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Biochemical evidence for the autophosphorylation and transphosphorylation of transforming growth factor β receptor kinases

(transmembrane serine/threonine kinases/phosphorylation)

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ABSTRACT **Transforming growth factor β (TGF-β) signals through a receptor complex containing the type I (TGF-βRI) and type II (TGF-βRII) receptors. We describe here biochemical studies on early events in the TGF-β signaling pathway. TGF-βRII is highly phosphorylated when expressed alone in COS-1 cells; its autophosphorylation occurs via an intramolecular (cis) mechanism that is independent of ligand binding. TGF-βRI is also highly phosphorylated when expressed alone in COS-1 cells. Both wild-type TGF-βRI and a kinase-deficient mutant thereof are transphosphorylated by the coexpressed TGF-βRII kinase in a ligand-independent fashion in these cells. We propose that the association of TGF-βRI and TGF-βRII, induced by ligand binding or overexpression, leads to transphosphorylation of the TGF-βRI by the TGF-βRII kinase. This represents a mechanism of activation of receptors distinct from that of tyrosine kinase receptors and may apply to other serine/threonine kinase receptors.**

Proteins belonging to the transforming growth factor β (TGF-β) family are prototypes of a large superfamily of polypeptides that also include Müllerian inhibiting substance, inhibins, activins, and several other morphogens (Vg, Dpp, BMPs) (1). The most well documented of TGF-β's effects on cells is its ability to act as a potent growth-inhibitory compound for a wide variety of cell types (2, 3). While this and other biological functions of TGF-β have been intensively described over the past decade, the biochemical mechanisms that underly these effects remain poorly understood.

Genetic studies on mutant cell lines expressing defective TGF-β receptors (TGF-βRs) and biochemical evidence from crosslinking experiments have suggested that the most widely distributed high-affinity type II (TGF-βRII) and type I (TGF-βRI) receptors are the key players in the complex that relays signals from TGF-β through the plasma membrane into the cell (4–6). Recent characterization and molecular cloning of these two receptors have now provided a basis for understanding the signal transduction pathways through which TGF-β acts (7–13). Among other insights, these studies have shown that both TGF-βRI and TGF-βRII belong to a subfamily of cell surface proteins with a cysteine-rich extracellular domain, a single transmembrane region, and a putative cytoplasmic serine/threonine kinase domain (14–16).

The structure of these receptors suggests analogies with the well-studied mitogen receptors that are similarly structured. One essential difference, however, is the cytoplasmic kinases associated with mitogen receptors, which are invariably tyrosine-specific. Ligand-induced autophosphorylation and resulting functional activation have been established as the basis of the transduction of biological signals through such tyrosine kinase receptors (17). For this reason, we undertook to examine whether TGF-βRs signal in a mode analogous to that of the tyrosine kinase receptors.

Others have reported that both type I and type II receptor kinases are capable of autophosphorylation *in vitro* (8, 13). Here we characterize the phosphorylation of both receptors *in vivo*. Recently, Wrana et al. (18) reported a study on the mechanism of activation of the TGF-βR; however, our present results differ in certain aspects from their results.

MATERIALS AND METHODS

Construction of Expression Vectors. The human TGF-βRII cDNA (H2-3FF clone; ref. 8) and its hemagglutinin (HA)-tagged version (19) were subcloned into expression vector Rc/CMV (Invitrogen). The human TGF-βRI cDNA (ALK-5 clone; ref. 10) was HA-tagged at the C terminus and subcloned into expression vector CMV7 (20). The kinase-deficient mutant receptors, TGF-βRIIK277R and TGF-βRIK232R, were generated by site-directed mutagenesis using PCR-based strategies. The coding regions containing the mutations and epitope tags were verified by DNA sequencing. pGEX-RIICt and pET-RIICt encoding fusion proteins containing the intracellular domain of TGF-βRII were generated by using vectors pGEX-2T (21) and pET-5 (Novagen).

Cell Culture and Transfection. COS-1 cells (CRL 1650; American Type Culture Collection) were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) (GIBCO/BRL). Cells were transiently transfected with the expression vectors described above using a DEAE-dextran method (22) and metabolically labeled as indicated 36–48 hr after transfection.

Generation of Monoclonal Antibodies. The fusion protein pGEX-RIICt was purified from *Escherichia coli* as described by Smith and Johnson (21) and subsequently used to immunize BALB/c mice. The fusion protein pET-RIICt purified from inclusion bodies was used in Western blot analysis to test tail bleeds. Hybridomas were made and screened by ELISA. Monoclonal antibody 5c specifically reacted with TGF-βRIICt and was further characterized and used in this study.

Immunoprecipitation and Deglycosylation. Monoclonal antibodies, 5c (anti-TGF-βRII) described above and 12CA5 (anti-HA) purchased from Babco (Richmond, CA), were used for immunoprecipitation of TGF-βRs. For ^{35}S-labeling, transfected COS-1 cells were incubated with [^{35}S]methionine at 100 μCi/ml (1 Ci = 37 GBq) at 37°C for 4 hr and lysed in 1 ml of ice-cold buffer A [50 mM Tris·HCl, pH 7.4/150 mM NaCl/1% Nonidet P-40/0.5% deoxycholate/aprotinin (10 μg/ml)/

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Abbreviations: TGF-β, transforming growth factor β; TGF-βR, TGF-β receptor; HA, hemagglutinin; endo H, endoglycosidase H; GST, glutathionine *S*-transferase; EGFR, epidermal growth factor receptor.

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leupeptin (10 μg/ml)/1 mM phenylmethylsulfonyl fluoride/pepstatin A (1 μg/ml)/soybean trypsin inhibitor (10 μg/ml)/1 mM dithiothreitol]. Supernatants were collected by centrifugation and SDS was added from a stock solution to give a final concentration of 0.1%. Lysates were precleared with protein A-Sepharose and incubated with appropriate antibodies at 4°C for 2 hr. Immunocomplexes were recovered on protein A-Sepharose saturated with rabbit anti-mouse IgG (Jackson ImmunoResearch) and then subjected to SDS/PAGE on 7.5% gels and autoradiography. For ^{32}P-labeling, cells were incubated in phosphate-free medium containing [^{32}P]orthophosphate at 500 μCi/ml at 37°C for 4 hr. Immunoprecipitation was conducted as described above except that phosphatase inhibitors (10 mM β-glycerophosphate/5 mM NaF/10 mM pNpp/500 μM Na_3VO_4) were included. For deglycosylation, immunoprecipitates were treated with endoglycosidase H (endo H) or PNGase F (also known as N-glycosidase F; New England Biolabs) as described by the manufacturer.

Tryptic Phosphopeptide Mapping. Tryptic phosphopeptide mapping was performed as described by Boyle *et al.* (23) on an LKB 2117 Mutiphor II electrophoresis unit. Thin-layer electrophoresis and chromatography were developed in pH 1.9 buffer and phosphochromatography buffer, respectively.

RESULTS

***In Vivo* Phosphorylation of TGF-βRII in Transiently Transfected COS-1 Cells.** The expression level of TGF-βRs in mammalian cells is normally very low, complicating most biochemical studies. For this reason, both full-length TGF-βRII and mutant derivatives were transiently overexpressed in COS-1 African green monkey kidney cells by using cytomegalovirus-based vectors. To distinguish between autophosphorylation of this receptor and its phosphorylation by other cellular kinases, a kinase-deficient mutant receptor was also constructed by introducing a single amino acid substitution (K277R) at the ATP-binding site of the type II receptor kinase domain. When immunoprecipitated from ^{32}P-labeled transfected COS-1 cells, only the wild-type TGF-βRII appeared as a highly phosphorylated species although the mutant TGF-βRIIKR species were equally well expressed, indicating that the kinase activity of TGF-βRII is required for its own phosphorylation. Furthermore, the overall level of TGF-βRII phosphorylation was unaffected by the treatment with 400 pM TGF-β_1 for various times prior to lysis, suggesting that the TGF-βRII can function as a constitutively active ligand-independent autophosphorylating kinase *in vivo* (Fig. 2 and data not shown).

It remained possible that TGF-β was able to induce some changes in the spectrum of sites phosphorylated on TGF-βRII; therefore, tryptic phosphopeptide mapping was performed. The initial analysis of TGF-βRII in the absence of ligand revealed eight major phosphopeptides as indicated in Fig. 1*D*, with spots 2, 3, and 5 having higher intensities than the others. We repeatedly noted that spot 1 disappeared when cells had been treated with TGF-β prior to lysis (Fig. 1 *A* and *B*), suggesting that the phosphorylation of a minority of residues (represented by spot 1) may well be influenced by the presence of ligand.

Previous work (8) had shown that a glutathione *S*-transferase (GST) fusion protein containing only the intracellular domain of the TGF-βRII (GST–RIICt) was capable of autophosphorylation *in vitro*. This afforded us the opportunity of comparing the residues of the TGF-βRII labeled *in vitro* with those phosphorylated in the living cells. As shown in Fig. 1*C*, the major phosphopeptides seen upon *in vitro* labeling (spots 2–5) are very similar to those derived from the full-length TGF-βRII phosphorylated *in vivo*. We also noted the absence of spots 1 and 6 on GST–RIICt. This reinforced the earlier conclusion (8) that most of the phosphorylation of the TGF-βRII observed *in vivo* is due to autophosphorylation.

Biochemical Mechanism of TGF-βRII Autophosphorylation. The apparent autophosphorylation of the TGF-βRII intracellular domain may in fact be explained by two distinct mechanistic models. On the one hand, two kinase domains brought in close juxtaposition may be able to phosphorylate one another in a bimolecular reaction. Such transphosphorylation is postulated to play a central role in the ligand-mediated activation of tyrosine kinase mitogen receptors (17). Alternatively, a single kinase domain may be able to phosphorylate itself in a monomolecular reaction.

To resolve these two possibilities, we attached an epitope tag (HA) to the inactive mutant kinase as described earlier and studied the interactions between this tagged mutant receptor and the wild-type receptor. Immunoprecipitation with mono-

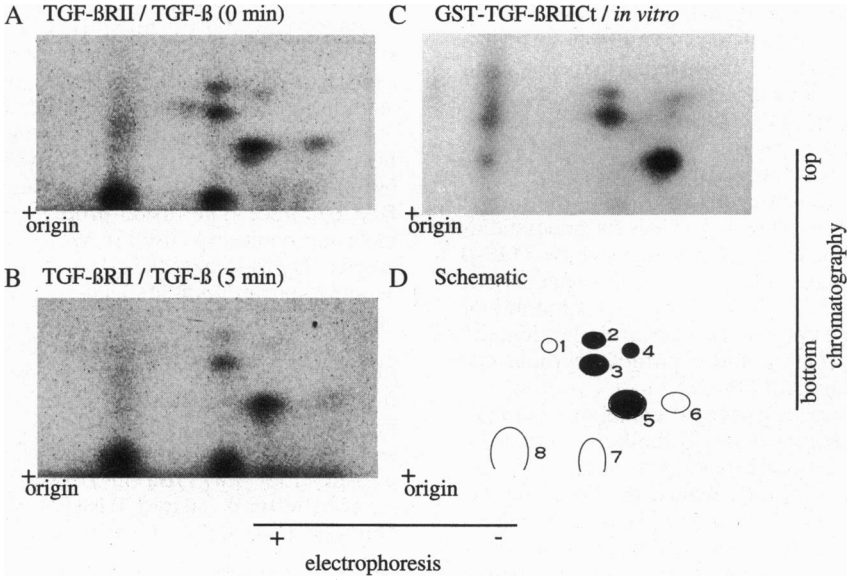

FIG. 1. Tryptic phosphopeptide maps of TGF-βRII. (*A*) ^{32}P-labeled TGF-βRIIWT molecules were immunoprecipitated from transfected COS-1 cells with monoclonal antibody 5c and subjected to phosphopeptide analysis (PPA). (*B*) Same as in *A*, except that cells were treated with 400 pM TGF-β_1. (*C*) GST fusion protein (GST–TGF-βRIICt) containing kinase domain of TGF-βIIRWT was labeled with [γ-^{32}P]ATP *in vitro* and subjected to PPA. (*D*) Schematic for tryptic phosphopeptide map of TGF-βRII. Specific phosphopeptides referred to in the text are indicated.

clonal antibody 5c, which recognizes the tagged mutant (IIKR-HA) and untagged wild-type (IIWT) receptor equally well, revealed equivalent high levels of both proteins in transfected COS-1 cells (Fig. 2 *A* and *B*). Only a small portion of each of the two receptor forms was digested by endo H, indicating that the side chains of both were efficiently processed into complex carbohydrate-containing forms (Fig. 2*A*, lanes 2, 3, 4, and 5). The slower migration rate of the tagged receptor allowed us to resolve it from the wild-type receptor upon gel electrophoresis. As shown in Fig. 2*B*, treatment with N-glycosidase F resulted in a sharpening of the bands representing the two receptor forms.

Cells expressing the untagged wild-type and/or the tagged mutant receptors were labeled metabolically with $^{32}P_i$. Lysates were then incubated with monoclonal antibody 5c (anti-TGF-βRII) or 12CA5 (anti-HA). As shown in Fig. 2*C*, lanes 9 and 10, the mutant receptor was not phosphorylated by the coexpressed wild-type receptor, even in the presence of ligand. Control experiments (Fig. 2*C*, lanes 11–20) indicated that addition of an HA tag to the wild-type receptor had no effect on its state of phosphorylation. Moreover, other amino acid substitutions in the ATP-binding site of TGF-βRII yielded identical results (data not shown).

Thus, these results strongly suggested that autophosphorylation of the TGF-βRII occurs via a cis (intramolecular) mechanism even though the TGF-βRII is capable of homooligomerization when overexpressed in COS-1 cells (19, 24). This indicates fundamental differences that distinguish the mechanism of activation of the TGF-βRII from that used by mitogen-activated tyrosine kinase receptors.

Transphosphorylation Between TGF-βRI and TGF-βRII in COS-1 Cells. Genetic studies on chemically mutagenized cells (4, 5) and tumor cell lines (6) resistant to TGF-β have suggested that both functional type I and type II receptors are essential for TGF-β signal transduction. In addition, a physical association between type I and type II receptors has been demonstrated by coprecipitation after chemical crosslinking with radiolabeled TGF-β (5, 6, 10, 11). The recent cloning of type I receptor has enabled study of the interactions between TGF-βRI and TGF-βRII in detail.

As was done earlier (5, 13) with the TGF-βRII, we generated a defective kinase of the TGF-βRI by introducing a single amino acid substitution in its ATP-binding site. Both the wild-type and mutant receptor (TGF-βRIWT and TGF-βRIKR) were HA-tagged at their C termini and expressed in COS-1 cells. As shown in Fig. 3*A*, only the wild-type TGF-βRI was highly phosphorylated, indicating TGF-βRI, like TGF-βRII, is capable of autophosphorylation *in vivo* (lanes 1–3). Furthermore, the specific activity of TGF-βRI autophosphorylation was comparable to that of TGF-βRII when the [^{32}P]orthophosphate and [^{35}S]methionine labeling intensities were compared (data not shown). These data contrast with a recent report (18) that the level of phosphorylation of TGF-βRI singly transfected in Mv1Lu R-1B cells was very low.

The presence of the mutant or wild-type TGF-βRI had no apparent effect on the constitutive autophosphorylation of TGF-βRII in the presence or absence of ligand (Fig. 3 *B* and *C* and data not shown), suggesting that TGF-βRII is unlikely to be a substrate of the TGF-βRI kinase. We then attempted to explore the alternative possibility, namely, that TGF-βRI can be phosphorylated by the TGF-βRII kinase. As shown in Fig. 3*B*, TGF-βRIWT-HA was coexpressed with either TGF-βRIIWT or TGF-βRIIKR in COS-1 cells. The kinase-deficient TGF-βRIIKR did not alter the autophosphorylation pattern of TGF-βRI detectably (Fig. 3*B*, lanes 3, 4, 7, and 8) compared to that of TGF-βRI expressed alone in COS-1 cells (Fig. 3*A*, lane 2 and 5), suggesting that the kinase activity of TGF-βRII did not contribute to the autophosphorylation of TGF-βRI. Interestingly, coexpression of wild-type TGF-βRII gave rise to a slowly migrating form of TGF-βRI in addition to its autophosphorylated species in the presence or absence of ligand (Fig. 3*B*, lanes 1 and 2), indicating that TGF-βRI could be transphosphorylated by the TGF-βRII kinase. Furthermore, the autophosphorylation of wild-type TGF-βRI was reduced upon coexpression of wild-type TGF-βRII (Fig. 3*B*, lanes 1–4). This phenomenon, which we did not explore further, suggests that type II receptor is capable of suppressing the intrinsic autophosphorylation activity of the type I receptor. One possible explanation for this is that TGF-βRI can serve as a substrate both for TGF-βRI kinase itself in an autophosphorylation reaction and for the TGF-βRII kinase in a transphosphorylation reaction; accordingly, what we observed here may represent a competition between the two kinases.

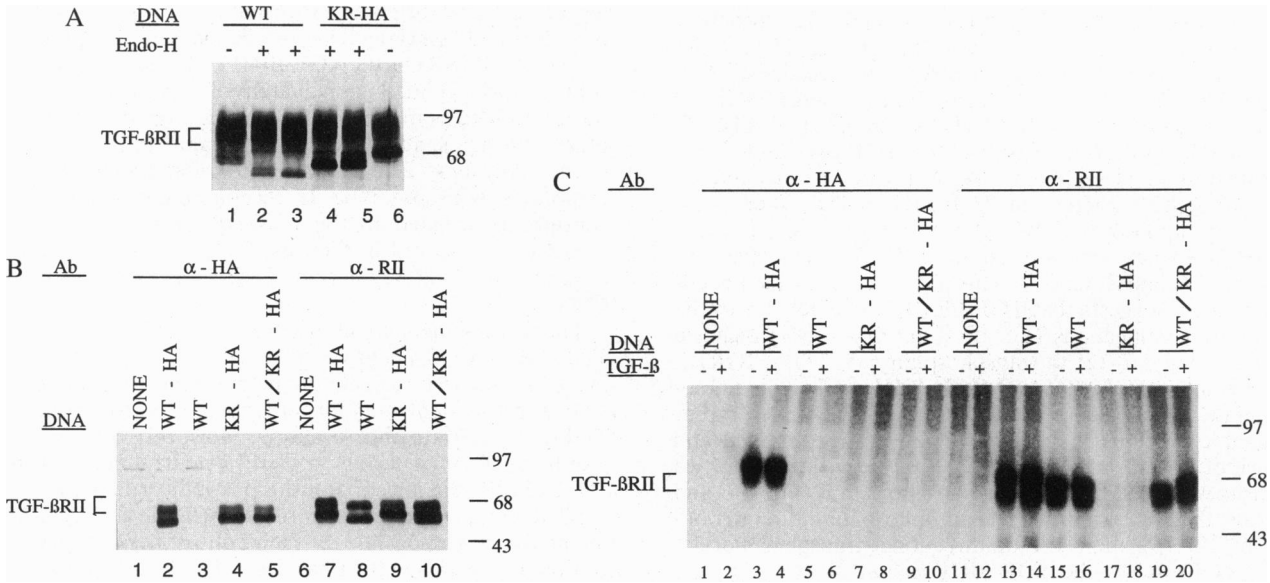

FIG. 2. Biochemical mechanism of TGF-βRII autophosphorylation. (*A*) Immunoprecipitates of TGF-βRIIWT and TGF-βRIIKR-HA from [^{35}S]methionine-labeled COS-1 cells were treated with (+) or without (−) endo H as indicated. (*B*) Coexpression of TGF-βRIIWT and TGF-βRIIKR-HA in [^{35}S]methionine-labeled COS-1 cells assayed by immunoprecipitation and N-glycosidase F treatment. (*C*) *In vivo* phosphorylation of TGF-βRIIWT and TGF-βRIIKR in the presence (+, 10 min) or absence (−, 0 min) of TGF-β_1 (400 pM). None, mock transfection; Ab, antibody; α, anti.

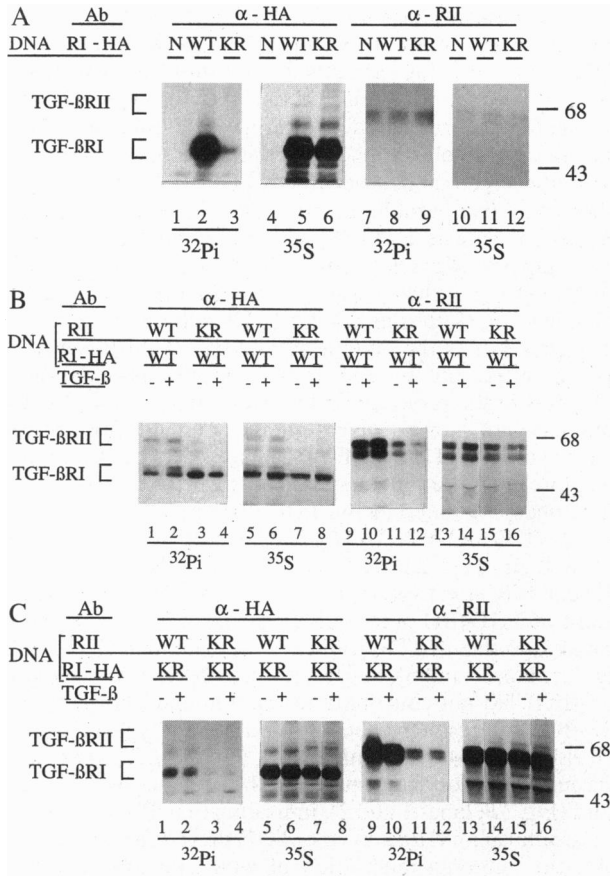

FIG. 3. Transphosphorylation between TGF-βRI and TGF-βRII. (*A*) COS-1 cells transfected with HA-tagged TGF-βRIWT or TGF-βRIKR alone were metabolically labeled with [^{35}S]methionine or [^{32}P]orthophosphate and immunoprecipitation was performed. (*B*) HA-tagged wild-type TGF-βRIWT was cotransfected with either wild-type (WT) or kinase-deficient (KR) TGF-βRII in COS-1 cells. Metabolic labeling was followed by TGF-β_1 treatment (400 pM; +, 10 min; −, 0 min) and immunoprecipitation. (*C*) Same as in *B*, except that HA-tagged kinase-deficient TGF-βRIKR was used in the cotransfections.

To eliminate the background level of TGF-βRI autophosphorylation in the experiment described in Fig. 3*B*, the defective kinase TGF-βRIKR-HA construct was used instead. It is clear that TGF-βRIKR-HA could be transphosphorylated by wild-type TGF-βRII even in the absence of ligand (Fig. 3*C*, lanes 1 and 2), indicating, as before, that TGF-βRI could serve as a substrate of TGF-βRII kinase. We also noted a decrease in the phosphate content of TGF-βRIKR-HA when coexpressed with TGF-βRIIKR compared to that of TGF-βRIKR-HA when expressed alone in COS-1 cells (Fig. 3*C*, lanes 3 and 4, and *A*, lane 3). This phenomenon, which needs further study, suggests that TGF-βRIIKR interferes with the ability of the endogenous TGF-βRII in COS-1 cells (as shown in Fig. 3*A*, lanes 7–12) to transphosphorylate TGF-βRIKR.

Importantly, TGF-βRI and TGF-βRII could coimmunoprecipitate when coexpressed in COS-1 cells under rather stringent conditions of cell lysis both in the presence and absence of ligand; moreover, the kinase activities of the two receptors were not required for this association (Fig. 3 *B* and *C*). Thus, these data indicate the autophosphorylation of both types of TGF-βRs and the unidirectional transphosphorylation of TGF-βRI by the TGF-βRII kinase.

DISCUSSION

The results presented above revealed significant differences in the biochemical mechanism(s) of phosphorylation and activation that distinguish the TGF-βRs from receptors with tyrosine kinase activity. TGF-βRII is able to autophosphorylate in a ligand-independent manner; this is in agreement with a recent study using Mv1Lu cells (18). Indeed, ligand-independent autophosphorylation of the activin type II receptor has also been reported (25), suggesting that this mechanism will apply to all receptors of this class. Use of both epitope tagging and site-directed mutagenesis has yielded evidence indicating that TGF-βRII autophosphorylation occurs as an intramolecular reaction, a process quite distinct from that of tyrosine kinase receptors, in which bimolecular transphosphorylation appears to operate (26, 27).

We have also found that ligand binding does not affect the autophosphorylation of TGF-βRII but does cause a decrease in its phosphorylation presumably by other cellular kinases. This indicates that the type II receptor undergoes a functional change in response to ligand. The physiologic significance of this in the signaling process is unclear at present.

The two components involved in initiating the TGF-β signal transmission, TGF-βRI and TGF-βRII, appear to interact asymmetrically, in that TGF-βRII is able to transphosphorylate TGF-βRI whereas the reverse mechanism does not seem to operate (Fig. 3). This contrasts with the behavior of the tyrosine kinase receptors, either homogenous receptors, such as those for epidermal growth factor (EGFR) or fibroblast growth factor (FGFR) (17), or closely related receptors of mixed subunit composition such as InsR/EGFR (27) and Neu/EGFR (28), whose activation appears to involve bidirectional transphosphorylation by both associated partners. This evidence provides support to the recent work in Mv1Lu cells by others (18).

Our results are discordant with the report by Wrana *et al.* (18) in two important respects. (*i*) We have found that TGF-βRI is autophosphorylated *in vivo* when expressed alone in COS-1 cells with a specific activity comparable to that of TGF-βRII (Fig. 3*A*). In contrast, in transfected Mv1Lu R-1B cells, the basal level of phosphorylation of TGF-βRI expressed alone was reported to be very low (18). We are unable to explain this discrepancy at present.

(*ii*) This other report (18) provided evidence that the transphosphorylation of the type I receptor by the type II kinase occurs only in a heteromeric complex that is assembled and stabilized by ligand. However, we have observed the ligand-independent complex formation of type I and type II receptors and associated ligand-independent transphosphorylation of TGF-βRI by the TGF-βRII kinase (Fig. 3). The other studies (18) depended upon analysis of only the fraction of the receptor proteins that are purified as detergent-resistant complexes through sequential chromatography in the presence of ligand. Any type I receptors that would fail to form such complexes with the type II receptors but might well be transphosphorylated in the presence or absence of ligand would not be scored in their assay. In contrast, our analysis examined the bulk population of the TGF-βRs in transfected COS-1 cells.

The ligand-independent association and phosphorylation of TGF-βRs in COS-1 cells we observed here might be explained in two ways. (*i*) The cell culture medium used to propagate the COS-1 cells might contain sufficient background levels of TGF-β to activate the receptors. However, control experiments performed using a TGF-β-neutralizing antibody, 1D11.16 (29), did not support this possibility (data not shown). (*ii*) The great overexpression of TGF-βRs in the COS-1 cells might also be responsible for their constitutive autophosphorylation; just such an effect has been observed with the erbB2/neu receptor (30, 31).

Others have reported (4–6) that both types of TGF-βRs are required for downstream signaling. Moreover, TGF-βRI appears to be capable of binding ligand only in the presence of TGF-βRII (10, 11). Thus, these observations indicate that

TGF-β induces ternary (or higher order) complexes that include ligand and receptor subunits of both types. At low physiologic levels of receptor expression, TGF-β may be required to bring the two receptor molecules into close proximity to assemble and stabilize the complexes; such association may be achieved in a ligand-independent fashion at high levels of receptor expression used in our assays. We note that work using a yeast two-hybrid expression system indicates an intrinsic affinity of type I and type II kinase domains for each other in the absence of extracellular domains and ligand (R. Perlman and R.A.W., unpublished observations).

Our present results and those of others (18) cause us to propose that the association of TGF-βRI and TGF-βRII, induced either by ligand binding or overexpression, leads to transphosphorylation of the TGF-βRI by the TGF-βRII kinase. This in turn may allow one or both of the associated kinases to direct their activities toward downstream substrates. Such a mechanism will only be fully vindicated when we are able to measure receptor kinase activities and the phosphorylation of receptor molecules when they are expressed at physiologic levels.

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