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12 April 1993; accepted 14 July 1993

Determination of Type I Receptor Specificity by the Type II Receptors for TGF-β or Activin

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Transforming growth factor–β (TGF-β) and activin signal primarily through interaction with type I and type II receptors, which are transmembrane serine-threonine kinases. Tsk 7L is a type I receptor for TGF-β and requires coexpression of the type II TGF-β receptor for ligand binding. Tsk 7L also specifically bound activin, when coexpressed with the type IIA activin receptor. Tsk 7L could associate with either type II receptor and the ligand binding specificity of Tsk 7L was conferred by the type II receptor. Tsk 7L can therefore act as type I receptor for both activin and TGF-β, and possibly other ligands.

Peptide growth and differentiation factors exert their effects by interacting with specific cell surface receptors. Several receptors, including the type II activin (*1*) and TGF-β (*2*) receptors and a type I receptor for TGF-β, Tsk 7L (*3*), have been shown to be transmembrane serine-threonine kinases. Within the TGF-β superfamily of growth and differentiation factors (*4*), the receptor-ligand interactions have been best studied for TGF-β and activin. In the case of TGF-β, the type I and type II receptors, defined on the basis of the size of the ^{125}I–TGF-β–cross-linked receptors on polyacrylamide gels, mediate most biological activities of TGF-β (*5*, 6). The type II receptor, possibly in conjunction with the type I receptor, is required for the antiproliferative effect of TGF-β, whereas the type I receptor mediates TGF-β–induced expression of genes involved in cell-matrix interactions (*7*). Cross-linking experiments similarly revealed type II and type I receptors for activin, corresponding in size to the respective TGF-β receptors (*1*, *8*). Ligand competition experiments suggested that activin and TGF-β bind to distinct and specific type II receptors (*1*, *2*). Overexpression of truncated type II receptors for TGF-β (*7*) and activin (*9*) inhibit ligand-induced responses in a dominant negative fashion, suggesting that oligomerization of receptor subunits is critical for function. A direct physical interaction of the TGF-β type I and type II receptors was proposed based on coimmunoprecipitation experiments (6).

After transfection, the type II TGF-β and activin receptors can bind their respective ligands (*1*, *2*). In contrast, Tsk 7L binds TGF-β only when coexpressed with the type II TGF-β receptor (*3*). To further evaluate the ligand specificity of Tsk 7L, we coexpressed Tsk 7L or its truncated form, which is missing most of the cytoplasmic domain (*3*), with the isogenic type IIA activin receptor (*1*) (Fig. 1A, left). In this case, ^{125}I-activin, but not ^{125}I–TGF-β (*10*), bound to both the type II activin receptor and Tsk 7L as shown by cross-linking. Coexpression of truncated Tsk 7L resulted in the appearance of a faster migrating band that was cross-linked to ^{125}I-activin as predicted. Tsk 7L did not bind ligand in the absence of the type II activin receptor. Coexpression of a control transmembrane protein such as TGF-α with the type II activin receptor resulted in binding of ^{125}I-activin only to the type II activin receptor. A chimeric type II receptor, containing the extracellular domain of the activin receptor and the transmembrane and cytoplasmic domains of the TGF-β receptor, by itself bound activin and not TGF-β and also conferred binding of activin and not TGF-β to the truncated Tsk 7L (*11*). Thus, the ligand binding specificity of the type I receptor is determined by the extracellular domain of the coexpressed type II receptor.

When cotransfected with the type II TGF-β receptor, Tsk 7L and its truncated form bound ^{125}I–TGF-β (Fig. 1A, right), as reported (*3*). Thus, Tsk 7L is a type I receptor that can specifically bind TGF-β or activin, depending on the nature of the cotransfected type II receptor. As reported for TGF-β binding to the type II TGF-β receptor (*3*), coexpression of Tsk 7L with the type II activin receptor inhibited binding of ^{125}I-activin to the type II receptor, an effect not readily seen with the truncated Tsk 7L. Most subsequent experiments will be illustrated only with the truncated Tsk 7L, but the truncated and full-size receptor had a similar ability to bind ligand (*10*).

Competition experiments showed that unlabeled activin, but not TGF-β, could displace ^{125}I-activin bound to Tsk 7L in the presence of the type II activin receptor (Fig. 1B, left). Similarly, only TGF-β, but not activin, could displace ^{125}I–TGF-β bound to the type I receptor in the presence of the type II TGF-β receptor (Fig. 1B, right). To compare the relative affinities of activin and TGF-β binding to the type I receptor, the radiolabeled ligands were cross-linked in the presence of increasing concentrations of unlabeled ligands. In the case of both activin and TGF-β, labeled ligand bound to the type I receptor and the type II receptor was displaced at approximately the same concentration (Fig. 1, C and D). The 50% displacement was at 160 to 320 pM for TGF-β, whereas for activin a similar displacement was observed at 800 to 1600 pM of ligand. Thus, the type I receptor bound ligand with a similar apparent affinity as the type II receptor, as observed for endogenous receptors (*5*).

Under certain conditions, the type I TGF-β receptor coimmunoprecipitates with the type II receptor, suggesting a physical interaction between both receptors (6). To evaluate whether the Tsk 7L type I receptor interacts with the type II TGF-β and activin receptors, we coexpressed the full-size or truncated type I receptor containing a COOH-terminal epitope tag with the type II TGF-β or activin receptor lacking this tag. After binding and cross-linking to ^{125}I-labeled ligand, a tag-specific antibody immunoprecipitated the type I receptor and coimmunoprecipitated the type II receptor, suggesting a physical interaction between these two receptor types (Fig. 2A). The intensity of the type II receptor band was frequently less than the type I receptor and was relatively lower in the case of the activin bound type II activin receptor (Fig. 2C, lane 2) than the TGF-β bound type II receptor (Fig. 2B, lane 2). This may be the result of a somewhat weaker interaction of Tsk 7L with the type II activin receptor than with the type II TGF-β recep-

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tor. However, no firm conclusions can be drawn about the stoichiometry of these interactions, because we do not know the relative cross-linking efficiency of the ligand to the type I and type II receptors.

On the basis of previous (*3*) and present data, we propose that Tsk 7L functions as a type I receptor for both TGF-β and activin, and that its ligand binding and specificity depends on the extracellular domain of the type II receptor. The binding is specific because even at high concentrations of unlabeled ligand, activin cannot compete with TGF-β binding and vice versa. The type I receptor may also bind other ligands of the TGF-β superfamily in the presence of the corresponding type II receptor, but this can as yet not be tested without cloned type II receptors and radiolabeled ligands. Our immunoprecipitations with an antibody to the epitope-tagged type I receptor and similar experiments using type II receptor antibodies (*6*) suggest a physical interaction between both receptors.

Thus, the ligand specificity of the type I receptor is defined by the type II receptor and a single receptor of the serine-threonine kinase receptor family can participate in two distinct receptor complexes. An analogy can be drawn between the type I receptor and gp130, a common signaling transmembrane component of several cytokine receptor complexes. Although different cytokines bind specific receptor complexes, signaling through gp130 leads to similar activities. However, gp130 does not bind ligand and a separate receptor in the complex determines ligand specificity (*12*). We have proposed that signaling through the type I receptor, but not the type II receptor, might regulate TGF-β–induced changes in extracellular matrix deposition (*10*). If the analogy with gp130 is valid, the use of a common type I receptor in different receptor complexes might explain the similarity in effects of TGF-β and activin on extracellular matrix formation. Another similarity is apparent with the heterodimeric integrin receptors, which consist of α and β chains in various combinations. Whereas both chains interact with the ligand, the ligand specificity of the heterodimer is determined by the combination of both transmembrane polypeptides (*13*). The use of a single receptor in different receptor combinations underscores the complexity of the biology and the ligand-receptor interactions of TGF-β–related factors. The receptor binding and biological activities of these factors may thus not only be determined by the abundance of specific receptors at the cell surface but also by their combinatorial interactions. This complexity may also resemble the interactions between transcription factors, whereby the net effect on transcription results from the integrated qualitative and quantitative consequences of these interactions (*14*).

Note added in proof: Tsuchida *et al.* similarly showed dual binding of activin or TGF-β to Tsk 7L and inhibition of type II receptor binding (*22*).

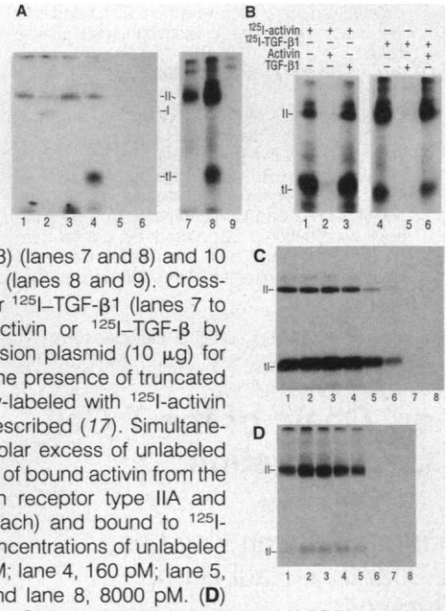

Fig. 1. Binding of ^{125}I-activin or ^{125}I–TGF-β to 293 cells transfected with Tsk 7L expression plasmids in the presence of type II activin or TGF-β receptor expression plasmids. (**A**) In the left panel, cells were transfected (*15*) with expression plasmid (10 μg) for the activin receptor IIA (*1*, *3*) (lanes 1 to 4), for Tsk 7L (*3*) (lanes 2 and 6), or for the truncated form of Tsk 7L (*3*) (lanes 4 and 5), or for TGF-α (*16*) (lane 3). In the right panel, the cells were transfected with 10 μg of type II TGF-β receptor expression plasmid (2, 3) (lanes 7 and 8) and 10 μg of the truncated Tsk 7L expression plasmid (lanes 8 and 9). Cross-linking was done with ^{125}I-activin (lanes 1 to 6) or ^{125}I–TGF-β1 (lanes 7 to 9). (**B**) Displacement of receptor-bound ^{125}I-activin or ^{125}I–TGF-β by unlabeled ligands. Cells transfected with expression plasmid (10 μg) for activin receptor IIA or type II TGF-β receptor in the presence of truncated Tsk 7L expression plasmid (10 μg) were affinity-labeled with ^{125}I-activin (lanes 1 to 3) or ^{125}I–TGF-β1 (lanes 4 to 6) as described (*17*). Simultaneously with the radiolabeled ligand, a 400-fold molar excess of unlabeled ligand was added as indicated. (**C**) Displacement of bound activin from the receptors. Cells were cotransfected with activin receptor type IIA and truncated tsk 7L expression plasmids (10 μg each) and bound to ^{125}I-activin (150 pM) in the presence of increasing concentrations of unlabeled activin: lane 1, 0 pM; lane 2, 16 pM; lane 3, 80 pM; lane 4, 160 pM; lane 5, 800 pM; lane 6, 1600 pM; lane 7, 3200 pM; and lane 8, 8000 pM. (**D**) Displacement of bound TGF-β1 from the receptors. Cells cotransfected with type II TGF-β receptor and truncated type I receptor expression plasmids (10 μg each) were incubated with ^{125}I–TGF-β1 (100 pM) in the presence of increasing concentrations of unlabeled TGF-β1: lane 1, 0 pM; lane 2, 16 pM; lane 3, 32 pM; lane 4, 80 pM; lane 5, 160 pM; lane 6, 320 pM; lane 7, 800 pM; and lane 8, 1600 pM. Cross-linking was done using ^{125}I-activin or ^{125}I–TGF-β1 as described (*17*) and samples were analyzed by denaturing gel electrophoresis. Radiolabeled ligands were prepared as described (*18*). I and II denote type I and II receptors; tI marks the truncated type I receptor.

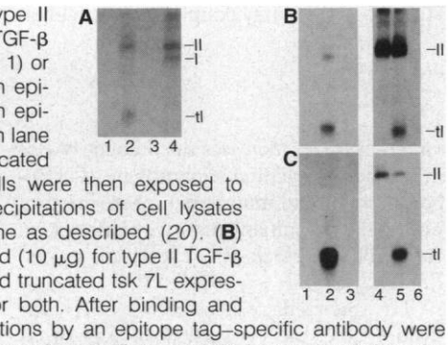

Fig. 2. Coimmunoprecipitation of type I and type II receptors. (**A**) Cells were transfected with type II TGF-β receptor expression plasmid (10 μg) alone (lane 1) or together with expression plasmid (10 μg) for an epitope-tagged truncated Tsk 7L (*3*) (lane 2) or an epitope-tagged full-size Tsk 7L (*19*) (lane 4). Cells in lane 3 were transfected with epitope-tagged truncated tsk 7L expression plasmid (10 μg) alone. Cells were then exposed to ^{125}I–TGF-β1 and cross-linked (*17*). Immunoprecipitations of cell lysates using epitope tag–specific antibodies were done as described (*20*). (**B**) Cells were transfected with an expression plasmid (10 μg) for type II TGF-β receptor (lanes 1, 2, 4, and 5), an epitope-tagged truncated tsk 7L expression plasmid (10 μg) (lanes 2, 3, 5, and 6), or both. After binding and cross-linking with ^{125}I–TGF-β1, immunoprecipitations by an epitope tag–specific antibody were done (*20*) and samples were analyzed by gel electrophoresis (lanes 1 to 3). Lanes 4 to 6 show the pattern without immunoprecipitation. (**C**) Cells were transfected with an expression plasmid (10 μg) for type IIA activin receptor (lanes 1, 2, 4, and 5), an epitope-tagged truncated tsk 7L expression plasmid (10 μg) (lanes 2, 3, 5, and 6), or both. After binding and cross-linking with ^{125}I-activin, immunoprecipitations by an epitope tag–specific antibody were done (*20*) and samples were analyzed by gel electrophoresis (lanes 1 to 3). Lanes 4 to 6 show the pattern without immunoprecipitation. I and II denote type I and II receptors; tI marks the truncated type I receptor.

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mains of the type II TGF-β receptor was constructed by a polymerase chain reaction (PCR)–based strategy. The extracellular domain of the type II activin receptor was amplified with primers to introduce a Bam HI site at the 5′ end and a Spe I site at the 3′ end. We generated the fragment encoding the transmembrane and cytoplasmic domains of the type II TGF-β receptor as a Spe I–Xba I fragment using a PCR reaction. Both fragments together were subcloned as a Bam HI–Xba I fragment into the vector pRK7 (*21*). The sequences of the primers used were as follows: 5′ activin receptor primer, 5′-GCTAGAATTCGGGAAAATGGGAGCTGCTGCA-3′; 3′ activin receptor primer, 5′-GGCGGACTAGTAACAAGGGTGGCTTCGGTGTAACAGG-3′; 5′ TGF-β receptor primer, 5′-TTGTTACTAGTCATATTTCAAGTG-3′; 3′ TGF-β receptor primer, 5′-GGCTCTAGAGCTATTTGGTAGTGTTTAG-3′.

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20. Immunoprecipitations were carried out as follows: After cross-linking and lysis in the presence of protease inhibitors, cell extracts were solubilized in Triton X-100, clarified by centrifugation, and were diluted tenfold in 0.5× phosphate-buffered saline with 0.4% gelatin (Bio-Rad) and 0.2% of both Tween 20 and Thimerosal (Serva). After incubating with 5 to 15 μg of specific antibody for 6 to 12 hours at 4°C, 10 mg of protein A–Sepharose CL-4B beads (Pharmacia), preswollen in the same buffer, were added and incubation continued for another 4 to 8 hours. Beads were harvested by centrifugation, washed extensively in the immunoprecipitation buffer, and radiolabeled complexes were then dissociated from the beads by boiling in electrophoresis sample buffer.
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4 August 1993; accepted 11 October 1993

Interaction of Shc with the ζ Chain of the T Cell Receptor upon T Cell Activation

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The *shc* oncogene product is tyrosine-phosphorylated by Src family kinases and after its phosphorylation interacts with the adapter protein Grb2 (growth factor receptor–bound protein 2). In turn, Grb2 interacts with the guanine nucleotide exchange factor for Ras, mSOS. Because several Src family kinases participate in T cell activation and Shc functions upstream of Ras, the role of Shc in T cell signaling was examined. Shc was phosphorylated on tyrosine after activation through the T cell receptor (TCR), and subsequently interacted with Grb2 and mSOS. The Src homology region 2 (SH2) domain of Shc directly interacted with the tyrosine-phosphorylated ζ chain of the TCR. Thus, Shc may couple TCR activation to the Ras signaling pathway.

The activation of Ras proteins appears to be a crucial early event in the intracellular signaling pathways initiated by a number of receptor tyrosine kinases (RTKs) (*1*). Activation of RTKs increases the amount of Ras in the active guanine triphosphate (GTP)–bound state, and mutational inactivation of *ras* genes or dominant negative inhibitors of Ras proteins block the effects of several RTKs (*2*, *3*).

The intermediary steps between RTK activation and the conversion of Ras to its active GTP-bound state have been delineated (*4*). Grb2 is an adapter protein that lacks a catalytic domain and is composed of one SH2 domain and two SH3 domains (*5*). The SH2 domains bind to specific phosphopeptide sequences, whereas SH3 domains bind to proline-rich sequences (*6*, *7*). The protein Grb2 binds to the autophosphorylated RTK through its SH2 domain, and it simultaneously associates through its SH3 domains with mSOS, a guanine nucleotide exchange protein that activates Ras by inducing exchange of guanine diphosphate for GTP on Ras (*4*, *8*). Thus, in RTK signaling, Ras (which is normally membrane-bound) is activated when Grb2 shuttles mSOS to the membrane.

Receptors that are not tyrosine kinases but signal through activation of associated tyrosine kinases also induce Ras activation. Another protein that interacts with Grb2 is the *shc* oncogene product. Shc is also an adapter protein that is widely expressed in all tissues and contains an SH2 domain and a collagen-like domain but no obvious catalytic domain (*9*). This protein is phosphorylated on tyrosine in cells transformed by nonreceptor tyrosine kinases such as v-Src and v-Fps (*9*). Through its SH2 domain Grb2 associates with tyrosine-phosphorylated Shc (*10*). Like Grb2, Shc appears to function upstream of Ras because the differentiation of PC12 pheochromocytoma cells induced by overexpression of Shc is blocked by the dominant inhibitory N17Ras mutant (*10*). Because several nonreceptor Src family tyrosine kinases (namely Lck, Fyn, and ZAP-70) participate in T cell signaling, and TCR stimulation leads to Ras activation (*11*, *12*), we speculated that Shc might couple the TCR-CD3 complex to the Ras activation pathway.

To evaluate the role of Shc in T cell activation, we determined whether Shc is tyrosine-phosphorylated during T cell activation. A murine T cell hybridoma By155.16 (By) (*13*), expressing a Vβ8$^+$ TCR and CD4, was activated by antibody-mediated cross-linking of the TCR, either alone or with the CD4 coreceptor. Shc was immunoprecipitated with antibodies to Shc (anti-Shc) from activated T cell lysates and immunoblotted for phosphotyrosine. Marked tyrosine phosphorylation of both the 48- and 52-kD isoforms of the Shc protein was detected after the cross-linking of TCR and CD4 (TCR×CD4) and was only weakly detected after cross-linking TCR alone or CD4 alone (Fig. 1A). Our analysis of the time course of phosphorylation indicated that Shc phosphorylation occurred early, as it could be detected within 15 s (Fig. 1B). The Shc phosphorylation peaked at about 5 min and its phosphorylation started to diminish by 15 min. Similarly, immunoprecipitation of proteins from By cell lysates after TCR activation with antibody to phosphotyrosine (anti-phosphotyrosine) and immunoblotting for Shc showed activation-dependent tyrosine phosphorylation of Shc (Fig. 1, C and D). Anti-Shc also immunoprecipitated an unidentified 140-kD tyrosine-phosphorylated protein.

We assessed whether Shc would interact

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