Biochemical characterization of zinc-dependent interactions between $p56^{lck}$ and CD4, and DTT-sensitive interactions between TGF-beta receptor subunits

by

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To Mom, Dad and Albert

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Abstract

Binding of the protein tyrosine kinase $p56^{lck}$ to T-cell co-receptors CD4 and CD8α is necessary for T-lymphocyte development and activation. Here we demonstrate that Zn^{2+} is essential for complex formation. In an *in vitro* binding reaction, Zn^{2+} mediates $p56^{lck}$ association with a GST fusion protein containing the cytosolic domains of CD4 or CD8α; no other metals tested support binding. Treatment of preformed GST-CD4/$p56^{lck}$ dimers with Zn^{2+} chelators results in dissociation of GST-CD4 from $p56^{lck}$ suggesting that Zn^{2+} is contained within these complexes. Furthermore, we show that within live cells, CD4/$p56^{lck}$ and CD8α/$p56^{lck}$ interactions occur in a zinc dependent fashion. Specifically, pretreatment of the human T-cell Jurkat cell line with membrane-permeable zinc chelators disrupts CD4/$p56^{lck}$ complexes, and treatment of COS cells co-expressing CD8α and $p56^{lck}$ with such chelators likewise leads to dissociation of CD8α/$p56^{lck}$ complexes. CD4/$p56^{lck}$ and CD8α/$p56^{lck}$ represent the first examples of intracellular proteins that require zinc as a bridge for heterodimerization.

The TGF-βs mediate a diversity of biological responses through the binding of a types I (TβR1) and II (TβRII) receptor. Specifically, these cytokines bring about the formation of a signalling complex consisting of a TβRI and TβRII heterocomplex - most likely a heterotetramer. (Gilboa et al., 1998; Henis et al., 1994; Moustakas et al., 1993; Yamashita et al., 1994) However, it has been shown that pre-treatment of cells with DTT abrogates functional recruitment of TβRI to TGF-β1-bound TβRII rendering the resulting complex unable to signal. (Cheifetz et al., 1990; Wells et al., in press) We further report that similar pre-treatment blocks binding of both TβRI and TβRII to TGF-β2, a ligand distinct from TGF-β1 in that it requires co-expression of both receptors to bind. To identify the properties of DTT required to elicit this phenomenon, we screened a number of chemically related reducing agents for a similar inhibitory effect upon complex formation. Here, we find that 2,3 DMP (2,3 dimercaptopropanol) likewise blocks functional interactions between TβRI and TβRII. Both it and DTT are di-thiol based compounds that

are membrane-permeable. Other thiol-containing compounds lacking either one of these properties exert no effect on complex formation. In addition, DTT does not affect TβRI homodimerization, an upstream event required for TβRI•TβRII hetero-oligomerization. Hence, this would suggest that mechanistically, DTT and similarly, 2,3 DMP, act upon intracellular factor(s) and through chemical properties related to the presence of di-thiols, inhibit TβRI•TβRII association. The cytoplasmic domain of TβRI plays a critical role in these interactions as we find that a cytoplasmically truncated TβRI fails to associate with ligand-bound TβRII. In contrast, the TβRII cytoplasmic domain is dispensable for complex formation, nor is it required to manifest the DTT effect. Therefore, we propose that DTT directly abrogates functional TβRI•TβRII interactions by acting upon structural elements lying within TβRI's intracellular domain that are necessary for it to be recruited by TβRII.

It is thought that DTT, by reducing critical disulfide bonds in the extracellular domain of TβR1, alters the receptor's conformation ultimately preventing TGF-β binding.(Vivien and Wrana, 1995) Other membrane permeable dithiols exerted the same effect. Because in contrast to monothiols, dithiols are metal chelators, we thought it possible that chelation rather than disulfide bond reduction explained their findings. Hence, our model predicts that Zn^{2+} is essential for the cytoplasmic domain of the type I receptor to assume a particular conformation necessary for interactions with the II receptor. We test this model by examining a number of predictions it makes regarding the structural nature of the type I receptor. First, it predicts that the type I receptor cytosolic domain should bind Zn^{2+} and its removal by pre-treatment with membrane permeable Zn^{2+} chelators should block formation of a TGF-β1/types I and II receptor complex. Furthermore, mutations resulting in the loss of zinc binding by the type I receptor should cause loss of recruitment to the ligand bound type II receptor complex and therefore render it unable to signal. Our experimental data however are only consistent with a subset of these predictions. Therefore, our conclusions ultimately do not support an essential role for a divalent metal, namely zinc, in formation of the TGF-β receptor complex.

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Chapter 1

Literature review:

(1) Role of zinc in protein-protein interactions
(2) CD4/CD8 mediated T-cell activation
(3) TGF-β receptor signal transduction

This thesis discusses two as of yet largely unstudied problems in protein/protein interactions. First we characterize a novel role for zinc in the mediation of protein-protein interactions. In particular, we examine the role of zinc in bridging the cytoplasmic domain of T-cell co-receptors, CD4 and CD8α, to the lymphocyte specific tyrosine kinase, $p56^{lck}$. Secondly, we are interested in the process in which transmembrane proteins recognize and ultimately associate with each other to form larger order complexes. Specifically, with respect to the formation of the TGF-β signaling receptor complex, it has been demonstrated that the types I and II TGF-β receptors homodimerize in the absence of ligand and upon ligand binding, form a types I and II heterotetramer. (Gilboa et al., 1998; Henis et al., 1994; Moustakas et al., 1993; Yamashita et al., 1994) In this thesis, we examine the structural determinants necessary for their oligomerization as well as DTT's mode of action in blocking their formation. This introductory chapter discusses the biochemistry and physiology of zinc and then reviews current models of CD4/CD8 mediated T-cell activation and TGF-β receptor signal transduction.

Zinc

Man's use of metals such as copper and iron for medicine and tool-making have long been recognized as critical to the rise of human civilization. Copper for example was discovered in ~ 9000BC and became widely used by the Greeks as medication for disease as well as nearly all types of tools and jewelry.(Pena et al., 1998) In contrast, the identification and importance of zinc emerged much later. In the production of metal works for example, there is no evidence of zinc being intentionally used prior to the Pax Romana. Indeed, ranking as one of their greatest achievements in metallurgy, the Romans introduced large scale production of brass through the use of calamine (zinc carbonate); this represented zinc's first use as a component for metal production.(Aitchison, 1960)However, elemental zinc remained unknown for at least another thousand years. Because of its low boiling temperature relative to other metals (927°C), it easily vaporized during the smelting process, and hence remained obscure to early metallurgists. It was not until the Chinese developed a method in the thirteenth century to rapidly condense zinc vapour (ZnO) to its liquid state that elemental zinc was first produced. Remarkably, during the Chinese Ming Dynasty (1368-1644), the process became refined enough to produce coins containing over 98% zinc.(Aitchison, 1960; Tylecote, 1992) During the proceeding 400 years, Europeans imported zinc from the Far East for producing brass. In fact, their dependence upon Asian imported zinc did not end until 1738 when the Englishman William

Champion patented a smelting method for producing metallic zinc.(Aitchison, 1960; Tylecote, 1987)

Biochemistry of zinc

Zinc was first characterized as an essential element for plants just over a century ago.(Raulin, 1869) Its role in biochemistry was first recognized in 1940 when Keilin and co-workers purified carbonic anhydrase and showed that the presence of zinc was necessary for catalytic activity.(Keilin and Mann, 1940) In the subsequent 50 years, zinc has emerged as the most ubiquitous metal of the transition group IIB elements in biological systems. Within proteins, it is able to assume a diversity of structural configurations allowing it to play roles in both structural and enzymatic biochemistry. For example, zinc metalloenzymes can be found in all six classes of enzymes established by the International Union of Biochemistry. Secondly, in a structural role, Zn^{2+} serves as the predominant metal ion stabilizing cross-links within proteins. In fact, it has been estimated that approximately 0.5 - 1% of all human cDNAs encode proteins containing zinc finger motifs, a subset of all zinc binding domains.(Hoovers et al., 1992)

Two intrinsic properties of Zn^{2+} make it uniquely suitable for the tasks nature assigns to it. First, like many group IIB metals, zinc has an oxidation state of +2 corresponding to the removal of two electrons from their least stable 4s orbitals. Based on hard-soft acid base theory, this positive charge lends it Lewis acid properties. And for Zn^{2+} in particular, the positive charge yields a borderline Lewis acid capable of ligating a diversity of electron donors including oxygen, nitrogen and sulfur. Secondly, by virtue of its filled d-shell orbital, it is electronically inert and as a result redox stable. In contrast to other divalent metals such as copper and iron, no other oxidation states exist other than +2. Consequently, zinc is uniquely suited to stabilizing cross-links within protein domains without introducing undesired chemical reactivity.

Hence, zinc's versatility arises from two seemingly opposing properties: 1) flexible Lewis acid properties and 2) its high redox stability. In fact, it is the nature of the binding site constructed around the zinc ion that determines which of these two properties becomes manifest. When fulfilling a structural role, zinc is always fully coordinated in a tetrahedral configuration. On the other hand, with an open coordination site ligated to a water molecule, zinc's Lewis acid properties are brought to bear upon enzymatic catalysis.(Vallee and Auld, 1990) For example, in enzymes such as carbonic anhydrase and carboxypeptidase A, it is believed that zinc can lower the pKa of the bound water molecule

hence facilitating formation of a metal-bound hydroxide ion. The resulting hydroxide ion is then capable of nucleophilic attack on a substrate molecule. In other enzymes such as alcohol dehydrogenase, substrate displaces the zinc-bound water. Then zinc, as a Lewis acid, polarizes the substrate molecule thereby priming it for nucleophilic attack. The identity of the other three zinc bound ligands and their spacing appears to determine which mechanistic pathway is used. Indeed, enzymes utilizing zinc to stabilize a metal bound hydroxide ion lack coordinating cysteines and are generally characterized by at least two histidine ligands. In contrast, most zinc alcohol dehydrogenases are characterized by a single histidine and two cysteine ligands. This particular configuration may alter the bound Zn^{2+}'s Lewis acid properties and consequently determine the pathway utilized.

Types of zinc binding domains

Zinc serves as a template around which proteins can form defined tertiary structures. In general, energetic considerations dictate that polypeptides be of at least 50 residues in order to form stable tertiary domains.(Creighton, 1993) However, by using zinc as a cross-link, polypeptides as short as 15 residues are able to fold stably. Nature has put these domains to use in a wide diversity of functions including protein-protein, DNA, RNA and DNA/RNA hybrid binding. In many cases, they participate in the sequence specific binding of nucleic acids as illustrated by the enormous family of zinc finger binding proteins. Numerous transcription factors contain zinc fingers that extend into the major groove of the double helix where residues within the zinc finger interact with DNA bases and backbone phosphates groups.(Pavletich and Pabo, 1991) Each zinc finger can bind 3 contiguous bases of DNA.(Pavletich and Pabo, 1991) Greater sequence specificity is achieved by simply assembling multiple zinc fingers each independently binding their cognate DNA target sequence.(Desjarlais and Berg, 1992; Desjarlais and Berg, 1992)

Aside from their almost exclusive use of cysteines and histidines as coordinating residues, the families of zinc binding domains are structurally diverse. The topologies of the zinc ion ligands is a clear illustration of this structural diversity. Among zinc finger domains, three proteins represent the configurations found in nature thus far. In the primary sequence of the transcription factor TFIIIA, a single zinc ion is coordinated by a pair of cysteines followed by a loop region and a second pair of histidines.(Miller et al., 1985) Variations on this theme include steroid receptors and LIM domains characterized by two zinc finger domains placed successively in close proximity to each other with the first through fourth coordinating residues binding the first zinc ion and the fifth through eighth binding the second.(Evans, 1988) In a more complex scheme, RING domains also possess

two zinc binding sites ligated by eight residues. However, the two modules are not discrete, and instead overlap each other.(Barlow et al., 1994; Borden et al., 1995; Freemont, 1993; Saurin et al., 1996) Specifically, the first and third pairs of cysteines coordinate one zinc ion and the intervening second and fourth pairs coordinate a second zinc ion. Third, the transcription activator GAL4, by virtue of having two cysteines each ligating two zinc ions, contains only six residues to coordinate two zinc ions.(Marmorstein et al., 1992)

With respect to function, LIM and RING domains appear to be zinc binding structures specialized for protein/protein interactions. RING domain proteins constitute a family of proteins containing conserved pairs of zinc ligands; however their intervening sequences and spacing are highly variable perhaps reflecting the myriad functions assumed by RING domain proteins. This family of proteins are involved with cell cycle, signal transduction, and viral pathogenesis. Unlike other zinc binding motifs, RING finger proteins appear to be monomeric and hence may not able homo-oligomerize in the absence of other factors. LIM domain proteins are less well characterized biochemically.(Dawid et al., 1998) Many are found in homeodomain proteins and cytoskeletal proteins. Partner proteins include other LIM proteins as well as a variety of other structural motifs.(Jurata and Gill, 1998) While their structures suggest that they may be able to bind DNA, any function in transcription has yet to shown.

Zinc as a protein-protein bridge

Although the paradigm of zinc binding domains forming structures involved in protein-protein interactions has been well established, evidence of zinc directly bridging two proteins has just begun to emerge. In such a role, the interacting interface would possess a zinc binding site containing coordinating residues from each monomer. The HIV Tat protein is a case in point.(Frankel et al., 1988; Frankel et al., 1988) Specifically, Tat *in vitro* can form homodimers bridged by zinc. Biophysical measurements suggest the presence of four zinc ions per dimer. Each monomer contains a cysteine rich domain highly reminiscent in sequence to that of the metal binding sites in metallothionein. In addition, presence of zinc appears to induce structural changes in this cysteine rich region presenting further indication of this region's involvement with metal binding. Overall global folding however appears to be unaffected. In another example, Wells and co-workers show that human growth hormone binds human prolactin receptor with relatively low affinity. However, in the presence of 50μM Zn^{2+}, binding affinity increases by ~8000 fold.(Cunningham et al., 1990; Cunningham and Wells, 1991) X-ray crystallographic data

reveals a single zinc ion bridging prolactin receptor residues Asp 217 and His 218 to growth hormone residues His 18 and Glu 174 deep in the binding interface. (Somers et al., 1994) Although the physiological relevance of growth hormone binding the prolactin receptor has yet to be established, this work nevertheless raises the possibility of engineering interfacial zinc binding interfaces to enhance the affinity of protein-protein interactions. In fact, Wells and co-workers, based on sequence alignment and crystallographic data, deduced the corresponding residue in the growth hormone receptor to a zinc coordinating residue in prolactin receptor, His218. Mutation of this specific residue to histidine led to a mutant growth hormone receptor exhibiting similar zinc dependent enhancement (20 fold increase) in ligand binding, lending credence to the feasibilty of such an approach to protein engineering. (Matthews and Wells, 1994)

Zinc physiology

An average adult human contains 2 to 3 grams of zinc, making it one of the most abundant physiological "trace" elements. Furthermore, largely attributed to its lack of chemical reactivity as well as its apparently efficient physiological homeostasis, no known human disorders have been associated with zinc toxicity. Additional evidence pointing to efficient zinc homeostasis has come from studies of the effects of zinc deficiency.(Golub et al., 1995; Golub et al., 1986) Symptoms of moderate zinc deficiency include hypogeusia and a suppressed immune function, and with more severe deficits, anemia and anorexia. However, very low levels of zinc intake are required to manifest these symptoms and as a result, occurrences of such deficiencies are rare. In mice, even the most extreme of dietary regimens induce only transiently decreased levels of zinc in tissues such as the brain. The mechanisms of homeostasis remain unknown. Several putative zinc transporters have been cloned. However, at this time, none have been directly demonstrated to facilitate zinc transport. Total cellular and serum concentrations of zinc are in the 10-100μM range.(Falchuk et al., 1995; Hocke et al., 1995; Nomizu et al., 1993; Reyes, 1996; Swanson and Sharp, 1992; Tiran et al., 1993; Whitehouse et al., 1982) However, most of it is bound to proteins. On the other hand, the free concentration of zinc is reported to be in the picomolar range.(Maret and Vallee, 1996) Under such concentrations, many zinc binding proteins, given their observed affinities for zinc, would incorporate very little of the metal in their structure thus raising the question of how zinc becomes incorporated into proteins. Recently, Vallee and co-workers have proposed that metallothioneins take on the role of zinc storage and its transfer from one protein to another.(Fischer and Davie, 1998; Jacob et al., 1998; Jiang et al., 1998; Maret and Vallee, 1998) Metallothioneins comprise a family of proteins that bind zinc with extremely high affinity (Kd ~ 10^{-13}M) and high

stochiometry (up to 7 zinc atoms/molecule). Their model rests on a key observation. Specifically, because of metallothionein's relatively low redox potential (<-366mV), its sulfur ligands can be readily oxidized by mild cellular oxidants such as oxidized glutathione (GSSG) and protein disulfide isomerase.(Maret and Vallee, 1998) Such an oxidation state would destabilize the zinc bound complex, causing its release and presumed transfer to another protein. Therefore, zinc release from metallothionein and its subsequent transfer to another zinc acceptor would be governed by the ratio of reduced to oxidized disulfides. A high reduced/oxidized ratio maintains MT's high affinity for zinc whereas a low one would cause oxidation of MT's sulfur ligands therefore favoring release of bound zinc. Furthermore, their data suggests that this reaction is not strictly reversible suggesting additional factors of regulation.

CD4 and CD8-mediated T-lymphocyte activation

T-cell activation

T-cell activation is the process by which a mature T-lymphocyte responds to the presence of an antigen. The changes induced in the T-cell are determined by the developmental history of the cell as well as the nature of the antigen. In particular, these responses include proliferation, programmed cell death, growth factor production, cell migration, cell-cell contact, and an ill-defined process called "memory." (Crabtree and Clipstone, 1994)

Elucidation of CD4 and CD8 function

The development of two technologies in the 1970's, namely monoclonal antibodies and fluorescence activated cell-sorting (FACS), dramatically accelerated efforts aimed at understanding T-lymphocyte biology. Used in concert, they led to the isolation and characterization of T-cell sub-populations based on their expression of a number of cell surface markers. Shortly thereafter, it became evident that these markers could be correlated with a T-lymphocyte's state of differentiation and function.(Reinherz and Schlossman, 1980) Among mature T-cells in particular, two sub-populations of cells exist, each characterized by exclusive expression of a specific membrane glycoprotein: T-helper cells are CD4 positive and T-killer cells are CD8 positive. Because blocking antibodies against CD4 and CD8 could inhibit a variety of functions related to antigen recognition and stimulation, it was clear that they played an essential role in T-cell function.(Dialynas et al., 1983; Haskins et al., 1984; Haskins et al., 1983; Marrack et al., 1983; Marrack et al., 1983; Swain et al., 1984; Wilde et al., 1983) Among such studies, S. Swain provided

evidence suggesting that they interact with specific classes of MHC proteins.(Swain, 1981) She observed that CD8 blocking antibodies inhibited functions specific for the class I major histocompatibility complex (MHC), but had no such effect on class II MHC mediated responses. Similar studies demonstrated CD4 involvement with class II MHC specific functions. Beyond antigen stimulated T-cell activation, these MHC interactions also bear implications in T-cell development as mice homozygously deficient for either glycoprotein exhibit specific deficiencies in the maturation of their respective lineage specific T-cells.(Fung-Leung et al., 1991; Rahemtulla et al., 1991)

Work extending the details of these interactions culminated in the demonstration of physical association betweeen CD4 and class 2 MHC protein by Doyle and co-workers.(Doyle and Strominger, 1987) These results led to the model proposing that CD4 and CD8 act as accessory molecules that increase adhesion between T-cell and APC. It implies that such an accessory molecule and TCR would bind independently to different MHC molecules and as a result increase cell-cell adhesion. CD4 and CD8 then presumably allows the TCR to respond to lower levels of antigen. However, the "accessory molecule" hypothesis fails to explain the strict association of CD4 and CD8 to their respective MHC classes. Indeed, most professional APCs express both classes of MHCs and consequently, either CD4 or CD8 binding to their respective MHCs should fulfil the cell adhesion requirement for T-cell activation. Given that the model limits their roles to cell-cell adhesion, strict segregation of CD4 and CD8 to specific MHC classes would not be necessary.

In 1989, Janeway and co-workers propose an alternative model referred to as the "co-receptor hypothesis."(Janeway, 1991; Janeway, 1992) In contrast to the "accessory molecule hypothesis," they propose that in addition to an adhesion function, these glycoproteins constitute required components of TCR-mediated signal transduction. Janeway and co-workers also predict that upon antigen stimulation, CD4 and CD8 must associate with TCR and bind the same MHC ligand resulting in a complex competent to initiate T-cell signal transduction. Shortly thereafter, physical association between CD4 and TCR was demonstrated providing the first evidence lending credence to the co-receptor model.(Dianzani et al., 1992; Dianzani et al., 1992; Kupfer and Singer, 1988; Kupfer et al., 1987; Kupfer et al., 1987)

Additional evidence of a signalling role emerged from the discovery that the cytosolic domains of both CD4 and CD8α (a subunit of the CD8 complex) associate with the non-receptor tyrosine kinase, Lck ($p56^{lck}$).(Rudd et al., 1988; Veillette et al., 1988)

Particularly, T-cells expressing CD4 intracellular truncations that do not bind Lck show significantly reduced responses to antigen stimulation.(Miceli and Parnes, 1991; Miceli et al., 1991) Under these circumstances, CD4 is incapable of placing Lck within close proximity of the TCR thus abrogating any co-receptor mediated signal transduction. However, in contrast to mice lacking CD4 or CD8α, transgenic mice expressing truncated forms of CD4 and CD8α do not display complete impairment in T-cell development.(Fung-Leung et al., 1993; Fung-Leung et al., 1991; Killeen and Littman, 1993; Rahemtulla et al., 1991) This residual response in the organism would suggest the existence of Lck^- independent functions for CD4 and CD8, namely cell-cell adhesion. And indeed, ensuing models of co-receptor function incorporate Lck mediated signal transduction as well as MHC binding as factors essential for full T-cell activation.

Structural make-up of CD4 and CD8

While both co-receptors have similar functions, they are structurally different. CD4 is a single transmembrane glycoprotein possessing an extracellular domain folded into four immunoglobulin-like domains, followed by a transmembrane domain and a 38 residue cytoplasmic tail.(Maddon et al., 1985; Wu et al., 1997) Most studies are consistent with CD4 being a monomer on the surface of cells. Under conditions optimized for crystallization however, CD4 can form dimers.(Wu et al., 1997) Recent observations suggest that TCRs can also dimerize raising the possibility of forming a heterotetramer upon binding MHC.(Reich et al., 1997) In such cases, CD4 dimers may be formed.(Vignali et al., 1996) However, whether these structures bear any relevance to signal transduction in T-cells has yet to be determined.

CD4 is also a receptor for HIV. A number of reviews detail its role in infection.(Baltimore, 1995; Chan and Kim, 1998; Choe et al., 1998; Finzi and Silliciano, 1998; James et al., 1996; McCune, 1991; McMichael, 1998)

CD8, on the other hand, exists as a di-sulfide linked dimer.(Norment and Littman, 1988; Snow and Terhorst, 1983) Although homodimers containing two α chains can form, the major dimeric species expressed in mature T-cells consists of an α and β chain heterodimer. Both chains are single transmembrane proteins characterized by an extracellular domain containing a single immunoglobulin-like domain.(Kavathas et al., 1984; Littman et al., 1985; Norment and Littman, 1988) CD8α is clearly required for CD8-mediated signal transduction as it is the only subunit that interacts with Lck.(Rudd et al., 1988; Veillette et al., 1988) The role of CD8β, on other hand, is not entirely clear. Physical

measurements indicate that it exhibits a faster 'on' rate of MHC binding than CD8α.(Garcia et al., 1996) Hence it is thought that CD8β contributes to the stability of the MHC-peptide/TCR complex and in fact, this increased stability correlates with the α/β heterodimer more efficiently responding to antigen stimulation than the α chain homodimer.(Renard et al., 1996)

Association of Lck with CD4 and CD8α requires two conserved cysteine residues in the cytosolic domain of either co-receptor and two in the amino-terminus of lck.(Turner et al., 1990) In particular, both CD4 and CD8α contain a CXCP motif. These cysteines and their spacing within each domain are highly conserved, yet mutation of adjacent residues has no effect on their association. In CD4, mutation of anyone of these two cysteines results in failure to form a complex with Lck. In contrast, only the mutation of both conserved cysteines in CD8α entirely abolishes its interactions with Lck.

Lck protein tyrosine kinase

Lck was first isolated as a membrane-associated phosphoprotein expressed at high levels in a Moloney Murine Leukemia Virus transformed T-cell lymphoma (LSTRA).(Casnellie et al., 1983; Casnellie et al., 1982; Gacon et al., 1982) It is expressed in all immature and mature T-cell types.(Marth et al., 1985) Other cell types expressing Lck include natural killer cells and activated B-cells.(Einspahr et al., 1990; Taieb et al., 1993) Lck-deficient mice exhibit severe thymic atrophy accompanied by an almost total lack of peripheral T-cells.(Molina et al., 1992) Such profound defects underscore its essential role in thymocyte development. In addition, this phenotype is markedly broader in scope than the phenotype caused by the combined disruption of both CD4 and CD8α. Together with the finding that Lck also associates with the IL-2 receptor β chain, it is likely that Lck possesses functions in T-cell development that are co-receptor-independent.(Hatakeyama et al., 1991)

Lck is a member of the Src family of protein tyrosine kinases. And as such, it bears a number of stereotypical stuctural features: (1) A myristylated N-terminal domain required for association to the inner aspect of the plasma membrane; (2) an SH3 motif that binds proline-rich peptides; (3) an SH2 motif that binds phosphotyrosine containing peptides; (4) a catalytic tyrosine kinase region; (5) a negative regulatory COOH domain.

However, as mentioned previously, the Lck N-terminal domain is required for association with CD4 and CD8α.(Shaw et al., 1989; Shaw et al., 1990; Turner et al., 1990) Specifically, it possesses two highly conserved cysteines spaced as a CXXC motif,

a feature unique to Lck. Mutation of either cysteine abrogates co-receptor binding. Moreover, although wildtype Src kinase is unable to interact with either co-receptor, a chimeric kinase tagged with only the unique Lck N-terminal domain does. Therefore, this domain is both required and sufficient to mediate CD4 and CD8 association.

Lck activity is regulated by the opposing actions of the $p50^{csk}$ kinase and the CD45 phosphatase.(Bergman et al., 1992; Gervais et al., 1993; Koretzky et al., 1990; Nada et al., 1991; Okada et al., 1991; Pingel and Thomas, 1989; Volarevic et al., 1992; Weaver et al., 1991) Their equilibrium ultimately determines the phosphorylation state of tyrosine 505 located in Lck's negative regulatory COOH domain. While it is phosphorylated, the kinase is inactive. In mechanistic terms, it is believed that the SH2 domain binds intramolecularly to the C-terminal phosphotyrosine 505 thereby rendering the kinase domain closed to external substrates. Indeed, deletion of either the SH2 domain or tyrosine 505 results in the loss of repression and as a consequence, constitutive kinase activity.(Amrein et al., 1993; Marth et al., 1988; Reynolds et al., 1992; Veillette et al., 1992)

A model has been proposed to describe the molecular details of Lck involvement in T-cell signal transduction. First, by virtue of associating with CD4 and CD8, antigen stimulation places it within close proximity of the TCR. This in turn leads to CD45-mediated desphosphorylation and hence activation of Lck. In concert with Fyn, another src kinase, Lck is believed to then phosphorylate a number of TCR subunits including the TCR ζ chain and the CD3 γ, δ, and ε chains.(Mustelin et al., 1990; Qian et al., 1993; Samelson et al., 1986; Straus and Weiss, 1993; Weiss, 1993) This rapid generation of phosphotyrosines is believed to provide a scaffolding to bind a large array of SH2-containing molecules required for the first step of signal amplication including downstream effectors such as Zap70, PLCγ, Vav, and Grb-2.(Crabtree and Clipstone, 1994; Ravichandran et al., 1996; Weil and Veillette, 1996) As a consequence of antigen receptor-induced tyrosine phosphorylation, two major signalling pathways are activated leading to transcriptional events necessary for T-cell activation: (1) calcium/calcineurin (Beals et al., 1997; Clipstone and Crabtree, 1992; Timmerman et al., 1996) and (2) ras/raf signal transduction (Cantrell, 1996; Genot et al., 1996; Rayter et al., 1992; Woodrow et al., 1993) (Figure 1). Briefly, calcineurin, a calcium/calmodulin dependent phosphatase, is activated by a rise in intracellular calcium, allowing it to dephosphorylate the transcription factor, NFAT (nuclear factor of activated T-cells). Upon its dephosphorylation, NFAT then translocates to the nucleus, ready to complex with other transcriptional factors. Its transcriptional partners are composed of Fos- and Jun-related protein heterodimers and in contrast to NFAT, their expression is induced via the ras/raf pathway. Indeed, it has

become evident that activation of both the calcium/calcineurin and ras/raf pathways results in the formation of an NFAT/Fos/Jun complex that is competent to activate transcription of an array of genes such interleukins-2 and 4.

Transforming Growth Factor - β (TGF-β) receptors

TGF-β receptor structure and function

In 1981, Roberts and Sporn isolated a class of growth factors identified as acid-stable polypeptides potentiated by epidermal growth factor to induce transformation upon fibroblasts on soft agar. These initial observations led to their christening as transforming growth factors-β (TGF-β1, -β2, -β3) Subsequently however, this initial designation proved to belie its more prevalent effect on epithelial cells: potent growth inhibition.(Holley et al., 1980; Ikeda et al., 1987; Roberts et al., 1985; Wrann et al., 1987) Experimental data collected over the subsequent decade has revealed the complexity of the TGFs-β; they represent one of the most multifunctional peptide growth factors yet described, playing roles in growth inhibition, wound healing, immune response, angiogenesis, embryonic development and apoptosis. Moreover, they are the prototypical (founding) members of what has become to be known as the TGF-β superfamily of growth factors including the inhibins, activins, bone morphogenetic proteins, and Müllerian inhibitory substance.

These growth factors bind to two classes of surface receptors termed types I and II. Each ligand binds and activates a particular pair of type I and type II receptors. The types I and II receptors share a common structural design. Both classes of receptors possess a short cysteine-rich extracellular domain followed by a single transmembrane domain and a serine-threonine kinase-containing cytoplasmic domain. Unique to the type I receptors is a glycine/serine rich region in the juxtamembrane region, termed the GS domain. To date, six type I receptors, termed Alk1 through 6, have been identified.Although all can bind TGFs-β when co-expressed with TβRII, only Alk-5 can signal. (ten Dijke et al., 1994)

The TGF-βs also exert their effect through the binding of a type III receptor (betaglycan) and its closely related sibling, endoglin. Both are transmembrane proteoglycans with relatively short cytoplasmic domains. In contrast to the types I and II receptors, their cytoplasmic domain lack any similarity to known signalling domains. Hence it has been assumed that these receptors do not play a role in signalling; instead evidence suggests that they bind ligand and subsequently present it to the types I and II receptors, thereby increasing binding affinity of the receptor complex to TGFs-β.(Sankar et al., 1995) Nevertheless, it is clear that they play an essential role in human development, in

particular with respect to angiogenesis. Individuals afflicted with hereditary hemorrhagic telangectasia (HHT) type I possess mutations in the gene encoding endoglin. These individuals suffer from multisystemic vascular dysplasia resulting in gastro-intestinal hemorrhaging and arterio-venous malformations, implicating endoglin involvement in vascular development.(McAllister et al., 1995; McAllister et al., 1994)

Whether there are type III receptors for other TGF-β ligands has yet to be determined. However, there is evidence consistent with the existence of a type III-like receptor expressed in vascular endothelial cells for activin-A .(McCarthy and Bicknell, 1994)

Employing orthogonal approaches, a number of studies have elucidated to a large extent the sequence of events required for activation of the TGF-β receptor complex. Specifically, the types I and II receptors appear to have evolved into two interdependent signalling molecules. Each receptor is required for ligand-induced signalling. In particular, type II is required for ligand binding whereas type I is necessary for initiation of intracellular signalling. In binding and cross-linking studies, it has been established that TβRI (Alk-5) requires the presence of the type II receptor to bind the TGF-β ligands.(Wrana et al., 1992) In the case of TGF-β1 and TGF-β3, TβRII first binds ligand which then forms a ternary complex by recruiting TβRI into the TβRII/ligand complex. On the other hand, TGF-β2 appears to require co-expression of both types I and II to bind at high affinities.(Rodriguez et al., 1995)

Studies measuring receptor kinase activity point to how recruitment of TβRI leads to signal initiation.(Wrana et al., 1994) In particular, TβRII maintains high constitutive kinase activity independent of the presence of ligand. In contrast, the TβRI kinase becomes activated only after recruitment to the TβRII/ligand complex. This would suggest that TβRII may be mediating transphosphorylation of TβRI upon formation of the ternary complex. Indeed, when co-expressed with a kinase-defective TβRII, TβRI fails to become phosphorylated. Yet, kinase-deficient and wild-type TβRI phosphorylation patterns are indistinguishable when co-expressed with wild-type TβRII. Therefore, type II kinase activity is necessary for TβRI phosphorylation most likely by direct transphosphorylation of TβRI by RII. Signalling studies further demonsrate that TβRI phosphorylation and therefore its activation constitute events downstream of initial TβRII/ligand binding.(Franzen et al., 1993) Specifically, a clone (R1B) of a TGF-β responsive mink lung cell line expresses the type II, but not type I receptors; it fails to respond to TGF-β stimulation as measured by growth inhibition or induction of early response genes such as

plasminogen activator inhibitor-1 (PAI-1). However, transfection of TβRI cDNA into R1Bs is sufficient to restore signalling. Furthermore, transfection of a constitutively active TβRI (threonine 204 to aspartate) is sufficient to exert TGFβ signalling in the absence of TβRII or ligand further exemplifying the effector role of TβRI in the TGF-β receptor complex.

The types I, II and III receptor all form ligand-independent homo-oligomers, most likely dimers and upon TGF-β binding, form a types I:II heterotetramer. (Figure 1) (Gilboa et al., 1998; Henis et al., 1994; Moustakas et al., 1993; Yamashita et al., 1994) Two studies demonstrate the functional consequences of this particular receptor stoichiometry. First, Luo and co-workers constructed chimeric receptors consisting of the extracellular domain of the erythropoietin (Epo) receptor fused to the intracellular domain of either the types I or II receptor. They show that Epo-induced hetero-dimerization of a type I chimera with that of a type II can signal growth arrest.(Luo and Lodish, 1996) Moreover, a type I chimera encoding for a constitutively active kinase still requires Epo-induced homodimerization to signal. These results are consistent with the notion that the requirements for TGF-β signal transduction include both type I homodimerization as well as its heterodimerization with type II. In another approach, Weis-Garcia et al. observe that a kinase-defectve type I receptor mutant complements an activation-defective mutant when co-expressed in cells. These results further confirm that type I homodimerization is indeed essential for signalling (Weis-Garcia and Massague, 1996).

TGF-β signal transduction

Largely due to classical genetic analysis in *Drosophila* and *Caernorhabtidis elegans*, key components of the signalling pathways emanating from the TGF-β receptors have been identified. Most notably, members of the SMAD family of proteins were initially discovered from *Drosophila* by a genetic screen in which genes that enhanced the phenotype of a mutation in a BMP2/4 homolog, Decapentaplegic (Dpp), were isolated.(Raftery et al., 1995; Sekelsky et al., 1995) This screen yielded two highly homologous genes: *Mothers against dpp (MAD)* and *Medea.* Both encode proteins that act genetically downstream of *dpp*. Other members of this apparently growing family were later isolated in *C. elegans* in a similar screen for mutants that exhibited phenotypes similar to that of a type II receptor mutant.(Savage et al., 1996)

Within the past three years, these initial findings have led to rapid progress in the cloning and characterization of eight mammalian SMAD genes.In particular, three sub-

classes of SMADs have emerged: (1) pathway-restricted Smads (Smads 1,2,3,5 and 8), (2) a partner protein that associates with receptor-activated SMADs (Smad 4) and (3) inhibitory SMADs acting as negative regulators of TGF-β signalling (Smads 6,7). Mechanistically, an activated type I receptor phosphorylates a specific SMAD. These receptor-associated SMADs are pathway restricted as each one interacts with only a particular subset of type I receptors. For example, only the TGF-β and activin type I receptors can phosphorylate Smads 2 and 3 whereas only the BMP receptors can phosphorylate Smads 1 and presumably 5 and 8.(Baker and Harland, 1996; Chen et al., 1996; Eppert et al., 1996; Holley et al., 1980; Kretzschmar et al., 1997; Liu et al., 1996; Liu et al., 1997; Nakao et al., 1997; Suzuki et al., 1997; Watanabe et al., 1997; Zhang et al., 1996) Following their phosphorylation, these SMADs then associate with Smad4 and translocate to the nucleus.(Lagna et al., 1996; Zhang et al., 1996) By presumably associating with other nuclear factors, the resulting complex then activates transcription of ligand-responsive genes.

Although Smads 2 and 3 are highly homologous and can both associate with the TGF-β type I receptor, differences do emerge within the context of the developing organism. Homozygous null *Smad 2* mutant mice die early during embryogenesis.(Nomura and Li, 1998; Waldrip et al., 1998) *Smad3* mutant mice on the other hand are viable and fertile until four months of age when they begin to develop metastatic colorectal cancer.(Zhu et al., 1998)

Negative regulators, Smads 6 and 7, possess an amino terminal domain highly divergent from other signalling Smads. They inhibit signalling by competing with pathway-restricted SMADs in binding activated type I receptors.(Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997) It has been proposed that by stably binding to these receptors, they then prevent transphosphorylation of the downstream pathway-restricted SMADs. Smads 6 and 7 can bind a number of type I receptors consistent with their inhibition of multiple TGF-β superfamily signalling pathways. Intriguingly, Smad 6 and 7 mediated inhibiton of TGF-β signalling may be autoregulatory as TGF-β stimulation induces their expression. An additional mechanism of inhibition has been proposed for Smad6 as it is capable of binding Smad1 and therefore may act as a non-functional Smad 4 decoy.

Several key aspects of Smad signalling require further study. For instance, the SMAD proteins can homo as well as hetero-oligomerize. However, the precise stoichiometry of these complexes remains to be fully determined.*In vitro*, Smad4 has been

shown to form homotrimers.(Shi et al., 1997) Kawabata and co-workers find that in live cells however, pathway-restricted Smads are monomers and undergo both homo- and hetero-oligomerization upon activation.(Kawabata et al., 1998)

In addition, based on crystallographic data, Shi and co-workers demonstrate that Smad3 binds a specific 4 base pair DNA sequence.(Shi et al., 1998) Because such low DNA binding selectivity would be insufficient for specifically activating transcription of TGF-β-responsive genes, it is believed that additional transcription factors act in combination with the SMAD complex to elicit gene expression. A model calling for such a combinatorial approach would allow for the mediation of a wide range of ligand-induced responses despite relatively low DNA sequence binding specificity for the SMAD family of proteins. To date, two such candidate factors appear to fulfill this role. First, the winged helix transcription factor FAST-1 has been shown to mediate activin-induced gene expression.(Chen et al., 1996; Chen et al., 1997; Chen et al., 1998) Specifically, FAST-1 is a nuclear protein and upon activin stimulation, associates with incoming Smads 2 and 4 to form a complex termed activin-response factor (ARF). The newly formed ARF then activates transcription by binding to DNA sequence elements found within the promoter of the *Mix.2* gene, an activin early-response gene. Within the promoter, FAST-1 binds a six base pair sequence thereby augmenting the DNA binding specificity of the ARF complex. A number of other activin responsive promoters appear to lack FAST-1 binding sites in close proximity to those of Smad2. This would suggest that other as of yet unidentified factors may also complex with Smads2 and 4 to induce remaining aspects of the activin induced response. Secondly, with respect to the TGF-β pathway, Hua and co-workers show that an E-box binding transcription factor called TFE3 cooperates synergistically with Smads 3 and 4 to activate transcription of the TGF-β responsive plasminogen activator inhibitor-1 (PAI-1) promoter.(Hua et al., 1998; Westerhausen et al., 1991) By studying the transcriptional activity of PAI-1 promoter mutants, they find that TFE3 and Smads3 and 4 bind to adjacent sites within a 36 bp element to induce TGF-β dependent trancription. Moreover, TFE-3 binds a specific six base pair DNA sequence within this element. This further demonstrates the involvement of transcription factors that confer greater DNA binding selectivity to SMAD containing complexes, a property presumably necessary for mediating pathway specific responses.

Other molecules have been implicated to mediate TGF-β signalling. The immunophilin, FKBP12, is one of the best characterized examples. Better known as a target for various clinically relevant immunosuppressants, it also binds to a leucine-proline sequence within the type I receptor GS domain preventing transphosphorylation by the type

II receptor. Upon ligand-induced TβRI/TβRII association, it is then thought that FKBP12 dissociates from TβRI.(Chen et al., 1997; Wang et al., 1994; Wang et al., 1996) Mutant type I receptors that are unable to bind FKBP12 exhibit high basal signalling activity consistent with FKBP12's role as a negative regulator of TGF-β signalling.(Chen et al., 1997) Therefore, its function appears to lie in protecting the cell from spontaneous signalling in the absence of ligand.

Three other proteins have been shown to be capable of directly associating with the TGF-β receptors. $p21^{RAS}$ farnesyl protein-transferase α has been shown to bind the type I receptor and both TRIP-1, a WD-domain containing protein, and apolipoprotein J have been shown to bind to the type II receptor.(Chen et al., 1995; Kawabata et al., 1995; Reddy et al., 1996; Ventura et al., 1996; Wang et al., 1996) However, the functional significance of these interactions have yet to be elucidated.

TGF-β responses

The multipotent TGF-β signals can be classified into immediate and secondary effects. With regard to immediate effects, the TGFs-β inhibit growth by arresting the cell cycle at the G1 to S transition. Specifically, inhibitory signals are transmitted through two independent pathways: (1) the upregulation of CDK inhibitors, p15 together with either p21 or p27, and (2) the repression of a CDK tyrosine phosphatase, CDC25a.(Figure 2) (Datto et al., 1995; Hannon and Beach, 1994; Iavarone and Massague, 1997) Both pathways apparently converge to the negative regulation of cyclin-dependent kinases (CDKs) 4 and CDK6. Activation of CDKs-4 and 6 are necessary for phosphorylation of the retinoblastoma (Rb) protein and subsequent entry to S phase. Therefore, it appears to be at the level of CDK activity, a key junction of the cell cycle machinery, that TGF-β exerts it primary negative growth control.

With certain cell types, TGFs-β also impinge second order effects including extracellular matrix production, immunosuppresion, and induction of angiogenesis.(Huber et al., 1992; Khanna et al., 1998; Roberts et al., 1986; Tashjian et al., 1985) Under particular circumstances, opposing secondary effects such as the induction of platelet derived growth factor secretion can overcome this block in cell cycle progression hence explaining earlier seemingly contradictory observations documenting the TGF-β induction of transformation upon fibroblasts.

TGF-βs and human pathogenesis

The most striking validation of the current model for TGF-β signalling can be illustrated by mutations of genes implicated in the signalling pathway and their resulting disease manifestations. It is believed that cells harboring mutations that render them unresponsive to TGF-β are endowed with a significant growth advantage over their responsive counterparts. Indeed, loss of TGF-β responsiveness is a common hallmark of various cancers.

On the receptor level, mutations in the type II receptor have been isolated from tumors of the colon, head and neck, T-cell lymphomas and gastric cancers, substantiating their role as tumor suppressors in a variety of cancers.(Garrigue-Antar et al., 1995; Knaus et al., 1996; Lu et al., 1998; Park et al., 1994) Furthermore, type II receptor mutations are found in 90% of all colon cancers exhibiting microsatellite instability.(Markowitz et al., 1995; Wang et al., 1995) The high mutation frequency observed does not appear to be due to an inherent susceptibility of the type II receptor gene to mutations. Rather, only gastric/colon cancers exhibit high mutation frequencies whereas other cancers arising from microsatellite instability do not. This would suggest that type II receptor mutations are somehow selected for in gastric and colon cancers. Mutations appear to occur late in tumorigenesis suggesting that the TGF-β pathway plays a role in malignant progression rather than tumor initiation.(Grady et al., 1998) Whether the type I receptor is likewise a primary target for inactivating tumors in cancers is not as well established. However, Schiemann and co-workers have recently isolated a type I receptor mutation from a TGF-β-resistant T-cell lymphoma [manuscript in preparation]. And indeed, these mutations were demonstrated to result in the loss of type I receptor expression.

The relevance to cancer of the SMAD family of proteins became apparent shortly after their isolation in *Drosophila* and *C. elegans*. As one of the first vertebrate SMADs to be identified, SMAD4 was discovered as a gene in which both alleles were deleted in 90% of human pancreatic carcinomas.(Hahn et al., 1996) Subsequent studies in mice reinforce the essential role played by SMAD4 in the TGF-β pathway. Specifically, Takaku and co-workers show that tumors homozygously deficient for both SMAD4 and another tumor suppressor gene, APC, caused larger and more rapidly progressing carcinomas than in mice homozygously deficient for only APC(Hahn et al., 1996) These results therefore are consistent with SMAD4 playing a key role in the rate of tumor progression. Among pathway specific SMADs, SMAD2 has also been shown to be a target of inactivating mutations in colon cancers.(Eppert et al., 1996; Riggins et al., 1997; Uchida et al., 1996)

Intriguingly, despite the fact that SMAD3 knock-out mice develop metastatic colorectal cancer, no mutations have been identified in human cancers thus far.

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Figures

Figure 1:
Downstream signalling pathways activated upon TCR stimulation

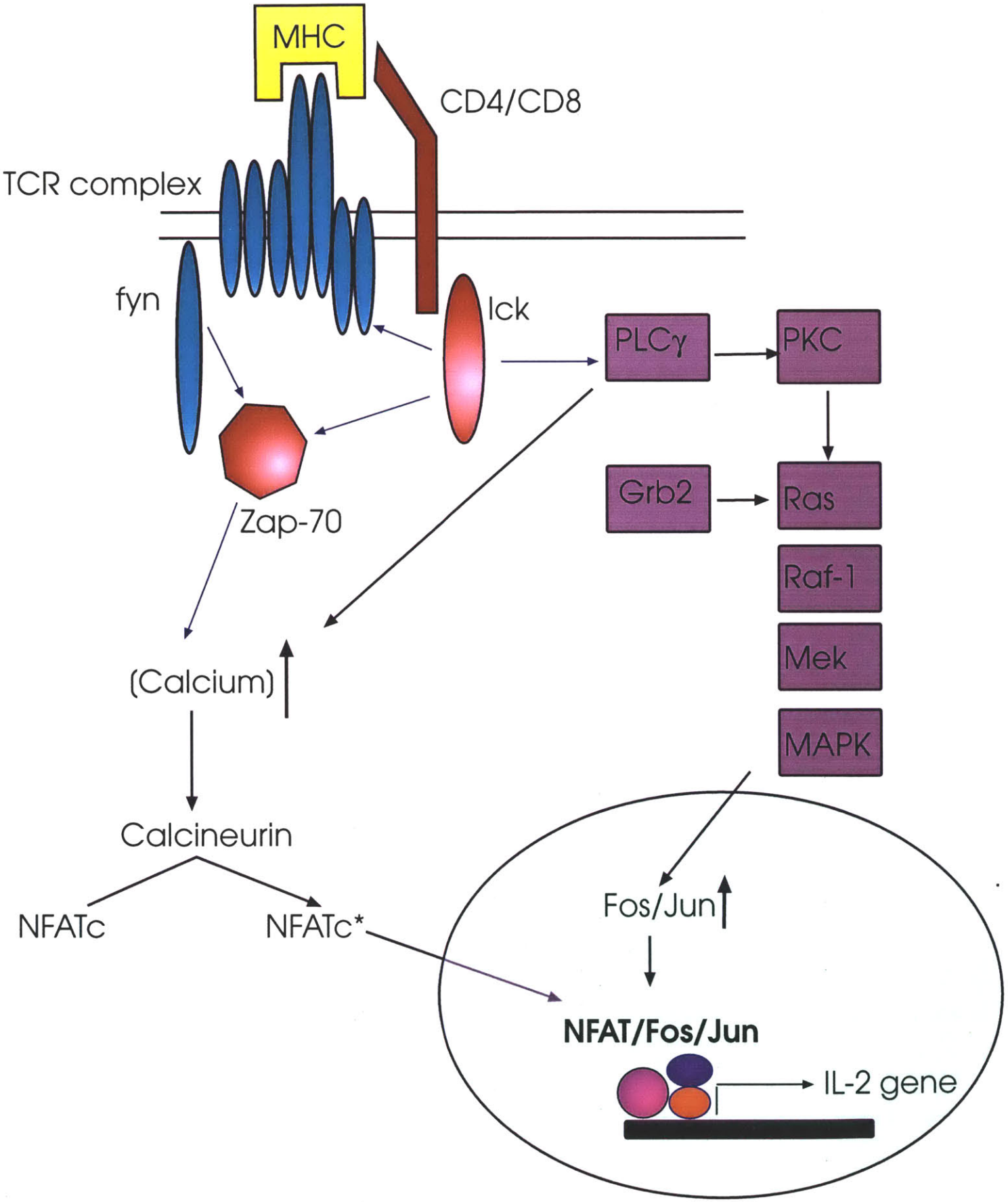
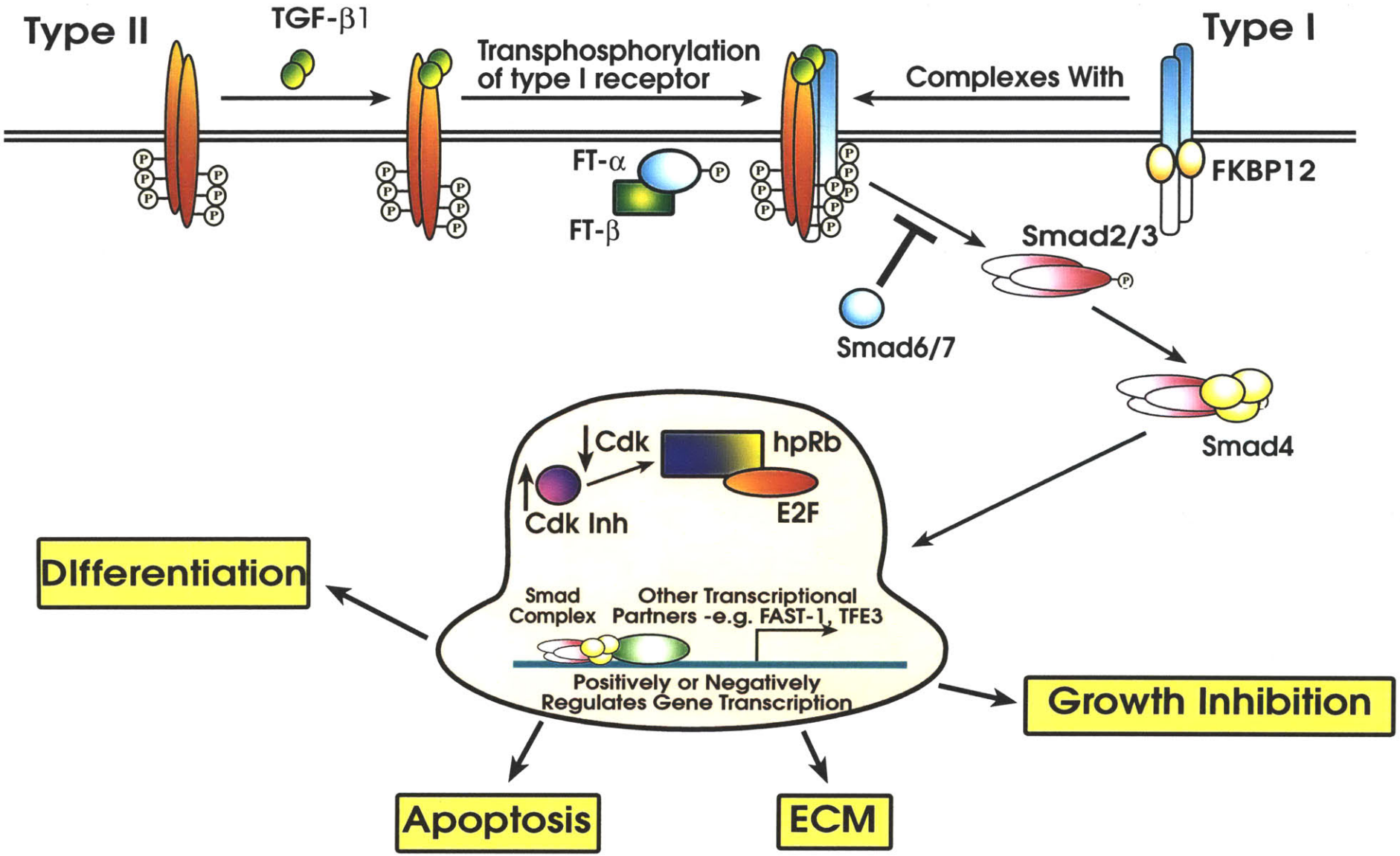


Figure 2: Overview of TGF-β Signaling



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Chapter 2

Zinc is essential for binding of $p56^{lck}$ to CD4 and CD8α

Preface

With the exception of figure 3(c) and its accompanying text, the entirety of this chapter has been published in the Journal of Biological Chemistry.

Ralph S. Lin, Carlos Rodriguez, André Veillette and Harvey F. Lodish (1998) Zinc is essential for binding of $p56^{lck}$ to CD4 and CD8α *J. Biol.Chem.* 273:32878-32882.

Abstract

Binding of the protein tyrosine kinase $p56^{lck}$ to T-cell co-receptors CD4 and CD8α is necessary for T-lymphocyte development and activation. Association of $p56^{lck}$ with CD4 requires two conserved cysteine residues in the cytosolic domain of CD4 and two in the amino-terminus of $p56^{lck}$, consistent with the notion that these four residues coordinate a single metal atom (Huse et al., 1998; Rudd et al., 1988; Shaw et al., 1989; Turner et al., 1990; Veillette et al., 1988). Here we demonstrate that Zn^{2+} is essential for complex formation. In an *in vitro* binding reaction, Zn^{2+} mediates $p56^{lck}$ association with a GST fusion protein containing the cytosolic domains of CD4 or CD8α; no other metals tested support binding. Treatment of preformed GST-CD4/$p56^{lck}$ dimers with the Zn^{2+} chelators 1,10 O-phenanthroline or 8-hydroxyquinoline-5-sulfonic acid results in dissociation of GST-CD4 from $p56^{lck}$, consistent with the finding of (Huse et al., 1998) that Zn^{2+} is contained within similar complexes. Furthermore, we show that within live cells, CD4/$p56^{lck}$ and CD8α/$p56^{lck}$ interactions occur in a zinc-dependent fashion. Specifically, pretreatment of the human T-cell Jurkat cell line with membrane permeable zinc chelators disrupts CD4/$p56^{lck}$ complexes, and treatment of COS cells co-expressing CD8α and $p56^{lck}$ with such chelators likewise leads to dissociation of CD8α/$p56^{lck}$ complexes. CD4/$p56^{lck}$ and CD8α/$p56^{lck}$ represent the first examples of intracellular proteins that require zinc as a bridge for heterodimerization.

Introduction

T-lymphocyte activation requires the coordination of a large complex of proteins collectively termed the T-cell receptor (TCR). Within the TCR, activation of $p56^{lck}$, a lymphocyte-specific member of the Src family of non-receptor protein tyrosine kinases, is necessary for initiation of signal transduction. By binding to the cytosolic domains of CD4 and CD8α, $p56^{lck}$ is placed in close proximity to its downstream substrates, CD3, TCR ζ chain and Zap70. They, in turn, activate subsequent signaling events (Weil and Veillette, 1996; Weiss and Littman, 1994).

Association of $p56^{lck}$ with CD4 requires two conserved cysteine residues in the cytosolic domain of CD4 and two in the amino-terminus of $p56^{lck}$ (Rudd et al., 1988; Shaw et al., 1989; Turner et al., 1990; Veillette et al., 1988). Mutation of any of these four cysteines results in failure to form a CD4/$p56^{lck}$ complex (Barber et al., 1989; Shaw et al., 1989; Veillette and Fournel, 1990). These cysteines and their spacing within each domain are highly conserved, yet mutation of adjacent residues has no effect on CD4/$p56^{lck}$ association (Shaw et al., 1989; Shaw et al., 1990; Turner et al., 1990). Given the strongly reducing environment in the cytosol of eukaryotic cells, formation of intermolecular disulfide bonds between CD4 and $p56^{lck}$ is unlikely (Hwang et al., 1992). Treatment of cell lysates with iodoacetamide, a reagent that alkylates free cysteines, prevents binding of $p56^{lck}$ to the cytosolic domain of CD4. This finding suggests that the cysteines involved in association remain reduced in the CD4/$p56^{lck}$ complex and raises the possibility of a metal ion stabilizing the complex (Shaw et al., 1990). Recently, Huse et al. (1998) co-expressed in *Escherichia coli* a fusion protein consisting of maltose binding protein fused to the CD4 cytoplasmic domain and a portion of the N-terminus of $p56^{lck}$; the resulting 1:1 molar complex contained one atom of zinc and formation of the complex required cysteine residues both in the CD4 cytoplasmic domain and the N-terminus of $p56^{lck}$.

Here, we show that Zn^{++} is essential for CD4/$p56^{lck}$ complex formation, both in cell-free binding assays and in live cells. In addition, we present evidence showing that formation of a CD8α/$p56^{lck}$ complex is similarly dependent on Zn^{2+}.

Experimental Procedures

Cells

Jurkat cells (American type culture collection; Clone E6-1, human acute T-cell leukemia) were grown to a density of ~10^6 cells/ml in RPMI-1640 medium containing 10% fetal calf serum, 5 mg/ml glucose, 100 units/ml penicillin and 100 µg/ml streptomycin. Fifty ml of culture was processed for each data point.

Construction of GST-CD4 and GST-CD8α fusion proteins and in vitro binding reactions.

By PCR a cDNA encoding the 37 residue cytoplasmic domain of murine CD4 was generated and inserted into the EcoR1 and Not1 sites of the Pharmacia pGEX-4T1 vector. The resulting ligation produced an in-frame fusion protein with glutathione S-transferase (GST). Forward and reverse primers mutating cysteines 418 (C418A) and 420 (C420A) to alanine were generated. Using these primers, double and single cysteine to alanine mutations in the cytoplasmic domain of CD4 were generated by overlap PCR. Similarly, a cDNA encoding the 27 amino acid cytoplasmic domain of orangutan CD8α was inserted in-frame with that of GST at the same site in the above Pharmacia vector. GST-CD4, GST-CD8α, and GST alone were expressed in *E. Coli* DH10B. After one hour induction with 1 mM isopropyl ß-D thiogalactopyranoside (IPTG) at 37°C, proteins were purified as previously described (Smith and Johnson, 1988) and immobilized to glutathione-Sepharose 4B (Pharmacia) at 4°C for 1 hour. After washing 15 µg of immobilized protein three times with TBS-GT (137 mM NaCl, 20 mM Tris-HCl [pH 8.0], 1% Triton X-100, 10% glycerol) (Turner et al., 1990), the slurry was resuspended in 500 µl of TBS-GT containing 1 µg of purified bovine $p56^{lck}$ (10 µl solution containing ~100 µg/ml or 500 units/ml; Upstate Biotechnology, catalog #14-106), and the indicated concentrations of $ZnCl_2$ or other metals. These binding reactions contain GST-CD4 or GST-CD8α at an approximately 20 to 1 molar excess over $p56^{lck}$. Following incubation at 4°C for 8 hours, samples were washed with TBS-GT three times and TBS once. Samples were analyzed by 8% SDS-PAGE (Laemmli, 1970) and transferred by semi-dry electrophoresis to 0.22 µm porosity nitrocellulose. The membrane was blocked with Buffer A [1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, 0.5% Tween-20, and 3% non-fat powdered milk, pH 7.0] followed by incubation with 0.5 µg/ml of an anti-human $p56^{lck}$ monoclonal antibody (Santa Cruz Biotechnology, catalog #SC-433) for 3 hours at room temperature. After washing 5 times with Buffer A, a 1:5000 dilution of goat anti-mouse secondary horse radish peroxidase antibody (Amersham) was added for 2 hours at room

temperature. The membrane was washed three times with Buffer A and two times with PBS; bound antibodies were detected by addition of an ECL substrate as specified by the manufacturer (Pierce). Autoradiographic films were analyzed by using a Bio-Rad GS-700 flatbed scanner together with Molecular Analyst v2.1 software (Bio-Rad).

Immunoprecipitation (IP) and immunoblotting

About 5 x 10^7 Jurkat cells or 10^8 COS-7 cells were lysed in 1 ml of TBS-GT containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Antibodies were preincubated with 50 µl/IP of protein-A sepharose for 15 minutes at 4°C. After a 5 minute centrifugation, cleared lysates were incubated with the antibody/protein-A sepharose mixture for ~ 2 hours at 4°C. Antibodies used were: anti-human $p56^{lck}$ polyclonal antibody (Upstate Biotechnology, catalog #06-583, 2 µl per IP), anti-human CD4 polyclonal antibody (Immunodiagnostics, catalog #7301, 5 µl per IP), and anti-mouse CD8α polyclonal antibody (5 µl per IP) (Sarmiento et al., 1980). After incubation at 4°C, immunoprecipitates were washed three times with TBS-GT followed by one wash with TBS. Immunoprecipitates were separated by 8% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with an anti-$p56^{lck}$ antibody as described above.

COS cell transfections

Using the DEAE/dextran method, COS-7 cells (American Type Culture Collection) were transiently transfected (Seed and Aruffo, 1987) with 10 µg of DNA: either murine $p56^{lck}$ in the vector pXM-139 (unpublished data, Veillette et al.), 10 µg of $p56^{lck}$ (C20/23S) in pcDNA3.1 (Invitrogen), or 10 µg of CD8α (Lyt2a) in pCDNA3.1. $p56^{lck}$(C20/23S) contains mutations in which cysteines 20 and 23 are mutated to serine (Gervais and Veillette, 1995). Forty-eight to 72 hours after transfection, the cells were washed once with PBS and lysed in TBS-GT containing 1 mM PMSF. After a 5 minute centrifugation at 4°C, cleared lysates were incubated with 15 µg of immobilized GST-CD4 overnight at 4°C. Samples were then washed and assayed for bound $p56^{lck}$ as described above. In other studies, cleared lysates were immunoprecipitated with anti-$p56^{lck}$ or anti-CD8α antibodies and processed for $p56^{lck}$ immunoblotting as described above.

Results

Figure 1A shows the results of an *in vitro* binding reaction, demonstrating that indeed Zn^{++} mediates the association of the cytoplasmic domain of CD4 and $p56^{lck}$ in a concentration-dependent manner. Recombinant glutathione S-transferase (GST) fused to the cytoplasmic domain of murine CD4 was produced and purified from *Escherichia coli* followed by immobilization on glutathione sepharose. As detected by immunoblotting with an anti-$p56^{lck}$ antibody, association of purified bovine $p56^{lck}$ with GST-CD4 depends upon the presence of 100 μM $ZnCl_2$. At this $ZnCl_2$ concentration almost 100% of the introduced $p56^{lck}$ becomes bound to GST-CD4. The detected $p56^{lck}$ band is not a contaminant of the GST-CD4 preparation as no binding is seen in the absence of $p56^{lck}$, and GST alone does not bind $p56^{lck}$. Furthermore, other metals tested at 100 μM, including Ca^{2+}, Ni^{2+}, Cu^{+}, Cu^{2+}, and Fe^{2+}, do not support complex formation (Figure 1B), suggesting that association of CD4 with $p56^{lck}$ specifically requires zinc.

Consistent with previous findings, our *in vitro* binding reaction recapitulates the critical role in association played by the four conserved cysteines. In particular, Figure 2A shows that immobilized GST-CD4 binds murine $p56^{lck}$ expressed in lysates of transfected COS cells, but fails to interact with a mutant $p56^{lck}$ in which the two essential cysteines, implicated in binding to CD4 and CD8α, were mutated to serine. Additionally, mutants of the CD4 cytoplasmic domain in which either one or both conserved cysteines were changed to alanine lose significant binding to $p56^{lck}$ (Figure 2B). Nevertheless, all mutants exhibited some residual binding. Furthermore, the presence of 1 mM β-mercaptoethanol in the binding reaction had no effect on formation of a CD4/ $p56^{lck}$ complex (data not shown), consistent with the notion that the cysteines necessary for association are reduced within the formed complex.

Moreover, the presence of Zn^{2+} chelators both prevents complex formation as well as disrupts preformed ones. As shown in Figure 2A, GST-CD4/$p56^{lck}$ complex formation is blocked by addition of the zinc chelator 1,10 O-phenanthroline to COS cell lysates expressing wild type $p56^{lck}$. Furthermore, pre-formed CD4/$p56^{lck}$ complexes are disassociated by similar treatment. In the study in Figure 3A, GST-CD4/$p56^{lck}$ complexes were formed and then treated with the zinc chelators 8-hydroxyquinoline-5-sulfonic acid or 1,10 O-phenanthroline or a non-chelating analog, 1,7 phenanthroline. Only 1,10 O-phenanthroline and 8-hydroxyquinoline-5-sulfonic acid disassociated pre-formed CD4/$p56^{lck}$ dimers. These findings suggest that removal of Zn^{++} disrupts CD4/$p56^{lck}$

complexes; disruption cannot be attributed to non-chelating properties of 1,10 O-phenanthroline such as its hydrophobicity, a characteristic shared with 1,7 phenanthroline (Auld, 1988). Thus Zn^{++} is required for CD4/$p56^{lck}$ interaction and its removal from the complex results in their dissociation.

Figure 3B shows that, in signaling lymphoid cells, CD4/$p56^{lck}$ complexes are disrupted by addition of the membrane permeable zinc chelators TPEN and 1,10 O-phenanthroline; importantly, 1,7 phenanthroline, a non-chelating analog, or EDTA, a membrane-impermeable chelator, has no effect on CD4/$p56^{lck}$ complexes. Specifically, as judged by co-immunoprecipitation with CD4, treatment of living Jurkat cells with TPEN or 1,10 O-phenanthroline eliminated binding of $p56^{lck}$ to CD4. However, 1,7 phenanthroline or EDTA exhibited no such effect. The total amount of $p56^{lck}$ in the cell was unaffected by any of the treatments, as shown by immunoprecipitation with and immunoblotting with anti-$p56^{lck}$ antibodies. We have obtained the same results with murine $p56^{lck}$ and CD4 co-transfected in COS cells (Figure 3C). Thus, both *in vitro* and in living cells, zinc chelators disrupt association of CD4 with $p56^{lck}$, presumably by chelating an essential Zn^{2+} ion present in the complex.

The cytosolic domains of the T-cell CD8α and CD4 co-receptors possess a homologous pair of cysteine residues, suggesting that they dimerize with $p56^{lck}$ in a similar manner. Indeed, in COS cells transiently transfected with CD8α and $p56^{lck}$, $p56^{lck}$ binds to CD8α and this interaction is disrupted by 1,10 O-phenanthroline, but not 1,7 phenanthroline (Figure 4A). Importantly, CD8α does not associate with the mutant $p56^{lck}$(C20,23S) lacking the two cysteines essential for interaction with CD4 (Figure 4A). This control also establishes that CD8α/$p56^{lck}$ interactions cannot be attributed to non-specific aggregations that are often associated with protein over-expression.

As with that of CD4, the cytosolic domain of CD8α also associates with $p56^{lck}$ *in vitro* but requires a ten-fold higher $ZnCl_2$ concentration to support binding - 1 mM *versus* 100 μM (Figure 4B). At the same concentration, other metals such as Cu^{2+}, Ca^{2+}, and Co^{2+} do not support binding (data not shown).

Discussion

Our work builds on previous studies implicating a role for Zn^{2+} or another metal ion in mediating an interaction between the short cytoplasmic domains of CD4 and CD8α with $p56^{lck}$. First, the cytoplasmic domains of CD4 and CD8α and the N-terminal region of $p56^{lck}$ each contain a pair of cysteines that are essential for CD4/$p56^{lck}$ and CD8α/$p56^{lck}$ interactions (Rudd et al., 1988; Shaw et al., 1989; Turner et al., 1990; Veillette et al., 1988). Second, alkylating agents that inactivate free cysteine sulfhydryl groups destroy CD4/$p56^{lck}$ interactions (Shaw et al., 1990). Third, Huse et. al. (Huse et al., 1998) identified a 1:1 molar complex of the CD4 cytoplasmic domain and a portion of the N-terminus of $p56^{lck}$ when these were co-expressed in *Escherichia coli* . Complex formation required cysteine residues both in the CD4 cytoplasmic domain and the N-terminus of $p56^{lck}$, and each protein complex contained one atom of zinc. However, Huse and colleagues were unable to assemble the CD4/$p56^{lck}$ complex *in vitro* and thus were unable to determine whether zinc or another metal is required for complex formation (S. Harrison, personal communication).

Here, we show that formation of a complex - presumably a dimer - of the cytoplasmic domain of CD4 and the complete $p56^{lck}$ protein specifically requires the presence of zinc. We first developed an *in vitro* binding assay which recapitulates the critical role in association played by the two cysteines in CD4 and two at the N- terminus of $p56^{lck}$. Only Zn^{2+}, and none of the other metals tested - Ca^{2+}, Ni^{2+}, Cu^{+}, Cu^{2+}, and Fe^{2+} - supported CD4/$p56^{lck}$ association. Moreover, Zn^{2+} chelators both prevent association as well as disrupt preexisting CD4/$p56^{lck}$ complexes. Likewise, in live signaling cells, membrane permeable zinc chelators dissociate CD4/$p56^{lck}$ complexes, presumably by removing zinc. In conjunction with (Huse et al., 1998), our data firmly establish a role for Zn^{++} in mediating CD4/$p56^{lck}$ association through the coordination of two cysteines in the CD4 cytoplasmic domain and two in the N-terminus of $p56^{lck}$.

The N-terminus of $p56^{lck}$ contains a strikingly high density of acidic, negatively charged residues whereas the cytoplasmic domains of both CD4 and CD8α contain a high density of basic residues. This charge complementarity (Bramson et al., 1991; Vega et al., 1990) may stabilize the interactions of $p56^{lck}$ with CD4 and CD8α and may explain the weak but specific interactions we detected *in vitro* between $p56^{lck}$ and mutants of CD4 in which one or both of the essential cysteines were changed to alanine. The elevated

concentrations of both GST-CD4 and $p56^{lck}$ used in these binding assays could favor formation of such complexes.

The relatively high zinc concentrations required for binding of $p56^{lck}$ to GST-CD4 (10-100 μM Zn^{++}) or GST-CD8α (100 μM to 1 mM Zn^{++}) are comparable to that required for HIV Tat homodimerization - 100 μM Zn^{2+}; this is the only other intracellular protein-protein interaction thus far characterized that requires zinc as a bridge for dimerization (24, 25). In addition, we found (unpublished) that glutathione S-transferase alone can bind zinc.Thus, the actual concentrations of free Zn^{2+} in our GST-CD4/$p56^{lck}$ and CD8α/$p56^{lck}$ binding assays may be lower than the added Zn^{2+} concentrations. Nevertheless, these high concentrations may reflect differences in association that occur *in vitro* versus in live cells. In live cells, factors such as myristylation of $p56^{lck}$ and its consequent insertion into the plasma membrane may contribute to the stability of the complex, as could association of $p56^{lck}$ with the TCR ζ chain. More intriguingly, Vallee and co-workers showed that, under oxidizing conditions similar to those in the endoplasmic reticulum, zinc can be transferred from its binding site in metallothioneins to those of lower affinity in other zinc proteins. Hence, inside the cell where the concentrations of free zinc are in the picomolar range, metallothioneins may play a role in transferring zinc to proteins during their biosynthesis (Jacob et al., 1998; Jiang et al., 1998; Maret et al., 1997; Maret and Vallee, 1998).

Furthermore, we show that CD8α interacts with $p56^{lck}$ in an analogous zinc-dependent manner to CD4. *In vitro*, association of GST-CD8α with $p56^{lck}$ specifically requires zinc; other metals tested do not support binding. In living cells, CD8α/$p56^{lck}$ complexes are disrupted by membrane-permeable zinc chelators. The higher zinc concentration required for formation of the CD8α/$p56^{lck}$ than the CD4/$p56^{lck}$ complex leads us to speculate that, under limiting zinc concentrations, $p56^{lck}$ binding to CD8α would be of lower affinity than to CD4. This is supported by studies showing that a greater proportion of total cellular $p56^{lck}$ associates with CD4 than with CD8α (50-90% versus 10-25% for CD4 and CD8α, respectively) (Rudd et al., 1988; Veillette et al., 1988). Furthermore, only the mutation of both conserved cysteines in CD8α entirely abolishes $p56^{lck}$ association (Turner et al., 1990). These observations suggest that the nature of zinc binding in the CD8α/$p56^{lck}$ complexes may not be identical to that in CD4/$p56^{lck}$.

Finally, our studies show that membrane-permeable Zn^{2+} chelators rapidly and completely disrupt CD4/$p56^{lck}$ complexes in lymphoid cells. Thus, TPEN and 1,10 O-

phenanthroline may serve as useful tools to dissect TCR signaling in the absence of CD4-mediated $p56^{lck}$ activation.

The CD4/$p56^{lck}$ and CD8α/$p56^{lck}$ dimers are, to our knowledge, the first examples of intracellular protein heterodimers bridged by zinc. CD4/$p56^{lck}$ complexes form in the endoplasmic reticulum within ten minutes of CD4 biosynthesis (Shaw et al., 1989). As yet we do not know what role, if any, zinc might play in folding of CD4, CD8α, and $p56^{lck}$, nor do we know the stage of biosynthesis where zinc links the two proteins together. Structural studies are needed to elucidate the details of the Zn^{2+}-containing interface between $p56^{lck}$ and either CD4 or CD8α; the HIV Tat protein may serve as an informative model with which to begin such structural studies. In the presence of 100μM $ZnCl_2$,Tat forms a homo-dimer *in vitro* bridged by 4 Zn^{2+} ions (Frankel et al., 1988; Frankel et al., 1988). Biophysical measurements indicate that little change in secondary structure occurs upon complex formation, suggesting that metal binding does not significantly alter global protein folding. With respect to association of $p56^{lck}$ with CD4 or CD8α, it is possible that zinc may likewise simply serve as a molecular "adhesive" by which to bridge two proteins.

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We are grateful to Drs. Stefan Constantinescu, Bill Schiemann, Merav Socolovsky, and Rebecca Wells for critical comments and discussions.

Figure Legends

Figure 1: Interaction of GST-CD4 and $p56^{lck}$ *in vitro* requires zinc.

A) GST-CD4/$p56^{lck}$ interaction requires the presence of $ZnCl_2$. Reactions contained 1 μg purified bovine $p56^{lck}$, either 15 μg of immobilized GST-CD4 or GST alone, and the indicated concentration of $ZnCl_2$. $p56^{lck}$ bound to the glutathione beads was subjected to Western blotting with an anti-$p56^{lck}$ antibody. The right most lane contains 1 μg of $p56^{lck}$ as a control for efficiency of binding; the first lane on the left depicts a control reaction containing GST-CD4 and 100 μM $ZnCl_2$, but no $p56^{lck}$. Samples were processed and assayed for bound $p56^{lck}$ as described in Experimental Procedures.

B) The GST-CD4/$p56^{lck}$ complex forms only in the presence of zinc.

Fifteen μg of immobilized GST-CD4 or GST alone was incubated in 500 μl of TBS-GT containing 1 μg $p56^{lck}$ and 100 μM of the indicated divalent metal for 8-12 hours at 4°C. Samples were processed and assayed for bound $p56^{lck}$ as described in Experimental Procedures.

Figure 2: Association of $p56^{lck}$ with GST-CD4 requires two conserved cysteine residues in the cytosolic domain of CD4 and two in the amino-terminus of $p56^{lck}$

A) Interaction of $p56^{lck}$ expressed in COS-7 cells with GST-CD4.

COS-7 cells were transiently transfected with either murine $p56^{lck}$ in pXM-139 or $p56^{lck}$ (C20/23S) in pcDNA3.1. Forty-eight hours after transfection, the cells were washed once with PBS and lysed; in the indicated sample, 5 mM 1,10 O-phenanthroline was added. After centrifugation at 4°C the cleared lysates were incubated with 15 μg immobilized GST-CD4 overnight at 4°C. Samples were then washed and assayed for bound $p56^{lck}$.

B) Interaction of $p56^{lck}$ with cysteine mutants of GST-CD4.
In 500 μl of TBS-GT containing 100 μM $ZnCl_2$, 1 μg of $p56^{lck}$ was incubated with 15 μg of GST, GST-CD4, GST-CD4 (C418A,C420A), GST-CD4 (418A) or GST-CD4 (C420A) for 8 hours at 4°C. Samples were processed and assayed for bound $p56^{lck}$ as described in Experimental Procedures.

Figure 3: Interaction of CD4 and $p56^{lck}$ is disrupted by zinc chelators

A) Zinc chelators disrupt preformed GST-CD4•$p56^{lck}$ complexes.

As described in Figure 1A, GST-CD4/$p56^{lck}$ complexes were formed at 100 μM $ZnCl_2$. After washing three times with TBS-GT, bound complexes were resuspended in 500 μl of TBS-GT alone, with 5 mM 1,7 phenanthroline, with 5 mM 1,10 O-phenanthroline, or with 5 mM 8-hydroxyquinoline-5-sulfonic acid. After a ten minute incubation at 4°C, samples were washed three times with TBS-GT and once with TBS. Samples were washed and assayed for bound $p56^{lck}$ as described in Experimental Procedures.

B) Membrane-permeable chelators disrupt $p56^{lck}$•CD4 complexes in Jurkat cells.

Jurkat cells were concentrated by centrifugation and resuspended in 10 ml of RPMI-1640 alone or with either 5 mM EDTA, 5 mM TPEN, 5 mM 1,7 phenanthroline or 5 mM 1,10 O-phenanthroline. Cells were incubated at 37°C for 10 minutes, recovered by centrifugation, washed with PBS and recovered once more prior to lysis. Of the cleared lysates 200 μl was immunoprecipitated with an anti-human $p56^{lck}$ polyclonal antibody and the remaining 800 μl was immunoprecipitated with an anti-human CD4 polyclonal antibody. Immunoprecipitations and subsequent anti-$p56^{lck}$ immunoblotting were performed as described in Experimental Procedures.

C) Treatment of COS cells co-expressing CD4 and $p56^{lck}$ with 1,10 O-phenanthroline disrupts their association.

COS-7 cells were transiently co-transfected with murine CD4 in pcDNA3.1 and either $p56^{lck}$ in pXM-139 (unpublished data, Veillette et al.). Forty-eight hours after transfection the cells were washed once with PBS and incubated in DME alone or with 5 mM 1,10 O-phenanthroline for 10 minutes at 37°C. Cells were then washed once in PBS and lysed. Half of the lysate was subjected to immunoprecipitation with an anti-mouse CD4 polyclonal antibody (T4-4 (Deen et al., 1988; Willey et al., 1992); NIH AIDS Research and Reference Reagent Program; catalog#805) and half with an anti-human $p56^{lck}$ polyclonal antibody. Immunoprecipitations and immunoblotting were performed as described in Experimental Procedures.

Figure 4: CD8α/$p56^{lck}$ interaction is zinc dependent

A) Treatment of COS cells co-expressing CD8α and $p56^{lck}$ with 1,10 O-phenanthroline disrupts their association.

COS-7 cells were transiently co-transfected with CD8α (Lyt2a) in pcDNA3.1 and either murine $p56^{lck}$ in pXM-139 (unpublished data, Veillette et al.) or murine $p56^{lck}$(C20/23S) in pCDNA3.1. Forty-eight hours after transfection the cells were washed once with PBS and incubated in DME alone or with either 5 mM 1,7 phenanthroline or 5 mM 1,10 O-phenanthroline for 10 minutes at 37°C. Cells were then washed once in PBS and lysed. Half of the lysate was subjected to immunoprecipitation with an anti-mouse CD8α polyclonal antibody (Sarmiento et al., 1980) and half with an anti-human $p56^{lck}$ polyclonal antibody. Immunoprecipitations and immunoblotting were performed as described in Experimental Procedures.

B) Association *in vitro* of GST-CD8α with $p56^{lck}$ requires zinc

GST-CD8α and GST were produced and isolated as described in Experimental procedures. Binding was performed in the same fashion as with GST-CD4 with the exception that at 1 mM $ZnCl_2$, we readjusted the pH of the buffer with 1M Tris-HCl, pH 8.0. Lane 6 is a control containing 1 μg $p56^{lck}$. Samples were processed and assayed for bound $p56^{lck}$ as described in Experimental Procedures.

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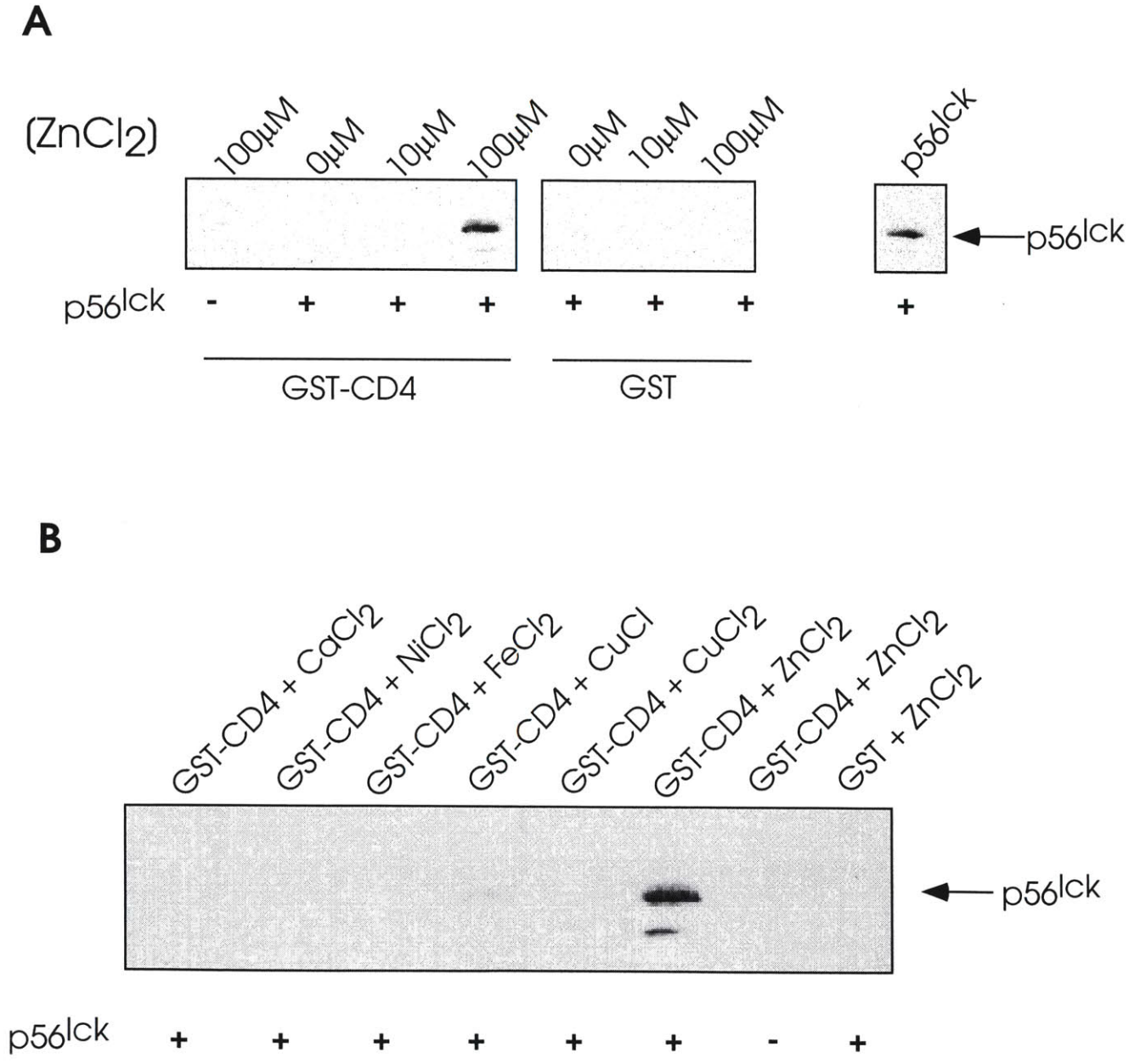
Figures

Figure 1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0μM
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p56lck
p56lck
p56lck - + + + + + +
+
GST-CD4
GST
B
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GST-CD4 + NiCl2
GST-CD4 + FeCl2
GST-CD4 + CuCl
GST-CD4 + CuCl2
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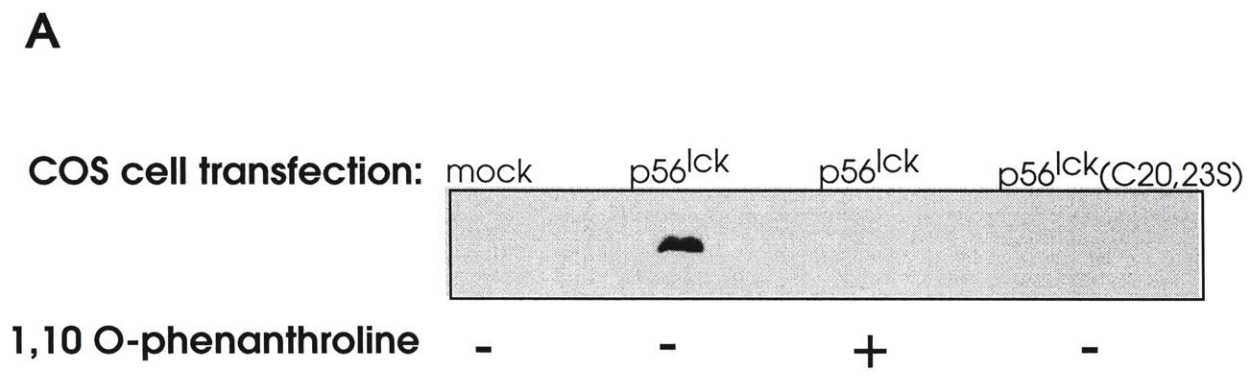
Figure 2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COS cell transfection:
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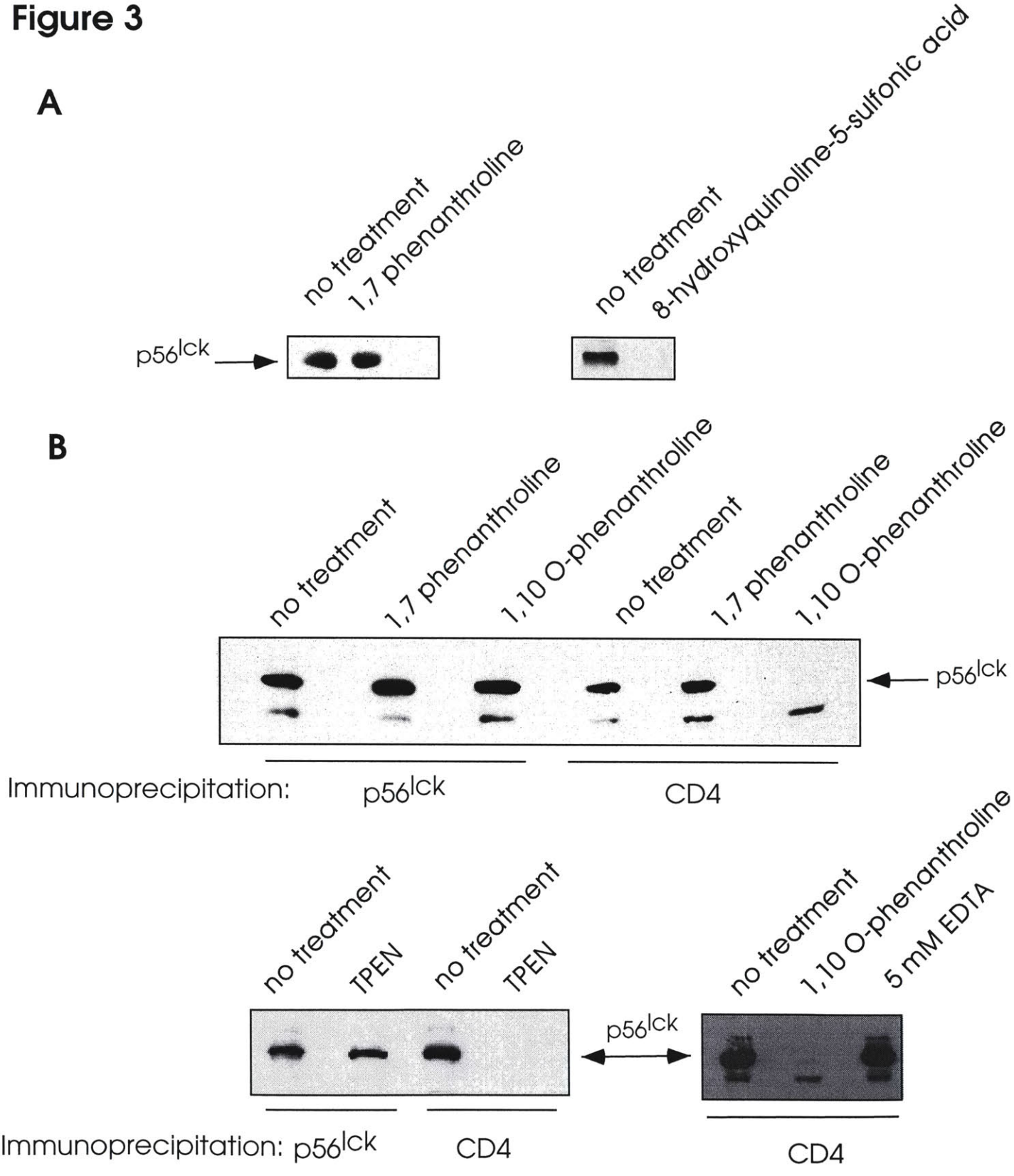
p56lck
p56lck(C20,23S)
1,10 O-phenanthroline
-
-
+
-
B
GST
GST-CD4
GST-CD4 (C418A,C420A)
GST-CD4 (C418A)
GST-CD4 (C420A)
p56lck

Figure 3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p56lck
B
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1,7 phenanthroline
1,10 O-phenanthroline
no treatment
1,7 phenanthroline
1,10 O-phenanthroline
p56lck
Immunoprecipitation:
p56lck
CD4
no treatment
TPEN
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TPEN
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Immunoprecipitation: p56lck
CD4
CD4

Figure 3 (continued)

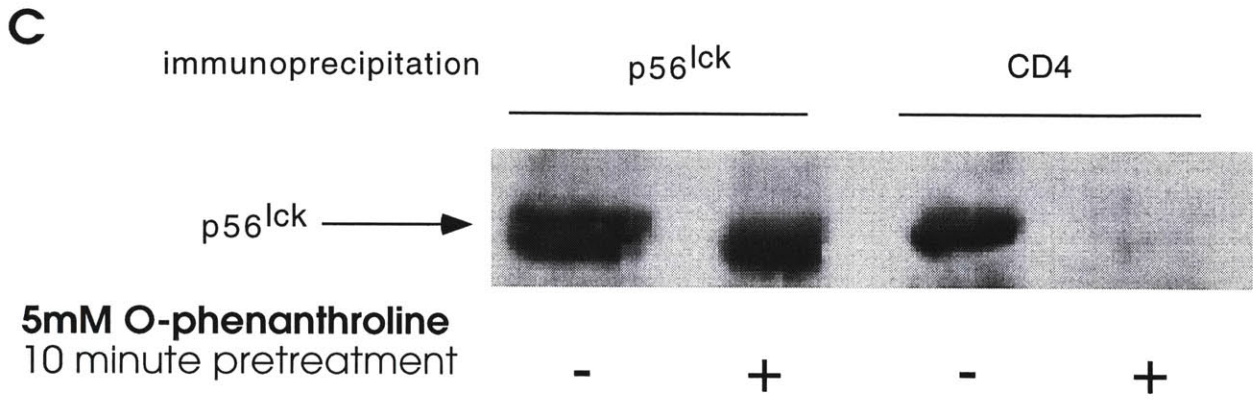
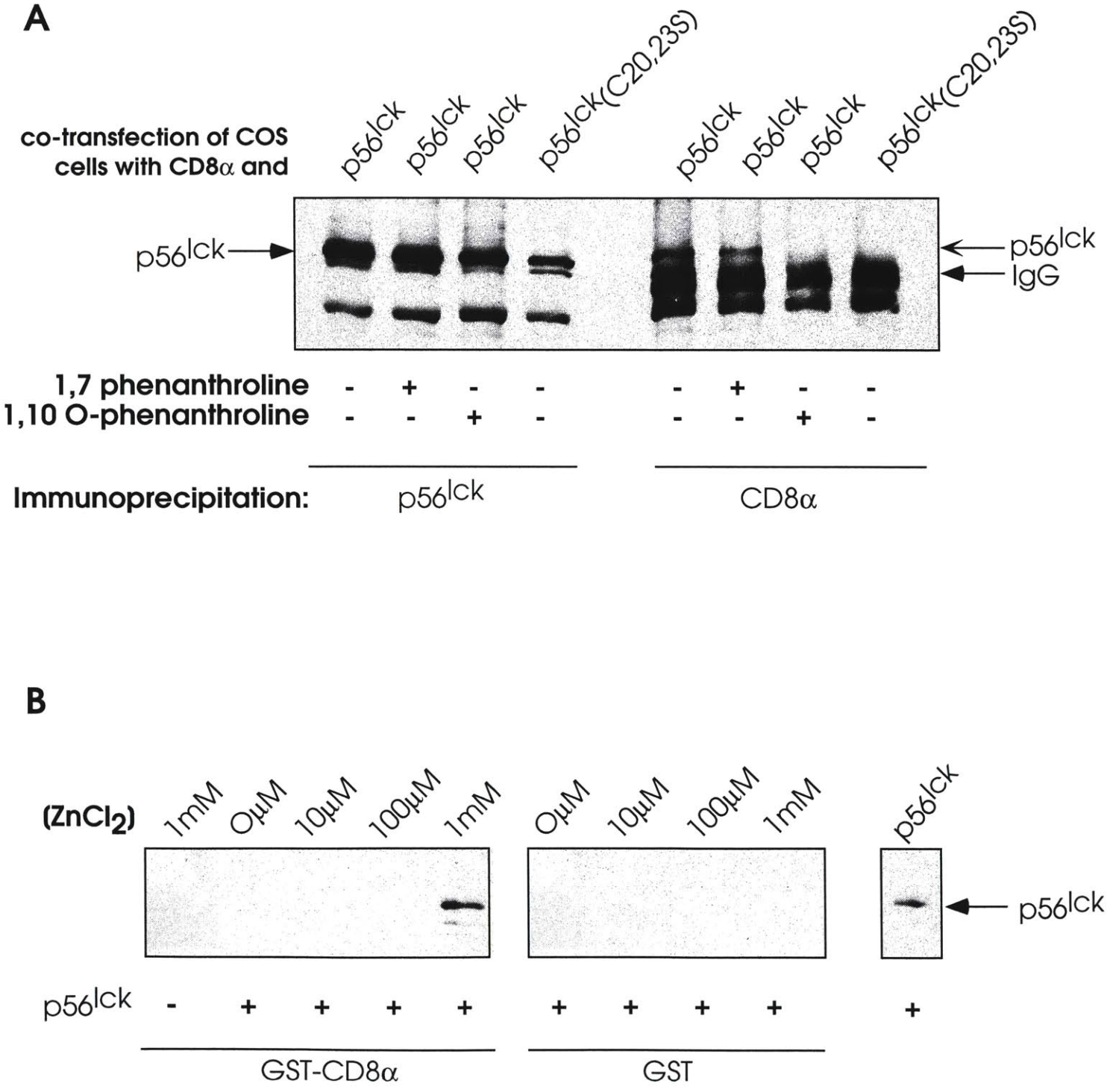


Figure 4


A
co-transfection of COS cells with CD8α and
p56lck
p56lck
p56lck
p56lck(C20,23S)
p56lck
p56lck
p56lck
p56lck(C20,23S)
p56lck
p56lck
IgG
1,7 phenanthroline - + - - - + - -
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Immunoprecipitation: p56lck CD8α
B
(ZnCl2) 1mM 0μM 10μM 100μM 1mM 0μM 10μM 100μM 1mM
p56lck
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GST-CD8α
GST

Chapter 3

Chemical dissection of TGF-β receptor complex formation

Preface

Figure 1A was contributed by Dr. Rebecca Wells; Figure 1B was conducted together with Dr. Carlos Rodriguez and Figure 4 was conducted together with Drs. Yin Sun and Xue-Dong Liu.

Abstract

The TGF-βs mediate a diversity of biological responses through the binding of a types I (TβR1) and II (TβRII) receptor. Specifically, these cytokines bring about the formation of a signalling complex consisting of a TβRI and TβRII heterocomplex - most likely a heterotetramer. (Gilboa et al., 1998; Henis et al., 1994; Moustakas et al., 1993; Yamashita et al., 1994) It has been shown that pre-treatment of cells with DTT blocks functional recruitment of TβRI to a TGF-β1 bound TβRII resulting in a complex unable to signal. (Cheifetz et al., 1990; Wells et al., in press) In this study, we further report that formation of pre-formed complexes is also blocked by DTT as similar pre-treatment abrogates binding of both TβRI and TβRII to TGF-β2, a ligand distinct from TGF-β1 in that it requires co-expression of both receptors to bind.(Rodriguez et al., 1995) To identify the properties of DTT required to elicit this phenomenon, we screened a number of chemically related reducing agents for a similar inhibitory effect upon complex formation. Here, we find that 2,3 DMP (2,3 dimercaptopropanol) likewise blocks functional interactions between TβRI and TβRII. Both it and DTT are di-thiol based compounds that are membrane-permeable. However, other thiol-containing compounds lacking either one of these properties exert no effect on complex formation. In addition, DTT does not affect TβRI homodimerization, an upstream event required for TβRI•TβRII hetero-oligomerization. Hence, this would suggest that mechanistically, DTT and similarly, 2,3 DMP, act upon intracellular factor(s), and through chemical properties related to the presence of di-thiols, inhibit TβRI•TβRII association. The cytoplasmic domain of TβRI plays a critical role in these interactions as we find that a cytoplasmically truncated TβRI fails to associate with ligand-bound TβRII. In contrast, the TβRII cytoplasmic domain is dispensable for complex formation (Wieser et al., 1993), nor is it required to manifest the DTT effect. Therefore, we propose that DTT directly acts upon structural elements contained within TβRI's intracellular domain that are required for functional TβRI•TβRII interactions.

Introduction

The TGF-βs (TGF-β1, -β2, -β3) constitute one of the most multifunctional peptide growth factors yet described, playing roles in growth inhibition, wound healing, immune response, angiogenesis, embryonic development and apoptosis. (Roberts, 1990) They are also the founding members of what has become to be known as the TGF-β superfamily of growth factors including the inhibins, activins, bone morphogenetic proteins, and Müllerian inhibitory substance.

The TGF-βs bind to two surface receptors termed types I (TβRI) and II (TβRII). The types I and II receptors share a common structural design. Both classes of receptors possess a short cysteine rich extracellular domain followed by a single transmembrane domain and a serine-threonine kinase-containing cytoplasmic domain. Unique to the type I receptors is a glycine/serine rich region in the intracellular juxtamembrane region, termed the GS domain.

Each receptor is required for ligand-induced signalling as TβRII is required for ligand binding whereas TβRI is necessary for initiation of intracellular signalling. Binding of the ligand TGF-β1 to the type II receptor causes it to associate with and phosphorylate the type I receptor. This interaction is essential for TGF-β-activated signal transduction.(Wrana et al., 1994) Both TβRI and TβRII form ligand-independent homo-oligomers, most likely dimers and upon TGF-β binding, pairs of types I and II receptor are presumably brought together to form a heterotetramer. (Gilboa et al., 1998; Henis et al., 1994; Moustakas et al., 1993; Yamashita et al., 1994) Homodimerization of the type I is receptor required for TGF-β signalling. Two studies demonstrate the functional consequences of its dimerization. First, Luo and co-workers constructed chimeric receptors consisting of the extracellular domain of the erythropoietin (Epo) receptor fused to the intracellular domain of either the types I or II receptor.(Luo and Lodish, 1996) They show that Epo-induced hetero-dimerization of type I and II chimeras can signal growth arrest. Moreover, a type I chimera encoding for a constitutively active kinase still requires Epo-induced homodimerization to signal. These results are consistent with the notion that the requirements for TGF-β signal transduction include both type I homodimerization as well as its heterodimerization with type II. In another approach, Weis-Garcia et al. observe that a kinase-defective type I receptor mutant complements an activation defective mutant when co-expressed in cells. These results further confirm that type I homodimerization is indeed essential for signalling.(Weis-Garcia and Massague, 1996)

Whether ligand plays a role in recruiting both receptors into a functional complex and/or stabilizing pre-existing complexes remains unresolved. Despite evidence pointing towards ligand-induced TβRI•TβRII association, a number of overexpression studies also detect the existence of ligand independent complexes on the cell surface.(Chen and Weinberg, 1995; Ventura et al., 1994; Wells et al., in press) Moreover, TGF-β2 binding requires the presence of both types I and II receptors providing further evidence for pre-formed complexes on the cell surface.(Rodriguez et al., 1995)

It is known that DTT abrogates binding and cross-linking of TGF-β1 to TβRI. (Cheifetz et al., 1990) Wells and co-workers suggest that DTT induces conformational changes in the complex rendering it incapable of signalling and more readily dissociated in the presence of detergent. (Wells et al., in press)

In this study, we wish to first further elucidate the mechanism by which DTT elicits this phenomenon, and secondly, to use this compound as a means to identify structural elements contained within TβRI and TβRII that are necessary for formation of a complex competent to signal. Our findings provide additional evidence that DTT significantly compromises functional interactions between both receptors as measured by both ligand binding and consequent downstream signal transduction. Secondly, we find that 2,3 dimercaptopropanol (2,3 DMP), a thiol-based compound chemically related to DTT, also exerts an inhibitory effect upon ligand binding to TβRI and downstream phosphorylation events. Both compounds are membrane permeable and contain dithiols. Results from analyzing other classes of thiol-based reducing agents suggest that both properties are involved in manifesting the observed effects. We also report that DTT does not block type I receptor homodimerization, an event upstream of ligand induced hetero-oligomerization. In conjunction with work of others, this would suggest that DTT blocks a step in complex formation further downstream, presumably interactions between structural domains involved in direct TβRI and TβRII association. The intracellular domain of TβRI appears to contain at least one of these key structural elements as cytoplasmic truncations of TβRI reach the cell surface, but fail to associate with a wildtype TβRII.

Experimental Procedures

Cell culture

Mv1Lu (American Type Culture Collection catalog# CCL-64) mink lung epithelial cells were cultured in modified Eagles medium (MEM) supplemented with 10% fetal bovine serum, 2mM glutamine, 100 units/ml of penicillin, 0.1 mg/ml streptomycin and MEM non-essential amino acids (Life Technologies). HYB2 cells are a gift of Dr. A. Geiser (National Institutes of Health) and were grown in Dulbecco's modified Eagle medium supplemented as above, except for MEM non-essential amino acids.

Construction of TGF-β receptor cytoplasmic truncations

HA-tagged full-length TβRI (Gilboa et al., 1998) or myc-tagged full-length TβRII (Henis et al., 1994) were subcloned into pcDNA3.1 (Invitrogen). Cytoplasmic truncations of these tagged receptors were constructed by polymerase chain reaction (PCR). With respect to TβRI, primers introducing a stop codon after either cysteine 148 (using starting methionine as 1) or histidine 358 followed by a Not I site together with a T7 primer were used to generate PCR fragments encoding TβRI truncations. The resulting DNAs were then subcloned into the Hind III and Not I sites of pcDNA3.1. Similarly, a primer introducing a stop codon after serine 199 in the TβRII sequence followed by a Not I site were used together with T7 to generate a cytoplasmically truncated TβRII receptor. The PCR fragment was then subcloned into the EcoRI and NotI sites of pcDNA3.1. This truncation is identical to one characterized by (Wieser et al., 1993)

[^{125}I] TGF-β binding and cross-linking studies

After two washes with KRH buffer (50mM Hepes, pH 7.4, 8mM NaCl, 5mM $MgSO_4$, 0.5mM $CaCl_2$, 0.5mM KCl), confluent plates of Mv1Lu or HYB2 cells were incubated for 10 minutes at 37°C with KRH-BSA (KRH containing 0.5% BSA). Afterwards, they were then pre-treated with KRH-BSA or in some experiments, KRH-BSA containing a particular compound for 10 or 15 minutes at 37°C. Cells were then placed on ice and after four washes with KRH buffer, binding and cross-linking with [^{125}I] TGF-β1 or [^{125}I] TGF-β2 was then performed as described in (Cheifetz et al., 1990). Briefly, 10^7 cells were incubated with 150 pM [^{125}I] TGF-β1 or [^{125}I] TGF-β2 in KRH buffer containing 1% fatty acid free serum albumin for 2 hours on ice. Following cross-linking with 500μg/ml disuccinimidyl suberate (DSS), the cells were washed three times with PBS (1 mM

KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.0), and lysed in TBS-GT buffer (137 mM NaCl, 20 mM Tris-HCl [pH 8.0], 1% Triton X-100, 10% glycerol).

Immunoprecipitations

After a 5 minute centrifugation, cleared lysates were incubated with an antibody/protein-A sepharose mixture at 4°C. Antibodies used include 12CA5 (α-HA; 1μl/IP) mouse ascites raised against an epitope of the influenza hemagglutinin (HA) protein (Wilson et al., 1984), a monoclonal *myc* antibody (9E10, 3μl/IP) (Evan et al., 1984), a polyclonal anti-peptide antibody recognizing the C-terminus of Tβ-RII (antibody 260; 5μl/IP) (Moustakas et al., 1993), and a polyclonal antibody raised against the cytoplasmic juxtamembrane region of TβRI (VPN;5μl/IP). (Franzen et al., 1993). After a 2 hour incubation at 4°C, immunoprecipitates were washed three times with TBS-GT followed by one wash with PBS. Immunoprecipitates were separated by SDS-PAGE and exposed to a Fuji phosphoimager screen for quantitation and analysis.

Purification of GST-Smad3

GST-Smad3 fusion proteins were produced in DH10B *E. coli* harboring cDNA encoding GST-SMAD3 subcloned into the pGEX-2T bacterial expression vector. (Zhang et al., 1996) The method of purification was adapted from (Bitter and Roeder, 1979; Frangioni and Neel, 1993). Cultures were grown overnight at 37°C and then diluted 1:10 into 6 liters of Luria Broth (LB). After incubation for 4 hours at 37°C, cells were induced for 2 hours with 1 mM isopropyl -β–D thiogalactopyranoside (IPTG) at 30°C. Bacteria were pelleted, resuspended in 120 mls of NETN (100mM NaCl, 20mM Tris (pH 8.0), 1mM EDTA, 0.5% NP-40) with 1mM PMSF, and sonicated for 15 seconds, 5-6 times in an ice bath. After clearing the lysates with a 15 minute centrifugation, fusion proteins were isolated by incubation with 500μl of a GST-Sepharose4B 1:1 slurry (Pharmacia) for 30 minutes at 4°C. After washing the beads three times with NETN, GST-fusion proteins were eluted at room temperature with 5mM reduced glutathione in NETN. The resulting eluate was then dialyzed against buffer D (20mM Tris (pH 7.9), 20% glycerol, 0.1M KCl, 0.2mM EDTA, 0.5mM phenylmethylsulfonyl fluoride (PMSF), 0.5mM DTT) overnight at 4°C and then stored at -70°C.

GST-Smad3 transphosphorylation assay

An *in vitro* assay for ligand induced transphosphorylation of GST-Smad3 is described in a previous study.(Wells et al., in press) Briefly, Mv1Lu or transfected COS cells at ~ 50-

80% confluence were washed three times with MEM followed by a 10 minute incubation at 37°C in MEM or MEM containing 1mM DTT or TCEP. Afterwards, cells were washed four times with MEM and then treated with 100pM TGF-β1 for 15 minutes at 37°C. Cells were washed three times with pre-chilled PBS and then lysed in lysis buffer (50mM Tris (pH 8.0), 150 mM NaCl, 1mM EDTA, 50mM NaF, 1mM $NaVO_3$, 1% NP40, 10% glycerol) and 1mM PMSF. 1 μg of GST-Smad3 (Zhang et al., 1996) was added to cleared lysates and incubated for 1 hour at 4°C followed by addition of glutathione sepharose 4B (Pharmacia; 20μl of 1:1 slurry) and another 1 hour incubation at 4°C. Beads were then washed three times with lysis buffer and once with kinase buffer (50mM Tris (pH 7.4), 10mM $MgCl_2$, 1mM DTT). To 20μl of the glutathione bead mixture, 10μl of a mix containing 1 μl 10X kinase buffer, 0.4 μl of 10mM ATP, 0.5 μl γ^{32}P-ATP (6000mCi/mmol), and 8μl of H_2O. After a 30 minute incubation at 30°C, samples were separated by 8% SDS-PAGE (Laemmli, 1970), transferred to 0.22 μM porosity nitrocellulose and then exposed to Kodak XAR film.

COS cell transfections

Using the DEAE/dextran method, COS-7 cells were transiently transfected (Seed and Aruffo, 1987) with 3 μg of human type II TGF-β receptor in the pcDNA1 vector (Invitrogen).(Lin et al., 1992) and 10 μg of either a constitutively active TβRI mutant (T204D) (Wieser et al., 1995) subcloned in pcDNA3.1 (Invitrogen) or wild-type TβRI in CMV-7.(Chen and Weinberg, 1995)(Chen and Weinberg, 1995; Andersson et al., 1989) Both type I receptors are HA-tagged at the C-terminus. In Figure 1B, the following two cDNAs were co-transfected: 10μg's of an N-terminal Myc-tagged human type II TGF-β receptor (Henis et al., 1994) in the pcDNA1 vector (Invitrogen) and a C-terminal HA-tagged human type I receptor expressed in the CMV-7 vector. In Figure 4A, 3μg of wild-type TβRII in pcDNA1 were co-transfected with 10μg of either HA-tagged full-length TβRI, a partial cytoplasmic truncation (HA-TβRI(T358)) or a complete cytoplasmic truncation (HA-TβRI(T148)) subcloned in pcDNA3.1. In Figure 4B, 3μg of wild-type TβRI in CMV-7 were co-transfected with 10μgs of either myc-tagged full-length TβRII (myc-TβRII) in pcDNA3.1 or a cytoplasmically truncated TβRII (myc-TβRII(T932)). Forty-eight to 72 hours after transfection, cells were then assayed for the relevant receptor activity.

Results

Previous studies have documented the loss of cell surface TβRI binding to TGF-β1 as a consequence of DTT pre-treatment. (Cheifetz et al., 1990; Wrana et al., 1992) In Figure 1A, we show that in both mink lung epithelial (Mv1Lu) cells, and HYB2 cells (a TGF-β-responsive line resulting from a fusion of a human EJ bladder carcinoma and an SW480 colon carcinoma cell) (Geiser et al., 1989; Geiser et al., 1992), antisera specific for TβRI immunoprecipitate [^{125}I] TGF-β1 cross-linked to TβRII as well as to Tβ-RI (lanes 5 and 7). Similarly, antisera recognizing TβRII immunoprecipitate [^{125}I] TGF-β1 cross-linked to TβRI as well as to Tβ-RII (lanes 1 and 3). Hence, TGF-β1 binding correlates with formation of a stable types I and II receptor complex. Pre-treatment of either cell line with DTT not only abrogates binding of [^{125}I] TGF-β1 to TβRI, but also blocks co-immunoprecipitation of TβR1 with [^{125}I] TGF-β1-bound TβRII (lanes 6 and 8). This would suggest that in addition to abolishing ligand binding to the type I receptor, DTT also blocks types I and II association. In contrast, [^{125}I] TGF-β1 binding to the type II receptor remains intact after DTT pre-treatment (lanes 1 - 4). Similar results were observed in L6 rat skeletal muscle myoblasts as well as when using antisera specific for the C-terminus of TβRI.[1]

To further examine how DTT affects interactions between TβRI and TβRII, we took advantage of the distinct binding requirements of TGF-β2. In contrast to TGF-β1, TGF-β2 cannot bind to TβRII unless TβRI is co-expressed, consistent with it binding to pre-formed types I and II receptor complexes.(Rodriguez et al., 1995) Hence, should DTT pre-treatment compromise interactions between TβRI and TβRII, one would observe the loss of TGF-β2 binding and cross-linking to both receptor types. And indeed in transiently transfected COS cells, DTT pre-treatment of L6 myoblasts blocks [^{125}I] TGF-β2 binding and cross-linking to both TβRII as well as to TβRI (Figure 1B, lanes 2 and 4). Similar results were obtained in L6 myblasts (Figure 1C).

We then screened a number of related thiol-based compounds for their ability to block complex formation. As shown in other studies, DTT pre-treatment not only prevents TβRI binding and cross-linking to TGF-β1, but also blocks downstream signalling as demonstrated by its inhibition of TGF-β dependent *in vitro* transphosphorylation of Smad3, a known *in vivo* TβRI substrate. (Figure 2A and B, lane 3).(Liu et al., 1997; Nakao et al., 1997; Wells et al., in press; Zhang et al., 1996) More importantly, Figure 2A, lane 7 shows that among the compounds tested, only 2,3 dimercaptopropanol (2,3 DMP) similarly inhibits type I receptor binding and cross-linking, albeit with lower efficacy

than DTT. This partial effect is consistently observed when immunoprecipitating with either TβRI or TβRII antisera. In addition, the residual type I receptor recruitment results in a corresponding partial loss of TGF-β induced *in vitro* transphosphorylation of Smad3. (Figure 2A and 2B, lane 7) Among the compounds tested, DTT and 2,3 DMP are unique in that they are both membrane-permeable and contain dithiols. In contrast, neither a membrane-impermeable dithiol such as diethyldithiocarbamic acid (DDC), nor monothiols such as β-mercaptoethanol or 2-mercapto-ethanesulfonic acid (MESA) exert any detectable effect upon formation of a functional types I and II complex (Figures 2A and 2B, lanes 4-6). These compounds all lack one or both properties shared by 2,3 DMP and DTT.

Mechanistically, it is possible that DTT and 2,3 DMP block direct interactions between TβRI and TβRII. Alternatively, they may inhibit an upstream step necessary for formation of a functional TβRI and TβRII heterotetramer, most notably TβRI homodimerization.(Luo and Lodish, 1996; Weis-Garcia and Massague, 1996) Analysis of the ability of the TβRI T204D mutant to signal with or without DTT pre-treatment would allow us to distinguish between these two possibilities. Specifically, T204D encodes for a constitutively active kinase that signals independently of the type II receptor; however, like its wild-type counterpart, it still requires homodimerization to signal.(Luo and Lodish, 1996) In Figure 3 we co-transfected DNA encoding the wild-type TβRII together with either the constitutively active T204D or wild-type TβRI and compared the resulting patterns of *in vitro* Smad3 phosphorylation with or without DTT pretreatment. DTT inhibition of type I homodimerization should manifest as the loss of Smad3 transphosphorylation by both T204D and wild-type TβRI. However, Figure 3 shows clearly that this is not the case. Although DTT can inhibit Smad3 transphosphorylation in cells expressing wild-type TβR1, no effect is observed in those expressing the constitutive T204D mutant. On the other hand, like wild-type TβR1, T204D is also sensitive to DTT pre-treatment as assayed by [^{125}I] TGF-β1 binding and cross-linking. (Figure 3B) Therefore, DTT exerts no deleterious effect upon homodimerization, but rather acts elsewhere in the sequence of events leading to complex formation.

Furthermore we find that both partial and complete deletions of the TβRI cytoplasmic domain result in truncated type I receptors that cannot bind [^{125}I]-TGFβ1 suggesting that they are unable to associate with TβRII. Specifically, binding and cross-linking of [^{125}I] TGF-β1 were performed on COS cells co-transfected with wild-type TβRII and either full-length or truncated versions of an HA-tagged TβRI. Both HA-TβRI (T358) and (T148) are expressed on the surface of COS cells as determined by immunofluorescence (data not shown). Nevertheless, as depicted in Figure 4A, antibodies

specific for the HA epitope immunoprecipated a full-length tagged TβRI, but failed to immunoprecipitate either truncated receptor. This is consistent with the notion that the intracellular domain contains structural elements necessary for association with TβRII. In contrast, a cytoplasmically truncated TβRII is indistinguishable to its full length counterpart in that it maintains the ability to bind [^{125}I]-TGFβ1 and associate with TβRI in a DTT-sensitive manner. Similar to Figure 4A, binding and cross-linking of [^{125}I] TGF-β1 was performed on COS cells co-transfected with wild-type TβRI and either a myc-tagged full-length TβRII or a myc-tagged TβRII truncated of its intracellular domain. With both type II receptors, anti-sera specific for the myc epitope co-immunoprecipitates [^{125}I] TGF-β1-bound TβRI as depicted in Figure 4B (lanes 1 and 3). This is consistent with previous findings demonstrating that the cytoplasmic domain of TβRII is dispensable for its association with TβRI.(Wieser et al., 1993) Moreover, like its full-length counterpart, a truncated TβRII recruits TβRI in a DTT-sensitive manner as DTT pretreatment blocks co-immunoprecipitation of both a full-length and truncated TβRII with TGF-β1-bound TβRI (Figure 4B, lanes 2 and 4).

Lastly, we note that TβRI•TβRII interactions when observed in transfected COS cells exhibit characteristics distinct to what is observed in endogeneously expressing cell lines. In particular, they produce distinct patterns of [^{125}I]-TGF-β1 bound receptors. In Mv1Lu, L6[1], and HYB2 cells, DTT brings about the complete loss of ligand-bound TβRI as well as TβRII co-immunoprecipitating with TβRI. (Figures 1A and 2A) However, under the same conditions, COS cells do exhibit detectable amounts of both receptors. (Figure 3B).

Discussion

In this report, our findings are four-fold: 1) In contrast to TGF-β1, TGF-β2 binding to both TβRI and TβRII is disrupted by DTT pre-treatment ;2) 2,3 DMP similarly inhibits TGF-β receptor complex assembly possibly allowing identification of chemical properties shared with DTT that correlate with the observed effects and; 3) DTT exerts no effect upon type I receptor homodimerization; 4) The cytoplasmic domain of TβRI, but not TβRII, is necessary for receptor complex formation.

We cannot rule out the possibility that DTT simply weakens these receptor complexes rendering them sensitive to dissociation after detergent lysis as observed in our immunoprecipitation studies. Nevertheless, further evidence for DTT-induced disruption of functional TβRI•TβRII interactions is provided by analysis of TGF-β2 binding and cross-linking. In light of its requirement for pre-formed types I and II complexes on the cell surface to bind, failure to bind either receptor type would suggest that the integrity of such pre-existing complexes becomes functionally compromised subsequent to DTT pre-treatment and prior to detergent lysis.

However, based on a technique using antibody-mediated immunofluorescence copatching of TGF-β receptors in live COS cells, Wells and co-workers report that ligand induced Tβ-RI/RII oligomerization is not DTT sensitive.(Wells et al., in press) Hence it is possible that DTT may not bring about complete complex dissociation. Nevertheless, their resulting stuctural integrity is such that they are unable to function in TGF-β signalling as measured by at least three criteria: (1) it prevents TGF-β1 binding to the type I receptor, and as a consequence; (2) ligand-induced Smad3 transphosphorylation is blocked; (3) Neither receptor type in the complex bind TGF-β2. Thus, DTT compromises the complex to the extent that it is unable to signal in response to ligand stimulation.

Furthermore, our data indicate that at least in a subset of cell-surface receptor complexes, the nature of their interactions in COS cells is distinct from that observed in endogeneously expressing cell lines. This would suggest that in COS cells, at least a subset of cell-surface complexes remain intact after dithiol pretreatment and therefore possess properties distinct from what is observed in endogenously expressing cell lines. It is possible that overexpression may alter the oligomeric or conformational state of these complexes and, as a consequence, alter their subsequent DTT sensitivity.

A previous study reports that in Mv1Lu cells, antisera specific for the type I receptor immunoprecipitate [^{125}I]-TGF-β1 bound type II receptor after DTT pre-treatment.(Vivien and Wrana, 1995) Wells and co-workers propose that the discrepancy between this and our observations could be due to the different concentrations of detergents used during lysis.(Wells et al., in press)

Furthermore, among a number of thiol-based reducing agents tested, only 2,3 DMP exhibits a similar inhibition of types I and II receptor association. Both 2,3 DMP and DTT are membrane-permeable and possess dithiols. Compounds lacking one or both characteristics exert no effect on complex formation suggesting that both properties are somehow involved in DTT's mode of action. DTT's effects has been attributed to the reduction of critical disulfide bonds in the extracellular domain of TβR1, altering its conformation and ultimately preventing TGF-β binding.(Vivien and Wrana, 1995)

We also show that DTT does not disrupt type I receptor homodimerization and hence must act elsewhere in the sequence of events leading to complex formation. In light of our data, we propose that through properties related to its dithiol group, DTT crosses the membrane and acts upon an intracellular factor, possibly the cytoplasmic domain of either receptor, to abolish functional TβRI•TβRII interactions. Nevertheless, it has been shown that the type II receptor cytoplasmic domain is dispensable for complex formation.(Wieser et al., 1993) We further report that it has no role in conferring DTT sensitivity to receptor complex formation. On the other hand, type I receptors truncated of their intracellular domain are expressed on the cell surface, but fail to interact with TβRII suggesting that this cytoplasmic region of TβRI plays an essential role in TβRI•TβRII interactions. We therefore propose that DTT does not directly affect TβRII, but instead acts upon structural elements contained with the cytoplasmic domain of TβRI to block functional receptor complex formation.

It is clear that additional work is required to further elucidate how DTT and other similar agents act to inhibit functional TGF-β receptor oligmerization. Specifically, work focusing on the identification of domains within the TβRI cytoplasmic domain as well as possibly other structural determinants required for their interactions would be particularly fruitful. Through this approach, we hope to obtain further insights into the series of events necessary for receptor complex assembly.

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Figure Legends

Figure 1: DTT blocks functional interactions between TβRI and TβRII.

A) Binding and cross-linking of [^{125}I] TGF-β1 to Mv1Lu and HYB2 cells. Mv1Lu cells (lanes 1, 2, 5, and 6) and HYB2 cells (lanes 3, 4, 7, and 8) were pre-incubated with (lanes 2, 4, 6 and 8) or without 1 mM dithiothreitol (DTT) (lanes 1, 3, 5 and 7) for 15 minutes at 37 °C, and then upon washing cells 5 times with KRH buffer subjected to [^{125}I] TGF-β1 binding and cross-linking as described in Experimental Procedures. After cell lysis, half of the sample was subjected to immunoprecipitation with an anti-peptide antibody raised against the C-terminus of TβRII (antibody 260; lanes 1 - 4); the other half was immunoprecipitated with an anti-peptide antibody specific for the cytoplasmic juxtamembrane region of TβRI (VPN; lanes 5 - 8). The resulting immunoprecipitates were then resolved by SDS-PAGE, dried and then exposed to a Fuji phosphoimager screen.

B) DTT sensitivity of [^{125}I] TGF-β2 binding to COS cells co-expressing types I and II TGF-β receptors

COS cells co-transfected with cDNA encoding N-terminal terminally *myc* tagged TβRI and C-terminal HA tagged Tβ-RII. Forty eight to 72 hours after transfection, plates were preincubated with either KRH (lanes 1 and 3) or KRH containing 1mM DTT (lanes 2 and 4) for 15 minutes at 37°C followed by binding and cross-linking to [^{125}I] TGF-β2. Samples were then split into two and immunoprecipitated with either an HA or *myc* monoclonal antibody. The resulting immunoprecipitates were then resolved by SDS-PAGE, dried and then exposed to a Fuji phosphoimager screen. Methods for both binding and cross-linking, and immunoprecipitations are described in Experimental Procedures.

C) DTT sensitivity of [^{125}I] TGF-β2 binding to L6 myoblasts.

L6 rat skeletal muscle myoblasts were pre-incubateed with or without 1mM DTT (lanes 2,4,6 and 8) for 10 minutes at 37°C, then upon washing cells 5 times with KRH buffer subjected to [^{125}I] TGF-β2 binding and cross-linking as described in Experimental Procedures. After cell lysis, half of the sample was subjected to immunoprecipitation with an anti-peptide antibody raised against the C-terminus of TβRII (antibody 260; lanes 3,4,7 and 8); the other half was immunoprecipitated with an anti-peptide antibody specific for the cytoplasmic juxtamembrane region of TβRI (VPN; lanes 1,2,5 and 6). The resulting

immunoprecipitates were then resolved by SDS-PAGE, dried and then exposed to a Fuji phosphoimager screen.

Figure 2: Only membrane permeable dithiols can disrupt types I and II receptor complexes.

A. [^{125}I] TGF-β1 binding and cross-linking to Mv1Lu cells after pre-treatment with various thiol based reducing agents

Mv1Lu were pre-incubated in KRH-BSA or KRH-BSA containing 1mM of the following reducing agents for 10 minutes at 37°C: dithiothreitol (DTT)(lane 3); β-mercaptoethanol (lane 4); 2-mercaptoethane sulfonic acid (MESA) (lane 5); diethyldithiocarbamic acid (DDC) (lane 6); 2,3 dimercaptopropanol (2,3 DMP) (lane 7). Lanes 1 and 2 remained untreated. Cells were then washed four times with KRH and then bound and cross-linked to [^{125}I] TGF-β1. Cell were then lysed, split in half and then immunprecipitated with antibodies specific for either Tβ-RI or Tβ-RII. Immunoprecipitates were then resolved by 8% SDS-PAGE, dried and then exposed to a Fuji phosphoimager screen. Both binding and cross-linking and immunoprecipations were performed as described in Experimental Procedures.

B. *In vitro* analysis of TGF-β induced GST-Smad3 phosphorylation after pre-treatement with various thiol based reducing agents.

Mv1Lu plates were grown and pre-treated as described above. Following washes with KRH, all plates except for lane 1 were treated with 200pM TGF-β1 for 15 minutes at 37°C. Cells were then assayed for Smad3 transphosphorylation activity as described in Experimental Procedures.

Figure 3: T204D transphosphorylation of GST-Smad3 occurs independently of DTT pretreatment. However, it exhibits similar DTT sensitivity to that of the wild-type Tβ-RI in binding and cross-linking to [^{125}I] TGF-β1.

A. T204D and wild-type type I TGF-β receptor transphosphorylation of GST-Smad3 after DTT pretreatment.

COS cells were co-transfected in duplicate plates with pcDNA1 containing cDNA encoding human TβRII and pcDNA3.1 containing cDNA encoding the constitutively active TβRI T204D mutant or wild-type TβRI. Both type I receptors are C-terminally tagged with an HA epitope tag.(Chen and Weinberg, 1995; Wieser et al., 1995) Forty-eight to 72 hours

after transfection, cells were washed once with KRH and then pre-treated with KRH-BSA or KRH-BSA containing 1mM DTT for 10 minutes at 37°C followed by four washes with KRH. Afterwards, all plates were stimulated with 100pM TGF-β1 for 15 minutes at 37°C. Cells were then assayed for Smad3 transphosphorylation as detailed in Experimental Procedures.

B. Binding and cross-linking of [^{125}I] TGF-β1 to COS cells expressing Tβ-RII with either T204D or wild-type TβRI after DTT pretreatment.

COS cells were transfected and cells were pre-treated with DTT as described in Figure 3A. Subsequent binding and cross-linking to [^{125}I] TGF-β1 was performed and then immunoprecipitated with an HA antibody as described in Experimental Procedures.

Figure 4: The cytoplasmic domain of TβRI is required for interactions between TβRI and TβRII.

A. Cytoplasmic truncations of TβRI are unable to bind and cross-link [^{125}I] TGF-β1

COS cells were co-transfected with wildtype TβRII expressed in pcDNA1 and either wildtype HA-tagged TβRI (HA-TβRI), or a partially (HA-TβRI(T358)) or fully (HA-TβRI(T148)) cytoplasmically truncated HA-tagged TβRI expressed in pcDNA3.1. 48-72 hours after transfection, binding and cross-linking to 400pM [^{125}I] TGF-β1 was performed and their subsequent lysates were immunoprecipitated with an anti-HA antibody as described in Experimental Procedures.

B. Cytoplasmic truncation of TβRII can still bind [^{125}I] TGF-β1 and associate with TβRI in a DTT sensitive manner.

COS cells were co-transfected in duplicate plates with wild-type TβRI expressed in pCMV-7 and either myc-tagged full-length TβRII (myc-TβRII) or a cytoplasmic truncation (myc-TβRII(T932)). 48-72 hours after transfection, plates were pre-treated with KRH-BSA (lanes 1 and 3) or KRH-BSA with 1mM DTT at 37C for ten minutes. After 4 washes with KRH, binding and cross-linking to 100pM [^{125}I] TGF-β1 was performed and subsequent cell lysates were immunoprecipitated with an anti-myc antibody as described in Experimental Procedures.

Footnotes

[1] Wells, R. and Lodish, H.F., unpublished observations.

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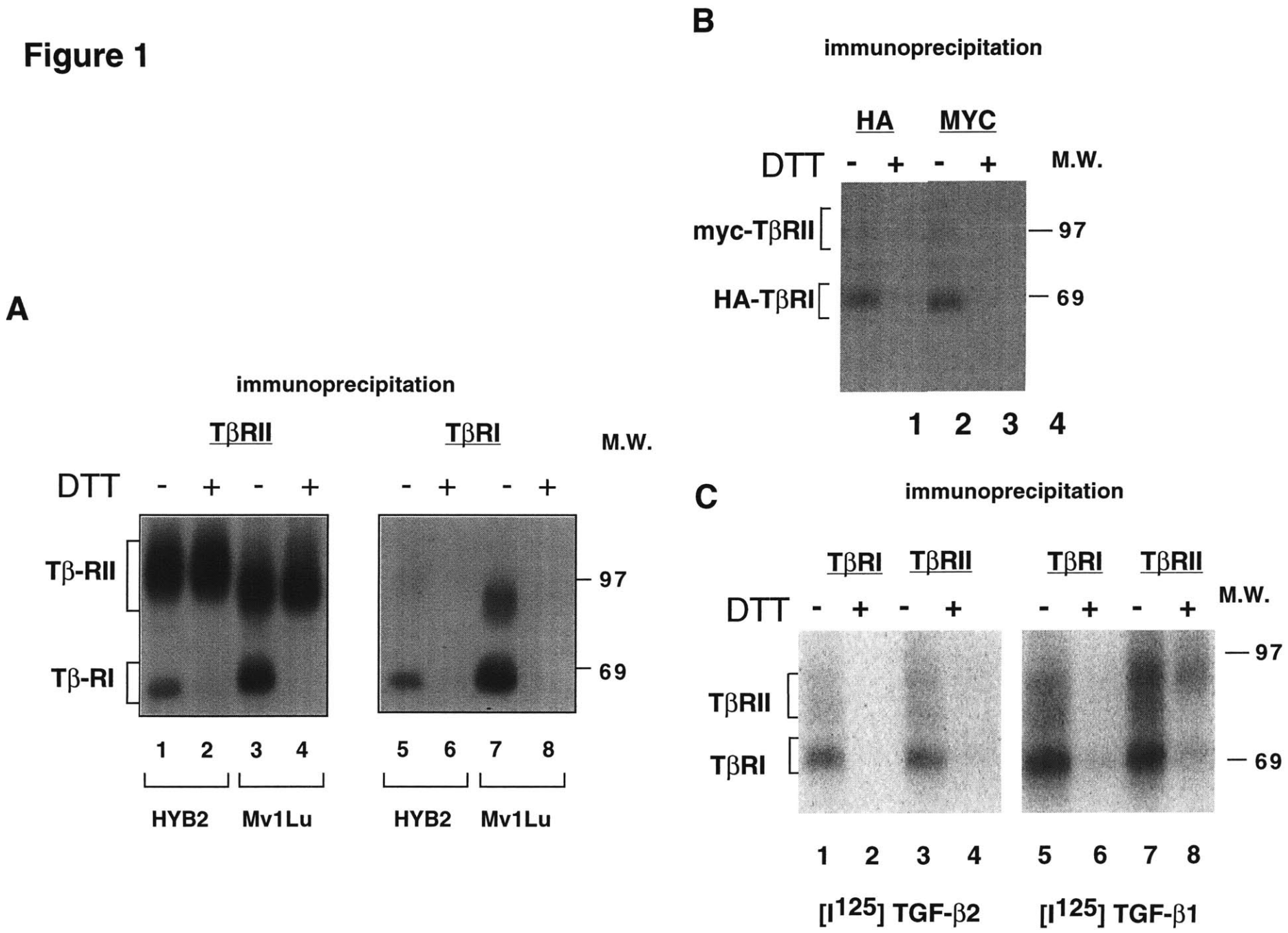
Figures

Figure 1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TβRI
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DTT - + - + - + - +
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HYB2 Mv1Lu HYB2 Mv1Lu
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HA MYC
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myc-TβRII
HA-TβRI
97
69
1 2 3 4
C
immunoprecipitation
TβRI TβRII TβRI TβRII
DTT - + - + - + - + M.W.
TβRII
TβRI
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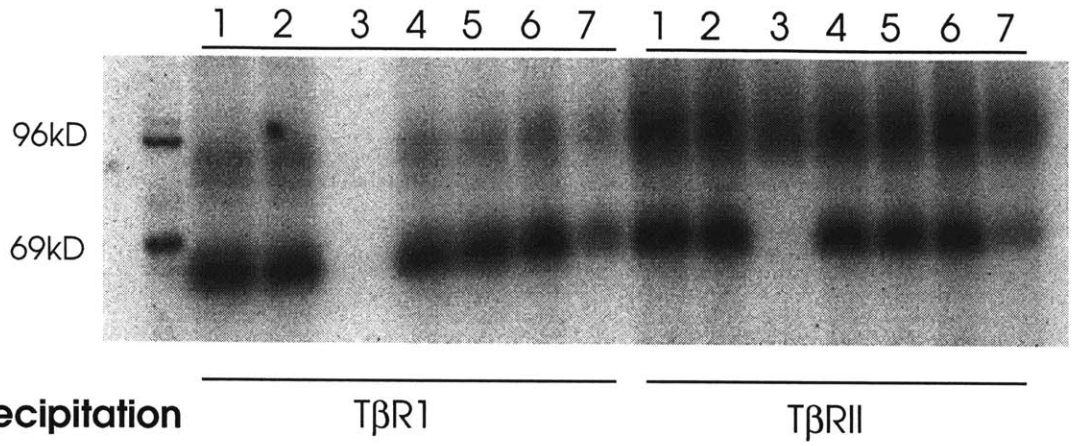
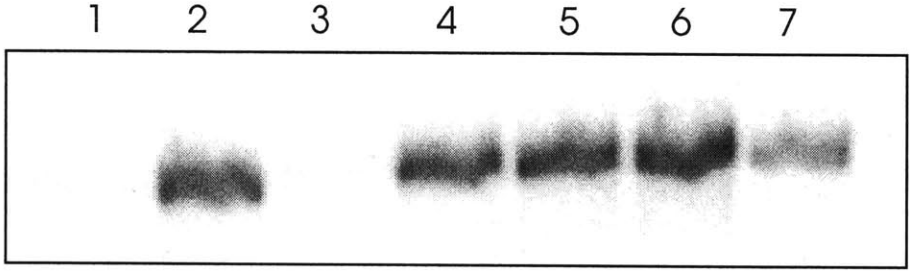
1 2 3 4 5 6 7 8
[I125] TGF-β2 [I125] TGF-β1

Figure 2

A. (I^{125}) TGF-β1 binding and crosslinking



B. GST-Smad3 phosphorylation



TGF-β	-	+	+	+	+	+	+
membrane permeability			+	+	-	-	+
dithiol			+	-	-	+	+

Figure 3

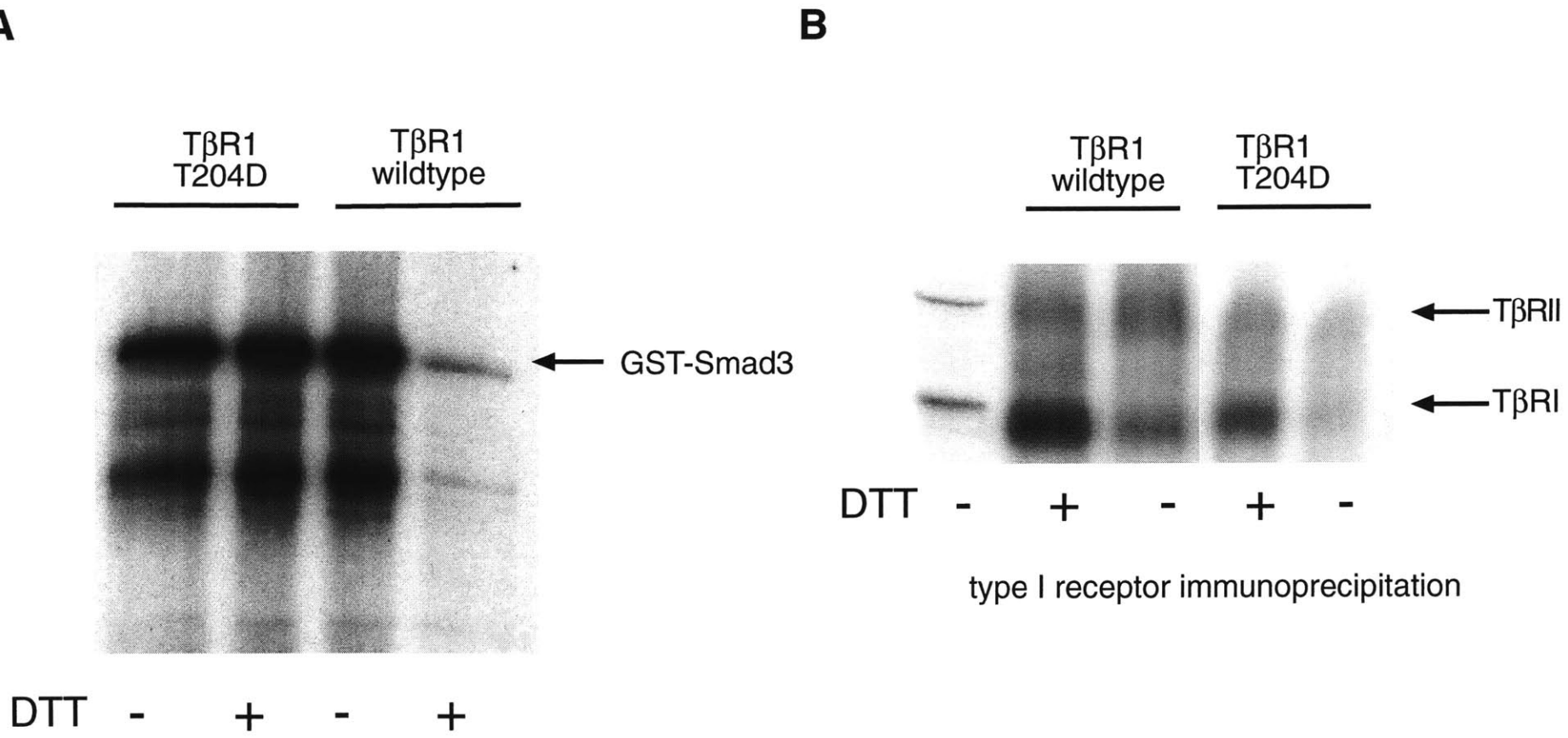
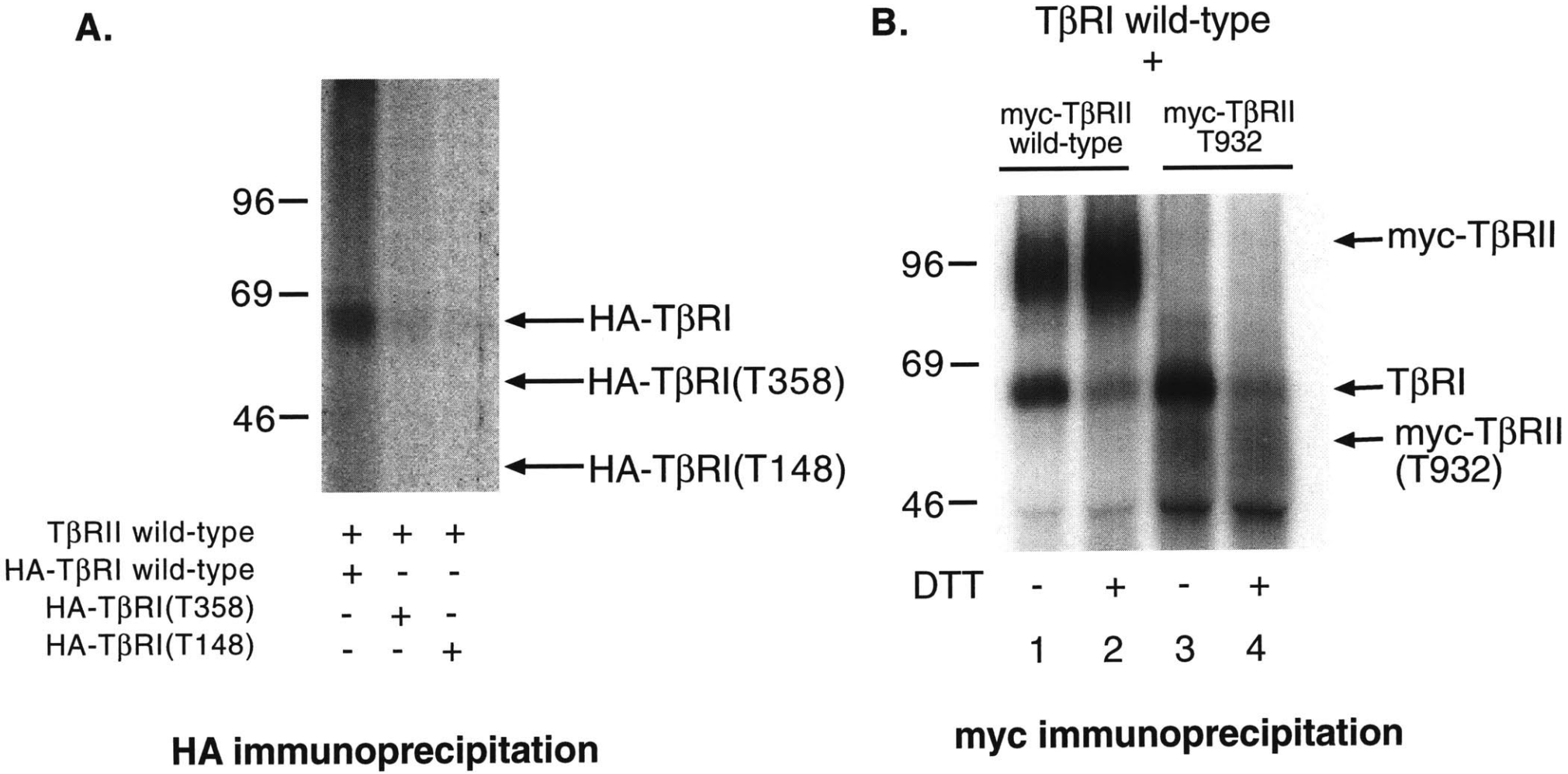

A
TβR1
T204D
TβR1
wildtype
GST-Smad3
DTT - + - +
B
TβR1
wildtype
TβR1
T204D
TβRII
TβRI
DTT - + - + -
type I receptor immunoprecipitation

Figure 4


A.
96
69
46
HA-TβRI
HA-TβRI(T358)
HA-TβRI(T148)
TβRII wild-type + + +
HA-TβRI wild-type + - -
HA-TβRI(T358) - + -
HA-TβRI(T148) - - +
HA immunoprecipitation
B.
TβRI wild-type
+
myc-TβRII wild-type
myc-TβRII T932
96
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myc-TβRII
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myc-TβRII (T932)
DTT - + - +
1 2 3 4
myc immunoprecipitation

Chapter 4

Investigation of a putative role for zinc as an essential factor for functional TGF-β receptor complex formation

Preface

Experiments described in Figures 1 and 3 were conducted with Dr. Carlos Rodriguez.

Abstract

The TGF-β receptor signalling complex is composed of two receptor serine/threonine kinases: type I (TβRI) and type II (TβRII). Binding of the ligand TGF-β1 to TβRII causes TβRII to associate with and phosphorylate TβRI. These interactions are essential for TGF-β1-activated signal transduction. Cheifetz and co-workers showed that dithiothreitol (DTT) abolished the ability of TGF-β1 to bind TβRI, yet binding to TβRII remained intact.(Cheifetz et al., 1990) As shown by Chapter 3 and others (Wells et al., in press), DTT not only prevents ligand binding, but also prevents the formation of a ligand-induced complex competent to signal. It is thought that DTT, by reducing critical disulfide bonds in the extracellular domain of TβRI, alters the receptor's conformation, thus preventing TGF-β binding.(Vivien and Wrana, 1995) Only membrane-permeable dithiols exert the same effect (Chapter 3) suggesting that an intracellular factor could be their target. Furthermore, because DTT, like other dithiols, is also a metal chelator, we thought it possible that chelation rather than disulfide bond reduction explained these observations. Hence, our model predicts that a divalent metal, most likely Zn^{2+}, is essential for the cytoplasmic domain of the type I receptor to assume a particular conformation necessary for interactions with the type II receptor. In this chapter, we test this model by examining a number of predictions it makes regarding the structural nature of the type I receptor. First, it predicts that the type I receptor cytosolic domain should bind Zn^{2+} and its removal by pre-treatment with membrane-permeable chelators should block formation of a TβRI/TβRII complex. Furthermore, mutants of the type I receptor that cannot bind zinc should correspondingly fail to associate with the ligand bound type II receptor and therefore would be unable to signal. We report experimental results demonstrating that the intracellular domain of TβRI binds zinc *in vitro*. However, membrane-permeable zinc chelators fail to exert any effect upon TβRI:TβRII interactions and moreover, TβRI mutants defective in zinc binding do not exhibit any significant defiencies in activating TGF-β dependent PAI-1 promoter activity. Therefore, our conclusions ultimately do not support an essential role for a divalent metal ion, namely zinc, in TGF-β signal transduction.

Introduction

As described in Chapter 3, membrane permeable dithiols specifically abrogate functional recruitment of the type I TGF-β receptor to the ligand bound/type II receptor complex. In particular, DTT and 2,3 DMP prevent both [^{125}I]TGF-β1 binding and cross-linking to the type I receptor as well as type I receptor mediated Smad3 transphosphorylation. In contrast, neither a membrane-impermeable dithiol such as diethyldithiocarbamic acid (DDC), nor monothiols such as β-mercaptoethanol or 2-mercapto-ethanesulfonic acid (MESA), exerted any effect. Hence, two properties appear to be necessary for type I receptor recruitment: (1) membrane permeability and (2) the presence of dithiols. In this chapter, we describe a model explaining the molecular mechanism underlying the DTT effect and test several predictions it makes regarding the structural make-up of the type I receptor.

In light of the requirements for membrane permeability, we thought it possible that these dithiols may be affecting intracellular domain(s) of the types I and/or II receptor cytoplasmic domain. However, it has been shown that the type II receptor cytoplasmic domain is dispensable for ligand induced association with the type I receptor. (Wieser et al., 1993) Since dithiols disrupt the formation of a functional types I and II complex, it follows that the more likely target of these reagents is the type I receptor cytoplasmic domain.

The DTT effect was first observed by Cheifetz and co-workers.(Cheifetz et al., 1990) Vivien and co-workers attribute this effect to DTT reducing disulfide bonds in the extracellular domain of Tβ-RI, rendering it unable to bind ligand.(Vivien and Wrana, 1995) In most proteins, however, disulfide bonds are buried in the interior and are not accessible to reducing agents in the absence of denaturation.

On the other hand, DTT , is also a potent chelator, a property distinguishing dithiols from monothiols. This often overlooked property of DTT led us to propose that it may be DTT's chelating rather than reducing properties that underlie this phenomenon. Zinc is the predominant metal found in protein structural domains. In this role, zinc serves as a template around which proteins can form defined tertiary structures. In general, energetic considerations dictate that polypeptides be of at least 50 residues in order to form stable tertiary domains.(Creighton, 1993) However, by using zinc as a cross-link, polypeptides as short as 15 residues are able to fold stably. Nature has put zinc binding domains to use in a wide diversity of functions including protein-protein, DNA, RNA and DNA/RNA

hybrid binding. Involvement in type I receptor function would nevertheless represent a novel role for zinc in the oligomerization of cell surface receptors.

As shown in Chapter 3, DTT does not affect type I receptor homodimerization, but rather appears to exert an effect specifically upon interactions between the types I and II receptor. Therefore, our model proposes that the TβRI cytoplasmic domain requires zinc for its proper folding which in turn, may be necessary for it to be recognized and recruited by the TGF-β bound TβRII.

This model requires that the type I receptor cytoplasmic domain binds zinc and moreover, its recruitment to the TGF-β bound type II receptor complex be susceptible to inactivation by zinc chelators. In addition, mutations in the type I receptor resulting in the loss of zinc binding should correlate with failure to interact with the ligand bound-type II receptor and therefore an inability to signal. Our experimental conclusions are two-fold: (1) the type I receptor binds zinc *in vitro*; (2) However, we fail to find evidence of zinc being necessary for type I receptor signalling. Therefore, although we cannot completely rule-out the possibility of the type I receptor binding zinc, ultimately our data does not support an essential role for it in TGF-β mediated signal transduction.

Experimental Procedures

Cell lines

Mv1Lu and R-1B cells (Boyd and Massague, 1989) were cultured in modified Eagles medium (MEM) supplemented with 10% fetal calf serum, 2mM glutamine, 100 units/ml of penicillin, 0.1 mg/ml streptomycin with non-essential amino acids (NENAA).

Binding of [$^{65}Zn^{2+}$] to GST fusions proteins immobilized on glutathione sepharose or nitrocellulose

By PCR, cDNA encoding the 356 residue cytoplasmic domain of the human type I TGF-β receptor (GST-TβRIcyt) was subcloned into the Sal1 and Not1 sites of pGEX4T-1 (Pharmacia). Using this DNA as a template, we employed the USE method (Unique Site Elimination) to mutate histidine and cysteine residues to alanine. (Deng and Nickoloff, 1992) The selection primer used mutates an AatII site contained within the pGEX4T-1 vector to a Sac I site. All GST-TβR1cyt fusion proteins as well as GST, GST-TβRIIcyt (Chen and Weinberg, 1995), and GST-EpoR (Wu et al., 1995) were produced in DH10B *E. Coli.* following induction for 1 hour with 1 mM isopropyl-β–D thiogalactopyranoside (IPTG) at 37°C. Proteins were purified as previously described (Smith and Johnson, 1988) and isolated by incubation with glutathione sepharose 4B (Pharmacia# 17-0756-01). Proteins (0.4 to 1.2 μg) immobilized on agarose were washed three times with metal binding buffer (MBB) (100 mM Tris, pH 6.8, 50 mM NaCl, 1 mM β-mercaptoethanol) and resuspended in 1 ml of MBB. 2.5 μCi of [$^{65}ZnCl_2$] (Dupont NEZ109, 5 mCi/ml, 239 mCi/mg) was then added to each sample and incubated at room temperature for one hour. The beads were washed three times in MBB and counted in a Packard Cobra model 5002 gamma counter. Samples were analyzed on a 12.5% SDS-PAGE gel (Laemmli, 1970) and stained with Coomassie Blue for protein quantitation. All data points were collected in duplicate.

Zinc blots were performed in a similar manner to that described in (Schiff et al., 1988). Briefly, proteins were purified as detailed above. Approximately, 5 μg of protein was used for each sample. Certain fusion protein samples were digested with 1 unit of thrombin (Sigma catalog #T6634) for eight hours at room temperature. Following separation by 10% SDS-PAGE, proteins were transferred onto a nitrocellulose membrane. Protein quantitation was determined by Ponceau-S staining (Sigma). Proteins were partially renatured by incubation for several hours at room temperature in TNET (20mM Tris, pH 8.0, 100mM

NaCl, 1mM EDTA, 1% Triton X-100) and washed three times in MBB. The filter was then incubated in MBB for 1 hour at room temperature with 1μCi/ml of [$^{65}ZnCl_2$]. After several fifteen minute washes at 4°C with MBB, the blot was exposed to Kodak XAR film. For quantitation, the blots were exposed to a Fuji phosphoimager screen and analysed using MacBas v2.2 software.

[^{125}I] TGF-β1 binding and cross-linking studies

After two washes with KRH buffer (50mM Hepes, pH 7.4, 8mM NaCl, 5mM $MgSO_4$, 0.5MM $CaCl_2$, 0.5mM KCl), confluent plates of Mv1Lu or R-1B cells were incubated for 10 minutes at 37°C with KRH-BSA (KRH containing 0.5% BSA). Afterwards, they were then pretreated with KRH-BSA or in some experiments, KRH-BSA containing a particular compound for ten minutes at 37°C. Cells were then placed on ice and after four washes with KRH buffer, binding and cross-linking with ^{125}I-TGF-β1 was then performed as described in (Cheifetz et al., 1990). Briefly, 10^7 cells were incubated with 150 pM [^{125}I] TGF-β1 in KRH buffer containing 1% fatty acid free serum albumin for 2 hours on ice. Following cross-linking with 50 mM disuccinimidyl suberate (DSS), the cells were washed with PBS (1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.0), and lysed in lysis buffer (TBS-GT (137 mM NaCl, 20 mM Tris-HCl [pH 8.0], 1% Triton X-100, 10% glycerol)).

Immunoprecipitations

After a 5 minute centrifugation, cleared lysates were incubated with an antibody/protein-A sepharose mixture at 4°C. Antibodies used include a polyclonal anti-peptide antibody raised against the C-terminus of Tβ-RII (antibody 260; 5μl/IP) (Moustakas et al., 1993) and a polyclonal antibody raised against a cytoplasmic juxtamembrane region of Tβ-RI (VPN;5μl/IP). (Franzen et al., 1993). After a 2 hour incubation at 4°C, immunoprecipitates were washed three times with TBS-GT followed by one wash with PBS. Immunoprecipitates were separated by SDS-PAGE and exposed to a Fuji phosphoimager screen for quantitation and analysis.

Purification of GST-Smad3

GST-Smad3 fusion proteins were produced in DH10B *E. coli* harboring the pGEX -2T-GST-smad3 bacterial expression vector. (Zhang et al., 1996) Method of purification is adopted from (Bitter and Roeder, 1979; Frangioni and Neel, 1993). Cultures were grown overnight at 37°C and then diluted 1:10 into 6 liters of Luria Broth (LB). After incubation

for 4 hours at 37°C, cells were subjected to induction for 2 hours with 1 mM isopropyl -β–D thiogalactopyranoside (IPTG) at 30°C. Bacteria were pelleted, resuspended in 120 mls of NETN (100mM NaCl, 20mM Tris (pH 8.0), 1mM EDTA, 0.5% NP-40) with 1mM PMSF, and sonicated for 15 seconds, 5-6 times in an ice bath. After clearing the lysates with a 5 minute centrifugation, fusion proteins were isolated by incubation with 500µl of a GST-Sepharose 4B (Pharmacia) 1:1 slurry for 30 minutes at 4°C. After washing the beads three times with NETN, GST-fusion proteins were eluted at room temperature with 5mM reduced glutathione in NETN. The resulting eluate was then dialyzed against buffer D (20mM Tris (pH 7.9), 20% glycerol, 0.1M KCl, 0.2mM EDTA, 0.5mM PMSF, 0.5mM DTT) overnight at 4°C and then stored at -70°C.

GST-Smad3 transphosphorylation assay

Mu1Lv and R-1B cells at ~ 50-80% confluence were washed three times with MEM followed by a 10 minute incubation at 37°C in MEM or MEM containing 1mM DTT or TCEP. Afterwards, cells were washed four times with MEM and then treated with 200pM TGF-β1 for 15 minutes at 37°C. Cells were washed three times with pre-chilled PBS and then lysed in lysis buffer (50mM Tris (pH 8.0), 150 mM NaCl, 1mM EDTA, 50mM NaF, 1mM $NaVO_3$, 1% NP40, 10% glycerol) and 1mM phenylmethylsulfonyl fluoride (PMSF). After a 5 minute centrifugation, cleared lysates were immunoprecipitated with 5µl of antibody 260 and 15 µl of a one to one protein A sepharose slurry for 1 hour at 4°C as detailed above. Immunoprecipitates were then washed three times with lysis buffer and once with kinase buffer (50mM Tris (pH 7.4), 10mM $MgCl_2$, 1mM DTT). To 20µl of the immunoprecipitate-protein A sepharose mixture, 10µl of a mix containing 1 µl 10X kinase buffer, 0.4 µl of 10mM ATP, 0.5 µl γ^{32}P-ATP (6000mCi/mmol), 2µg of GST-smad3 (Zhang et al., 1996) (1µg/µl), and 6µl of H_2O. After a 30 minute incubation at 30°C, samples were separated by 8% SDS-PAGE (Laemmli, 1970), transferred to 0.22 µM porosity nitrocellulose and then exposed to a Kodak phosphoimager screen.

Cell-free [^{125}I] TGF-β1 binding assay

10^7 Mv1Lu cells were lysed in 0.2 ml of 50 mM Hepes buffer, pH 7.4, containing 1% Triton-X-100 and 1 mM PMSF. After centrifugation to eliminate insoluble nuclear material, supernatants were incubated with 700 pM [^{125}I] TGF-β1 for 2 hours at 4°C in the presence or absence of the indicated chemical agent. The samples were then subjected to crosslinking with 50 µM disuccinimidyl suberate (DSS) for 15 minutes at 4°C. The cross-linking reaction was stopped by adding Tris (pH 7.4) to 1 M and the samples were

submitted to immunoprecipitation for 2 hours at 4°C with polyclonal antibodies specific either for TβRI (Franzen et al., 1993)(panel B) or TβRII (Moustakas et al., 1993)(panel A). The immunocomplexes were resolved by 10% SDS PAGE gel and exposed to a Fuji phosphoimager screen.

Mutagenesis

Forward and reverse oligonucleotides mutating cytoplasmic histidines or cysteines within the cytoplasmic domain of the human type I receptor TGF-β receptor to alanine were synthesized. Using these primers, mutations in the full length human type I TGF-β receptor were then generated by using overlap polymerase chain reaction (PCR). The template used was an N-terminal HA-tagged human type I receptor (Gilboa et al., 1998) subcloned to the HindIII/NotI sites of pcDNA3.1 (Invitrogen). The bacterial T7 primer (Invitrogen) and an anti-sense oligonucleotide encoding the last seven codons of the type I receptor cDNA fused to a NotI restriction site served as flanking primers for the overlap PCR reaction. Resulting PCR products were digested by HindIII and Not I and then inserted to these sites in pcDNA3.1.

Luciferase assay

Mu1Lv cells (R1B) were plated in six well dishes at a density of 5 x 10^5 cells/well. 24 hours later, cells were transfected by Lipofectamine Plus (Life Technologies, catalog # 10964013) with 1μg of p3TP-luc (Boyd and Massague, 1989) , pSVβ (Clontech), and either wildtype or a particular mutant type I receptor subcloned into pcDNA3.1 (Invitrogen). 24 hours later, cells are treated in the absence or presence of 10 or 100pM TGF-β1. 24 hours later, cells are lysed and measured for luciferase (D-luciferin from Analytical Luminescence, catalog #1600) and β-galactosidase activities (Tropix, catalog # ABL120RG) in an Autolumat LB953 EG & G Wallac luminometer. All measurements of p3TP-luc reporter activity for luciferase activity are divided by β-galactosidase activity readings to normalize for variable transfection efficiencies.

Results

We use two methods to show that the cytoplasmic domain of the type I receptor binds zinc. In Figure 1, a fusion protein of glutathione-S transferase (GST) with the cytosolic domain of the type I receptor (GST-TβRIcyt) is denatured, separated by SDS-PAGE, transferred to a nitrocellulose filter, subjected to a standard renaturation protocol, and then incubated with $^{65}Zn^{2+}$. Both the GST-TβRIcyt fusion as well as a known zinc-binding protein, carbonic anhydrase, bind $^{65}Zn^{2+}$ (lanes 1 and 3). Lysozyme, a protein not known to bind zinc, is negative in this assay (lane 2). However, since GST-protein alone binds $^{65}Zn^{++}$ weakly, we cleaved GST from the fusion protein by thrombin digestion. The isolated type I receptor cytoplasmic domain retains its ability to bind $^{65}Zn^{++}$ (lane 4).

Because of the denaturation and renaturation steps used in this assay, we cannot determine whether the native type I receptor is able to bind zinc. In another assay, we address this issue by incubating GST-TβRIcyt and GST-TβRIIcyt immobilized on glutathione sepharose with $^{65}Zn^{2+}$, yielding results similar to those of Figure 1. Importantly, over 80% of the type I receptor cytoplasmic domains immobilized on GST beads are able to bind $^{65}Zn^{2+}$ (Table 1). Using this more quantitative assay, we determined the affinity of the type I receptor cytoplasmic domain for $^{65}Zn^{2+}$ to be between 0.5 to 1 μM (Figure 2). With the exception of Cu^{2+} and Co^{2+}, other divalent metals tested bound with at least 10-fold lower affinity, as measured by displacement of $^{65}Zn^{2+}$ bound to GST-TβR1cyt both immobilized on glutathione sepharose and on a gel blot (data not shown). In this assay, about half of the immobilized GST-TβRIIcyt also bound $^{65}Zn^{2+}$ (Table 1).

Some cell surface receptors can bind ligand after detergent solubilization of cell membranes. (Hoang et al., 1993) Since a number of metals chelators, including ethylenediamine tetraacetic acid (EDTA)(Richardson et al., 1994) or ethylene glycol N,N,N'N' tetraacetic acid (EGTA) (Nishio et al., 1995) are unable to cross the plasma membrane, we studied their effects on the ability of [^{125}I] TGF-β1 to bind to the types I and II receptor in a cell-free extract. When added to detergent solubilized MuLv cells, [^{125}I] TGF-β1 bound and could be cross-linked to both receptors. They form a heteromeric complex since antisera to the type I receptor immunoprecipitate [^{125}I] TGF-β1 cross-linked to the type II receptor and *vice-versa.* (left lanes in Figures 3A and 3B). In this assay, several chelators, including broad specificity chelators like EDTA or BAPTA

(Aballay et al., 1995) and relatively zinc-specific chelators such as 1,10 O-phenanthroline (Auld, 1988; Schiavo et al., 1993) and TPEN (Auld, 1988; Iuliano et al., 1992) specifically inhibit binding of [^{125}I] TGF-β1 to the type I receptor (panel B) but exert no effect on binding of [^{125}I] TGF-β1 to the type II receptor (panel A). DTT only partially inhibits receptor oligomerization (panels A and B). This is consistent with its ability to only partially remove $^{65}Zn^{2+}$ from the GST-TβR1cyt fusion protein. (Table 2B)

However, in assays using live cells, a series of repeated washings separates chemical pre-treatment from subsequent [^{125}I] TGF-β1 addition. In contrast, the cell-free assay requires co-incubation of [^{125}I] TGF-β1 with these chemical agents. As a consequence, we cannot exclude possible changes exerted by these agents on the ligand and its ability to form a heteromeric complex. To address this issue,we tested whether pre-treatment of intact cells with two membrane-permeable zinc chelators (described in Chapter 2) can block subsequent [^{125}I] TGF-β1 binding and cross-linking to the type I receptor. As shown in figures 4A and B, neither 1,10 O-phenanthroline nor TPEN have any effect, in contrast to the results obtained in the cell-free assay.

Zinc structural domains are characterized by the almost predominant use of histidines and cysteines as coordinating residues. The type I receptor cytoplasmic domain contains an unusually high density of histidines (~5%) and cysteines, both of which could participate in binding putative zinc atom(s). Indeed Figures 5A and B show that mutations to alanine of these residues result in the loss of $^{65}Zn^{2+}$ binding. In most cases, these mutants exhibit 40-60% binding relative to the wildtype receptor cytoplasmic domain.

We proceeded to examine the functional consequences of these mutants with respect to their abilty to induce TGF-β-dependent activation of the PAI-1 promoter.(Westerhausen et al., 1991; Wrana et al., 1992) Specifically, we constructed similar mutations in the context of the full length type I receptor and transiently co-transfected them with a TGF-β inducible luciferase reporter construct (p3TP-luc) containing portions of the PAI-1 promoter fused to three TPA response elements into R-1B cells, a type I receptor defective cell line obtained by mutagenesis of the mink lung epithelial cell line Mv1Lu. (Boyd and Massague, 1989) Because R-1Bs lack a functional type I receptor, we can examine the functional activity of these mutants in the absence of endogeneous receptors. Consistent with published studies of R-1B cells, they do not respond to TGF-β unless transfected with the type I receptor cDNA.(Figure 6) As shown in Figures 6A, B and C, virtually all of the mutants tested qualitatively exhibit similar signalling phenotypes to the wildtype type I receptor. An exception is TβR1-H256A which exhibits slightly lowered TGF-β-induced

signalling relative to the wildtype type I receptor (Figure 7). However, we find little correlation between mutations resulting in the loss of *in vitro* $^{65}Zn^{2+}$ binding, and their subsequent ability in the context of the full length receptor to activate TGF-β-dependent PAI-1 promoter activity.

Also contained within or near the transmembrane domain are three cysteines and one histidine. These residues were mutated to either alanine or serine and analyzed for their ability to induce PAI-1 promoter activity. Again, to a first approximation, these mutants do not exhibit any significant signalling defects. (Figure 6D).

Figures 8A and B shows that pre-treatment of Mv1Lu cells with a non-thiol based reducing reagent, Tris (2-carboxyethyl) phosphine (TCEP), followed by [^{125}I] TGF-β1 binding and cross-linking recapitulates the same effects as that of pre-treatment with membrane-permeable dithiols. Like DTT, it prevents TGF-β1 from binding/cross-linking TβRI, yet binding to TβRII remained unaffected. (Figure 8A) More importantly, when Mv1Lu cells undergo TCEP pre-treatment, a type II receptor immunoprecipitate can no longer induce *in vitro* transphosphorylation of Smad3. This transphosphorylation is type I receptor-mediated as R-1B cells in contrast exhibit no ligand-dependent Smad3 transphosphorylation. Hence, TCEP also abolishes type I receptor-mediated transphosphorylation of Smad3, suggesting that it abrogates functional type I and II interactions in a similar manner to that of membrane permeable dithiols.

Discussion

Two observations form the basis of the hypothesis that zinc constitutes an essential factor for functional interactions between the types I and II TGF-β receptors. First, among the classes of thiol-based reducing agents tested in Chapter 3, only membrane permeable dithiols abrogate recruitment of type I receptor to the ligand bound/type II receptor complex. This would suggest that properties relating to both membrane permeability and dithiols are necessary to elicit this effect. Metal chelation is one property that distinguishes dithiols from monothiols. Hence, our data would be consistent with the notion that DTT affects a cytoplasmically localized factor and by chelating a metal, most likely zinc, prevents formation of a functional types I and II complex. Second, as shown by Wieser and co-workers, the type II receptor cytoplasmic domain is dispensable for ligand-induced association between the types I and II receptor. Together with this observation, the type I receptor cytoplasmic domain would be the more likely target of these compounds. Hence our model proposes that zinc binds to the type I receptor and is required for their functional recruitment to the ligand bound/type II receptor complex.

Our evaluation of this hypothesis rests on four major findings: (1) when bacterially expressed, the type I receptor cytosolic domain can bind zinc *in vitro* with an affinity of 1μM.(2) In a cell-free assay, Zn^{2+} chelators can block type I receptor binding and cross-linking to TGF-β1. However, this assay cannot exclude the possibility that these chemical agents adversely affect in some manner a ligand's ability to form a types I and II hetero-complex. Furthermore, with membrane permeable zinc chelators, we fail to obtain similar results in intact cells suggesting that these results should be interpreted with caution. (3) Histidine and cysteine TGF-β type I receptor mutants can interact with the type II receptor to form a complex competent to signal TGF-β-dependent PAI-1 promoter activity. Therefore, at least when mutated individually, histidine and cysteine residues are not essential for types I and II interactions. Some mutants however do activate the reporter construct at weaker levels relative to the wildtype type I receptor, particularly at lower TGF-β concentrations. This could reflect lower efficiency of folding in the endoplasmic reticulum and consequently fewer surface receptors. However, the involvement of zinc is unclear as our data lacks any obvious correlations between impaired signalling and loss of *in vitro* $^{65}Zn^{2+}$ binding. (4) Type I receptor recruitment can also be blocked by pre-treatment with TCEP which to our knowledge does not possess any significant zinc chelating properties.

In summary, our findings suggest that *in vitro* , the type I receptor indeed does bind zinc. However, there is little or no correlation between our *in vitro* Zn^{2+} binding data and the signalling ability of full length receptors mutated in the corresponding cysteine or histidine residues. Moreover, TCEP, which to our knowledge is a reagent that does not chelate zinc, can recapitulate the same effects exerted by DTT, raising the question whether the previously asserted dithiol requirement is indeed related to zinc chelation. Hence, while we cannot rule out the possibility that the type I receptor binds zinc, our data do not support an essential role for it in the formation of a functional types I and II TGF-β receptor complex.

The FK506 family of immunosuppressants represent another class of compounds known to affect TβRI/RII interactions. (Chen et al., 1997; Wang et al., 1994; Wang et al., 1996) Wang and co-workers demonstrated that the immunophilin FKBP12 binds the TβRI GS domain preventing transphosphorylation by the type II receptor. Upon ligand-induced TβRI/TβRII association, it is then thought that FKBP12 dissociates from TβRI. Pretreatment of cells with immunosuppressants such as FK506 or rapamycin also cause FKBP12 dissociation and as a result, induce elevated basal TGF-β signalling activity. It is possible that DTT may act to stabilize FKBP12 binding to the type I receptor and consequently prevent functional interactions between the types I and II receptor.

Nevertheless, reinterpretation of the data available to date is clearly required. We believe that an investigation of what structural determinants of the type I receptor are affected by DTT would be fruitful. Details of future research directions is discussed in the proceeding chapter.

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Figure Legends

Figure 1: The cytoplasmic domain of Tβ-R1 binds [$^{65}Zn^{2+}$] in an *in situ* assay.

5μg of protein are contained within each lane. Carbonic anhydrase serves as a positive control for $^{65}Zn^{2+}$ binding (lanes 1 and 5) and lysozyme serves as a negative control (lanes 2 and 6). In lanes 4 and 8, GST-Tβ-RIcyt fusion protein was digested with 1 U thrombin as detailed in the Materials and Methods section. Following separation by 10% SDS-PAGE, proteins were blotted onto a nitrocellulose membrane. The blot was stained with Ponceau-red for protein quantitation as shown in Panel A. Proteins on the blot were then partially renatured by incubation for several hours at room temperature in TNET (20 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100) and washed three times in MBB (see Table 1). The filter was then incubated in MBB for 1 hour at 20°C with 1 mCi/ ml of [$^{65}ZnCl_2$]. After several 15 minute washes at 4°C with MBB, the blot was exposed to Kodak XAR film (Panel B). For quantitation, the blots were exposed to a Fuji phosphoimager screen and the amount of radioactivity analyzed using MacBas v2.2 software.

Table 1: Binding of [$^{65}Zn^{2+}$] to GST fusion proteins immobilized on glutathione sepharose

Fusion proteins (0.4 to 1.2 μg) immobilized on glutathione sepharose 4B were incubated with 2.5 μCi of [$^{65}Zn^{2+}$] in 1 ml of MBB as detailed in Experimental Procedures.

Table 2: Binding of [$^{65}Zn^{2+}$] to GST-Tβ-RIcyt

Zinc binding was conducted in a manner similar to that detailed in Experimental Procedures except in the presence of 300 μM salts (A). In (B), following incubation of GST-Tβ-RIcyt beads with [$^{65}Zn^{++}$] and washing with MBB, samples were further incubated for 20 minutes at 20°C with the indicated concentration of each chelator dissolved in MBB. Samples were again washed three times in MBB and quantitated in a gamma counter.

Figure 2: Affinity of GST-Tβ-RIcyt for [$^{65}Zn^{2+}$]

Zn^{2+} binding was conducted as detailed in Experimental Procedures, except reactions contained increasing concentrations of unlabeled $ZnCl_2$.

Figure 3: Metal chelators block interactions between the types I and II TGF-β receptors in a cell-free [^{125}I] TGF-β1 binding and cross-linking assay.

In a *cell-free* assay, detergent solubilized Mv1Lu cells were incubated in the presence of [^{125}I] TGF-β1 or [^{125}I] TGF-β1 with 2 mM dithiothreitol (DTT), 25 mM ethylenediamine tetraacetic acid (EDTA), 25 mM ethylene glycol N,N,N',N', tetraacetic acid (EGTA), 2 mM 1,1 0-Phenanthroline (OP), 2 mM N,N,N',N'-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN), 5 mM 2-mercaptoethanol or 2 mM 1,2-bis-(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA). Upon completing binding and cross-linking reactions, lysates were split into samples and the immunoprecipitated with antibodies specific for either the types I or II receptor. Upon washing with lysis buffer, these immunoprecipitates were resolved by 10% SDS-PAGE and exposed to a Fuji phosphoimager screen.

Figure 4: Membrane permeable zinc chelators fail to block interactions between the types I and II receptor in intact cells.

Mv1Lu or R-1B cells were pretreated with either KRH-BSA or KRH-BSA with 5 mM 1,10 O-phenanthroline, 1,7 phenanthroline, or TPEN for 10 minutes at 37°C. Cell were then placed on ice and washed four times with pre-chilled KRH. Binding and cross-linking with 100pM [^{125}I] TGF-β1 was then performed as described in Experimental Procedures.

Figure 5: Mutations to alanine of cysteine and histidine residues contained within the type I receptor cytoplasmic domain exhibit partial loss of [$^{65}Zn^{2+}$] binding.

A) Cysteine to alanine mutations introduced in the type I receptor cytoplasmic domain result in partial loss of [$^{65}Zn^{2+}$] binding.

Mutations are analyzed within the context of the GST-Tβ-R1cyt fusion protein. Approximately 1 μg of GST-Tβ-R1cyt fusion protein or GST alone was incubated with [$^{65}Zn^{2+}$] as described in Experimental Procedures. Binding of [$^{65}Zn^{2+}$] by mutant fusion proteins was calculated relative to wildtype GST-Tβ-R1cyt and normalized to -per mole of protein.

B) Histidine to alanine mutations introduced in the type I receptor cytoplasmic domain result in partial loss of [$^{65}Zn^{2+}$] binding.

Binding of [$^{65}Zn^{2+}$] to GST-Tβ-R1cyt histidine to alanine mutant fusion proteins was conducted in the same manner as described above.

Figure 6: Histidine and cysteine TGF-β type I receptor mutants can interact with the type II receptor to form a complex competent to signal TGF-β dependent PAI-1 promoter activity.

A) Histidine to alanine full length type I TGF-β receptor mutants activate PAI-1 promoter activity.

R-1B cells were transfected with the histidine to alanine mutants of the full length type I receptor, the TGF-β inducible p3TP-luc reporter construct and pSVβ, as a control for transfection efficiency. Data reflects the normalized luciferase activity of cell lysates after 24 stimulation at the given TGF-β concentrations.

B,C) Cysteine to alanine full length type I TGF-β receptor mutants activate PAI-1 promoter activity.

Cysteine to alanine full length type I TGF-β receptor mutants were tested in the same manner as above.

D) Tβ-R1-H256A exhibits impaired signalling relative to the wildtype type I receptor.

Transfected into R-1B cells in the same manner as described above, Tβ-R1-H256A and wildtype I receptor were assayed for their signalling activity at various concentrations of TGF-β1.

Figure 7: Cysteines and histidines that lie in the transmembrane signal are not necessary for TGF-β dependent PAI-1 promoter activity.

Histidines and cysteines within or neighboring the transmembrane domain of the type I were singly mutated to either serine or alanine and by the assay outlined in Experimental Procedures, tested for their ability to activate TGF-β dependent PAI-1 promoter activity.

Figure 8: Pre-treatment with TCEP blocks formation of a functional TGFβ1/types I and II receptor complex.

A. TCEP blocks type I receptor binding and cross-linking to [^{125}I] TGF-β1

Mv1Lu and R-1B cells were pretreated with KRH-BSA or KRH-BSA containing 1mM DTT or 1mM TCEP for ten minutes at 37°C and then rinsed four times with prechilled KRH. Then as detailed in Experimental Procedures, cells were bound and cross-linked to 100pM [^{125}I] TGF-β1, immunoprecipitated with both types I and II receptor antisera, and then resolved by 8% SDS-PAGE. The gel was then dried and exposed to a Fuji phosphoimaging plate.

B. TCEP preatment blocks TGF-β induced type I receptor mediated Smad3 transphosphorylation.

50-80% confluent Mv1Lu and R-1B cells were washed with MEM two times and then pretreated with MEM or MEM containing 1mM DTT or 1mM TCEP for ten minutes at 37°C. Plates were subsequently washed with MEM four times followed by stimulation with 200pM TGF-β1 for 15 minutes at 37°C. Then as described in Experimental Procedures, the lysates of treated cells were assayed for GST-Smad3 transphorylation. R-1B cells serve as a negative control to verify that Smad3 transphosphorylation requires the presence of the type I receptor.

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Figures

Figure 1

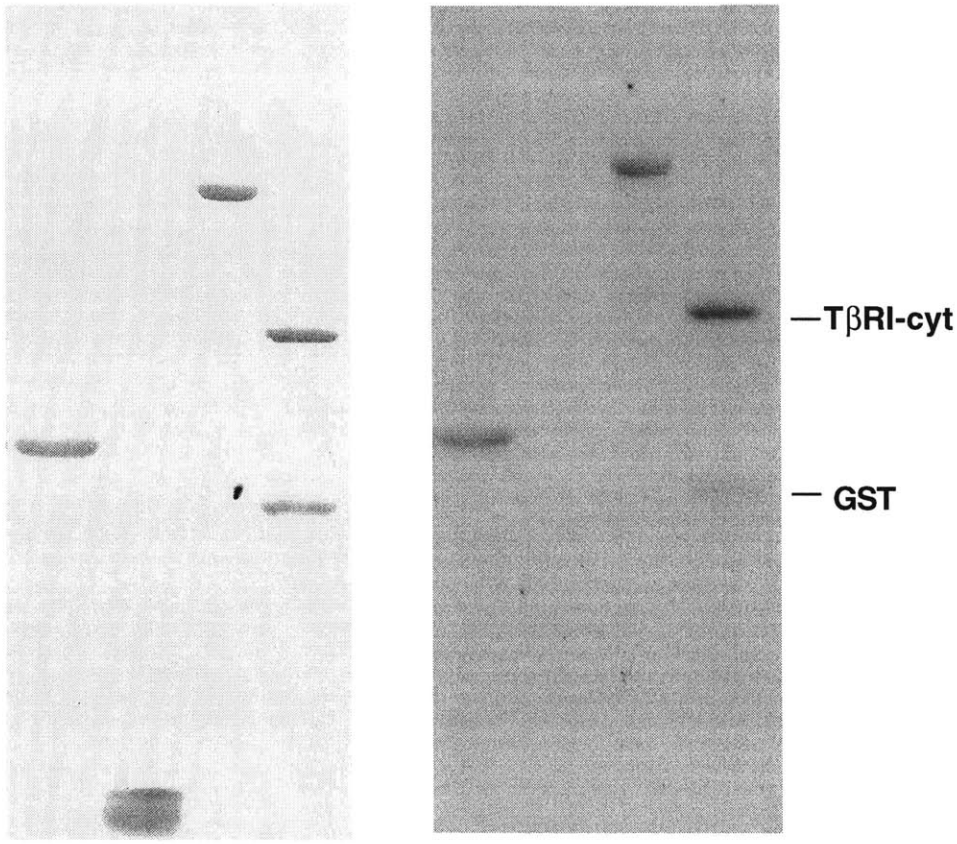


Table 1 Binding [^{65}Zn] to GST fusion proteins immobilized on glutathione agarose

Protein:	μg protein bound to column	picomoles [^{65}Zn] bound	moles [^{65}Zn] bound / moles protein
GST	0.4	0.065	0.004
GST-TβRIcyt	1.2	10	0.84
GST-TβRIIcyt	0.8	4.84	0.42
GST-EpoR	0.75	0.089	0.006

Table 2 Binding of [^{65}Zn] to GST-TβRI-cyt immobilized on glutathione- agarose beads

A) In the presence of 300μM metal salts

	Relative amount of [^{65}Zn] bound ± SD
no addition	1.00
$CaCl_2$	0.90 ± 0.21
$ZnCl_2$	0.018 ± 0.006
$CoCl_2$	0.51 ± 0.03
$CuCl_2$	0.62 ± 0.078
$FeCl_2$	1.10 ± 0.15
$MgCl_2$	0.98 ± 0.034

B) In the presence of chelators

	Relative amount of [^{65}Zn] bound ± SD
no addition	1.00
2mM DTT	0.424 ± 0.042
25mM EDTA	0.067 ± 0.018
1mM O-phenanthroline	0.078 ± 0.011
1mM TPEN	0.048 ± 0.003

Figure 2

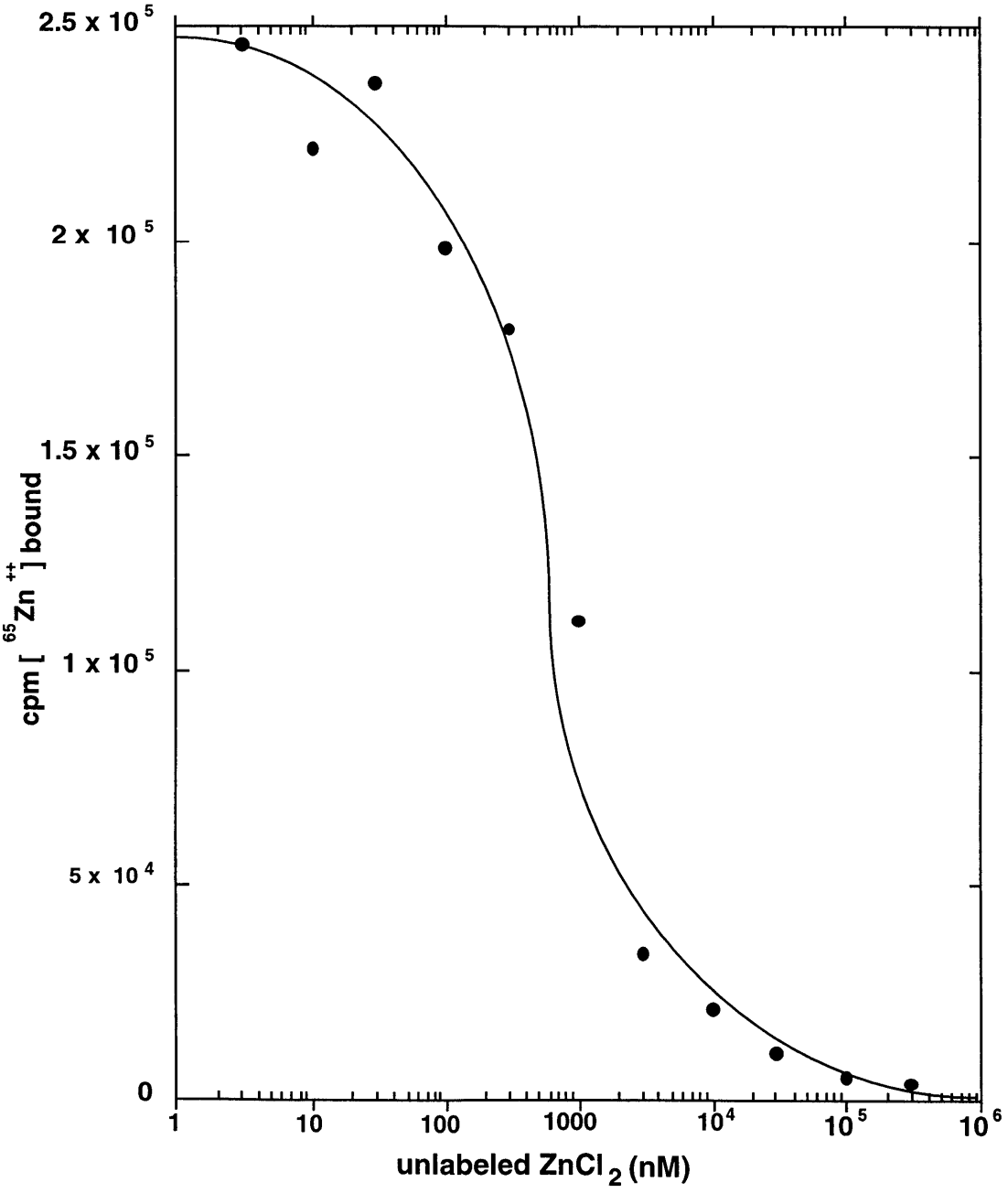
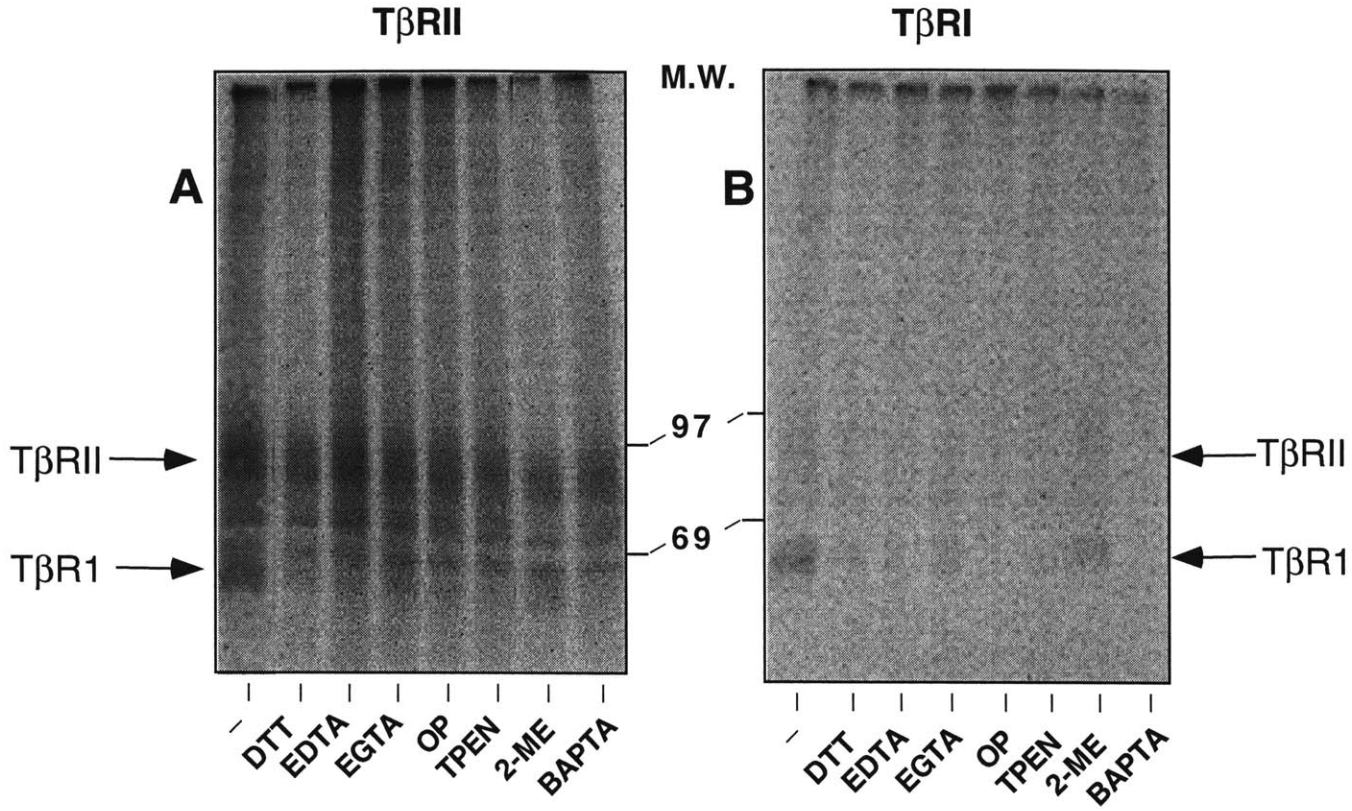

2.5 x 10 5
2 x 10 5
1.5 x 10 5
1 x 10 5
5 x 10 4
0
cpm [65Zn ++] bound
1
10
100
1000
10 4
10 5
10 6
unlabeled ZnCl 2 (nM)

Figure 3


TβRII
TβRI
M.W.
A
B
TβRII
TβR1
97
69
TβRII
TβR1
-
DTT
EDTA
EGTA
OP
TPEN
2-ME
BAPTA
-
DTT
EDTA
EGTA
OP
TPEN
2-ME
BAPTA

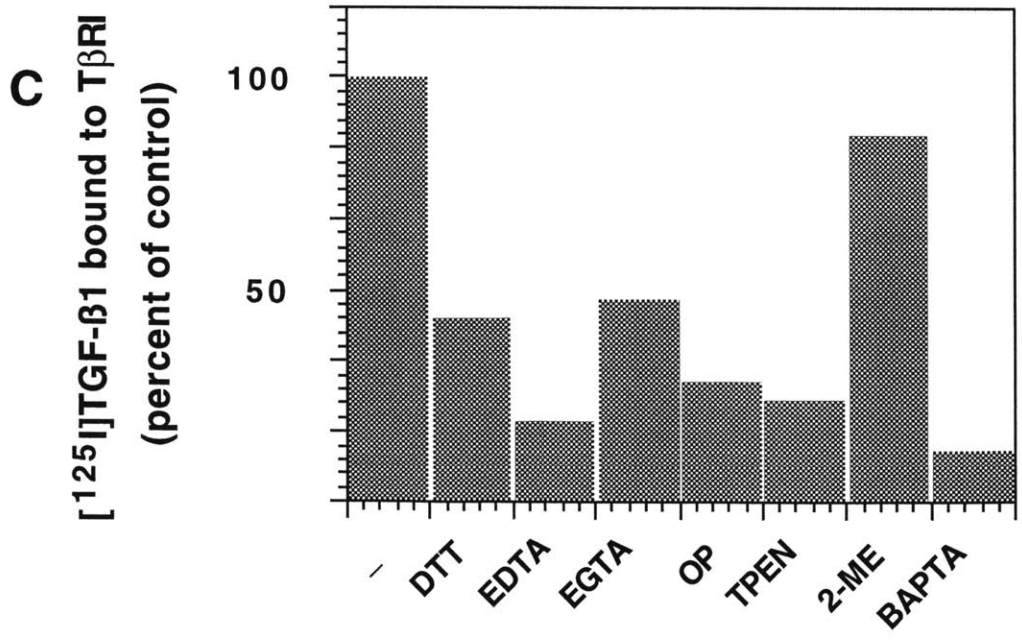

C
[125I]TGF-ß1 bound to TβRI
(percent of control)
100
50
-
DTT
EDTA
EGTA
OP
TPEN
2-ME
BAPTA

Figure 4

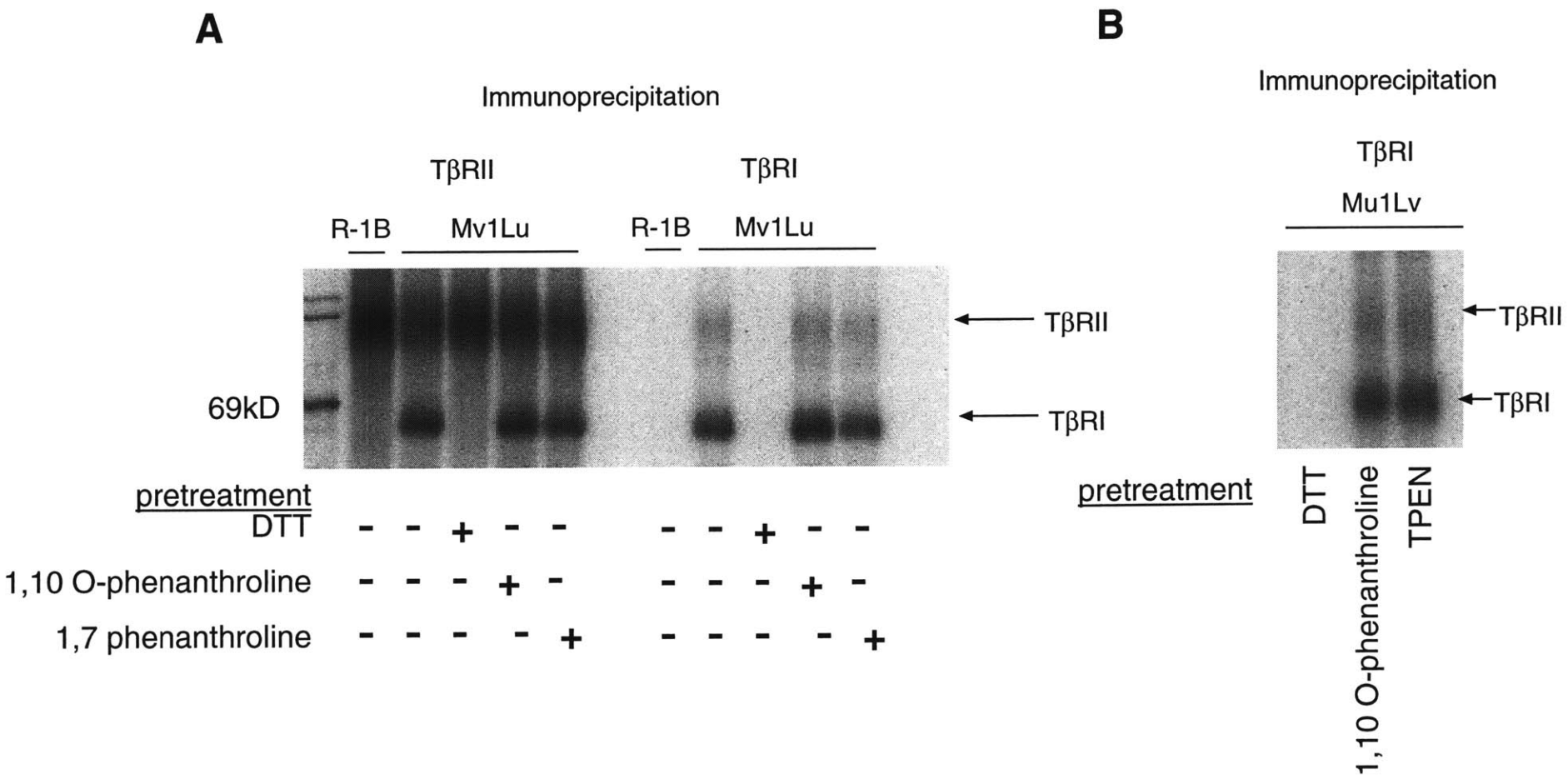

A
Immunoprecipitation
TβRII
TβRI
R-1B
Mv1Lu
R-1B
Mv1Lu
69kD
TβRII
TβRI
pretreatment
DTT
1,10 O-phenanthroline
1,7 phenanthroline
B
Immunoprecipitation
TβRI
Mu1Lv
TβRII
TβRI
pretreatment
DTT
1,10 O-phenanthroline
TPEN

Figure 5A

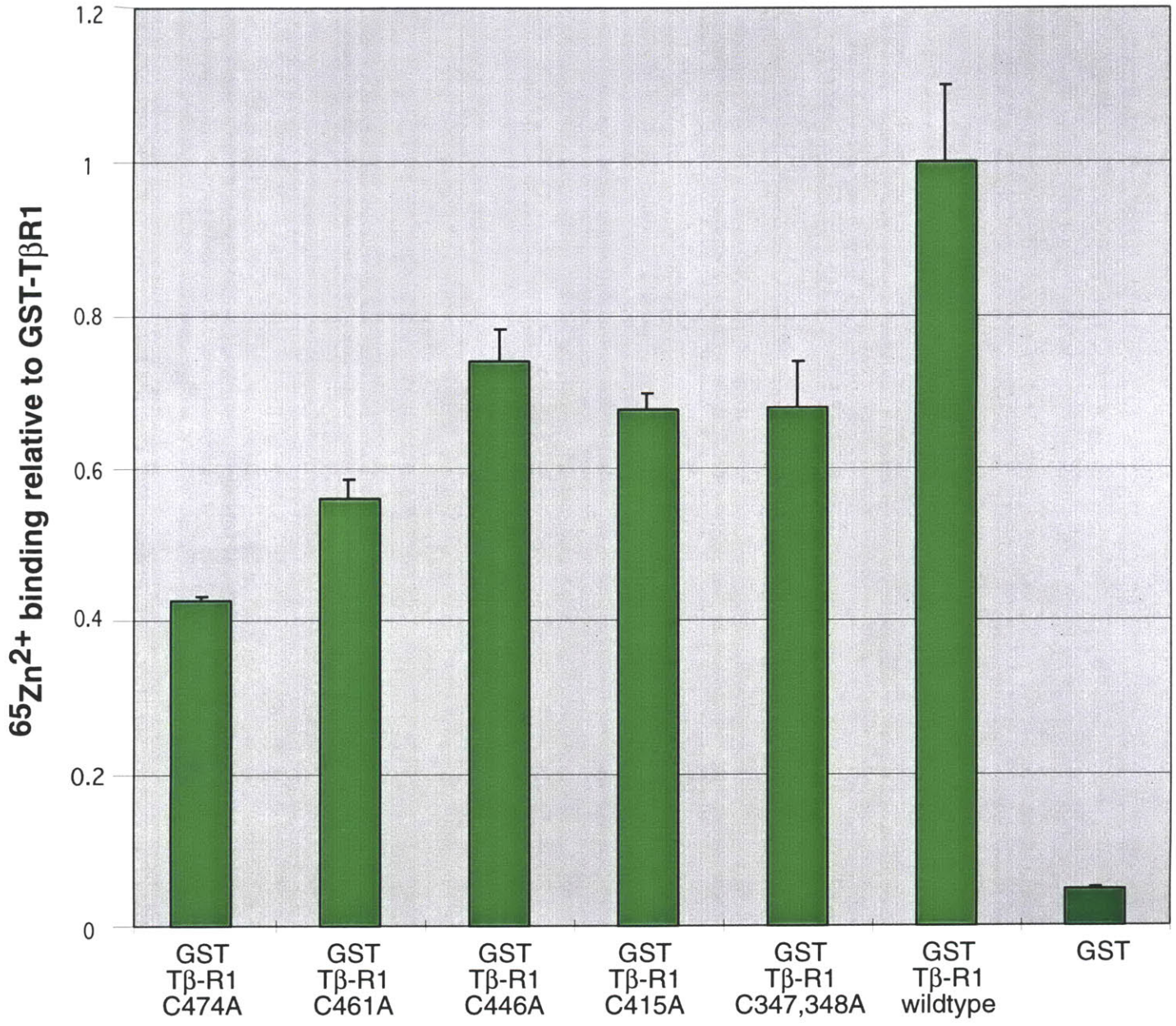

$^{65}Zn^{2+}$ binding relative to GST-TβR1
1.2
1
0.8
0.6
0.4
0.2
0
GST Tβ-R1 C474A
GST Tβ-R1 C461A
GST Tβ-R1 C446A
GST Tβ-R1 C415A
GST Tβ-R1 C347,348A
GST Tβ-R1 wildtype
GST

Figure 5B

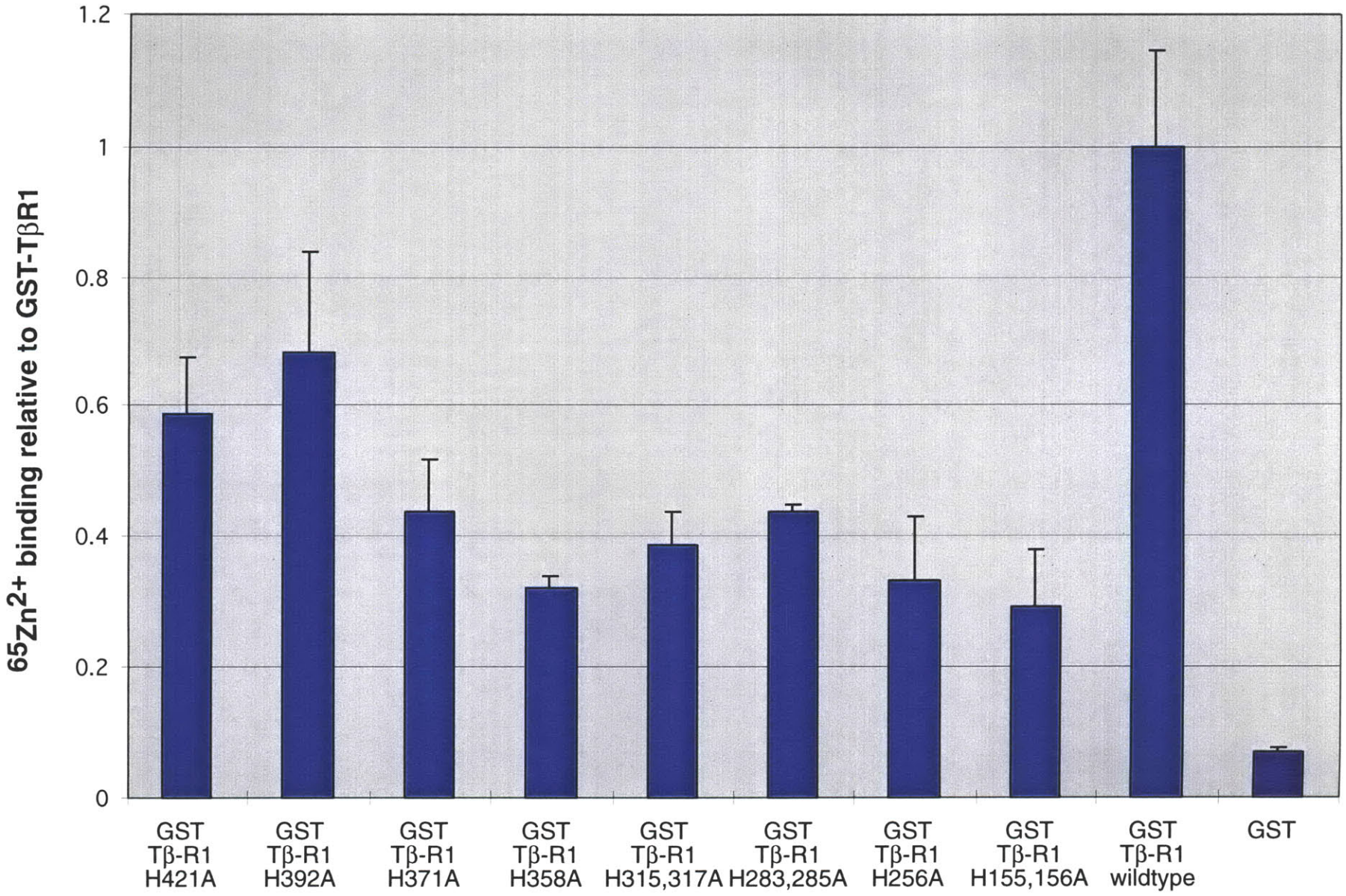

$^{65}Zn^{2+}$ binding relative to GST-TβR1
1.2
1
0.8
0.6
0.4
0.2
0
GST Tβ-R1 H421A
GST Tβ-R1 H392A
GST Tβ-R1 H371A
GST Tβ-R1 H358A
GST Tβ-R1 H315,317A
GST Tβ-R1 H283,285A
GST Tβ-R1 H256A
GST Tβ-R1 H155,156A
GST Tβ-R1 wildtype
GST

Figure 6A

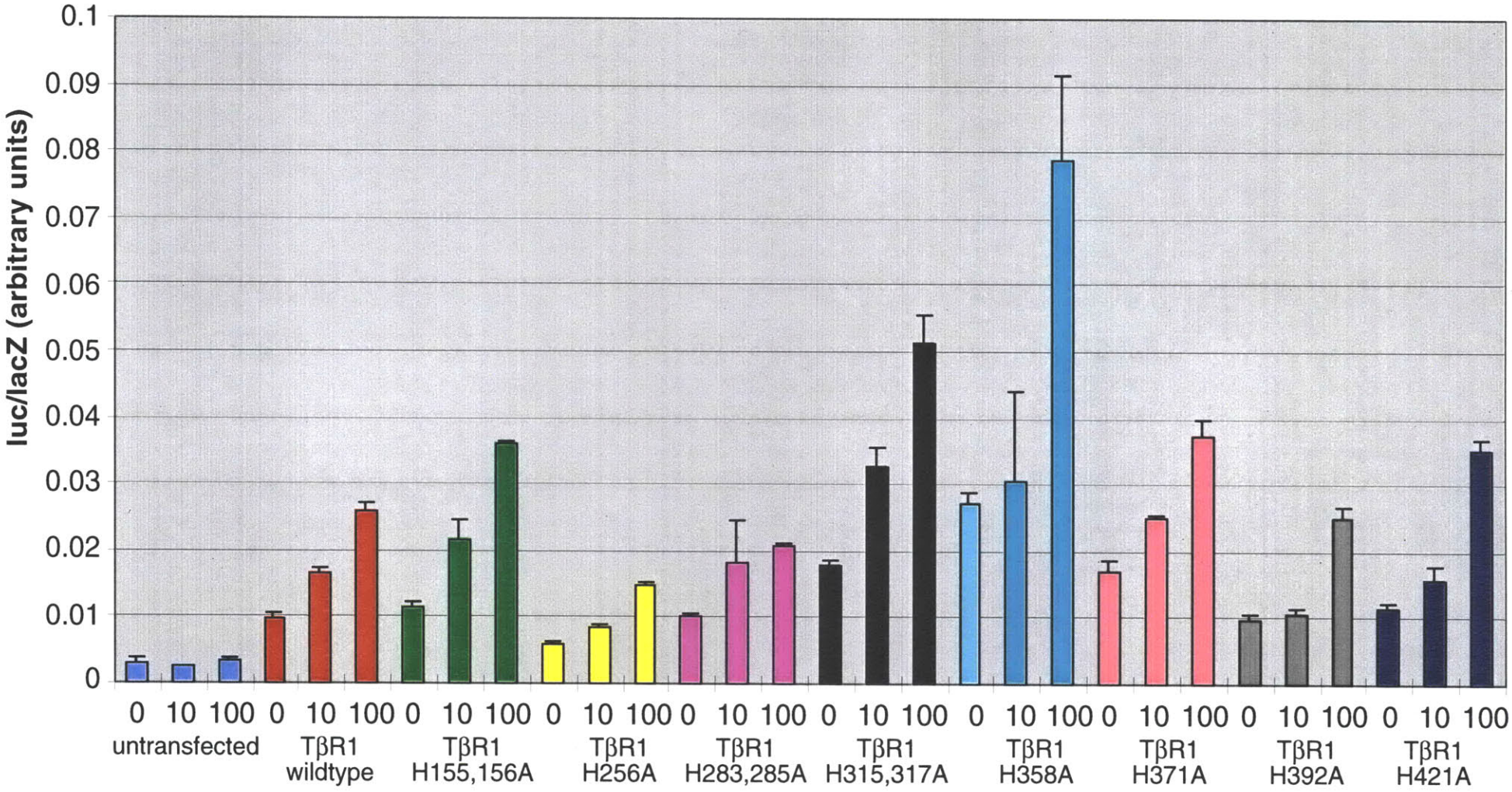

luc/lacZ (arbitrary units)
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0 10 100 0 10 100 0 10 100 0 10 100 0 10 100 0 10 100 0 10 100 0 10 100 0 10 100 0 10 100 0 10 100 0 10 100 0 10 100
untransfected
TβR1 wildtype
TβR1 H155,156A
TβR1 H256A
TβR1 H283,285A
TβR1 H315,317A
TβR1 H358A
TβR1 H371A
TβR1 H392A
TβR1 H421A

Figure 6B

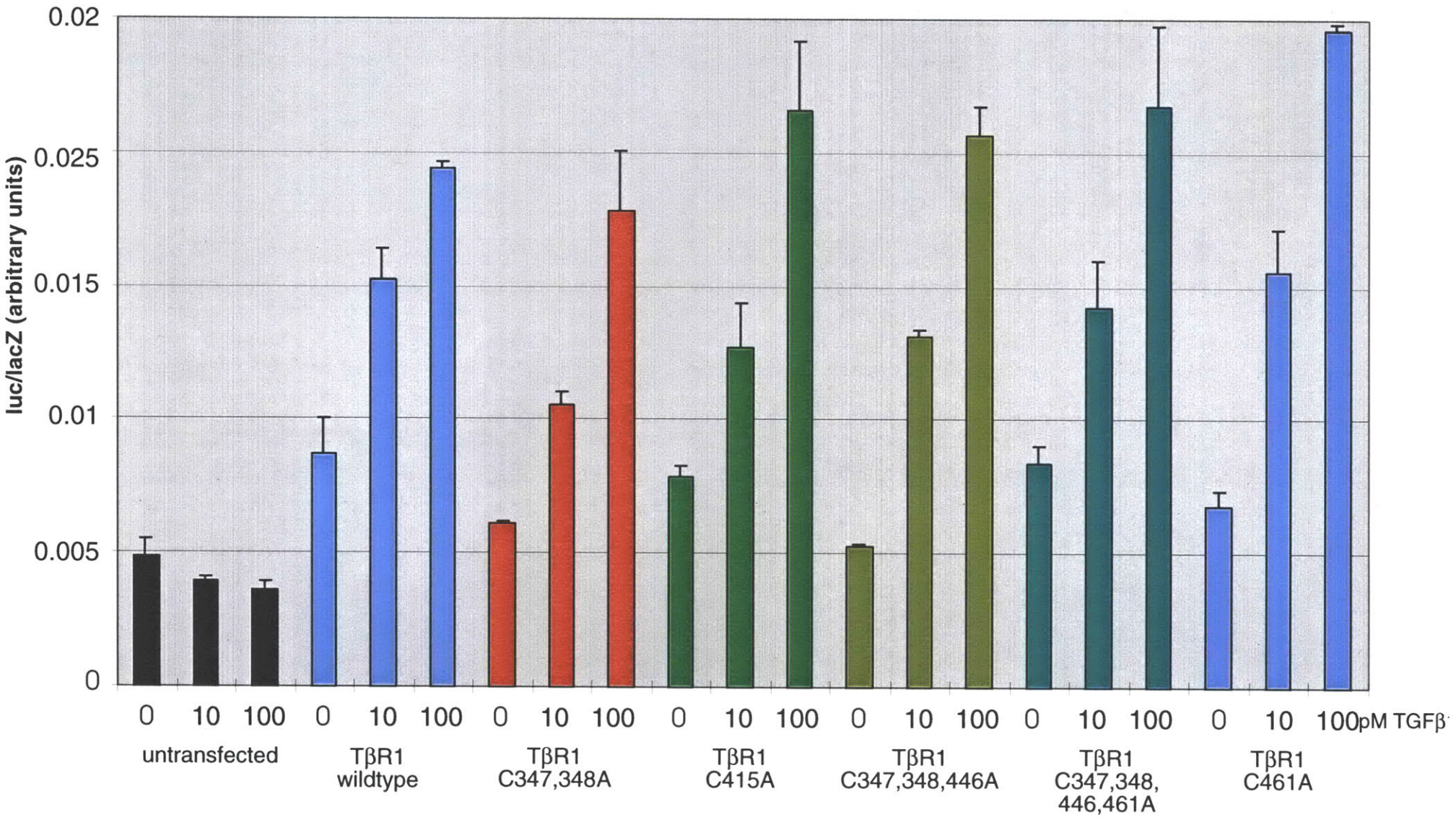
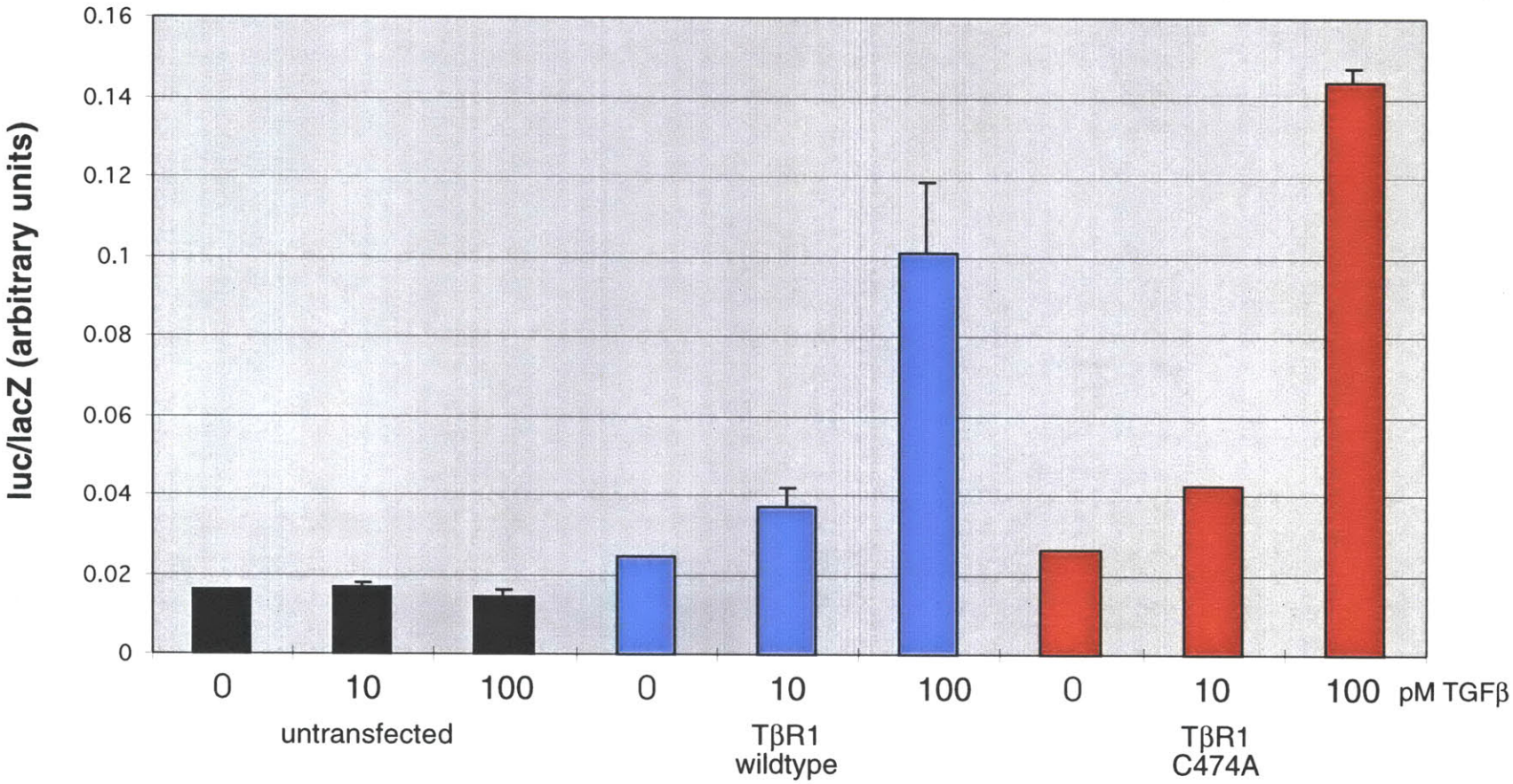

0.02
0.025
0.015
0.01
0.005
0
luc/lacZ (arbitrary units)
0 10 100 0 10 100 0 10 100 0 10 100 0 10 100 0 10 100 0 10 100 0 10 100pM TGFβ
untransfected
TβR1 wildtype
TβR1 C347,348A
TβR1 C415A
TβR1 C347,348,446A
TβR1 C347,348, 446,461A
TβR1 C461A

Figure 6C


luc/lacZ (arbitrary units)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
10
100
0
10
100
0
10
100
pM TGFβ
untransfected
TβR1
wildtype
TβR1
C474A

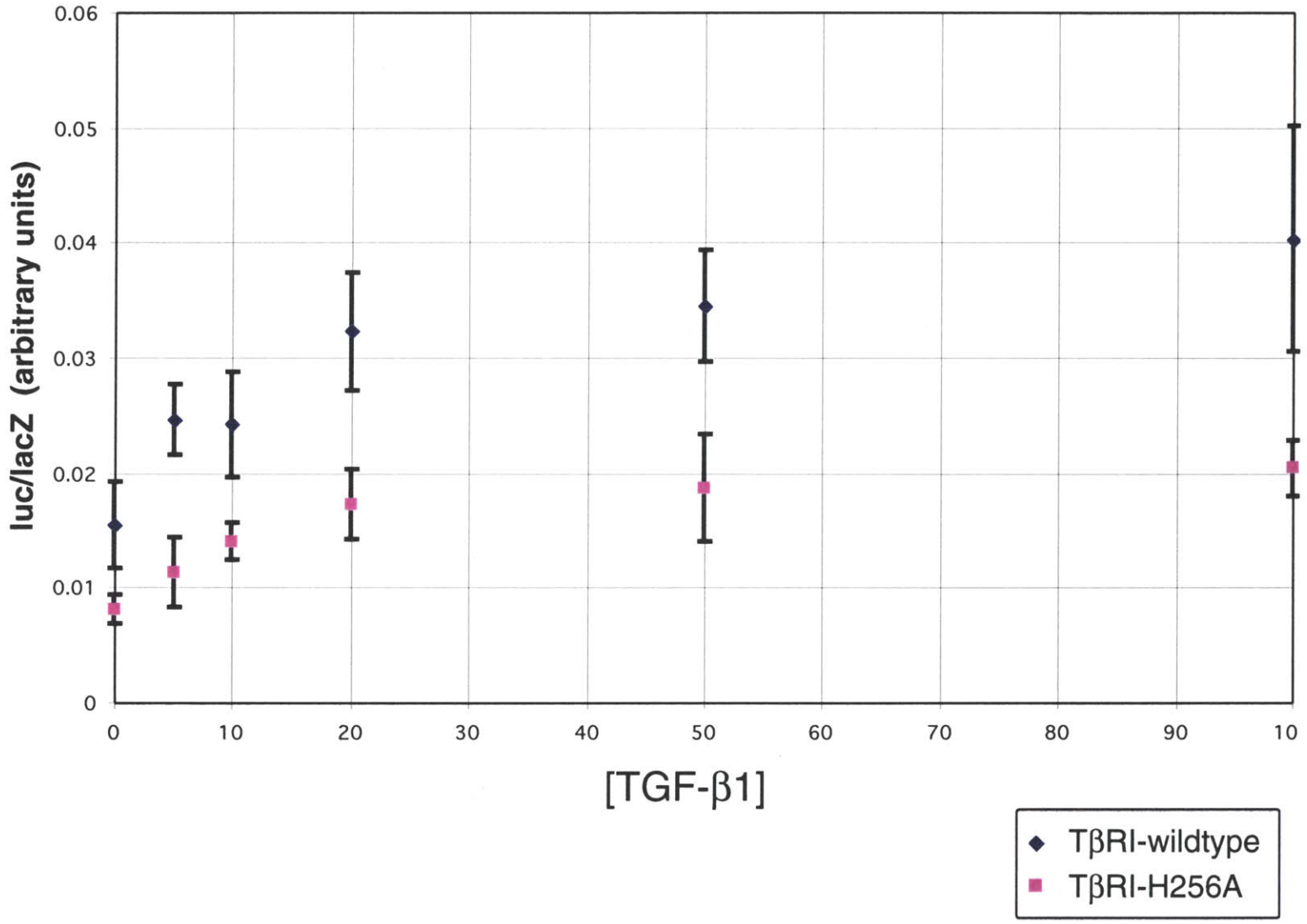
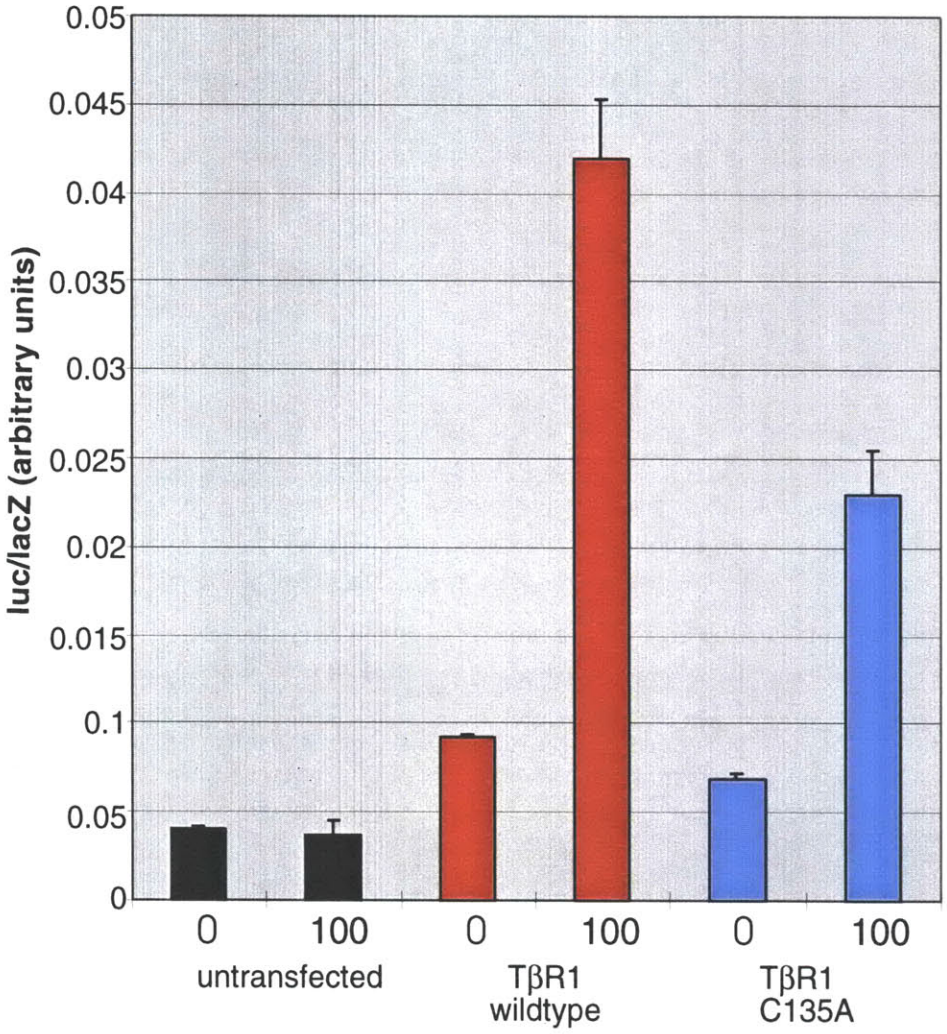

0.06
0.05
0.04
0.03
0.02
0.01
0
luc/lacZ (arbitrary units)
0
10
20
30
40
50
60
70
80
90
10
[TGF-β1]
TβRI-wildtype
TβRI-H256A

Figure 7


luc/lacZ (arbitrary units)
0.05
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.1
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100
0
100
untransfected
TβR1
wildtype
TβR1
C135A

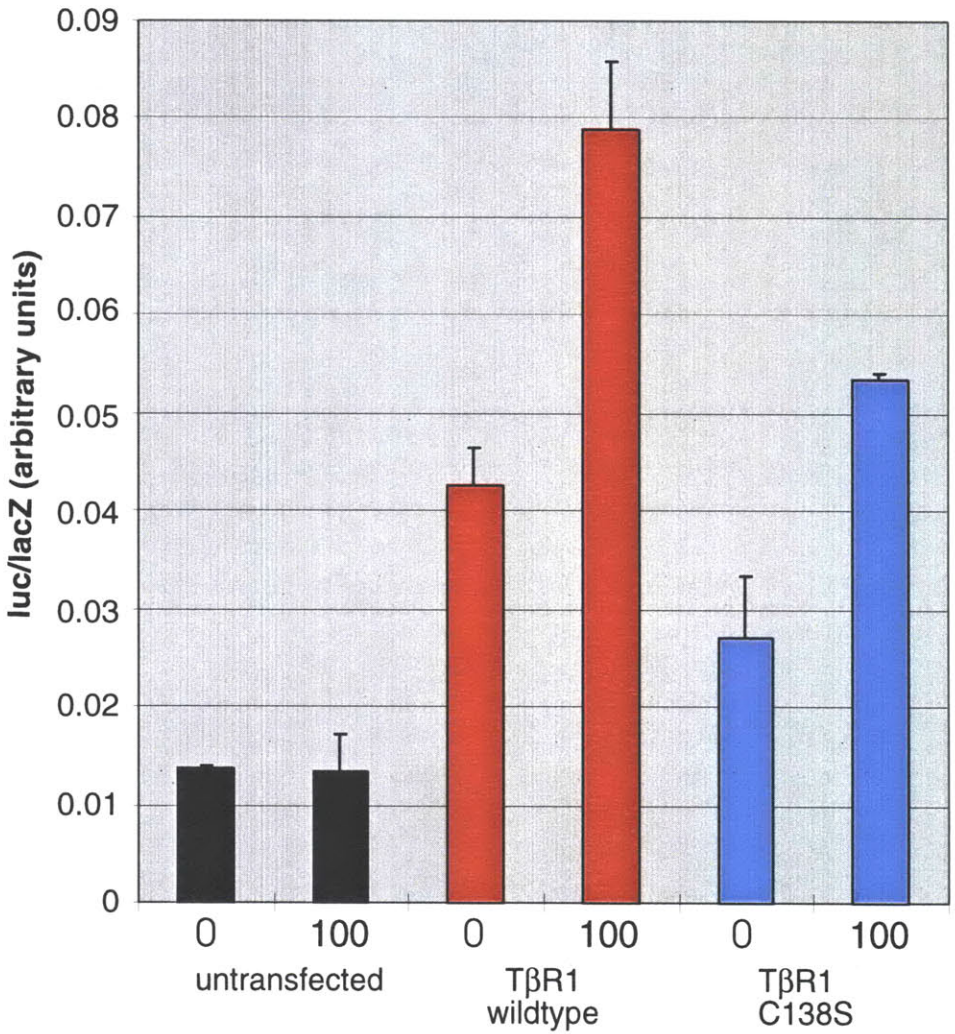

luc/lacZ (arbitrary units)
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
100
0
100
0
100
untransfected
TβR1
wildtype
TβR1
C138S

Figure 7 (continued)

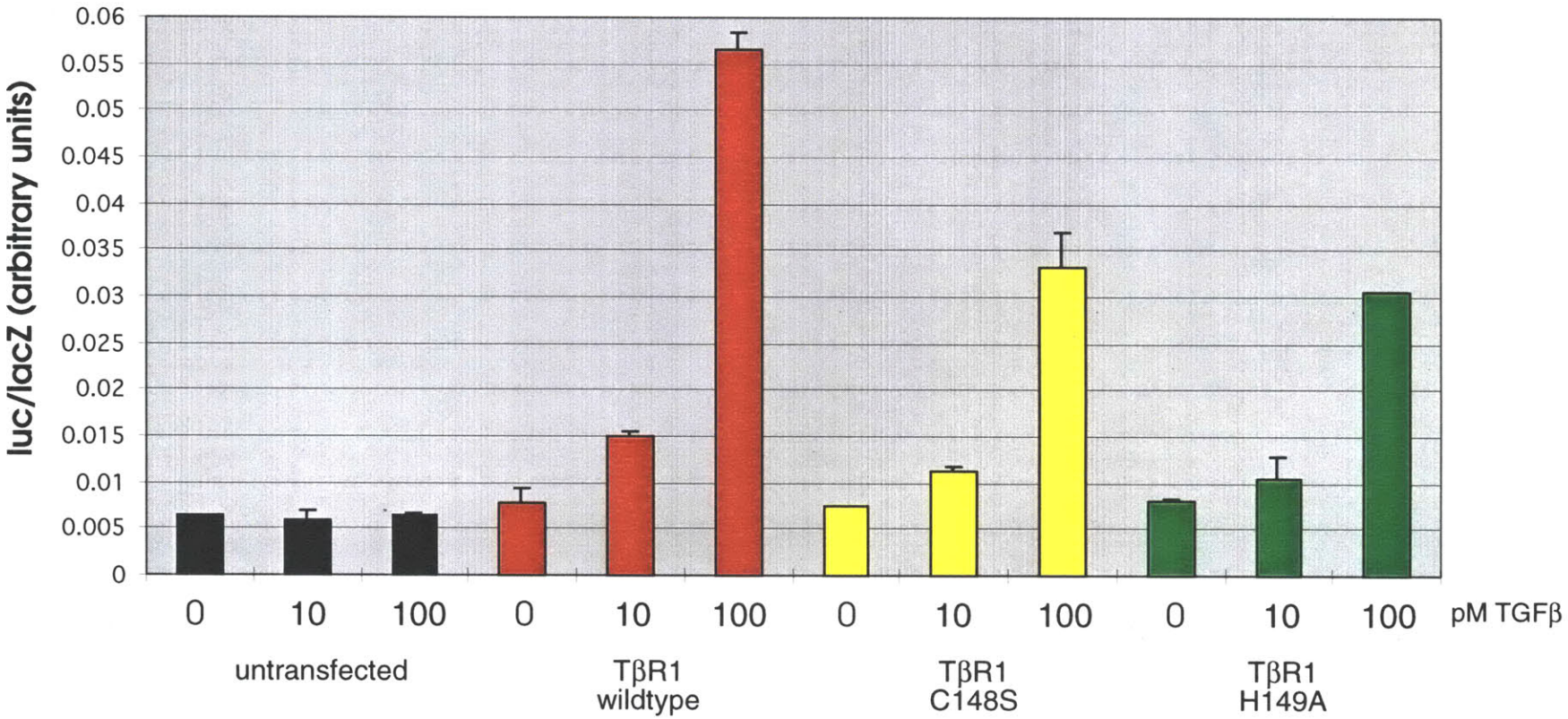
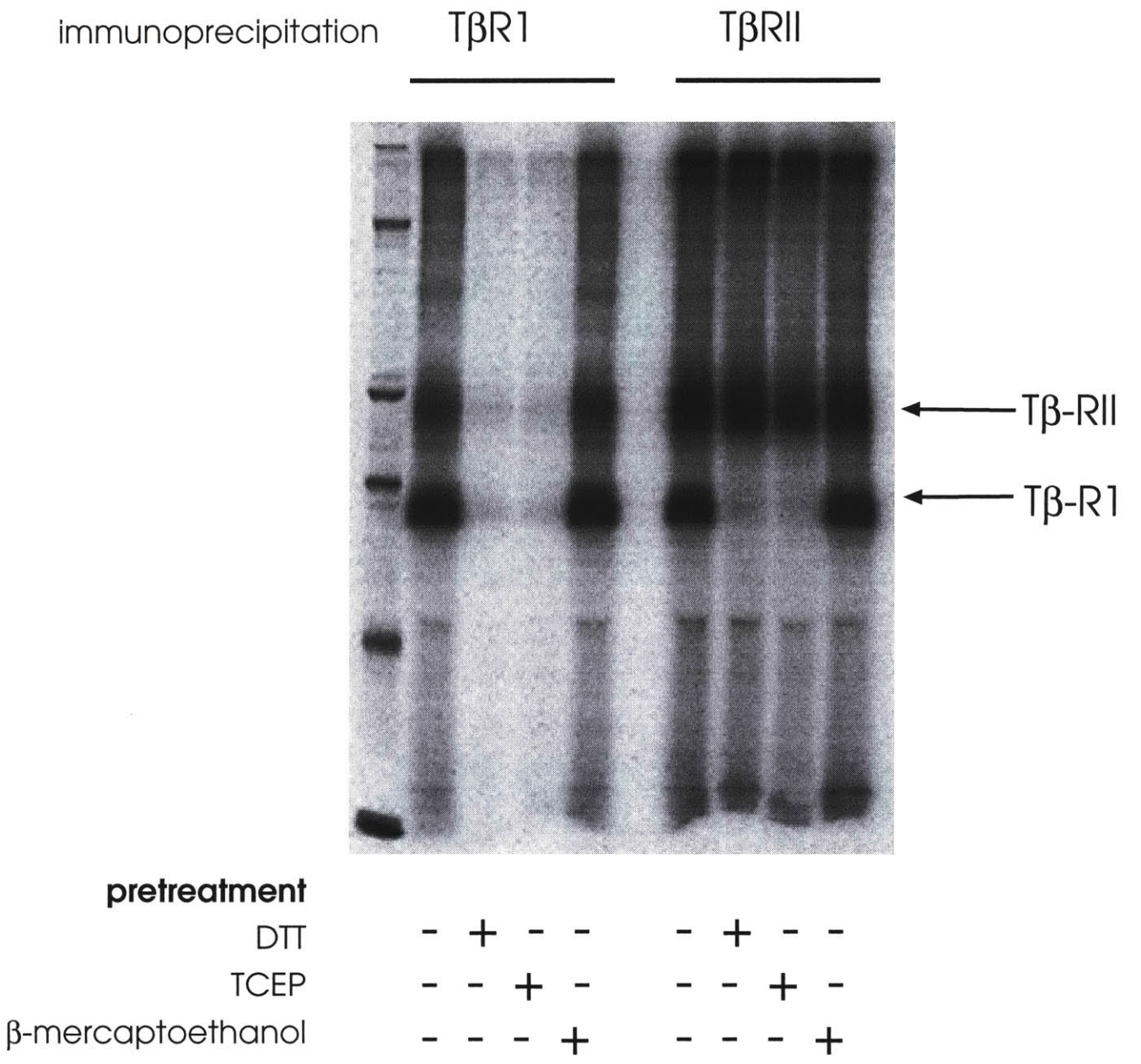
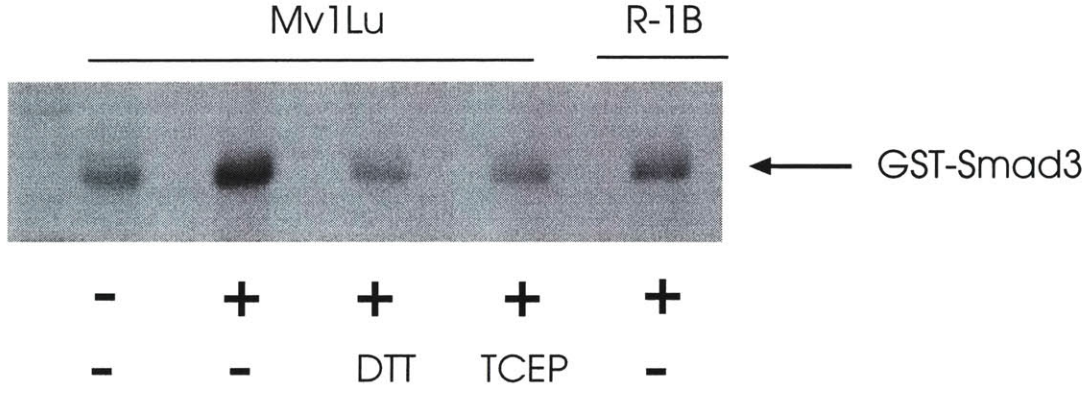


Figure 8

A.


immunoprecipitation
TβR1
TβRII
Tβ-RII
Tβ-R1
pretreatment
DTT - + - - - + - -
TCEP - - + - - - + -
β-mercaptoethanol - - - + - - - +

B.


Mv1Lu
R-1B
GST-Smad3
TGF-β - + + + +
pretreatment - - DTT TCEP -

Chapter 5

Concluding remarks

CD4/CD8 interactions with $p56^{lck}$

With respect to $p56^{lck}$, zinc confers to CD4 and CD8α the ability to specifically "fish out" one particular downstream effector among multiple Src kinases expressed within the intracellular milieu. As a result, $p56^{lck}$ is placed within immediate proximity of the TCR and other downstream substrates relevant to T-cell activation.

Possible lines of future research would center around structural characterization of the interface between CD4/CD8α and $p56^{lck}$. Crystallographic or NMR data would provide the first picture at atomic resolution of how zinc bridges two intracellular proteins together. Both Tat and CD4/CD8α possess zinc coordinating cysteines similar in spacing and possessing adjacent sequences similar to those of metallothionein. Therefore, whether the structure of the zinc-coordinating sites are at all similar may have implications to how such structures evolved to play a role in mediating protein/protein interactions. Furthermore, with respect to the Tat homodimer, zinc binding appears to exert structural changes limited to the cysteine rich region of each monomer and do not appear to affect global folding.[Frankel, 1988 #434; Frankel, 1988 #433] Biophysical measurements could determine whether this is also the case for CD4/CD8α and $p56^{lck}$. Given more structural information, it may also be possible to identify mutations that either strengthen or weaken the affinity of binding. Whether this could be correlated to any functional consequences in T-cell activation may be informative.

Previous studies have demonstrated that when fused to another protein, a 6 amino acid segment of the CD4 cytoplasmic tail is sufficient to confer association with $p56^{lck}$. (Shaw et al., 1990) Conversely, a 34 residue region at the N-terminal domain of $p56^{lck}$ was sufficient to specify interactions with CD4. (Shaw et al., 1989) From a protein engineering standpoint, inserting such sequences to proteins as tags to create *de novo* metal-linked dimers may be feasible.

Aside from structural issues, one of the most challenging problems may be determining how zinc is introduced into the CD4/$p56^{lck}$ dimer. Previous studies suggest that CD4/$p56^{lck}$ exit the endoplasmic reticulum as a dimer. An *in vitro* biochemical system in which one can examine the translation of both proteins in the presence of membranes should enable us to determine whether the presence of zinc and other factors are necessary to reconstitute metal-dependent association. Such studies may shed light towards the longstanding question of how zinc and other metals are incorporated into proteins.

TGF-β receptor complex formation

In many respects, details describing the sequence of events leading from ligand binding to final TGF-β receptor complex assembly and activation remain largely uncharacterized. I believe that the identification of domains in both receptors necessary for their mutual association would provide a starting point towards achieving this aim. The type II receptor cytoplasmic domain has already been demonstrated to be dispensable for ligand induced recruitment of the type I receptor. (Wieser et al., 1993) However, as described in Appendix I, a type I receptor truncated of the cytoplasmic domain is expressed on the cell surface, but cannot detectably bind and cross-link TGF-β1. Together with previous studies demonstrating that both types I and II receptor form homodimers in the ER (Gilboa et al., 1998), this would suggest that the type I cytoplasmic domain is not essential for receptor homodimerization, but rather plays a role in functional association with the ligand bound/type II receptor. Determination of whether the truncation in the presence of the full-length type I receptor can complex with the type II receptor would be informative.

It is possible that the TβRI cytoplasmic domain is involved in maintaining a specific orientation of the homodimer necessary for it to be recognized by and associate with the type II receptor. A relationship between the cytosolic domain of a receptor and extracellular ligand binding has been previously linked to the efficiency of erythropoietin (Epo) mediated signalling. (Livnah et al., 1996; Syed et al., 1998) In particular, Epo-mimetic ligands that alter the orientation of the Epo receptor's extracellular region exert profound effects upon the signalling potency of their corresponding intracellular domains. It is possible that the type I receptor cytoplasmic domain conversely alters the conformation of the transmembrane and/or extracellular domain allowing the receptor to assume an appropriate orientation for functional interactions with the ligand bound type II receptor. Again, structural data of each receptor's extracellular domain would enable us to make predictions regarding the orientation and conformation of individual components within the TGF-β receptor complex.

With respect to recruitment of the type I receptor to the ligand bound/type II receptor, we cannot absolutely rule out the possibility of zinc binding the type I receptor. Nevertheless forthcoming crystallographic data of the type I cytoplasmic domain should unambiguously establish the presence or absence of any putative metal binding sites.

Although $p56^{lck}$ binding to CD4/CD8α and association between the types I and II TGF-β receptors appear to reflect disparate phenomena, they do however exemplify an approach nature commonly brings to bear towards achieving highly specific and avid protein/protein interactions. Enlisted as molecular "third parties", zinc and TGF-β essentially serve to bridge and consequently stabilize complex formation between two proteins. As molecular details are elucidated from the study of these and other examples, "important principles (will) emerge that explain the need for complexity of the overall system and the unique requirements of individual proteins." (Yuk, M.H., Thesis 1995)

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