Processing of the Transforming Growth Factor β Type I and II Receptors

BIOSYNTHESIS AND LIGAND-INDUCED REGULATION*

(Received for publication, November 22, 1996, and in revised form, December 19, 1996)

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Three cell surface transforming growth factor β (TGFβ) receptor (R) proteins regulate the effects of TGFβ isoforms on growth and differentiation. TGFβ-IR and -IIR are transmembrane serine/threonine kinases directly mediating the signaling across the plasma membrane. Both TGFβ and its receptors are ubiquitously expressed, hence the fine regulation of the multiplicity of responses most likely involves several levels of control including the regulation of expression, complex formation, and down-regulation of the receptor proteins. In mink lung epithelial cells, TGFβ-IR was first synthesized as a ~60-kDa endoglycosidase H-sensitive precursor protein, which was converted to a mature ~70-kDa protein. The half-life of metabolically labeled mature TGFβ-IR was estimated to be 60 min and was further reduced to ~45 min in the presence of exogenous TGFβ1. Minimal internalization of 125I-TGFβ1 at 37 °C was detected suggesting that the rapid turnover was not due to endocytosis and degradation of the ligand-receptor complexes. TGFβ-IR was synthesized as a ~53-kDa precursor protein, which was processed to a mature ~55-kDa receptor protein. The half-life of TGFβ-IR was >12 h. A fraction of tunicamycin-treated type I and II receptors that reach the cell surface was able to associate in the presence of ligand suggesting that heteromeric complexes can form in a post-endoplasmic reticulum compartment before full glycosylation is achieved. These results show differential processing and turnover of TGFβ-IR and TGFβ-II providing a potential additional mechanism for modulation of cellular responses to TGFβs.

The TGFβ family of proteins participates in the regulation of a variety of biological activities including regulation of cellular growth and phenotype (1–3). Most cells can produce latent forms of TGFβ, and their activation plays an important regulatory role in TGFβ actions (4, 5). In epithelial cells, TGFβ treatment leads to inhibition of growth, regulation of the production of extracellular matrix proteins, and modulation of proteolysis (4). The cell surface signaling receptor complex is composed of two transmembrane serine/threonine kinases named type I (~55 kDa) (6) and type II (~70 kDa) (7) TGFβ receptors. TGFβ-IIIR binds the ligand first, after which TGFβ-IR is recruited to a heteromeric complex most likely containing several receptor molecules (8, 9). Ligand-dependent phosphorylation of the GS-domain of TGFβ-IR leads to the propagation of the signal downstream (10, 11). Type III TGFβ receptor, a proteoglycan also known as betaglycan, functions mostly as a storage protein as well as in presenting the ligand for the signaling receptors (12). Both TGFβ-IR and TGFβ-IIIR are needed to mediate the biological effects of TGFβ ligands. Recent reports, however, suggest separate signaling pathways for the antiproliferative and the matrix modulatory effects of TGFβ with the latter only requiring TGFβ-IR signaling in some cell systems (13, 14).

Since most cell types can produce both TGFβ receptors and ligand(s), the regulation of cellular responsiveness relies on the production of active TGFβ and its presentation to signaling receptors. Therefore, the role of receptor protein associations, turnover, and down-regulation is likely critical for the control of TGFβ signals and the modulation of overall cellular responsiveness. TGFβ-IIIR levels have been shown to correlate with TGFβ responsiveness (15, 16). Cancer cells refractory to TGFβ’s antiproliferative action have often lost TGFβ-IIIR expression (17–19). Although TGFβ-IR can bind ligand only in association with TGFβ-IIIR, it is indispensable for TGFβ responses since phosphorylation of its GS domain provides possible binding sites for intracellular substrates (10, 20). Interestingly, cellular transformation by Ha-ras oncogene as well as trophic hormones can down-regulate cell surface binding sites for TGFβ, thus altering TGFβ responsiveness (21–23). We have examined the biosynthesis and ligand-induced modulation of naturally expressed TGFβ-IR and TGFβ-IIIR in CCL-64 mink lung epithelial cells, which are known to express abundant amounts of all three TGFβ receptors and are potently growth inhibited by exogenous TGFβ.

EXPERIMENTAL PROCEDURES

Cell Culture and Metabolic Labeling—CCL-64 mink lung epithelial cells (American Type Culture Collection, Rockville, MD) were cultured in McCoy’s medium supplemented with 5% fetal bovine serum (JRH Biosciences, Lenexa, KS). Subconfluent (~80%) cell monolayers were labeled with 500 μCi/ml Tran35S-label for 2.5 h in improved minimal essential medium
lacking cysteine and methionine. Following labeling the cell monolayers were prepared as described below.

Cell Lysis and Immunoprecipitation—Cell monolayers in 100-mm tissue culture plates were solubilized with 1 ml of lysis buffer (20 mM Tri-HCl, pH 7.4, 2 mM EDTA, 25 mM NaF, 1% Triton X-100, 1 mM dithiothreitol, 2 mM NaMo4, 2 mM NaVO4, 1 µg/ml aprotinin, 1 µg/ml leupeptin) for 30 min at 4°C. After a 12,000 x g centrifugation for 15 min, the lysates were precleared with protein A-Sepharose (Sigma) for 30 min at 4°C and precipitated overnight with a polyclonal TGF β1 (Genentech, South San Francisco, CA), TGF β1 (C-16, Santa Cruz Biotechnology), or TGF β1 antibodies. The latter was raised against the extracellular domain of the human type II receptor overexpressed in S9 cells as a HIS-tagged protein and provided by Dr. Xiao-Fan Wang (Duke University, Durham, NC). This incubation was followed by a 1.5-h incubation with protein A-Sepharose. The Sepharose particles were washed three times with radioimmunoprecipitation buffer (50 mM Tri-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) and the immune complexes dissociated with lysis buffer containing 1% SDS for 5 min at 95°C. The concentration of SDS was diluted to 0.1% with lysis buffer and a second immunoprecipitation with the same antibody was performed for 2.5 h at room temperature, the last 1.5 h in the presence of protein A-Sepharose. The immune complexes were eluted with Laemmli sample buffer, boiled, resolved by 8% SDS-PAGE, and visualized by autoradiography.

Deglycosylation Procedures—Cell lysates were subjected to overnight immunoprecipitation with TGF β1 or TGF β2 antibodies as described above. After washing the immune complexes bound to protein A-Sepharose beads were treated with 50 milliunits of endoglycosidase H (endo H) or a combination of NANase II, O-glycosidase, and PNGase F (deglyc) overnight followed by a second immunoprecipitation with the same antibodies. The immune complexes were analyzed by 8% SDS-PAGE and autoradiography. The arrows indicate the migration of receptor proteins. Molecular mass markers in kDa are shown at the left of each panel.

Processing of the TGFβ Type I and II Receptors

FIG. 1. Biosynthesis of TGFβ1-IIR. Cells were labeled with 500 µCi/ml Tran35S-label for 20 min and chased for the indicated times with growth medium containing unlabeled methionine (300 µg/ml) and cysteine (500 µg/ml). Lysis of cells was followed by double immunoprecipitation with TGFβ-IIR antibodies as described under “Experimental Procedures.” A, chase time ranging from 0 to 4 h. B, after a 30-min chase and immunoprecipitation, the immune complexes bound to protein A-Sepharose were treated with 50 milliunits of endoglycosidase H (endo H) or a combination of NANase II, O-glycosidase, and PNGase F (deglyc) overnight followed by a second immunoprecipitation with the same antibodies. The immune complexes were analyzed by 8% SDS-PAGE and autoradiography. The arrows indicate the migration of receptor proteins. Molecular mass markers in kDa are shown at the left of each panel.

FIG. 2. Biosynthesis of TGFβ1-IR. A, cells were labeled with 500 µCi/ml Tran35S-label for 20 min and chased for the indicated times with growth medium containing unlabeled methionine (300 µg/ml) and cysteine (500 µg/ml). Lysis of cells was followed by double immunoprecipitation with TGFβ-IR antibodies. B, cells were labeled with 50 µCi/ml Tran35S-label for 2.5 h, lysed, and precipitated with TGFβ-IR antibodies in the presence (+) or absence (−) of the immunizing peptide. Immune complexes bound to protein A-Sepharose beads were deglycosylated (deglyc) as in Fig. 1B followed by a second immunoprecipitation with the same antibody. For panels A and B, the immune complexes were analyzed by 8% SDS-PAGE and autoradiography. The arrows indicate the migration of receptor proteins. Molecular mass markers in kDa are shown at the left of each panel.
The Biosynthesis and Turnover of TGFβ Type I and II Receptors

The Biosynthesis and Turnover of TGFβ Type I and II Receptors—Recent studies using cycloheximide to block cellular protein synthesis have suggested that the turnover rate of TGFβ binding sites in bone cells is relatively fast (25). Since TGFβ-II cannot bind TGFβ-I alone, these affinity binding studies provide clues only on the turnover of TGFβ-II protein. We have followed the fate of metabolically labeled receptors in CCL-64 mink lung epithelial cells. Subconfluent cultures were labeled with Tran35S-label for 20 min followed by chase with medium lacking label for variable times. The newly synthesized TGFβ-II first appeared as a ~60-kDa form with a half-life of <30 min (Fig. 1A). This form was sensitive to treatment with endoglycosidase H (Fig. 1B). Since endoglycosidase H cleaves only high mannose oligosaccharides but not more complex structures, this result suggests that this ~60-kDa form represents an ER, pre-Golgi precursor form. Within 15 min of the chase, a ~70-kDa endoglycosidase H-resistant smear appeared with a longer half-life of approximately 60 min. This form was sensitive to deglycosylation by enzymes that remove all N- and O-linked oligosaccharides (Fig. 1B), indicating it represents the mature type II receptor.

TGFβ-II was also first synthesized as a precursor form of molecular mass ~53 kDa that was chased to a mature ~55-kDa protein (Fig. 2A). The observed half-life of both precursor and mature forms was considerably longer than that of TGFβ-II. The longer persistence of the type I receptor precursor form indicates that a less efficient ER processing compared with that of TGFβ-II precursor. The steady-state Tran35S-labeled mature TGFβ-II was sensitive to N- and O-linked deglycosylation (Fig. 2B), thus indicating that the 55-kDa form represents the fully processed cell surface receptor. The higher molecular mass band was not blocked by competing immunizing peptide, indicating it is nonspecific (Fig. 2B).

Effect of TGFβ on TGFβ-II and -IIR Turnover—We analyzed next whether TGFβ could further influence the rapid turnover of TGFβ-II. For this purpose, subconfluent cells were labeled with Tran35S-label for 2.5 h in methionine/cysteine-free medium followed by chase with full medium. At the end of the labeling (time 0) both the precursor and the mature TGFβ-II could be immunoprecipitated from the cell lysates (Fig. 3A). The amount of the mature ~70-kDa TGFβ-II was higher compared with the precursor form indicating that it represents the predominant and/or more stable receptor species. In cells that were treated with 10 ng/ml TGFβ-I for the last 20 min of labeling as well as throughout the chase, two other proteins coprecipitated with TGFβ-II: a ~50-kDa and a ~37-kDa protein, the latter corresponding to the size of TRIP-1 (TGFβ receptor interacting protein-1 (26)). The identity of these proteins was not further confirmed, but their ability to coprecipitate with TGFβ-II antibodies in a ligand-dependent manner under stringent double immunoprecipitation conditions suggests a strong association induced by exogenous ligand. In the presence of TGFβ-I the half-life of the mature TGFβ-II was shortened to approximately 45 min (Fig. 3B).

Labeling with Tran35S-label for 2.5 h in methionine/cysteine-free medium followed by chase with full medium indicated that the ~55-kDa mature TGFβ-II form was the predominant one (Fig. 4). Some TGFβ-II protein could still be immunoprecipitated 18 h after the 2.5-h steady-state labeling indicating that TGFβ-II half-life was considerably longer than that of TGFβ-II. Contrary to the results with TGFβ-II, the half-life of

RESULTS AND DISCUSSION

The Biosynthesis and Turnover of TGFβ Type I and II Receptors—Recent studies using cycloheximide to block cellular protein synthesis have suggested that the turnover rate of TGFβ binding sites in bone cells is relatively fast (25). Since TGFβ-II cannot bind TGFβ-I alone, these affinity binding studies provide clues only on the turnover of TGFβ-II protein. We have followed the fate of metabolically labeled receptors in CCL-64 mink lung epithelial cells. Subconfluent cultures were labeled with Tran35S-label for 20 min followed by chase with medium lacking label for variable times. The newly synthesized TGFβ-II first appeared as a ~60-kDa form with a half-life of <30 min (Fig. 1A). This form was sensitive to treatment with endoglycosidase H (Fig. 1B). Since endoglycosidase H cleaves only high mannose oligosaccharides but not more complex structures, this result suggests that this ~60-kDa form represents an ER, pre-Golgi precursor form. Within 15 min of the chase, a ~70-kDa endoglycosidase H-resistant smear appeared with a longer half-life of approximately 60 min. This form was sensitive to deglycosylation by enzymes that remove all N- and O-linked oligosaccharides (Fig. 1B), indicating it represents the mature type II receptor.

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steady-state 35S-labeled TGFβ-IR was not considerably affected by TGFβ1 treatment (Fig. 4).

Internalization of 125I-TGFβ1—We addressed whether the rapid turnover of TGFβ receptors in the presence of ligand was due to endocytosis and degradation. To measure the rate of 125I-TGFβ1 internalization, cells were exposed to the radiolabeled ligand for different times at 37°C. Surface-bound ligand was released from the cell surface by an acid wash procedure. This buffer (pH 2.4) was shown to remove ~95% of specifically bound 125I-TGFβ1 after 2 h of binding at 4°C, conditions under which no ligand internalization should occur (Fig. 5A, bars 2). To confirm the efficacy of our receptor stripping procedure, binding was performed in intact cells after acid wash. Cell membranes were not affected by the transient exposure to low pH, since 125I-TGFβ1 binding was comparable with that in non-pretreated cells (Fig. 5A, bars 3 versus bar 1).

A time-dependent increase in acid-washable specific binding of 125I-TGFβ1 was observed (Fig. 5B). Very little ligand was internalized during the first 10 min of the experiment, and even after a 30-min incubation at 37°C the ratio of surface/internalized ligand was 0.25 (Fig. 5B). These results suggest that the rapid turnover of TGFβ receptors in the presence of ligand may not be explained by endocytosis and subsequent degradation. This was further supported by Western blot analysis of TGFβ1-treated cells. An overnight incubation with exogenous ligand did not alter TGFβ-IR content in CCL-64 cells (Fig. 5C).

The possibility that the short half-life results from the release of the extracellular domain of TGFβ1 into the medium was investigated by immunoprecipitation of the chase medium after labeling with Tran35S-label (see above) with the polyclonal TGFβ-IR antibody (2732) raised against the receptor’s extracellular domain. Even with a long exposure time no proteins were detected in the precipitated chase medium 1–3 h after cell labeling (data not shown), and small proteolytic fragments also were not detected in the precipitated cell lysates (Fig. 1A).

Association of TGFβ-IR with TGFβ-IR—Chen et al. (27) reported recently that the cytoplasmic domains of type I and II receptors have an inherent affinity for each other even in the absence of the ligand. The interaction was shown to require kinase activity and thus depended on phosphorylation. Part of the receptors at cell surface exist as hetero-oligomers although TGFβ-IR homo-oligomers predominate (28, 29). We studied the stage at which TGFβ-IR can associate with TGFβ-IR by stripping oligosaccharide chains from receptor proteins with tunicamycin followed by 125I-TGFβ1 binding and cross-linking as indicated under “Experimental Procedures.” Cell lysates were subjected to immunoprecipitation with TGFβ-IR or TGFβ-IR antibodies and immune complexes analyzed by 5–15% gradient SDS-PAGE. The labeled proteins were visualized by autoradiography either on x-ray film (left panel) or PhosphorImager screens (right panel). The molecular mass markers are shown on the left of each panel. Ip, immunoprecipitate.
bodies (24 h, lane 4). However, deglycosylated TGFβ-IR did not coprecipitate with TGFβ-IR antibodies (5 h, lane 4). This could well reflect a lower precipitation efficiency of the latter since TGFβ-IR antibodies coprecipitated both deglycosylated TGFβ-IIR and mature TGFβ-IR (5 h, lane 2). The lesser amounts of deglycosylated TGFβ-IR that could be detected in the presence of tunicamycin (24 h panel) may reflect diminished trafficking to the cell surface, an alternation in the half-life of the ER form of TGFβ-IR, and/or a critical need of N-linked glycosylation in TGFβ-IR for ligand binding. These not mutually exclusive possibilities will require further study.

In summary, native TGFβ type I and II receptors are processed differently and separately in mink lung epithelial cells, with the TGFβ-IR protein exhibiting a more efficient ER processing and a much shorter half-life (approximately 60 min versus >12 h) than type I receptor. Studies with exogenous labeled ligand suggested that the fast turnover of TGFβ-IR protein is not due to receptor endocytosis and subsequent degradation. This short metabolic half-life of native TGFβ-IR, as measured directly by metabolic labeling, agrees with a recent study in osteoblasts. In this study, suppression of protein synthesis with cycloheximide reduced 125I-TGFβ-IR binding. Our direct biosynthetic studies suggest a more prolonged half-life for TGFβ-IR in epithelial cells. It is possible perhaps that the turnover of TGFβ receptors may be different in cells of different lineage and/or altered by endogenous secretion of receptor ligands. This speculation requires further study. The short metabolic half-life of TGFβ-IR may have important implications for the reversible and rapid modulation of the many TGFβ-mediated cellular responses. In addition its different processing with that of TGFβ-IR allows for a possible additional mechanism of regulation of TGFβ actions.

Acknowledgments—We thank Teresa C. Dugger for expert technical assistance and Dr. Peter Nørgaard for helpful discussions.

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