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Transforming Growth Factor-ßs (TGFß)are polypeptides that are constitutively secreted and activated by many breast carcinomas. They contribute to the tumor's ability to invade and metastasize, to induce angiogenesis and to escape from immune destruction. These circumstances raise the question whether blocking the effects of tumor-derived TGFß on normal tissue (stroma, bloodvessels and immune cells) could be developed as a novel approach to the treatment of breast cancer. We propose to block TGFß action by developing small molecules that inhibit the type I TGFß receptor kinase, which is the key molecule that initiates and mediates TGFß signaling. We plan to develop a cell free ELISA-type assay for high-throughput screening for selective inhibitors of TßR-I kinase activity by using an antibody that specifically detects the phosphorylated form of its substrate, Smad2. Combinatorial libraries of small molecules will then be screened for potent and highly selective for the TßR-I kinase. These will then be tested against normal cells *in vitro* using a number of different assays for TGFß's biological effects. Promising compounds will then be tested for their antitumor activity against highly metastatic, - angiogenic and immunogenic varieties of transplantable breast cancers in mice.

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INTRODUCTION:

Transforming Growth Factor-ßs (TGFß)are polypeptides that are constitutively secreted and activated by many breast carcinomas. They contribute to the tumor's ability to invade and metastasize, to induce angiogenesis and to escape from immune destruction. These circumstances raise the question whether blocking the effects of tumor-derived TGFß on normal tissue (stroma, bloodvessels and immune cells) could be developed as a novel approach to the treatment of breast cancer. We propose to block TGFß action by developing small molecules that inhibit the type I TGFß receptor kinase, which is the key molecule that initiates and mediates TGFß signaling. We plan to develop a cell free ELISA-type assay for high-throughput screening for selective inhibitors of TßR-I kinase activity by using an antibody that specifically detects the phosphorylated form of its substrate, Smad2. Combinatorial libraries of small molecules will then be screened for potent and highly selective for the TßR-I kinase. These will then be tested against normal cells *in vitro* using a number of different assays for TGFß's biological effects. Promising compounds will then be tested for their antitumor activity against highly metastatic, -angiogenic and immunogenic varieties of transplantable breast cancers in mice.

BODY:

Even though this grant was awarded in 2000, it was only activated on April 1, 2001 because the principal Investigator and his laboratory moved from Yale University to The Cancer Institute of New Jersey (a part of Robert Wood Johnson Medical School). Therefore, we are just in the preparatory stages of the work proposed.

Task 1. Development of high-throughput assay for specific inhibitors of TßR-I kinase

a. Synthesis and purification of TßR-I and ActR-IB kinases (Months 0-6)

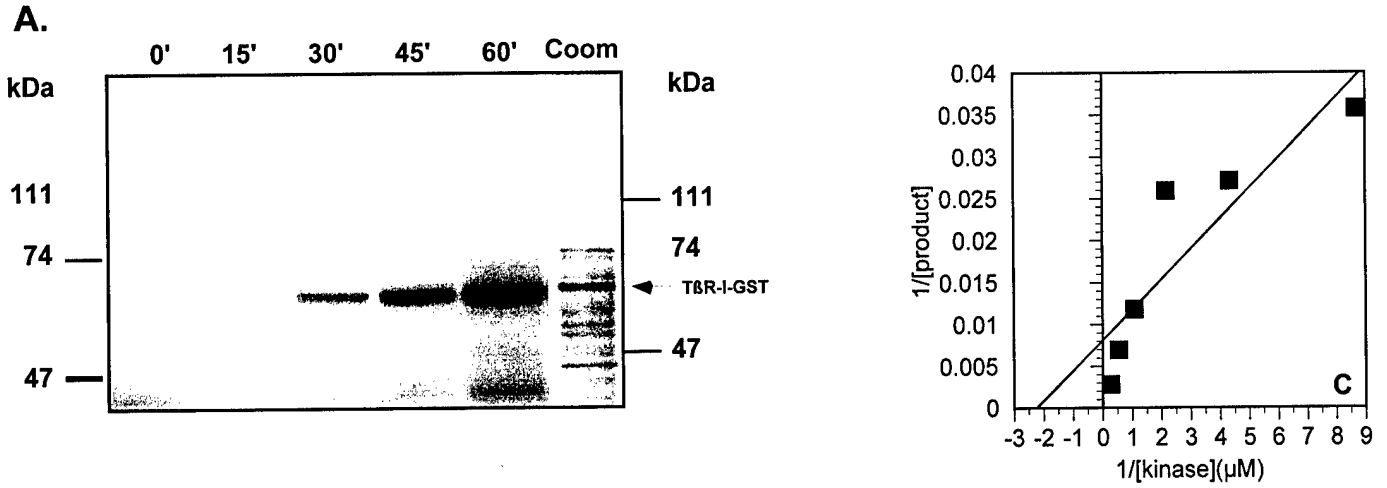

Figure 1. ***In vitro*** **autophosphorylation of GST-TßR-I kinase. A.** Approximately 3.5 µM purified GST-TßR-I was incubated with 20 µM [γ-^{32}P]ATP (5 Ci/mmol) in kinase buffer (50 mM Tris [pH 7.5], 100 mM NaCl, 10 mM $MnCl_2$, 10 mM DTT, 0.05% Triton X-100) at 25°C for the times indicated. Reaction products were resolved by 10% SDS-PAGE, and the radiolabeled bands visualized by phosphorimager after exposure to a phosphor screen (Molecular Dynamics). Coom: Coomassie blue stained gel of purified protein preparation. **B.** *In vitro* GST-TßR-I autophosphorylation reaction (60 min) as a function of GST-TßR-I concentration. **C. Lineweaver-Burke plot:** The relative intensities

of the radiolabeled protein band of 67 kDa corresponding to autophosphorylated GST-TßR-I were determined using NIH Image (version 1.62) software.The estimated Km for the autophosphorylation reaction is 0.45 µM, with an estimated Vmax of 2.5 µM.

At this point, we have conducted a number of feasibility studies that clearly indicate that the aims of this proposal are realistic and can reasonably be expected to be achieved within the next 3 years: We have been able to generate sufficient amounts of both the target enzyme (TßR-I) and its specific substrate (Smad2), and have shown that we can reliably detect kinase activity using both isotopic and non-isotopic methods. A key reagent in this regard is a rabbit antiserum that we have recently developed, which is highly specific for the phosphorylated forms of the physiological substrate of TßR-I, Smad2. Furthermore, in a preliminary screen of a series of staurosporine analogues, we have shown that we can detect and quantify kinase inhibitor activity *in vitro* using a sensitive and specific slot blot assay.

Production and purification of GST-TßR-I: The first requirement for the development of a successful *in vitro* kinase assay that can be used to screen for enzyme inhibitors is the availability of sufficient amounts of pure and biochemically active kinase. The TßR-I kinase is our primary target. It's closest structural and functional homologue, the type IB activin receptor (AcTR-IB) will be used as the most stringent control for selectivity. For the TßR-I cytoplasmic domain construct, the internal *EcoRI* site of the human TßR-I cDNA was removed with the peptide sequence unchanged. The full-length cytoplasmic domain (amino acids 148-503) was then amplified with an *Eco*RI site and a *Xho*I site attached at the 3' and 5' ends, respectively, and subcloned into pGEX-4F-1 (Pharmacia) to produce a vector that encodes afusion protein of GST and the TßR-I kinase domain. The same procedure was used for the construction of GST-ActR-IB expression vector. Moreover, we replaced the threonine at position 204 with aspartic acid to increase *in vitro* kinase activity of the TßR-I kinase more then 10-fold (1, 2). Similarly, we constructed the constitutively activated form of the ActR-1B kinase (ACD-T206D) (3).

Using glutathione sepharose 4B purification (Pharmacia), we have been able to obtain approximately 1.5 mg highly purified GST-TßR-I per 4 liter culture (**Figure 1A**). The activity of the GST-TßR-I kinase was confirmed in an *in vitro* autophosphorylation reaction. As shown in **Figure 1**, autophosphorylation of the GST-TßR-I fusion protein occurs readily when incubated in the presence of 20 µM [γ-^{32}P]ATP (spec. act. 5 Ci/mmol). A radiolabeled band of approximately 67 kDa first becomes detectable after a 30 min incubation at 25°C. Moreover, the frozen GST-TßR-I kinase retains its enzymatic activity for at least 8 weeks.

b. Synthesis and purification of substrate Smad2 (Months 0-6)

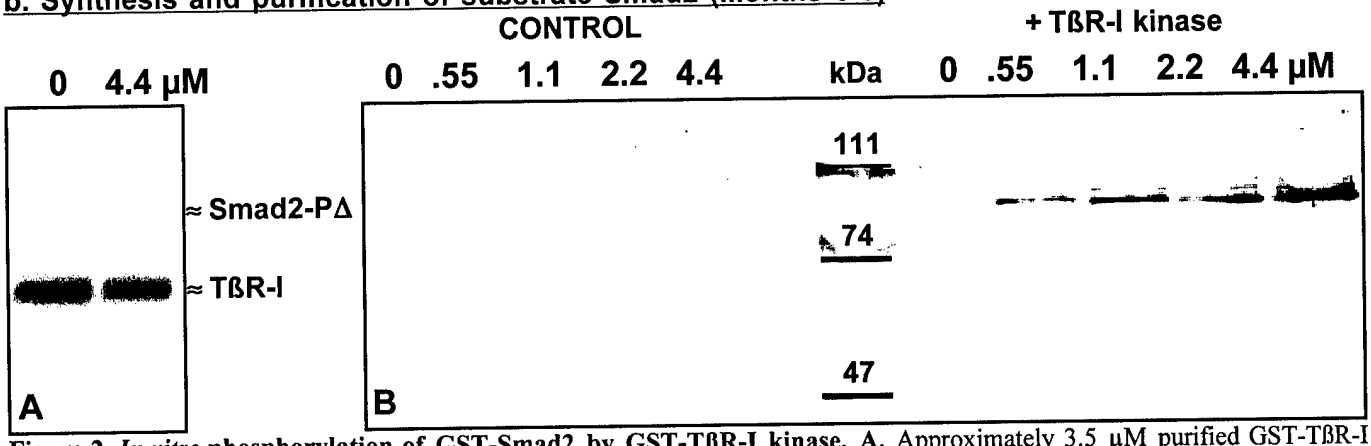

Figure 2. ***In vitro*** **phosphorylation of GST-Smad2 by GST-TßR-I kinase. A.** Approximately 3.5 µM purified GST-TßR-I was incubated with 20 µM [γ-^{32}P]ATP (5 Ci/mmol) in kinase buffer (50 mM Tris [pH 7.5], 100 mM NaCl, 10 mM MnCl2, 10 mM DTT, 0.05% Triton X-100)(total reaction volume 50 µl) at 25°C in the presence or absence of 4.4 µM purified GST-Smad2. The radiolabeled protein band of 91 kDa corresponds to phosphorylated GST-Smad2. **B.** Purified GST-Smad2 at the concentrations indicated was incubated with 20 µM cold ATP in the absence ("control") or presence ("+TßR-I kinase") of 3.5 µM GST-TßR-I for 60' at 25°C. The reaction products were resolved by SDS-PAGE and then subjected to Western blotting using a phospho-Smad2-specific rabbit antiserum and alkaline phosphatase-conjugated goat anti-rabbit-IgG + NBT/BCIP as detector system. The amount of detectable phosphorylated GST-Smad2 product increases as a function of the input GST-Smad2 substrate concentration in the reaction.

Purification of GST-Smad2: The cytoplasmic protein, Smad2, contains the characteristic C-terminal Ser-Ser-X-Ser(SSXS) motif in which the two most terminal Ser residues become phosphorylated in response to TGFß or activin signaling (4). Based on these observations, we have constructed an expression vector that encodes a 91 kDa fusion protein of GST and almost full-length Smad2. Purification of this GST-Smad2 protein was performed as described above for GST-TßR-I, and also yielded approximately 1.5 mg from a 4-liter bacterial culture. As can be seen in **Figure 2A**, co-incubation of GST-Smad2 with GST-TßR-I in the presence of 20 μM [_-^{32}P]ATP (5 Ci/mmol) results in 2 radiolabeled bands of 91 and 67 kDa, which represent phosphorylated GST-Smad2 and GST-TßR-I, respectively.

Production and purification of anti-phospho-Smad2-specific rabbit antiserum: Because our initial goal is to develop a high-throughput screening assay for potential inhibitors of TßR-I kinase activity, it would be highly advantageous to be able to avoid the use of radioisotopes. In addition, if a non-isotopic antibody-based detection system for phosphorylated product were available, one could increase the sensitivity of the assay by allowing the kinase reaction to run its course until the maximum amount of phosphorylated product has been produced. For these reasons, we have produced a polyclonal rabbit antibody that specifically recognizes Smad2 only in its phosphorylated state.

In order to be able to distinguish between the phosphorylated and inactive forms of Smad2, a synthetic 13 amino acid C-terminal peptide of Smad2 in which two phosphoserine residues were incorporated at the extreme C-terminus was coupled to keyhole limpet hemocyanin (KLH) as carrier protein. New Zealand white rabbits were immunized by subcutaneous injection with peptide-carrier protein complexes. The specificity and relative titers of the antibodies in the purified preparations were determined by ELISA, using the immunogenic peptide coupled to BSA as target. Maximal antibody titers were achieved within two weeks of immunization.

A multiple-step affinity chromatographic purification was then carried out in order to produce a final antibody preparation with the desired reactivity as described in detail by Dr. DiGiovanna et al. (5). First, the serum was passed over an KLH-agarose (Sigma) affinity column in order to remove the majority of the antibodies that were generated against the much larger carrier protein. A second negative selection step was carried out using an affinity column composed of Affigel 10 coupled to synthetic unphosphorylated Smad2 and Smad3 peptides. The final step in the purification consisted of positive selection using a column consisting of the phospho-Smad2 peptide immobilized onto Affigel-10 matrix. The sodium thiocyanate eluate from this last column using was immediately neutralized using 0.2 M Tris (pH 8.0), and dialyzed against PBS. Fractions with the highest specific anti-phospho-Smad2 activity were pooled and stored at –80°C. This procedure has yielded approximately 0.17 mg of purified antibody (2 μg/μl) from each ml of rabbit serum.

The specificity of the anti-phospho-Smad2 antibody is illustrated in **Figure 2B**: *In vitro* phosphorylated GST-Smad2 can easily be detected by Western blot. Most importantly, unphosphorylated GST-Smad2 is not detected by the anti-phospho-Smad2 antibody. Furthermore, the amount of detectable phosphorylated GST-Smad2 product increases as a function of the input GST-Smad2 substrate concentration in the reaction. To further determine the sensitivity of the anti -Smad2P antibody, we used the antibody to detect varying amounts of BSA-conjugated synthetic phospho-Smad2 peptide using a slot blot assay (**Figure 3**). This assay is able to detect as little as 1 pmole of phosphorylated protein and is linear up to 8 pmoles. Based on these preliminary studies, we are confident that we will be able to use this slot blot assay to identify potential inhibitors of the TßR-I kinase.

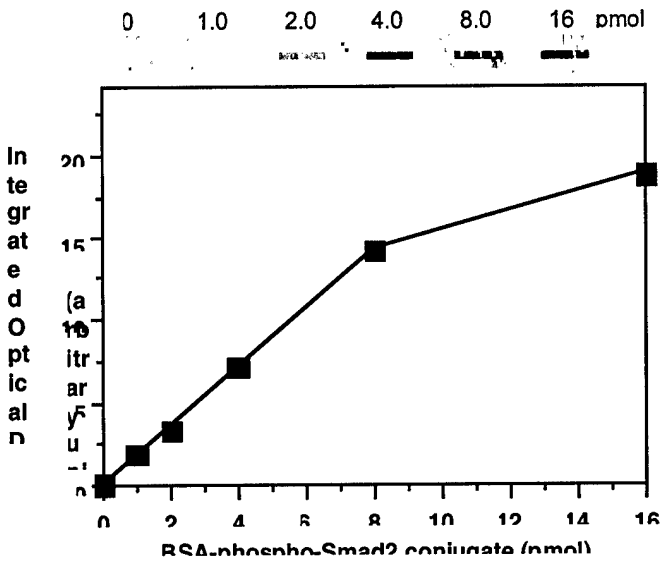

Figure 3. Sensitivity of anti-phospho-Smad2 alkaline phosphatase slot blot detection assay. Indicated amounts of synthetic phospho-Smad2 peptide conjugated to BSA were deposited onto nitrocellulose membrane using a BioRad slot blotter. The filter was then incubated with 1 µg/ml rabbit anti-Smad2P antibody, and the image developed using alkaline phosphatase-conjugated goat anti-rabbit IgG (Calbiochem) and NBT/BCIP (Boehringer-Mannheim). The assay is able to detect as little as 1 pmole of phosphorylated protein and is linear up to 8 pmoles (Densitometry: NIH Image 1.62).

Effects of staurosporine analogues on TßR-I kinase activity. In order to begin to characterize the properties of the TßR-I kinase, we have conducted a number of preliminary studies to characterize the effects of known serine-threonine kinase inhibitors on TßR-I kinase activity. Specifically, we have analyzed the effects of the potent staurosporine analog, K252a (6), and 3 different synthetic derivatives, on the activity of purified TßR-I kinase *in vitro*. All 4 agents were synthesized and provided by Dr. John Wood, Dept. of Chemistry, Yale University. As shown in **Figure 4**, K252a has only modest activity against the TßR-I kinase *in vitro*, whereas its 2-methyl derivative is highly active, with an IC_{50} in the low nannomolar range. Two additional derivatives (*s*- and *i*-butyl K252a) had no inhibitory activity at concetrations up to 250 μM. These results illustrate that relatively minor variations on the structure of known PK inhibitors can have profound effects on their activity against specific kinases. In addition, the 2-methyl K252a compound provides us with a potentially interesting lead compound for our studies.

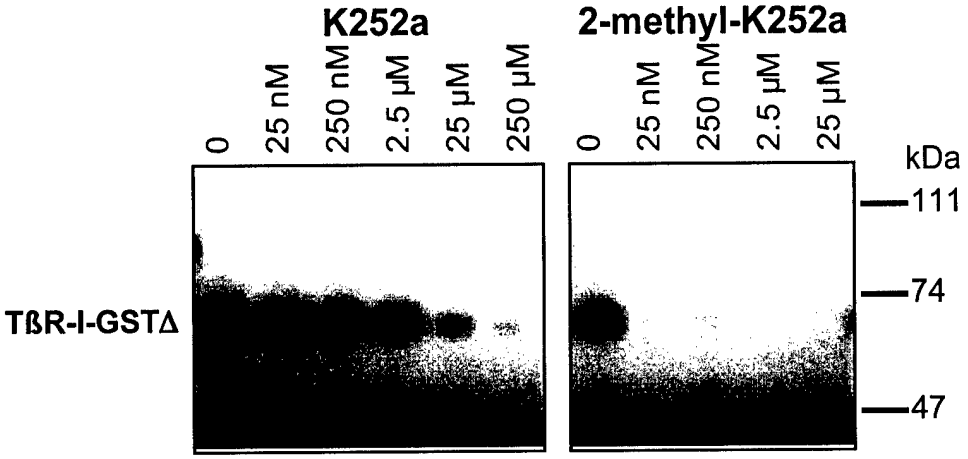

Figure 4. Effects of staurosporine analogs on *in vitro* autophosphorylation of TßR-I kinase. *In vitro* kinase reactions were performed as described for Figure 6, in the presence or absence of varying concentrations of staurosporine analogues, K252a and 2-methyl-K252a. K252a has an IC_{50} between 2.5 and 25 µM, which is significantly higher than expected based on its effects on other serine-threonine kinases, such as protein kinase-C. In contrast, the 2-methyl derivative of K252a has an IC_{50} of <25 nM. Thus, 2-methyl-K252a is a potent inhibitor of TßR-I kinase activity.

Table 1. Residues thought to interact with staurosporine in PKA and the homologous residues in the staurosporine-insensitive serine-threonine protein kinases.

		Residue in position							
Kinase	IC50 (µM)	49*	57	70	105*	120-127*	170*	173	183
PKA	0.015	Leu	Val	Ala	Lys	MEYVPGGE	Glu	Leu	Thr
CSK	1.015	Ile	Val	-	*Val*	TEYMAKGS	**Arg**	Leu	*Ser*
CK2	12-17	Leu	Val	Val	*Thr*	FEHVNNTD	**His**	Met	**Ile**
TßR-I	>25**	Ile	Val	Ala	*Gly*	SDYHEHGS	**Lys**	Leu	*Ala*

The residues that are smaller than their homologues in PKA are indicated in *italics*, those that are larger in **bold** type. The position numbers refer to the sequence of PKA. *: Critical residues derived from crystal structure of CSK/staurosporine complex (residues 120-127: hinge region)(8). **: Effect of staurosporine analogue, K252a, against TßR-I (our data).

Although no structural information is available for K252a complexed with a target enzyme, structures of three protein kinases, PKA, CDK2 and C-terminal Src kinase (CSK) co-complexed with the protein kinase inhibitor staurosporine (from which K252a was derived) have been recently solved (7-9). These studies provide a detailed account of inhibitor-kinase interactions and inhibitor selectivity. Although staurosporine exhibits nanomolar IC_{50} values against a wide range of protein kinases, such as PKA and CDK2 (10, 11), others, including CK2 and CSK, are relatively refractory to staurosporine inhibition, exhibiting IC_{50} values in the micromolar range (10, 11). Computer modeling based on PKA crystal structure in conjunction with sequence analysis suggest that the low sensitivity to staurosporine of CK2 may be accounted for by the bulky nature of three residues, Val66, Phe113 and Ile174 which are homologous to PKA Ala70, Met120 and Thr183, respectively (**Table 1**). In contrast these PKA residues are either conserved or replaced by smaller ones in protein kinases highly sensitive to staurosporine inhibition. On the other hand, His160 which is homologous to PKA Glu170, appears to be responsible for the unique behaviour of CK2 with respect to a staurosporine derivative (CGP44171A) bearing a negatively charged benzoyl substituent: while CGP44171A is 10- 100-fold less effective than staurosporine

against PKA and most of the other protein kinases tested, it is actually more effective than staurosporine for CK2 inhibition, but it looses part of its efficacy if it is tested on a CK2 mutant (H160D) in which His160 has been replaced by Asp. These studies illustrate that the catalytic sites of protein kinases are divergent enough as to allow a competitive inhibitor like staurosporine to be fairly selective, a feature that can be enhanced by suitable modifications designed based on the structure of the catalytic site of the kinase [Meggio, 1995 #35]. These types of differences might explain the selective potency of 2-methyl-K252a as compared to K252a against the TßR-I kinase (**Figure 4**).

KEY RESEARCH ACCOMPLISHMENTS:

- Bacterial synthesis and purification of recombinant constitutively active TßR-I kinase
- Bacterial synthesis and purification of recombinant Smad2
- Production of phospho-Smad2-specific rabbit antiserum
- Development of slot-blot assay for detection of TßR-I kinase activity
- Preliminary testing of effects of staurosporine analogs on TßR-I kinase activity.

REPORTABLE OUTCOMES:

None

CONCLUSIONS:

These preliminary studies as well as our ongoing studies of TGFß signaling in cancer have provided us with all of the tools and reagents necessary to proceed with a search for antagonists of the TGFß receptor system and to test their effects in meaningful biological systems.

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