Regulated Cell Surface Pro-EGF Ectodomain Shedding Is a Zinc Metalloprotease-dependent Process*

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Epidermal growth factor receptor (EGFR) ligands are synthesized as type I membrane protein precursors expressed at the cell surface. Shedding of the ectodomain of these proteins is the way cells regulate the equilibrium between cell-associated and diffusible forms of these growth factors. Whereas the regulated shedding of transforming growth factor-α (TGF-α), HB-EGF, and amphiregulin precursors have been clearly established, regulation of full-length pro-EGF shedding has not been clearly demonstrated. Here, using both wild-type and M2 mutant CHO-K1 as well as HeLa cell lines transiently transfected with epitope-tagged rat pro-EGF expression plasmid, we demonstrate that these cells synthesize EGF as a high molecular weight membrane-associated precursor. Glycoprotein expressed at the cell surface. All cell lines capable to release the entire ectodomain of pro-EGF in the extracellular medium following juxtamembrane cleavage of the precursor once it is present at the cell surface. More significantly we clearly established that CHO-M2 and HeLa cells only constitutively release low levels of pro-EGF. This shedding is a regulated phenomenon in wild-type CHO cells where it can be induced by different agents such as phorbol 12-myristate 13-acetate (PMA), pervanadate, and serum but not by calcium ionophores. Using specific inhibitors as well as protein kinase C (PKC) depletion, PMA stimulation was shown to be completely dependent on PKC activation whereas pervanadate and serum stimulation were not. Regulated ectodomain shedding involves the activity of a zinc metalloprotease as determined by inhibition with phenantroline and TAPI-2 and by the results obtained with the CHO-M2 shedding defective mutant cell line. Comparison of the ability of CHO and HeLa cell lines to shed pro-EGF and pro-TNF-α upon stimulation greatly suggests that TACE (ADAM 17) may not be the ectoprotease involved in the secretion of pro-EGF ectodomain and that this protease, which remains to be identified, shows a restricted cellular expression pattern.

Epidermal growth factor (EGF) is a small (about 6 kDa) diffusible polypeptide first discovered and purified from rodent

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1 The abbreviations used are: EGF, epidermal growth factor; EGFR, EGFR receptor; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin; HA, hemagglutinin; RIA, radioimmunoassay; WT, wild type; PMA, phorbol 12-myristate 13-acetate; CHO, chinese hamster ovary; PKC, protein kinase C; TGF-α, transforming growth factor-α.
appears to be mediated by members of the metzinin superfamily of zinc-dependent proteases that include the matrix metalloproteinases (MMPs) and ADAMs (for a Disintegrin And Metalloproteinase) (24, 26–28). The involvement of proteases such as ADAM 9 (MDC9/meltrin-γ) and more importantly ADAM 17 (TACE) in the regulation of EGFR ligand shedding as been proposed for TGF-α (24, 25, 28) HB-EGF (29, 30), and amphiregulin (29, 31). Unfortunately, the information concerning pro-EGF maturation and secretion does appear much more controversial.

In rodents submaxillary glands, the precursor molecule is not detectable and only the mature and diffusible 6 kDa form of EGF accumulates in the secretory granules of the regulated exocrine secretory pathway (12, 32, 33). In this case, the release of EGF in the biological fluid (saliva) involves exocytosis, i.e. fusion of the secretory granule membrane with the apical cellular membrane. This phenomenon is highly regulated and may be achieved at least through the activation of adrenergic receptors (34, 35).

In other tissues studied so far such as kidney (12, 36–39), mammary (40), and lacrimal glands (41), the fully mature 6 kDa EGF was undetectable, and EGF accumulated as its high molecular weight precursor associated with the apical membrane of epithelial cells. The presence of EGF-containing peptides of varying molecular masses (from 6 to 160 kDa) in urine (37, 42, 43) and milk (22) of different species indicates that EGF can be released (shed) from its membrane-anchored precursor. This occurs through proteolytic cleavage in the juxtamembrane region. The full-length precursor was the major substrate for shedding, leading to the initial release of the entire ectodomain. In order to lead to the ultimate, mature 6-kDa form of the molecule, the ectodomain needs to be further matured in the extracellular medium. It has already been shown that, in vitro, mature EGF could be released from its precursor through the action of different type of proteases; acidic protease such as pepsin (36) or serine-proteases such as trypsin (22, 39, 41) and plasmin (44). Moreover, membrane fractions of both kidney (45, 46) and mammary gland (47) contain a membrane-bound proteolytic activity that colocalized with pro-EGF and released soluble EGF from its membrane-associated precursor. Based on its sensitivity to a set of inhibitors, it was shown to belong to the serine-protease family. This experimental evidence is in conflict with results obtained in two independent studies performed with cultured cells. In these cells transfected either with a full-length (48) or truncated (49) human pro-EGF cDNA, EGF release was sensitive to metalloprotease inhibitors (batimatstat and BB-2116) and cat ion chelators (EDTA and EGTA) and insensitive to non-metalloprotease inhibitors (48). Another important feature concerning EGFR ligand shedding is its potential regulation. It is now clearly established that secretion of TGF-α, HB-EGF, and amphiregulin are regulated phenomena that can be triggered by PMA (through PKC activation), calcium ionophore (calcium influx), serum or pervanadate (tyrosine phosphatase inhibition) (9, 23, 25, 31, 50–53). Concerning EGF, its regulated shedding from cell surface is not so clearly established. Dempsey et al. (48) working with MDCK cells transfected with the full-length human pro-EGF cDNA failed to stimulate EGF secretion with serum or PMA whereas Dong and Wiley (49) working with mammary cells (HB2) transfected with a prodomain-truncated human pro-EGF cDNA also failed to stimulate secretion with PMA but observed a stimulation of EGF release with both calcium ionophore and a tyrosine phosphatase inhibitor.

From these studies, it appears that important discrepancies exist in the conclusions that can be drawn from the studies on pro-EGF shedding compared with other EGFR ligands. In order to provide insights into pro-EGF shedding properties, we decided to study pro-EGF secretion in CHO-K1 (both wild type and M2 mutant) and HeLa cell lines transiently transfected with a full-length rat pro-EGF cDNA. The CHO-K1 cellular model has been used previously to establish the mechanism of regulation of the secretion of other EGFR ligands (TGF-α, HB-EGF), and HeLa cells have also been used to study the shedding of various proteins including HB-EGF (64, 65). In the present study, we have been able to demonstrate that once pro-EGF is exposed at the cell surface, it can be shed to release its entire ectodomain in the extracellular medium. In CHO-K1, this ectodomain shedding can be stimulated by PMA, serum, or pervanadate. PMA acts through a PKC-dependent pathway whereas serum and pervanadate act through PKC-independent pathway(s). The use of specific protease inhibitors as well as of the CHO-M2 mutant cell line that displays impaired constitutive and regulated pro-EGF ectodomain shedding demonstrated that shedding was achieved through the activation of a zinc-dependent metalloprotease activity. More important is the fact that, whereas the zinc-dependent metalloprotease ADAM 17 (TACE) has an important role in the regulation of EGFR ligands shedding such as TGF-α, HB-EGF, and amphiregulin (24, 25, 28, 29, 30, 31), our results suggest that TACE may not be relevant in the shedding of pro-EGF since pro-EGF shedding was not regulated in HeLa cells, which are known to possess high amounts of TACE.

MATERIALS AND METHODS

Chemicals—Peroxidase-conjugated goat anti-mouse IgG, trypsin (10000 BAEE units/mg), extravidin-peroxidase, soybean trypsin inhib itor (SBTI), 1 mg inhibits 10000 units trypsin, aprotinin, peptatin A, E-64, phenantrolin, bisindolylmaleimide I (BIM I), EDTA, tunicamycin, protein molecular weight markers, DMEM without methionine and cysteine, A23187, ionomycin, PMA, ampicillin, LB broth, LB agar, and protein G-Sepharose were obtained from Sigma Chemical Co. QiAquick Gel Extraction and PCR purification kits were obtained from Qiagen. BCA protein assay kit and Sulfo-NHS-LC-Biotin were from Perbio. Triton X-100 was obtained from MERCK N- and O-deglycosylation as well as Quantum Prep Plasmid purification kits were from BioRad Laboratories. Desoxyribonucleotide trisphosphates, agaro, and DNA size markers were obtained from Amersham Biosciences. Upstream and downstream oligonucleotide primers were synthesized by MWG-biotech. pTargetT7™ mammalian expression vector system, T7-coupled reticulocyte lysate systems and JM-109 or DH5α competent cells were from Promega. Expand™ Reverse Transcriptase and Expand™ High Fidelity PCR System were from Roche Applied Science. 125I-Na (100 μCi/ml, 3.7 MBq/ml) and Renaissance® Western blot Chemiluminescence Reagent Plus were purchased from PerkinElmer Life Sciences. TRANSCRIPTS’Label was from ICN. Ro-31-8220 (BIM IX; methanesulfonate), polyclonal anti-human TACE (AB1) antibody, TAPI-2 and TIM1P and 3 were from Calbiochem. Phosphate-buffered saline (PBS), NUT. MIX.F12(HAM) Glutamax (HAM-F12), fetal calf serum (FCS), OptiMEM, Dulbecco’s modified Eagle’s medium (DMEM) Glutamax, fungizone, penicillin-streptomycin solution, random primers, FcDNA.13Mye-His-mamalian expression vector and monoclonal anti-HA antibody were from Invitrogen. Exgen 500 was from Euromedex. Monoclonal anti-HA antibody was from Covance. Polyclonal rabbit anti-human TGF-α antibody was from Promocell and TGF-α from PreproTech Inc.

Rat Pro-EGF cDNA Cloning and Construction of Epitope-tagged Pro EGF—Total RNAs from Sprague Dawley rats were prepared and de scribed previously (56). Kidney RNAs were first subjected to reverse transcription using random priming and Expand™ Reverse Transcriptase as described by the manufacturer’s protocol. PCR amplification of the cDNA was performed according to rat pro-EGF PCR protocol. Sequence as published by Saggi et al. (Ref. 13, GenBank™ accession number: M63585). The 5' sense primer, complementary to nucleotides 992–1010 of rat pro-EGF cDNA, carried a HindIII restriction site whereas the 3’ antisense primer, complementary to nucleotides 3881–3900, carries an EcoRI restriction site. Amplification was performed using the Expand™ high fidelity PCR system (Roche Applied Science). After an initial denaturation for 2 min at 94 °C, cDNA was first subjected to 10 cycles of amplification with...
lysates were used for EGF or TNF-α/H9251
were cultured in monolayers in HAM-F12 medium whereas HeLa cells
transcription using T NT
into JM-109 bacterial cells leading to the pT12 clone. The sequence of
cloned pro-EGF was confirmed by both restriction analysis and sequencing
using selected primers. Its ability to drive the synthesis of prepro-EGF protein of high molecular weight was further confirmed by in vitro transcription using Tnt®-coupled reticulocyte lysate systems (Promega). To introduce epitope tags into the cloned rat pro-pro-EGF cDNA sequence, the entire coding sequence (HindIII-EcoRI fragment) was first excised and cloned into PdEm methyl-MyccHis mammalian expression vector (Invitrogen). The prepro-EGF/Myc-His construct was generated by suppressing the stop codon using a PCR-based strategy. An antisense oligonucleotides complementary to nucleotides 3768–3787 and carrying an Apal restriction site was used, allowing the in-frame ligation of the prepro-EGF cDNA with both c-Myc and His6 epitopes sequences at the 3′-end of the cDNA. This cDNA was further modified by inserting the HA1 epitope (YFPDVPDYA) of influenza virus hemagglutinin (57) between amino acids Ser297 and Asn296 of rat prepro-EGF using PCR approach as described by Arribas and Massagué (51) for the insertion of the HA epitope into the rat TGF-α sequence. This final modification supplies the pcD12-Ha plasmid, which was confirmed DNA sequencing. PCR-based strategy was also used to generate a cDNA allowing the synthesis of a HA-tagged soluble form of EGF, which lacked most of its prodomain. Briefly, a stop codon was introduced in the extracellular domain of pro-EGF-HA following the Lys116 codon (i.e. the C-terminal end of the mature urinary EGF sequence) and the prodomain deleted between Leu32 and Val972 to generate the ΔPro–ΔTM-EGF expression plasmid. This expression plasmid allows the synthesis of a secreted form of rat pre-EGF that conserved the signal peptide and the first ten amino acids of the EGF prodomain (Met to Pro2) as well as the arginine residue (Arg297) that preceded the mature EGF sequence (Asn294 to Lys297) but lacks the downstream sequences encoding the junction between the transmembrane and cytoplasmic domains.

Cell Culture and Transfection—The CHO-K1 and CHO-M2 cells were cultured in monolayers in HAM-F12 medium whereas HeLa cells were cultured in DMEM. Both medium were supplemented with 10% FCS, 100 μg/ml streptomycin, 100 units/ml penicillin, and 1.25 μg/ml amphotericin B (fungizone) at 37 °C under 5% CO2 and used within ten passages. Cell monolayers were suspended by treatment with 5 mM EDTA in PBS (CHO) or trypsin (HeLa) and usually plated in 8-well plates (75,000 cells/plate) in culture medium the day before transfection.

CHO or HeLa cells (50–70% confluence) were transiently transfected with 2.5 μg of the appropriate expression plasmid containing either natural or ΔPro–ΔTM-soluble pro-EGF (plasmid Tg1, pcD34, or pcD34M2, respectively), human prepro-EGF cDNA (ΔPro–ΔTM-EGF) or else human pro-TNF-α cDNA in PcdNA3 (a kind gift from Dr. F. Peiretti) using Exgen 500 reagent as described by the manufacturer. Following transfection, cells were switched in HAM-F12 or DMEM supplemented with 10% FCS and antibiotics. Transfected cells were further incubated for 48 h to allow for pro-EGF or pro-TNF-α expression and analyzed either directly or after addition of incubations in varying conditions. At the end of these different types of experiments, cells and conditioned media were collected. Conditioned medium was cleared of contaminating cells by centrifugation (1000 × g for 5 min). Cells monolayers were washed twice with PBS and lysed for 30 min in detergent-containing buffer (50 mM sodium phosphate buffer, pH 7.4; EDTA 5 mM, Triton X-100 1%). Cell lysates were cleared of insoluble material by centrifugation at 15,000 × g for 20 min. Both incubation media and cell lysates were used for EGF or TNF-α content analysis as described below.

EGF and TNF-α Radioimmunoassay—Rat EGF or human TNF-α was labeled with carrier free 125I-TNa to a specific activity of 150–200 μCi/μg using the chlorine-T method.RIA analysis for human TNF-α contents was performed exactly as previously described for rat EGF (41). The IgG fraction of the rabbit polyclonal anti-rat EGF antibody (rEGF2, 1/5000) or of the anti-human TNF-α antibody (1/1000) were used as immunoprecipitating antibodies. Immune-reactive rat EGF (irEGF) standards and conditioned media and human TNF-α conditioned media were quantified 48 h after cells have been transiently transfected. As described previously (41), in order to adequately quantify EGF amounts, aliquots from both conditioned media and cell lysates were subjected to trypsin hydrolysis in order to release low molecular mass fully immunoreactive mature EGF from putative precursor molecules.

Metabolic Labeling of Cellular Proteins—Plated CHO (both wild type and M2 mutant) cells grown in 6-well plates as described above were transiently transfected with the pcD12-HA expression plasmid. 48 h later, monolayers were washed once with PBS and then preincubated in serum-free, methionine/cysteine-free DMEM (Met-Cys-free DMEM) supplemented with 1% lipid-free BSA for 30 min at 37 °C under 5% CO2. Cells were then labeled in the same medium containing 250 μCi/ml of 2 h of TRAnS® Label at 37 °C under 5% CO2. Labeling was stopped by washing the cells three times with PBS. Cells were then further incubated in the chase medium (complete DMEM containing either 1% BSA or 10% FCS) containing the appropriate drugs and for the time specified in the legends.

Biotinylation of Cell Surface Proteins—CHO (both wild type and M2 mutant) or HeLa cells were grown on 6-well plates for 48 h after transient transfection with the appropriate expression plasmid. Prior to biotinylation, cells were washed three times with ice-cold PBS containing 1 mM CaCl2 and 1 mM MgCl2 (PBS+), then biotinylated in the presence of 1 mg/ml sulfo-NHS-LC-Biotin in the same buffer for 30 min on ice. Biotinylation was stopped by incubating cells for 10 min at 4 °C in the presence of 100 mM glycine in PBS+ and washed once with PBS+. Following biotinylation, cells were incubated at 37 °C with the indicated chase medium (complete DMEM containing either 1% BSA or 10% FCS) containing the appropriate drugs and for the time specified in the legends.

Immunoprecipitation, Western Blotting Analysis, and Autoradiography—All immunoprecipitation protocols were performed at 4 °C, unless otherwise stated. After incubating cells in the different conditions specified in the legends of the figures, conditioned media and cell lysates were prepared as described above. Equal relative amounts of cell lysates and conditioned media were incubated overnight with either anti-rat EGF (rEGF2, 1/2000), anti-HA (HA-11, 1/5000) or anti-Myc (1/500) antibodies. In order to precipitate immune complexes, 40 μl of a 50% slurry of protein A (rEGF2) or protein G-Sepharose (HA-11 or Myc) were added for 2 h under constant shaking. Protein A- or G-Sepharose was pelleted by centrifugation and immune complexes washed three times with high salt lysis buffer (50 mM NaCl, 300 mM pH 7.4; EDTA 5 mM; Trition X-100 1%).

Immunoprecipitated incubation medium or cell lysates were solubilized in SDS containing electrophoresis sample buffer and separated by SDS-PAGE on a 7.5% Tris-HCl or 16.5% Tris-Tricine slab gel. Following electrophoresis, proteins from non-labeled, 35S-labeled or biotinylated samples were electrotransferred overnight to opti membrane (Molecular Dynamics). Quantification of the radioactivity of the bands in each lane was performed using ImageQuant software.

In the case of labeled sample, membranes were blocked with 5% nonfat milk in TBS containing 0.2% Tween 20 (TBST) for 1 h at 37 °C. Blots of biotinylated samples were directly probed with horseradish peroxidase-conjugated streptavidin (1/2500 in 0.5% milk containing TBST) for 1 h at room temperature. To reveal specific HA or Myc epitope-containing proteins, blots were immunostained with monoclonal antibodies (anti-HA or anti-Myc, 1/5000 in 0.5% milk containing TBST) for 3 h at room temperature and probed with 1/10000 dilution of goat anti-mouse IgG antibody linked to horseradish peroxidase as described previously (56). Blots were probed in Renaissance® Western blot Chemiluminescence Reagent Plus according to manufacturer’s recommendatons and visualized by exposure to Amersham Biosciences Hyperfilm-ECL.

Immunodegradation of TACE (ADAM 17) in cell lysates from CHO and HeLa cells was performed following 7.5% slab gel electrophoresis. Western blot analysis was performed with the rabbit polyclonal anti-TACE antibody (1/2000) exactly as described above.

RESULTS

In an attempt to gain insight into the mechanisms involved in the fate of rat EGF secretion, we first decided to clone the entire rat pro-EGF cDNA. To facilitate analysis of membrane-anchored pro-EGF shedding, the rat EGF cDNA was modified in order to introduce epitope tags in both the ectodomain (HA epitope) and the cytoplasmic tail (Myc epitope) of the molecule. The HA tag was introduced downstream of the prodomain, between Ser297 and Asn298, i.e. the second and third amino acid of the prodomain sequence.
Metallolprotease-dependent Regulated Pro-EGF Ectodomain Shedding

CHO-K1 cells were grown in culture medium (HAM F12 supplemented with 10% FCS) to about 70% confluency in 90-mm dishes. Plated cells were either not transfected (WT) or transiently transfected with unmodified (pT12) or epitope-tagged (pcD12-HA) pro-EGF-containing expression plasmids. Following transfection, cells were further incubated in 5 ml of culture medium at 37 °C under 5% CO2. 48 h later, conditioned media were harvested and cell lysates prepared. Aliquots of both cell lysates and conditioned media were subjected or not to trypsin hydrolysis before quantification of rat EGF by RIA as described previously (41). Total amounts of immunoreactive rat EGF in cell lysates and conditioned media are reported as well as the percentage of secreted EGF for each experimental condition.

| Amount of EGF in | Conditioned medium | Cell lysates |
|------------------|---------------------|--------------|
|                  | % of EGF in conditioned medium | µmol |
| CHO WT           | Trypsin ND          | ND           |
|                  | + Trypsin ND        | ND           |
| CHO pT12         | Trypsin 2.3         | 2.6          | 46.9 |
|                  | + Trypsin 6.8       | 9.5          | 41.5 |
| CHO pcD12-HA     | Trypsin 4           | 3.4          | 54.4 |
|                  | + Trypsin 14        | 16.3         | 46.1 |

* Not detected.

Acids of mature EGF. The Myc and His6 tags were C-terminal extension of the precursor molecule (see Fig. 1A for the schematic representation of the epitope-tagged rat pro-EGF construct). Both unmodified (pT12) and epitope-tagged (pcD12-HA) expression plasmids encoding the EGF precursor as well as the derived expression plasmid encoding the pro–domain-truncated HA-tagged soluble EGF (Apro-ATM-EGF) were used to transiently transfected established cell lines.

RIA Analysis of the Synthesis and Secretion of Rat EGF Immunoreactive Materials in Transiently Transfected CHO-K1 Cells—We first wanted to determine if a heterologous protein, which contains the EGF epitope, was expressed in CHO-K1 cells transfected either with the pT12 or pcD12-HA plasmids. EGF contents under different experimental conditions were first determined by RIA. As reported in Table I, EGF was undetectable in both lysate and conditioned medium of non-transfected cells both before and after trypsin treatment. In contrast, important and similar amounts of immunoreactive EGF could be detected in both lysate and conditioned medium following transfection of cells with either the untagged pro-EGF (pT12) or the pre-EGF-HA/Myc-tagged encoding plasmid (pcD12-HA). In the experiments realized with the EGF-precursor encoding plasmids, EGF immunoreactivity was increased by 3–4.8-fold following trypsin treatment. These results indicate that CHO cells do not naturally synthesize nor secrete detectable levels of EGF or that EGF synthesized by cells from Chinese hamster cannot be detected by our antibody, which is directed against rat EGF (41). The presence of immunoreactive EGF in both cell lysates and conditioned media indicates that cells transfected with either one of the two plasmids secreted EGF materials. Cells transfected with the two different pro-EGF plasmids did not only synthesize similar amounts of EGF but also secreted the same percentage of EGF (about 50% of total). This points out that the cellular behavior of the encoded proteins toward synthesis and secretion is very similar and suggests that epitope tagging of pro-EGF does not have any significant effect. Moreover, as stated in a previous study (41), the increase in EGF immunoreactivity following trypsin treatment may be an indication that EGF is synthesized and secreted as high molecular weight EGF-containing precursor proteins.

Western Blot Analysis of Expression and Secretion of Rat pro-EGF in Transiently Transfected CHO-K1 Cells—Our initial attempt to detect EGF-containing proteins by Western blot analysis using rEGF2 antibody failed because this antibody was not able to recognize the denatured and reduced forms of EGF (data not shown). That is why we decided to introduce epitope tags in both extracellular and intracellular parts of the precursor protein (see Fig. 1A for the schematic structure of EGF constructs). Western blot analysis of anti-EGF immunoprecipitates from pcD12-HA-transfected CHO cells show that pro-EGF is expressed as three high molecular weight species containing both HA and Myc epitopes (Fig. 1B, Cell), and we did not observe any protein lacking one of the two epitopes. Size analysis showed a major species of 176 kDa and two minor ones of 136 and 111 kDa. Three high molecular weight immunoreactive proteins, one major species of 165 kDa and two minor species of 121 and 99 kDa were observed in the anti-EGF immunoprecipitates from conditioned medium of pcD12 HA-transfected cells (Fig. 1B, Medium). These proteins only contained the HA epitope and not the Myc epitope. Performing electrophoresis with 16.5% Tris-Tricine acrylamide slab gels did not allow us to detect any other lower molecular weight immunoreactive species, especially mature 6 kDa EGF, either in cell lysates or in the conditioned medium (data not shown). As could be predicted, no EGF-containing protein could be detected either with anti-HA or anti-Myc antibodies in both cell lysate and conditioned medium from pT12-transfected CHO cells.

The presence of both extracellular (HA) and intracellular (Myc) epitopes in anti-EGF immunoprecipitates from cell lysates suggests that these species represent membrane-anchored forms of pro-EGF. The 176-kDa species would be the full-length pro-EGF whereas the two others may represent species shortened in the transmembrane domain. The loss of the intracellular C-terminal Myc tag in pro-EGF species from the conditioned medium as well as the shift in molecular weight led us to hypothesize that these proteins are derived from the membrane-anchored pro-EGF through proteolytic cleavage in the juxtamembrane domain, i.e. between EGF and the transmembrane domain. Moreover, the similar shift in molecular mass (respectively 11, 15, and 12 kDa) suggests that the different soluble pro-EGF species; 165, 121, and 99 kDa are respectively derived from the 176, 136, and 111 cellular species through proteolytic cleavage in the juxtamembrane domain.

As shown on Fig. 1C (upper panel), labeling of cell surface proteins with sulfo-NHS-LC-biotin demonstrated that all three different membrane-anchored pro-EGF species detected in Fig. 1B were exposed at the cell surface. In pcD12-HA transfected cells, biotinylated proteins of 176, 136, and 111 kDa could be immunoprecipitated with the anti-EGF, anti-HA, or the anti-Myc antibodies. These pro-EGF species were also observed in anti-EGF immunoprecipitates from pT12-transfected cells. Incubation of biotinylated cells for 4 h in culture medium supplemented with 10% serum resulted in the release of biotinylated pro-EGF species of 165, 121, and 99 kDa (Fig. 1C, lower panel). According to the results shown in Fig. 1B, these biotinylated proteins were immunoprecipitated with both anti-EGF and anti-HA antibodies but not with the anti-Myc antibody. All three soluble species were also observed in anti-EGF immunoprecipitates from pT12-transfected cells. Since all three species (176, 136, and 111 kDa) of cellular pro-EGF could be biotinylated at the cell surface, this greatly suggests that the lower molecular mass species (136 and 111 kDa) may not represent some non-glycosylated precursor forms of the 176 kDa species. This hypothesis was confirmed by performing short pulse labeling experiments (5 min) of newly synthesized proteins with 35S-methionine/cysteine followed by chase incubation (up to 3 h). Following 35S labeling, the radioactive 176 kDa anti-HA
immunoprecipitated protein is instantaneously and highly detectable in the cell lysate. At any time tested, this major pro-EGF showed the same high molecular weight. Lower molecular mass proteins of 136 and 111 kDa were only faintly detected later (data not shown), a result that argues against their role as precursor forms of the 176-kDa species.

Taken altogether, these results indicate that pT12- and pcD12-HA-transfected CHO cells contain one major (176 kDa) and two minor (136 and 111 kDa) pro-EGF species that are membrane-anchored proteins exposed at the cell surface. Once exposed at the cell surface, these pro-EGF species may undergo proteolytic cleavage in their juxtamembrane domain in order to release soluble pro-EGF of 165, 121, and 99 kDa. The protein pattern observed with CHO cells transfected with the epitope-

![Diagram](image-url)
tagged pro-EGF encoding pcD12-HA plasmid is clearly not a consequence of epitope tagging of the molecule. As a matter of fact, the same pattern of proteins was observed in both cell lysate and conditioned medium following biotinylation of untagged pro-EGF synthesized by pT12-transfected CHO cells.

Since the calculated size of the full-length epitope-tagged membrane-anchored EGF precursor, 128 kDa, as determined from cDNA sequence, is far smaller than the estimated size found by SDS-PAGE (176 kDa) we suggested that this protein might be glycosylated. This was confirmed following treatment of both secreted and cellular pro-EGF with different glycosidases (N-glycosidase, O-glycosidase, and N-acetyl-neuraminidase) or incubation of cells with tunicamycin (an inhibitor of endogenous glycosylation). These experiments show that both soluble (165, 121, and 99 kDa) as well as membrane-anchored pro-EGF (176, 136, and 111 kDa) were N-glycosylated proteins (data not shown). However, the shift in molecular mass following deglycosylation (15–20 kDa) was not sufficient to explain the discrepancy between the one calculated from the peptide backbone and the one measured on SDS-PAGE. Size analysis of the in vitro transcription/translation product from the pcD12-HA expression plasmid yielded only one product, whose estimated molecular mass of 154–159 kDa is very close to the estimated size (160 kDa) of the deglycosylated 176 kDa membrane-anchored pro-EGF. These results greatly favor the hypothesis that an unusual electrophoretic behavior of the EGF precursor molecules may explain the overestimation of both membrane-anchored and soluble high molecular weight pro-EGF found in transfected CHO cells but also in other cell types such as MDCK and HB2 cells.

Fig. 1D show the results obtained with CHO cells transiently transfected with the HA-tagged prodomain-truncated soluble EGF expression plasmid (Apro-ATM-EGF). As can be shown, following an incubation of 4 h in serum-containing medium, anti-EGF immunoprecipitates from both cell lysate and conditioned medium were shown to contain an anti-EGF immunoreactive protein of 8.4 kDa. This molecular mass is greater than the one that could be predicted for fully mature HA-anchored EGF (6.9 kDa) but perfectly matches the one predicted for an expressed protein that lacks the signal peptide but was not cleaved at the level of its residual prodomain. The identical molecular masses that are observed for both cellular and secreted EGF indicated that the last one is not derived from the first one through proteolytic cleavage. Thus, as observed with the full-length pro-EGF-encoding plasmids (Fig. 1, A–C), secreted EGF does not appear to be processed in its ectodomain and particularly at the Arg respectively level that would have led to the fully mature HA-tagged soluble 6.9-kDa EGF species as observed in rat urine (58). The way by which the 8.4 kDa soluble EGF is secreted appeared to be completely different from the one involved for full-length pro-EGF and must follow the secretory pathway for soluble proteins that do not involve cell surface shedding.

Shedding of Rat Membrane-anchored pro-EGF Is Regulated in CHO-K1 Cells—As shown in Fig. 2 (upper panel), pcD12-HA-transfected 35S-labeled CHO cells show an increased release of all three species of pro-EGF upon incubation in the presence of PMA, pervanadate (V$_{O_2}$), or serum (FCS). No stimulation of pro-EGF release was detected when cells were incubated with either Me$_2$SO (vehicle), the calcium ionophores A23187 (see Fig. 2) or ionomycin (data not shown). Following quantification by phosphorimaging, the amount of each [35S]pro-EGF species was expressed relative to the unstimulated condition (medium). As seen in Fig. 2 (lower panel), the stimulatory agents tested increased the release of the three pro-EGF to the same extent. This indicates that any of the soluble pro-EGF cannot be used to account for the properties of pro-EGF secretion. Thus, in the following experiments only results concerning the major pro-EGF kilodalton species (pro-EGF$_{165}$) will be reported and quantified.

We have demonstrated here that in contrast to previously published results (48), the release of soluble rat pro-EGF may be regulated at least in one cell type, the CHO-K1 cells. However, with that kind of experiment, the increased release of soluble forms of pro-EGF may be due to the stimulation of intracellular protein traffic in the secretory pathway and/or an increase in the shedding rate of the membrane precursor. In order to test for an increased cleavage of the cell surface pro-EGF, we analyzed pro-EGF secretion upon PMA stimulation following both 35S labeling and cell surface protein biotinylation. As shown in Fig. 3, compared with control experiments, PMA induced a time-dependent increase of both 35S-labeled (upper panel) and biotinylated pro-EGF$_{165}$ (middle panel) in the conditioned medium, suggesting that the HA-tagged proteins secreted came from a previously labeled intrinsic cell surface protein, i.e. the membrane-anchored pro-EGF. Quantification of the kinetics of 35S-labeled pro-EGF$_{165}$ release indicates that the effect of PMA was linear for up to 2 h (lower panel). Stimulation rapidly decreases thereafter probably as a result of both desensitization of the PKC-dependent pathway and down-regulation of the PKC activity. From these results, it is obvious that the rise in pro-EGF detected in the medium...
Metalloprotease-dependent Regulated Pro-EGF Ectodomain Shedding

35S-Labelling

Streptavidin-PO Labelling

Fig. 3. Kinetic of stimulation of cell surface pro-EGF secretion. CHO cells were transiently transfected with pcD12-HA expression plasmid. 48 h later, cells were labeled for 2 h with 35S label. At the end of the labeling, cells were extensively washed three times with PBS and cell surface proteins biotinylated using sulfo-NHS-LC-biotin as described under “Materials and Methods.” Labeled cells were chased for up to 180 min, in the absence or presence of 1 μM PMA as indicated on the figure. At each time point, conditioned media were immunoprecipitated with the anti-HA antibody and immunoprecipitated proteins separated by SDS-PAGE and blotted to nitrocellulose. 35S-labeled proteins were visualized by phosphorimaging using a STORM 840 instrument (upper panel) and biotinylated proteins visualized by ECL using peroxidase-conjugated streptavidin (middle panel) as described under “Materials and Methods.” Radioactivity associated with 165 kDa [35S]pro-EGF was quantified for each time point (lower panel), and results are expressed as the percentage of the amount of 35S-labeled pro-EGF165 present in the incubation medium upon 180 min of stimulation with PMA.

upon PMA stimulation is due to the stimulation of membrane-anchored precursor shedding.

This conclusion was further confirmed by the experiments performed with CHO cells transiently transfected with Δpro-ΔTM-EGF expression plasmid. RIA analysis of EGF content in both conditioned media and cell lysates show us that cells transfected with Δpro-ΔTM-EGF or pcD12-HA synthesized equal amounts of immunoreactive EGF (data not shown), however the rate of EGF secretion by cells expressing the truncated 8.4 kDa soluble form of EGF (11.2 ± 0.89% per h) was two times that of cells expressing the full-length membrane-anchored EGF precursor (4.9 ± 0.35% per h), suggesting that both EGF species do not follow the same pathway to be secreted into the extracellular medium. Moreover, compared with pro-EGF-transfected cells, incubation in the presence of PMA, serum, or pervanadate showed that secretion of the soluble form of EGF could not be up-regulated whereas the one resulting from expression of the full-length membrane-anchored pro-EGF could be (Fig. 4). This result, and the fact that no molecular weight shift between the cellular and secreted HA-EGF was detected (8.4 kDa, Fig. 1D) indicate that a general stimulation of intracellular protein trafficking does not account for the stimulation of pro-EGF ectodomain secretion and reinforces the hypothesis that the rise in pro-EGF165 detected in the medium upon stimulation is due to the stimulation of membrane-anchored precursor shedding.

Role of PKC in the Regulation of Membrane-anchored pro-EGF Shedding—Since PKC activation is often linked to protein secretion and was clearly involved in the PMA-dependent regulation of EGFR ligand shedding, we have tested its role in the control of rat pro-EGF secretion. Implication of phorbol ester activable PKC in pro-EGF secretion was first demonstrated by the use of specific PKC inhibitors such as Ro 31-8220 or BIM I. As can be seen on Fig. 5A, preincubation of CHO cells in the presence of PKC inhibitors either partially (Ro 31-8220) or completely (BIM I) abolished a subsequent stimulation of pro-EGF165 secretion by PMA. In the same conditions, basal secretion as well as pervanadate and serum stimulation was unaffected (data not shown). The involvement of PKC in the stimulatory effect of PMA was further confirmed following its down-regulation prior to cells stimulation. PcD12-HA-transfected cells were first incubated with 1 μM PMA for 18 h prior to 35S labeling. As shown in Fig. 5B, down-regulation of PKC completely abolished the stimulation of pro-EGF165 secretion by PMA. This inhibition is not the consequence of a general and nonspecific inhibitory effect of the treatment since pervanadate stimulation was only slightly affected and serum-induced secretion was not. Moreover, PMA pretreatment did not show any inhibitory effect on the incorporation of 35S-amino acids into cellular pro-EGF, i.e. pro-EGF synthesis (data not shown). Taken together, these results and those obtained with PKC inhibitors, clearly indicate that the stimulatory effect of PMA is completely dependent on PKC activities whereas both pervanadate and serum stimulation are not.

Pro-EGF Ectodomain Shedding in CHO-K1 Cells Is Associated with a Zinc Metalloprotease Activity—The following experiments were performed in order to determine the type of protease activity involved in membrane-anchored pro-EGF
shedding. Therefore, we tested the effects of different protease inhibitors on pro-EGF secretion from PcD12-HA transfected CHO-K1 cells. The inhibitory effect was assayed both on PMA-stimulated and -unstimulated [35S]labeled cells. As shown in Fig. 6A, none of the protease inhibitors tested had any significant inhibitory effect on the unstimulated secretion. However, the PMA-stimulated [35S]pro-EGF165 secretion was significantly reduced by the metalloprotease inhibitor phenantrolin, a zinc chelator molecule, and this inhibition can be overcome by the addition of an excess of zinc in the incubation medium (data not shown). Neither aprotinin nor SBTI (two serine protease inhibitors), nor pepstatin (an aspartyl protease inhibitor), nor E64 (a cysteine protease inhibitor) had any significant inhibitory effect either on basal or stimulated secretion. EGTA and EDTA, two commonly used metalloprotease inhibitors, which preferentially chelate Mg²⁺ and Ca²⁺, were also inefficient (data not shown).

These results indicate that the activity of the protease involved in pro-EGF cleavage is greatly dependent upon the presence of some divalent cations and suggest that it might be a Zn²⁺ metalloprotease. This hypothesis was further supported by the use of TAPI-2, a hydroxamate acid-based derivative, which inhibits zinc-dependent metalloprotease activities by binding to the catalytic site of the enzyme. As shown in Fig. 6B, TAPI-2 inhibited both unstimulated and PMA-stimulated [35S]pro-EGF165 secretion at concentrations below 1 μM. Half-maximal inhibition of the PMA stimulation was achieved for a concentration of 0.1 μM and complete inhibition reached at a concentration of 3 μM. The inhibitory effect of TAPI-2 was not restricted to the PMA stimulation since a 2 μM concentration completely inhibited the serum- and pervanadate-stimulated [35S]pro-EGF165 secretion (data not shown). Tissue inhibitors of matrix metalloprotease (TIMP 1 and 3) were also tested at concentrations up to 50 nM but failed to prevent the PMA stimulation of immunoreactive EGF secretion (data not shown).
The metalloprotease-dependent ectodomain shedding of pro-EGF was further confirmed by studying pro-EGF secretion in a CHO cell line (CHO-M2) defective in the metalloprotease-dependent shedding of many cell surface proteins (25, 28, 51).

Wild-type CHO (wt) and mutant CHO (CHO-M2) cells were transiently transfected with the pcD12-HA expression plasmid. 48 h later, cells were processed for cell surface proteins biotinylation using sulfo-NHS-LC-biotin (part A) or labeled for 2 h with TRANS5S Label (part B) as described under “Materials and Methods.” A, biotinylated cells were further incubated for 4 h in conditioning medium as in Fig. 1B. Cell lysates and incubation media were harvested and immunoprecipitated with the anti-HA antibody (HA-11) as described under “Materials and Methods.” Immunoprecipitated proteins were separated by SDS-PAGE on a 7.5% slab gel, and visualized by phosphorimaging using a STORM 840 instrument. Radioactivity associated with the pro-EGF165 species was quantified for each experimental condition, expressed as arbitrary units, and are the average ± S.E. of triplicate experiments.
CHO-wt and CHO-M2 cell were further compared for their ability to up-regulate pro-EGF secretion upon stimulation. pcD12-HA-transfected 35S-labeled CHO-wt and CHO-M2 were incubated in the presence of PMA, serum or pervanadate, and pro-EGF secretion analyzed as described in Fig. 2. Results reported in Fig. 7B showed that these agents were unable to increase pro-EGF shedding from the cell surface of CHO-M2 cells and demonstrate that both constitutive and regulated pro-EGF secretion was completely impaired in the CHO-M2 cell line compared with CHO-wt.

Taken as a whole, the results obtained with protease inhibitors as well as CHO-M2 clearly established that, at least in CHO cells, both constitutive and regulated EGF secretion involve one or more zinc-dependent metalloprotease(s) activities. This or these metalloprotease activities shed the ectodomain of pro-EGF from the membrane-anchored pro-EGF once exposed at the cell surface.

**Lack of Regulated pro-EGF Shedding in HeLa Cells**—The zinc metalloprotease TACE (ADAM 17) is now known to be involved in shedding of many different cell surface proteins and among them three members of the EGF growth factor family, i.e. TGF-α (24, 25, 28, 29), HB-EGF (29, 30), and amphiregulin (29, 31). So, in order to determine its possible involvement in the shedding of pro-EGF, we have used the established HeLa cell line, which is well known to possess high amounts of TACE (Ref. 59 and Fig. 8A) and was able to cleave one of the substrates of TACE, the β-amylloid precursor protein (βAPP) (60).

Using an anti-TACE antibody directed against the cytoplasmic domain of the protease, we have first verified the presence of the protease in both CHO-K1 and HeLa cell lines (Fig. 8A). As can be shown, HeLa cells contained high amount of both the inactive high molecular weight proenzyme and of 2 mature forms with lower masses. In contrast, when equal amounts of cellular proteins from our CHO-K1 cells were analyzed, they appeared to contain only low amounts of mature enzymes.

Like CHO-K1 cells, HeLa cells transiently transfected with the pcD12-HA expression plasmid synthesize EGF as its high molecular weight precursor. This precursor is membrane-anchored and is expressed at the cell surface as shown by biotinylation experiments. However, compared with CHO cells, the presence of HA-tagged pro-EGF in conditioned medium following incubation in the presence of serum was only barely detectable (data not shown). This result was confirmed when immunoreactive EGF secretion was measured by RIA. HeLa cells synthesized two to three times less EGF than CHO cells and with a basal EGF secretion rate, which was also 2× lower (2.2% per hour compared with 4.9% for CHO cells). In contrast to CHO, HeLa cells have EGF receptors (EGFR) expressed at the cell surface (61). As reported for TGF-α secretion by MDCK cells (62), we tested the possibility that secreted EGF could be lowered by a rapid consumption through receptor binding and internalization. However, this is clearly not the explanation in HeLa since none of the manipulations intended to inhibit this effect had any increasing effect on the amount of secreted EGF. The conditions tested were the following: incubation in the presence of an excess of human EGF or of an anti-EGF antibody (mAb 225) that blocked the binding of secreted EGF to the EGFR as well as blockade of receptor kinase activity by AG1478 that inhibited EGFR internalization (data not shown).

More importantly, in HeLa cells, EGF secretion did not show any strong up-regulation upon incubation in the presence of either PMA, serum, or pervanadate (Fig. 8B, upper left) under conditions that strongly increase EGF secretion in CHO cells (Fig. 8B, lower left). Only a small but significant increase could be observed upon PMA stimulation. One simple explanation may be that our HeLa cell line may be unable to achieve regulated shedding of cell surface proteins. To test this hypothesis, HeLa and CHO cells were transiently transfected with a pro-TNF-α-containing expression plasmid, and secretion of TNF-α in both cell lines was analyzed by RIA. Results obtained...
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allowed us to rule out this hypothesis since they clearly showed that in HeLa cells, shedding of pro-TNF-α was enhanced by the different treatments: PMA, FCS, or pervanadate (Fig. 5B, upper right). Moreover, both basal and PMA-stimulated shedding could be inhibited by the zinc metalloprotease inhibitor TAPI-2 (data not shown). In contrast, CHO cells show only a small increase in pro-TNF-α shedding upon stimulation with PMA and serum (∼140% of basal) when compared with the effect obtained on pro-EGF shedding in the same cell line (200–500% of basal) or to the release of TNF-α from HeLa cells (200–400% of basal).

Takemoto, together, these results greatly favor the hypothesis that HeLa cells may not have the enzymatic machinery (ecto-
protease) necessary to shed the EGF precursor. The ADAM 17 protease (TACE) was first identified as a protease that specifically cleaves full-length pro-TNF-α (63, 64). The fact that HeLa cells, which possess a high amount of TACE, did not efficiently shed pro-EGF, whereas these cells were able to cleave pro-TNF-α, questioned the role of TACE in the processing of pro-EGF. This conclusion is further corroborated by the ability of CHO cells that contain low amounts of TACE to efficiently shed pro-EGF while being unable to shed pro-TNF-α from the cell surface. Thus, while both pro-TNF-α and pro-EGF shedding are achieved by zinc-dependent metalloproteases, pro-TNF-α shedding appears to be correlated to the amount of TACE, while this correlation does not apply to pro-EGF shedding.

**DISCUSSION**

In the course of a recent study (41) we have developed an efficient polyclonal antibody (rEGF) directed against the native form of rat EGF. This antibody allowed us to demonstrate the presence of immunoreactive EGF in the exocrine rat lacri-
mal gland. In this tissue EGF was shown to be stored as its high molecular weight membrane-anchored precursor as already shown in kidney (12, 36, 37, 39). Immunoreactive rat EGF is also present in corresponding secretory fluids, i.e. tears and urine (18–20, 37, 42, 43). However, the nature of the regulatory pathways as well as the proteins involved in the shedding of membrane-anchored pro-EGF that may account for the release of soluble EGF are very poorly understood. The reasons may be that there was neither simple physiological model nor any immortalized cell line available to study the endogenous synthesis and secretion of EGF.

Since the tight regulation of shedding of EGFR ligands such as TGF-α (50, 53, 65) and HB-EGF (66) have been previously demonstrated in the CHO cell line, we were then interested in determining the possible regulation of rat pro-EGF secretion in this cellular model. In this way, we first cloned rat prepro-EGF cDNA and introduced epitope tags in both the extracellular and intracellular domain of the molecule. This was done in order to conveniently follow the different parts of the precursor molecule and also because our anti-rat EGF antibody does not recognize the denatured and reduced form of EGF, precluding any analysis by SDS-PAGE. RIA analysis of cell lysate and conditioned media indicates that CHO cells transfected with pro-EGF-containing expression plasmids efficiently synthesize and secrete EGF immunoreactive molecules.

Cellular pro-EGF was found to be expressed as three differ-
ent species that are all present at the cell surface. These pro-
teins that contain EGF as well as the intracellular and extra-
cellular epitopes, certainly represent the membrane-anchored pro-EGF. The size of the major pro-EGF species (176 kDa) is very similar to the one of the human pro-EGF expressed in MDCK (48) and in NIH 3T3 (67) cell lines (respectively, 185 and 170 kDa) and may be the full size rat pro-EGF. The lower and minor molecular mass species (136 and 121 kDa) are probably derived from the major 176 kDa full-length precursor through cleavage in the N-terminal distal part of the prodo-
main. They have not been observed with NIH 3T3 and MDCK cells transfected with the human pro-EGF (48, 67) but we previously reported a similar maturation of the membrane-
associated pro-EGF in rat kidney (41). Nevertheless, from the present experiments, this limited prodomain release does not seem to have any consequences on EGF secretion since all species appeared to be equally sensitive to juxtamembrane cleavage.

According to studies on MDCK (48) and NIH 3T3 (67) cells, we did not observe any proteolytic maturation of pro-EGF that would lead to the release of the entire EGF prodomain. These observations are quite different from those reported for the maturation process of other EGFR ligands such as TGF-α (50) and human HB-EGF (68) where the precursor prodomain appears to be cleaved very rapidly once it has reached the cell surface. However, our results are very similar to the ones obtained with mouse HB-EGF (68) and amphiregulin (31), where the full-length precursor preferentially accumulates at the cell surface. This may reflect some specific and intrinsic properties of the EGF prodomain that would be independent both from the origin of EGF and of the expressing cells. It is often thought that membrane-anchored EGFR ligands are able to interact with the EGF receptor (EGFR) located on an adja-
cent cell, thus acting as a juxtacrine growth factor (9, 71). This property of EGF ligands has been clearly demonstrated for both TGF-α (69–71) and human HB-EGF (3, 68), whose mem-
brane-anchored precursors are known to be rapidly subjected to prodomain release (50, 66). In contrast, the juxtacrine activity of the membrane-anchored mouse HB-EGF precursor is greatly reduced because of a decreased maturation of its prodo-
main (68). The physiological significance of the N-terminal processing still remains unknown. However, prodomain re-
lease leads to the exposure of the growth factor moiety to the cellular environment, thus potentially facilitating its interac-
tion with the EGFR on an adjacent cell. Under these condi-
tions, the observation that both human (48, 67) and rat mem-
brane-anchored pro-EGF (this study) do not undergo any significant cleavage of their prodomain led us to question whether it would act as a juxtacrine growth factor. The hypoth-
thesis that the EGF precursor may have juxtacrine potency has not been deduced from co-culture experiments but was inferred from the ability of both soluble (secreted) pro-EGF (43, 67) and detergent-solubilized membrane pro-EGF (36) to bind and ac-
tivate the EGF receptor. However, under these two conditions EGF is freed from the environmental constraint (steric hin-
drance) of the membrane. This would be sufficient to allow a low affinity interaction between the EGF moiety and the EGFR (high molecular mass pro-EGF is less biologically active than mature 6 kDa EGF) but it does not necessarily imply that the native membrane-anchored pro-EGF has this property. To more definitely answer this question, we think that the results that could be obtained from co-culture experiments with CHO cells expressing or not the membrane-anchored rat EGF pre-
cursor and EGFR-expressing cells will be valuable.

We did not observe any precursor form to the 176-kDa rat pro-
EGF species, and in contrast to the results obtained in MDCK cells, cell surface biotinylation experiments led to the efficient labeling of the 176-kDa pro-EGF (as well as the 136-
and 111-kDa species). This favors the hypothesis that they represent mature pro-EGF species that have reached the cell surface after trafficking through the cell. Thus, in CHO as in NIH 3T3 cells, intracellular post-translational maturation and trafficking of EGF must be much more rapid than in
MDCK, leading to an increased steady-state level of the mature precursor at the cell surface. As found previously with NIH 3T3 and MDCK cells, pro-EGF-expressing CHO cells release high molecular mass EGF into the extracellular medium. Secreted pro-EGF species (one major 165 kDa and minor 121 and 99 kDa species) are shorter in size and appear to directly derive from their membrane counterpart (respectively, the major 176 kDa and minor 136 and 111 kDa form). Because of the great similarity in the molecular size of cellular and secreted pro-EGF in NIH 3T3, Mroczkowski et al. (67) suggested that soluble pro-EGF might be the consequence of an inefficient stop-transfer sequence. Under these conditions, the non-inserted precursor may represent the secreted form. However, we can rule out this hypothesis since, at least in CHO cells, secreted pro-EGF lacks the intracellular Myc epitope indicating that it has been cleaved at its C-terminal domain. These shorter soluble species do not appear to be synthesized in this state since they were not observed in cell lysates. Nevertheless, we cannot exclude that intracellular processing of the C-terminal domain might lead to pro-EGF species immediately released into the extracellular medium. However, the fact that following cell surface biotinylation, pro-EGF released into the extracellular medium is biotinylated does not favor such an interpretation. To our knowledge, this is the first direct evidence that part if not all of the secreted pro-EGF arise from the proteolytic cleavage of membrane-anchored pro-EGF exposed at the cell surface. The determination of the exact cleavage site was not in the scope of this study. However, the size decrease between the different cellular and soluble pro-EGF species was comprised between 11 and 15 kDa. This is very close to the predicted value (13–14 kDa) when making the hypothesis that cleavage of cellular pro-EGF occurred in the extracellular juxtamembrane domain. Thus, our experimental results, if they do not directly demonstrate the juxtamembrane cleavage of membrane-anchored pro-EGF, significantly favors this hypothesis.

The regulated ectodomain shedding of EGFR ligands such as TGF-α, HB-EGF, and amphiregulin is now clearly established. It can be triggered by different agents such as PMA (through PKC activation), calcium ionophore (calcium influx), serum, or pervanadate (tyrosine phosphatase inhibition) (9, 23, 25, 31, 50), and inhibited by zinc-dependent metalloprotease inhibitors (24, 25, 28–31). Concerning EGF, its regulated shedding from cell surface is not so clearly established and experimental results are sparse and controversial. To our knowledge, only two studies have addressed this question. In an extensive study performed with MDCK cell line transfected with a full-length human pro-EGF cDNA-encoding plasmid, the authors failed to observe any stimulation of pro-EGF ectodomain cleavage and particularly upon phorbol ester (PMA) or serum treatments (48). However, in this cellular model, the constitutive basolateral and apical secretion of pro-EGF is partially sensible to the metalloprotease inhibitor batimastat. In a second study performed with a human mammary gland cell line (HB2) transfected with a truncated human pro-EGF cDNA construct lacking most of the prodomain region, EGF secretion was found to be stimulated by a calcium ionophore (A23187) and a tyrosine phosphatase inhibitor (phenylarsine oxide) but not by phorbol esters (49). The metalloprotease inhibitor BB-2116 inhibited the calcium ionophore-stimulated EGF release.

With regard to the above results, our finding that full-length rat pro-EGF ectodomain shedding was regulated in CHO-K1 was surprising. As for other EGFR ligands, PMA, serum, and a tyrosine phosphatase inhibitor stimulated shedding. However, whereas CHO cells expressing TGF-α showed an increased shedding upon calcium ionophore treatment (A23187, see Ref. 50), we did not find any stimulatory effect using two different calcium ionophores, namely A23187 and ionomycin. Additionally, we found that, as well as for TGF-α, both constitutive and regulated pro-EGF ectodomain shedding was sensitive to zinc-dependent metalloprotease inhibitors (phenantrolin and TAPI-2). Moreover, the PMA-stimulated pro-EGF ectodomain shedding was very sensitive to inhibition by the metalloprotease inhibitor TAPI-2. The IC50 value found in our study (0.1 μM) was 10–100 times less than those currently reported for the inhibition of membrane protein shedding (23, 25). The key role played by a metalloprotease in both constitutive and regulated pro-EGF shedding was also deduced from secretary experiments performed with the mutant CHO-M2 cell line. CHO-M2 were initially selected for their defect in TGF-α shedding but were subsequently shown to be defective in the metalloprotease-dependent shedding of many cell surface proteins (25, 28, 51). A defect that can now be extended to EGF since both constitutive and regulated pro-EGF secretion was completely impaired in the CHO-M2 cell line compared with CHO-wt.

We do not have any clear explanation for the inability of MDCK cells to secrete pro-EGF in a regulated manner (48). However, since intracellular maturation and/or traffic appeared to be limiting steps in the course of EGF secretion, an explanation could be that a too low amount of steady state level in cell surface pro-EGF would preclude any detection of cell surface precursor cleavage regulation. In addition, the constitutive metalloprotease activity of the basolateral membrane that prevents accumulation of pro-EGF in this cellular domain may be already too high to be further increased. At last, the possibility that the regulated metalloprotease that cleaves pro-EGF in CHO cells may not be present or correctly located nearby its substrate in MDCK cells cannot be ruled out. This hypothesis may also be applied to HB2 cells since they appeared to have different possibility to regulate pro-EGF shedding (49). As was stated by the authors, based on a differential sensitivity of proHB-EGF and pro-EGF shedding to PMA and calcium ionophore, different metalloproteases may be involved in the cleavage of EGF versus HB-EGF. Our results demonstrate that CHO cells cleave pro-EGF in a PMA but not calcium-dependent manner whereas HB2 cells exhibit the opposite regulation. As stated above, the difference between the two cell lines may come from the involvement of different proteases. This discrepancy may not necessarily imply different protease populations in the two cell lines, but could be the result of differential recruitment of specific proteases because of the different type of substrates involved in the shedding event. The deletion of the prodomain would allow the interaction of the substrate with one type of metalloprotease that can be activated by the calcium-dependent pathway whereas the full-length substrate would interact with a metalloprotease activated through the PMA/PKC pathway. So, in addition to the role that can be played by the stalk region, comparison of the results of both studies suggests that the prodomain of pro-EGF may contribute to the specificity of the interaction between the substrate and the protease(s).

An increasing amount of experimental evidence suggests that the ADAM metalloprotease TACE may be the shedding of TGF-α (24, 25, 28), HB-EGF (29), and amphiregulin (29, 31). However, the recent demonstration of the, in vitro, inability for purified TACE to cleave synthetic peptide presenting the cleavages sites of human pro-EGF (both N- and C-terminal) (29) would argue against a role for TACE as the pro-EGF sheddase and would favor the possibility that different metalloproteases could be involved in the release of EGF versus TGF-α. Our demonstration of the inability of HeLa cells to shed EGF from the cell surface in a regulated manner, whereas this cell line
appears to contain high amounts of TACE and secrete TNF-α also argue against a role for TACE in regulated pro-EGF secretion. This conclusion was reinforced by the observation that wild-type CHO cells we used in this study do efficiently regulate pro-EGF shedding whereas they contain low amounts of TACE and are not able to regulate TNF-α secretion, and that TIMP-3, which is known to be a potent inhibitor of TACE (72), failed to inhibit pro-EGF secretion. Borroto et al. (73) recently showed that the defect in ectodomain shedding of the mutant CHO-M2 cells was the consequence of a defective removal of the prodomain that keeps TACE in an inactive form in the early secretory pathway. Thus, our results obtained with this mutant CHO cells are in favor of a pro-EGF shedding controlled by TACE. However, despite their control experiments that show a normal processing for some other metalloproteases involved in activated ectodomain shedding, it is possible that the M2 cells are not only deficient in TACE activity but also in an other unsuspected ADAM or zinc metalloprotease-related activity, which will be responsible for the controlled shedding of pro-EGF. We tried to obtain more direct evidences about the involvement of TACE in pro-EGF shedding using fibroblasts expressing an inactive form of TACE (Tace−/−) cells. Unfortunately, we failed to obtain any expression of pro-EGF in this cell line despite the fact that we tested many different transfection protocols and were able to transfect a GFP-containing expression plasmid.

As was demonstrated previously, shedding of TGF-α, HB-EGF, and amphiregulin precursors are regulated phenomena that can be triggered by PMA (through PKC activation), calcium ionophore (calcium influx), serum, or pervanadate (tyrosine phosphatase inhibition) (9, 23, 25, 31, 50–53). We have been able to clearly demonstrate that pro-EGF shedding is also increased upon PMA, serum, and pervanadate treatment. However, comparison of the effect of specific PKC inhibitors and PKC depletion on the different ways of stimulation indicates that different intracellular pathways may be involved. In fact, the PKA stimulation is completely dependent on the activation of some PKC activities whereas both serum and pervanadate stimulation are not. The fact that a specific PKC can be involved in the PKA stimulation of pro-EGF shedding cannot be evidenced here but a study on proHB-EGF suggests that PKC-δ was the target for the phorbol ester-induced proHB-EGF shedding (30). It has been recently suggested that the MAP kinase signaling cascade could be involved in the regulation of the ectodomain shedding of both TGF-α (53) and HB-EGF (66). Our preliminary results suggest that it may be also involved in the regulation of pro-EGF shedding activated by PMA, serum, and pervanadate. Since both PKC-dependent and PKC-independent pathways can activate the MAP kinase pathway, MAP kinases such as ERK1/2 may be the target of these different types of stimulation. The recent discovery that ERK was able to phosphorylate the cytoplasmic tail of TACE at a specific site strongly suggests that it may modulate TACE activity through serine phosphorylation (74), and potentially act in the same way to regulate other metalloproteases.

In this study, we have clearly demonstrated that as other members of the EGFR ligand family, when expressed in CHO cells, EGF is synthesized as a membrane-anchored precursor present at the cell surface. The entire ectodomain of the precursor can be shed from the cell through a proteolytic cleavage that involved a zinc-dependent metalloprotease activity that appears not to be TACE but can be activated by both PKC-dependent and -independent pathways. However, these experimental results obtained with cultured cells, are in conflict with those obtained with membrane fractions of both kidney (45, 46) and mammary gland (47). Indeed, these preparations contain a membrane bound proteolytic activity that colocalizes with pro-EGF. However, this activity, which is able to release soluble EGF from its membrane-associated precursor, belongs to the serine protease family and not to the metalloprotease one. In order to resolve such a discrepancy, the establishment of a more physiologically relevant cells line from organ cells, which naturally secrete EGF appears necessary and is now under investigation.

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Regulated Cell Surface Pro-EGF Ectodomain Shedding Is a Zinc Metalloprotease-dependent Process

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