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Requirement for Geranylgeranyl Transferase I and Acyl Transferase in the TGF-β-Stimulated Pathway Leading to Elastin mRNA Stabilization

Umberto Kucich, Joan C. Rosenbloom, Gloria Shen, William R. Abrams, Michelle A. Blaskovich,* Andrew D. Hamilton,† Junko Ohkanda,† Saïd M. Sebti,* and Joel Rosenbloom[1]

*Department of Anatomy and Histology, University of Pennsylvania School of Dental Medicine, Philadelphia, Pennsylvania 19104; *Drug Discovery Program, H. Lee Moffitt Cancer Center and Research Institute, Department of Biochemistry and Molecular Biology, University of South Florida, Tampa, Florida 33612; and †Department of Chemistry, Yale University, New Haven, Connecticut 06520*

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The TGF-βs are multipotent in their biological activity, modulating cell growth and differentiation as well as extracellular matrix deposition and degradation. Most of these activities involve modulation of gene transcription. However, TGF-β1 has been shown previously to substantially increase the expression of elastin by stabilization of tropoelastin mRNA through a signaling pathway which involves a phosphatidylcholine-specific phospholipase and a protein kinase C. The present results, through the use of specific inhibitors of geranylgeranyl transferase I, farnesyl transferase, and acyl transferase, demonstrate that geranylgeranylated and acylated, but not farnesyslated protein(s) is required for this TGF-β1 effect. In addition, the general tyrosine kinase inhibitor genistein completely blocked this TGF-β1 effect. The results suggest that the TGF-β1 signaling pathway requires not only receptor ser/thr kinase activity, but also tyrosine kinase and small GTPase activities.

The transforming growth factor β family (TGF-β) of peptides consists of four closely related (70–80% homology at the protein sequence level) proteins that are synthesized as larger precursor molecules containing the mature form of TGF-β at the carboxy-terminal portion. After proteolytic cleavage, the two portions of the precursor remain together and are secreted as a biologically inactive, noncovalently-bound complex consisting of dimers of both the amino terminal precursor remainder, designated latency associated peptide (LAP), and mature TGF-β. In some cases, this complex is secreted bound to another protein termed latent TGF-β-binding protein (LTBP) (1,2). The various LTBP's have also been found associated in many cases with the extracellular matrix (3,4). The TGF-βs are multipotent in their biological activity, modulating cell growth and differentiation as well as extracellular matrix deposition and degradation (5). This wide range of activities is initiated through transmembrane receptors (Type I and Type II) which possess both constitutive (Type II) and activated (Type I) serine-threonine kinase activity (6–8). Recently a novel TGF-β receptor (Type V) has been described that is also a putative receptor for the insulin-like growth factor-binding protein 3 (9). It is possible that specific cellular changes may be modulated through this receptor distinct from those initiated by Type I and II receptors. However, the relationship between the Type V receptor and the more extensively studied Type I and Type II receptors remains to be determined.

Although the varied biological activities of the TGF-βs have been well documented, the mechanisms through which their multiple effects are transduced have been the subject of intensive investigations and remain only partially understood. The discovery that a new family of proteins, the Smad proteins (10–14), are key intermediates in the signaling process linking TGF-β receptors to cellular responses, has revealed important new information on the way cells respond to this cytokine. Most of the Smad-mediated effects are associated with transcriptional regulation, while the TGF-β up-regulation of the extracellular matrix protein, tropoelastin, is achieved at the post-transcriptional level through mRNA stabilization (15–17).

Our laboratories have begun to determine the mechanisms by which TGF-β regulates elastin expression in cultured diploid human fetal lung fibroblasts. The evidence has suggested that a protein kinase C (PKC)

[1] To whom correspondence should be addressed. Fax: 215 573 2324. E-mail: jrosen@biochem.dental.upenn.edu.

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and a phosphatidylcholine-specific phospholipase C (PC-PLC) are required for increased elastin expression (17). Previous reports that the small GTPase, $p21^{ras}$, is directly involved in TGF-β_1 and TGF-β_2 responses (18) and that Ras activation is directly coupled to PC-PLC activity in several systems (19,20), has led us to investigate the involvement of Ras and Ras-related GTP-binding proteins in the modulation of tropoelastin by TGF-β. The findings presented in this paper demonstrate that prenylated and acylated protein(s), but probably not $p21^{ras}$, is essential for effective mediation of the TGF-β-stimulated increase in elastin expression. In addition, one or more tyrosine kinases, but not trimeric G proteins, appear to be involved.

MATERIALS AND METHODS

Reagents. Cerulenin was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of reagent grade.

Cell culture, RNA, and tropoelastin analyses. Human fetal lung fibroblasts (GM05389; Coriell Institute for Medical Research, Camden, NJ) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) as previously described (17). Before the addition of TGF-β1, the medium of confluent cultures was replaced with DMEM containing 1% serum. After incubation with TGF-β1 for 24 h, total cellular RNA was extracted by the acid guanidine isothiocyanate method (21). 15 μg of RNA were electrophoresed on formaldehyde-1.2% agarose gels, transferred to Zeta-Probe membranes (Bio-Rad Richmond, CA), and hybridized to a 2.2-kbp human elastin cDNA probe labeled with ^{32}P by the random primer method (22). RNA loading and transfer were evaluated by probing with a glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA probe. Equivalent loading and transfer were also verified by quantitative image analysis of ethidium bromide staining of ribosomal RNA in the blots themselves. The filters were analyzed by phosporimaging and results were quantified to determine the relative amounts of mRNA (Image-Quant V3.1 software; Molecular Dynamics, Sunnyvale, CA). Elastin mRNA values were analyzed in duplicate and normalized to equivalent values for GAPDH to compensate for loading and transfer.

The tropoelastin in the cell supernatants was quantitated by a direct ELISA method, similar to the published procedure of Prosser et al. (23). The standard curve was linear between 1 and 50 ng/ml recombinant tropoelastin (24) and each sample was analyzed in triplicate.

Western blot analysis. Human fetal lung fibroblasts were resuspended in a small volume of lysis buffer (30 mM Hepes, pH 7.5, 10 mM NaCl, 5 mM $MgCl_2$, 25 mM NaF, 1 mM EGTA, 1% Triton-X, 10% glycerol, 2 mM sodium orthovanadate, 2 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 25 μg/ml leupeptin, and 6.4 mg/ml Sigma 104 Phosphatase Substrate (Sigma Chemical Co., St. Louis, MO) and lysed on ice for 30 min, vortexing occasionally. Lysates were cleared by spinning at 13,000*g* at 4°C for 15 min, then supernatants were analyzed for protein content using the Bradford assay (Bio-Rad Laboratories, Hercules, CA) and Western blots were performed to determine expression of prenylated proteins. Briefly, SDS–PAGE electrophoretic gels were run according to standard procedures, then proteins were transferred overnight to nitrocellulose and blocked for 1 h at room temperature in a 5% milk solution in phosphate-buffered saline containing 0.1% Tween 20 (PBS-T). After blocking, membranes were exposed to the appropriate primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in a 3% milk/PBS-T solution for 1 h at room temperature. After washing in PBS-T, the corresponding secondary antibody, HRP-conjugated goat anti-mouse (for Lamin B) or HRP-conjugated goat anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA) was added for 1 h at room temperature. After thorough washing, membranes were exposed to chemiluminescence reagents (NEN Life Science Products, Inc., Boston, MA) and exposed to X-ray film to visualize results.

RESULTS AND DISCUSSION

Effects of prenylation inhibitors. Several lines of evidence have suggested that modulation of growth and differentiation of various cell lines by TGF-β is mediated by the small GTPase, $p21^{ras}$ (18,25,26). In all these cases, the effects are ultimately achieved through modulation of transcription. Furthermore, in these systems, the activation of Ras was coupled to the hydrolysis of phosphatidylcholine by a specific phospholipase C (19,20). Of importance to the present studies, inhibition of this phospholipase C completely abolished the TGF-β stimulation of tropoelastin expression (17). These findings prompted us to pursue the effects of agents known to regulate the activity of Ras and Ras-related proteins and to determine whether a Ras superfamily member is involved in the signaling pathway leading to stabilization of elastin mRNA by TGF-β.

Prenylation of some proteins, including those belonging to the small G-proteins of the Ras superfamily, is required for their proper subcellular localization and function (27–29). Two major enzymes involved in this process are farnesyltransferase (FTase) which attaches a 15-carbon farnesyl group to cysteine residues located in the carboxyl portion of proteins, and geranylgeranyl transferases (GGTase I and II) which attach a 20-carbon geranylgeranyl group to key cysteine residues (28,29). The finding that these lipid modifications are essential for transforming activity (30) stimulated several laboratories to design specific inhibitors for potential anti-cancer therapy (31–33). Because farnesylated and geranylgeranylated proteins may function in distinct signaling pathways, we investigated the inhibitory effects of new specific FTase (FTI-277) and GGTase (GGTI-298) inhibitors on TGF-β1 regulation of elastin expression (34,35).

We first determined the effects of FTI-277 and GGTI-298, which were prepared as previously described (33), on the processing of two farnesylated (N-Ras and lamin B) and one geranylgeranylated (Rap1A) proteins. Human fetal lung fibroblasts were treated with various concentrations of FTI-277 or GGTI-298 for 24 h prior to addition of TGFβ1. The cells were then harvested, lysed and the lysate separated by SDS-PAGE and immunoblotted with various antibodies as described under Materials and Methods. Figure 1 shows that cells treated with vehicle or TGF-β1 alone had only fully processed farnesylated N-Ras and lamin B and geranylgeranylated Rap1A. Treatment with FTI-277 resulted in inhibition of farnesylation of N-Ras and lamin B which resulted in the slower migrating unprocessed bands. FTI-277 also resulted in an increased amount of these proteins in their unprocessed form which we

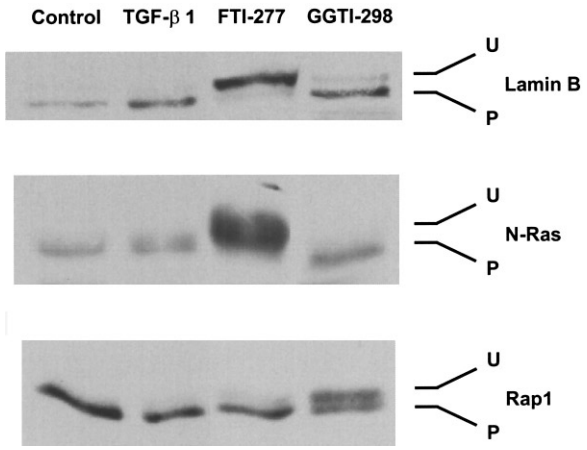

FIG. 1. Effects of FTI-277 and GGTI-298 on the processing of prenylated proteins in human fetal lung cells. Human fetal lung fibroblasts were treated with vehicle (control), TGF-β1 alone, and FTI-277 (20 μM) or GGTI-298 (20 μM) prior to TGF-β1. The cells were then lysed and the lysates immunoblotted with anti-Lamin B, N-Ras, and Rap1 antibodies as described under Materials and Methods. U and P designate unprocessed and processed forms of the indicated proteins.

have previously observed (34). FTI-277 had no effects on the processing of Rap1A (Fig. 1). In contrast, GGTI-298 inhibited the processing of Rap1A without affecting the processing of N-Ras and lamin B. These results demonstrate that the inhibitors are able to inhibit protein farnesylation and geranylgeranylation selectively in human fetal lung fibroblasts. We next investigated the ability of these inhibitors to modulate TGF-β1 regulation of elastin expression. The GGTase I inhibitor, blocked the upregulation by TGF-β1 of elastin expression at both the protein and mRNA levels in a dose-dependent manner, with near complete inhibition achieved at 20 μM (Fig. 2). In contrast, the FTase inhibitor had little effect on elastin expression (Fig. 3) at concentrations that blocked the farnesylation of both lamin B and N-Ras. (Fig. 1). These results suggest that one or more geranylgeranylated proteins are necessary to effect elastin mRNA stabilization, and protein farnesylation is not required.

Effect of acylation inhibition. Although prenylation of Ras proteins appears to be essential for biologic activity, acylation in which a fatty acyl group, usually 16-carbon palmitate, is attached by a labile thioester bond to cysteine residues is of more limited occurrence. For example, both H-Ras and N-Ras are farnesylated and palmitoylated whereas K_B-Ras is only farnesylated, but not palmitoylated (36). The added palmitate moiety appears to increase the avidity of Ras proteins for membranes and may increase their transforming activity (36). While prenylation of the Ras superfamily, including Rho, Rab and Rac proteins has been extensively studied (37–39), the degree of acylation in these proteins, as well as its effect on their biological function is presently unknown. Cerulenin is a relatively specific inhibitor of protein acylation (40). In rat adipocytes, cerulenin blocked palmitoylation of specific proteins and this specific inhibition correlated with a decreased response to insulin administration (41). In addition, other studies have shown that cerulenin impaired internalization of growth hormone releasing factor in rat anterior pituitary cells (42). In our system, cerulenin produced a concentration-dependent inhibition of the TGF-β1 stimulation such that at 20 μg/ml, elastin expression was reduced to close to the control basal values at both the protein and mRNA levels (Fig. 4). These

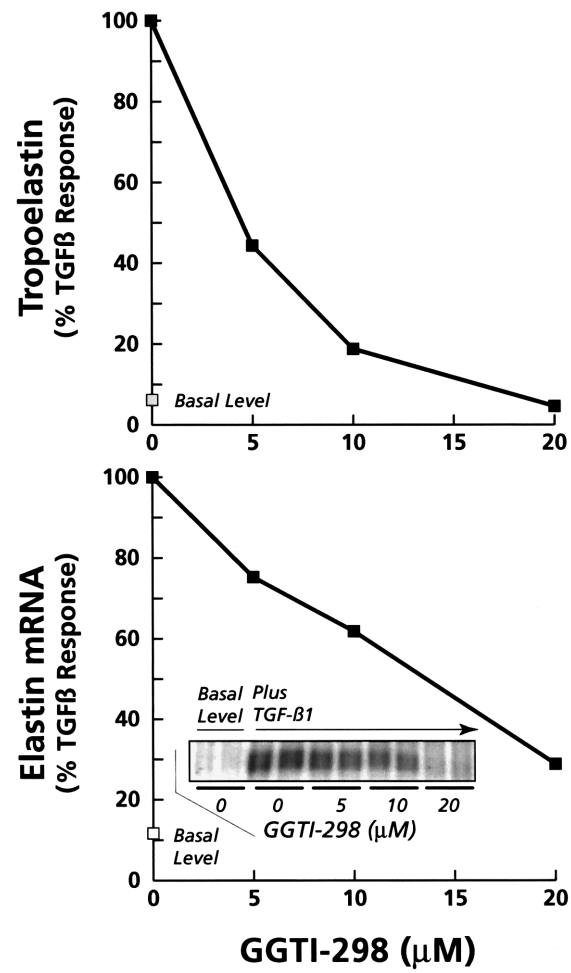

FIG. 2. Effect of inhibition of geranylgeranylation on the TGF-β1 modulation of elastin expression. Confluent cultures of human fetal lung fibroblasts were incubated for 24 h with the various indicated concentrations of the GGTase I inhibitor, GGTI-298), and TGF-β1 was then added to a final concentration of 3 ng/ml. (Top) The incubation media obtained after 24 h were analyzed for tropoelastin with a specific ELISA as described previously (23). The values represent the average of determinations from duplicate cultures. Values represent the average of duplicate cultures determined in triplicate. Basal level refers to the values obtained in the absence of TGF-β1. (Bottom) Total RNA was prepared from the cells and subject to Northern analysis using a human elastin cDNA probe as described in Materials and Methods.

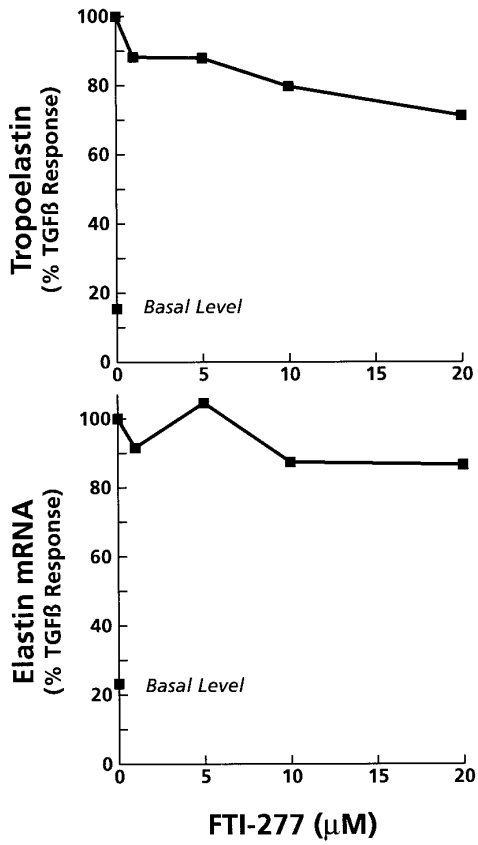

FIG. 3. Effect of inhibition of farnesylation on the TGF-β1 modulation of elastin expression. Confluent cultures of human fetal lung fibroblasts were incubated for 24 h with the various indicated concentrations of the FTase inhibitor, FTI-277, and TGF-β1 was then added to a final concentration of 3 ng/ml. After 24 h, total RNA was prepared from the cells and subject to Northern analysis and tropoelastin in the media was determined as described in Fig. 2 and Materials and Methods. The values represent the average of determinations from duplicate cultures.

results strongly suggest that acylation in addition to geranylgeranylation of protein is required to effect the elastin mRNA stabilization.

Acylation (palmitoylation) is limited mostly to membrane-associated proteins, and has the unusual property among lipid modification of proteins of being reversible (43). Therefore, acylation and deacylation may provide a regulatable way for a protein to shuttle between membranes and cytoplasm in order to carry out its function (44). Besides Ras and Ras-related proteins, heterotrimeric G proteins are common targets of this modification (45). To test the possibility that trimeric G proteins are involved in the signaling pathway, cells were preincubated with cholera and pertussis toxins, inhibitors of trimeric G-protein-mediated transduction signaling (46,47), before the addition of TGF-β1. The results demonstrated that in contrast to other effects of TGF-β (47), no effect of either toxin was observed on the elastin message stabilization elicited by TGF-β1 (Fig. 5), suggesting that trimeric G proteins are not involved in this process.

Role of tyrosine kinases. While, at present, we do not know whether the inhibition of GGTase I is affecting the prenylation of more than one protein involved in the control of elastin production, it is interesting to note that this inhibitor, but not FIT-277, inhibited platelet-derived growth factor (PGDP) receptor tyrosine phosphorylation (35). This result suggested that phosphorylation of this receptor may be modulated by a small GTP-binding protein, such as Rho, which had previously been found to be associated with the PDGF receptor type β (48) and also necessary for activation of a tyrosine kinase (49). Thus, it is possible that Rho or some other nonreceptor tyrosine kinase may be involved in the present effect of TGF-β1. To test this possibility, cells were preincubated with genistein, a potent inhibitor of tyrosine phosphorylation (50), before the addition of TGF-β1. Genistein inhibited the TGF-β1 stimulation of tropoelastin in a dose-dependent fashion, completely blocking the effect at 50

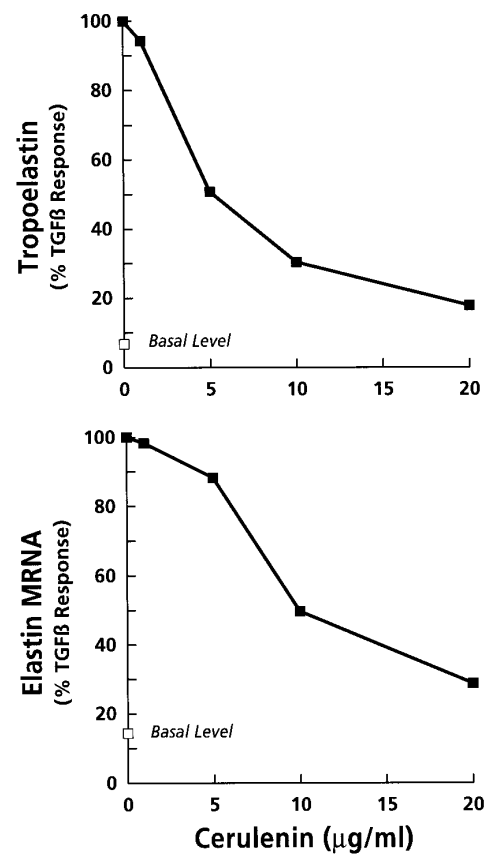

FIG. 4. Effect of inhibition of acylation on the TGF-β1 modulation of elastin expression. Confluent cultures of human fetal lung fibroblasts were incubated for 24 h with various concentrations of the acylation inhibitor, cerulenin, and TGF-β1 was then added to a final concentration of 3 ng/ml. After 24 h, total RNA was prepared from the cells and subject to Northern analysis and tropoelastin in the media was determined as described in Materials and Methods. The values represent the average of determinations from duplicate cultures.

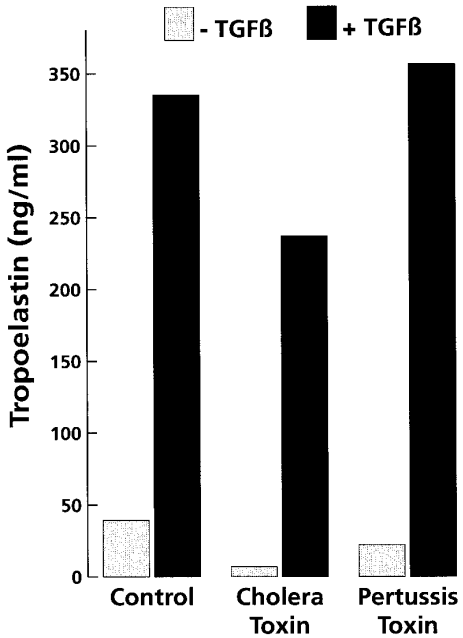

FIG. 5. Effect of cholera toxin and pertussis toxin, on the TGF-β1 modulation of tropoelastin synthesis. Confluent cultures of human fetal lung fibroblasts were incubated for 2 h with either one of the two toxins, and TGF-β1 was then added to a final concentration of 3 ng/ml. The incubation media obtained after 24 h were analyzed for tropoelastin with a specific ELISA. Values represent the average of duplicate cultures determined in triplicate.

μg/ml (Fig. 6). This result provides a possible link between protein tyrosine phosphorylation resulting in activation of a protein such as Rho and TGF-β1 signal transduction.

Reports that the TGF-β type I receptor interacted with farnesyl-protein transferase-α (51) and that the small GTPase protein, $p21^{ras}$, is involved in TGF-β signaling when coupled to a phosphatidylcholine-specific phospholipase C (19,20), an enzyme essential for the elastin upregulation by TGF-β (17), led us to investigate the effects of specific inhibitors known to regulate the biological activity of small GTP-binding proteins. Since farnesylation of $p21^{ras}$ is a primary requirement for its biological activity (27), promoting membrane localization for its pivotal role in cell signaling, we employed FTI-277 (a specific farnesyl transferase inhibitor). However, we observed no effect on the TGF-β up-regulation of tropoelastin at either the protein or mRNA level (Fig. 3). In contrast, GGT1-298, an agent which inhibits geranylgeranyl transferase I (GGTase I) but not farnesyl transferase (34,35), markedly inhibited the up-regulation of tropoelastin by TGF-β (Fig. 2). Taken together, these results suggest that $p21^{ras}$ is probably not involved, but rather that some other small GTP-binding protein plays a critical role in the TGF-β action. Members of the Rho or Rac family which are known to be modified by the addition of geranylgeranyl groups are strong candidates (27). The family of GTP-binding protein Rab, is probably not involved since these proteins are modified by geranylgeranyl transferase II (29), an enzyme which is not inhibited by GGTI-298 (34). We extended these studies by the use of the antibiotic cerulenin which blocks acylation, mainly palmitoylation (40,41), of target proteins. Addition of this agent to fetal lung fibroblasts abolished the TGF-β effect at both the mRNA (4A) and protein level (4B). Thus, our results demonstrate that geranylgeranylation and acylation are crucial for the regulation of elastin production by TGF-β.

Although the activities of many cytokines is very often dependent upon their ability to modulate gene transcription, there are also a number of instances in which post-transcriptional mechanisms are involved. While the transcriptional mechanisms have been heavily studied and are being defined in detail, much less is known about other levels of control. Recently, it has been shown that RasGAP Src homology 3 [SH3] binding protein (G3BP) harbors a phosphorylation-dependent RNase activity which specifically cleaves the 3′-untranslated region of human c-*myc* mRNA (52). It was postulated that G3BP could represent a link between a RasGTPase-activating protein-mediated signaling pathway and RNA turnover. The present work provides clues to the potential proteins which may be involved in the intersection of TGF-β signaling and capacity of this family of cytokines to regulate gene expression through alteration of mRNA stability.

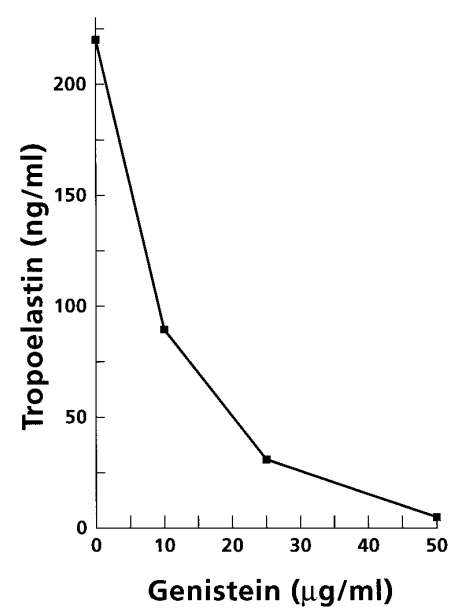

FIG. 6. Effect of the tyrosine kinase inhibitor, genistein, on the TGF-β1 modulation of elastin mRNA levels. Confluent cultures of human fetal lung fibroblasts were incubated for 2 h with the various indicated concentrations of genistein and TGF-β1 was then added to a final concentration of 3 ng/ml. The incubation media obtained after 24 h were analyzed for tropoelastin with a specific ELISA. Values represent the average of duplicate cultures determined in triplicate.

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