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PRINCIPAL INVESTIGATOR: Kunxin Luo, Ph.D.

CONTRACTING ORGANIZATION: Whitehead Institute for
Biological Research
Cambridge, Massachusetts 02142

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Cambridge, Massachusetts 02142

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FOREWORD

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Introduction:

The principal goal of this project is to understand the transforming growth factor-β (TGFβ) receptor signal transduction pathways and the molecular mechanism underlying the regulation of the activity of the TGFβ receptor kinases. TGFβ could suppress the growth of breast cancer cells both *in vivo* and *in vitro* (1, 2, 3), and this function requires the expression of functional TGFβ receptors (2, 3) and downstream signaling molecules (4).

The TGFβ family of cytokines has a wide range of biological functions including tumor suppression, extracellular matrix production, embryonic development, and regulation of differentiation(5). These functions are mediated by three specific surface receptors, Types I, II and III, all of which have been cloned (6, 7, 8, 9). The types I and II receptors for TGFβ, TβRI and TβRII, are members of the first known receptor serine/threonine kinase family, and share 40% homology between their kinase domains. TβRII contains an extracellular domain which binds TGFβ, a transmembrane domain and a cytoplasmic domain with serine/threonine kinase activity. TβRI also has an extracellular domain even though it does not bind TGFβ when expressed without TβRII. The cytoplasmic portion of TβRI contains a kinase domain and a membrane proximal region which contains a Gly-Ser rich sequence (GS box) that has been proposed to be important for the activation of TβRI (10). Both receptors exist normally as homodimers on the cell surface (11, 12) and their kinase activities are essential for signal transduction (6, 8, 9, 13). Binding of TGFβ1 to TβRII induces the formation of a heteromeric complex of TβRI and TβRII (6, 8, 9, 13), which results in transphosphorylation of TβRI by the constitutively active TβRII (14). Phosphorylation of TβRI presumably activates its kinase activity and allows phosphorylation of downstream substrates such as Smad2 (15, 16, 17) and Smad3 (18). The phosphorylated Smad proteins then translocate to nucleus (18) and presumably, with the help of a DNA-binding molecule, activate downstream gene expression. Phosphorylation of serine residues at the carboxyl-terminal of Smad proteins is required for the nucleus translocation of Smad and subsequent growth inhibition. Mutations of these serine residues to alanine result in a Smad protein that is dominant negative in growth inhibition (16). The carboxyl half of Smad has transcriptional activating activity. Overexpression of this transactivating domain results in a Smad that is constitutively active in signal transduction (4, 19, 20).

As an initial step towards a better understanding of the multiple signaling pathways of the TGFβ receptors, we have examined the regulation of the kinase activity and signal transduction of TβRII by autophosphorylation. We have shown that TβRII kinase is intricately regulated by autophosphorylation on at least three serine residues (21). Ser213, in the membrane-proximal segment outside the kinase domain, undergoes intra-molecular autophosphorylation which is

essential for the activation of TβRII kinase activity, activation of TβRI, and TGFβ-induced growth inhibition. In contrast, phosphorylation of Ser409 and Ser416, located in the substrate recognition T-loop region in a three-dimensional structure model of protein kinases, is enhanced by receptor dimerization and can occur via an inter-molecular mechanism. Phosphorylation of Ser409 is essential for TβRII kinase signaling, while phosphorylation of Ser416 inhibits receptor function. Mutation of Ser416 to alanine results in a hyperactive receptor that is more able than wild-type to induce TβRI activation and subsequent cell cycle arrest. Both S409 and S416 are located in the substrate binding T-loop in the kinase domain and may invovle in interaction with substrates.

In addition to these three sites, there is still another major site of autophosphorylation in TβRII whose identity was still not clear at the beginning of the fellowship period. This report focuses on the identification of the last major autophosphorylation site in TβRII and cahracterization of its function in signal transduction leading to growth inhibition. I also investigated downstream signaling events affected by autophosphorylation of TβRII.

Body

Experimental approach:

1. mapping the sites of autophosphorylation in TβRII: To map the last major autophosphorylation sites in TβRII, Point mutations of individual serine residues in the cytoplasmic domain of TβRII were also carried out by PCR, and each mutant was analyzed by two-dimensional tryptic peptide mapping (21).

2. Analysis of the ability of TβRII containing mutations in the autophosphorylation sites to mediate growth inhibition: Mutant receptors were stably introduced into the pro-B Ba/F3 cells as chimeric receptor with the extracellular and transmembrane domains of erythropoietin receptor (EpoR) and cytoplasmic domain of TβRII. Infected cells were incubated with various concentrations of Epo for 4 days and the growth of cells were measured by cell counting.

3. Analysis of the ability of mutant TβRII to transphosphorylate TβRI: Ba/F3 cells containing mutant receptors were stimulated with or without Epo, and the receptor complex was isolated by immunoprecipitation. The ability of mutant TβRII to phosphorylate TβRI was measured in an in vitro kinase assay (21).

4. Analysis of the abilities of mutant TβRII to induce phosphorylation of Smad2: Receptor complex containing wt and mutant TβRII and wt TβRI were isolated form transfected 293T cells by immunoprecipitation, and subjected to in vitro kinase assay in the presence of 32P-ATP and purified Smad2 protein. Phosphorylated Smad2 was then resolved by SDS-PAGE.

Results and Discussions:

In addition to S213, S409 and S416, TβRII kinase undergoes autophosphorylation on S486.

Using mutagenesis analysis, I have identified the fourth major site of autophosphorylation to be S486 (Figure 1) which is located in the kinase domain. Mutation of S486 t o alanine did not significantly affect the autophosphorylation activity of TβRII.

When compared in a three-dimensional structure of protein kinase, S486 is found to locate in a region unique to TβRII, indicating that this residue may have a regulatory function unique to TβRII. When compared with other kinases of the TGFβ receptor family, S486 is also unique to TβRII and not present in TβRI and activin receptor or BMP receptor kinases.

Function of autophosphorylation on S486 in growth inhibition:

In contrast to S213, S409 an S416 whose phosphorylation affects kinase activity and signal transduction of TβRII significantly, mutation of S486 to alanine did not affect the kinase activity of TβRII, nor its ability to mediate growth inhibition. The growth of cells expressing the S486A mutatn is indistinguishable from that of cells expressing the wt receptor (Figure 2).

Although S486 is not required for growth inhibition by TβRII, it may still be important for other aspects of TβRII signal transduction or regulation of TβRII activity. In addition to growth inhibition, TGFβ can also induce gene activation and regulation of differentiation. Phosphorylation at S486 may play a role in the signaling activities leading to these functions. Receptor autophosphorylation has also been shown to be invovled in receptor downregulation in the case of some tyrosine kinases. It is not clear whether receptor serine kinases employ similar mechanism of regulation. Since TβRII has very short half life on the cell surface (30 min), downregulation of TβRII is obviously a very important step of regulation. We will test the function of these autophosphorylated serine residues in the downregulation of TβRII.

Effects of TβRII autophosphorylation on the phosphorylation of Smad2: Since Smad2 has been shown to be a direct substrate of TGFβ receptor complex and is required for signal tranduction leading to growth inhibition, I decided to examine the effects of receptor autophosphorylation on the phosphorylation of Smad2 (Figure 3). Wild-type TβRII, when complexed with TβRI, can induce phosphorylation of Smad2 in an in vitro kinase assay. In contrast, receptor complex containing S213A failed to phosphorylate Smad2. This is consistent with the previous finding that S213A mutant is defective in kinase activity and can not transphosphorylate and activate TβRI. Therefore, the inactive receptor complex can not phosphorylate Smad2. Also consistent with the growth inhibition result, phosphorylation at S409 is also required for the phosphorylation of Smad2, since mutation of S409 to alanine failed to induce phosphorylation of Smad2. Although we have shown previously that S409A mutant is capable of transphosphorylating TβRI, this phosphorylation did not lead to activation of TβRI, as manifested by its inability of phosphorylate Smad2. This indicates that phosphorylation of TβRI does not always lead to activation of its kinase activity.

S416A mutant is hyperactive in promoting growth inhibition, and also induces a greater level of Smad2 phosphorylation. S486A mutant in indistinguishable from the wt receptor in its ability to mediate growth inhibition, and it can induce phosphorylation of Smad2 to a level similar to that induced by the wt TβRII. Therefore, the ability of these autophosphorylation mutants to induce Smad2 phosphorylation correlates perfectly with their abilities to mediate growth inhibition.

This again confirms the notion that Smad2 plays an important role in signal transduction leading to growth inhibition.

Autophosphorylation of TβRII occurs both intra- and inter-molecularly: To investigate whether autophosphorylation at S486 occurs via an intra- or inter-molecular machanism, a kinase inactive TβRII was coexpressed in cells with the S486A mutant. If autophosphorylation at S486 occurs via an intra-molecular mechanism, after in vitro autophosphorylation, no phosphorylation can be observed, since the kinase inactive TβRII can not phosphorylate itself and S486A mutant no longer has the site to be phosphorylated. However, if autophosphorylation occurs via an inter-molecular mechanism, the kinase activity of S486A mutant could transfer the phosphate to the serine residue still present on the kinase inactive mutant.

I have found that upon cotransfection of the kinase inactive and S486A TβRII, peptide containing S486 was detected on a tryptic peptid map, suggesting that phosphorylation at S486 can occur via an inter-molecular mechanism.

The inter-molecular mechanism suggests an important function for a TβRII homodimer on the cell surface (11, 12). Despite the fact that TβRII homodimer alone is not sufficient for signal transduction, homodimerization of TβRII may play an important role in the regulation of TβRI activation. Dimerization of the RII cytoplasmic domain leads to increased phosphorylation of Ser409 or Ser416, which, in turn, modulates TβRI activation. Since phosphorylation of the two serine residues have opposite effects and since both are potential substrates for other cellular kinases, it is possible that, by regulating the ratio of phosphorylation at these two serines in response to ever-changing cellular environment, the extent of substrate phosphorylation can be fine-tuned in an accurate and reversible manner. In particular, the ratio of phosphorylation at Ser409 and Ser416 could alter in response to TGFβ stimulation and this may provide a mechanism for the up or down regulation of receptor signal transduction.

Our results show that TβRII phosphorylation is intricately regulated and affects TGFβ receptor signal transduction both positively and negatively. Therefore, the regulation of TβRII is not as simple as had been thought before. Multiple autophosphorylation events have been observed in receptor tyrosine kinases including PDGF receptor (22) and CSF-1 receptor (23). These phosphorylated tyrosine residues mediate interactions with different downstream signaling molecules and thus provide a mechanism that enables one receptor to initiate multiple signaling pathways. Since TGFβ receptor can mediate complex biological responses, autophosphorylation on multiple serine residues may serve as a possible mechanism for the activation of different signal transduction pathways leading to these responses. Although identified as

autophosphorylation sites, these serine residues may also serve as targets for other cellular kinases, and thus the activity of TβRII could be up- or down-modulated in response to an array of intracellular signals. The identification of these important serine residues serves as a starting point to further dissect the regulatory mechanisms and signal transduction pathways of the TGFβ receptors.

Figure 1. TβRII is autophosphorylated on S486. Mutant TβRII containing a point mutation changing S486 to A was isolated from transfected 293T cells by immunoprecipitation, subjected to autophosphorylation in vitro in the presence of γ32P-ATP and analyzed by two dimensional tryptic peptide mapping. The arrow indicates the origin of loading. Spot c represent peptide containing S486.

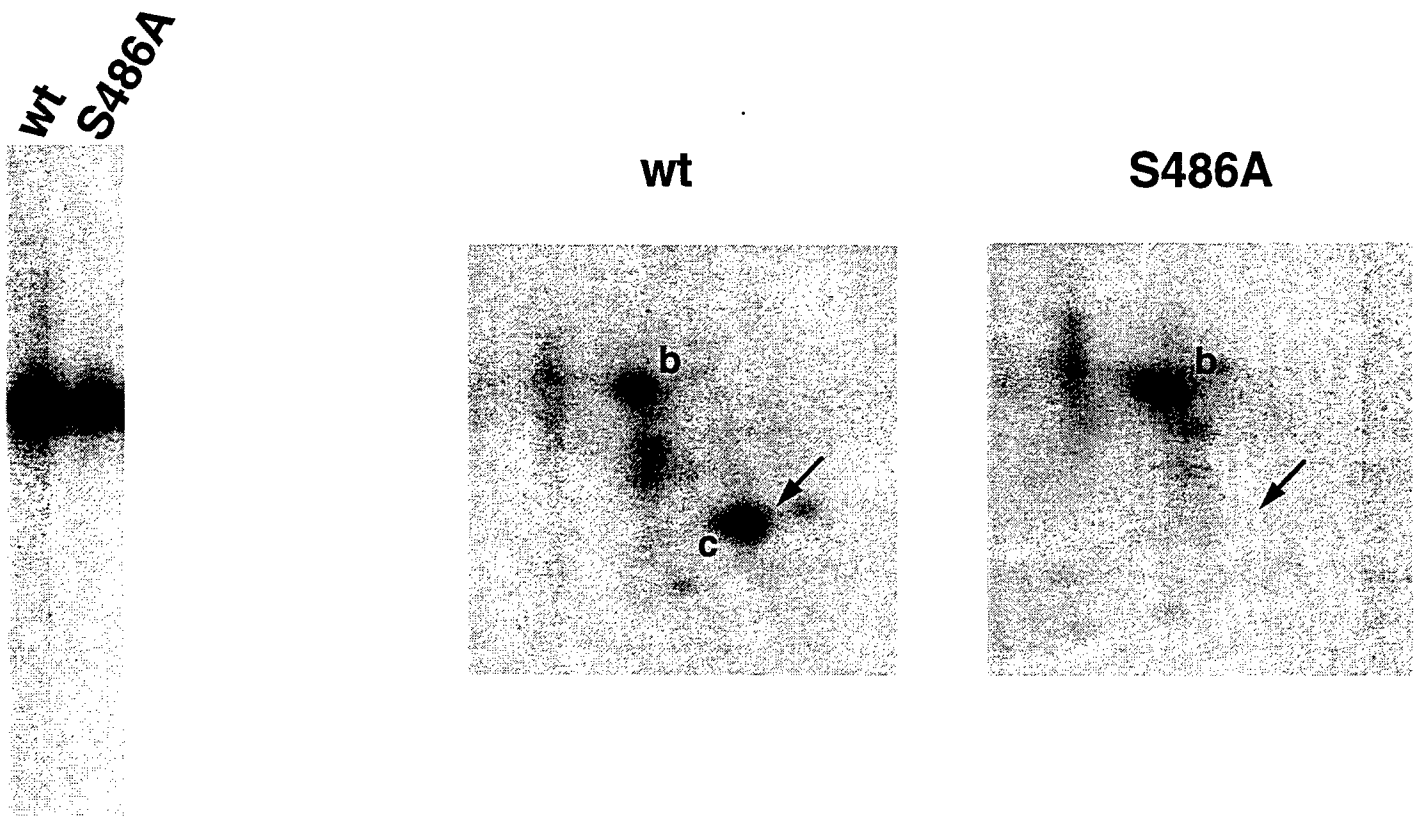

Figure 2: Growth inhibition assay

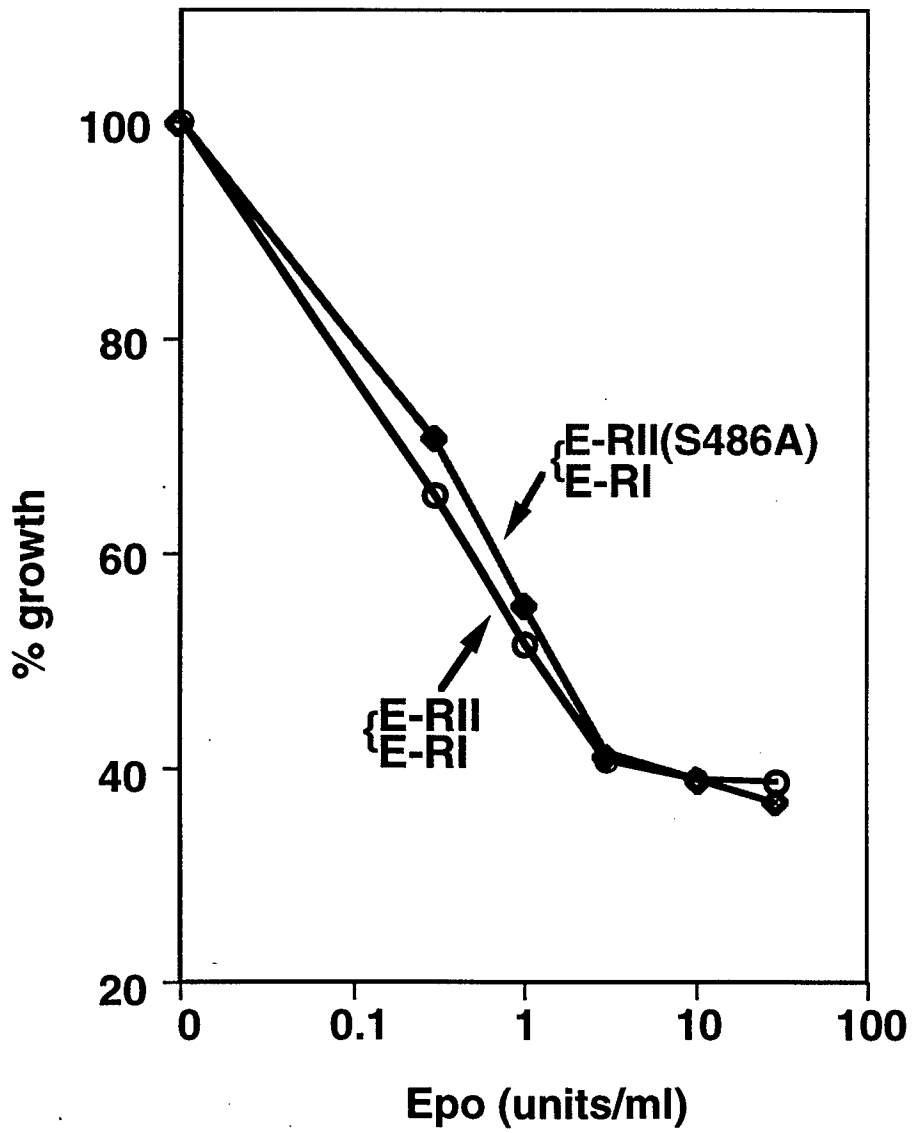

Figure 2. Growth inhibition assay. Ba/F3 cells expressing E-RII(S486A) and E-RI were incubated with increasing amount of Epo, and the growth of cells were examined by cell counting 4 days later.

Figure 3: Phosphorylation of Smad2 in vitro by mutant TβRIIs

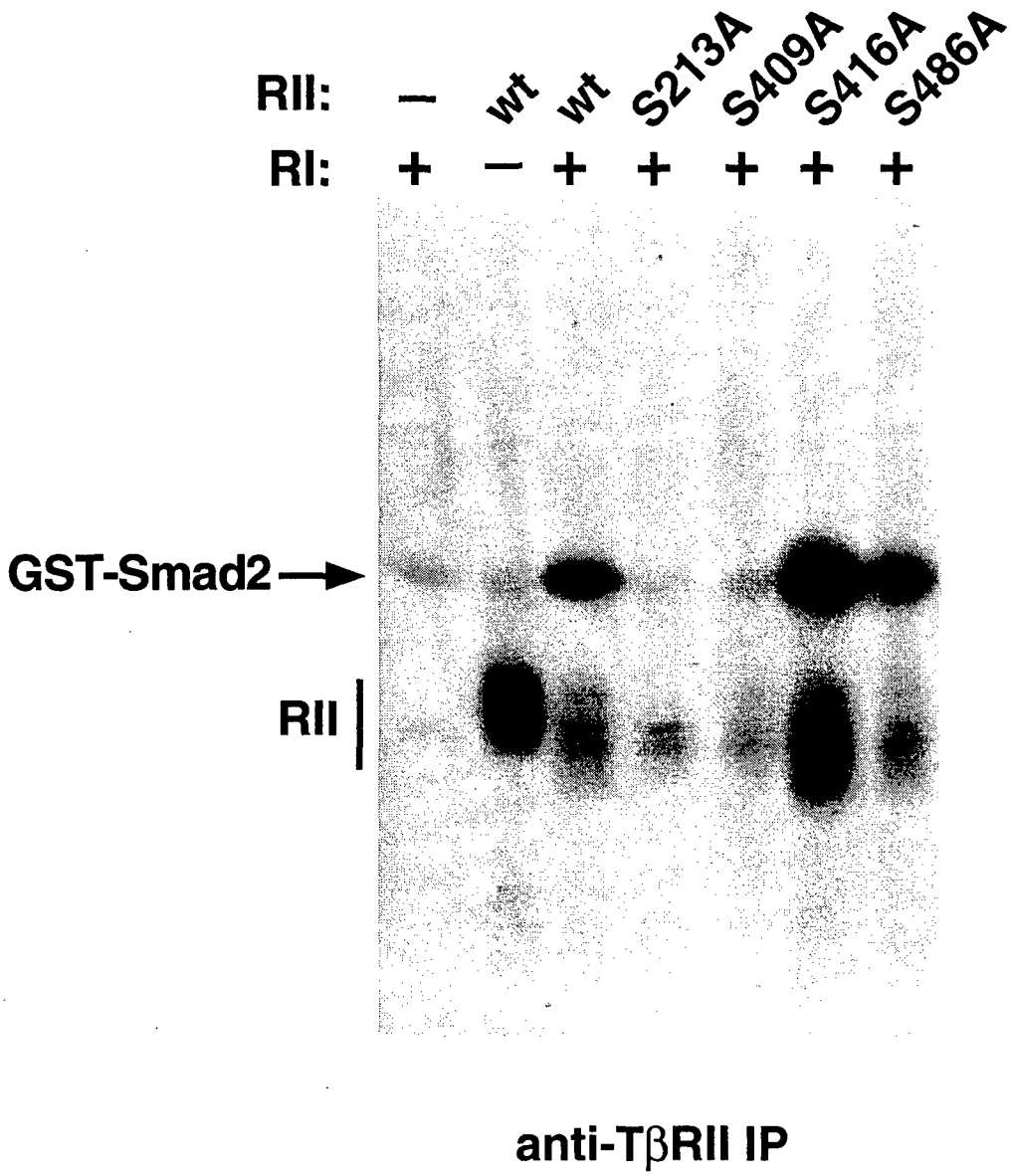

Figure 3. Phosphorylation of Smad2. Receptor complex containing wt E-RI and mutant E-RIIs were isolated from transfected 293T cells and incubated with 2 mg purified Smad2 in the presence of g32P-ATP.

Conclusion:

Combined with our previous studies, the studies presented here showed for the first time that TβRII kinase is regulated intricately by autophosphorylation on multiple sites. The identification of these site will no doubt help us dissect the complex signaling system of the TGFβ receptors and also provide a starting point for the isolation of interacting downstream signaling molecules. It also provides a handle to start understanding the mechanism of regulation for the activation of TGFβ receptors.

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