleton have also been implicated in ICAM-1-mediated signaling. ICAM-3 can stimulate a number of T cell functions such as activation and secretion of IL-2 by inducing phosphorylation of Fyn, Lck, and CD45 (Juan et al., 1994). Since ICAM-1 and -3 are similar in overall structure, the same phosphorylation targets were screened after ICAM-1 stimulation. Paradoxically, a transient inhibition of kinase activity in a T cell tumor line was observed, via tyrosine phosphorylation of cdc2, a posttranscriptional modification shown to inhibit this cell cycle progression regulating protein. (Chirathaworn et al., 1995).

In a murine B cell tumor line, antibody-mediated ICAM-1 crosslinking results in class II major histocompatibility complex upregulation as well as tyrosine phosphorylation of the src family kinase, lyn (Holland et al., 1997). ICAM-1 stimulation also results in Raf-1 and Erk-1 activation in this B cell line indicating that this MAP kinase pathway is activated as a result of ICAM-1 engagement. The ICAM-1-mediated ERK activation has also been observed in human vascular endothelial cells (HUVEC) and further shown to be involved in IL-8 (interleukin-8) and RANTES (regulated upon activation, normal T-cell expressed and secreted) production (Sano et al., 1998). IL-8 belongs to a group of molecules known as C-X-C chemokines, which are known to serve as chemoattractants and activators of neutrophils in vivo (Strieter et al., 1989). RANTES belongs to the C-C chemokine group, attracting T cells and eosinophils in vitro (Schall et

al., 1990; Rot et al., 1992). Thus, the ICAM-1 engagement by leukocyte β2 integrins (or other ligands) may amplify cell recruitment through triggering chemokine production.

The effect of ICAM-1 engagement on oxidative burst in neutrophils and mononuclear phagocytes has been investigated. In $CD14^+$ peripheral blood mononuclear cells (PBMCs), crosslinking membrane ICAM-1 with anti-ICAM-1 monoclonal antibodies plus $F(ab')_2$ fragments of goat anti-mouse IgG, in the presence of suboptimal levels of the bacterial peptide FMLP, results in oxidative burst (Rothlein et al., 1994). In human neutrophils, ICAM-1 crosslinking causes an increase in oxidative burst, an increase in Mac-1 expression , and a decrease in L-selectin expression (Vuorte et al., 1999).

Gene activation by ICAM-1 crosslinking has also been examined. ICAM-1 crosslinking triggers a rise in cytosolic free calcium, followed by a 2-3 fold increase in ICAM-1 mRNA levels (peaking at 1-3 hr.), and maximal protein expression between 18-48 hr (Clayton et al., 1998). Similar protein expression patterns have been observed for VCAM-1 after ICAM-1 crosslinking. Very recently, differential display after ICAM-1 stimulation was performed. At 2 hrs, induced and downregulated genes were clearly demonstrated, but the actual identities of the genes within each group were not identified. (Kohlmeier et al., 2000).

As with several other cell types, ICAM-1 engagement in rat astrocytes causes Erk activation by phosphorylation (Ettiene-Manneville et al., 1999). In addition, in these same cells, increased levels of both intracellular cAMP and the phosphorylated form of the transcription factor, cAMP response element-binding protein (CREB), are seen after

ICAM-1 stimulation. These events are partially dependent on protein kinase C, which can act as an indirect activator of cAMP/PKA and Erk pathways.

Most of the previous ICAM-1 signaling studies have used activating antibodies to promote membrane molecular clustering. There have, however, been a small number of studies done with soluble fibrinogen as a stimulating ligand. As previously mentioned, fibrinogen has been shown to bind to ICAM-1, as well as to other cell adhesion molecules such as Mac-1 and CR4 (CD11c/CD18), $\alpha_v\beta_3$, and $\alpha_{IIb}\ \beta_3$ (platelet gpIIb/IIIa) (Wright et al., 1988; Altieri et al., 1990; Rosales et al., 1995). Recently, the fibrinogen binding site on ICAM-1 has been examined in greater detail and found to be on Ig-like domain 1 (Duperray et al., 1997). This site is apparently distinct from the LFA-1 or Mac-1 binding sites. Fibrinogen has been found to increase relaxation in saphenous vein rings in lower concentrations (0-2 μM), an effect which can be blocked by addition of anti-ICAM-1 antibodies (Hicks et al., 1996). Interestingly, inhibiting nitric oxide synthesis with L-NAME or addition of the nitric oxide trap, hemoglobin, caused only a small change in the fibrinogen-mediated relaxation response, while inhibition of prostacyclins, another family of vasodilators synthesized by the endothelium produced similar results. Only a combination of L-NAME and indomethacin caused an appreciable change in the fibrinogen-mediated relaxation response.

Fibrinogen stimulation of human saphenous vein endothelial cells induces the secretion of MCP-1 (Harley et al., 1999). This effect was mimicked by activating anti-ICAM-1 antibodies. Fibrinogen also induces proliferation in Raji B cells, an effect blockable with peptides corresponding to an extracellular portion of ICAM-1 (Giranda et

al., 1990). This was attributed to fibrinogen-induced Erk-1 activation, inhibitable with the same extracellular ICAM-1 peptide.

1.4 ICAM-1 Cytodomain Interactions

While the work presented above describes several potentially important cellular responses to ICAM-1-mediated signaling, there remains little known about the most proximal events in these activation responses. That is, for these responses to occur, the cytoplasmic tail of ICAM-1 must be playing either a direct or indirect role. Attempts to define ICAM-1 cytodomain-interacting proteins have generally yielded a consistent pattern, in that many of the identified molecules are related to the cytoskeleton. Since ICAM-1's major function is to serve as an anchoring molecule between two cells, it is not surprising to find cytoskeletal adaptor proteins binding to the ICAM-1 cytodomain.

ICAM-1 cytodomain associates with α-actinin in a region close to the transmembrane domain where a cluster of basic residues resides (Carpen et al., 1992). Positive charge alone is not sufficient for this interaction, as several other positively charged peptides, when tested, failed to bind to α-actinin. β-tubulin and glyceraldehyde-3-phosphate dehydrogenase also interact with the ICAM-1 cytodomain (Federici et al., 1996). The β-tubulin association raises the possibility that ICAM-1 interacts with microtubules in addition to actin filaments, consistent with the general theme of ICAM-1 association with the cytoskeleton. The potential biologic meaning of the glyceraldehyde-3-phosphate dehydrogenase interaction is not clear. Glyceraldehyde-3-phosphate

dehydrogenase functions in one of the intermediate steps of the glycolysis pathway, converting glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate. This is the first redox reaction of glycolysis and requires NAD^+. Glyceraldehyde-3-phosphate dehydrogenase uses its C-terminal domain to interact with ICAM-1 cytodomain, which is also the site of its NAD+ binding site. Thus, NAD+ could potentially compete with ICAM-1 cytodomain for binding to glyceraldehyde-3-phosphate dehydrogenase. It is therefore possible that ICAM-1 engagement alters cellular metabolism, but this has not been investigated.

Ezrin, a member of the ERM (ezrin/radixin/moesin) family of proteins, which are involved in linking the actin-containing cytoskeleton to the plasma membrane, also binds ICAM-1 (Heiska et al., 1998). This interaction is direct, as determined by plasmon resonance, and depends on the presence of phosphatidylinositol 4,5-bisphosphate ($PtdIns(4,5)P_2$). This is interesting because ezrin and other ERM proteins are downstream components of the rho signaling pathway, which was previously shown to be involved in the ICAM-1-mediated formation of actin stress fibers. Since rho regulates phosphatidylinositol 4-phosphate 5-kinase, which synthesizes $PtdIns(4,5)P_2$, rho could control ezrin-ICAM-1 interactions as well (Chong et al., 1994).

1.5 ICAM-1 as a Pathological Basis of Disease

ICAM-1 has been implicated in a vast array of human diseases ranging from the common cold to cancer. Some examples of these ICAM-1-mediated diseases are as follows:

1.5.1 Infectious diseases:

Rhinoviruses have been described as the single most important etiologic agent of the common cold (Bella et al., 2000). The mechanism of their entry into human cells has been pieced together using a variety of ICAM-1/HRV fragments for crystallographic studies as well as electron micrography. The small icosahedral virus must first bind ICAM-1 (most serotypes of the human rhinovirus (HRV) use ICAM-1 as the surface receptor for entry into human nasal epithelial cells), which causes a conformational change in the virus that allows for uncoating of the protein capsid and injection of the RNA genome into the cytoplasm (Casasnovas, 2000). Rhinovirus attachment is confined to the BC, CD, DE, and FG loops of the amino-terminal Ig-like domain 1 (the end furthest from the membrane). Since domain 1 differs considerably between human ICAM-1 and -2, there is no HRV interaction with ICAM-2 (Bella et al., 1999).

Malaria caused by the most virulent of Plasmodia, P. falciparum, has a very unique property. Erythrocytes infected with P. falciparum gain the ability to bind vascular endothelial cells in an ICAM-1-mediated fashion at a site distinct from the LFA-

1 binding site, probably contributing to the clinical symptomatology of the disease as a result (Berendt et al., 1989; Ockenhouse et al., 1992).

1.5.2 Cancer:

The relationship between ICAM-1 and melanomas has been examined extensively. At first, it was thought that ICAM-1 could serve as a marker of progression to malignancy, since ICAM-1 expression increases as melanocytes transform into melanomas (Johnson et al., 1989). This is paradoxical because T-cell/target cell interactions are, in part, ICAM-1 mediated, potentially increasing the likelihood of T cell recognition. However, melanomas actually shed ICAM-1, generating a soluble form that can competitively inhibit leukocyte adhesion. In fact, soluble ICAM-1 and anti-CD11a monoclonal antibody equally inhibit the leukocyte-melanoma interactions (Becker et al., 1995). It has been proposed that, through leukocyte adhesion, melanoma cells can spread hematogenously, eventually adhering to the endothelium and diapedesing (Simmons, 1995).

ICAM-1 levels have been studied in both acute and chronic forms of lymphocytic leukemia. ICAM-1 expression in acute B-lymphocytic leukemia (ALL) is undetectable (Moller et al., 1993), while chronic lymphocytic leukemia (CLL) lymph nodes sections are strongly ICAM-1^+ (Csanaky, 1994). Therapy for CLL and hairy cell leukemia often includes interferon-α which induces ICAM-1 and is thought to provide clinical benefit by increasing the targeting of these cancers by cytotoxic T cells (Marotta et al., 1993).

Among other B-lymphoid cancers, higher ICAM-1 expression levels appear to correlate with relatively benign disease, whereas lower ICAM-1 levels appear to portend a less favorable prognosis (van de Stolpe et al., 1996). This is certainly the case for both Hodgkin's and non-Hodgkin's lymphomas. T-lymphoid cancers are more difficult to characterize relative to ICAM-1 expression since no trends have been identified to date. However, a recent report described the protective effects of ICAM-1 knockout on the dissemination of malignant T lymphoma cells (Aoudjit et al., 1998).

1.5.3 Autoimmune Diseases:

Several autoimmune disorders have been studied in terms of their ICAM-1 profiles, including rheumatoid arthritis, vasculitis, type-1 diabetes, sarcoidosis, lupus nephritis, autoimmune glomerulonephritis, autoimmune thyroid disease, and autoimmune hepatitis (van de Stolpe et al., 1996). ICAM-1 knockout mouse models have been useful in this arena. In these models, absence of ICAM-1 produces a dramatic increase in mouse survival with a normally rapidly fatal form of systemic autoimmune disease, characterized by glomerulonephritis and vasculitis, similar to severe cases of systemic lupus erythematosus (Bullard et al., 1997).

1.5.4 Atherosclerosis:

Since atherosclerosis is an inflammatory disease with chronic and superimposed acute features, the role of ICAM-1 in atherogenesis has been investigated. Monocytes and T cells adhere to the arterial endothelial surface and migrate into the subendothelial

space in animals fed an atherogenic diet, the same pattern seen in histological studies of human arterial plaque sections (Watanabe et al., 1998). In hypercholesterolemic rats, ICAM-1 upregulation is observed primarily in lesion-prone areas of aorta during the early stages of atherogenesis. Increased endothelial ICAM-1 expression was also thought to increase fibrinogen deposition due to fibrinogen's known capacity for binding ICAM-1 on its first Ig-like domain (Duperray et al., 1997) leading to plaque formation (Languino et al., 1993). In a murine venous bypass graft model, the normally observed neointimal hyperplastic response is reduced by 30-50% in ICAM-1 KO mice (Zou et al., 2000).

1.5.5 Septic Shock:

Not surprisingly, ICAM-1 also plays a role in septic shock, due to lipopolysaccharide's strong induction of various cytokines, e.g., IL-1 and TNFα, as well as ICAM-1 itself. These inflammatory cytokines induce the augmented expression of ICAM-1 and thereby increase neutrophil (through β2 integrins) adherence and fibrinogen binding. Increased neutrophil extravasation causes vascular hyperpermeability, edema, and septic shock (Xu et al., 1994), while fibrinogen has been implicated as a causative factor in disseminated intravascular coagulation (DIC) (van de Stolpe et al., 1996).

1.5.6 Neurological Disease:

An increasing number of studies are being performed on neural systems with respect to ICAM-1 expression. Besides studies of ICAM-1 intracellular signaling in astrocytes and glial cells mentioned previously, studies are being conducted on diseased

neural tissue as well. In one study, the differential expression of ICAM-1 in the β-amyloid containing plaques of Alzheimer brains demonstrated a striking amount of ICAM-1 immunohistochemical staining in granular and Purkinje cell plaques vs. those in the molecular layer (Verbeek et al., 1996).

1.5.7 Transplantation:

In acute allograft rejection, recipient T cells recognize transplanted, allogeneic tissue by way of T cell receptor-MHC (plus peptide) interactions. This primary allorecognition is insufficent to generate the full amplitude of alloactivation responses. Adhesion molecules and costimulatory receptor-ligand interactions are also required. ICAM-1 can serve both adhesion strengthening and costimulatory ligand functions. Through the engagement of its counter-receptor LFA-1, several mechanisms are triggered which amplify T cell activation (Wang et al., 1998). In the course of events preceding the actual placement of the graft in the recipient, ischemia followed by reperfusion of the grafted tissue can induce ICAM-1 expression on graft endothelium. When given as intraperitoneal injections four weeks after small intestine transplantation in mice, anti-ICAM-1 and anti-LFA-1 antibodies prolong allograft survival. (Kato et al., 1996). Six weeks after cessation of antibody treatments, these mice have normal mucosa without signs of rejection. This not only has implications for the parenchymal tissue within the graft, but also for its vasculature, since transplant-associated arteriosclerosis remains an obstacle to long-term graft survival. In a mouse allogeneic carotid artery graft model, neointima formation is reduced by 52%, 33%, and 38%, in ICAM-1, MHC II, or MHC I

plus MHC II KO mice, respectively, (Shi et al., 1999). ICAM-1 antibodies also reduce postsinusoidal WBC adherence following ischemia and reperfusion, in a rat liver transplantation model (Meyer et al., 1998).

1.6 TAXREB107

Human T cell leukemia virus (HTLV) type 1 is the etiological agent involved in two human diseases: Adult T cell leukemia (ATL) and HTLV-1-associated myelopathy / tropical spastic paraparesis (HAM/TSP) (Yoshida et al., 1995). Although the exact mechanisms of disease causation by HTLV-1 have not been entirely worked out, the pathological basis of these diseases is partially dependent on a viral protein named tax for its transactivating properties. HTLV-1 actually has two important gene regulatory proteins, tax and rex (Inoue et al., 1987). Tax was first shown to activate transcription of viral genes upon viral entry into the cell by binding to an enhancer in the LTR (long terminal repeat) region of the viral genome containing an imperfect triplicate of a 21-bp element (Shimotohno et al., 1986; Brady et al., 1987; Rosen et al., 1987). Of interest, each p21 element of the triplicate in the tax-responsive enhancer can be further subdivided into three highly conserved domains (A, B, and C) of approximately 4-5 bps each (Fujisawa et al., 1989). Tax absolutely requires domain B to bind, but the roles of A and C remain in question (Fujisawa et al., 1989). Rex, on the other hand, controls the processing of viral RNA.

Both proteins are vital to the normal replication of HTLV-1, but tax has garnered considerable attention due to its ability to activate human genes as well. In addition to its ability to bind the cAMP response element (CRE)-containing 21-bp enhancer in the long terminal repeat region of the viral genome, tax binds and activates enhancers containing the NF-κB binding site or serum response element (SRE) sites (Ruben et al., 1988). Among the human genes known to be activated by tax are several with known involvement in proliferative responses, e.g., IL-2 receptor α chain (CD25), GM-CSF, c-fos, c-egr (Fujii et al., 1988; Nagata et al., 1989). Activation of CD25 and c-fos are thought to contribute to the first step in the development of ATL (Maruyama et al., 1987). Through interactions with other DNA binding proteins, the gene activating function of tax is extremely versatile. Tax interacts with CRE-binding proteins, cAMP response element modulator (CREM), NF-κB p50, serum response factor (SRF), and the 36 kDa TRE-2 binding protein (Yoshida et al., 1995). Tax can also bind and activate an 11-bp palindromic sequence (TTTCCGGGAAA) homologous to IFN-γ and IL-6 response elements (Owen et al., 1997).

There is relatively little known about individual domains within the tax protein. Tax mutation studies demonstrate that the N-terminus, and possibly some portion in the middle, seems to be important in its ability to bind NF-κB sites and the 21-bp sequence in the long terminal repeat region, but which specific residues are involved and how they disrupt engagement at docking sites are not yet known (Hirai et al., 1992). Other enhancer activators, e.g. Gal4 and herpes virus VP16, contain a cluster of acidic residues which is a critical part of their activation domains (Ptashne, 1988). Tax contains such a

cluster of acidic residues in its C-terminus and deletion of a 69 bp region encompassing the cluster results in loss of DNA-binding capability (Smith et al., 1990).

Since tax does not directly bind DNA, early studies focusing on the nature of tax-DNA interactions were directed at finding proteins that could potentially serve as adaptors. Thus a series of new proteins named tax-responsive element binding (TAXREB) proteins were discovered by looking for binding to HTLV-1 long terminal repeat enhancer sequences. The vast majority of the proteins found belonged to the ATF and CREB families. TAXREB107 was isolated from a Jurkat cDNA library. It is a 288 amino acid molecule that binds to domain C of the tax-responsive enhancer region, instead of domain B as viral tax does (Morita et al., 1993). TAXREB107 is constitutively expressed in a wide range of tissues, and is upregulated beyond basal levels in TPA-activated Jurkat T cells. Sequence analysis of human TAXREB107 shows very little homology with HTLV-1 tax. However, the sequence of TAXREB107 is highly homologous to that of ribosomal protein L6, a protein found in the large subunit, p60, of eukaryotic systems.

Shortly after the discovery of TAXREB107, a 227 amino acid protein named C140 was described. C140 mRNA levels increase dramatically in malignant, transformed rat thyroid FRTL cells compared to nontransformed cells (Ohta et al., 1994). Neoplastic thyroid tissue (papillary carcinoma and adenoma) and thyroid tissue from a Grave's Disease patient contain much greater levels of C140 mRNA compared with the almost negligible levels found in normal tissue. TAXREB107 and C140 share over 90% amino acid sequence homology. Although no further studies have been done on C140, it is

interesting to speculate on its potential functional similarities to tax and in particular, TAXREB107, due to its homology.

In addition to upregulation in neoplastic thyroid tissue, TAXREB107 is an erythropoietin early response gene (Li et al., 1999). A subtractive hybridization strategy has been used on Rascher murine erythroleukemia cells to identify TAXREB107 and confirm the observation with Northern blot analysis, That is, a four-fold increase in TAXREB107 mRNA can be detected after 1 hour of erythropoietin treatment. Finally, TAXREB107 may also have a role in regulating cell growth by binding to both high and low molecular weight forms of FGF-2, as determined by yeast two-hybrid assays (Shen et al., 1998).

Chapter 2--Establishing the Existence of an Interaction Between ICAM-1 Cytodomain and TAXREB107

2.1 Goals and Strategy Design

The aim of this work is to study the role of ICAM-1 as an outside-in signaling molecule. A number of proteins have been shown to be activated upon ICAM-1 stimulation at the cell surface with the majority being downstream components of known signaling pathways. However, because the ICAM-1 cytodomain does not appear to have enzymatic activity, the mechanism by which ICAM-1 engagement leads to any biochemical activation event is unclear. It is thus likely that critical proteins are recruited to the ICAM-1 cytodomain, and that these recruited molecules ultimately transduce the ICAM-1 "outside-in" signals. The few molecules that have been shown to interact directly with ICAM-1 cytodomain are mostly cytoskeletal subunits or adapter proteins for cytoskeletal scaffolds. Although potentially important, it is unlikely that these proteins are the molecular triggers for signal transduction. Therefore, there must be other ICAM-1 cytodomain-interacting proteins. The approach used here began with a general screen for molecules capable of interacting with the ICAM-1 cytodomain, using a whole cell lysate from human umbilical vein endothelial cells, which are known to express ICAM-1. Using the GST pulldown technique, potential ICAM-1 cytodomain binding proteins were identified. After confirming the interactions with alternative techniques, we

established that these novel components can modulate known effector pathways of ICAM-1 stimulation.

In the course of our functional studies, an activating monoclonal antibody, R6.5, along with goat anti-mouse secondary antibodies, were generally used to engage ICAM-1 via "crosslinking". Besides the obvious advantage of specificity against ICAM-1, the primary plus secondary antibody system enables the formation of ICAM-1 microclusters at the cell surface, which are known to effect activation responses through other surface molecules capable of outside-in signaling. In at least one set of experiments, fibrinogen, one of the natural ligands, was also used and produced similar effects as that seen with antibody stimulation.

2.2 Screening for Interactions with ICAM-1 Cytodomain

Previous studies looking for ICAM-1 cytodomain interactions screened whole cell lysates with ICAM-1 cytodomain peptides CNBr-coupled to sepharose beads (Carpen et al., 1992; Federici et al., 1996). Although this provides a relatively pure system, a potential pitfall is that the CNBr activated beads will bind to any primary amino group in the peptide. Since ICAM-1 cytodomain contains an abundance of residues that contain primary amino groups (3 Arg + 5 Lys out of 31 total residues), this method may potentially crosslink only one out of nine peptides at the intended N-terminus. The peptides bound to the beads at any of the intermediate residues may have critical domains blocked, thus decreasing the yield of the pulldown technique.

In light of this, a GST-ICAM-1 cytodomain fusion protein was generated and used to insure the freedom of the cytodomain to interact without impeding any potential functional groups. This system incorporated an ICAM-1 cytodomain at the C-terminal end of the glutathione-S-transferase, so as to leave the C-terminal end of the ICAM-1 cytodomain free as well. The GST-ICAM-1 cytodomain fusion protein was expressed in DH5α E. coli bacteria and purified on glutathione beads. The potential disadvantage of this system is that a number of proteins may be pulled down by virtue of an interaction with GST rather than ICAM-1, thus creating a false positive. However, lysates were precleared on GST-coupled beads before incubation with GST-ICAM-1 cytodomain beads to deplete such proteins. Care was also taken to run an SDS-PAGE analysis of the preclearance with actual pull down samples to make sure that bands of interest were visible in the pull down samples only.

A human umbilical vein endothelial cell (HUVEC) lysate was used since previous literature had shown that this cell line has ICAM-1 signaling capacity (Watson et al., 1995; Sano et al., 1998). The pulldown proteins were analyzed by SDS-PAGE and silver stained according to the Mann protocol (Shevchenko et al., 1996), providing seven total bands of interest, ranging from 32-210 kDa. These bands were subjected to in-gel tryptic digest and eluted from the gel slices. Matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) was performed on the eluted peptide fragments using 100 fmol bradykinin and ACTH clip as internal calibrants. MALDI-MS provides a peak profile based on mass spectrometry of all peptides eluted from each sample, which is then compared with known peak profiles of other proteins in the database. The primary

program used was ProFound, which relied on the OWL database. As a secondary backup algorithm, PeptideSearch (relies on the EMBL/non-redundant database) was used. A 32 kDa band was identified by both algorithms as a human thyroid carcinoma cell protein, C140. Since C140 was predicted to only be 19 kDa in size, unlike the 32 kDa band, this seemed puzzling at first. However, since this technology is relatively new, only 50% of unknown, submitted samples analyzed via MALDI-MS peptide database are identified due to the relatively small size of the database. The fraction of samples identified increases substantially when the protein is from an organism such as E. coli, whose genome has been sequenced. Since the probability score using the primary algorithm, ProFound, was greater than 90%, it stands to reason that perhaps the real identity of the band was that of a protein with strong similarity to C140. A BLAST search subsequently revealed a 32 kDa protein known as TAXREB107 which has over 90% homology with C140 and contains a very similar pattern of tryptic digest sites.

To further confirm the identity of the protein, nanospray MS/MS analysis of the same sample was performed on a Q-Tof mass spectrometer. Due to some difficulties in running the sample through the nanovials, a manual analysis of the match was performed and showed a short potential sequence of EKY at the N-terminal end of one of the tryptic fragments. Considering that this is a tryptic digest, the residue preceding the first amino acid should be either lys or arg. As predicted, KEKY was found in position 237-240 of TAXREB107's amino acid sequence, helping to confirm the identity of the 32 kDa band.

2.3 Confirming the ICAM-1 cytodomain/ TAXREB107 Interaction

After identifying TAXREB107 as a likely ICAM-1 cytodomain-associated protein, the next step was to confirm this molecular interaction by co-immunoprecipitation. The initial approach was to design and make a c-myc epitope (myc-e) tagged TAXREB107 since there are no commercially available anti-TAXREB107 antibodies. A GFP-labeled ICAM-1 cytodomain was also made. The pCMV+TAXREB107 with or without pWAY5+ICAM-1 cytodomain construct was transfected into Chinese hamster ovary (CHO) cells and allowed to express for 24 hours. Figure 1 demonstrates immunoprecipitation of endogenous ICAM-1 followed by Western blotting for c-myc epitope, using whole cell lysates from CHO cells that had been transfected with the pCMV control vector or varying amounts of c-myc-epitope tagged TAXREB107. The anti-ICAM-1 polycolonal antibody H-108 recognizes hamster in addition to human, mouse and rat ICAM-1. Based upon the c-myc epitope blotting of a protein with the appropriate mass (32 kDa), TAXREB107 is easily identifiable in the ICAM-1 immunoprecipitates. The efficiency/amount of the interaction increases with a greater amount of TAXREB107 expressed. Although the transfection efficiency is less than in CHO cells (which is why most of these experiments were performed in CHO), similar results were obtained when expressing c-myc-epitope tagged TAXREB107 in the ICAM-1-positive human endothelial cell line EA.hy926 (data not shown).

To demonstrate more directly that TAXREB107 can become associated with the ICAM-1 cytodomain in cells, c-myc-epitope tagged TAXREB107 and GFP-ICAM-1

were co-expressed in CHO cells, and co-immunoprecipitations were performed in the opposite direction as in Figure 1. That is, TAXREB107 was immunoprecipitated with an anti-c-myc epitope antibody, followed by an anti-GFP antibody. Figure 2 demonstrates a TAXREB107-ICAM-1 cytodomain interaction in the co-transfected cells. GFP alone did not associate with the transfected TAXREB107.

In an attempt to duplicate the protein-protein interaction experiments in a non-transfected system, we generated a rabbit polyclonal anti-TAXREB107 antibody and repeated the co-immunoprecipitation experiments, investigating the endogenous molecules in the EA.hy926 endothelial cell line. Figure 3 demonstrates that this protein A/G-purified polyclonal serum recognizes an endogenous protein that co-migrates with overexpressed recombinant TAXREB107. Figure 4 demonstrates that endogenous TAXREB107 is detectable in ICAM-1 immunoprecipitates, and vice versa. In contrast, TAXREB107 was not detectable in PECAM-1 immunoprecipitates. These three experiments support the GST pull-down finding of an ICAM-1 cytodomain/ TAXREB107 interaction.

2.4 Yeast Two Hybrid Assay

In an attempt to determine whether the aforementioned molecular association is a *direct* protein-protein interaction, yeast two hybrid assays were performed. Plasmid constructs for a GAL4 binding domain-ICAM-1 cytodomain fusion protein and a GAL4

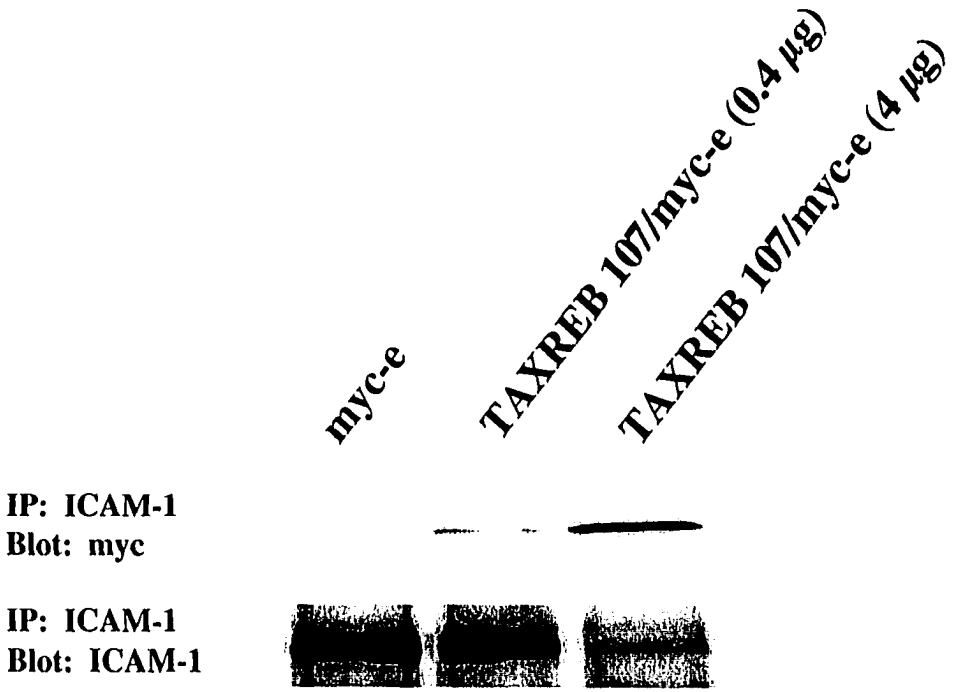

Figure 1. Co-immunoprecipitation of recombinant TAXREB107 with endogenous ICAM-1. CHO cells were transfected with the indicated amounts of myc-e/TAXREB107 plasmid DNA, after which endogenous ICAM-1 was immunoprecipitated. After SDS-PAGE and transfer, the blots were probed first with an anti-myc-e antibody (top panel) and reprobed with an anti-ICAM-1 antibody (bottom panel). myc-e/TAXREB107 and ICAM-1 blotted at the expected masses of 32 kDa and 100 kDa, respectively.

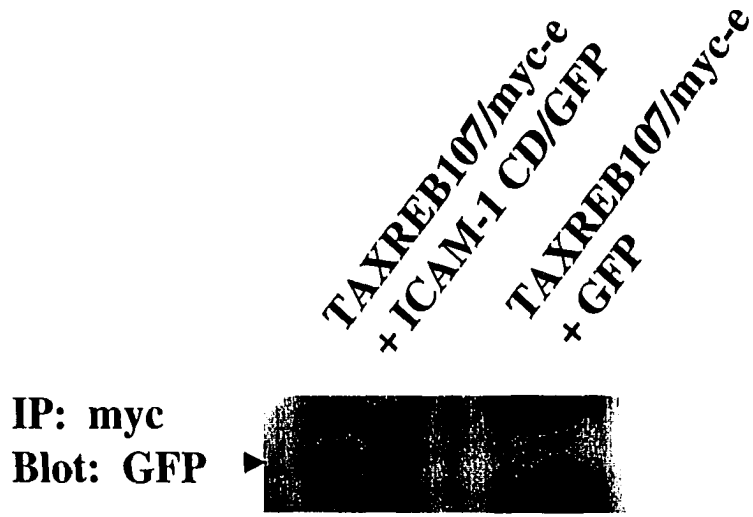

Figure 2. Co-immunoprecipitation of recombinant TAXREB107 with recombinant ICAM-1 CD. CHO cells were transfected with myc-e/TAXREB107 plus GFP/ICAM-1 CD or plus GFP alone, after which TAXREB107 was immunoprecipitated with an anti-myc antibody. After SDS-PAGE and transfer, the blots were probed with an anti-GFP antibody. GFP/ICAM-1 CD blotted at the expected mass of 32 kDa.

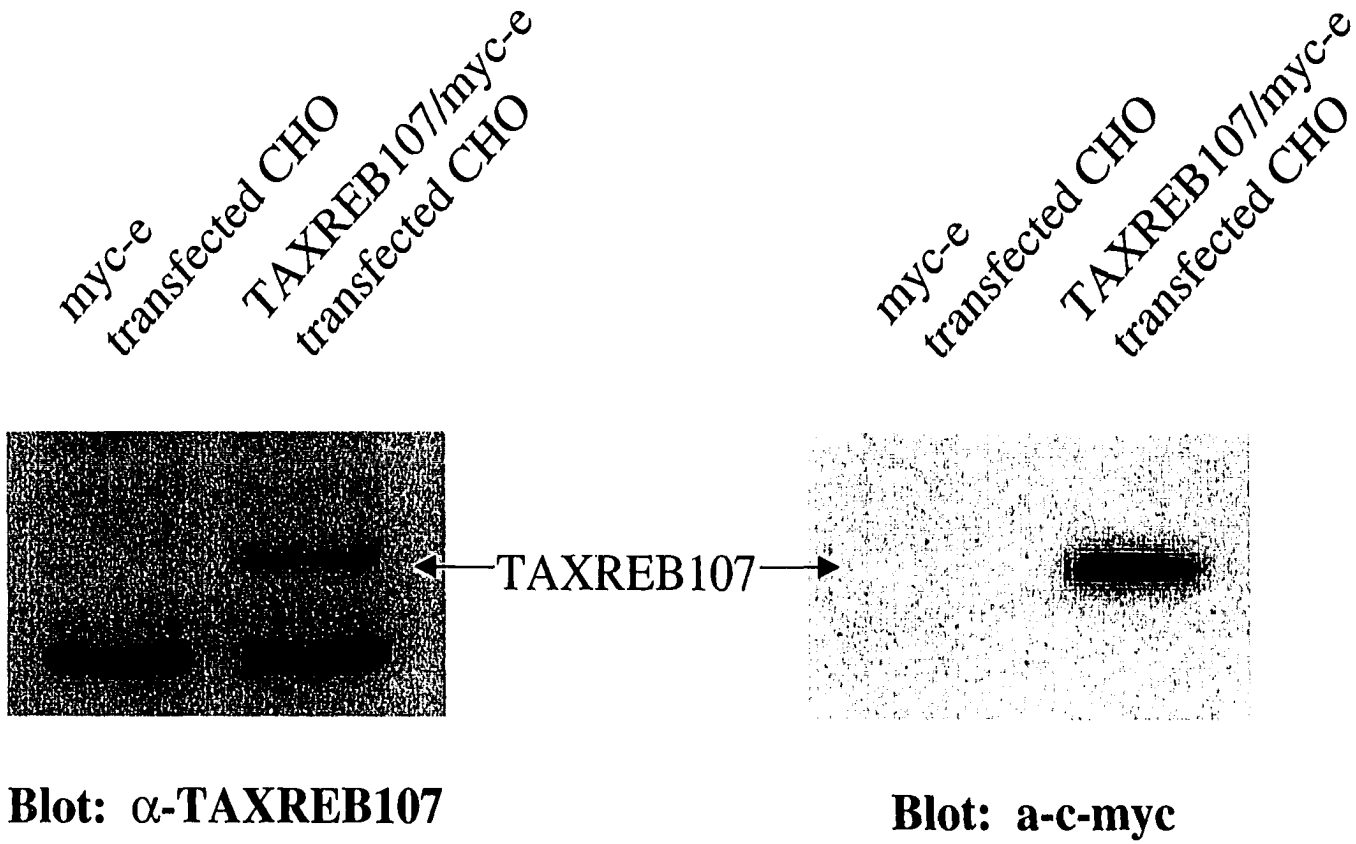

Figure 3. Generation of an anti-TAXREB107 polyclonal Ab. Protein A/G-purified polyclonal antibody, obtained from rabbits immunized with GST-TAXREB107, was used in Western blots with whole cell lysates prepared from CHO cells transfected with c-myc-tagged TAXREB107 (left panel). Blots were reprobed with anti-c-myc antibody (right panel), identifying the co-migrating band and confirming the TAXREB107 antibody specificity.

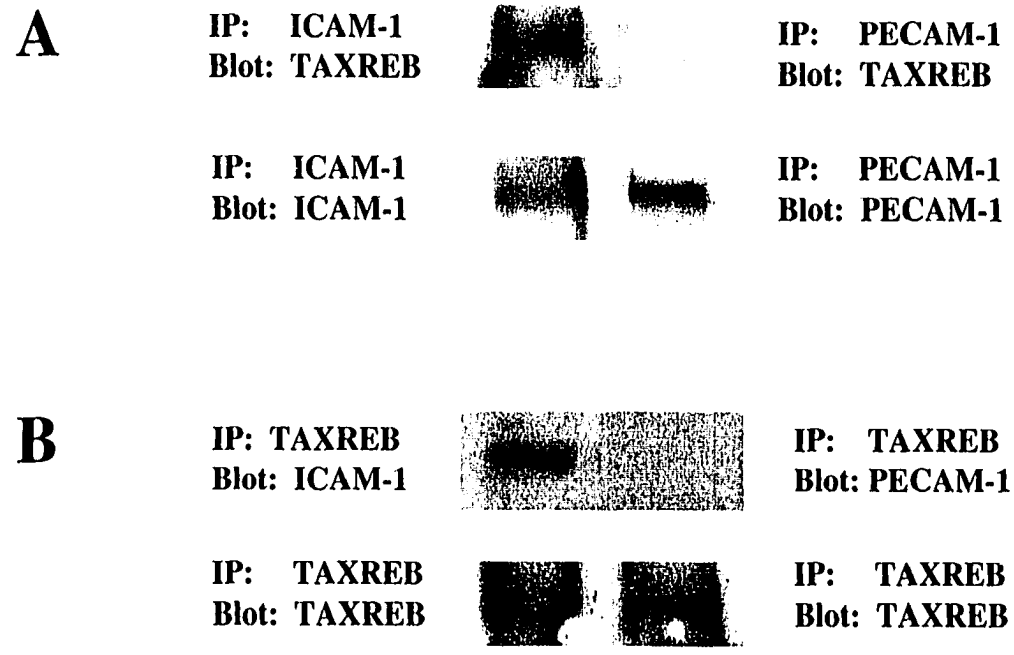

Figure 4. Co-immunoprecipitation of endogenous TAXREB107 with endogenous ICAM-1, but not PECAM-1. (A) Endogenous ICAM-1 or PECAM-1 were immunoprecipitated from EA.hy926 cells. After SDS-PAGE and transfer, blots were probed first with polyclonal anti-TAXREB107 antibody (upper panel), and reprobed with either anti-ICAM-1 or -PECAM-1 antibody. (B) Endogenous TAXREB107 was immunoprecipitated from EAhy.926 cells. After SDS-PAGE and transfer, blots were probed first with either anti-ICAM-1 or -PECAM-1 antibody and reprobed with polyclonal anti-TAXREB107 antibody. ICAM-1, PECAM-1 and TAXREB107 were blotted at the expected masses of 100 kDa, 120 kDa, and 32 kDa, respectively.

activation domain-TAXREB107 fusion protein were made by cloning both ICAM-1 cytodomain and TAXREB107 C-terminal to the DNA-BD and DNA-AD, respectively. These plasmid constructs were then transfected into the Y190 yeast strain for the assay. A twofold screen using both HIS3 and lacZ reporters was used to select for positive clones. Although several colonies did appear on His^- SD plates, subsequent testing of these colonies for β-galactosidase activity proved negative, indicating that a direct ICAM-1 cytodomain/TAXREB107 interaction did not occur. Parallel experiments for positive and negative controls included p53/Large T-antigen and Lamin C/Large T-antigen, respectively. These results suggest an indirect interaction between ICAM-1 cytodomain and TAXREB107, perhaps involving one or more adaptor proteins.

2.5 Discussion

Given the negative yeast two-hybrid assays, it is possible that the proteins of interest are not synthesized normally in yeast, such that their interaction domains may be altered. However, an indirect protein-protein interaction is the more likely explanation. Numerous examples of indirect interactions exist in current working models of signal transduction pathways. Perhaps one of the best known is that of the ras signaling pathway (Boriack-Sjodin et al., 1998). Transmembrane receptors acting through tyrosine kinases activate ras by stimulating the guanine-nucleotide exchange reaction controlled by a protein named Son of sevenless (Sos) which acts as a guanine-nucleotide exchange factor

for ras. Once a ligand binds the transmembrane receptor, the intracellular domain of the receptor is tyrosine phosphorylated, often through a dimerization and autophosphorylation process. Sos is then recruited from the cytoplasm to the phosphorylated receptor with the help of an adaptor protein named Grb2, which has the ability to bind other proteins via its SH2 and SH3 interaction domains. (Rudd, 1999). Grb2 binds constitutively to Sos via an SH3 domain and the Grb2/Sos pair is recruited to the cell membrane upon tyrosine phosphorylation of the receptor. At this point, the SH2 domain of Grb2 is now capable of binding to the receptor. This recruitment brings Sos adjacent to ras and promotes the exchange of GDP for GTP, thereby activating ras.

It is conceivable that an adaptor protein such as Grb2 could directly bind the ICAM-1 cytodomain and mediate interactions with other proteins. Careful examination of the ICAM-1 cytodomain amino acid sequence reveals that amino acids # 519-525 (approximately the middle portion of the cytodomain) not only fits the basic requirement of an SH3 ligand domain (e.g., PXXP motif), but actually resembles a particular class of SH3 domain ligands. In particular, the pattern KGXPXXP is found in both ICAM-1 cytodomain and the amphiphysin (I and II) SH3 ligand consensus sequence (Micheva et al., 1997). Amphiphysin is a protein intimately involved in endocytosis. By binding to partners such as endophilin, dynamin, and synaptojanin, it helps to mediate the fission of endocytic vesicles in nerve terminals. An SH3-mediated binding interaction with the ICAM-1 cytodomain has not been demonstrated. We performed *in vitro* binding assays using various candidate fusions proteins composed of GST-SH3 domains from Endophilin-1, -2, -3, lyn kinase, fyn kinase, PI3K, and c-src. None of these SH3 domain

peptides bound to the ICAM-1 cytodomain peptide. Since SH3 domains are known to bind with low affinity (Kd = 1-200 μM) (Nguyen et al., 1998), plasmon resonance was employed to increase the sensitivity of detection. However, this also failed to reveal any interactions between the ICAM-1 cytodomain and the several candidate SH3 domains tested. It remains possible that among the multitude of known and currently unknown SH3 domains, a binding partner for this putative SH3 ligand exists.

Chapter 3 -- Enhancement of ICAM-1-mediated MAPK Activity by TAXREB107

3.1 Establishing a Cell Line for Examination of TAXREB107 Effect on ICAM-1-mediated MAPK Response

Previous literature has established the existence of MAPK activation responses in various cell types after ICAM-1 stimulation, including the B cell lymphoma line A20 (Holland et al., 1997), and primary human umbilical vein cells (HUVEC) (Sano et al., 1998). Since neither of these cell lines is easily and highly transfectable, it would be convenient for MAPK assays to find other cell lines with higher levels of transfection efficiency and that also have low basal levels of resting MAPK activity. In an effort to find other cell lines possessing these qualities, a number of cell lines were screened, including Chinese hamster ovary carcinoma (CHO), Hela (human cervical epithelial line), and COS (monkey kidney line). Of the lines tested, all had relatively high transfection efficiencies, but only CHO consistently showed large amounts of TAXREB107 expression when transfected and also displayed relatively low basal MAPK activity.

3.2 Confirmation of R6.5 Monoclonal Antibody Binding to CHO Cells

As described above, CHO cells are a convenient model to work with due to their ease of transfectability, rapid growth rate, and low MAPK background levels during the resting state. However, since there are no commercially available anti-hamster ICAM-1 antibodies, we evaluated whether R6.5 (BIRR01, Enlimomab), an anti-human-ICAM-1 murine IgG_{2a} monoclonal antibody, previously shown to have ICAM-1 activating properties in human cells, could recognize hamster ICAM-1 on CHO cells. ICAM-1 engagement by R6.5 on human neutrophils has been shown to upregulate CD11b expression, decrease L-selectin expression, and increase oxidative burst. When considering the likelihood that R6.5 could recognize hamster ICAM-1, it was encouraging that R6.5 recognizes human Ig-like domain #2 (Diamond et al., 1990), which has high interspecies homology. When initially testing R6.5 reactivity on CHO cells by flow cytometry, all the stainings were negative (data not shown). However, because a signal-transducing molecule can be membrane-expressed at a level below the detection threshold for FACS, yet remain a competent signaling molecule when engaged by ligand, we utilized a fluorescence amplification system. By using protein A-conjugated, 0.4 μm diameter, fluorphore-containing microspheres (fluospheres), hamster ICAM-1 (R6.5 reactivity) was easily detectable above background (Fig. 5, IRR=isotype-matched anti-LFA-3 and anti-CD3 controls) on CHO cells (Fig. 5). Thus, it appeared reasonable to expect an activation response to monoclonal antibody R6.5 in CHO cells.

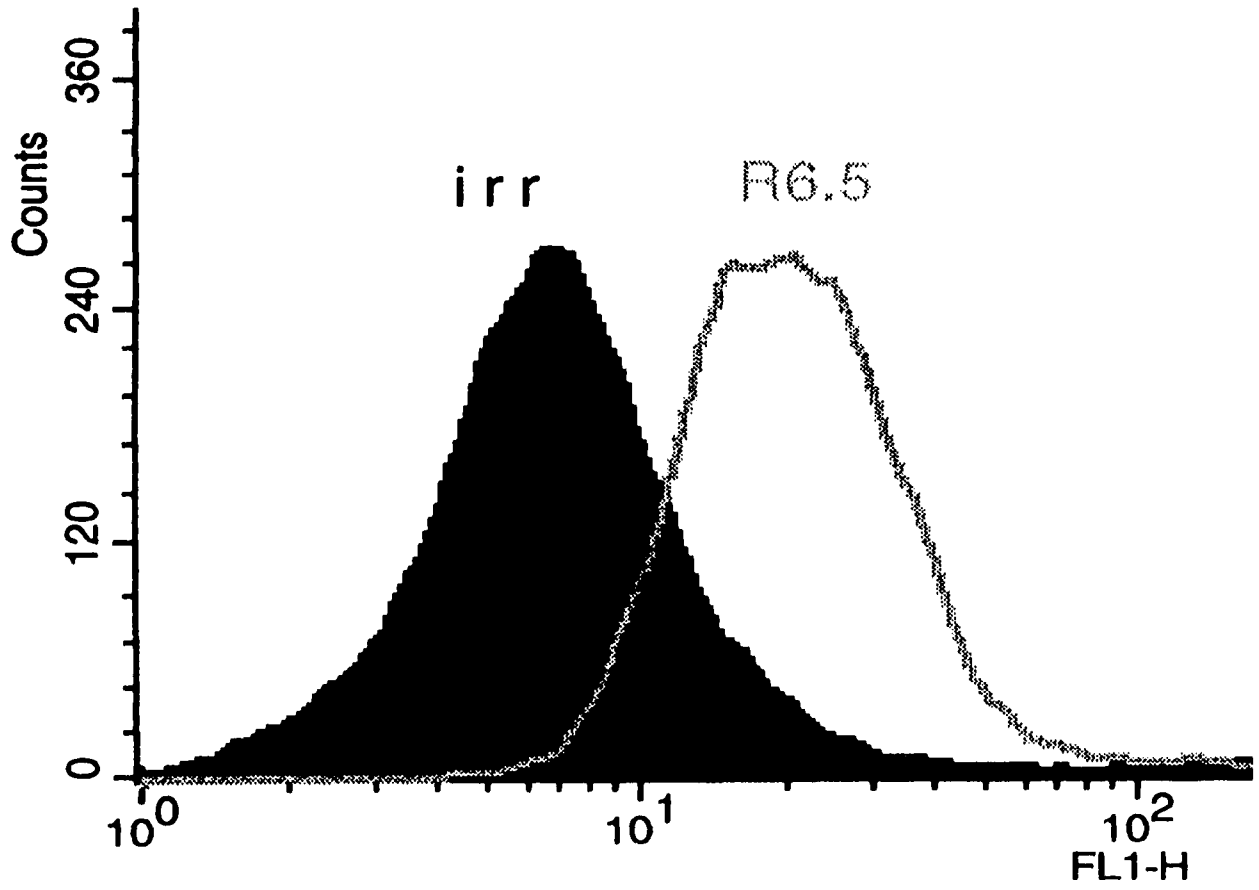

Figure 5. Flow cytometric analysis of mAb R6.5 recognition of CHO cells using fluospheres. CHO cells were incubated with R6.5 followed by staining with protein A-conjugated fluospheres. Irrelevant (IRR) controls included isotype-matched murine mAbs TS 2/9 (anti-human LFA-3) and OKT3 (anti-human CD3). 5000 cytometer-acquired events were analyzed per sample.

3.3 TAXREB107 Enhances ICAM-1-mediated ERK 1/2 Activity

To address whether the TAXREB107/ICAM-1 cytodomain interaction has any effect on ICAM-1-mediated signaling, a series of MAPK (ERK1 and 2) activation experiments were performed in R6.5-stimulated, vector control or TAXREB107-transfected CHO cells. Cells were transfected with either a GFP control, GFP-ICAM-1 cytodomain, or c-myc-epitope tagged TAXREB107 plasmids. After overnight transfection, cells were either treated with only goat anti-mouse secondary antibody alone (0 min, unstimulated), or treated with R6.5 + goat anti-mouse antibody for 1 or 5 minutes. Cell lysates were then immunoprecipitated with activation-specific anti-phospho-ERK 1/2 antibody and the immunoprecipitates were incubated with an Elk-1 peptide substrate. Figure 6 demonstrates a phospho-Elk-1 Western blot, in which after 5 min of ICAM-1 engagement in the setting of only control GFP expression, phospho-Elk-1 is detectable (lane 3), i.e. ERK activation occurs. This ICAM-1-mediated activation event was greatly augmented in TAXREB107-transfected cells (lane 9). In some experimental samples, recombinant ICAM-1 cytodomain was expressed, in an attempt to compete for cytodomain interactions, and thus, impart a "dominant negative" effect. In this set of experiments, recombinant ICAM-1 cytodomain expression abrogated the ICAM-1-mediated ERK activation (fig. 6, lane 6) at 5 min. It is important to note that, at the 5 min time point, ERK activation was variably induced by ICAM-1 engagement alone. However, the combination of ICAM-1 engagement and TAXREB107 expression always activated ERK 1 and 2 (see below). In an attempt to duplicate the above findings

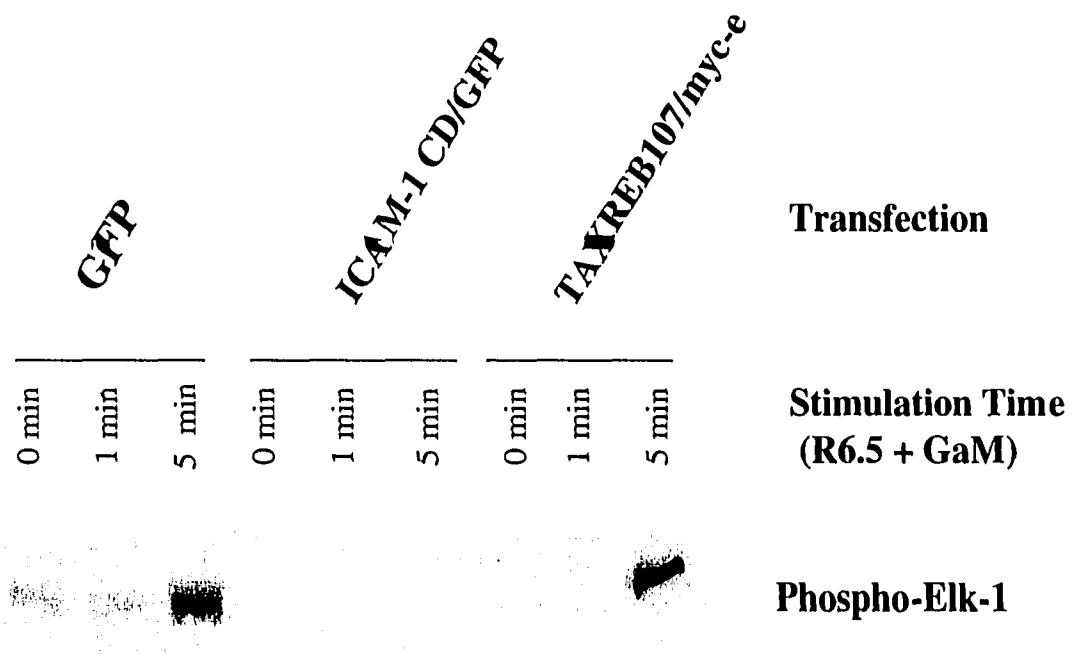

Figure 6. *In vitro* ERK assay in R6.5-stimulated cells. CHO cells were transfected overnight with plasmids for the indicated proteins, then stimulated for 0, 1 or 5 min using R6.5 α-ICAM-1 Ab + GaM Ab. *In vitro* kinase assays were performed on immunoprecipitated active Erk 1/2 by adding the Erk 1/2 substrate Elk-1 in the presence ATP. Phospho-Elk-1 Western blot is shown.

with a natural ICAM-1 ligand (as opposed to antibody-mediated activation), fibrinogen, which binds to Ig-like domain-1 of ICAM-1 (Duperray et al., 1997), was used. Figure 7 demonstrates that at the 5 min time point, fibrinogen induces ERK 1/2 activation. This was also inhibited by expression of recombinant ICAM-1 cytodomain (data not shown). The MAPK response to fibrinogen was augmented in TAXREB107-transfected cells. These results are absolutely analogous and parallel to those obtained with R6.5, i.e. antibody-mediated ICAM-1 clustering, and suggest that the TAXREB107-ICAM-1 cytodomain interaction is necessary for these potentially important MAPK responses.

3.4 ICAM-1 cytodomain Cotransfection Blocks TAXREB107 Enhancement of ICAM-1-mediated MAPK Response

Inhibition of the synergistic ICAM-1/TAXREB107-mediated ERK activation response with recombinant ICAM-1 cytodomain expression would further support that these molecules interact at the level of the ICAM-1 cytodomain. Figure 8 demonstrates a potent, 5 min induction of ERK 1/2 activation with the combination of ICAM-1 engagement and TAXREB107 expression. This activation is completely inhibited by concurrent expression of the ICAM-1 cytodomain (fig. 8, last lane). This finding rules out the possibility that antibody-mediated ICAM-1 crosslinking is simply recruiting other signaling molecules which, through their cytoplasmic tails, are triggering ERK activation. We cannot be certain that other important signaling molecules are not recruited but, at the very least, these recombinant cytoplasmic domain expression experiments

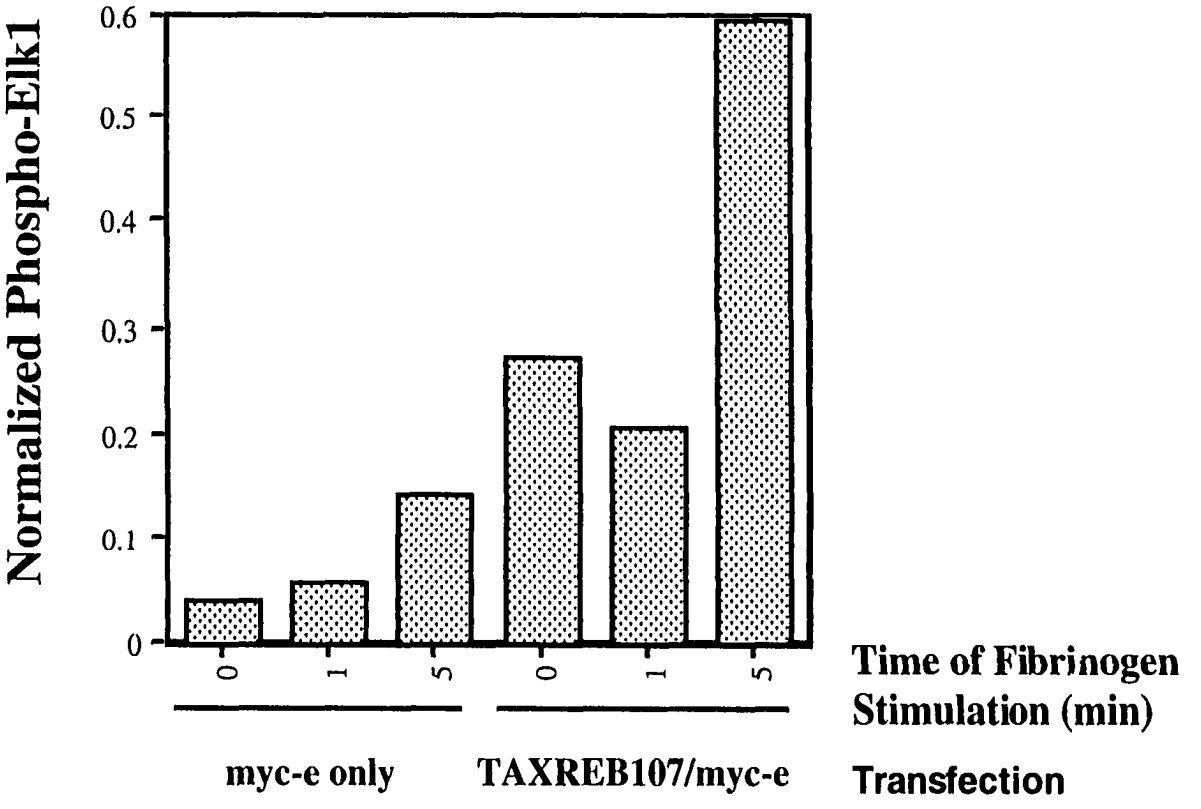

Figure 7. *In vitro* ERK assay in fibrinogen-stimulated cells. CHO cells were transfected overnight with plasmids for the indicated protein and stimulated with fibrinogen, a natural ligand for ICAM-1. *In vitro* ERK assay was performed as described in Figure 6. Phospho-ELK-1 signals were densitometrically normalized to IgG heavy chain to account for slight differences in the amount of IP Ab added.

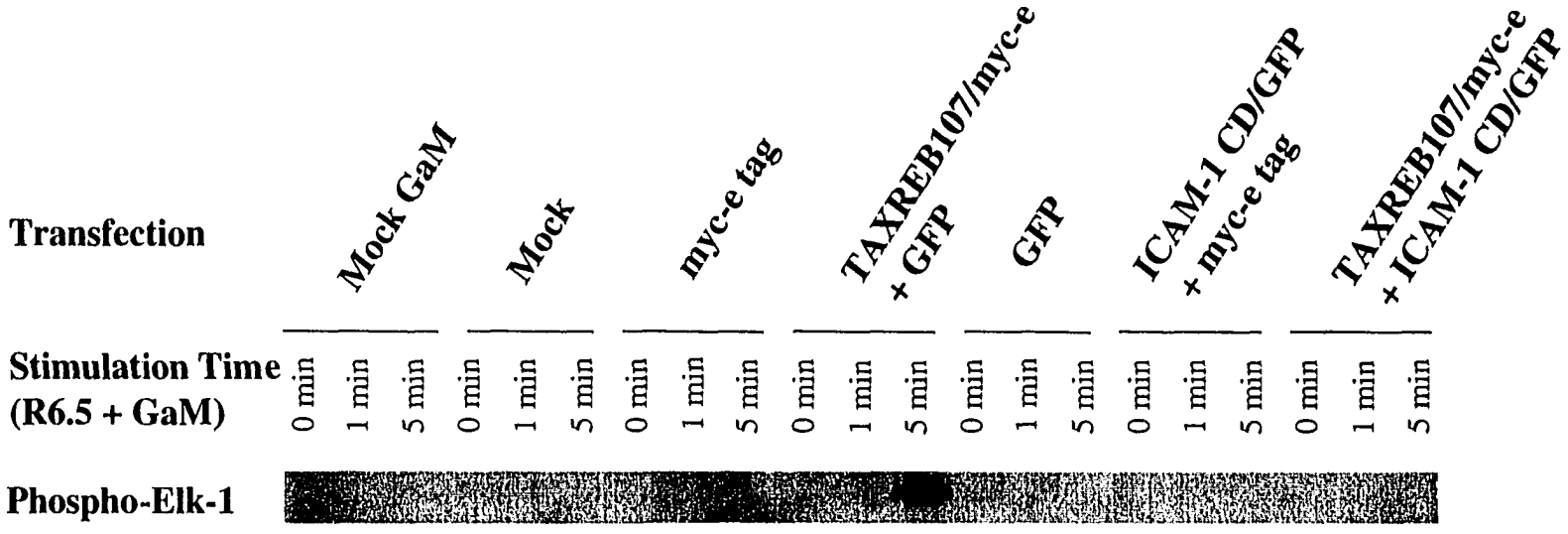

Figure 8. Effect of ICAM-1 CD expression on TAXREB107-enhanced, ICAM-1-stimulated ERK activation. CHO cells were transfected overnight with plasmids for the indicated proteins and stimulated for the noted time with R6.5 α-ICAM-1 +GaM Ab (except Mock GαM which was stimulated with GαM Ab alone), then assayed for *in vitro* ERK activity as described in Fig. 6. Cells labeled "Mock" underwent the same transfection protocol as other samples, but no plasmid was added.

confirm that ICAM-1, itself, is primary to the signaling event, especially given that other adhesion-molecule cytodomain overexpressions are not inhibitory (see below).

3.5 ICAM-1-mediated MAPK Activation in the Human Cell Line, HT-29

Although CHO ICAM-1 was clearly recognizable by R6.5 (fig. 4), attempts were made to duplicate the above findings in a human, ICAM-1 positive cell line, since R6.5 was generated against human ICAM-1. HT-29, a human colonic epithelial carcinoma cell line expresses R6.5-detectable (by FACS) membrane ICAM-1 without the requirement for fluorescence amplification, as in CHO cells. Figure 9 displays an ERK 1/2 activation assay performed by Western blot detection of activation-specific, phospho-ERK-1 and -2, instead of the kinase assays displayed in figures 6, 7, and 8. At 5 min of ICAM-1 engagement (R6.5 stimulation) in TAXREB107-transfected cells, activated ERKs were easily detachable. As in CHO cells, this prominent activation was abolished by cotransfection with the ICAM-1 cytodomain. Thus, although transfection and MAPK experiments could be done more efficiently in CHO cells, identical results were obtained in a human cell line.

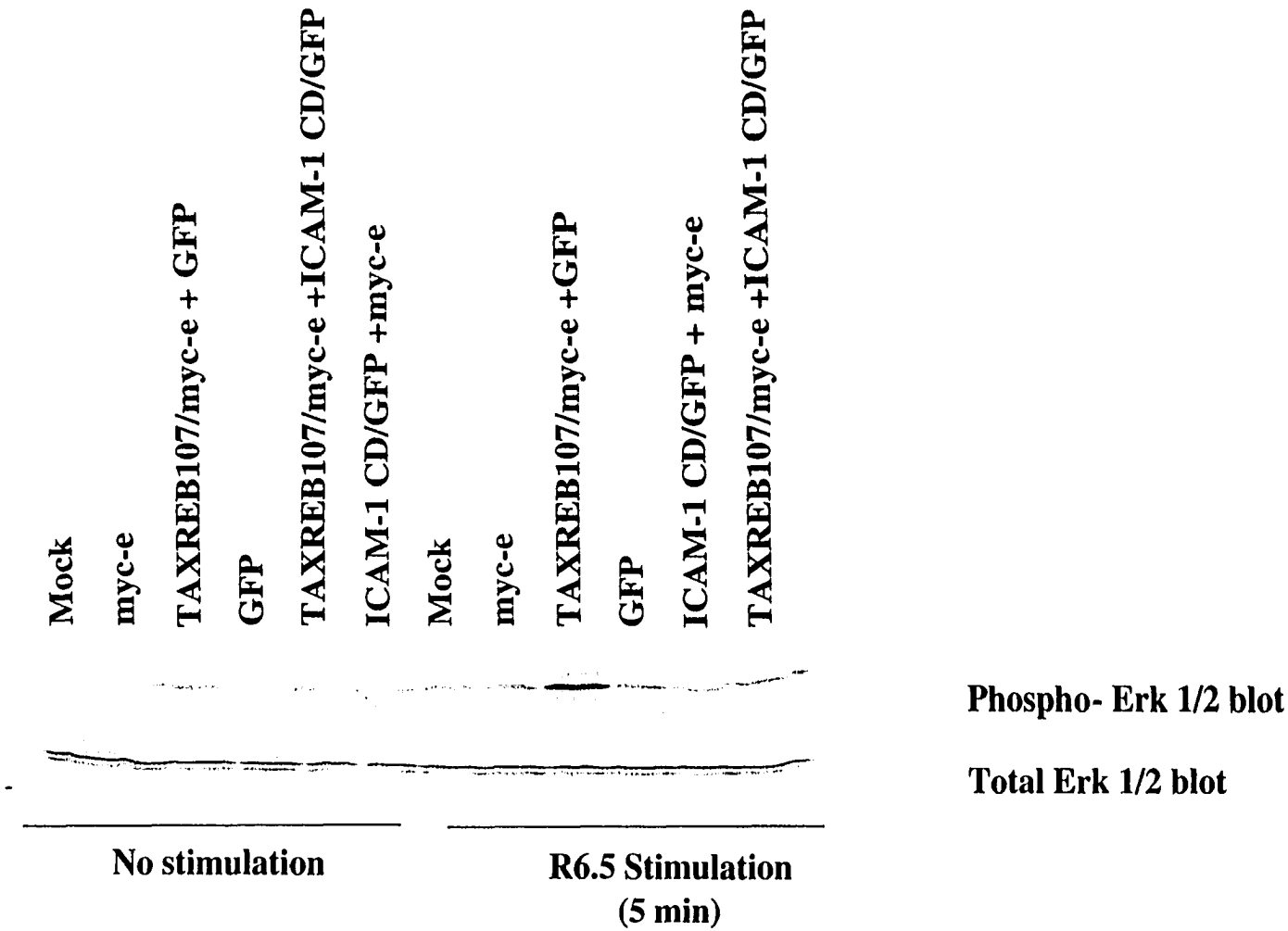

Figure 9. ICAM-1-mediated ERK activation in HT-29 human colonic carcinoma cells. HT-29 cells were transfected overnight with plasmids for the indicated proteins, then stimulated with R6.5 for 5 min. Western blotting for activated, phospho-ERK 1/2 (upper panel) was performed, followed by reprobing for total ERK 1/2 (lower panel).

3.6 VCAM-1 Cytodomain Cotransfection Does Not Block TAXREB107 Enhancement of ICAM-1-mediated MAPK Response

Vascular cell adhesion molecule (VCAM-1) is an endothelial cell adhesion molecule and is also a member of the immunoglobulin superfamily, similar to ICAM-1 in structure. Both possess relatively long extracellular domains containing multiple, in-series Ig-like domains, a single transmembrane region, and a very short cytoplasmic tail (Pigott et al., 1993). Due to the similarities in overall structure, the VCAM-1 cytodomain was used as a negative control for the ICAM-1 cytodomain in inhibition experiments. A plasmid construct was made for a GFP-VCAM-1 cytodomain fusion protein with the same approach as that used to make the GFP-ICAM-1 cytodomain fusion protein, i.e., insertion of VCAM-1 cytodomain sequence downstream of GFP in the pWAY5 vector. Figure 10 demonstrates, again, that ICAM-1 engagement (R6.5 stimulation for 10 min) in TAXREB107-transfected CHO cells results in a prominent ERK 1/2 activation, as detected in phospho-ERK 1/2 Western blotting. This activation was completed abrogated by cotransfection of the ICAM-1 cytodomain but *not* the VCAM-1 cytodomain, confirming specificity of the inhibitory effect. This further supports the importance of ICAM-1's cytoplasmic domain involvement in the noted signaling responses.

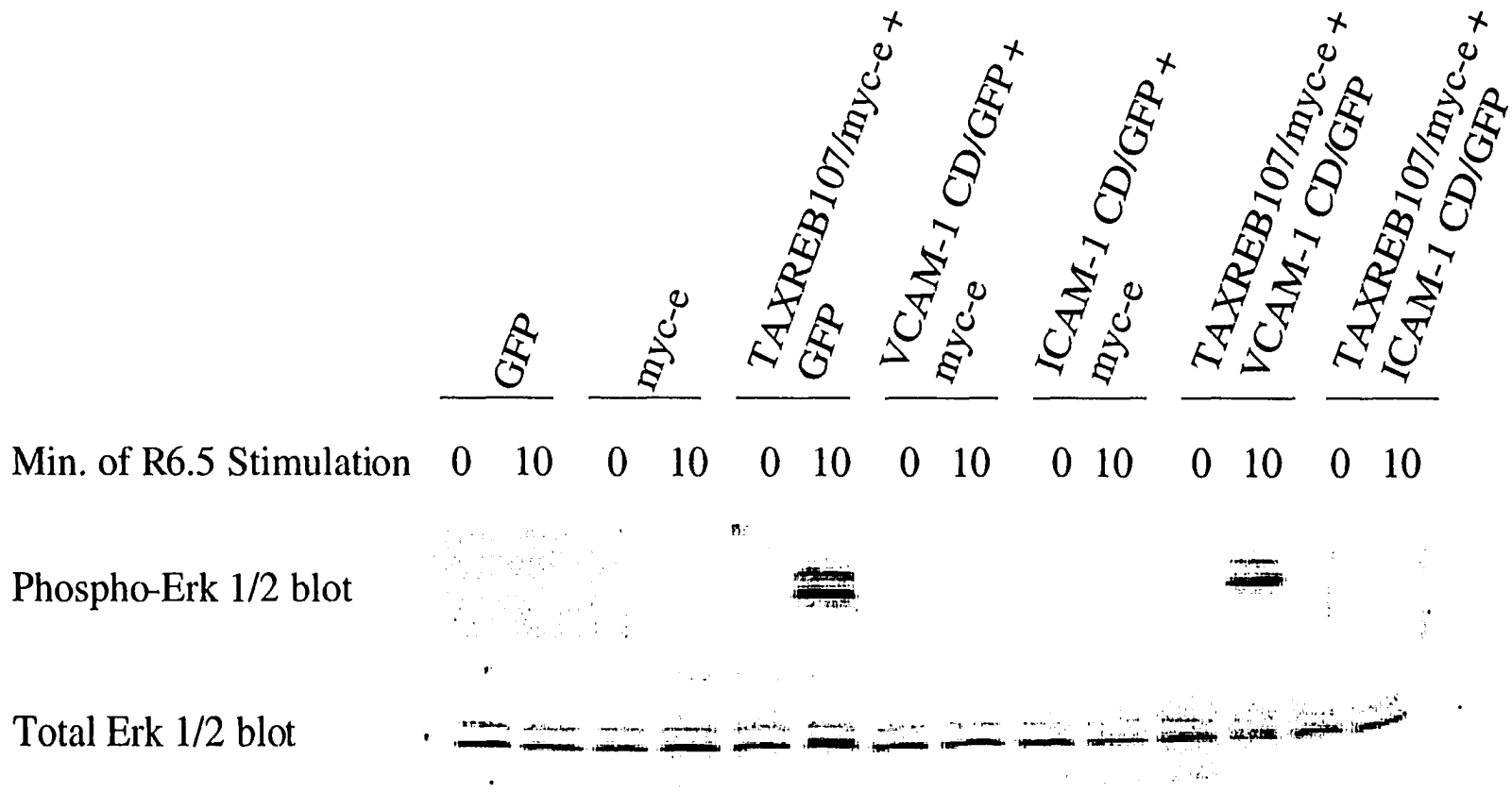

Figure 10. Effect of VCAM-1 CD on TAXREB107-enhanced ICAM-1-mediated ERK activation. CHO cells were transfected overnight with the plasmids for the indicated proteins and stimulated with R6.5 plus GaM Ab. Western blotting for activated, phospho-ERK 1/2 was performed (upper panel), followed by reprobing for total ERK 1/2 (lower panel).

3.7 ICAM-1-deficient Cell Line 2FTGH Lacks an R6.5-induced MAPK Response

As another important control for the ICAM-1 specificity of the R6.5 plus TAXREB107-induced responses, a series of experiments were performed in an ICAM-1-negative variant of the human fibroscaroma line 2FTGH. Both conventional FACS staining and use of fluosphere amplification yielded negative results with this line (data not shown). Figure 11 demonstrates that, despite a competent ERK activation response to phorbol 12-myristyl 13-acetate (PMA), neither R6.5 stimulation alone, nor R6.5 stimulation in TAXREB107-transfected cells resulted in 2FTGH ERK activation. TAXREB107 transfectants expressed levels of myc-epitope-TAXREB107 similar to that achieved in the CHO and HT-29 experiments, as detected by myc-epitope Western blotting (not shown).

3.8 Discussion

These experiments reproducibly demonstrate that TAXREB107 overexpression is capable of enhancing the MAPK activation response resulting specifically from ICAM-1 engagement, and that the ICAM-1 cytodomain is involved in this potentiating effect. Many *in vitro* targets of MAP kinases have been described. At this time, we do not know which major classes of downstream effectors are activated by ICAM-1 stimulation. Examples of MAPK targets include nuclear transcription factors, metabolic enzymes, cytoskeletal proteins, and other signaling proteins (Lewis et al., 1998). Of

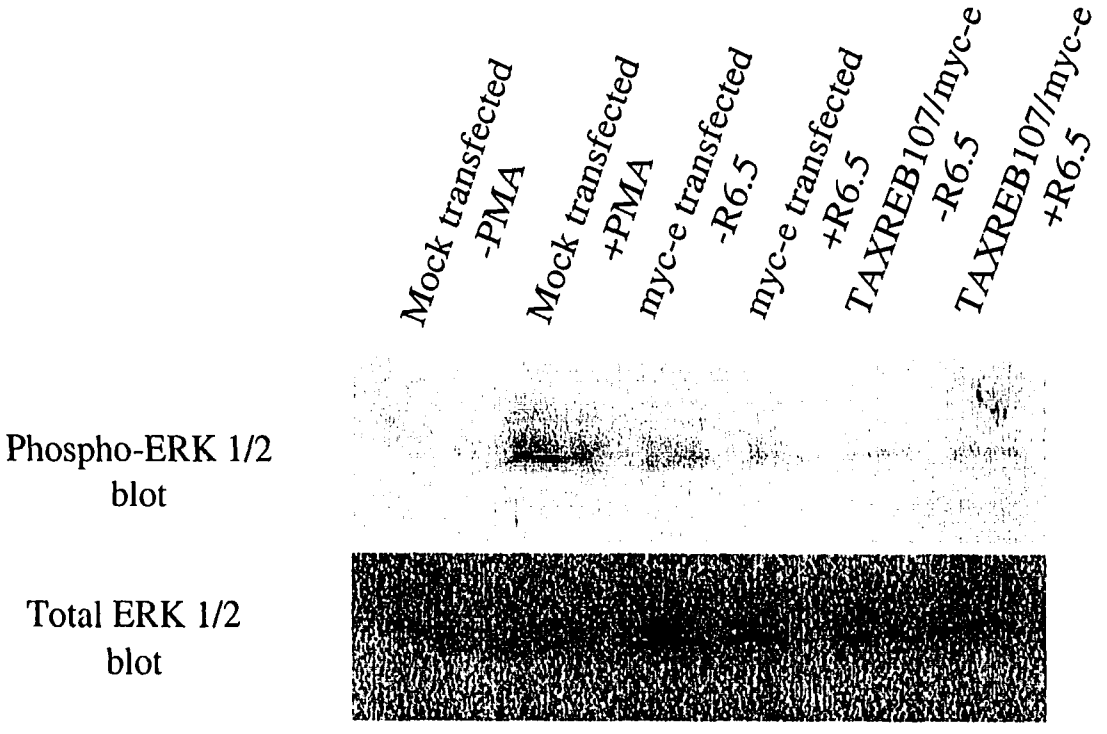

Figure 11. Effect of anti-ICAM-1 R6.5 stimulation in an ICAM-1-deficient cell line. 2FTGH fibrosarcoma cells were transfected overnight with plasmids for the proteins indicated, then treated for 5 min with R6.5 (or antibody control) plus GaM Ab. Alternatively, cells were mock transfected (no DNA) and activated with PMA (1.62 nM) for 5 min. Western blot for activated phospho-ERK 1/2 was performed (upper panel), followed by reprobing for total ERK 1/2 (lower panel).

note, a family of protein kinases called MAPKAP kinases (MAPK-activated protein kinases) was among the first group of proteins shown to be activated by ERKs. MAPKAPs activate numerous transcription factors including cAMP response element-binding protein (CREB), CREB-binding protein (CBP), c-fos, Nur77, and serum response factor (SRF) (Lewis et al., 1998). There is a remarkable similarity between this list of transcriptional activators and those activated by viral tax. As mentioned in the introduction, viral tax is capable of activating CREB, c-fos, and SRF. It is tempting to speculate that, despite the lack of significant sequence homology between viral tax and human TAXREB107, they may serve similar functional roles, either through involvement in enzymatic cascades, and/or direct gene activation (see below). One of the major transcription factors activated by viral tax is NF-κB, but this apparently is not activated by the ERK signaling pathway (Bocco et al., 1996). We have begun to investigate whether other MAPK family members are activated by ICAM-1 engagement, with or without TAXREB107 interaction. Preliminary JNK/SAPK activation experiments in ICAM-1 stimulated CHO cells have been negative. The p38 pathway was shown to be activated by ICAM-1 crosslinking in rat astrocytes (Lee et al., 2000).

ERK activation has also been shown to mediate cytoskeletal events. This may be an important, ICAM-1-mediated alternative mechanism to regulate cytoskeletal rearrangement. Addition of active ERK to cell-free extracts of Xenopus oocytes results in microtubule polymerization (Gotoh et al., 1991). In addition, neuronal cell function can be altered by activated ERKs, in that ERK phosphorylates microtubule-associated proteins 1/2, Tau, and synapsin I (an actin-binding protein involved in cross-linking

synaptic vesicles with the cytoskeleton (Lewis et al., 1998). To date, no ERK-mediated cytoskeletal studies have been performed in endothelial cells, which have generally focused on rho-activated pathways (Adamson et al., 1999; Hirase et al., 2000).

Chapter 4 -- TAXREB107 Translocates to the Nucleus After ICAM-1 Stimulation

4.1 Cellular Localization of TAXREB107

There are recent reports of ICAM-1 modulation of gene expression, including activation of the ICAM-1 gene itself. Although we have described above how TAXREB107 and ICAM-1 interact to induce a rapid signaling event (ERK activation), it remains possible that, even in this ICAM-1-stimulated context, TAXREB107 has an additional role, specifically in gene expression. Viral tax can bind to NF-κB DNA elements (Ruben et al., 1988). Since TAXREB107 can bind tax-responsive elements, perhaps if TAXERB107 is located in the nucleus after ICAM-1 engagement, it could be involved in NF-κB-dependent gene activation, e.g., in the ICAM-1 gene itself.

TAXREB107 has only recently been cloned and its subcellular localization has not been defined. In order to evaluate TAXREB107's subcellular localization, with and without ICAM-1 stimulation, c-myc-epitope tagged TAXREB107-transfected CHO cells were R6.5 or isotype-matched control antibody + Goat anti-mouse secondary antibody treated for 30 min, fixed, permeabilized and stained with a rhodamine-conjugated anti-c-

myc-epitope monoclonal antibody. As negative controls, rhodamine-conjugated irrelevant antibody staining in TAXREB107-transfected cells, and vector control (without TAXREB107)-transfected cells both yield negligible background staining (Figure 12). Figure 13 demonstrates a diffuse, cytoplasmic TAXREB107 distribution in TAXREB107-transfected, non ICAM-1-stimulated cells. In addition, there may be a small amount of punctate, intranuclear (possibly nucleolar) staining. Upon stimulation for 30 minutes, a majority of TAXREB107 translocates to the nucleus (Figure 14). These data demonstrate, for the first time, the subcellular localization of TAXREB107, and that it rapidly translocates to the nucleus upon cell activation. This also raises the possibility that one of the consequences of ICAM-1 engagement could be to promote nuclear localization of a protein that, although not defined as such, may be an important transcription factor.

4.2 TAXREB107 Sequence Analysis

Analysis of TAXREB107 sequence reveals the presence of a bipartite nuclear localization signal (NLS) in the N-terminal portion of the molecule at positions 14-31 and 70-87. However, control of nuclear import is not dependent purely on the presence of an NLS. Rates of nuclear import have been shown to be dependent on two other important factors: nuclear membrane-associated proteins and phosphorylation of sequences within or flanking the NLS. A classic example is the NLS of SV40 Large T antigen (Dingwall et al., 1991). When this NLS (aa 126-132) and the 15 bps of sequence upstream (aa 111-

Figure 12. Negative control immunofluorescence staining for TAXREB107 localization studies. CHO cells were transfected overnight with myc-epitope alone, fixed, permeabilized, and stained with a rhodamine-conjugated α-myc-e Ab. 400X magnification.

Figure 13. TAXREB107 Cellular Localization In Unstimulated CHO cells. CHO cells were transfected overnight with TAXREB107/myc-e, fixed, permeabilized, and stained with a rhodamine-conjugated α-c-myc mAb. 400X magnification.

Figure 14. TAXREB107 Cellular Localization In ICAM-1 Stimulated CHO cells. CHO cells were transfected overnight with TAXREB107/myc-e, stimulated with R6.5 anti-ICAM-1 mAb for 30 minutes, fixed, permeabilized, and stained with a rhodamine-conjugated α-c-myc mAb. 400X magnification.

125) are fused with β-galactosidase, nuclear import of the fusion protein occurs. The fifteen base pair upstream sequence contains several Ser/Thr phosphorylation sites that are substrates for casein kinase II. When these sites are mutated, fusion protein uptake is slowed dramatically. TAXREB107 localization could be under control of the same enzymatic events. Within the downstream element of the bipartite nuclear localization signal (aa 78-81), there is a casein kinase II phosphorylation site. Also, there is a protein kinase C phosphorylation site directly upstream (aa 64-66) of this same bipartite NLS. Either or both of these phosphorylation sites could play a role in the nuclear import of TAXREB107 after ICAM-1 stimulation. It is also possible that some other, non-kinase activation event is required to conformationally alter TAXREB107 from a three-dimensional configuration that conceals its NLS, to one in which it is exposed. It is tempting to speculate that TAXREB107 may be sequestered in the cytoplasm by its interaction with the ICAM-1 cytodomain, and translocates upon release or conformational change induced by stimulation.

In an attempt to visualize the translocation of TAXREB107 from the cytoplasm to the nucleus in real time and without the requirement for fixation and antibody staining, a GFP-TAXREB107 fusion construct was made and transfected into CHO cells. The goal was to track TAXREB107 translocation by recording images of live cells immediately after ICAM-1 engagement. However, when the fusion protein was expressed, it was invariably found in the nucleus, even prior to R6.5 stimulation. Addition of R6.5 caused no change in the nuclear localization of GFP-TAXREB107.

Why the fusion protein constitutively localizes to the nucleus is unclear at this time, although problems with cellular localization have been observed with other GFP fusion proteins (Cubitt et al., 1995; Gerdes et al., 1996). When CHO cells were transfected with GFP alone, the majority of the GFP remained cytosolic, with a small amount residing in the nucleus. It is possible that GFP causes a conformational change in TAXREB107, especially since its NLS is in the N-terminal portion of the molecule. Attachment of GFP to the N-terminus of TAXREB107 may have resulted in a conformational exposure of the NLS, and enhanced nuclear import.

4.3 Discussion

It is intriguing that a DNA-binding molecule such as TAXREB107 affects the activation of a MAPK pathway. Other known examples of DNA-binding proteins interacting with surface molecules include the STAT (Signal Transducers and Activators of Transcription) family of proteins. The STATs work in a novel way to transduce signals from cytokine receptors by going directly from their membrane-associated location to DNA, without any intermediate kinase cascade components as seen with many other outside-in signaling pathways. A basic model of this pathway is as follows (Leonard et al., 1998): cytokine receptors such as IL-2 and IL-15 receptors interact constitutively with Janus kinases (Jaks). The presence of the appropriate cytokine ligands activates the Jaks which phosphorylate tyrosine residues on the receptor. The STATs, which reside in the cytosol in their latent form, possess SH2 domains (phospho-tyrosine recognizing

domains) that are able to bind the phosphorylated cytokine receptor, to which they are recruited. Jaks then phosphorylate the STATs which form homo or heterodimers, translocate to the nucleus and bind specific DNA target sequences.

It is interesting to speculate on the mode of action of TAXREB107 after ICAM-1 stimulation, for it seems that there are both similarities to and differences from the mechanism by which STAT molecules transduce signals. One major difference is that TAXREB107 can enhance the phosphorylation of ERK 1/2, suggesting that TAXREB107 can interact with other cytosolic molecules after initial ICAM-1 stimulation, rather than go directly to the nucleus as STATs do to activate specific genes. On the other hand, the observation that a DNA-binding protein such as TAXREB107 may bind the ICAM-1 cytodomain in an inactive state implies that it may act like a STAT. Both begin their signal transducing pathway at the cell membrane and end in the nucleus, likely at a specific promoter site, except that TAXREB107 may interact with the MAPK pathway at some intermediate point. It is not known whether TAXREB107 forms dimers in order to translocate to the nucleus.

If TAXREB107 does indeed translocate to the nucleus and activate gene expression, it would be interesting to see what these targets are. TAXREB107 was discovered using a screen for proteins capable of binding to a specific stretch of DNA previously shown to act as a binding site for the HTLV-1 transactivator tax. It stands to reason that perhaps genes that could be upregulated by viral tax, may also be targets for TAXREB107. One of the earliest known tax-regulated genes is the IL-2 receptor α-subunit (CD25), a protein critically involved in the proliferative T cell response (Ruben et

al., 1988). A short segment within the CD25 promoter, GGGGAATCTCCC, interacts with viral tax. We tested the possibility that ICAM-1 stimulation could upregulate CD25 expression in Jurkat T cells. CD25 transcriptional activation was first tested using both RT-PCR and a CD25 promoter/luciferase reporter system. Although there were positive experiments, neither experimental system produced sufficiently consistent results that allowed us to make a conclusion. FACS analysis using anti-CD25 antibodies was also performed with similar inconsistent results. In light of the MAPK enhancement activity seen in cells stimulated with ICAM-1 antibodies after transfecting with TAXREB107, we measured CD25 expression after transfection with TAXREB107. Unlike the MAPK effect, TAXREB107 expression did not significantly change CD25 levels in ICAM-1 stimulated cells.

Chapter 5 -- Conclusion and Future Studies

5.1 Summary

In the work described here, the recently discovered molecule, TAXREB107, was determined to interact with the ICAM-1 cytoplasmic domain. As one of the few instances where a non-cytoskeletal associated protein was found to bind the ICAM-1 cytodomain, these studies describe a novel mechanism involving the proximal ICAM-1 signaling pathway. Initially discovered by the GST pulldown technique followed by MALDI spectrometry, the ICAM-1 cytodomain/TAXREB107 interaction was then confirmed by co-immunoprecipitation studies. An attempt to establish whether the interaction is direct, using the yeast two-hybrid assay, suggested that it may not be. However, lack of reporter expression in these studies does not exclude the possibility of known pitfalls in the yeast two-hybrid system, including those involving nuclear import. Therefore, it may very well be that the ICAM-1 cytodomain/TAXREB107 interaction is direct, but was not detectable by the yeast two-hybrid assay.

We have not defined the exact role of TAXREB107 in ICAM-1-mediated signaling. It may well be one of several (or many) proteins that can directly or indirectly interact with the short cytodomain of this important cell adhesion molecule. In our studies, we examined TAXREB107's effect on a known downstream ICAM-1-mediated signaling event, ERK activation. TAXREB107 overexpression in CHO cells was able to

significantly enhance the ICAM-1-mediated ERK activation response. It is important to note that this induction occurred only upon ICAM-1 engagement, and not in non-activated cells, even when TAXREB107 was overexpressed. In an effort to demonstrate the ICAM-1 specificity of this event, recombinant GFP-ICAM-1 cytodomain fusion protein was co-expressed with TAXREB107 in CHO cells, resulting in complete inhibition of the MAPK activation after ICAM-1 engagement. A control GFP-VCAM-1 cytodomain fusion protein was unable to block the ICAM-1-mediated MAPK response, demonstrating ICAM-1 specificity.

Indeed, the cellular function of TAXREB107 is currently unknown, although there is evidence that it can translocate to the nucleus due to the presence of a bipartite nuclear localization signal. Once inside the nucleus, it can potentially bind specific DNA sequences as shown by its ability to interact with the long terminal repeat region of the HTLV-1 genome. For the first time, we now demonstrate that TAXREB107 has a predominantly cytoplasmic distribution in non-activated cells, but rapidly translocates to the nucleus upon ICAM-1 engagement. Mechanisms governing this translocation require additional study.

5.2 Model of ICAM-1 Signaling Through TAXREB107

Undoubtedly, there remains much work to be done to sort out the molecular details of the ICAM-1/TAXREB107 interaction and its potential role in a novel signaling pathway. Based on the information presented in this work, we can, however, provide a

hypothetical model describing the role of TAXREB107 in the proximal portion of the ICAM-1 signaling pathway (Figure 15). In this model, the ICAM-1 cytodomain binds to TAXREB107 either directly or via adaptor proteins in the unstimulated state. This may be one of the cytosolic anchors by which TAXREB107 is localized to the cytoplasm rather than the nucleus, despite its possession of a bipartite nuclear localization signal. The interaction with the ICAM-1 cytodomain (and other cytosolic proteins) may promote a conformation that either conceals its NLS, or dramatically slows the rate of its nuclear import. When ICAM-1 is engaged at the cell surface, TAXREB107 detaches from ICAM-1. Whether it leaves with one or more adaptor proteins or alone has yet to be determined. In either case, it now has the ability to activate ERK 1/2, probably indirectly, since sequence analysis of TAXREB107 does not reveal the presence of any known kinase domains. Another possibility would be that, upon ICAM-1 dissociation, the ICAM-1 cytodomain can then interact with other cytosolic proteins, creating a signaling scaffold leading to ERK activation. Although possible, this is less likely because the profound potentiation of ERK activation by TAXREB107 overexpression cannot be explained in this fashion. Finally, TAXREB107 translocates across the nuclear membrane, presumably to activate specific genes whose promoter regions resemble the TAX responsive enhancer found in the HTLV-1 long terminal repeat region. Potential candidate genes for TAXREB107-mediated activation have yet to be determined.

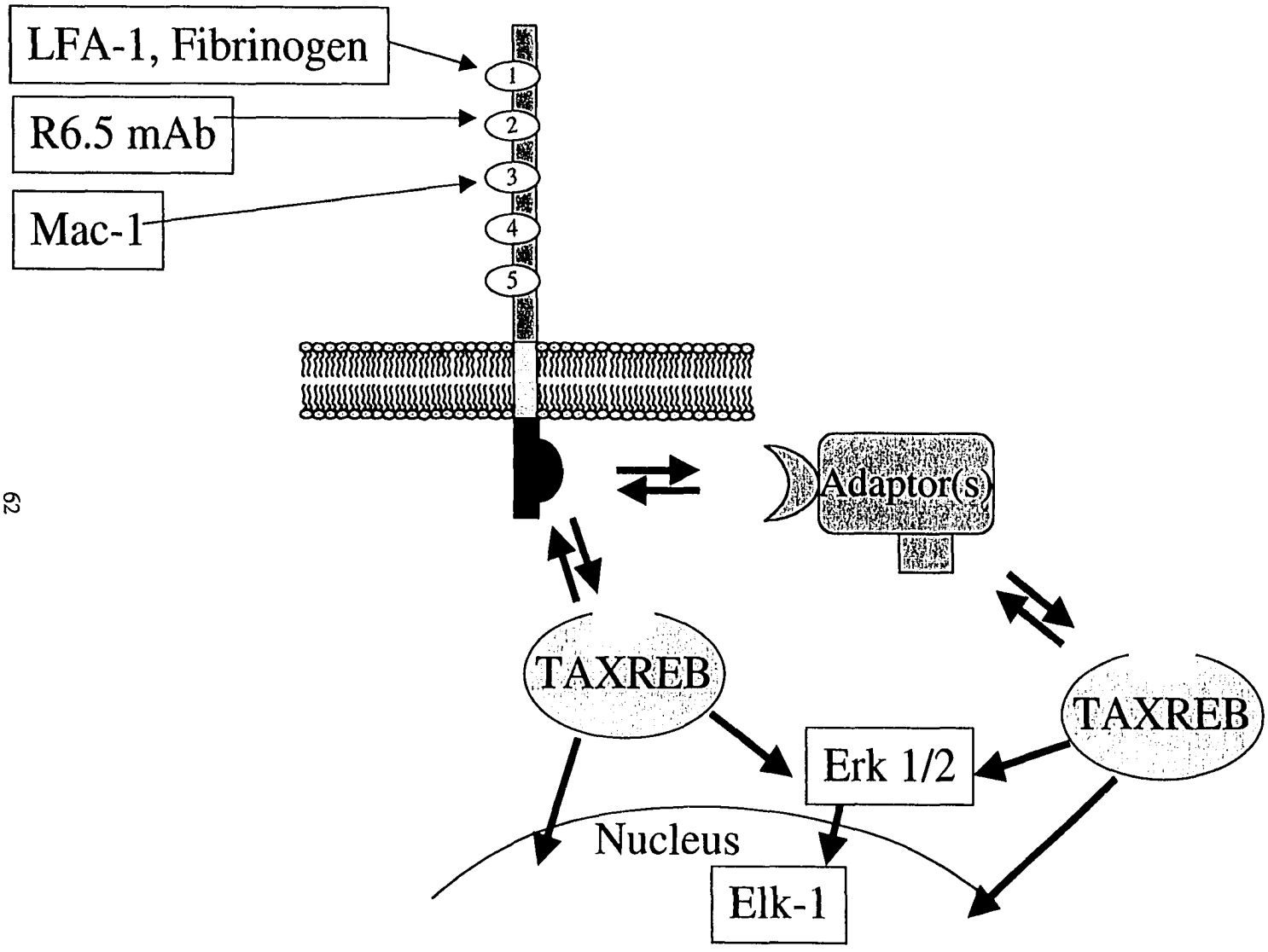

Figure 15. Theoretical model of an ICAM-1-mediated signaling pathway showing either a direct or indirect ICAM-1 CD/TAXREB107 interaction, leading to Erk 1/2 activation and TAXREB107 translocation to the nucleus.

5.3 Future Studies

Future studies will include pinpointing the critical sequences in TAXREB107 and ICAM-1 cytodomain that mediate their interaction, screening for adaptor molecules that promote the indirect TAXREB107/ ICAM-1 cytodomain association, screening for TAXREB107-modulated genes, and defining further cellular/functional effects of ICAM-1 engagement, especially those that require the noted TAXREB107 interaction.

Mutagenesis studies will be performed to pinpoint the sequences/domains within TAXREB107 and the ICAM-1 cytodomain that are required for the molecular interaction. TAXREB107 has a stretch of charged residues that may be important for ionic interactions with the basic region of the ICAM-1 cytodomain. One may either start with mutagenizing these regions or systematically proceed with deletions of TAXREB107 portions in series. With regard to the ICAM-1 cytodomain, it contains an SH3 consensus binding sequence which includes the classical PXXP motif preceded by a basic residue at the –2 position. Although a number of SH3 domains were found to be non-interactive with this region, these were but a few of hundreds of possible SH3 domains. It is possible that an SH3-containing adaptor protein requires this region to mediate the ICAM-1/TAXREB107 binding interaction. The short length of the ICAM-1 cytodomain is an advantage. Since only 30 residues are involved, it is entirely possible to perform an alanine scanning experiment on the entire ICAM-1 cytodomain amino acid sequence. The recombinant mutants will be screened in ICAM-1 co-immunoprecipitation and ERK activation assays.

Defining the associated/adaptor proteins that mediate the indirect interaction between TAXREB107 and ICAM-1 cytodomain could provide insight into their dissociation after ICAM-1 surface engagement. Also, because TAXREB107 does not have any known kinase domains, it may interact with another kinase that is capable of phosphorylating ERK 1/2, perhaps Raf or MEK. Thus, future experiments will include screening for other TAXREB107-interacting proteins, initially by the yeast two-hybrid approach. One potential difficulty, however, besides inherent to the technique, is that some interactions may take place only very transiently and thus may not be easily detectable. In order to define these interactions, it may be necessary to perform time series co-immunoprecipitation studies, looking for the transient associations occurring after ICAM-1 stimulation.

Future plans also include to search for TAXREB107-modulated genes. As described above, TAXREB107 was first discovered as a DNA-binding protein that recognizes domain C of the HTLV-1 long terminal repeat. Planned approaches include differential analysis with gene chip array and utilizing the ever-expanding genome database to systematically search for potential TAXREB107-activatable genes based on identifying sequences similar to its known binding site.

Finally, plans also include to examine the effects of TAXREB107 expression on a variety of described ICAM-1-stimulated cellular responses, both ERK-dependent and –independent. These include endothelial cell proliferation, junctional complex formation, alterations in permeability and regulation of the actin cytoskeleton.

Chapter 6 Materials and Methods

Cell lines

The Chinese hamster ovarian cancer cell line (CHO) was obtained from the American Type Culture collection (Rockville, MD). CHO cells were cultured in F12/Ham. HT-29 human colonic carcinoma cells were kindly provided by Dr. David Rimm and cultured in DMEM. Fibrosarcoma cell line 2FTGH, obtained from the Cleveland Clinic Foundation, was cultured in high-glucose DMEM. All culture media were supplemented with 10% FBS, 1 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate (all from Life Technologies, Gaithersburg, MD).

Antibodies and Chemicals

Mouse anti-human ICAM-1 monoclonal antibody (clone R6.5) was protein A purified from ascites generated in our laboratory. Goat anti-mouse IgG (H+L) was purchased from Zymed Laboratories, Inc. (San Francisco, CA). Rabbit anti-human ICAM-1 polyclonal antibody (clone H-108) and mouse anti-c-myc epitope monoclonal antibody (clone 9E10) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-TAXREB107 antibody was produced by injecting rabbits with full-length TAXREB107 protein (made first as a GST fusion protein) and protein A purifying the serum. Anti-ACTIVE MAPK polyclonal antibody (α-phospho-Erk 1/2) was purchased from Promega (Madison, WI). Anti-total Erk 1/2 polyclonal antibody was purchased from New England Biolabs (Beverly, MA). Anti-GFP monoclonal antibodies were

obtained from Clontech (Palo Alto, CA). Rhodamine-conjugated anti-c-myc monoclonal antibody was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Protein A-conjugated Fluospheres were purchased from Molecular Probes (Eugene, OR). Secondary goat anti-mouse antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Fibrinogen was purchased from Sigma (St. Louis, MO).

Anti-TAXREB107 Antibody

Polyclonal anti-TAXREB107 antibody was generated by immunizing rabbits with a purified GST-TAXREB107 fusion protein (prepared from lysed DH10B cells) coupled to Freund's adjuvant. This polyclonal antibody reacted specifically against a c-myc-tagged full length TAXREB107 produced in CHO cells, but not against the c-myc tag itself. Briefly, CHO cells transfected with a plasmid containing c-myc tagged TAXREB107 were lysed in sample buffer and the whole cell lysate was analyzed by Western blotting with 10% SDS-PAGE. Total immunoglobulin was purified from rabbit serum with Protein A agarose beads and used to probe the blot in a 1:1000 dilution in 5% nonfat milk TBST. After several washes in TBST, a secondary goat-anti-rabbit-HRP antibody from Jackson Immunologicals was used at 1:10,000 in 5% nonfat milk TBST.

GST Pulldown

GST-ICAM-1 cytodomain fusion protein was bound to glutathione-conjugated agarose beads (Sigma, St. Louis, MO). GST alone was conjugated to glutathione beads as a negative control. A human umbilical vein endothelial cell lysate was run over the bead

columns and washed 3X with lysis buffer. Beads were then boiled in a reducing buffer, run on 10% SDS-PAGE, and silver stained. Protein bands present in the GST+ICAM-1 cytodomain sample, but not in the negative control, were excised, tryptic-digested and sent out for mass spectrometry analysis to determine the protein identity.

MALDI Spectrometry

Bands isolated by GST pulldown were subjected to in-gel tryptic digests and sent for matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) at Yale University's William Keck facility for identification by match-up of the peak profile against known peak profiles using two different search algorithms on two separate databases (OWL and EMBL).

Co-immunoprecipitation

Depending on whether endogenous or recombinant proteins were being examined, lysates were made from either transfected CHO cells or non-transfected EA.hy926 cells. Lysates were obtained from transfectants 16 hr after transfection. Lysis buffer consisted of 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X-100, 2.5 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 μg/ml leupeptin, 1 tablet Boehringer-Mannheim protease inhibitor tablet per 10 mL. Immunoprecipitation was performed with 2 μg antibody/400 μg protein at 4°C O/N with gentle rocking, plus 50 μl Protein A/G sepharose slurry (Santa Cruz Technologies, Santa Cruz, CA) for 1 hr at 4°C. Immunoprecipitates were then washed 3X with 1 mL lysis buffer and 50 μl 8X sample

buffer was added, run on a 10% SDS- PAGE, followed by Western blotting (see Western blotting, below).

ERK Assay

ERK activation assays were performed using two methods, *in vitro* kinase assays and direct Western blotting for activated ERKS. For *in vitro* kinase assays, cells were serum starved overnight and then treated with either R6.5 monoclonal antibody or fibrinogen for the required amount of time, washed once with ice-cold PBS, and lysed in 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X-100, 2.5 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 μg/ml leupeptin, 1 tablet Boehringer-Mannheim protease inhibitor tablet per 10 mL. Lysates were sonicated 4 x 5 sec each on ice, followed by 10 min centrifugation at 12000 x g. 200 μg of cell lysate was taken from each sample and added to 15 μl of immobilized phospho-Erk 1/2 monoclonal antibody (NEB). Since activated p44/42 MAPK is phosphorylated at Thr202 and Tyr204, this antibody was designed to recognize the pTEpY sequence, thus binding to only the activated MAPK molecules. Samples were incubated at 4°C for 4 hrs with gentle rocking, then microcentrifuged for 30 sec at 4°C, washed 2x with 0.5 mL ice-cold lysis buffer, followed by 2x 0.5 mL ice-cold kinase buffer (25 mM Tris (pH7.5), 5 mM β-glycerolphosphate, 2 mM DTT, 0.1 mM sodium vanadate, 10 mM $MgCl_2$). Washed beads were suspended in 50 μl kinase buffer supplemented with 200 μM ATP and 2 μg Elk-1 fusion protein, incubated 30 min at 30°C, and terminated with 25 μl 8X sample buffer. Samples were then boiled for 5 min and analyzed by 10% SDS-PAGE. Western

blotting was performed as described below, with an α-phospho-Elk-1 specific monoclonal antibody.

For direct phospho-ERK assays, CHO cells transfected with the indicated constructs were lysed in 8X sample buffer, scraped into Eppendorf tubes and boiled 5 min. 20 μl sample was then run on 10% SDS-PAGE, transferred and Western blotted with α-phospho-ERK 1/2 antibody (1:3000 in 5% non-fat milk TBST O/N 4°C) plus goat-anti-rabbit-HRP secondary antibody (1:10,000 in 5% non-fat milk TBST 1 hr RT). This antibody recognizes the identical pTEpY sequence described above. The blots were then stripped and reprobed for total ERK 1/2.

Transfections

Cells were transfected using Lipofectamine PLUS (Life Technologies, Gaithersburg, MD). Briefly, for a 60 mm culture dish, approximately 2 μg plasmid DNA were incubated with 8 μl PLUS reagent for 20 minutes RT, then mixed with a diluted sample of Lipofectamine (12 μl Lipofectamine in 2 ml serum free media), and incubated at RT for another 20 minutes. The adherent cell monolayer was washed once with serum free medium and then incubated in the transfection mixture for 3.5 hrs. 37°C, 5% CO_2. The transfection media was then aspirated and fresh serum free media was added to the cells and placed at 37°C, 5% CO_2 O/N.

Plasmid Constructs

A fusion protein containing an N-terminal Glutathione-S-Transferase and C-terminal ICAM-1 cytodomain (aa 502-532) was constructed in the pGEX-2T vector (Pharmacia Biotech, Piscataway, NJ) by first generating ICAM-1 cytodomain insert by PCR with primers followed by digestion and insertion into the BamHI/EcoRI sites of pGEX-2T.

Upper primer: 5'-CTGGATCCATGGCGGGTGAAAAAGTTGAGAAG-3'

Lower primer: 5'-CCGGAATTCTTAGAACACCAATTTGTGAGG-3'

c-myc tagged vectors were produced using the pCMV-Tag3 system (Stratagene, La Jolla, CA). TAXREB107 insert was generated by PCR,

upper primer: 5'-GGAATTCATGGCGGGTGAAAAAGTTGAGAAG-3'

lower primer: 5'-CCGCTCGAGTTAGAACACCAATTTGTGAGG-3'

digested with EcoRI and XhoI, then inserted into the pCMV-T3 vector. N-terminal GFP tagged vectors were produced using the pWAY5 vector (kind gift from Dr. Tom Hughes). For the GFP-ICAM-1 cytodomain construct, ICAM-1 cytodomain was again generated by PCR.

upper primer: 5'-CTCAGCAAGCTTCTCTATAACCGCCAG-3'

lower primer: 5'-CCGCTCGAGTCAGGGAGGCGTGGCTTG-3'

PCR product ends were polished with mung bean nuclease and subjected to EcoRI digestion, then inserted into pWAY5. The GFP-VCAM-1 cytodomain insert was made as separate single stranded 5'-phosphorylated DNA oligomers,

upper strand: 5'-AGAAAAGCCAACATGAAGGGGTCATATAGTCTTGTAG
AAGCACAGAAATCAAAAGTGTAGC-3'

lower strand: 5'-TCGAGCTACACTTTGATTTCTGTGCTTCTACAAGACTA
TATGACCCCTTCATGTTGGCTTTTCT-3'

which were then annealed and ligated into SmaI/XhoI predigested pWAY5 vector.

Immunofluorescence Staining

Cells grown on coverslips were incubated in methanol at -20°C x 5 min., washed 1X with CMF-PBS, then blocked with 1% BSA CMF-PBS + 0.1% NaN_3 x 20 min at RT. After another CMF-PBS wash, cells were probed with primary, fluorochrome-conjugated antibody at the indicated concentrations, then washed 3X with CMF-PBS at RT x 5 min each. Cover slip stainings were then mounted on glass slides for visualization and photography on a Microphot Immunofluorescence microscope (Nikon, Japan).

Flow Cytometry

Cells were harvested by trypsinization (Sigma, St. Louis, MO) and staining with primary then fluorophore-conjugated secondary monoclonal antibodies in a 1% BSA CMF-PBS solution containing 0.1% sodium azide. Approximately 5000 cells were analyzed per sample on a FACSort machine (Becton Dickinson & Co., San Jose, CA), using the Cellquest program. Isotype-matched controls were used for all experimental samples.

Fluosphere Detection of R6.5 binding to CHO Cells

Briefly, protein A-conjugated 0.4 μm diameter microspheres (fluospheres) were washed twice with 50 mM sodium borate pH 8.2, added to R6.5 (130 μg/mL) for 45 minutes RT, then washed again in 1% BSA D-PBS. After resuspending the cells in blocking solution, the R6.5 + fluospheres were added to 1 X 10^6 CHO cells in suspension for 30 minutes at 4°C. A final wash with 1% BSA D-PBS, followed by resuspension in 0.5 mL D-PBS, prepared the cells for FACS analysis. Since the emission spectra of the fluorophore in the beads has peak intensity at approximately 505-515 nm, a standard FITC channel was used to detect signals in the FACS analysis.

Western Blot Analysis

Cell lysates were boiled for 10 min in sample buffer (0.35 M Tris-Cl, 10.28% SDS, 30% glycerol, 10% DTT, 0.012% bromophenol blue) then loaded onto 10% SDS-polyacrylamide gels. Total protein loaded onto each lane was identical, as determined by

Bradford Assay (Bio-Rad, Hercules, CA). Gels were run in running buffer (0.124 M Tris base, 0.96 M glycine, 17.3 mM SDS) at 100 V while the dye remained in the stacking gel, and 200 V for the remainder of the run. Transfer onto 0.45 μm pore size nitrocellulose was performed in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) at 4°C x 1 hr with 100 V using a Bio-Rad transfer apparatus. Samples were then blocked x 1 hr RT with 5% milk TBST (50 mM Tris-HCl, 750 mM NaCl, 0.25% Tween) and incubated with primary antibody overnight at 4°C on a rocker. Blots were subsequently washed 3X with TBST x 10 min each, incubated with secondary antibody x 1 hr at RT in 5% milk TBST and washed again 3X for 10 min with TBST. Finally, blots were reacted with chemiluminescence solution (NEN, Boston, MA) for 1 min at RT, followed by exposure to film (Amersham, Buckinghamshire, England).

Purification of ICAM-1 Cytodomain Peptides on SCX Columns

SCX columns (Millipore Corp., Bedford, MA) were washed in methanol and distilled water before loading the ICAM-1 cytodomain peptides 2392 and 2393. Columns were spun 1200 x g 1 min, washed with 0.5 ml 10 mM HCl. Elution was performed with 50 μl 1.4 N NH4OH/MeOH and spinning 12000 x g. Samples were then dessicated under vacuum for 15 min and resuspended in HBS buffer (Biacore Inc., Piscataway, NJ).

References

Adamson, P., S. Etienne, P. Couraud, V. Calder and J. Greenwood (1999). "Lymphocyte migration through brain endothelial cell monolayers involves signaling through endolthelial ICAM-1 via a rho-dependent pathway." J Immunol **162**: 2964-2973.

Altieri, D. C., F. R. Agbanyo, J. Plescia, M. H. Ginsberg, T. S. Edgington and E. F. Plow (1990). "A unique recognition site mediates the interaction of fibrinogen with the leukocyte integrin Mac-1 (CD11b/CD18)." J Biol Chem **265**: 12119-22.

Aoudjit, F., E. Potworowski, T. Springer and Y. St-Pierre (1998). "Protection from lynmphoma cell metastasis in ICAM-1 mutant mice: a posthoming event." J Immunol **161**: 2333-2338.

Becker, J. C. and E. B. Brocker (1995). "Lymphocyte-melanoma interaction: role of surface molecules." Recent Results Cancer Res **139**: 205-14.

Bella, J. and M. G. Rossmann (1999). "Review: rhinoviruses and their ICAM receptors." J Struct Biol **128**: 69-74.

Bella, J. and M. G. Rossmann (2000). "ICAM-1 receptors and cold viruses." Pharm Acta Helv **74**: 291-7.

Berendt, A. R., D. L. Simmons, J. Tansey, C. I. Newbold and K. Marsh (1989). "Intercellular adhesion molecule-1 is an endothelial cell adhesion receptor for Plasmodium falciparum." Nature **341**: 57-9.

Bocco, J. L., A. Bahr, J. Goetz, C. Hauss, T. Kallunki, C. Kedinger and B. Chatton (1996). "In vivo association of ATFa with JNK/SAP kinase activities." Oncogene **12**: 1971-80.

Boriack-Sjodin, P. A., S. M. Margarit, D. Bar-Sagi and J. Kuriyan (1998). "The structural basis of the activation of Ras by Sos." Nature **394**: 337-43.

Brady, J., K. T. Jeang, J. Duvall and G. Khoury (1987). "Identification of p40x-responsive regulatory sequences within the human T-cell leukemia virus type I long terminal repeat." J Virol **61**: 2175-81.

Bullard, D., P. King, M. Hicks, B. Dupont, A. Beaudet and K. Elkon (1997). "Intercellular adhesion molecule-1 deficiency protects MRL/MpJ-Faslpr mice from early lethality." J Immunol **159**: 2058-2067.

Carpen, O., P. Pallai, D. Staunton and T. Springer (1992). "Association of intercellular adhesion molecule-1 (ICAM-1) with actin-containing cytoskeleton and alpha-actinin." J Cell Biol **118**: 1223-1234.

Casasnovas, J. M. (2000). "The dynamics of receptor recognition by human rhinoviruses." Trends Microbiol **8**: 251-4.

Chirathaworn, C., S. Tibbetts, M. Chan and S. Benedict (1995). "Cross-linking of ICAM-1 on T cells induces transient tyrosine phosphorylation and inactivation of cdc2 kinase." J Immunol **155**: 5479-5482.

Chong, L. D., A. Traynor-Kaplan, G. M. Bokoch and M. A. Schwartz (1994). "The small GTP-binding protein Rho regulates a phosphatidylinositol 4- phosphate 5-kinase in mammalian cells." Cell **79**: 507-13.

Clayton, A., R. Evans, E. Pettit, M. Hallet, J. Williams and R. Steadman (1998). "Cellular activation through the ligation of intercellular adhesion molecule-1." J Cell Sci **111**: 443-453.

Csanaky, G. (1994). "High endothelial venules and cell adhesion molecules in B-cell chronic lymphocytic leukaemia and related low grade B-cell lymphoma/leukaemia: II. Expression of cell adhesion molecules in lymph nodes biopsies." Histol Histopathol **9**: 577-82.

Cubitt, A. B., R. Heim, S. R. Adams, A. E. Boyd, L. A. Gross and R. Y. Tsien (1995). "Understanding, improving and using green fluorescent proteins." Trends Biochem Sci **20**: 448-55.

de Fougerolles, A. R., X. Qin and T. A. Springer (1994). "Characterization of the function of intercellular adhesion molecule (ICAM)-3 and comparison with ICAM-1 and ICAM-2 in immune responses." J Exp Med **179**: 619-29.

Diamond, M. S., D. E. Staunton, A. R. de Fougerolles, S. A. Stacker, J. Garcia-Aguilar, M. L. Hibbs and T. A. Springer (1990). "ICAM-1 (CD54): a counter-receptor for Mac-1 (CD11b/CD18)." J Cell Biol **111**: 3129-39.

Diamond, M. S., D. E. Staunton, S. D. Marlin and T. A. Springer (1991). "Binding of the integrin Mac-1 (CD11b/CD18) to the third immunoglobulin- like domain of ICAM-1 (CD54) and its regulation by glycosylation." Cell **65**: 961-71.

Dingwall, C. and R. A. Laskey (1991). "Nuclear targeting sequences--a consensus?" Trends Biochem Sci **16**: 478-81.

Duperray, A., L. Languino, J. Plescia, A. McDowall, N. Hogg, A. Craig, B. AR and D. Altieri (1997). "Molecular identification of a novel fibrinogen binding site on the first domain of ICAM-1 regulating leukocyte-endothelium bridging." J Biol Chem **272**: 35-441.

Durieu-Trautmann, O., N. Chaverot, S. Cazaubon, A. Strosberg and P. Couraud (1994). "Intercelular adhesion molecule 1 activation induces tyroine phosphorylation of the cytoskeleton-associated protein cortactin in brain microvessel endothelial cells." J Biol Chem **269**: 12536-12540.

Dustin, M. L. and T. A. Springer (1989). "T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1." Nature **341**: 619-24.

Ettiene-Manneville, S., N. Chaverot, A. Strosberg and P. Couraud (1999). "ICAM-1-coupled signaling pathways in astrocytes converge to cyclic AMP response element-binding protein phosphorylation and TNF-alpha secretion." J Immunol **163**: 668-674.

Federici, C., L. Camoin, M. Hattab, A. Strosberg and P. Couraud (1996). "Association of the cytoplamsic domain of intercellular-adhesion molecule-1 with glyceraldehyde-3-phosphate dehydrogenase and beta-tubulin." Eur J Biochem **238**: 173-180.

Fujii, M., P. Sassone-Corsi and I. M. Verma (1988). "c-fos promoter trans-activation by the tax1 protein of human T-cell leukemia virus type I." Proc Natl Acad Sci U S A **85**: 8526-30.

Fujisawa, J., M. Toita and M. Yoshida (1989). "A unique enhancer element for the trans activator (p40tax) of human T- cell leukemia virus type I that is distinct from

cyclic AMP- and 12-O- tetradecanoylphorbol-13-acetate-responsive elements." J Virol **63**: 3234-9.

Gerdes, H. H. and C. Kaether (1996). "Green fluorescent protein: applications in cell biology." FEBS Lett **389**: 44-7.

Giranda, V. L., M. S. Chapman and M. G. Rossmann (1990). "Modeling of the human intercellular adhesion molecule-1, the human rhinovirus major group receptor." Proteins 7: 227-33.

Gotoh, Y., E. Nishida, S. Matsuda, N. Shiina, H. Kosako, K. Shiokawa, T. Akiyama, K. Ohta and H. Sakai (1991). "In vitro effects on microtubule dynamics of purified Xenopus M phase- activated MAP kinase." Nature **349**: 251-4.

Harley, S. and J. Powell (1999). "Fibrinogen up-regulates the expression of monocyte chemoattractant protein 1 in human saphenous vein endothelial cells." Biochem J **341**: 739-744.

Heiska, L., K. Alfthan, M. Gronholm, P. Vilja, A. Vaheri and O. Carpen (1998). "Association of ezrin with intercellular adhesion molecule-1 and -2 (ICAM-1 and ICAM-2)." J Biol Chem **273**: 21893-21900.

Hicks, R. C., J. Golledge, R. Mir-Hasseine and J. T. Powell (1996). "Vasoactive effects of fibrinogen on saphenous vein." Nature **379**: 818-20.

Hirai, H., J. Fujisawa, T. Suzuki, K. Ueda, M. Muramatsu, A. Tsuboi, N. Arai and M. Yoshida (1992). "Transcriptional activator Tax of HTLV-1 binds to the NF-kappa B precursor p105." Oncogene 7: 1737-42.

Hirase, T., S. Kawashima, T. Ueyama, Y. Rikitake, N. Inoue and K. Hirata (2000). "Intercellular adhesion molecule 1 signaling increases tight juction permeability via rhoA and rho kinase in endothelial cells." Circulation **102 (supplement)**: II-174.

Holland, J. and T. Owens (1997). "Signaling through intercellular adhesion molecule 1 (ICAM-1) in a B cell lymphoma line." J Biol Chem **272**: 9108-9112.

Inoue, J., M. Yoshida and M. Seiki (1987). "Transcriptional (p40x) and post-transcriptional (p27x-III) regulators are required for the expression and replicationof human T-cell leukemia virus type-I genes." Proc Natl Acad Sci **84**: 3635-3657.

Johnson, J. P., B. G. Stade, B. Holzmann, W. Schwable and G. Riethmuller (1989). "De novo expression of intercellular-adhesion molecule 1 in melanoma correlates with increased risk of metastasis." Proc Natl Acad Sci U S A **86**: 641-4.

Juan, M., O. Vinas, M. R. Pino-Otin, L. Places, E. Martinez-Caceres, J. J. Barcelo, A. Miralles, R. Vilella, M. A. de la Fuente, J. Vives and et al. (1994). "CD50 (intercellular adhesion molecule 3) stimulation induces calcium mobilization and tyrosine phosphorylation through p59fyn and p56lck in Jurkat T cell line." J Exp Med **179**: 1747-56.

Kato, Y., A. Yamataka, H. Yagita, K. Okumura, T. Fujiwara and T. Miyano (1996). "Specific acceptance of fetal bowel allograft in mice after combined treatment with anti-intercellular adhesion molecule-1 and leukocyte function-associated antigen-1 antibodies." Ann Surg **223**: 94-100.

Kita, Y., T. Takashi, Y. Iigo, T. Tamatani, M. Miyasaka and T. Horiuchi (1992). "Sequence and expression of rat ICAM-1." Biochim Biophys Acta **1131**: 108-10.

Kohlmeier, J., B. Wick, L. Rumsey, M. Chan and S. Benedict (2000). "ICAM-1 stimulation modulates gene expression in T cells." AHA meeting abstracts: 148.10 p. A1141.

Languino, L. R., J. Plescia, A. Duperray, A. A. Brian, E. F. Plow, J. E. Geltosky and D. C. Altieri (1993). "Fibrinogen mediates leukocyte adhesion to vascular endothelium through an ICAM-1-dependent pathway." Cell **73**: 1423-34.

Lee, S. J., K. Drabik, N. J. Van Wagoner, S. Lee, C. Choi, Y. Dong and E. N. Benveniste (2000). "ICAM-1-Induced expression of proinflammatory cytokines in astrocytes: involvement of extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways." J Immunol **165**: 4658-66.

Leonard, W. J. and J. J. O'Shea (1998). "Jaks and STATs: biological implications." Annu Rev Immunol **16**: 293-322.

Lewis, T., P. Shapiro and A. NG (1998). "Signal transduction through MAP kinase cascades." Advances in cancer research **74**: 49-139.

Li, R., H. Madden and A. Sytkowski (1999). "Identification of TAXREB107 as an erythropoietin early response gene." AIDS Res and Human Retrovir **15**: 375-379.

Lollo, B. A., K. W. Chan, E. M. Hanson, V. T. Moy and A. A. Brian (1993). "Direct evidence for two affinity states for lymphocyte function- associated antigen 1 on activated T cells [published erratum appears in J Biol Chem 1994 Apr 1;269(13):10184]." J Biol Chem **268**: 21693-700.

Manning, A. M., H. F. Lu, G. L. Kukielka, M. G. Oliver, T. Ty, C. A. Toman, R. F. Drong, J. L. Slightom, C. M. Ballantyne, M. L. Entman and et al. (1995). "Cloning and comparative sequence analysis of the gene encoding canine intercellular adhesion molecule-1 (ICAM-1)." Gene **156**: 291-5.

Marlin, S. D. and T. A. Springer (1987). "Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1)." Cell **51**: 813-9.

Marotta, G., V. Zagonel and A. Pinto (1993). "Induction of LFA-1/CD11a and ICAM-1/CD54 adhesion molecules on neoplastic B cells during in vivo treatment of chronic lymphocytic leukemia with interferon-alpha 2 [letter; comment]." Blood **81**: 267-9.

Maruyama, M., H. Shibuya, H. Harada, M. Hatakeyama, M. Seiki, T. Fujita, J. Inoue, M. Yoshida and T. Taniguchi (1987). "Evidence for aberrant activation of the interleukin-2 autocrine loop by HTLV-1-encoded p40x and T3/Ti complex triggering." Cell **48**: 343-50.

McCourt, P. A., B. Ek, N. Forsberg and S. Gustafson (1994). "Intercellular adhesion molecule-1 is a cell surface receptor for hyaluronan." J Biol Chem **269**: 30081-4.

Meyer, K., M. Brown, G. Zibari, J. Panes, R. McMillan, J. McDonald and D. Granger (1998). "ICAM-1 upregulation in distant tissues after hepatic ischemia/reperfusion: A clue to the mechanism of multiple organ failure." J Pediatr Surg **33**: 350-353.

Micheva, K., A. Ramjaun, B. Kay and P. McPherson (1997). "SH3 domain-dependent interactions of endophilin with amphiphysin." FEBS **414**: 308-312.

Moller, P., C. Henne, F. Leithauser, A. Eichelmann, A. Schmidt, S. Bruderlein, J. Dhein and P. H. Krammer (1993). "Coregulation of the APO-1 antigen with intercellular adhesion molecule- 1 (CD54) in tonsillar B cells and coordinate expression in follicular center B cells and in follicle center and mediastinal B-cell lymphomas." Blood **81**: 2067-75.

Morita, T., T. Sato, H. Nyunoya, A. Tsujimoto, J. Takahara, S. Irino and K. Shimotohno (1993). "Isolation of a cDNA clone encoding DNA-binding protein (TAXREB107) that binds specifically to Domain C of the tax-responsive enhancer element in the long terminal repeat of human T-cell leukemia virus type I." AIDS Res and Human Retrovir **9**: 115-121.

Nagata, K., K. Ohtani, M. Nakamura and K. Sugamura (1989). "Activation of endogenous c-fos proto-oncogene expression by human T- cell leukemia virus type I-encoded p40tax protein in the human T-cell line, Jurkat." J Virol **63**: 3220-6.

Nguyen, J., C. Turck, F. Cohen, R. Zuckermann and W. Lim (1998). "Exploiting the basis of proline recognition by SH3 and WW domains: Design of N-substituted inhibitors." Science **282**: 2088-2092.

Ockenhouse, C. F., R. Betageri, T. A. Springer and D. E. Staunton (1992). "Plasmodium falciparum-infected erythrocytes bind ICAM-1 at a site distinct from LFA-1, Mac-1, and human rhinovirus [published erratum appears in Cell 1992 Mar 6;68(5):following 994]." Cell **68**: 63-9.

Ohta, K., T. Endo, K. Gunji and T. Onaya (1994). " Isolation of a cDNA whose expression is markedly increased in malignantly transformed FRTL cells and neoplastic human thyroid tissues." J Mol Endocrin **12**: 85-92.

Owen, S., D. Rudolph, C. Dezzutti, N. Chibata, S. Naik, S. Caughman and R. Lal (1997). "Transcriptional activation of the intercellular adhesion molecule 1 (CD54) gene by human T lymphotrophic virus types I and II tax is mediated through a palindromic response element." AIDS Res and Human Retrovir **13**: 1429-1437.

Pigott, R. and C. Power (1993). The Adhesion Molecules. San Diego, Academic Press London.

Ptashne, M. (1988). "How eukaryotic transcriptional activators work." Nature **335**: 683-9.

Rosales, C. and R. L. Juliano (1995). "Signal transduction by cell adhesion receptors in leukocytes." J Leukoc Biol **57**: 189-98.

Rosen, C. A., R. Park, J. G. Sodroski and W. A. Haseltine (1987). "Multiple sequence elements are required for regulation of human T-cell leukemia virus gene expression." Proc Natl Acad Sci U S A **84**: 4919-23.

Rosenstein, Y., J. K. Park, W. C. Hahn, F. S. Rosen, B. E. Bierer and S. J. Burakoff (1991). "CD43, a molecule defective in Wiskott-Aldrich syndrome, binds ICAM-1." Nature **354**: 233-5.

Rot, A., M. Krieger, T. Brunner, S. C. Bischoff, T. J. Schall and C. A. Dahinden (1992). "RANTES and macrophage inflammatory protein 1 alpha induce the migration

and activation of normal human eosinophil granulocytes." J Exp Med **176**: 1489-95.

Rothlein, R., M. L. Dustin, S. D. Marlin and T. A. Springer (1986). "A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1." J Immunol **137**: 1270-4.

Rothlein, R., T. Kishimoto and E. Mainolfi (1994). "Cross-linking of ICAM-1 induces co-signaling of an oxidative burst from mononuclear leukocytes." J Immunol **152**: 2488-2495.

Rothlein, R. and T. A. Springer (1986). "The requirement for lymphocyte function-associated antigen 1 in homotypic leukocyte adhesion stimulated by phorbol ester." J Exp Med **163**: 1132-49.

Ruben, S., H. Poteat, T. Tan, K. Kawakami, R. Roeder, W. Haseltine and C. Rosen (1988). "Cellular transcription factors and regulation of IL-2 receptor gene expression by HTLV-1 tax gene product." Science **241**: 80-92.

Rudd, C. E. (1999). "Adaptors and molecular scaffolds in immune cell signaling." Cell **96**: 5-8.

Sanchez-Madrid, F., A. M. Krensky, C. F. Ware, E. Robbins, J. L. Strominger, S. J. Burakoff and T. A. Springer (1982). "Three distinct antigens associated with human T-lymphocyte-mediated cytolysis: LFA-1, LFA-2, and LFA-3." Proc Natl Acad Sci U S A **79**: 7489-93.

Sano, H., N. Nakagawa, R. Chiba, K. Kurasawa, Y. Saito and I. Iwamoto (1998). "Cross-linking of intercellular adhesion molecule-1 induced interleukin-8 and RANTES

production through the activation of MAP kinases in human vascular endothelial cells." Biochem Biophys Res Communic **250**: 694-698.

Schall, T. J., K. Bacon, K. J. Toy and D. V. Goeddel (1990). "Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES." Nature **347**: 669-71.

Shen, B., M. Arese, A. Gualandris and D. Rifkin (1998). "Intracellular association of FGF-2 with the ribosomal protein L6/TAXREB107." Biochem Biophys Res Commun **252**: 524-528.

Shevchenko, A., M. Wilm, O. Vorm and M. Mann (1996). "Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels." Anal Chem **68**: 850-8.

Shi, C., M. W. Feinberg, D. Zhang, A. Patel, C. U. Sim, Z. M. Dong, S. M. Chapman, J. C. Gutierrez-Ramos, D. D. Wagner, N. E. Sibinga and E. Haber (1999). "Donor MHC and adhesion molecules in transplant arteriosclerosis." J Clin Invest **103**: 469-74.

Shimotohno, K., M. Takano, T. Teruuchi and M. Miwa (1986). "Requirement of multiple copies of a 21-nucleotide sequence in the U3 regions of human T-cell leukemia virus type I and type II long terminal repeats for trans-acting activation of transcription." Proc Natl Acad Sci U S A **83**: 8112-6.

Simmons, D. (1995). "The role of ICAM expression in immunity and disease." Cancer Surveys **24**: 141-155.

Simmons, D., M. W. Makgoba and B. Seed (1988). "ICAM, an adhesion ligand of LFA-1, is homologous to the neural cell adhesion molecule NCAM." Nature **331**: 624-7.

Siu, G., S. M. Hedrick and A. A. Brian (1989). "Isolation of the murine intercellular adhesion molecule 1 (ICAM-1) gene. ICAM-1 enhances antigen-specific T cell activation." J Immunol **143**: 3813-20.

Sligh, J., C. Ballantyne, S. Rich, H. Hawkins, C. Smith, A. Brandly and A. Beaudet (1993). "Inflammatory and immune responses are impaired in mice deficient in intercellular adhesion molecule 1." Proc Natl Acad Sci **90**: 8529-8533.

Smith, M. R. and W. C. Greene (1990). "Identification of HTLV-I tax trans-activator mutants exhibiting novel transcriptional phenotypes [published errata appear in Genes Dev 1991 Jan;5(1):150 and 1995 Sep 15;9(18):2324]." Genes Dev **4**: 1875-85.

Staunton, D. E., M. L. Dustin, H. P. Erickson and T. A. Springer (1990). "The arrangement of the immunoglobulin-like domains of ICAM-1 and the binding sites for LFA-1 and rhinovirus [published errata appear in Cell 1990 Jun 15;61(2):1157 and 1991 Sep 20;66(6):following 1311]." Cell **61**: 243-54.

Staunton, D. E., M. L. Dustin and T. A. Springer (1989). "Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1." Nature **339**: 61-4.

Strieter, R. M., S. L. Kunkel, H. J. Showell, D. G. Remick, S. H. Phan, P. A. Ward and R. M. Marks (1989). "Endothelial cell gene expression of a neutrophil chemotactic factor by TNF-alpha, LPS, and IL-1 beta." Science **243**: 1467-9.

Tsukita, S., A. Nagafuchi and S. Yonemura (1992). "Molecular linkage between cadherins and actin filaments in cell-cell adherens junctions." Curr Opin Cell Biol **4**: 834-9.

van de Stolpe, A., N. Jacobs, W. J. Hage, L. Tertoolen, Y. van Kooyk, I. R. Novakova and T. de Witte (1996). "Fibrinogen binding to ICAM-1 on EA.hy 926 endothelial cells is dependent on an intact cytoskeleton." Thromb Haemost **75**: 182-9.

van de Stolpe, A. and P. van der Saag (1996). "Intercellular adhesion molecule-1." J Mol Med **74**: 13-33.

Verbeek, M. M., I. Otte-Holler, P. Wesseling, D. J. Ruiter and R. M. de Waal (1996). "Differential expression of intercellular adhesion molecule-1 (ICAM-1) in the A beta-containing lesions in brains of patients with dementia of the Alzheimer type." Acta Neuropathol **91**: 608-15.

Volberg, T., B. Geiger, R. Dror and Y. Zick (1991). "Modulation of intercellular adherens-type junctions and tyrosine phosphorylation of their components in RSV-transformed cultured chick lens cells." Cell Regul **2**: 105-20.

Vuorte, J., P. Lindsberg, M. Kaste, S. Meri, S. Jansson, R. Rothlein and H. Repo (1999). "Anti-ICAM-1 monoclonal antibody R6.5 (enlimomab) promotes activation of neutrophils in whole blood." J Immunol **162**: 2353-2357.

Wang, G. J., M. Collinge, F. Blasi, R. Pardi and J. R. Bender (1998). "Posttranscriptional regulation of urokinase plasminogen activator receptor messenger RNA levels by leukocyte integrin engagement." Proc Natl Acad Sci U S A **95**: 6296-301.

Wang, Q. and C. M. Doerschuk (2000). "Neutrophil-induced changes in the biomechanical properties of endothelial cells: roles of ICAM-1 and reactive oxygen species." J Immunol **164**: 6487-94.

Watanabe, T. and J. Fan (1998). "Atherosclerosis and inflammation mononuclear cell recruitment and adhesion molecules with reference to the implication of ICAM-1/LFA-1 pathway in atherogenesis." Int J Cardiol **66 Suppl 1**: S45-53; discussion S55.

Watson, C. A., P. Petzelbauer, J. Zhou, R. Pardi and J. R. Bender (1995). "Contact-dependent endothelial class II HLA gene activation induced by NK cells is mediated by IFN-gamma-dependent and -independent mechanisms." J Immunol **154**: 3222-33.

Wright, S. D., J. I. Weitz, A. J. Huang, S. M. Levin, S. C. Silverstein and J. D. Loike (1988). "Complement receptor type three (CD11b/CD18) of human polymorphonuclear leukocytes recognizes fibrinogen." Proc Natl Acad Sci U S A **85**: 7734-8.

Xu, H., J. A. Gonzalo, Y. St Pierre, I. R. Williams, T. S. Kupper, R. S. Cotran, T. A. Springer and J. C. Gutierrez-Ramos (1994). "Leukocytosis and resistance to septic shock in intercellular adhesion molecule 1-deficient mice." J Exp Med **180**: 95-109.

Yoshida, M., T. Suzuki, J. Fujisawa and H. Hirai (1995). "HTLV-1 oncoprotein tax and cellular transcription factors." Microbiol Immunol **193**: 79-89.

Zou, Y., Y. Hu, M. Mayr, H. Dietrich, G. Wick and Q. Xu (2000). "Reduced neointima hyperplasia of vein bypass grafts in intercellular adhesion molecule-1-deficient mice." Circ Res **86**: 434-440.