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13. ABSTRACT ***(Maximum 200 Words)***
Transforming growth factor-ß (TGFß) is an important regulator of tumor growth and metastasis formation in breast carcinomas. TGFß has a dual role in tumor progression, initially acting as a tumor suppressor by inhibiting the proliferation of normal epithelial cells and early stage tumor cells, and in later stages of the disease acting as a tumor promoter by inducing a more invasive tumor cell phenotype with elevated metastatic potential. This phenotypic change is often correlated with an epithelial-mesenchymal transition (EMT). The molecular basis for the switch in tumor cell responsiveness to TGFß is mostly unclear. The importance of the Smad signaling pathway in mediating the growth inhibitory response to TGFß in normal epithelial cells is well established. However, the TGFß signaling events leading to EMT and enhanced tumorigenic properties are poorly understood. The aims of this project are to analyze these signaling events, and to identify novel signaling molecules that interact with TGFß receptor complexes in invasive breast carcinoma cells. We have generated cell lines expressing different forms of the TGFß receptor that are now being characterized and used for the isolation of receptor complexes from the plasma membrane. Preliminary data indicate the feasibility of this approach and identify candidate receptor interacting polypeptides.

14. SUBJECT TERMS
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Final Report

Introduction.

The major cause of mortality in breast cancer is the formation of metastases, the spread of tumor cells to distant sites in the body via the lymph system or the bloodstream. The inhibition of metastasis formation is therefore an important goal for therapeutic intervention.

A critical regulator of breast tumor growth and metastasis is the transforming growth factor-ß (TGFß). TGFß initially acts as a tumor suppressor by inhibiting the proliferation of normal epithelial cells and early stage tumor cells. During the course of the disease a switch in TGFß-responsiveness of the tumor cells often occurs. Instead of causing growth-inhibition TGFß can then induce the acquisition of a more invasive and tumorigenic cell phenotype with an elevated potential to form metastases. This phenotypic change is often correlated with an epithelial-to-mesenchymal transition (EMT). Together with effects of TGFß on stromal cells, the immune response, and the angiogenic process these direct actions on the tumor cells play a critical role in the promotion of malignant progression by TGFß. TGFß is abundantly expressed in most primary human breast tumors.

The molecular basis for the switch in tumor cell responsiveness is mostly unclear. It has been suggested, however, that a cooperation between TGFß and Ras signaling pathways is required [1]. TGFß regulates cellular processes via a signaling system that includes two distinct transmembrane receptor kinases, the type I receptor (TßR-I/Alk-5) and the type II receptor (TßR-II). Binding of TGFß to a TßR-II/TßR-I complex leads to the activation of the receptors and the propagation of the signal through phosphorylation of the cytoplasmic signaling mediators Smad2 and Smad3 by TßR-I. The importance of the SMAD signaling pathway in mediating the growth inhibitory response to TGFß in normal epithelial cells is well established. However, TGFß signaling events leading to EMT and enhanced tumorigenic properties in breast carcinoma cells are mostly unknown.

Based on previous studies using mammary epithelial cells as a model system I hypothesized that TGFß-induced EMT and gain of tumorigenic properties may depend on SMAD-independent TGFß signaling events [2]. The aims of the proposed project were to determine the contribution of SMAD-independent signaling to TGFß induction of EMT and gain of tumorigenic properties, and to identify novel signaling molecules that interact with TGFß receptor complexes in invasive breast carcinoma cells.

The potential significance of these studies relate to the multifunctional role of TGFß during tumor development and progression. This multifunctional role represents a major obstacle for the development of drugs aimed at manipulating TGFß functions in a beneficial way. It would be desirable to eliminate tumor promoting functions of TGFß without affecting its tumor suppressive functions. The identification of SMAD-independent signaling events involved in the tumor promoting actions will provide novel targets for the development of such selective drugs.

Studies and Results.

A growing body of genetic and biochemical evidence has accumulated suggesting that activated TGFβ receptors signal through effector molecules that are activated independently of the SMAD signaling mediators and that elicit responses either with or without the cooperation of SMADs. These studies argue in favor of the hypothesis underlying the current project, namely that in human breast cancer cells a SMAD-independent TGFβ signaling pathway may exist which is essential for TGFβ-mediated induction of EMT and the gain of tumorigenic properties. This postulated pathway therefore may play a critical role in the tumor-promoting function of TGFβ in breast cancer.

The evidence for SMAD-independent TGFβ signaling includes the demonstration that Smad4-defective cell lines retain certain responsiveness to TGFβ [3]. Furthermore, constitutively active forms of TGFβ family receptors that all signal through the same subgroup of receptor-regulated SMADs can elicit distinct biological responses in various experimental systems, depending on the receptor utilized [4-6]. These experiments suggest that either the activation of SMADs by the various receptors is qualitatively different, possibly involving unknown receptor-specific interacting molecules, or that the receptors signal through additional, SMAD-independent and receptor-specific signaling pathways.

Recently it was reported that TGFβ can induce the interaction of its receptor with the protein phosphatase 2A (PP2A), leading to subsequent association and inactivation of the $p70^{S6}$ kinase [7]. This signaling pathway appears to function independently and in complementation to the SMAD pathway in the induction of cell cycle arrest. Finally, multiple studies have suggested that TGFβ can signal through the mitogen-activated protein kinases (MAPKs) JNK and p38 [8-10]. However, the observed effects vary strongly in kinetics, magnitude, and MAPK subtype depending on the cell type and the experimental conditions used. The physiological relevance of these observations remains to be clarified.

We have investigated whether TGFβ can activate MAPKs in the cell lines (i.e. EpH4 and EpRas) that are used as a model system for the analysis of TGFβ signaling in non-transformed versus transformed mammary epithelial cells. As illustrated in a representative experiment TGFβ was capable of activating JNK in EpH4 cells transiently and in a very rapid fashion, with a peak of activity at about 5 to 10 minutes post-TGFβ treatment (Fig.1). This result supports the idea that TGFβ signals through SMAD-independent mechanisms in mammary epithelial cells and that such mechanisms might play a role in TGFβ-induced EMT and the acquisition of malignant properties in breast tumor cells. A biochemical link between MAPKs and the TGFβ receptors has not been established to date.

We have made significant progress in the establishment of the stable cell lines that are key to the success of the project. All the necessary constructs were produced, utilizing both an eukaryotic expression vector (pCMV5) that is suitable for transient transfections and a retroviral vector (pBI) that is suitable for the establishment of stable cell lines. These constructs include the TGFβ type I receptor (TβR-I/Alk-5) as a kinase-dead single point mutant, a constitutively-active single point mutant, or a double mutant. These receptor derivatives were tagged with a double Flag-epitope at their carboxy-terminal ends.

We have started the production of stable cell lines with the successful introduction of a retroviral vector (pRevTet-Off) into EpH4 cells that leads to the expression of a tetracycline-controlled transactivator (tTA). In the presence of tetracycline (or the analog doxicycline; Dox) in the growth medium tTA is inactive, while in the absence of the antibiotic tTA is active. Several independent cell clones were isolated and tested for Dox-dependent inducibility of a reporter construct that

contains a tTA-responsive promoter (Fig.2). Clone #9 was selected for further experiments based on its low basal activity in the presence of Dox and its strong (at least 50-fold) inducibility in the absence of Dox. Clone #9 was subsequently used to establish a number of stable cell lines that express the various mutant forms of the epitope-tagged TGFβ receptor, either alone or in combination with a H-RasV12 oncogene. These cell lines are currently being characterized with respect to the inducibility and expression of the exogenous proteins.

In parallel to creating the stable cell lines we have established biochemical conditions for the affinity purification of epitope-tagged TGFβ receptors. For this purpose COS cells were transiently transfected with the receptor expression constructs and the exogenous receptors were then immuno-affinity purified from whole cell lysates. Various detergents and buffer conditions were tested to obtain optimal conditions for the isolation of the receptor from cell cultures. Figure 3 shows an example of a receptor preparation obtained by this method and analyzed by SDS-PAGE and silverstaining. Several polypeptides appear to be co-purified in a receptor-specific fashion, indicating that they may represent receptor-interacting polypeptides. Whether these interactions are physiologically significant or a consequence of receptor overexpression in COS cells remains to be determined. Nevertheless, these preliminary results suggest that the biochemical approach taken in this project might be successful.

Furthermore, we are devising a method that will allow to separate the fraction of receptor that is present in the plasma membrane from the remaining fraction of receptor that is present in the endoplasmatic reticulum, the endosomes, or other intracellular compartments. This approach will ensure that identified polypeptides are likely to be signaling molecules that interact with the receptor in its plasma membrane location. This method uses covalent biotinylation of cell surface proteins, thereby allowing their streptavidin-mediated affinity purification subsequent to the initial Flag-epitope immuno-purification.

Using one of the stable cell lines that expresses exogenous Alk-5 we have now carried out first experiments to isolate the receptor for the identification of associated polypeptides. Analysis of the receptor preparation by SDS-PAGE and silverstaining revealed several polypeptides that appear to be specific for the receptor expressing cells (when compared to the parental control cells) (Fig. 4). We are now in the process of comparing the patterns of putative receptor-associated polypeptides among the different cell lines created, and to obtain sufficient material for the identification of polypeptides by mass spectrometry.

Another goal of our studies is the delineation of the structural motifs that are required for critical protein-protein interactions in the tumor-promoting response to TGFβ in breast cancer cells. To dissect the structural requirements in TβR-I we are developing an assay system that will allow us to determine the domains of the receptor that are required for a particular cellular response, e.g. EMT. For this purpose adenoviral vectors were constructed that express either the kinase-dead mutant form of TβR-I or a constitutively-active form of TβR-I. Adenoviruses were produced that can efficiently infect EpH4 and EpRas cells, as visualized by the co-expression of the green fluorescent protein (GFP) (Fig. 5). The exogenous receptor is expressed in a similar time course upon virus infection (data not shown). Expression of activated TβR-I should allow to induce EMT (or other cellular responses) in the mammary epithelial cells and will therefore provide a system in which to delineate the structural requirements within the TGFβ receptor.

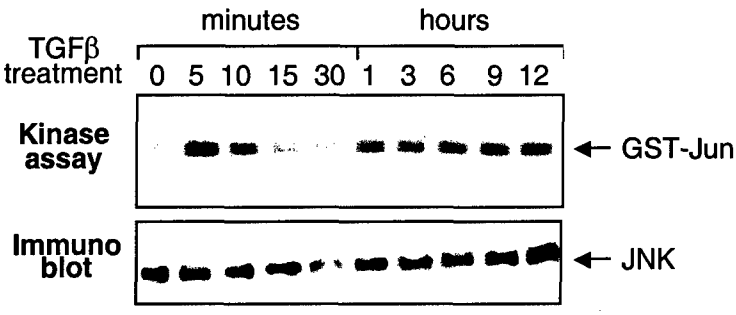


Figure 1. Activation of JNK by TGFβ1 in mammary epithelial cells. EpH4 mammary epithelial cells were serum starved for 12h and then treated with TGFβ1 (50 pM) for the indicated times. Cells were lysed, endogenous JNK1 was immuno-precipitated with an α-JNK1 antibody (Santa Cruz Biotechnology), and *in vitro* kinase assays were performed using GST-Jun as a substrate. Kinase reactions were analyzed by SDS-PAGE and autoradiography. Aliquots of the same cell lysates were also subjected to SDS-PAGE and subsequent immuno-blotting with the α-JNK1 antibody and an HRP-conjugated secondary antibody. Blots were developed using ECL.

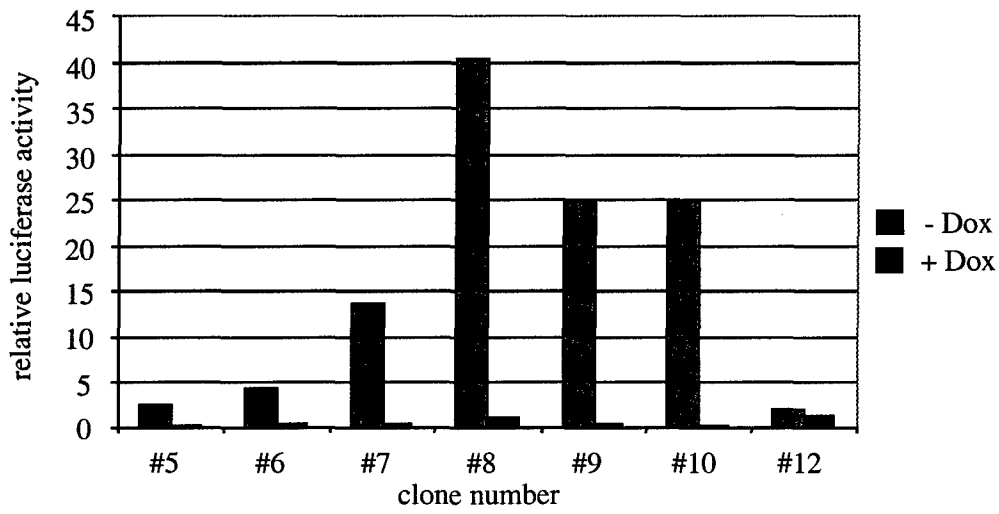


Figure 2. Inducibility of a tTA-responsive luciferase reporter gene in individual cell clones. EpH4 cells were stably transfected with a retroviral vector expressing the doxicycline regulated transactivator tTA. Several individual clones were selected and tested by transient transfection with a tTA responsive reporter gene and an internal control gene. Cells were then treated with or without Dox for 18 hrs, lysed, and luciferase activity was measured by luminometry. Normalized luciferase activity is blotted.

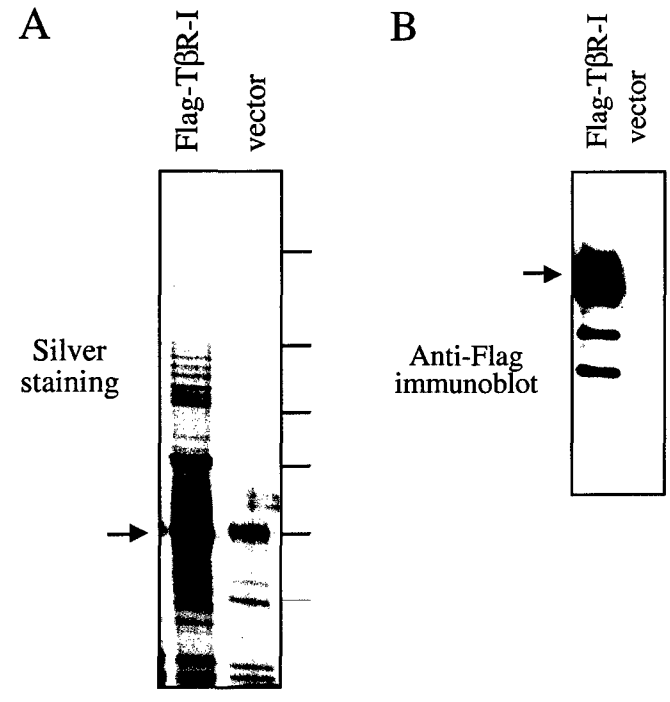


Figure 3. Immuno-purification of TβR-I via the Flag-epitope. COS cells were transiently transfected with an expression vector encoding the Flag-epitope tagged TGFβ receptor type I or the empty vector as a control. Two days post-transfection the cells were lysed and immuno-purification was carried out using anti-Flag monoclonal antibodies (M2) covalently coupled to agarose beads (Sigma). Bound material was eluted using Flag peptide and then analyzed by SDS-PAGE and silverstaining (A) and SDS-PAGE and anti-Flag immunoblotting (B). The arrows indicate the position of the main form of the tagged receptor.

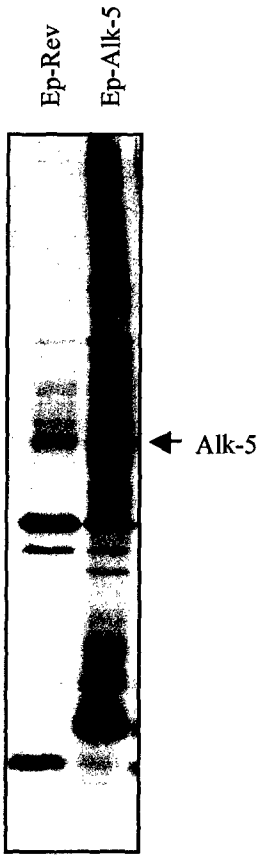
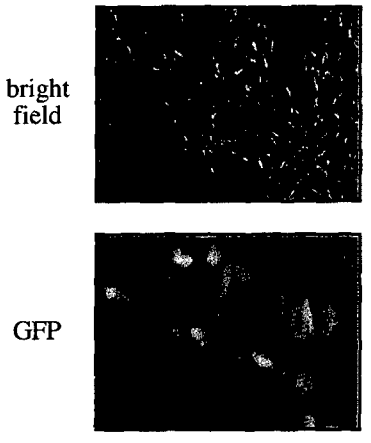


Figure 4. Isolation of Alk-5 receptor associated polypeptides from stable cell lines. Mammary epithelial cell lines stably expressing an epitope-tagged form of the Alk-5 receptor were used to co-purify associated polypeptides. A cell line stably transfected with the empty vector was used as a control. Receptor preparations were analyzed by SDS-PAGE and silverstaining. The location of the full length Alk-5 receptor is indicated.

Figure 5. Infection of EpH4 mammary epithelial cells with GFP-expressing adenoviruses. EpH4 cells were grown in flasks up to a confluency of about 50% and then infected with recombinant adenoviruses that carry expression cassettes for the green fluorescence protein (GFP) and the TGFβ type I receptor. Two days post-infection cells were analyzed by phase-contrast microscopy and photographed. The identical field of cells is shown for bright field and immuno-fluorescence microscopy.



Key research accomplishments.

- Establishment of mammary epithelial cell lines that express an epitope-tagged form of the TGFβ receptor under tetracycline control
- Establishment of biochemical conditions for the isolation of TGFβ receptor complexes from the plasma membrane of these cell lines
- Preliminary identification of polypeptides that copurify specifically with the TGFβ receptor

Reportable outcomes.

Several mammary epithelial cell lines expressing various forms of epitope-tagged TGFβ receptor have been generated and are currently being characterized and utilized for the identification of receptor-associated polypeptides.

Conclusions.

The growth factor TGFβ plays an important role in the malignant progression of certain forms of carcinoma, including breast carcinoma. In tumor cells TGFβ can cause epithelial-to-mesenchymal transition and the gain of tumorigenic and metastatic properties. The signaling events that mediate these functions of TGFβ are poorly understood. The current project addresses the nature of these signaling events and attempts to identify and characterize the proteins that interact with TGFβ receptors to initiate these critical events. The described results represent a significant step towards the successful completion of the project and suggest that the experimental approach taken will lead to important new discoveries. These discoveries have the potential of contributing to a new intellectual framework for the development of drugs that specifically target tumor promoting functions of TGFβ in late stage breast cancer progression.

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