Autocrine Regulation of Membrane Transforming Growth Factor-α Cleavage*

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Transforming growth factor α (TGF-α) is biosynthesized as a membrane-bound precursor protein, pro-TGF-α, that undergoes sequential endoproteolytic cleavages to release a soluble form of the factor. In the present study, we have analyzed the biosynthesis and regulation of TGF-α production in human tumor-derived cell lines that endogenously express pro-TGF-α and the epidermal growth factor (EGF) receptor. These cells biosynthesized membrane-anchored forms of the TGF-α that accumulated on the cell surface. Membrane-bound pro-TGF-α interacted with the EGF receptor, and complexes of receptor and pro-TGF-α contained tyrosine-phosphorylated receptor. Activation of the EGF receptor by soluble EGF or TGF-α had a dual effect on TGF-α production: an increase in pro-TGF-α mRNA levels and an increase in pro-TGF-α cleavage. These effects were largely prevented by preincubation with an anti-EGF receptor monoclonal antibody that blocked ligand binding. Growth factor autoduction of cleavage could be stimulated by several second messenger pathways that are activated by the EGF receptor, including protein kinase C and intracellular calcium, and by other alternative mechanisms. EGF-stimulated cleavage of pro-TGF-α could be partially blocked by inhibition of these second messenger pathways. These results suggest that juxtacrine stimulation takes place in human tumor cells that coexpress both the EGF receptor and membrane-anchored TGF-α and that TGF-α is able to induce its own endoproteolytic cleavage by activating the EGF receptor.

Transforming growth factor α (TGF-α) is a 50-amino-acid single polypeptide, initially isolated from the culture medium of several oncogenically transformed cell lines (1), that is structurally and functionally related to the epidermal growth factor (EGF). TGF-α binds to the 170-kDa EGF receptor, a transmembrane glycoprotein with an extracellular ligand-binding domain and an intracellular domain that contains a tyrosine-specific protein kinase (2). Upon binding of TGF-α to the EGF receptor, the tyrosine kinase is activated, resulting in a cascade of biochemical and physiological responses that are involved in the mitogenic signal transduction pathway of cells (2). TGF-α has gained attention because of its predominant expression in tumor-derived cell lines and in human tumors (3, 4), suggesting that TGF-α contributes to neoplastic growth through autocrine and paracrine mechanisms (5). In fact, coexpression of high levels of TGF-α and the EGF receptor leads to a transformed cellular phenotype (3, 6), and increased expression of the precursor for TGF-α in transgenic mice causes hyperplasia of several tissues and even neoplastic transformation (7–9). Furthermore, the expression of EGF receptors is elevated in many epithelial tumor-derived cell lines (3), and many types of epithelial malignancies display increased EGF receptors on their cell surface membranes (5). This overexpression correlates with a poor clinical outcome in a number of malignancies (10).

TGF-α is derived from a larger 20–22-kDa transmembrane precursor (11, 12), pro-TGF-α, which undergoes several post-translational modifications that include N- and O-linked glycosylation (13–15) and palmitoylation (13). Several molecular forms of soluble TGF-α have been reported, and this heterogeneity appears to be due to both the type and degree of ectoglycosylation and the preference for different sites of proteolytic cleavage of the precursor (14, 16, 17). In transfected fibroblasts, proteolytic maturation of TGF-α occurs in two steps (13, 15, 18). At the plasma membrane or in a cellular compartment very close to it, the first cleavage of pro-TGF-α occurs between Ala89 and Val90 (by an activity referred to as pro-TGF-αase-1). This removes the NH2-terminal glycosylated extension leaving a cell-associated 17-kDa pro-TGF-α form that contains the mature sequence of TGF-α within the precursor. This cleavage step occurs rapidly (t½ = 15 min) (18). Release of soluble forms of TGF-α occurs only after a second enzymatic activity (referred to as pro-TGF-αase-II) cleaves the Ala89–Val90 peptide bond that links TGF-α to the rest of the precursor molecule. This cleavage results in the generation of a 6-kDa soluble fragment, which accumulates in the culture medium and corresponds to mature TGF-α, and a cell-associated 15-kDa residual terminal fragment often referred to as a tail.

In transformed cells such as retrovirally transformed fibroblasts (14, 16) and hepatoma cells (17), indirect evidence indicates that the basal activity of both enzymatic activities can be relatively high. In these cells, primary activity of pro-TGF-αase-II can lead to production of an additional soluble 20-kDa form of TGF-α, designated meso-TGF-α, which retains the glycosylated NH2 extension (14).

Endoproteolytic cleavage by pro-TGF-αase-II is highly regulated and depends on the activity of several second messenger systems such as protein kinase C (PKC), free cytosolic calcium ([Ca2+]), and other still undefined pathways that can be stimulated by serum factors (18, 19). The pro-TGF-αase-II cleaving activity is quite distinct from other protein maturation or degradation processes (20). Biochemical evidence suggests that the
enzyme belongs to the serine protease family (21) and requires ATP and membrane association for activity (22), and topologically, its regulated activity depends on the presence of the enzyme and substrate at the plasma membrane. This endoproteolytic system is likely to be involved in the cleavage of other transmembrane proteins that undergo regulated release of their ectodomains (23).

Since knowledge of the molecular properties and factors regulating release of pro-TGF-α and other membrane-anchored growth factors is of significant biological and potentially therapeutic interest, we have analyzed the expression, biosynthesis, and cleavage of pro-TGF-α in several different tumor cell lines that endogenously express both the EGF receptor and TGF-α. We show that membrane-bound pro-TGF-α accumulates at the plasma membrane and is able to interact with the EGF receptor. Moreover, TGF-α or EGF, by acting through the EGF receptor, is able to increase TGF-α production by a dual mechanism that involves mRNA increase and cleavage of the membrane-bound precursor into a soluble factor. Some of these effects are prevented by a monoclonal antibody (mAb) that binds to the extracellular domain of the EGF receptor and blocks ligand binding (24, 25). This antibody induces tumor xenograft regression in nude mice, which bear neoplasias caused by injection of human tumor cells that overexpress both the EGF receptor and its ligand TGF-α (26, 27).

EXPERIMENTAL PROCEDURES

Materials—Anti-EGF receptor mAbs 225 and 528 and anti-pro-TGF-α antibodies have been described previously (24, 25, 28). EGF was from Collaborative Research (Waltham, MA) and TGF-α from Intergen Company (Purchase, NY). Anti-phosphotyrosine 4G10 antibody was from Upstate Biotechnology Inc. (Lake Placid, NY); phorbol 12-myristate 13-acetate (PMA) and EGTA were from Sigma; A23187 was from Calbiochem.

Cell Lines—Chinese hamster ovary cells (CHO) had been previously transfected with a cDNA that encodes the rat pro-TGF-α (CHO-TGF-α cells) (15). A431 squamous carcinoma cells, MDA-468 breast adenocarcinoma cells, SK-R-29 renal carcinoma cells, ME-180 cervix carcinoma cells, C4I cervix carcinoma cells, and DU-145 prostate adenocarcinoma cells, were obtained from ATCC. DiI-coated cells were generously provided by Dr. B. Boman (Creighton University, Omaha, NE). MCF-10A cells were obtained from the Michigan Cancer Foundation. Unless otherwise specified, cells were grown at 37°C in monolayer culture with Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium (1:1). When otherwise specified, cell cultures became near confluent, the medium was replaced with cysteine and PMA and incubated for 15 min. Where indicated, cells were fed with 10% fetal bovine serum added to the medium for 6 h.

Metabolic Labeling—Total cellular RNA was extracted from A431 cells using guanidium thiocyanate as previously described (29). Total cellular RNA (10 μg/lane) prepared as above was electrophoresed on a 1% formaldehyde-agarose gel and transferred onto nitrocellulose (Schleicher and Schuell) membranes. The membranes were hybridized to DNA probes that were radiolabeled by random priming. Hybridizations and autoradiography were performed as previously described (29). At least 106 [35S]dCTP total counts were added for each hybridization. The radiolabeled probe was isolated from a plasmid containing a 1.35-kilobase pair EcoRI cut human TGF-α (11).

Western Immunoblotting—Three confluent dishes (100 mm) of A431 cells were washed twice with PBS containing Ca2+ and Mg2+ and then lysed with 1 ml of immunoprecipitation buffer. Lysates were cleared by a 15-min centrifugation (10,000 × g, 4°C), followed by immunoprecipitation with either the anti-pro-TGF-α antibody or anti-EGF receptor mAb 528 for 2 h at 4°C. Immunocomplexes were harvested by the addition of 50 μl of a 1:1 slurry of protein A-Sepharose in PBS, washed four times with the immunoprecipitation buffer, and boiled into 30 μl of electrophoresis sample buffer. The samples were subjected to SDS-PAGE followed by overnight transfer to polyvinyl difluoride membranes. Membranes were blocked with a solution containing 150 mM NaCl, 1% bovine serum albumin, 0.1% Tween 20, and 20 mM Tris, pH 7.4, for 1 h, then incubated with the anti-phosphotyrosine antibody (1:5000) for an additional hour, washed four times for 5 min each in the same solution, and incubated for 45–60 min with a 1:10,000 dilution of a horseradish peroxidase-conjugated secondary antibody. The membrane was vigorously washed four times for 5 min each in the same solution, followed by a 1-min incubation with a luminol-based solution and chemiluminescent detection.

RESULTS

Molecular Forms of TGF-α in Human Tumor Cell Lines—The presence of high M, soluble TGF-α forms in the culture medium from oncogenically transformed cells (14, 16) led us to address whether human tumor-derived cell lines have distinctive biosynthetic and/or enzymatic cleaving activity. Monolayers of different cell lines were metabolically labeled with [35S]methionine, lysed at different chase times, and immunoprecipitated with an antisera raised against the last 14 amino acids of the cytosolic domain of pro-TGF-α. In the human epidermoid carcinoma cell line A431 (Fig. 1A), three major pro-TGF-α forms were detected at the beginning of the chase: an 18-kDa form together with two lower mobility forms of 20 and 22 kDa. When compared to CHO-TGF-α biosynthetic products, these forms comigrated with the nascent (18 kDa) and heterogeneously glycosylated (20–22 kDa) pro-TGF-α, respectively. Within the first 30 min of chase, the glycosylated 20–22-kDa forms rapidly disappeared to become a 17-kDa membrane-bound form, which remained cell-associated for up to 4 h of chase. Analysis of pro-TGF-α from the other tumor cell lines showed that they followed a similar pattern of pro-TGF-α biosynthesis (Fig. 1B and data not shown) and also predominantly accumulated the 17-kDa form. A 15-kDa band, which corresponds to the COOH-terminal fragment (tail), was seen in the CHO-TGF-α cells after 2 h of chase. In some experiments, this 15-kDa band could also be detected in the tumor cell lines 1 h or more after the chase, but this always represented a small fraction of the total. Those bands were all true pro-TGF-α biosynthetic products was verified by performing the immunoprecipitations in the presence of an excess (1 μM) of competing peptide against which the anti-pro-TGF-α antibody was raised (Fig. 1C).

Interaction of Pro-TGF-α with the EGF Receptor in A431 Cells—In cocultures of cells artificially engineered to overexpress either the EGF receptor or mutant pro-TGF-α resistant to endoproteolytic cleavage, these two molecules have been found to be biologically interactive, resulting in activation of receptor tyrosine kinase (30–32). The situation with cells that endogenously express both proteins may be significantly different. First, these cells typically have a lower complement of pro-TGF-α than transfected cell lines; second, they produce pro-TGF-α that can be readily cleaved; and third, the receptor
and the ligand may be present in the same cell. To analyze whether membrane-anchored pro-TGF-α could interact with the EGF receptor, lysates from confluent cultures of A431 cells were immunoprecipitated with either anti-pro-TGF-α or anti-EGF receptor antibodies, and Western blots of these immunoprecipitates were probed with an anti-phosphotyrosine antibody. As shown in Fig. 2A (left lane), a tyrosine-phosphorylated 170-kDa band was immunoprecipitated using the anti-pro-TGF-α antibody. This band comigrated with the EGF receptor immunoprecipitated from the same cells (Fig. 2B, left lane). Treatment of these cells with exogenous EGF induced further phosphorylation of the 170-kDa EGF receptor band (Fig. 2B, right lane) but, as anticipated, did not appreciably affect the phosphotyrosine content of EGF receptor coimmunoprecipitated with the anti-pro-TGF-α antibodies (Fig. 2A, right lane). These results suggest that cell membrane-bound pro-TGF-α partially activates EGF receptors on the A431 cells. Receptors that are not activated by endogenous pro-TGF-α can be activated by addition of exogenous ligand.

Autocrine Regulation of TGF-α Production—In several cell lines that biosynthesize both the EGF receptor and pro-TGF-α, the mRNA coding for these proteins is usually up-regulated by increasing the level of activity of the receptor (33–35). We asked whether there was a dual regulation of pro-TGF-α production, i.e., if certain treatments (stimulatory or inhibitory) could affect both synthesis on the one hand and cleavage to produce soluble factor on the other. The effects of receptor activation on pro-TGF-α regulation were studied by adding either EGF or TGF-α. As an inhibitor of receptor activity, anti-receptor mAb 225 was used. This antibody binds with high affinity to the extracellular domain of the EGF receptor, blocks binding of ligand(s) to the receptor, and decreases ligand-induced receptor phosphorylation (24, 25).

First, the effects of EGF receptor stimulation on pro-TGF-α mRNA accumulation were analyzed. Addition of saturating concentrations of TGF-α (30 nM) increased the production of a 4-kilobase pair mRNA that hybridized with a specific human pro-TGF-α probe in A431 cells (Fig. 3). Having established that EGF receptor stimulation resulted in increased pro-TGF-α mRNA, we next analyzed the effects of EGF receptor inhibition on pro-TGF-α levels. Addition of mAb 225 (10–100 nM) decreased the basal pro-TGF-α mRNA level in A431 cells (Fig. 3). Thus, the prevention of ligand-induced activation of the receptor by a receptor-blocking antibody resulted in down-regulation of TGF-α mRNA expression.

Studies with metabolically radiolabeled A431 cells showed that addition of exogenous TGF-α induced cleavage of membrane pro-TGF-α (Fig. 4). As expected from a precursor-product relationship, either a decrease of the 17-kDa pro-TGF-α form or an increase in the 15-kDa tail form is indicative of pro-TGF-α cleavage, and the kinetics can be followed by analyzing the labeled 17-kDa/15-kDa ratio. Treatment with EGF or TGF-α for as short as 15 min induced the appearance of the 15-kDa cytosolic tail of pro-TGF-α with a concomitant increase in the intensity of the 17-kDa precursor form (Fig. 4B). This cleavage did not increase by further prolonging incubation times with either growth factor for up to 1 h. The persistence of a significant proportion of the 17-kDa form indicates that cleavage was not complete. Incubation with TGF-α also induced cleavage of membrane pro-TGF-α in other tumor-derived cell lines (Fig. 4B).
cells was next investigated. Treatment for 45 min with the tumor-promoting phorbol ester PMA, which is known to directly activate PKC isozymes (40), provoked 17 to 15 kDa conversion (Fig. 5A). This was prevented by prior prolonged incubation of A431 cell cultures with PMA for 24 h, a treatment that causes down-regulation of PKC activity (not shown). These PKC and Ca\(^{2+}\) pathways are independent of each other, since, on the one hand, down-regulation of PKC did not prevent Ca\(^{2+}\) ionophore-induced cleavage (Fig. 5A) and, on the other hand, treatment with EGTA did not prevent PMA-induced cleavage of pro-TGF\(-\alpha\) (not shown). When the activation of cleavage by the two second messenger systems was compared, raising intracellular Ca\(^{2+}\) was more efficient than increasing PKC activity (Fig. 5A, first 4 bars), and either pharmacological treatment was found to be more efficient than EGFTGF\(-\alpha\) in provoking processing of the 17-kDa form.

Cleavage of membrane-anchored pro-TGF\(-\alpha\) by activation of the EGF receptor with EGF or TGF\(-\alpha\) was only partially inhibited by pharmacological inhibition of these second messenger systems (Fig. 5A, last 4 bars, and Fig. 5B, lanes 3 and 4). Although the combined pharmacological inhibition of both second messenger systems markedly reduced growth factor-induced cleavage (Fig. 5B, lane 5), there was still a residual cleavage activity that neither the Ca\(^{2+}\) nor the PKC systems could account for (Fig. 5B, compare the 17 kDa (uncleaved) to 15 kDa (cleaved) pro-TGF\(-\alpha\) ratio in lanes 1 and 5).

Treatment with the anti-EGF receptor blocking mAb did not affect the general machinery responsible for pro-TGF\(-\alpha\) biosynthesis or cleavage since (i) incubation with the mAb prior to and during the chase did not affect the normal pattern of pro-TGF\(-\alpha\) biosynthesis (Fig. 4A) and (ii) cleavage induced by pharmacological activation of second messenger systems with PMA or A23187 was insensitive to mAb 225 (Fig. 6).

**DISCUSSION**

In experiments with cells expressing genetically engineered mutant forms of pro-TGF\(-\alpha\) resistant to cleavage by pro-TGF\(-\alpha\)-ase-I, the membrane-bound 17-kDa form has been shown to be biologically active by a proposed juxtacrine mechanism (30–32). Although the mechanism of activation of the EGF receptor by soluble ligand has been well characterized (41, 42), little is known about the expression and activity of pro-TGF\(-\alpha\) in human cancer cells with an active EGF receptor/TGF\(-\alpha\) autocrine pathway. For this reason, we investigated the molecular forms of pro-TGF\(-\alpha\) in a series of tumor-derived cell lines with putative active EGF receptor autocrine loops and high levels of receptor expression.

In transfected fibroblasts, generation of soluble TGF\(-\alpha\) from the 17-kDa membrane-anchored form depends upon the activity of the transmembrane endoprotease pro-TGF\(-\alpha\)-ase-II, which cleaves the Ala\(^{180}\)–Val\(^{190}\) peptide bond, thus eliminating the role of membrane-bound pro-TGF\(-\alpha\) as a juxtacrine molecule (18). In retrovirally transformed embryo fibroblasts (14, 16) and hepatocellular carcinoma cells (17), the culture medium accumulates mature 6-kDa TGF\(-\alpha\) as well as heterogeneous 15-kDa soluble meso-TGF\(-\alpha\). Although pulse-chase analyses of pro-TGF\(-\alpha\) in these cell lines have not been reported, the high amount of large meso-TGF\(-\alpha\) reflects a high pro-TGF\(-\alpha\)-ase-I activity, which releases some of the pro-TGF\(-\alpha\) molecules from the cell membrane before cleavage by pro-TGF\(-\alpha\)-ase-I can occur. In the tumor-derived cells that we have analyzed, the biosynthesis of pro-TGF\(-\alpha\) initially followed a pattern analogous to that described for transfected cellular models (13, 15, 18). However, these cell lines accumulated the 17-kDa membrane-anchored precursor form, suggesting that the activity of pro-TGF\(-\alpha\)-ase-I was low. In addition, the rapid disappearance of the NH\(_2\)-terminal glycosylated extension to produce the 17-
kDa molecular form indicates a considerable pro-TGF-\(\alpha\)-ase-I activity. The data presented here suggest that membrane-anchored pro-TGF-\(\alpha\) represents a significant proportion of biosynthesized pro-TGF-\(\alpha\) in the tumor cells that we have studied and, in this conformation, is associated with tyrosine-phosphorylated EGF receptor. It is possible, therefore, that membrane-anchored forms could carry out juxtacrine stimulation that would continually enhance growth of receptor-containing cells, and cleavage would terminate this function and facilitate the clearance of the factor. This could be an efficient form of receptor activation, since down-regulation of ligand and receptor, and their subsequent catabolism, would be precluded. The response to the addition of saturating concentrations of exogenous EGF (Fig. 4A) demonstrates that most EGF receptors remain inactivated when these tumor cells are only exposed to endogenous sources of ligand. Furthermore, soluble forms of TGF-\(\alpha\) were reported to be more active than membrane-bound TGF-\(\alpha\) (30).

Nevertheless, in these rapidly growing A431 cell cultures it is the membrane-bound form that predominates, and interestingly, exogenous soluble ligand in saturating amounts actually inhibits proliferation (24, 43, 44).

Cleavage of membrane pro-TGF-\(\alpha\) is highly regulated (37). Mechanisms that can trigger the release of soluble TGF-\(\alpha\) include rises in the intracellular free Ca\(^{2+}\) concentration (19), activation of PKC (18), and other still undefined pathways switched on by serum factors (19). Although these mechanisms are different, the cleavage event activated by all of them is probably the same, since identical sets of protease inhibitors block cleavage activated by the different pathways (21) and genetic mutants of pro-TGF-\(\alpha\) in CHO fibroblasts are resistant to cleavage activated by several alternative mechanisms (23). The Ca\(^{2+}\)-dependent and PKC mechanisms of cleavage appear to be quite universal, since we have also found them operative in human tumor cell lines. In A431 cells, pharmacological increases in cytosolic Ca\(^{2+}\) or PKC activity increased the conversion of 17-kDa pro-TGF-\(\alpha\) to the 15-kDa terminal fragment tail. Since a certain degree of cross-talk between these two second messenger systems exists, it was possible that their effect was mediated by a shared pathway. This has been ruled out by using treatments that neutralize one pathway but not the other. Thus, elevated Ca\(^{2+}\) activates pro-TGF-\(\alpha\) cleavage in cells that have been desensitized to PMA action. On the other hand, PMA is able to induce cleavage in the presence of the Ca\(^{2+}\) chelator EGTA in the culture medium, a treatment that completely prevents calcium ionophore-induced cleavage (19).

An interesting finding that comes out of our data is the relative effectiveness of these second messenger systems in inducing pro-TGF-\(\alpha\) cleavage. In A431 cells, Ca\(^{2+}\) is more efficient in

![Fig. 5. Effects of A23187 and PMA on pro-TGF-\(\alpha\) cleavage.](image)

A, 17 kDa ( uncleaved) to 15 kDa (cleaved) pro-TGF-\(\alpha\) ratio in A431 cells. Cells were labeled with \[^{35}S\]cysteine for 30 min and chased for 45 min in complete medium containing EGF (10 nM), PMA (1 \(\mu\)M), or A23187 (1 \(\mu\)M). Where indicated, EGTA (10 mM) was added to the cultures 5 min before the addition of these agents. In lanes 7 and 8, cells had been depleted of protein kinase C by a 24-h preincubation with 1 \(\mu\)M PMA (PKC, -). B, control A431 cells (PKC, +) and A431 cells that had been depleted of protein kinase C by 24-h preincubation with 1 \(\mu\)M PMA (PKC, -) were labeled as above and chased for 30 min in the presence of EGF ± EGTA as described under A.

![Fig. 6. EGF receptor blocking antibody mAb 225 prevents TGF-\(\alpha\) mediated pro-TGF-\(\alpha\) cleavage but does not prevent A23187- and PMA-mediated pro-TGF-\(\alpha\) cleavage.](image)

A431 cells were metabolically labeled with \[^{35}S\]cysteine for 30 min and chased for 45 min. During the last 30 min of the chase TGF-\(\alpha\) (10 nM), A23187 (1 \(\mu\)M), or PMA (1 \(\mu\)M) was added as indicated. Paired cultures were incubated with saturating amounts of mAb 225 (100 nM), as indicated during the 45 min of the chase period.
provoking cleavage than PMA, while the opposite is the rule for cells of fibroblastic origin (18, 19). This suggests potential mechanisms for specificity, since in some tissues a cell could be highly sensitive to the stimulation of one pathway and induce release of soluble TGF-α, while other cell types could be largely refractory to the activation of this pathway.

What are the mechanisms by which EGF receptor activation triggers pro-TGF-α cleavage? EGF receptor activation is known to induce the hydrolysis of membrane polyphosphoinositides, inducing increases in [Ca^{2+}], and PKC activity (39, 45). Pharmacological manipulations of these second messenger systems support the conclusion that both may participate to a certain extent. Yet, the residual activity of pro-TGF-α cleavage after neutralization of the [Ca^{2+}] and PKC pathways suggests that additional unidentified mechanisms are triggered by EGF receptor activation.

We find that TGF-α production is autoregulated by a dual mechanism in human tumor cell lines. On the one hand, activation of the EGF receptor increased the level of pro-TGF-α mRNA, as has been reported in other cells (33–35), and, on the other hand, receptor activation rapidly induced cleavage of the membrane-anchored factor. The latter is of special interest for other mechanisms for specificity, since in some tissues a cell could be largely sensitive to the stimulation of one pathway and induce release of soluble TGF-α, while other cell types could be largely refractory to the activation of this pathway.

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