Recycling of Cell Surface Pro-transforming Growth Factor-α Regulates Epidermal Growth Factor Receptor Activation

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Impairments in signal transduction, leading to the regulation of cell proliferation, differentiation, or migration are frequently the cause of cancer. Since the accurate spatial and temporal location of their components is crucial to ensure the correct regulation of these signaling pathways, it could be anticipated that defects in intracellular trafficking are at the base of certain neoplasias. However, the trafficking of many components of pathways frequently up-regulated in cancers, such as the epidermal growth factor receptor (EGFR) pathway, are largely unknown. Here, we show that the pro-transforming growth factor-α (pro-TGF-α), a prototypical EGFR ligand, is endocytosed from the cell surface via a clathrin-dependent pathway. Internalized pro-TGF-α does not progress to the lysosome; instead, it is delivered to the cell surface via recycling endosomes. To analyze the functional meaning of the internalization of pro-TGF-α, we used a deletion construct that is normally transported to the cell surface but is deficiently endocytosed. Due to this impairment, the levels of this construct at the cell surface are dramatically augmented. Consequently, the deletion construct displays a higher EGFR-activating ability, revealing a link between the trafficking of pro-TGF-α and the signaling by the EGFR and opening the possibility that defects in the trafficking of the growth factor may contribute to the development of tumors.

The spatial and temporal distribution (i.e. the trafficking) of the components of different signaling pathways is exquisitely controlled. In this way, the correct regulation and integration of the myriad of processes that ultimately determine cell differentiation, proliferation, survival, and migration is assured (1). Impairments in signaling pathways are frequently the cause of cancer. Thus, it could be anticipated that defects in intracellular trafficking are at the base of certain neoplasias. This hypothesis has been recently supported by data showing that defects in the endocytosis of certain receptor tyrosine kinases may be relevant to the development of different tumors (2).

The epidermal growth factor receptor (EGFR) signaling cascade has been intensively studied due to its frequent up-regulation in tumors of epidermal origin (3). Initial work focused largely on mechanisms and components downstream of the EGFR and related receptors; currently, the mechanisms and components regulating the biosynthesis and activity of the EGFR ligands are also intensively studied.

At least seven different ligands can bind and activate the EGFR (4); the so-called transforming growth factor-α (TGF-α) has been frequently used as a model. Like the rest of the EGFR ligands, TGF-α is synthesized as a transmembrane molecule (known as pro-TGF-α). The extracellular domain of pro-TGF-α can be proteolytically released by the tumor necrosis factor α-converting enzyme (also known as ADAM17) (5) in a process known as ectodomain shedding. Shedding is a tightly regulated process that occurs at the cell surface and can be activated by several signaling pathways, the best characterized of which involves protein kinase C (reviewed in Ref. 6). Inhibition of pro-TGF-α shedding effectively blocks its ability to activate the EGFR and, thus, inhibits the growth of certain tumors in preclinical models (7, 8).

It is conceivable that, in addition to ectodomain shedding, other mechanisms participate in the control of the activity of pro-TGF-α. Among these, the regulation of its intracellular traffic is a likely one, since it has been shown for EGFR itself (9). In agreement with this hypothesis, several reports have revealed the complexity of the trafficking of newly synthesized pro-TGF-α to the cell surface. In Drosophila, the pro-TGF-α ortholog Spitz requires a type 2 transmembrane protein, named Star, to exit the endoplasmic reticulum (10). In mammals, the cytoplasmic tail of pro-TGF-α contains several redundant motifs for sorting to the basolateral compartment in polarized cells (11). Furthermore, the correct progression of pro-TGF-α through the secretory pathway involves the interaction of its cytoplasmic domain with a PDZ domain-containing protein (12–14). Mutations in the carboxyl-terminal motif (ETVY), which interacts with PDZ domains, severely impair the transport of pro-TGF-α to the cell surface (15, 16). Much less is known about the dynamics of cell surface pro-TGF-α. A previous report partially addressed this point by comparing the fate of cell surface wild type pro-TGF-α with that of a pro-TGF-α deletion construct lacking the whole cytoplasmic domain (17). However, since this deletion prevents the interaction with PDZ proteins, it in turn impairs the transport of pro-TGF-α to the cell surface, making it a questionable tool to characterize the fate of the growth factor located at the cell surface that, therefore, remains largely unknown.

In this study, we show that cell surface pro-TGF-α is efficiently internalized via a clathrin-dependent pathway. Internalized pro-TGF-α escapes the lysosomal pathway and is delivered to the plasma membrane via recycling endosomes. A pro-TGF-α deletion mutant that lacks most of the cytoplasmic domain but retains the PDZ-interacting motif is efficiently transported to the cell surface but shows severely impaired endocytosis. Finally, we show that the impairment of internalization augments the levels of cell surface pro-TGF-α and, thus, the activation of the EGFR, showing that the dynamics of the growth factor at the cell surface have a direct impact on its activity. In summary, our
results expand our knowledge of the trafficking of pro-TGF-α and reveal a further link between trafficking and signaling by the EGFR.

**EXPERIMENTAL PROCEDURES**

Clones, Antibodies, and Reagents—Rat pro-TGF-α cDNA tagged at the N terminus, after His\(^2\), with the HA epitope has been described (18). Rat pro-TGF-α cDNA tagged at the same position with the Myc epitope was produced by PCR. The mutant pro-TGF-α/DCT -PDZ contains the Myc epitope and lacks the cytoplasmic tail from His\(^{128}\) but keeps the last four residues, Glu\(^{156}\), Thr, Val, and Val\(^{159}\). The GFP fusion of Eps15\(^{Δ95-295}\) was a kind gift from A. Benmerah (19).

Polyclonal antibody anti-Myc (ab9106) was from Abcam (Cambridge, UK). Monoclonal antibody anti-EGFR (non-phospho-Tyr\(^{173}\)) was from Upstate Biotechnology, Inc. (Lake Placid, NY). Polyclonal antibody anti-phospho-EGFR (Tyr\(^{1068}\)) was from Cell Signaling (Beverly, MA). Polyclonal antibody GRASP 65 was as described in Ref. 20. All fluorescent secondary antibodies were from Molecular Probes, Inc. (Eugene, OR). Recombinant human EGF was from R&D Systems (Minneapolis, MN). Fluorescent EGF (E-35350) and transferrin (T-23365) were from Molecular Probes, Inc. (Eugene, OR). Recombinant human EGFR was from Biotechnology, Inc. (Lake Placid, NY). Fluorescent EGF (E-35350) and transferrin (T-23365) were from Molecular Probes, Inc. (Eugene, OR). BS-95 was from British Biotech.

**Cell Lines and Transfections**—MDCK, HeLa, and CHO cells were grown in DMEM supplemented with 10% fetal calf serum and glutamine. MDCK cells stably expressing GFP-cellubrevin were a kind gift of T. Galli and have been described (33). For obtaining the stable clones, cells were transfected with calcium phosphate, and individual clones were selected with 500 cells were transfected with calcium phosphate, and individual clones grown in DMEM supplemented with 10% fetal calf serum and glutamine (Eugene, OR). BB-94 was from British Biotech.

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**Experimental Procedures**—CHO cells stably expressing wild-type or mutant pro-TGF-α were seeded in regular medium at 2 × 10⁵ cells in 60 plates. The day after, cells were washed twice with DMEM without Met and Cys and incubated for 1 h in this medium before adding them with 1 mg/ml 35S Translabel (Biolink 2000; Arlington Heights, IL). Immediately after the pulse, cells were lysed in lysis buffer (PBS with 1% Nonidet P-40, 10 mM EDTA, and protease inhibitors), and the extracts were precleared by incubation with Protein A–agarose for 1 h at 4°C. Precleared extracts were immunoprecipitated overnight at 4°C with polyclonal anti-Myc antibody, followed by a 1-h incubation at 4°C with Protein A–agarose. The immunoprecipitated labeled proteins were resolved by SDS-PAGE and detected by fluorography.

**Flow Cytometry Analysis**—CHO cells stably expressing wild-type or mutant pro-TGF-α were seeded 1 day before the experiment in non-tissue culture plastic p10 plates. The day after, cells were resuspended mechanically and counted, and 2 × 10⁶ cells/point were incubated with monoclonal anti-Myc antibody in PBS with 2.5% BSA (PBS-BSA) for 1 h at 4°C, washed three times in PBS-BSA, incubated with fluorescein isothiocyanate-conjugated anti-mouse antibody for 1 h at 4°C, washed again in PBS-BSA, and resuspended in PBS-BSA with 2 mg/ml propidium iodide. For the internalization experiments, after the incubation with anti-Myc antibodies for 1 h at 4°C, cells were washed, resuspended in PBS-BSA, and left at 37°C for the indicated times before being incubated with the fluorescein isothiocyanate-conjugated anti-mouse antibody for 1 h at 4°C. Only living cells (i.e., negative for propidium iodide staining) were included in the analysis.

**Immunofluorescence**—For immunofluorescence in permeabilized cells, cells were fixed with 4% PFA for 20 min at room temperature, washed with PBS containing 50 mM ammonium chloride, and permeabilized with 0.1% saponin in PBS with 1% BSA. All antibodies were diluted in permeabilization buffer and left for 1 h at room temperature. When indicated, living cells were incubated with primary antibodies diluted in DMEM plus 15 mM Hepes, pH 7.4, before fixation.

Pro-TGF-α Internalization Assay—To analyze the potential role of clathrin in pro-TGF-α endocytosis, MDCK cells stably expressing HA-tagged pro-TGF-α were transiently transfected with GFP or GFP-Eps15Δ95-295. 20 h after transfection, cells were starved for 1 h at 37°C and incubated with monoclonal anti-HA antibody (10 ng/ml) for 15 min at 37°C prior to fixation and permeabilization. Internalized pro-TGF-α labeled with anti-HA antibodies was detected with Alexa Fluor 568-conjugated anti-mouse secondary antibody. The experiment was performed four times, and in each experiment, between 50 and 100 cells of each type expressing comparable levels of GFP protein were counted.

Pro-TGF-α Recycling Assay—Pro-TGF-α recycling to the plasma membrane was performed based on a protocol by Mitchell et al. (21). Briefly, cells were incubated for 1 h either at 4°C or at 37°C with monoclonal anti-HA antibody (10 ng/ml) diluted in DMEM–Hepes (DMEM plus 15 mM Hepes, pH 7.4). Any remaining surface antibody was removed by acid wash (0.5 mM acetic acid, 0.2 M NaCl, 2 min/4°C). Cells were then incubated for 1 h at 37°C in DMEM–Hepes with EGF (100 ng/ml) and the ectodomain shedding inhibitor BB-94 (25 μM), allowing recycling of the internalized pro-TGF-α-anti-HA complexes to the cell surface. Before fixation and direct mounting, cells were incubated in the same medium containing Alexa Fluor 488-conjugated anti-mouse antibody for 1 h at 37°C, allowing labeling of the recycled pro-TGF-α complexes.

EGFR Activation Assay—A431 cells were seeded at day 1 at 1 × 10⁶ cells in 60 plates. The following day (2), cells were washed twice with DMEM/F-12 and left one more day in medium without serum. Parental CHO cells or CHO cells stably expressing wild-type or mutant pro-TGF-α were seeded at day 2 at 5 × 10⁵ cells in non-tissue culture plastic p10 plates in order to facilitate later mechanical detachment. On day 3, the CHO cells were mechanically resuspended, washed twice in DMEM/F-12, and counted, and 5 × 10⁶ cells of each type were added to one p6 well of A431 for the indicated times. One well of A431 was incubated with EGF (100 ng/ml) as a control. Treated A431 were washed twice in cold PBS and lysed in lysis buffer. A431 whole extracts were centrifuged at 15,000 × g for 20 min at 4°C, and the resulting supernatant was analyzed by SDS-PAGE and Western blot.

**RESULTS**

Cell Surface Pro-TGF-α Is Efficiently Endocytosed—To monitor the fate of cell surface pro-TGF-α, we used a version with an epitope (HA) in the extracellular domain. As expected, when intact stably transfected MDCK cells were incubated in the presence of anti-HA antibody at 4°C, the plasma membrane was labeled (Fig. 1A). In agreement with a previous report (22), incubation of anti-HA-stained cells at 37°C induces a decrease in the levels of cell surface signal and the appearance of intracellular vesicles (Fig. 1A). These vesicles are not observed in parental cells (data not shown), arguing that they are not generated by uptake of the antibody not bound to the growth factor. The same overall distribution and internalization pattern was observed in two additional cell lines (CHO and HeLa) and with pro-TGF-α constructs tagged with different epitopes (data not shown); thus, the generation of vesicles is not specific for a particular cell line or pro-TGF-α construct.

Soluble TGF-α is released to the medium through the proteolytic shedding of pro-TGF-α (reviewed in Ref. 4). In MDCK cells, it has been shown that, following secretion, soluble TGF-α is rapidly consumed by...
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interaction with its receptor, EGFR (23). Therefore, the question arose as to whether the internal vesicles detected contained soluble TGF-α shed from the plasma membrane and bound with the EGFR or, alternatively, internalized transmembrane pro-TGF-α. To distinguish between these possibilities, the internalization experiment was repeated in the presence of the metalloprotease inhibitor BB-94, a well characterized hydroxamate-based compound that blocks ectodomain shedding (reviewed in Ref. 6). As expected, cells pretreated with BB-94 displayed higher levels of pro-TGF-α at the cell surface, as judged both by immunofluorescence (Fig. 1A) and by flow cytometry analysis (data not shown). Interestingly, the number of intracellular vesicles does not seem to be affected by the presence of BB-94 (Fig. 1A), indicating that most, if not all, vesicles detected contain transmembrane pro-TGF-α.

To confirm that the internalization of pro-TGF-α is independent of its interaction with the EGFR, we performed further experiments. As previously described (24), labeled EGF is endocytosed in HeLa cells through interaction with the EGFR (Fig. 1B, right). A saturating excess of unlabeled EGF, which efficiently competes the internalization of labeled EGF, had no effect on the internalization of pro-TGF-α labeled with anti-HA antibodies, in stably transfected HeLa cells (Fig. 1B, left). Since EGF and TGF-α are indistinguishable in their ability to bind the EGFR (25), this result indicates that the internalization of pro-TGF-α is independent of the EGFR. Thus, these experiments clearly show that pro-TGF-α is endocytosed from the plasma membrane in a variety of cells and that this endocytosis is not dependent on the interaction of the growth factor with the EGFR.

Pro-TGF-α Is Internalized in a Clathrin-dependent Fashion—Endocytic vesicles can be formed via clathrin-dependent or clathrin-independent mechanisms. The former is well characterized and involves the selective uptake of certain plasma membrane proteins through their interaction with clathrin adaptors, such as AP2 (26). To analyze the potential involvement of this pathway in the internalization of pro-TGF-α, we first compared the distribution of the internalized growth factor at early time points with that of the transferrin receptor, a well established marker for clathrin-mediated endocytosis. As shown in Fig. 2A, internalized pro-TGF-α partially co-localizes with the transferrin receptor as judged by fluorescent transferrin uptake. Thus, this result suggests that pro-TGF-α is endocytosed via a clathrin-dependent mechanism.

To confirm the contribution of the clathrin-dependent pathway to the endocytosis of pro-TGF-α, we analyzed the effect on the internalization assay of Eps15Δ95–295, a dominant negative form of the AP2-binding protein Eps15, which inhibits clathrin-coated pit assembly (19, 27). As shown in Fig. 2B, 75% of GFP-transfected control cells efficiently internalized pro-TGF-α, whereas this value decreased to 25% in the case of GFP-Eps15Δ95–295-transfected cells (paired t test: two-tailed p value is 0.0003). Comparable results were obtained when the experiment was performed in the presence of the metalloprotease inhibitor BB-94 (data not shown), showing that the detected signal corresponds largely to transmembrane pro-TGF-α. Therefore, these data show that the internalization of pro-TGF-α is mediated, at least partially, by clathrin-mediated endocytosis.

Internalized Pro-TGF-α Escapes the Lyosomal Pathway and Recycles to the Plasma Membrane—To characterize the fate of internalized pro-TGF-α, we analyzed its co-localization with different endocytic markers. At early time points, internalized pro-TGF-α partially co-localized with markers of early endosomes such as EGFR or transferrin (Fig. 3A; see also Fig. 2A). However, at longer time points, little or no co-localization between pro-TGF-α and endocytosed EGF could be detected (Fig. 3A), indicating that they follow different endocytic routes.

It has been previously shown that endocytosed EGF-EGFR complexes are directed to lysosomes for degradation (28); thus, the lack of co-localization between pro-TGF-α and endocytosed EGFR indicates that the former escapes the lysosomal pathway. Supporting this conclusion, endocytosed pro-TGF-α was found to colocalize with cellubrevin (Fig. 3B), a marker of recycling endosomes (29). These results show that internalized pro-TGF-α escapes the lysosomal pathway and open the possibility that it recycles to the plasma membrane.

To directly document potential recycling, we used a modification of a procedure by Mitchell et al. (21). Cells expressing HA-tagged pro-TGF-α were incubated with anti-HA antibodies at 37 °C. Since the protocol used consists of several steps, staining at 4 °C does not allow the
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FIGURE 2. Internalization of pro-TGF-α is clathrin-dependent. A, MDCK cells stably expressing HA-tagged pro-TGF-α were incubated at 37 °C with mouse monospecific anti-HA antibodies and fluorescent transferrin (TF-Alexa Fluor 568; 1 mg/ml) before fixation, permeabilization, and incubation with anti-mouse antibodies coupled to Alexa Fluor 488. Note the partial co-localization of internalized pro-TGF-α and transferrin. Bar, 10 μm. B, the same cells as in A were transiently transfected with either GFP or a GFP-Eps15Δ95–295 dominant negative mutant. Transfected cells were allowed to internalize mouse monoclonal anti-HA antibodies, fixed, permeabilized, and incubated with labeled anti-mouse antibodies. The percentage of cells expressing either GFP or GFP-Eps15Δ95–295 displaying intracellular signal for pro-TGF-α was quantified. The histogram represents the mean values and S.D. of four independent experiments.

accumulation of enough signal to follow it during the whole procedure. After this first incubation, cells were acid-washed in order to remove any cell surface antibody, therefore leaving only those that have been internalized. The effectiveness of the acid wash was assessed by showing that cells incubated with the anti-HA antibody at 37 °C lose cell surface signal but not intracellular vesicles (Fig. 3C and supplemental Fig. 1). Furthermore, as expected, cells stained at 4 °C lose all detectable signal (supplemental Fig. 1). Non-control acid-washed cells stained at 37 °C were then returned to the same temperature to allow the recycling of internalized pro-TGF-α coupled to anti-HA antibodies to the cell surface. Then cells were exposed for 1 h at 37 °C to Alexa Fluor 488-conjugated anti-mouse antibodies before fixation and direct mounting. The whole experiment was done in the presence of the metalloprotease inhibitor BB-94 for two purposes. One the one hand, this rules out the possibility that the internalized pro-TGF-α corresponds to soluble growth factor in complexes with EGFR under the conditions used for this experiment. On the other hand, the addition of BB-94 during the recycling washes prevents the shedding of recycled pro-TGF-α, enhancing the signal. As shown in Fig. 3C, a clear staining, mostly concentrated at the cell-cell contacts was revealed by the Alexa Fluor 488-conjugated anti-mouse antibodies, showing direct recycling of internalized transmembrane pro-TGF-α to the cell surface. In addition, a clear signal was detected in punctate structures, showing that recycled cell surface pro-TGF-α can be internalized again and indicating that pro-TGF-α can be subjected to several rounds of recycling. In a parallel control experiment, parental cells did not show any detectable signal (data not shown); therefore, we concluded that all of the primary and secondary antibodies are taken via pro-TGF-α. Altogether, our data suggest that pro-TGF-α is internalized from the cell surface and can be recycled, via recycling endosomes, to the plasma membrane.

Deletion of Pro-TGF-α Cytoplasmic Tail Results in Higher Plasma Membrane Expression Due to Impaired Internalization—Clathrin-mediated endocytosis relies on the recognition of specific sorting sequences localized on the cytoplasmic domains of the cargo proteins (30). Thus, we reasoned that by removing the cytoplasmic tail of pro-TGF-α, we might impair its endocytosis and thus analyze its functional importance. Because the cytosolic domain of pro-TGF-α harbors a PDZ-binding motif important for its transport through the early secretory pathway (12, 13), we designed a pro-TGF-α construct (pro-TGF-α-ΔCT-PDZ) lacking most of its cytosolic domain but retaining the last four residues (ETVV), which mediate binding to PDZ domains (Fig. 4A). Next, we stably transfected CHO cells with either wild type pro-TGF-α or the pro-TGF-α-ΔCT-PDZ construct, both tagged with a Myc epitope in the extracellular domain (Fig. 4A), and selected clones that expressed similar levels of both constructs, as judged by immunoprecipitation of metabolically labeled cells (Fig. 4B). It is important to note that, in contrast to pro-TGF-α constructs with mutations affecting the PDZ-binding domain (13, 15), the band pattern of the pro-TGF-α-ΔCT-PDZ construct is similar to that of wild type pro-TGF-α, indicating normal glycosylation and, thus, normal progression of this deletion mutant through the secretory pathway.

To further characterize the pro-TGF-α-ΔCT-PDZ construct, we compared its subcellular localization with that of wild type pro-TGF-α by immunofluorescence. The distribution of pro-TGF-α in CHO cells is similar to that in MDCK and HeLa cells, with strong intracellular staining, particularly enriched in the perinuclear area, and a weak cell surface signal (Fig. 4C). By contrast, the pro-TGF-α-ΔCT-PDZ deletion construct displayed, in addition to the perinuclear distribution, a stronger cell surface staining (Fig. 4C). This difference was more evident when living cells were incubated at 4 °C with anti-Myc antibodies (Fig. 4D). When quantified by flow cytometry, the plasma membrane signal for pro-TGF-α-ΔCT-PDZ was 4-fold higher than that of wild-type pro-TGF-α (Fig. 4E). The increased levels of cell surface pro-TGF-α-ΔCT-PDZ were not a clonal artifact, because three independent clones gave the same pattern, and comparable results were obtained in transiently transfected HeLa and MDA-MB-435S cells (data not shown).
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The high levels of cell surface pro-TGF-α-ΔCT-PDZ could be due to impaired ectodomain shedding or, alternatively, to impaired endocytosis. To quantitatively compare the shedding of the deletion construct with that of wild-type pro-TGF-α, we treated cells with the phorbol ester phorbol 12-myristate 13-acetate, a potent activator of protein kinase C-induced ectodomain shedding, and determined the levels of both constructs at the cell surface at different time points. The reduction in the cell surface levels induced by phorbol 12-myristate 13-acetate, as judged by flow cytometry, in cells expressing the deletion construct was comparable with that observed in cells expressing the full-length construct (Fig. 4F), indicating that the shedding of pro-TGF-α is not affected by deletion of the cytoplasmic tail. Furthermore, long term incubation of cells expressing both constructs with the inhibitor of ectodomain shedding BB-94 led, as expected, to higher cell surface levels due to inhibition of basal shedding (Fig. 4, compare E and G). However, the difference observed in the absence of the inhibitor did not change (4-fold higher for pro-TGF-α-ΔCT-PDZ, compared with wild-type pro-TGF-α) (Fig. 4G). Thus, these results show that the high levels of cell surface pro-TGF-α-ΔCT-PDZ are not due to an impairment of ectodomain shedding.

To determine the possible contribution of deficient endocytosis to the high levels of cell surface pro-TGF-α-ΔCT-PDZ, we performed internalization assays. Unsurprisingly, the endocytosis of wild-type pro-TGF-α leads to the uptake of anti-Myc antibodies and, as judged by immunofluorescence, to a widespread vesicular staining with only faint plasma membrane signal (Fig. 5A). By contrast, in cells expressing pro-TGF-α-ΔCT-PDZ, although some vesicular staining is detectable, most of the Myc immunoreactivity remained at the cell surface (Fig. 5A), indicating that the endocytosis of the deletion construct is impaired. Furthermore, the few endocytic vesicles detected in cells transfected with pro-TGF-α-ΔCT-PDZ colocalized with internalized EGF at long time points (data not shown), indicating that, in contrast with full-length pro-TGF-α, pro-TGF-α-ΔCT-PDZ follows the lysosomal pathway. To quantify the internalization rate, we determined the levels of both constructs at the cell surface by flow cytometry at different time points. Confirming the analysis by immunofluorescence, we found a significant statistical difference between pro-TGF-α and pro-TGF-α-ΔCT-PDZ at all three time points analyzed (paired t test; two-tailed p value is 0.0005). Collectively, these results indicate that endocytosis is an effective way to control the level of cell surface pro-TGF-α; deletion of the cytoplasmic tail impairs endocytosis and, as a consequence, increases dramatically the level of the growth factor at the plasma membrane.

Activation of the EGFR by Internalization-defective Pro-TGF-α—Since the endocytic trafficking of pro-TGF-α determines the levels of the growth factor at the cell surface, it could, in turn, determine its functionality (i.e. its ability to activate the EGFR). To test this hypothesis, we performed an activation assay comparing the capacity of CHO cells expressing either wild-type or mutant pro-TGF-α to activate the EGFR of serum-starved A431 cells. Parental CHO cells induced a modest activation of the EGFR, conceivably due to endogenous ligands. As expected, expression of pro-TGF-α led to a higher level of EGFR phosphorylation (Fig. 6). Cells expressing the internalization-defective pro-TGF-α-ΔCT-PDZ mutant induced a statistically significant difference in the level of EGFR activation at all three time points analyzed (Fig. 6). Thus, as hypothesized, the internalization of pro-TGF-α has a clear impact on its ability to activate the EGFR. This result unveils a novel mechanism leading to the regulation of the EGFR through the control of the trafficking of pro-TGF-α.

DISCUSSION

The results presented here illustrate the tight links between intracellular traffic and signal transduction. Previous reports have established the impact of endocytosis on signaling by receptor tyrosine kinases, such as the EGFR, beyond the well known inactivation by down-regulation. G-protein-coupled receptors can deeply modulate the activity of RTK by altering components of the endocytic apparatus, leading to formation of “receptor-specialized” coated pits, through phosphorylation or ubiquitylation (reviewed in Ref. 31). We now show that the endocytic trafficking of pro-TGF-α, a prototypic ligand for the EGFR, modulates the expression of the growth factor at the cell surface and, hence, the ability to modulate the activation of the EGFR. The anterograde transport of pro-TGF-α has been partially characterized; it depends on its interaction with a protein containing a PDZ domain (12–14). Thus, we focused our efforts on the potential regula-
tion of the endocytic transport, a poorly characterized issue. Our results with a dominant negative mutant of the AP2-binding protein Eps15 clearly show that pro-TGF-α is internalized via clathrin-coated vesicles. Furthermore, soon after internalization, pro-TGF-α co-localizes with the transferrin receptor and the endocytosed EGF/EGFR complex, two well characterized markers of internalization via clathrin. However, at longer time points, there is no significant co-localization between pro-TGF-α and endocytosed EGF/EGFR, indicating that after the initial events, they follow different routes. Since it has been well established that the internalized EGF/EGFR complexes are directed to lysosomes, we hypothesized that endocytosed pro-TGF-α escape the degradative pathway. Supporting this hypothesis, the internalized growth factor colocalized with recycling endosomes. This result points to a potential recycling of endocytosed pro-TGF-α to the cell surface. Indeed, an experiment designed to directly address this possibility confirmed the cell surface recycling of internalized pro-TGF-α. Thus, the levels of cell surface pro-TGF-α are controlled by a complex equilibrium between several components, including the rate of synthesis, transport to the cell surface, endocytosis, and recycling.

In order to determine the functional significance of the endocytosis of pro-TGF-α, we sought to design a mutant with a normal anterograde transport but an impaired endocytosis. As expected, a construct with a deletion that encompasses most of the cytoplasmic domain but conserves the PDZ-binding motif showed no detectable deficits in post-translational modifications, traffic to the cell surface, or ectodomain shedding. However, the internalization of this deletion construct was severely impaired, leading to a profound increase in its cell surface levels, compared with the wild type molecule. Thus, the analysis of this

**FIGURE 4.** Deletion of the cytoplasmic tail results in increased levels of pro-TGF-α at the cell surface. A, schematic of wild type (WT) pro-TGF-α and a deletion mutant lacking the cytoplasmic tail but retaining the PDZ-binding motif (-ETVV). B, parental CHO cells or the same cells permanently transfected with the constructs depicted in A were metabolically labeled and lysed, and cell lysates were immunoprecipitated with anti-Myc antibodies. C, fixed/permeabilized CHO cells stably expressing the same constructs were incubated with monoclonal anti-Myc antibodies followed by Alexa Fluor 488-conjugated secondary antibodies. D, the same cells were incubated at 4 °C with monoclonal anti-Myc antibodies, washed, fixed, and incubated with Alexa Fluor 488-conjugated secondary antibodies. E, the same cells as in B were treated with (G) or without (E) BB-94 and analyzed by flow cytometry with monoclonal anti-Myc antibody. The indicated values show the mean fluorescence. F, the same cells as in B were incubated for the indicated times with phorbol 12-myristate 13-acetate (1 μM) prior to flow cytometry analysis. Shown are the mean and S.E. values for the mean fluorescence of three independent experiments.
mutant indicates that endocytosis is indeed a relevant mechanism to control the levels of cell surface pro-TGF-α and, hence, its functionality, as judged by its ability to activate the EGFR. In addition, characterization of the small fraction of mutant molecules that are internalized showed that they followed the lysosomal pathway, indicating that the cytoplasmic tail also is required for the intracellular routing of internalized pro-TGF-α. This result is in agreement with a recent report by Dempsey et al. (11), showing the presence of several sorting motifs in this domain.

In steady-state conditions, an equilibrium between pro-TGF-α at the cell surface and intracellular compartments (i.e., the Golgi and recycling endosomes) may be a convenient way for the cell to control the amount of pro-TGF-α at the cell surface. Under certain circumstances, the pool of pro-TGF-α at the Golgi network could be mobilized to the cell surface, and, as shown here, modulation of the endocytosis and/or recycling could lead to increased or decreased levels of cell surface pro-TGF-α and, thus, its EGFR-activating ability.

Previous reports have shown that cell surface pro-TGF-α can act in a juxtacrine fashion, binding EGFRs in adjacent cells. Alternatively, pro-TGF-α can be proteolytically shed, producing the soluble form of the growth factor, which acts in a paracrine fashion reaching EGFRs located at a distance from the pro-TGF-α-producing cells (6). Lack of endocytosis increases the levels of cell surface pro-TGF-α and, therefore, probably enhances both modes of signaling. On one hand, it increases the number of cell surface molecules that can bind adjacent EGFRs. On the other hand, since tumor necrosis factor α-converting enzyme, the protease responsible for the shedding of pro-TGF-α, is not limiting, an increment in the levels of the cell surface growth factor will result in augmented levels of soluble growth factor.

Our results might be particularly interesting, considering the implication of TGF-α in tumors (22, 32) and taking into account the emerging evidence linking endocytosis and cancer, with a growing list of endocytic proteins found as oncogenic fusion proteins (1, 2). A possible scenario is that in addition to increased pro-TGF-α expression and/or increased shedding, altered endocytic pro-TGF-α traffic might also be responsible in part for increased EGFR-dependent signaling, leading to tumorigenesis. Exploring this hypothesis will be our next goal.

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Recycling of Cell Surface Pro-TGF-α Regulates EGFR Activation

FIGURE 5. Deletion of the cytoplasmic domain impairs the internalization of pro-TGF-α. A, CHO cells stably expressing pro-TGF-α or pro-TGF-αΔCT were incubated with mouse monoclonal antibody anti-Myc at 37 °C, fixed, permeabilized, and revealed with Alexa Fluor 488-conjugated anti-mouse antibody. Bar, 10 μm. B, the same cells as in A were incubated in suspension at 4 °C with mouse monoclonal antibody anti-Myc, washed, and left at 37 °C for the indicated times, and the percentage of cell surface pro-TGF-α was quantified by flow cytometry. Shown are the mean and S.D. values for the mean fluorescence of four independent experiments (unpaired t test; two-tailed p value is 0.02, 0.003, and 0.04 for 10, 3, and 60 min, respectively). WT, wild type.

FIGURE 6. Activation of the EGFR by cells expressing wild type (WT) pro-TGF-α and pro-TGF-αΔCT-PDZ deletion construct. Serum-starved A431 cells were incubated with parental CHO cells or CHO cells stably transfected with full-length pro-TGF-α or pro-TGF-αΔCT-PDZ. One well of A431 was incubated with EGF (100 ng/ml) for 5 min as a control (Cont). After the indicated time points, CHO cells were washed, A431 cells were lysed, and cell lysates were analyzed by Western blot with antibodies against the EGFR, phosphorylated EGFR, or, as a control, actin (A). Representative Western blots of total and phosphorylated EGFR are shown. B, quantification of phosphorylated EGFR from three independent experiments. Shown is the percentage of phosphorylated EGFR induced by the different cells, taking the value of phosphorylated EGFR in nontreated A431 cells as 100% and normalizing for the total amount of EGFR. The two-tailed p values for CHO cells expressing pro-TGF-α compared with CHO cells expressing pro-TGF-αΔCT-PDZ are <0.0001, 0.007, and 0.0036 for 5, 10, and 30 min, respectively.
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