The Types II and III Transforming Growth Factor-β Receptors Form Homo-Oligomers

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Abstract. Affinity-labeling experiments have detected hetero-oligomers of the types I, II, and III transforming growth factor β (TGF-β) receptors which mediate intracellular signaling by TGF-β, but the oligomeric state of the individual receptor types remains unknown. Here we use two types of experiments to show that a major portion of the receptor types II and III forms homo-oligomers both in the absence and presence of TGF-β. Both experiments used COS-7 cells co-transfected with combinations of these receptors carrying different epitope tags at their extracellular termini. In immunoprecipitation experiments, radiolabeled TGF-β was bound and cross-linked to cells co-expressing two differently tagged type II receptors. Sequential immunoprecipitations using anti-epitope monoclonal antibodies showed that type II TGF-β receptors form homo-oligomers. In cells co-expressing epitope-tagged types II and III receptors, a low level of co-precipitation of the ligand-labeled receptors was observed, indicating that some hetero-oligomers of the types II and III receptors exist in the presence of ligand.

Antibody-mediated cross-linking studies based on double-labeling immunofluorescence explored co-patching of the receptors at the cell surface on live cells. In cells co-expressing two differently tagged type II receptors or two differently tagged type III receptors, forcing one receptor into micropatches by IgG induced co-patching of the receptor carrying the other tag, labeled by noncross-linking monovalent Fab'. These studies showed that homo-oligomers of the types II and III receptors exist on the cell surface in the absence or presence of TGF-β1 or -β2. In cells co-expressing types II and III receptors, the amount of heterocomplexes at the cell surface was too low to be detected in the immunofluorescence co-patching experiments, confirming that hetero-oligomers of the types II and III receptors are minor and probably transient species.

THE receptors for transforming growth factor β (TGF-β)[1] regulate important cellular functions, among which are cell proliferation, differentiation, and motility (Roberts and Sporn, 1990; Massagué, 1990; Moses et

1. *Abbreviations used in this paper:* HA, influenza hemagglutinin; TGF-β, transforming growth factor β; TGF-βRI, TGF-βRII, and TGF-βRIII, TGF-β receptor types I, II and III, respectively; DSS, disuccinimidyl suberate; α-myc, mouse monoclonal antibody which recognizes a specific c-myc sequence; α-HA, mouse monoclonal antibody which recognizes an epitope of the influenza hemagglutinin (HA) protein; FITC-IgG GαRIgG, fluorescein-coupled goat IgG anti-rabbit IgG; GαMIgG, goat IgG anti-mouse IgG; Cy3-Fab' GαMIgG, goat Fab' anti-mouse IgG conjugated to the cyanine dye Cy3.18.

al., 1990; Lin and Lodish, 1993). Chemical cross-linking to ^{125}I-TGF-β reveals numerous proteins that bind the ligand with high affinity, including cell-surface receptors as well as soluble and extracellular matrix proteins (Massagué, 1992; Lin and Lodish, 1993). Among the cell-surface receptors, the most widely distributed are the types I (55 kD), II (80 kD), and III (280 kD) TGF-β receptors (Cheifetz et al., 1987; Segarini et al., 1989; Massagué, 1992; Lin and Lodish, 1993). The TGF-β receptors type I (TGF-βRI) and type II (TGF-βRII) are thought to mediate the multiple effects of TGF-β, while type III receptors (TGF-βRIII) are involved in presentation of the ligand to the signaling receptors (reviewed in Massagué, 1992; Lin and Lodish, 1993).

TGF-βRIII is a transmembrane proteoglycan with a short, highly conserved, cytoplasmic domain that has no apparent signaling motif (Wang et al., 1991; López-Casillas et al., 1991; Morén et al., 1992). In most cell types TGF-βRIII is the most abundant TGF-β receptor subtype (Segarini et al., 1989; Massagué, 1992). TGF-βRIII and its soluble secreted ectodomain (Andres et al., 1989) may act as "reservoir" molecules for ligand presentation to or retention from the sig-

naling TGF-βRII and TGF-βRI receptors (Andres et al., 1989; Massagué, 1992; Lin and Lodish, 1993). Expression of TGF-βRIII enhances binding of TGF-β ligands, particularly TGF-β2, to TGF-βRII and thus some regulatory interactions exist between the types II and III TGF-β receptors (López-Casillas et al., 1993; Moustakas et al., 1993). Immunoprecipitation experiments provided evidence for cell-surface complexes containing both types II and III TGF-β receptors. That the TGF-βRIII primarily has a modulatory role is in accord with its structural features and is also consistent with the observation that TGF-βRIII is absent in several cells that are responsive to TGF-β (Ohta et al., 1987; Segarini et al., 1989).

TGF-βRII is a transmembrane serine/threonine kinase (Lin et al., 1992; Wrana et al., 1992; Tsuchida et al., 1993a). It belongs to the family of receptor serine/threonine kinases that includes the mammalian, Xenopus and Drosophila activin type II receptors (Mathews and Vale, 1991; Attisano et al., 1992; Legerski et al., 1992; Mathews and Vale, 1992; Nishimatsu et al., 1992; Shinozaki et al., 1992; Childs et al., 1993) and the daf-1 and daf-4 gene products of Caenorhabditis elegans. The latter two receptors control development of the dauer larva (Georgi et al., 1990); the daf-4 gene product can act as a type II receptor for bone morphogenetic protein (BMP) (Estevez et al., 1993). Recently, specific cDNA clones were identified as encoding type I TGF-β receptors (Attisano et al., 1993; Bassing et al., 1993; Ebner et al., 1993a,b; Franzén et al., 1993) and the closely related type I activin receptors (Attisano et al., 1993; Ebner et al., 1993b; Matsuzaki et al., 1993; Tsuchida et al., 1993b). These integral membrane proteins are shorter than the TGF-βRII or activin type II receptors, but possess a serine/threonine kinase domain and share conserved sequences with the type II receptors in both their extra- and intracellular domains. Thus, types II and I receptors belong to homologous branches of the receptor serine/threonine kinase family. Several members of this family with presently unidentified ligand specificity have recently been reported (He et al., 1993; ten Dijke et al., 1993).

Studies using chemically mutated mink lung epithelial cells (Boyd and Massagué, 1989; Laiho et al., 1990, 1991) and tumor cell lines (Kimchi et al., 1988; Segarini et al., 1989; Siepl et al., 1991; Geiser et al., 1992) that have lost the growth-inhibitory response to TGF-β and concomitantly lost cell-surface receptors TGF-βRI, TGF-βRII, or both, have led to the notion that both TGF-βRI and TGF-βRII are required for TGF-β-mediated signaling. Functional interactions between TGF-βRII and TGF-βRI are suggested by studies showing that TGF-βRII requires TGF-βRI for signaling, while binding of TGF-β1 to TGF-βRI can be detected only when this receptor is co-expressed with TGF-βRII (Wrana et al., 1992; Attisano et al., 1993; Bassing et al., 1993; Ebner et al., 1993a, b; Franzén et al., 1993; Inagaki et al., 1993; Wieser et al., 1993). Moreover, some heteromeric complexes of the types II and I receptors exist, since some TGF-βRI, affinity labeled with ^{125}I-TGF-β1, can be co-immunoprecipitated with TGF-βRII.

The important implications of functional interactions among cell-surface TGF-β receptors emphasize the need for a thorough investigation of their oligomeric structure, which is still largely unknown. In the current work, we investigated the formation of homo- and hetero-oligomers among TGF-

βRII and TGF-βRIII at the cell surface under native conditions. Using cells that were co-transfected with various combinations of epitope tagged and/or wild-type TGF-βRII and TGF-βRIII, we performed double-labeling immunofluorescence experiments based on the forced patching of one TGF-β receptor and following the distribution of the other co-expressed receptor. Complemented with immunoprecipitation studies, our data show that both the TGF-βRII and TGF-βRIII are present at the cell surface as homo-oligomers (possibly dimers in the case of the type II receptors) even prior to the addition of ligand. Furthermore, we show that heteromeric TGF-βRII/TGF-βRIII complexes which form following ligand binding involve only a minor fraction of the cell-surface receptor population.

Materials and Methods

Materials

Expression Vectors for Epitope-tagged TGF-β Receptors

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of the TGF-βRII clone). The 3.4-kb piece from the wild-type, untagged receptor encoding most of the coding region of the cDNA was ligated with the 4.2-kb piece from a preparation containing HA- or c-myc-tagged NH$_2$-terminal TGF-βRII sequences and the cloning vector pcDNA-I. The resulting constructs contained as insert all of the TGF-βRII coding region, most of which was derived from the wild-type, untagged receptor clone, and the NH$_2$-terminal region (including the tag) derived from the tagged receptor clone. The coding regions derived from the tagged cDNAs (nucleotides 1–206 in the TGF-βRII sequence; Lin et al., 1992) were verified by DNA sequencing.

The rat TGF-βRIII cDNA (R3-OFF clone; Wang et al., 1991) was inserted into the SV-40 expression vector pcDNA-I/neo (Invitrogen) via the Hind III polylinker site. Mutagenesis was carried out as described above for TGF-βRII, except that the epitope-tag sequences were flanked by sequences corresponding to nucleotides 61–75 (on the 5' end) and 76–90 (3' end) of the rat TGF-βRIII cDNA sequence (counting the A of the methionine codon of the cDNA as 1, Wang et al., 1991). This yielded in-frame insertions of the HA or c-myc epitope-tags six bases downstream of the putative signal peptide sequence (Wang et al., 1991). Double-stranded preparations of pcDNA-I/neo with wild-type, untagged rat TGF-βRIII or with epitope-tagged rat TGF-βRIII inserts were digested by XbaI, which cut the plasmids at the polylinker, and BstEII, which cut at position 255 of the cDNA's coding sequence. The 5.5-kb fragment from the wild-type, untagged receptor encoding most of the coding region and 3' untranslated sequences of the cDNA was ligated to the 7.6-kb fragment from a preparation containing HA- or c-myc-tagged NH$_2$-terminal TGF-βRIII sequences and the cloning vector pcDNA-I/neo. The resulting plasmids contained inserts with all of the TGF-βRIII coding region, most of which was derived from the wild-type, untagged receptor clone but with nucleotides 1–255 originating from the tagged receptor cDNAs. This region was verified for each tagged receptor by DNA sequencing.

In the case of human TGF-βRIII, a cDNA clone from HepG2 cells, identical in sequence to the one cloned by Morén et al. (1992), was inserted into pcDNA-I/neo via the HindIII and NotI polylinker sites. Site-directed mutagenesis was performed using oligonucleotides encoding the epitope-tag sequences flanked by the sequences corresponding to nucleotides 672–686 (on the 5' end) and 687–701 (3' end) of the human TGF-βRIII sequence (counting as 1 the first nucleotide of the published sequence; Morén et al., 1992). The epitope-tags were inserted in-frame at the equivalent position to that in the rat type III receptor (between Pro[22] and Glu[23] in the human TGF-βRIII). The entire coding regions of the epitope-tagged TGF-βRIII cDNAs were sequenced, to verify that they included the tags and did not contain any other mutations.

COS-7 Cell Transfections

COS-7 cells (CRL 1651; American Type Culture Collection, Rockville, MD) were grown in DME supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO BRL, Gaithersburg, MD). They were transiently transfected by the DEAE-dextran method (Seed and Aruffo, 1987) using pcDNA-I or pcDNA-I/neo containing the TGF-βRII or III cDNAs (tagged or untagged). Immunofluorescence and ligand binding and/or immunoprecipitation assays were performed 44–48 h after transfection.

Receptor Cross-linking

Binding and cross-linking of 100 pM ^{125}I-TGF-β1 to cells grown on 6-well trays or 100 mm dishes (Corning Inc., Corning, NY) was as described (Moustakas et al., 1993). Cross-linked proteins were resolved by 5–10% linear gradient SDS-PAGE under reducing conditions and exposed to Kodak XAR film at −70°C.

Receptor Immunoprecipitation

IgG fractions of 9E10 (α-myc) and 12CA5 (α-HA) monoclonal antibodies were described above. A rabbit pre-immune serum was also used as a nonspecific control for immunoprecipitations. Cell-surface TGF-β receptors were labeled with 100 pM ^{125}I-TGF-β1 as described above. 5 × 10^6 cells were dissolved in 1 ml of ice-cold immunoprecipitation (IP) buffer (PBS containing 1% Triton-X-100, 1 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride). Extracts were precleared by incubation with 50 μg/ml protein A-Sepharose (Sigma Chemical Co., St. Louis, MO) or protein G-Sepharose (Pharmacia LKB). Then 5–10 μg/ml of the appropriate IgG was added and reactions were incubated at 4°C for 4–12 h. This was followed by addition of 50 μg/ml protein A-Sepharose (for α-HA and preimmune serum) or 50 μg/ml protein G-Sepharose (for α-myc) to precipitate the immunocomplexes. The pellets were thoroughly washed in IP buffer. For sequential immunoprecipitations of single or double epitope-tagged receptors, the immunocomplexes from the first immunoprecipitation reactions were dissolved in a small amount of a solution containing 1% SDS, 50 mM DTT, and 10% β-mercaptoethanol, boiled for 5 min, and mixed thoroughly. The eluted proteins were diluted in IP buffer such that the final SDS concentration was less than 0.1%. Monoclonal IgG's were added at the above-noted concentrations, and reactions were incubated at 4°C for 12 h. Finally, immunocomplexes were precipitated with protein A- or protein G-Sepharose as above. These secondary immunopellets were thoroughly washed with IP buffer, dissolved in Laemmli loading buffer, and subjected to 5–10% linear gradient SDS-PAGE prior to autoradiography.

Immunofluorescence

44–48 h after transfection, COS-7 cells were washed twice with serum-free DME, and incubated 30 min at 37°C to allow digestion of serum-free DME, and incubated 30 min at 37°C to allow digestion of serum-derived ligands. After washing twice with cold HBSS (GIBCO BRL) supplemented with 20 mM Hepes and 1% fatty acid-free BSA (Sigma Chemical Co.) (HBSS/Hepes/BSA, pH 7.2), the cells were incubated in the same buffer (1 h, 4°C) with normal goat IgG (200 μg/ml) to block nonspecific binding. This was followed by successive incubations (45 min, 4°C, with three washes between incubations) with anti-tag or α-IIN Fab' or IgG (alone or in various combinations), followed by various combinations of goat anti-rabbit Fab' or IgG (unlabeled, or labeled with FITC or with the Cy3 cyanine dye). Cy3, whose excitation and emission wavelengths resemble tetramethylrhodamine, was preferred over tetramethylrhodamine due to its higher fluorescence yield, especially important in Fab' labeling. The details of each successive labeling protocol depend on the specific experiment and are given in the figure legends. The samples were washed three times with cold HBSS/Hepes, fixed successively in methanol (−20°C, 5 min) and acetone (−20°C, 2 min), and mounted with Slowfade (Molecular Probes, Eugene, OR) mounting solution. Fluorescence micrographs were taken using rhodamine (for Cy3 fluorescence) or FITC (with a narrow band-pass filter) conditions with a 63× objective, immersed in oil, on a Zeiss Axiophot fluorescence microscope.

Results

To date, only heteromeric complexes of the types I and II, and the types II and III, TGF-β receptors have been detected. In these studies, radiolabeled TGF-β was bound and cross-linked to cell–surface receptors, followed by detergent solubilization, immunoprecipitation with an antireceptor antibody, and gel electrophoresis. While this approach is highly sensitive and can detect low levels of heterocomplexes (Wrana et al., 1992; Inagaki, et al., 1993; López-Casillas et al., 1993; Moustakas et al., 1993), it is not applicable to the study of homo-oligomers due to the inability to distinguish between identical subunits. Furthermore, detergent solubilization is potentially disruptive for certain noncovalent protein complexes.

To develop a system and method capable of detecting both homo- and hetero-oligomeric complexes containing TGF-βRII and TGF-BRIII under native conditions, we have tagged each receptor with two different epitopes. The epitope tags were introduced at the NH$_2$ termini of the extracellular domains, to enable double-labeling immunofluorescence studies on live cells (Henis et al., 1990) as well as immunoprecipitation studies on affinity-labeled receptors. These types of experiments are performed best at a high receptor density at the cell surface, particularly because this enables good visualization by immunofluorescence. For this reason, transient expression in COS-7 cells was chosen. The human TGF-βRII cDNA (Lin et al., 1992) in the SV-40 expression vector pcDNA-I was modified such that either a

c-myc sequence, recognized by the monoclonal antibody 9E10 (Evan et al., 1985), or the HA epitope, recognized by the monoclonal antibody 12CA5 (Wilson et al., 1984), was inserted three amino acids downstream of the putative site of signal peptidase cleavage (the epitope tags are located between Pro[26] and His[27]). In the case of TGF-βRIII, both the rat (Wang et al., 1991) and human (Morén et al., 1992) cDNAs in the expression vector pcDNA-I/neo were modified to include either of the c-myc or HA epitope tags. The tags were inserted two amino acids downstream of the putative site of signal peptidase cleavage in the rat sequence (between Pro[25] and Glu[26]) or in the equivalent position in the human TGF-βRIII sequence (between Pro[22] and Glu[23]). When transiently expressed in COS-7 cells using the SV-40 vectors, the tagged receptors were synthesized, delivered to the cell surface, and bound ligand indistinguishably from the wild-type receptors (Fig. 1 A; and data not shown). The insertion of the epitope tag in these specific locations does not interrupt the biological activities of the receptors, as shown for HA-tagged TGF-βRII in a growth-inhibition assay (Wrana
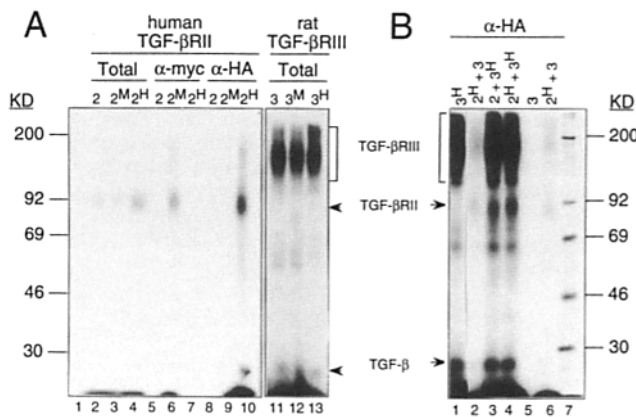

Figure 1. Affinity labeling of wild-type and epitope-tagged types II and III TGF-β receptors. Specificity of anti-epitope immunoprecipitation and co-immunoprecipitation of receptor type II with III. (A) COS-7 cells were transiently transfected with human TGF-βRII, either wild-type (lanes 2, 5, and 8, labeled 2), c-myc-tagged (lanes 3, 6, and 9, 2^M), or HA-tagged (lanes 4, 7, and 10, 2^H), or with wild-type (lane 11, 3), c-myc-tagged (lane 12, 3^M), or HA-tagged (lane 13, 3^H) rat TGF-βRIII. Cells were incubated with 100 pM of 125I-TGF-β1 and bound ligand was cross-linked to cell-surface receptors. Detergent extracts of the transfected cells were analyzed directly by 5-10% SDS-PAGE (lanes 2-4 and 11-13, total) or after immunoprecipitation with the anti-myc antibody (lanes 5-7, α-myc) or with the anti-HA antibody (lanes 8-10, α-HA). Molecular weight markers are in lane 1 and their positions are indicated on the left margin. The positions of the types III and II receptors and cross-linked dimeric TGF-β are noted on the right margin. (B) COS-7 cells were transfected with combinations of wild-type or HA-tagged receptors, affinity labeled with 100 pM of 125I-TGF-β1, and immunoprecipitated with the α-HA mAb. Lane 1, HA-tagged rat TGF-βRIII; lane 2, HA-tagged human TGF-βRII plus wild-type rat TGF-βRIII; lane 3, wild-type human TGF-βRII plus HA-tagged rat TGF-βRIII; lane 4, HA-tagged human TGF-βRII plus HA-tagged rat TGF-βRIII; lane 5, wild-type human TGF-βRIII; lane 6, HA-tagged human TGF-βRII plus wild-type human TGF-βRIII. Molecular weight markers are in lane 7 and their positions are indicated on the right margin. The positions of the types III and II receptors are marked on the left margin.

et al., 1992) and the c-myc-TGF-βRIII in an assay based on modulation of the growth-inhibitory response to TGF-β2 (López-Casillas et al., 1993).

Co-immunoprecipitation Studies Identify Affinity-labeled TGF-βRII Homo-Oligomers and TGF-βRII-TGF-βRIII Hetero-Oligomers

125I-TGF-β1 was bound and cross-linked to COS-7 cells transfected with various combinations of epitope-tagged or -untagged TGF-βRII (human) and TGF-βRIII (rat or human). Immunoprecipitation on singly transfected cells using α-HA or α-myc IgG showed that these antibodies precipitate only the type II receptors carrying the appropriate epitope tag (Fig. 1 A, lanes 6 and 10, and B, lane 1), but not untagged receptors or receptors carrying the other tag (Fig. 1 A, lanes 5, 7, 8 and 9, and B, lane 5). The same specificity applied to the epitope-tagged type III receptors (Fig. 1 B; and data not shown), the only complication being that the α-myc IgG cross-reacted weakly with affinity-labeled HA-tagged type III receptors (Moustakas, A., data not shown). This limited our analysis of the type III receptors using the affinity-labeling/immunoprecipitation approach and only the appropriate, noncross-reacting combinations of type III receptors were further tested.

The endogenous TGF-β receptors in COS-7 cells were not detectable under the conditions of our binding and cross-linking assay (not shown). Transfection with the cDNA encoding the human TGF-βRII resulted in specific binding and cross-linking of TGF-β to this receptor only (Fig. 1 A, lanes 2-4) and no type I TGF-β receptors co-immunoprecipitated with the type II receptors (Fig. 1 A, lanes 5-10). Transfection with cDNAs encoding the rat (Fig. 1 A, lanes 11-13, and B, lanes 1-4 and 6) or human (not shown) TGF-βRIII resulted in specific binding and cross-linking of TGF-β to the type III receptor as well as to two additional, closely migrating TGF-β-binding proteins of ~45-52 kD. We have consistently observed these low molecular weight binding proteins in this and other cell systems where the type III receptor is overexpressed (Inagaki et al., 1993). These affinity-labeled proteins co-precipitate with antibodies specific for the type III receptor (Moustakas et al., 1993) or with the monoclonal antibodies specific for the appropriate epitope tags used, even under denaturing conditions, suggesting that these species are antigenically related to the type III receptor. These proteins are distinct from the TGF-β type I receptor since in this gel system the type I receptor co-migrates exactly with the 69-kD molecular weight marker whereas the doublet migrates faster. Further, neither of the affinity-labeled doublet proteins is precipitated by antibodies specific for the TGF-βRI (Moustakas, A., unpublished results). Nor do these two proteins exhibit the characteristic sensitivity of the TGF-βRI to pretreatment of cells with DTT (Inagaki et al., 1993) and, unlike affinity-labeled type I receptor, they do not co-immunoprecipitate with the type II receptor. These two proteins most probably represent membrane bound forms of the type III receptor whose exact molecular nature remains unknown.

In cells cotransfected with TGF-βRII and TGF-βRIII and labeled with 125I-TGF-β1, immunoprecipitation of one receptor type, carrying a specific epitope tag, was accompanied by detectable co-precipitation of the other receptor type (either untagged or carrying the other tag) (Fig. 1 B, lanes
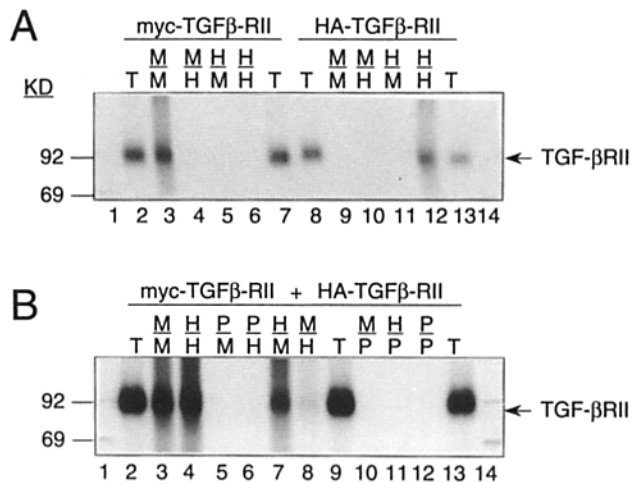
2, 3, and 6). Co-precipitation of affinity-labeled TGF-βRIII was also detected when TGF-βRII (tagged or untagged) was precipitated using a rabbit polyclonal anti–peptide antibody directed against an NH$_2$-terminal epitope (Lys^{30} to Ala^{44}) of the human TGF-βRII (α-IIN; Moustakas et al., 1993; and data not shown). Co-precipitation of affinity-labeled TGF-βRII was detected when TGF-βRIII (tagged or untagged) was precipitated using a rabbit polyclonal anti–peptide antibody directed against an exoplasmic domain epitope (Gly^{507} to Tyr^{520}) of the rat TGF-βRIII (Moustakas, A., unpublished results). These findings are in accord with recent observations on co-immunoprecipitation of TGF-βRII and TGF-βRIII in several cell types (López-Casillas et al., 1993; Moustakas et al., 1993). Addition of any of the above antibodies, at the concentrations used in the immunoprecipitation or immunofluorescence experiments, during the incubation with ^{125}I-TGF-β1 did not affect ligand binding or cross-linking, indicating that these antibodies do not inhibit TGF-β1 binding to either receptor (not shown).

Formation of homo-oligomeric complexes among a single type of TGF-β receptor was not studied previously. In order to examine the formation of such complexes, sequential immunoprecipitation experiments were performed on cells coexpressing HA- and c-myc-tagged type II receptors. Fig. 2 shows that these receptors, affinity labeled with ^{125}I-TGF-β1, can be co-immunoprecipitated demonstrating that homo-oligomers of the type II receptor exist on the cell surface, at least in the presence of ligand. In the key samples (Fig. 2 B, lanes 7 and 8) cell extracts were immunoprecipitated with one monoclonal anti–epitope antibody, the immunoprecipitates were boiled in a solution containing SDS, and eluted polypeptides were then re-immunoprecipitated with the other monoclonal antibody. The c-myc-tagged type II receptor cross-linked to TGF-β1 can be co-immunoprecipitated by the α-HA monoclonal antibody (Fig. 2 B, lane 7). It is not co-immunoprecipitated if the cells do not express the HA-tagged receptor (Fig. 2 A, lane 5), nor if a preimmune serum is used for the first immunoprecipitation (B, lane 5). Similarly, HA-tagged type II receptor cross-linked to TGF-β1 can be co-immunoprecipitated with the α-myc monoclonal antibody (Fig. 2 B, lane 8). It is not co-immunoprecipitated if the cells do not express the c-myc-tagged receptor (Fig. 2 A, lane 10) or if the first immunoprecipitation is with a preimmune serum (B, lane 6). Thus, affinity-labeled HA- and c-myc-tagged type II receptors are in mutual complexes on the cell surface.

We were unable to demonstrate co-immunoprecipitation of HA- and c-myc-tagged type III receptors using a similar protocol. Since the immunofluorescence copatching experiments (see below) demonstrate that type III homo-oligomers do exist, one explanation for this discrepancy is that homo-oligomeric type III complexes do not withstand detergent solubilization and thus are not detected by the immunoprecipitation protocol. A second is that, while primary immunoprecipitation of epitope-tagged TGF-βRIII after binding cross-linking was very efficient (Fig. 1 B), the efficiency of the secondary immunoprecipitation of the denatured type III receptor (after boiling in SDS) is low.

The immunoprecipitation studies (or any other biochemical assay) cannot quantify the fraction of the receptors that participate in homomeric complexes, since only a subpopulation of the cells coexpress the two cotransfected receptors.


Figure 2. Co-immunoprecipitation of affinity-labeled type II receptors: type II receptors form homo-oligomers in the presence of TGF-β1. COS-7 cells were transiently transfected with c-myc-tagged type II receptors alone (A, lanes 2–7), HA-tagged type II receptors alone (A, lanes 8–13), or both epitope-tagged receptors together (B, lane 2–13). Cells were affinity labeled with 100 pM ^{125}I-TGF-β1. The total extracts (T; A, lanes 2, 7, 8, and 13, and B, lanes 2, 9, and 13) were analyzed by 5–10% SDS-PAGE. Cells were solubilized in detergents and subjected to immunoprecipitation with the antibody listed in the numerator: α-myc (M; A, lanes 3, 4, 9, and 10; B, lanes 3, 8, and 10); α-HA (H; A, lanes 5, 6, 11, and 12; B, 4, 7, and 11) or a control rabbit pre-immune serum (P; B, lanes 5, 6, and 12). As detailed in Materials and Methods, the immunoprecipitates were dissolved by boiling in SDS, and subjected to immunoprecipitation using the antibody listed in the denominator: α-myc (M; A, lanes 3, 5, 9, and 11; B, 3, 5, and 7); α-HA (H; A, lanes 4, 6, 10, and 12; B, 4, 6, and 8); or the control pre-immune serum (P; B, lanes 10, 11, and 12). Immunoprecipitates were analyzed by 5–10% SDS-PAGE. Molecular weight markers are in lanes 1 and 14 and are indicated on the left margin of each panel. The position of the type II receptor is marked on the right margins. Only the relevant portions of the autoradiograms are indicated. Autoradiography was performed at −70°C with an intensifying screen for 3 d (A) or 7 d (B).

Furthermore, the sequential immunoprecipitation experiments examine only a small percentage of the cell–surface receptor population, due to the low efficiency of chemically cross-linking the labeled ligand to the receptors. In addition, heterocomplexes containing both types II and III TGF-β receptors may be preferentially labeled in such studies, since their formation appears to be mediated by the TGF-β ligand. Therefore, although they are highly sensitive, the immunoprecipitation studies cannot determine whether the complexes represent a major or a minor fraction of the cell–surface receptors. In spite of these limitations, it is apparent that the fraction of TGF-βRIII that coprecipitates with HA-tagged TGF-βRII is much smaller than the fraction of c-myc-tagged TGF-βRII that coprecipitates with HA-tagged TGF-βRII (compare Fig. 1 B, lanes 1 and 2 with Fig. 2 B, lanes 4 and 7). To further explore these questions, we restored to cell–surface immunofluorescence studies.

Epitope-tagged Receptors Can be Patched on the Surface of Living Cells

Immunofluorescence copatching experiments, designed to
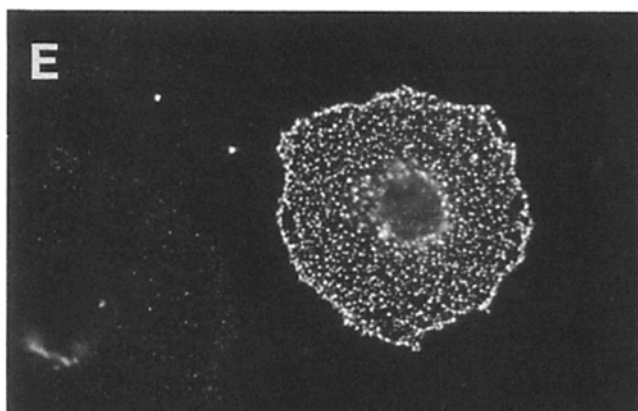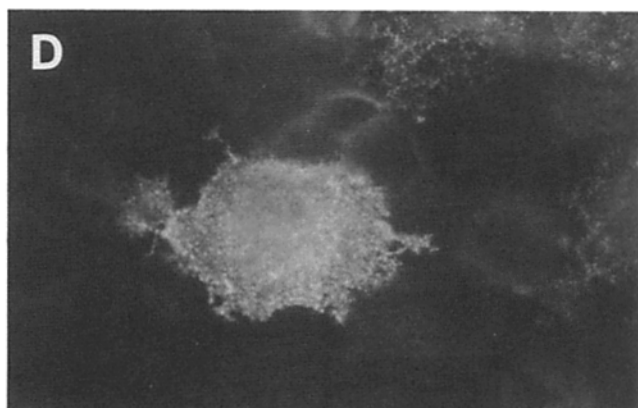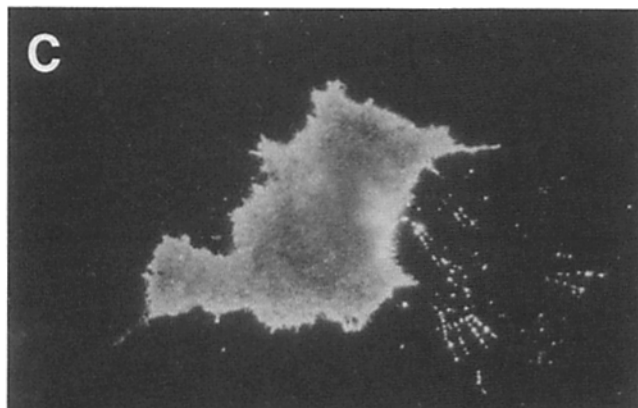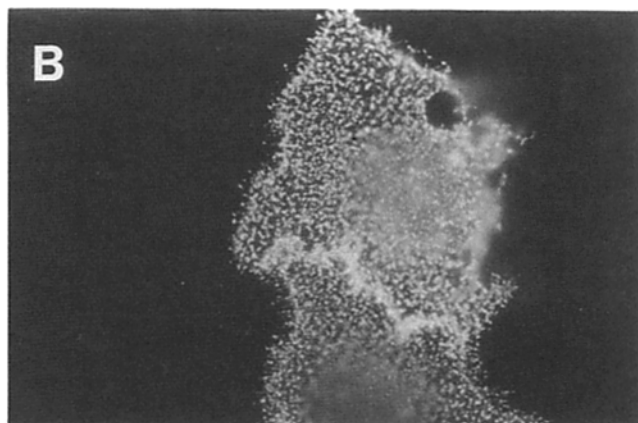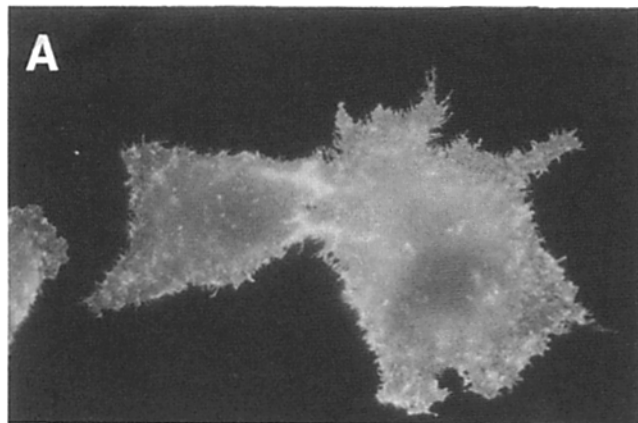
detect oligomeric receptors at the surface of live cells, are based on IgG-mediated patching of one polypeptide chain. The copatching of another chain (carrying a different epitope), labeled exclusively with noncross-linking monovalent Fab' fragments, is then followed. These experiments have several advantages: (a) they examine the bulk of the cell–surface receptors; (b) the use of microscopy enables one to select exclusively the coexpressing cells for analysis; (c) sensitivity of receptor complexes to detergents, as may be the case for type III receptor homo-oligomers, does not present a problem as there is no detergent-solubilization step; and (d) receptor interactions can be measured in the absence or presence of ligand using the same analytical techniques, unlike biochemical experiments where the method of analysis varies depending on the presence or absence of ligand.

Surface labeling of live COS-7 cells (performed in the cold, to avoid internalization) expressing tagged or untagged TGF-βRII or TGF-βRIII with fluorescent antibodies is shown in Fig. 3, which also depicts controls for the labeling specificity. For both TGF-βRII and TGF-βRIII, labeling with noncross-linking monovalent, Fab' fragments resulted in a diffuse fluorescent pattern (Fig. 3, A and C). In contrast, labeling with bivalent primary and secondary IgG's led to the formation of conspicuous micropatches, presumably due to IgG-mediated cross-linking (Fig. 3, B, D, and E). This shift indicates that the receptors are laterally mobile at the cell surface; otherwise they would not be incorporated into patches. This situation is ideal for copatching experiments, since it is relatively easy to follow the shift of the Fab'-labeled receptors from a homogeneous to a patchy distribution. It should be noted that the α-IIN rabbit IgG was more effective in inducing patching than α-HA or α-myc, probably due to its polyclonal nature (Fig. 3, compare B and E). TGF-βRIII underwent IgG-mediated patching less readily than TGF-βRII (Fig. 3, compare B and D), for which reason patching of TGF-βRII was preferred whenever possible. Fig. 3 (F–H) are controls for the specificity of the antibodies used.

The Majority of Types II and III Receptors Are Not in Hetero-Oligomers

Experiments showing the lack of copatching of TGF-βRIII with TGF-βRII in the absence of ligand are depicted in Fig. 4. These experiments employed COS-7 cells cotransfected with TGF-βRII (tagged or untagged) and TGF-βRIII carrying another epitope-tag. About 40% of the positive cells expressed both receptor types, and these cells were examined for copatching. As shown in Fig. 4, the TGF-βRII patches mediated by FITC-IgG (A and E, green) do not overlap with the red fluorescence of the epitope-tagged TGF-βRIII, monitored by Cy3-Fab' (B and F). In the double-exposure photographs (Fig. 4, C, D, and G) the TGF-βRII patches are green, while the Cy3 fluorescence appears as orange, due to the green FITC background. Most of the epitope-tagged TGF-βRIII is smoothly distributed on the cell surface; only occasional FITC- and Cy3-labeled patches coincided, giving rise to a yellow color upon double exposure (Fig. 4, lower part of G). Qualitatively similar results (not shown) were obtained when epitope-tagged TGF-βRIII was induced into micropatches by appropriate IgG's and epitope-tagged TGF-βRII was labeled with monovalent Fab'. Importantly, no significant copatching of TGF-βRIII with TGF-βRII was observed even on double-labeled cells that expressed low levels (just above the limit of detection needed for fluorescence microscopy) of one or both receptors. These results indicate that only a small fraction—below the level that can be unambiguously detected in the immunofluorescence copatching experiments—of the type III receptors may reside in mutual complexes with TGF-βRII in live cells and in the absence of ligand. The lack of copatching is not due to incompatibility between human type II and rat type III TGF-β receptors, as evidenced by the failure of epitope-tagged human TGF-βRIII to copatch with human TGF-βRII (Fig. 4 D).

In view of the findings of co-immunoprecipitation of the types II and III receptors following TGF-β binding (see Fig.

Figure 3. Expression of wild-type and epitope-tagged types II and III TGF-β receptors on the surface of transfected COS cells, and patching by bivalent but not monovalent antibodies specific for the NH₂ termini. COS-7 cells were transfected with the types II or III TGF-β receptors, untagged or tagged at the NH₂ terminus with the HA or c-myc epitope, in the vectors pcDNA-I or pcDNA-I/neo. 44 to 48 h after transfection the intact cells were labeled at 4°C, in order to label exclusively the cell surface, with the appropriate monovalent Fab' or IgG. The cells were then washed and labeled with a fluorescent secondary Fab' or IgG, depending on whether or not IgG-mediated patching was desired. The results shown for TGF-βRIII were obtained with the rat clone; similar results (not shown) were obtained for the human receptor clone. Bar, 20 μm. (A) Cells transfected with the human type II receptor carrying the c-myc epitope at the NH₂ terminus (c-myc-TGF-βRII) were labeled with 50 μg/ml Fab' derived from the α-myc monoclonal antibody followed by 20 μg/ml Cy3-tagged Fab' goat anti-mouse antibody (Cy3-Fab' GαMIgG). Cell surface tagged type II receptors are uniformly distributed. (B) Cells transfected as in A were labeled with 20 μg/ml intact IgG α-myc followed by 20 μg/ml FITC-tagged intact goat anti-mouse IgG (FITC-IgG GαMIgG). Cell-surface tagged type II receptors are aggregated into small patches. (C) Cells transfected with the rat type III receptor carrying the HA epitope at the NH₂-terminus (HA-TGF-βRIII) were labeled with 50 μg/ml Fab' derived from the α-HA monoclonal antibody followed by 20 μg/ml Cy3-Fab' GαMIgG. Cell-surface tagged type III receptors are uniformly distributed on the cell surface. (D) Cells transfected as in C were labeled with 20 μg/ml intact α-HA IgG followed by 20 μg/ml FITC-IgG GαMIgG. Many of the cell-surface tagged type III receptors are aggregated into small patches. (E) Cells were transfected with the wild-type (untagged) TGF-βRII, and labeled with 150 μg/ml of intact IgG purified from a rabbit antipeptide antibody raised against an NH₂-terminal peptide of the type II receptor (α-IIN IgG) followed by 30 μg/ml of FITC-labeled goat anti-rabbit IgG (FITC-IgG GαRIgG). The cell surface type II receptors are aggregated into small patches. (F) Cells transfected with the wild-type (untagged) rat type III receptor were labeled with 150 μg/ml of intact α-IIN IgG followed by 30 μg/ml FITC-GαRIgG. No surface labeling is evident on any cells. (G) Cells transfected with c-myc-TGF-βRII were labeled with 20 μg/ml intact α-HA IgG followed by 20 μg/ml FITC-IgG GαMIgG. No surface labeling is evident on any cells. Similarly, no surface labeling was obtained using cells transfected with untagged TGF-βRII or TGF-βRIII. (H) Cells transfected with rat HA-TGF-βRIII were labeled with 20 μg/ml intact α-myc IgG, followed by 20 μg/ml FITC-IgG GαMIgG. No surface labeling is evident on any cells. Similarly, no surface labeling was obtained using cells transfected with untagged TGF-βRII or TGF-βRIII.
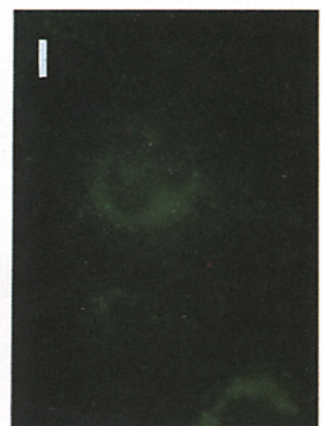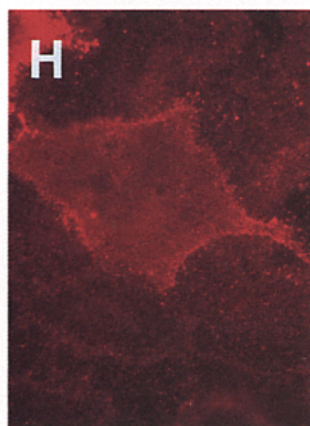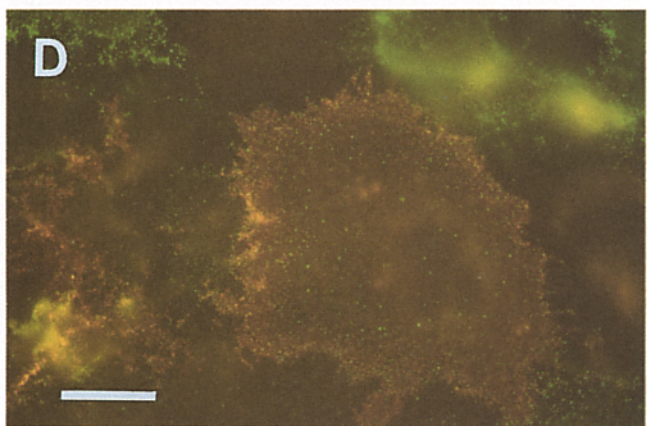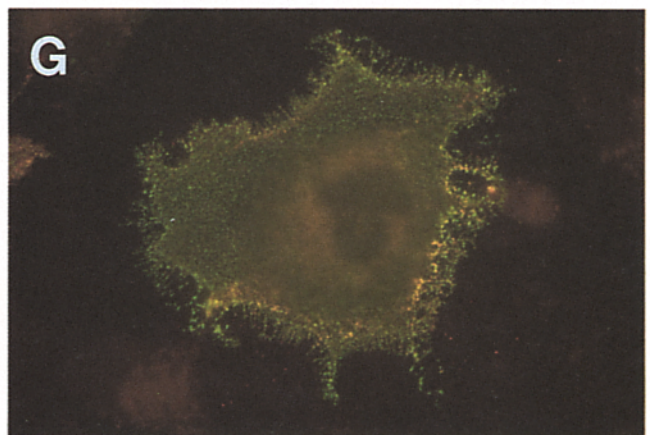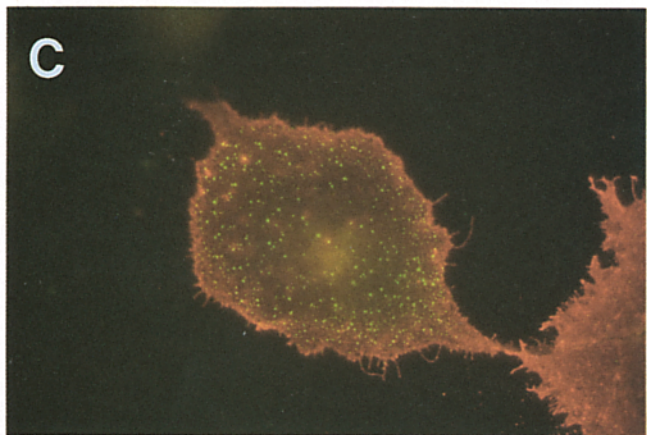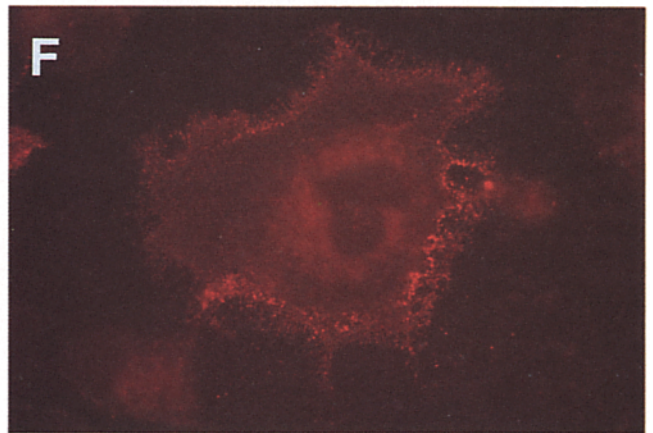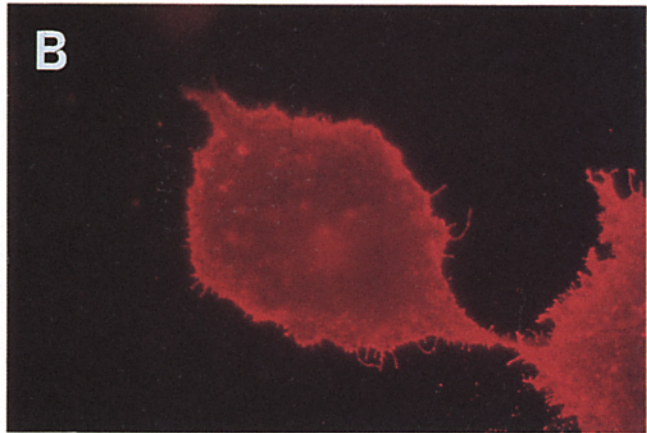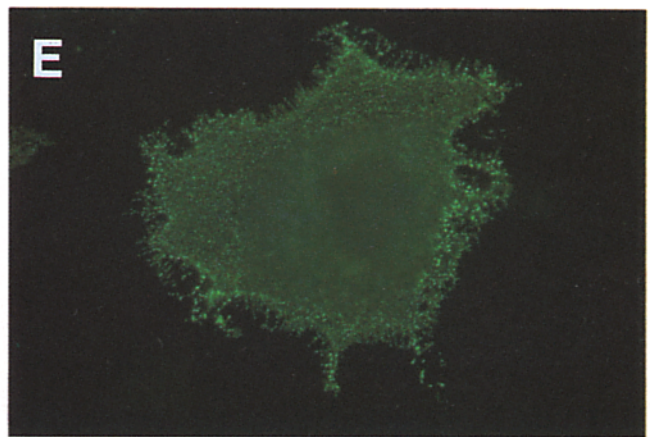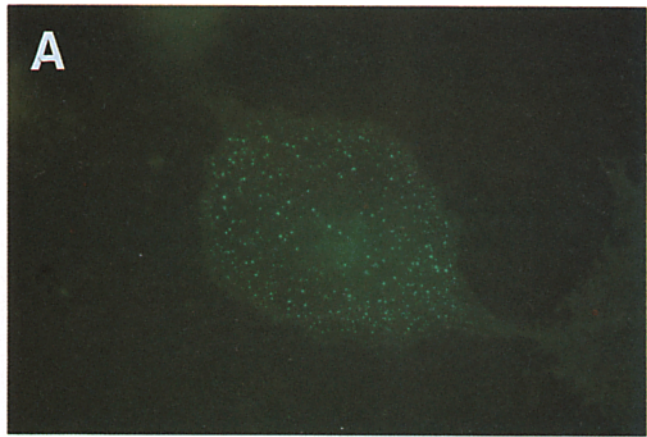
1; and López-Casillas et al., 1993), we examined the effects of TGF-β1 and TGF-β2 on copatching of the types II and III receptors. The results (Fig. 5) clearly show that even in the presence of these ligands, IgG-mediated patching of human TGF-βRII (A and E) does not lead to appreciable copatching of epitope-tagged rat or human TGF-βRIII (B and F). The nonoverlap of the fluorescence is evident in the double exposures in Fig. 5, C and G. The lighter shade of green in Fig. 5 A is due to a longer exposure time than in E and not due to leakage of Cy3 fluorescence; such leakage is negligible as evidenced by the control experiment in H and I of Fig. 4. Even addition of disuccinimidyl suberate (DSS) after ligand binding, to cross-link some ligand to its receptor, prior to the IgG-mediated patching did not result in distinctive copatching (Fig. 5, D and H). The same results—absence of copatching of the types III and II receptors—were obtained at several concentrations of TGF-β1 or TGF-β2 (50 pM, 200 pM, 1 nM; data not shown), excluding the possibility that sub- or over-saturation of the receptors by the ligand interfered with its ability to induce heterocomplexes. Analogous observations—absence of copatching of the types III and II receptors—were made when epitope-tagged TGF-βRIII was induced to patch instead of TGF-βRII (not shown). It should be noted that the antibodies used in these studies did not affect binding of TGF-β to its receptors, as evidenced by the absence of effects of these antibodies on affinity labeling by ^{125}I-TGF-β1 of the types II or III receptors expressed in transfected COS cells (not shown). Similarly, the presence of concentrations of TGF-β1 or TGF-β2 up to 1 nM (the highest tested) had no effect on labeling by the fluorescent antibodies (Fig. 5). Thus, even in the presence of ligand, the level of type II/type III TGF-β receptor heterocomplexes must be low. We conclude that only a minor fraction of the cell-surface TGF-βRII and TGF-βRIII are localized in heteromeric complexes.

TGF-βRII and TGF-βRIII Exist As Homo-Oligomers on the Plasma Membrane

The experiment in Fig. 6 shows that HA- and c-myc-tagged type II TGF-β receptors copatch together on the cell surface in the absence (A–C) or presence (D–F) of TGF-β1 or TGF-β2. COS-7 cells were cotransfected with c-myc- and HA-tagged TGF-βRII. The receptors carrying one epitope tag were forced into patches using primary IgG and FITC-IgG secondary antibodies (Fig. 6, A and D) while the receptors carrying the other tag were labeled with monovalent Fab' and secondary Cy3-Fab' (B and E). As is apparent in the double exposures (Fig. 6, C and F), a very high percentage of the cell-surface receptors copatch. There are many coincident green and red patches that become yellow upon double exposure (e.g., Fig. 6, long arrows). Copatching was observed in the absence of ligand (Fig. 6, A–C) and was not enhanced significantly by the addition of 50 pM to 1 nM TGF-β1 or TGF-β2 ligand (D–F; and data not shown). Similar distributions were observed on individual cells expressing high or low levels of the receptors (not shown). Addition of DSS after ligand binding, to cross-link some ligand to its receptor, prior to the IgG-mediated patching, did not result in increased copatching (data not shown). Interestingly, not all of the green FITC-IgG patches coincided with red Cy3-Fab' patches of receptor carrying the other tag (e.g., Fig. 6, short arrows). Such a phenomenon is expected in the case of small homo-oligomers (dimers or trimers), where the probability is high that some homo-oligomers will consist entirely of receptor polypeptides bearing the same epitope tag and that

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Figure 4. Types III and II TGF-β receptors do not copatch in the absence of ligand. Transfection and live cell immunofluorescence were as described under Materials and Methods. (A–C) COS-7 cells were cotransfected with rat HA-TGF-βRIII and wild-type (untagged) human type II receptor. 44 h after transfection the intact cells were labeled at 4°C by the following consecutive steps: (a) 50 μg/ml Fab' fragments derived from the mouse anti-HA monoclonal antibody (α-HA Fab') together with 150 μg/ml intact rabbit α-IIN IgG, (b) 30 μg/ml FITC-IgG GαRIgG (to patch the type II receptor) together with 20 μg/ml Cy3-Fab' GαMIgG (to label the type III receptor). (A) The fluoroscein image shows the patches of (untagged) type II TGF-β receptors induced by the anti-peptide IgG's. (B) The Cy3 image shows that the HA-tagged type III TGF-β receptors are uniformly distributed on the cell surface. (C) A double exposure of the fluoroscein and Cy3 images shows that most of the patches are green and contain only patched type II receptors. The reddish-orange color (*red,* becoming *orange* upon double exposure due to the contribution of green background by the photography under FITC conditions) is from the Cy3-tagged type III receptors and clearly does not overlap with the type II receptors. Identical results (not shown) were obtained using c-myc- or HA-tagged TGF-βRII, or c-myc-TGF-βRIII. (D) A double exposure of the fluoroscein and Cy3 images from a cell from an experiment similar to that in C, except that transfection was with an HA-tagged human type III receptor together with wild-type (untagged) human type II receptor. As in C, most of the patches are green and contain only patched type II receptors. The reddish-orange color is from the Cy3-tagged type III receptors and clearly does not overlap with the type II receptors. Similar results (not shown) were obtained using epitope-tagged TGF-βRII or the human c-myc-TGF-βRIII. (E–G) COS-7 cells were cotransfected with rat HA-TGF-βRIII and human c-myc-TGF-βRII. At 44 h the intact cells were labeled at 4°C by the following consecutive steps: (a) 50 μg/ml α-HA Fab' followed by 20 μg/ml Cy3-Fab' GαMIgG, (b) saturating amounts (200 μg/ml) of unlabeled Fab' fragments of goat anti-mouse IgG (Fab' GαMIgG); this ensures saturation of the α-HA Fab' by secondary Fab'; (c) 20 μg/ml α-myc IgG, followed by 20 μg/ml FITC-IgG GαMIgG. (E) The fluorescein image shows the patches of c-myc-tagged type II TGF-β receptors induced by the α-myc IgG's. (F) The Cy3 image shows that the HA-tagged type III TGF-β receptors are distributed throughout the entire surface, concentrated at the cell periphery and near the nucleus. They do not overlap the patches of type II receptors. (G) A double exposure of the fluorescein and Cy3 images shows that most of the patches are green and contain only patched c-myc-tagged type II receptors. The reddish-orange color is from the Cy3-tagged type III receptors and clearly does not overlap with the type II receptors. Similar results (not shown) were obtained using HA-TGF-βRII and c-myc-TGF-βRIII. (H and I) Controls showing that the addition of unlabeled Fab' GαMIgG after the Cy3-Fab' GαMIgG (step b in E–G) is sufficient to block subsequent nonspecific binding in step c. The cells were transfected with HA-TGF-βRIII only, and then labeled exactly as in E–G above. (H) Cy3-Fab' labeling, illustrating the uniform distribution of the HA-tagged type III TGF-β receptors. (I) Shows the nonspecific FITC staining of the same field. Bar, 20 μm.

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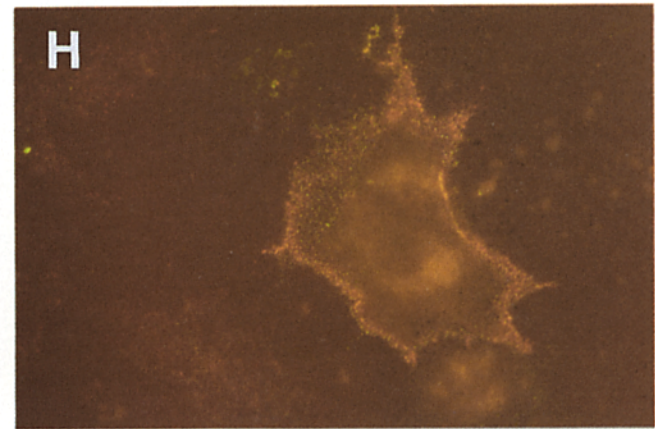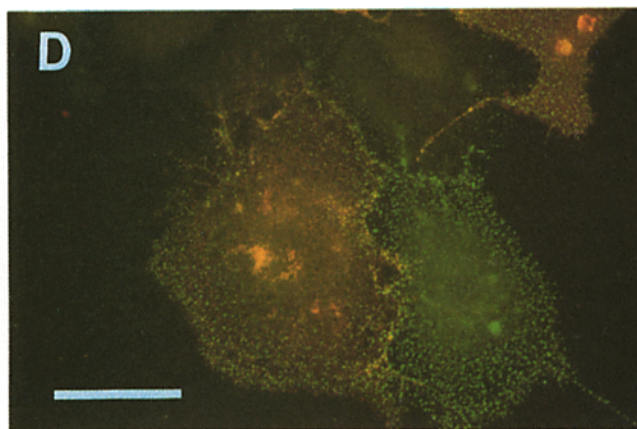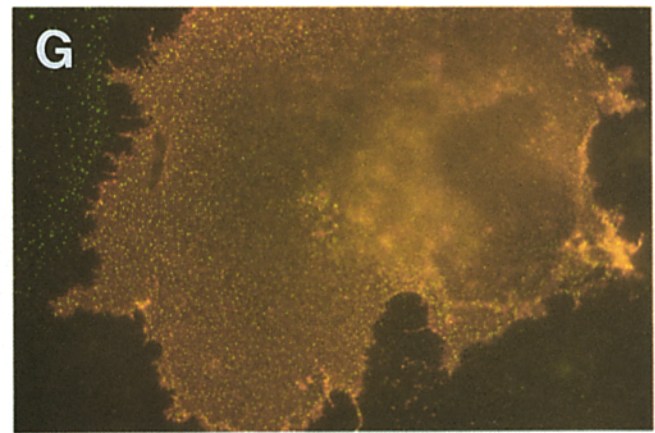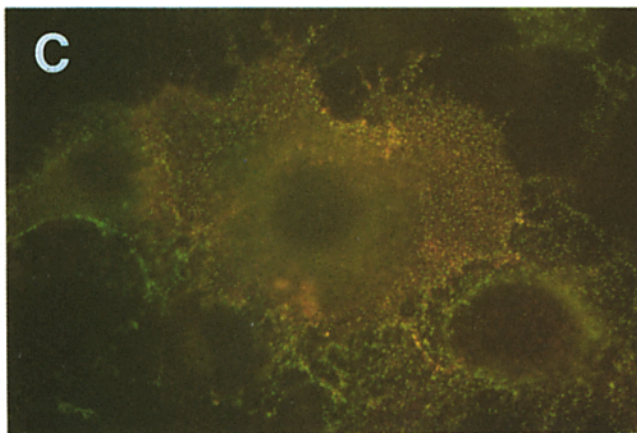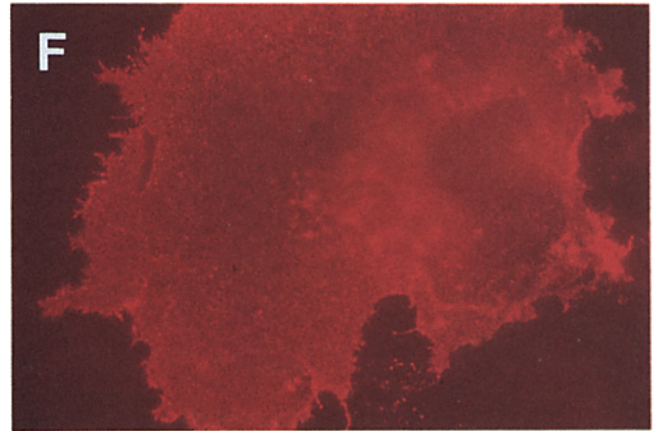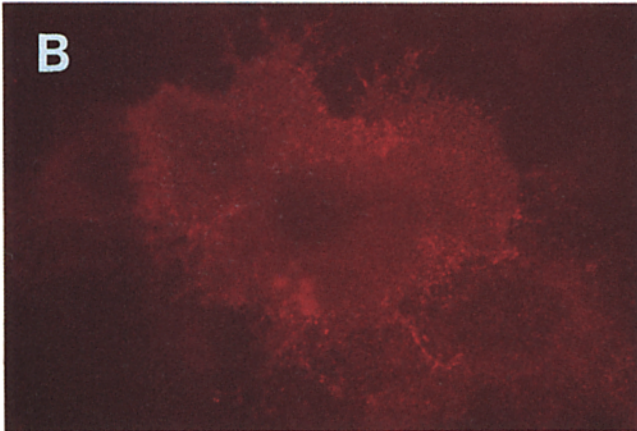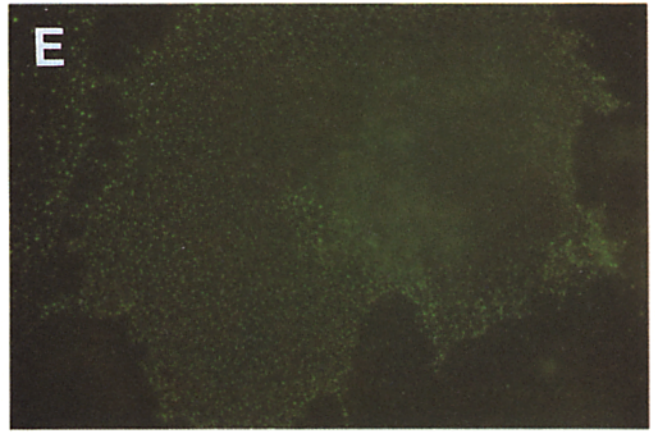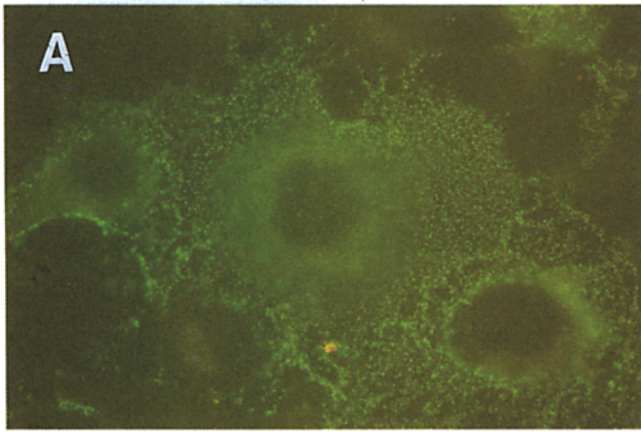
therefore will not copatch with the other epitope-tagged receptor polypeptide (see Discussion).

Similar studies demonstrated copatching of HA- and c-myc-tagged type III TGF-β receptors on the cell surface in the absence (Fig. 7, A–C) or presence (D–F) of TGF-β1. In this case, virtually all of the green patches of tagged type III TGF-β receptors induced by the α-HA IgG's (Fig. 7, A and D) contained red fluorescence due to the copatched receptors bearing the opposite epitope tag (B and E), as can be seen in the double exposures, where all of the patches are yellow (C and F). However, the inefficient patching of the type III receptors reduces the resolution in these experiments. Similar results (not shown) were obtained using other TGF-β1 concentrations (50 pM or 1 nM), replacing TGF-β1 by TGF-β2, or cross-linking the ligand to the surface receptors with DSS prior to labeling with antibodies. As in the former cases, no significant differences could be noticed between cells with high versus low surface expression of the receptors.

Discussion

TGF-β has a broad range of activities, controlling cell growth, differentiation, and extracellular matrix composition (Massagué, 1990; Roberts and Sporn, 1990; Moses et al., 1990; Lin and Lodish, 1993). These responses are elicited and modulated via high-affinity cell–surface receptors, principally the types I, II, and III receptors. Formation of homo- or hetero-oligomeric receptor complexes may play an important role in regulating the multiple intracellular signals generated by TGF-β. Several studies have demonstrated functional and physical interactions among the different receptor subtypes (Wrana et al., 1992; Inagaki et al., 1993; López-Casillas et al., 1993; Moustakas et al., 1993), but the molecular composition of putative receptor hetero-oligomers on the cell surface under native conditions is still unknown. Furthermore, the existence of receptor homo-oligomers has not been explored. Here we investigated homo- and hetero-oligomer formation among types II and III TGF-β receptors using cells co-expressing various combinations of epitope-tagged types II and III receptors. We used both antibody-mediated cross-linking and immunofluores-

cence microscopy on live cells, as well as sequential immunoprecipitation reactions on affinity-labeled cell–surface receptors. These studies demonstrated that both TGF-βRII and TGF-βRIII form homo-oligomers at the cell surface, and that type II/type III receptor heterocomplexes comprise only a minor fraction of the population of cell–surface receptors, implying that the heterocomplexes may represent transient species.

The immunofluorescence copatching experiments on cells coexpressing HA-TGF-βRII and c-myc-TGF-βRII, or HA-TGF-βRIII and c-myc-TGF-βRIII (Figs. 6 and 7), corroborated by the results of the sequential immunoprecipitation experiments on affinity-labeled TGF-βRII (Fig. 2), clearly demonstrate for the first time that both types II and III TGF-β receptors form homo-oligomers. These homo-oligomers are detected in the intact cell plasma membrane, and exist prior to ligand binding. Although our experiments used cells overexpressing the transfected receptors, a situation that might increase nonspecific interactions, there are several indications that the homo-oligomers are not generated as a result of high receptor expression. First, many cell types normally express high physiological levels of the type III TGF-β receptor — ~200,000 per cell (Massagué, 1990) — approximately that found on transfected COS cells that express moderate levels of the type III receptor. Second, even for the type II receptor, which is normally expressed at levels of several thousands per cell, the microscopic nature of the immunofluorescence copatching experiments enabled examination of transfected COS cells expressing as low as 20,000 type II receptors per cell. (The amount of TGF-βRII at the surface of specific transfected COS-7 cells was evaluated by measuring the fluorescence intensity on single cells labeled by the α-IIN antibody and a secondary fluorescent IgG, using a photomultiplier tube attached to the microscope. The intensity was compared to that obtained using similarly labeled H2-16 cells, an SW480 colon carcinoma cell line stably transfected with the human TGF-βRII [Lin, H. Y., A. Moustakas, P. Knaus, R. Wells, Y. I. Henis, and H. F. Lodish, manuscript in preparation]. The TGF-βRII number at the surface of the H2-16 cells [~20,000 receptors/cell] was independently measured by binding of ^{125}I-TGF-β1 and by binding–cross-linking assays). Yet the results — co-patching

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Figure 5. TGF-β1 and TGF-β2 do not mediate appreciable copatching of the types II and III TGF-β receptors. (A–C) Cells were cotransfected with human c-myc-TGF-βRII and human HA-TGF-βRIII. 44 h after transfection the cells were preincubated for 2 h at 4°C with 1 nM TGF-β1. The cells were labeled exactly as in A–C of Fig. 4, except that 1 nM TGF-β1 was added to all of the antibody solutions. (A) The fluorescein image shows the patches of c-myc-tagged type II TGF-β receptors induced by the anti-peptide IgG's. (B) The Cy3 image shows that the HA-tagged type III TGF-β receptors are uniformly distributed on the cell surface. (C) A double exposure of the fluorescein and Cy3 images shows that most of the patches are green and contain only patched type II receptors. The reddish-orange color is from the Cy3-tagged type III receptors and clearly does not overlap with the type II receptors. Similar results (not shown) were obtained with 50 or 200 pM TGF-β1, or when patching was mediated by α-myc IgG instead of α-IIN IgG. (D) Double exposure of cells transfected and labeled as in A–C, except that prior to labeling with the antibodies TGF-β1 was cross-linked to the cell–surface receptors using DSS. (E–G) Cells were cotransfected with human c-myc-TGF-βRII and rat HA-TGF-βRIII. 44 h after transfection that cells were preincubated for 2 h at 4°C with 200 pM TGF-β2. Except for the addition of TGF-β2 to all antibody solutions, rather than TGF-β1, the experiment was otherwise identical to that in A–C. (E) The fluorescein image shows the patches of c-myc-tagged type II TGF-β receptors induced by the anti-peptide IgGs. (F) The Cy3 image shows that the HA-tagged type III TGF-β receptors are uniformly distributed on the cell surface. (G) A double exposure of the fluorescein and Cy3 images shows that most of the patches are green and contain only patched type II receptors. The reddish-orange color is from the Cy3-tagged type III receptors and clearly does not overlap with the type II receptors. Similar results (not shown) were obtained with TGF-β2 at 50 pM or 1 nM, or when patching of the type II receptors was induced by α-myc IgG. (H) Double exposure of cells transfected and labeled as in E–G, except that prior to labeling with the antibodies TGF-β2 was cross-linked to the cell–surface receptors using DSS. Bar, 20 μm.
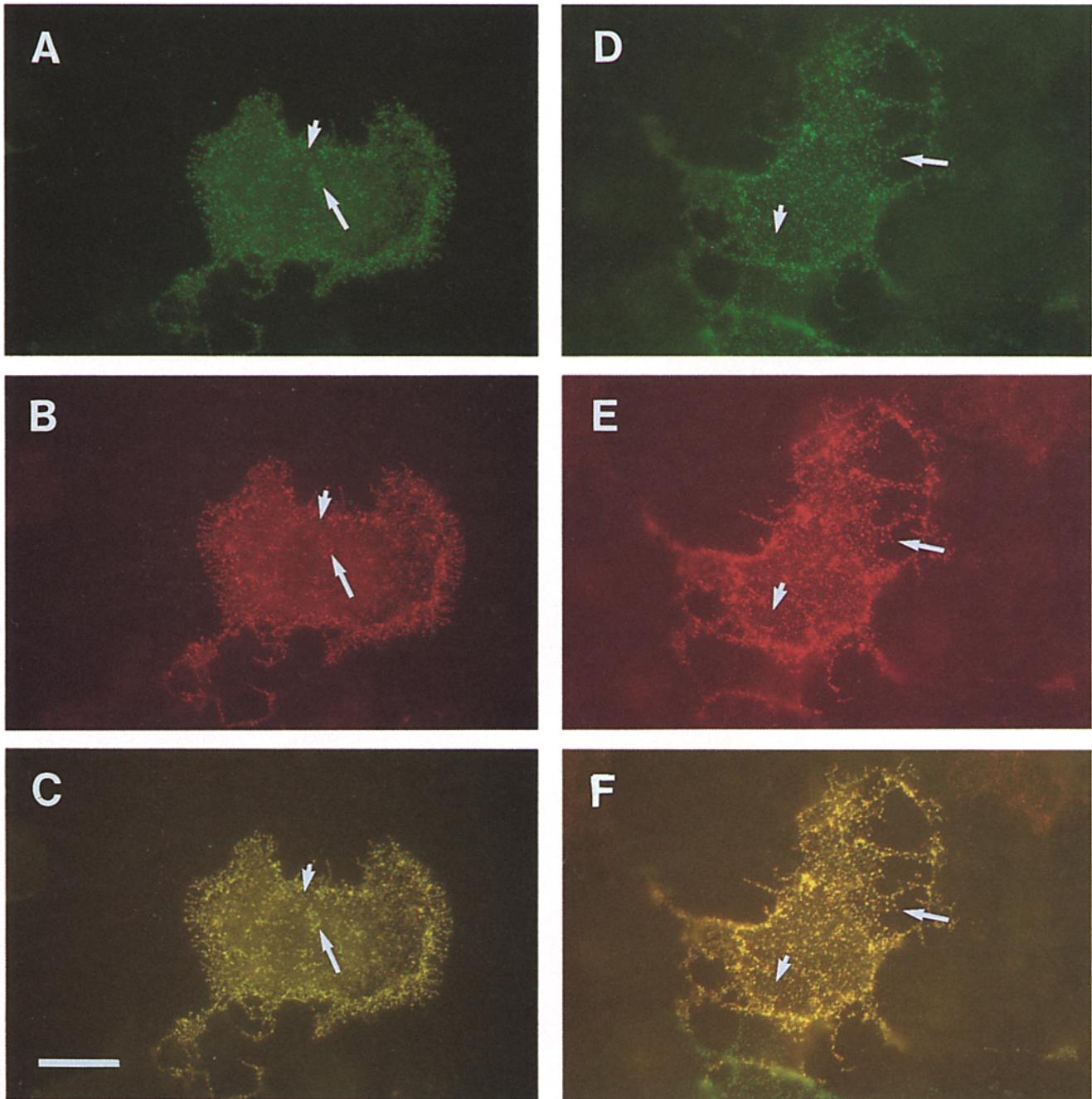
Figure 6. HA- and *c-myc*–tagged type II TGF-β receptors copatch together on the cell surface in the absence (*A–C*) or presence (*D–F*) of TGF-β1. (*A–C*) COS-7 cells were cotransfected with HA- and *c-myc*–tagged type II TGF-β receptors. 44 h after transfection the intact cells were labeled at 4°C by the following consecutive steps: (*a*) 50 μg/ml α-myc Fab' followed by 20 μg/ml Cy3-Fab' GαMIgG, (*b*) saturating amounts (200 μg/ml) of unlabeled Fab' GαMIgG, and (*c*) 20 μg/ml α-HA IgG followed by 20 μg/ml FITC-IgG GαMIgG. (*A*) The fluorescein image shows the patches of HA-tagged type II TGF-β receptors induced by the anti-HA IgG and the FITC-IgG GαMIgG. (*B*) The Cy3 image shows that the *c-myc*–tagged type II TGF-β receptors are also localized in patches. The long arrow points to examples of patches that contain both HA- and *c-myc*–tagged type II TGF-β receptors, and the short arrow indicates patches that contain only fluorescein-labeled HA-tagged type II TGF-β receptors. (*C*) A double exposure of the fluorescein and Cy3 images shows that many patches are yellow (e.g., long arrow) and contain both HA- and *c-myc*–tagged type II receptors. Some (*short arrow*) are green and contain only fluorescein-labeled HA-tagged type II TGF-β receptors whose patching was induced by the IgG's. (*D–F*) COS-7 cells were co-transfected with HA- and *c-myc*–tagged type II TGF-β receptors. 44 h after transfection 200 pM TGF-β1 was added and kept in all of the subsequent labeling solutions. After a 2-h preincubation at 4°C the intact cells were labeled at 4°C by the following consecutive steps: (*a*) 50 μg/ml α-HA Fab' followed by 20 μg/ml Cy3-Fab' GαMIgG; (*b*) saturating amounts (200 μg/ml) of unlabeled Fab' GαMIgG; and (*c*) 20 μg/ml α-myc IgG followed by 20 μg/ml FITC-IgG GαMIgG. (*D*) The fluorescein image shows the patches of *c-myc*–tagged type II TGF-β receptors induced by the α-myc IgG and secondary FITC-IgG GαMIgG. (*E*) The Cy3 image shows that the HA-tagged type II TGF-β receptors are also localized in patches. The long arrow points to examples of patches that contain both *c-myc*– and HA-tagged type II TGF-β receptors, and the short arrow indicates patches that contain only fluorescein-labeled *c-myc*–tagged type II TGF-β receptors. (*F*) A double exposure of the fluorescein and Cy3 images shows that many patches are yellow (e.g., *long arrow*) and contain both *c-myc*– and HA-tagged type II receptors. Some (*short arrow*) are green and contain only fluorescein-labeled *c-myc*–tagged type II TGF-β receptors. Similar results (not shown) were obtained with 50 pM or 1 nM TGF-β1, or when TGF-β2 (same concentrations) was used as the ligand. Additions of a DSS cross-linking step after ligand binding and prior to labeling with the antibodies also did not alter the results (not shown). Bar, 20 μm.
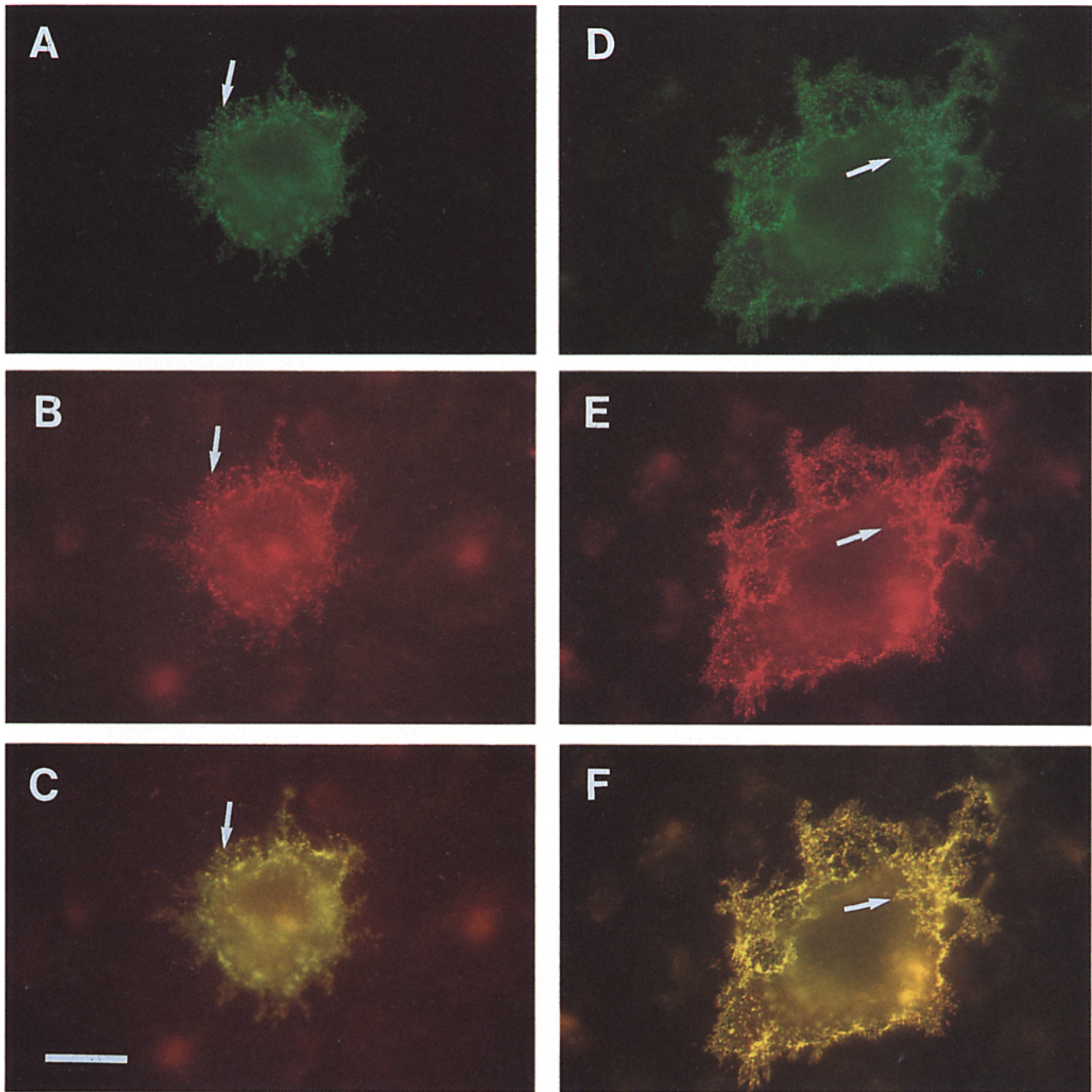
Figure 7. HA- and *c-myc*–tagged type III TGF-β receptors copatch together on the cell surface in the absence (*A–C*) or presence (*D–F*) of TGF-β1. COS-7 cells were cotransfected with rat HA-TGF-βRIII and rat *c-myc*-TGF-βRIII. (*A–C*) Labeling with antibodies was exactly as in Fig. 6 (*A–C*). (*A*) The fluorescein image shows the patches of HA-tagged type III TGF-β receptors induced by the anti-HA IgG and the secondary FITC-IgG GαMIgG. (*B*) The Cy3 image shows that the *c-myc*–tagged type III TGF-β receptors are also localized in patches. The long arrow points to examples of patches that contain both HA- and *c-myc*–tagged type III TGF-β receptors. (*C*) A double exposure of the fluorescein and Cy3 images shows that most of the patches are yellow (e.g., *long arrow*) and contain both HA- and *c-myc*–tagged type III receptors. (*D–F*) Preincubation with 200 pM TGF-β1 and labeling with antibodies was exactly as in Fig. 6 (*D–F*). (*D*) The fluorescein image shows the patches of *c-myc*–tagged type III TGF-β receptors induced by the α-myc IgG and the secondary FITC-IgG GαMIgG. (*E*) The Cy3 image shows that the HA-tagged type III TGF-β receptors are also localized in patches. The long arrow points to examples of patches that contain both *c-myc*– and HA-tagged type III TGF-β receptors. (*F*) A double exposure of the fluorescein and Cy3 images show that most of the patches are yellow (e.g., *long arrow*) and contain both *c-myc*– and HA-tagged type III receptors. Similar results were obtained using 50 pM or 1 nM TGF-β1 or TGF-β2, or after adding a DSS cross-linking step prior to labeling with antibodies (not shown). Bar, 20 μm.

of c-myc- and HA-tagged type II receptors—were the same as in COS cells expressing ~10 times more type II receptor. Third, studies on co-immunoprecipitation of affinity-labeled types II and type III receptors expressed in transfected COS-7 cells gave results similar to those obtained using cells expressing normal amounts of the two receptors (López-Casillas et al., 1993; Moustakas et al., 1993). Furthermore, the fact that no significant amounts of type II/type III heterocomplexes were detected by immunofluorescence copatching experiments under identical conditions (Figs. 4 and 5) implies that the copatching is specific, and that the experimental conditions used do not lead to the generation of nonspecific aggregates of the TGF-β receptors. Our failure to detect homo-oligomers of affinity-labeled type III receptors by sequential immunoprecipitation, presumably due to the disruption of such complexes by the detergent used to solubilize the cell membranes, also attests to the specificity of formation of the observed complexes of the type II receptor on the surface of COS-7 cells and argues against the notion of induction of receptor complex formation by the molecular probes used here. Furthermore, the same experiments demonstrate that the oligomers formed are not simply mediated by the presence of the epitope tags or by the anti-tag antibodies, since otherwise they would also mediate the formation of type II/type III complexes, which were not observed in the immunofluorescence studies.

The homo-oligomeric state of TGF-βRIII is in accord with the report that the related glycoprotein endoglin (López-Casillas et al., 1991; Wang et al., 1991) is a disulfide-linked homodimer (Gougos and Letarte, 1990; Cheifetz et al., 1992). In the case of the type II receptor, formation of homo-oligomers is in agreement with reports that overexpression of a truncated kinase-deficient form of the receptor had dominant negative effects on TGF-β signaling (Brand et al., 1993; Chen et al., 1993; Wieser et al., 1993). These studies prompted the suggestion that the impairment in TGF-β signaling may be mediated by trans inhibition of autophosphorylation, implying that the receptor can form homo-oligomers. Thus, homo-oligomerization of the type II receptor may enable receptor trans autophosphorylation. Analogous findings were reported for a corresponding kinase-negative type II activin receptor (Hemmati-Brivanlou and Melton, 1992).

In view of the evidence for functional interactions between TGF-βRII and TGF-βRI (Wrana et al., 1992; Inagaki et al., 1993), it could be argued that type I receptors may stabilize the type II receptor oligomers. However, the low level of TGF-βRI in the COS-7 cells, relative to the overexpressed type II and/or type III receptors (Fig. 1 A) rules out this possibility, and indicates that formation of neither type II nor type III receptor homo-oligomers require TGF-βRI. Similarly, the type II/type III receptor heterocomplexes cannot contain stoichiometric amounts of TGF-βRI, in accord with recent reports (López-Casillas et al., 1993; Moustakas et al., 1993). It could also be argued that some other native cellular protein is associated with the observed receptor complexes and is essential for their formation (such a possibility pertains to any type of co-immunoprecipitation or copatching experiment, and therefore also applies to all previous work on TGF-β receptor complexes). In any case, such a candidate protein(s) would have to be expressed normally at high levels, and would have to remain associated with the types II and III receptors in the detergent solutions used for immunoprecipitation. Such a candidate protein could not be part of the extracellular matrix or cytoskeleton, since membrane proteins that stably interact with these structures are laterally immobile (Edidin, 1987; Jacobson et al., 1987), while the IgG-mediated shift of TGF-βRII and TGF-βRIII from a homogeneous to a patchy distribution demonstrates that the majority of these receptors are laterally mobile.

In the immunofluorescence copatching experiments demonstrating TGF-βRII homo-oligomerization, a large fraction of HA-TGF-βRII copatched with c-myc-TGF-βRII, but a significant amount of receptors that did not copatch was also evident (Fig. 6). This is suggestive of small oligomers, probably dimers or trimers. The reasoning is as follows: (a) If the receptor polypeptides were monomers, no copatching would be seen. (b) If the receptor polypeptides form dimers, and then 50% of the receptors would contain polypeptides with different epitope tags. Only such dimers will undergo copatching. (c) The fraction of oligomers capable of co-patching will increase with the number of subunits in the oligomer (75% for trimers, 87.5% for tetramers), since the only receptors that will not copatch are those in which all of the receptor subunits bear the same epitope tag. Although the copatching experiments are semi-quantitative, the fraction of green FITC-IgG patches that does not coincide with red Cy3-Fab' patches is estimated to be between 1/4 and 1/2 (e.g., Fig. 6), clearly above the very low level expected for a tetramer or a higher-order oligomer. Thus, if the cell–surface TGF-βRII population is homogeneous, these receptors most likely form dimers or trimers. However, the type II receptor population might be heterogeneous with a subpopulation that does not form homo-oligomers and thus does not copatch. In this case, it is possible that higher order receptor oligomers comprise the fraction that shows copatching. In the immunofluorescence copatching experiment demonstrating the oligomeric structure of the type III receptors (Fig. 7), it was not possible to discern non-coincident patches; however, the resolution in this particular experiment is lower in view of the inherent low efficiency of the anti-epitope monoclonal antibodies in inducing patching of the type III receptors. It is therefore difficult to draw conclusions concerning the possible size of the TGF-βRIII homo-oligomers.

The immunoprecipitation experiments on cells coexpressing types II and III TGF-β receptors demonstrated coprecipitation of the two receptor types affinity-labeled by TGF-β ligand (Fig. 1 B). These findings are in agreement with similar recent studies (López-Casillas et al., 1993; Moustakas et al., 1993) which detected ligand-induced TGF-βRII/TGF-βRIII heterocomplexes. The present study further explores these heterocomplexes by examining the bulk population of the receptors at the cell surface—and not only those cross-linked to the ligand—using immunofluorescence copatching experiments (Fig. 4 and 5). These studies revealed for the first time that the vast majority of the types II and III receptors do not reside in mutual complexes, even in the presence of ligand. Indeed, a 1:1 stoichiometry is unlikely in view of the fact that in most cells TGF-βRIII is much more abundant than TGF-βRII (Wang et al., 1991; López-Casillas et al., 1991). The percentage of the types II and III receptors residing in heterocomplexes is low—probably below 10%—since it cannot be detected in the copatching experiments. In any case, it must be well below the percentage of type II and type III receptors present as homo-oligomers, where copatching
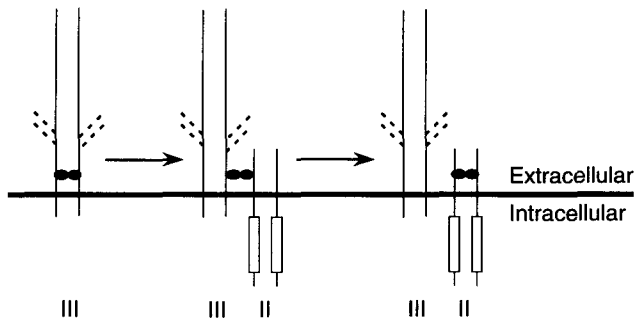
Figure 8. Model for interactions between homodimers of the type II and type III TGF-β receptors. The two receptors are drawn as dimers in the plasma membrane, in the absence or presence of TGF-β, and are labeled with Roman numerals. TGF-β is drawn as a filled double ellipse; the type II receptor is drawn with its cytoplasmic kinase domain as an open box; and the type III receptor is drawn with its extracellular glycosaminoglycan chains as dotted lines. The heteromeric type II/type III receptor complex is depicted in the middle and arrows represent a possible route of ligand-receptor interactions during initiation of the signal transduction pathway.

could be clearly detected. The presence of only a minor fraction of types II and III receptors in heterocomplexes is consistent with the proposal that the type III receptor enhances binding of TGF-β to the type II receptors, and that a complex comprised of TGF-βRIII, TGF-βRII, and ligand is an intermediate in "presentation" of the TGF-β ligand from the type III to the signaling type II and/or type I receptors (Fig. 8; López-Casillas et al., 1993). Our results suggest that the ternary complex of TGF-βRIII, TGF-βRII, and TGF-β may be a transient species in the plasma membrane, one whose steady state concentration is always very low.

Although the type I receptor does not appear to be required for the formation of either types II or III homo-oligomers or type II/type III heterocomplexes, it may interact with some of these preformed complexes, and these interactions are likely to play a role in signaling by the receptor complex. For example, displacement of TGF-βRIII from an intermediate complex, containing the type II and the type III receptors as well as TGF-β, by TGF-βRI could represent the pathway for formation of a stable signaling complex containing the type II and the type I receptors as well as TGF-β, as proposed by López-Casillas et al. (1993), except that the individual receptors may already be part of a homo-oligomer. That is, what were assumed in earlier work to be complexes of TGF-β with one type II and one type III (or one type II and one type I) receptor could actually be complexes of one TGF-β with one homodimer of a type II and one homodimer of a type III receptor. Direct association of TGF-βRI with preformed TGF-βRII homo-oligomers is also possible. Further experiments on the oligomeric structure of TGF-βRI and on its interactions with TGF-βRII and TGF-βRIII are required to address these issues. The existence of TGF-β receptor complexes of different compositions offers the potential for a wide repertoire of responses to the same family of ligands, depending on the equilibrium between the different receptor complexes. These in turn may regulate the multiple intracellular signals generated by TGF-β.

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