Membrane-anchoring Domains

Ligands that bind to the epidermal growth factor (EGF) receptor are initially synthesized as integral membrane proteins that are released from the cell surface by regulated proteolysis. To study the role of the membrane-anchoring domain in ligand release, we made two artificial ligands. The first possessed the membrane-anchoring domain from EGF whereas the second had the corresponding domain from heparin binding EGF-like growth factor (HB-EGF). Both ligands lacked amino-terminal extensions, and were epitope-tagged at the carboxyl terminus. Following stable expression in human mammary epithelial cells, their cellular localization and rate of proteolytic release were examined. We found that constructs with the membrane-anchoring domain from EGF were found primarily at the cell surface and displayed a relatively high rate of constitutive release. Constructs with the HB-EGF membrane-anchoring domain displayed a higher internalized fraction and a very low rate of constitutive release. The two ligand constructs also displayed different patterns of stimulated release. Proteolysis of the chimera with the HB-EGF membrane-anchoring domain was stimulated by activation of protein kinase C, but release of EGF from constructs with the EGF membrane-anchoring domain was insensitive to this. Calcium ionophores, calmodulin antagonists, and tyrosine phosphatase inhibitors stimulated the release of both ligands. Furthermore, the release of the two constructs showed different sensitivity to metalloprotease inhibitors. Despite a large fold-increase in ligand proteolysis following cell stimulation, only a small fraction of total cell-associated ligand was released per hour. Our results show that the membrane-anchoring domain of EGF-like ligands can specify both their localization and proteolytic processing. The structures of the membrane-anchoring region of this class of ligands can thus regulate their activity.

There are currently six known ligands made by mammalian cells that bind to the epidermal growth factor receptor (EGFR): EGF (1), TGFα (2), heparin-binding EGF (3), amphiregulin (4), betacellulin (5), and epiregulin (6). These EGFR ligands are initially synthesized as integral membrane precursor proteins consisting of an N-terminal extension, EGF-like domain, transmembrane region, and a cytoplasmic tail. The EGF-like motif, shared by all EGFR ligands, contains six conserved cysteine residues and forms the three-loop structure essential for EGFR binding (7). Except for the EGF-like domain, the various EGFR ligand precursors show no strong homologies with one another.

Mature, soluble EGFR ligands are released from the cell surface by proteolytic cleavage (8). Soluble EGFR ligands act as autocrine or paracrine growth factors for EGFR-expressing cells. The membrane-bound forms of EGF-like ligands have been proposed to possess juxtacrine activity and are thought to directly stimulate neighboring cells expressing EGFR (9–13). There is evidence that the membrane-bound and soluble forms of HB-EGF have different potencies in activating the EGF receptor and have different effects on cell growth and survival (14–16). Few studies, however, have addressed the juxtacrine activity of other EGFR ligands. Recently, other investigators and we have shown that some EGFR ligands require proteolytic release for their biological activity (17, 18). Regulated release of soluble ligands from the cell surface could thus be an important step in controlling the availability of different EGFR ligands.

Metalloproteases are thought to be responsible for cleaving TGFα (19), HB-EGF (20), amphiregulin (21), and EGF (22). Recent evidence implicates the involvement of the ADAM (a disintegrin and metalloprotease) family of proteins in the release of these ligands (23, 24). The metalloprotease activity of ADAM family members was first shown for TACE/ADAM 17 (25, 26), which was initially cloned as the tumor necrosis factor-α converting enzyme. Later studies indicated that TACE also cleaves TGFα and l-selectin. Interestingly, TACE knockout mice have a phenotype very similar to that observed following EGFR knockout (18, 27, 28). TACE could be involved in the processing of other EGFR ligands as well because TGFα knockout mice show a much milder phenotype (29). Both MMP-9 and MDC9 (ADAM9) have been reported to be involved in the proteolytic release of HB-EGF (30, 31). Interestingly, TACE/ADAM17 and MDC9/ADAM9 exhibit different specificity for cleaving candidate substrate peptides and display different sensitivities to hydroxamic acid-type metalloprotease inhibitors in vitro (32). Therefore, there may be multiple metalloproteases involved in EGFR ligand release. The specificity of metalloproteases for different EGFR ligands remains to be clarified.
Several studies have focused on the regulated release of EGFR ligands. For example, there is substantial evidence that PKC activation stimulates TGFα, HB-EGF, and amphiregulin release (12, 21, 33–35). In contrast, EGF release does not appear to be stimulated by PKC activation (22). A recent report suggests that MDC9/ADAM9 interacts with PKC and a complex of the two molecules is involved in PMA-induced cleavage of HB-EGF (31). This may represent a common mechanism for PKC-regulated release of other cell surface proteins, such as TGFα, t-selectin, and amyloid precursor protein (19, 36, 37). Other factors, including calcium influx and tyrosine phosphatase activity, have also been shown to regulate the release of EGFR ligands independent of PKC activity (20, 38, 39).

The membrane-anchoring domain (membrane spanning plus cytoplasmic tail) appears to influence the processing of EGFR ligands. The cytoplasmic domain of TGFα dictates its basolateral distribution in polarized Madin-Darby canine kidney cells (40). The cytoplasmic terminal valine of TGFα is required for its efficient maturation and intracellular routing (41) in R1 cells, and is required for PKC-induced proteolytic release in Chinese hamster ovary cells (42). Although it has been proposed that the cytoplasmic tails of HB-EGF and amphiregulin are unimportant in PKC-induced ligand release (20, 39), the structural requirements for constitutive release are unknown. In addition, TGFα has been reported to reside primarily at the cell surface whereas HB-EGF has been shown to be efficiently internalized (12, 40, 41, 43). Because these studies were performed with individual ligands in different cell types, it is unclear whether differences in ligand behavior are due to ligand structure or the cell types expressing them.

A complicating factor in the analysis of ligand processing is the use of different assays for measuring the release of the different ligands. Assays used to measure ligand appearance in the extracellular medium are typically cumbersome and of poor sensitivity because of the low amounts of released ligand and the lack of high affinity antibodies for most of them. This greatly complicates structure-function studies of EGFR ligands.

To investigate the structural determinants of ligand behavior in cells, we used a domain swapping strategy to study the role of the membrane-anchoring region of EGFR ligands in the regulation of ligand release. We compared the behavior of artificial ligands containing the membrane-anchoring domain from either EGF or HB-EGF. We chose this pair for our initial studies because the processing of EGF and HB-EGF has been reported to be quite disparate (12, 22, 35, 36). Each of the four ligands studied was purified from hybridoma supernatants, and the antigenic epitope shared between EGF and HB-EGF was used for immunochemical staining. The EGFR portion was PCR-amplified using primers 1 and 2 with EGF-ct as template, while the HB-EGF membrane-anchoring region was PCR amplified from HB-EGF using primers 3 and 4. The above two PCR products were used as templates for PCR with primers 1 and 4 to generate the chimera EGF-hc. A FLAG epitope (DYKDDDDK) tag was added to the carboxyl termini of both EGF-ct and EGF-hc using a similar PCR based strategy. Primers 5 and 6 contain sequences of the 5′ and 3′ ends of the FLAG sequence preceding the stop codon. Primers 1 and 5 were used to PCR amplify EGF-ctF from EGF-ct. Primers 1 and 6 were used to generate EGF-hcF by PCR from EGF-hc. PCR products were then digested with NcoI and BamHI restriction enzymes and cloned into the MFG retrovirus-based expression vector (48). All constructs were confirmed by DNA sequencing.

**Cell Culture and Transduction**—The mammary epithelial cell line HB2 (49) was obtained from Joyce Taylor-Papadimitriou. Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% horse calf serum (Hyclone Laboratories), 5 μg/ml insulin, 5 μg/ml dexamethasone, 100 μg/ml streptomycin, and 100 units/ml penicillin. The ψ-CRIP retrovirus packaging cell line (50) was maintained in Dulbecco’s modified Eagle’s medium containing 10% calf serum, 100 μg/ml streptomycin, and 100 units/ml penicillin.

The MFG retroviral vectors containing EGF-ctF or EGF-hcF were co-transfected into ψ-CRIP cells with pmCIneo (Stratagene) expressing the G418 resistance gene as described previously (48). Transfected ψ-CRIP cells were selected in the presence of 600 μg/ml G418 for 2 weeks, and then infected for cells producing retrovirus. Supernatant from these cells was added to MFG packaging cells producing the high-titer viral titers was used to transduce HB2 cells for 4 h in the presence of 4 μg/ml Polybrene for 4 h. EGF expression level was determined by staining transduced HB2 cells with antibodies to human EGFR, then stained with fluorescein isothiocyanate goat anti-mouse IgG conjugate. After phasing at clonal density, stable clones were isolated and screened by immunofluorescence microscopy as described below.

**Immunofluorescence Microscopy**—Cells were plated on fibronectin coated coverslips at a 1:20 dilution. After 2 days, cells were fixed in freshly made 3.6% paraformaldehyde and 0.024% saponin for 10 min. Cells were then incubated with 1 μg/ml anti-EGF monoclonal antibody Z-12 and 5 μg/ml anti-FLAG monoclonal antibody M2 for 1 h, and stained with fluorescein isothiocyanate goat anti-mouse IgG conjugate (1:100) and Texas Red goat anti-rabbit IgG conjugate (1:200) for 45 min. The coverslips were mounted on slides in Prolong antifade medium (Molecular Probes, Inc.) and viewed with a Nikon inverted fluorescence microscope. Images were captured using a Photometrics cooled CCD camera with a Macintosh workstation running Openlab software (Improvision, Inc., Boston, MA).

**Internalization of Cell Surface Membrane-bounded EGF Ligands**—Cells were plated on coverslips at a 1:20 dilution and grown for 2 days. Surface EGF ligand was labeled by incubating the cells with anti-EGF antibody Z-12 (2 μg/ml) in phosphate-buffered saline on ice for 1 h. Unbound anti-EGF antibody Z-12 was removed by washing cells three times with ice-cold phosphate-buffered saline. Labeled cells were incubated in standard Dulbecco’s modified Eagle’s medium at 37 °C for 1 h to allow the surface proteins to be internalized. Cells were then fixed in...
3.6% paraformaldehyde containing 0.024% saponin for 10 min and stained with Texas Red goat anti-rabbit IgG conjugate for 45 min. The coverslips were mounted on slides, and images were taken as described above.

As an alternative method for measuring ligand internalization, we used $^{125}$I-labeled anti-EGF monoclonal antibody HA. Cells were plated into 35-mm dishes and grown to near confluence. 1 mg/ml $^{125}$I-labeled anti-EGF antibody HA was incubated with cells on ice for 3 h to label cell surface EGF ligands. Cells were washed with ice-cold phosphate-buffered saline three times and incubated in HEPES-buffered medium containing 1% bovine serum albumin at 37 °C for 1 h. Cells were then washed again with ice-cold phosphate-buffered saline three times. Cell surface-bound HA was collected by incubating cells with a stripping buffer (50 mM glycine-HCl, pH 3.0, 2 mM urea, 100 mM NaCl, 1 mg/ml polyanlypyrrolidone) on ice for 5 min (52). Internalized HA was extracted with 2% SDS. The radioactive counts were measured using a $\gamma$-counter. The ratio of inside to surface radioactivity was calculated to indicate the fraction of internalized ligand.

**Treatments of Cells with Pharmacological Agents**—Cells were plated into 6-well dishes and grown to near confluence. Twenty mg/ml anti-EGFR mAb 225 was incubated with cells for at least 45 min before each experiment to block EGF binding to EGFR. One ml of serum-free medium containing 20 mg/ml 225 antibody was added to each well in the absence or presence of 1 mM PMA or A23187, or 25 mM calmidazolium chloride. After 1 h incubation at 37 °C, the medium was harvested and EGF concentration was determined using an EGF ELISA as described previously (47). Cells in each well were counted and EGF concentration was normalized to picograms per million cells. To test the effect of phenylarsine oxide, cells were treated with 0 or 10 mM phenylarsine oxide in phosphate-buffered saline for 5 min before adding 1 ml of 225 antibody-containing medium. For the metalloprotease inhibitor studies, the indicated concentrations of batimastat or BB-2116 were used in combination with control medium, medium containing PMA, A23187, or calmidazolium chloride, or following phenylarsine oxide treatment.

**Cell Surface Biotinylation, Immunoprecipitation, and Western Blot Analysis**—Cells were plated in 60-mm dishes and grown to near confluence. Cells were washed three times with ice-cold phosphate-buffered saline and biotinylated with 0.5 mg/ml NHS-LC-Biotin (Pierce) for 30 min on ice. The reaction was quenched by incubating cells in 0.1 M glycine in phosphate-buffered saline for 10 min. Cells were washed three times with phosphate-buffered saline. Then cells were incubated at 37 °C in standard Dulbecco's modified Eagle's medium containing 1 mM A23187 for different lengths of time. At the indicated time points, cells were lysed in Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris buffer, pH 8.0) supplemented with 10 mM each of chymostatin, leupeptin, aprotinin, pepstatin, and 4 mM iodoacetate. Equal amounts of protein were immunoprecipitated with 2 mg/ml anti-EGF Z-12 at 4 °C overnight, followed by protein A-Sepharose for 2.5 h. Immunoprecipitates were washed three times with a high salt buffer containing 300 mM NaCl. Immunoprecipitates were then solubilized in SDS-electrophoresis sample buffer, separated on a 10–15% gradient SDS gel and transferred to a nitrocellulose membrane (Bio-Rad). The membrane was probed with streptavidin-horseradish peroxidase conjugate. The Renaissance enhanced luminol detection kit was used to develop the blot on films. The densities of bands on the films were analyzed using a densitometer (Bio-Rad). The membrane was probed with streptavidin-horseradish peroxidase conjugate. The Renaissance enhanced luminol detection kit was used to develop the blot on films. The densities of bands on the films were analyzed using a densitometer (Bio-Rad).

**RESULTS**

**Construction and Expression of Engineered EGFR Ligands**—To study the regulatory contribution of the membrane-anchoring regions to the trafficking of EGF and HB-EGF, we constructed two artificial ligands (Fig. 1). Each ligand contained the mature receptor-binding domain of EGF. One ligand, EGF-ct, is a truncated ligand in that it lacks the prepro domain of both ligands was indistinguishable. Both were found at the cell surface and at the edges of spreading cells. The FLAG epitope displayed the identical distribution in cells expressing EGF-ctF.

We next compared the rate of release of EGF-ct and EGF-ctF in a number of independently isolated clonal lines. The amount of EGF released into the medium was quantified by ELISA (47). Similarly, cell-associated EGF was measured by ELISA following detergent extraction. As shown in Fig. 2B, the range of ligand expression was similar for cells expressing either EGF-ct or EGF-ctF. Although the absolute level of ligand expressed by different clones varied almost 10-fold (compare clones ctF2 and ct20), there was no significant difference in the percentage of cell-associated ligand that was released during the 1-h incubation. Thus, the addition of a FLAG epitope does not appear to affect either transport or release of our artificial EGF ligand. Furthermore, our observation that a constant fraction of EGF is released at all expression levels suggests that we are not saturating the ligand proteolysis system.

**Distribution and Trafficking of EGF-ctF and EGF-hcF Chimeras Are Distinct**—We next compared the distribution of our engineered EGF ligands by immunofluorescence. Cells expressing similar levels of either EGF-ctF or EGF-hcF were fixed, permeabilized, and stained using antibodies against EGF and the FLAG epitope. As shown in Fig. 2A, the distribution of the EGF domain of both ligands was indistinguishable. Both were found at the cell surface and at the edges of spreading cells. The FLAG epitope displayed the identical distribution in cells expressing EGF-ctF.

We next compared the rate of release of EGF-ct and EGF-ctF in a number of independently isolated clonal lines. The amount of EGF released into the medium was quantified by ELISA (47). Similarly, cell-associated EGF was measured by ELISA following detergent extraction. As shown in Fig. 2B, the range of ligand expression was similar for cells expressing either EGF-ct or EGF-ctF. Although the absolute level of ligand expressed by different clones varied almost 10-fold (compare clones ctF2 and ct20), there was no significant difference in the percentage of cell-associated ligand that was released during the 1-h incubation. Thus, the addition of a FLAG epitope does not appear to affect either transport or release of our artificial EGF ligand. Furthermore, our observation that a constant fraction of EGF is released at all expression levels suggests that we are not saturating the ligand proteolysis system.

**Distribution and Trafficking of EGF-ctF and EGF-hcF Chimeras Are Distinct**—We next compared the distribution of our engineered EGF ligands by immunofluorescence. Cells expressing similar levels of either EGF-ctF or EGF-hcF were fixed, permeabilized, and stained using an anti-EGF antibody. As shown in Fig. 3, the pattern of EGF staining was different between cells expressing EGF-ctF and EGF-hcF. EGF-ctF was primarily confined to the cell surface and the junctions between the cells, whereas EGF-hcF was found both at the cell surface and in a collection of punctuate spots which appeared to be intracellular vesicles. Their intracellular localization was confirmed both by confocal microscopy and by the necessity for
membrane permeabilization to detect them (data not shown). These observations are consistent with previous reports that HB-EGF undergoes endocytosis (43, 53).

To determine whether EGF-hcF was internalized significantly faster than EGF-ctF, cell surface ligands were first tagged by incubating them on ice for 1 h with a primary anti-EGF antibody. The cells were then warmed to 37 °C for 1 h to allow for endocytosis of the ligand-bound antibody. The cells were fixed and permeabilized and the subcellular location of the antibody was then determined by staining with a fluorescein-conjugated secondary antibody (54). As shown in Fig. 4A, antibody bound to EGF-ctF was primarily confined to the cell surface and the margin of the cells, whereas a large fraction of the antibody prebound to EGF-hcF was found in intracellular vesicles. This suggests that EGF-hcF undergoes endocytosis while EGF-ctF is primarily confined to the cell surface.

To quantify the difference in endocytosis between EGF-ctF and EGF-hcF, we used an 125I-labeled anti-EGF mAb to label cell surface EGF-ctF and EGF-hcF at 0 °C. Following a 1-h incubation at 37 °C, surface-associated antibody was collected by acid stripping. Internalized antibody was collected following cell lysis. The ratio of internalized and surface radioactivity was then calculated. As shown in Fig. 4B, about 50% of surface-labeled EGF-hcF was found inside the cells within 1 h, while only about 20% of EGF-ctF was internal. This indicates either that EGF-hcF is internalized more rapidly than EGF-ctF or that it is recycled more slowly back to the cell surface. The fraction of ligand released per hour is less than the fraction internalized (Fig. 2B), indicating that internalization is faster than ligand release.

**FIG. 2.** Distribution and release of EGF-ct and EGF-ctF are indistinguishable. A, cells were stained with polyclonal rabbit anti-EGF antibody (Z-12) and monoclonal mouse anti-FLAG antibody (M-2), followed by Texas Red-conjugated goat anti-rabbit (left panels) and fluorescein-conjugated goat anti-mouse antibodies (right panels). Camera exposure time for each secondary antibody was constant. B, the clonal cell lines indicated were incubated with 20 μg/ml anti-EGFR 225 for 1 h. The conditioned medium and cell lysates were analyzed using the EGF ELISA. EGF concentration was normalized to picograms per million cells. Dark bars represent the amount of soluble ligand. Light bars represent the total ligand extracted from the cells. Percentage values represent the fraction of total ligand that was released into the medium.

The Membrane-anchoring Domain Specifies Regulation of Ligand Release—To determine how the membrane-anchoring domains of our ligands regulate their release, we compared the secretion rate of EGF from cells expressing EGF-ctF and EGF-hcF. We used an EGF ELISA and included antagonistic anti-EGFR mAb 225 in the medium to block the uptake of released EGF by the endogenous EGFR. The release rate was expressed as the percentage of total cellular EGF that was released into the medium per hour. Four clones of each type were examined. As shown in Fig. 5, the rate of constitutive release of EGF-ctF was between 9 and 13% per hour. This was much higher than the range of 1–3% per hour observed for cell expressing EGF-hcF. These data indicate that the membrane-anchoring domain can influence the constitutive rate of ligand release.

It is known that pharmacological agents, such as PMA (33, 35), can stimulate the release of several EGFR ligands. However, it has been reported that the release of other ligands, such as EGF and betacellulin, is not affected by PMA treatment (22, 55). To determine whether the membrane-anchoring domains of EGFR ligands confer specificity in stimulated ligand release, we measured the release rates of EGF from cells expressing...
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EGF-ctF and EGF-hcF in response to a variety of agents. Presented in Table I is a summary of this survey. We found calcium ionophores, such as A23187, and calmodulin antagonists, such as calmidazolium chloride, to be potent stimulators of both EGF-ctF and EGF-hcF release. Tyrosine phosphatase inhibitors, such as phenylarsilne oxide, were also potent stimulators of EGF release. A variety of other agents, such as elevators of intracellular cAMP, were without effect. Significantly, PMA stimulated the release of EGF-hcF, but had little effect on EGF-ctF release. These data indicate that there are both general and specific mechanisms that regulate the release of different EGF ligands.

The ability of different agents to stimulate release of EGF-ctF versus EGF-hcF was examined in more detail, as shown in Fig. 6. Following addition of PMA, the extracellular level of EGF-hcF was greatly increased, by more than 7-fold (Fig. 6A). In contrast, PMA had little effect on EGF-ctF release. Addition of the specific PKC inhibitor GF109203 to the medium abrogated PMA-induced effects (Fig. 6A, dark bar), confirming that the PMA effect was due to activation of PKC.

The calcium ionophore A23187 displayed a similar effect on the release of both EGF-ctF and EGF-hcF (Fig. 6B). This effect was dependent on extracellular calcium because 2 mM EGTA blocked the effect of A23187 (Fig. 6B, dark bar). In contrast, EGTA had no effect on PMA-stimulated release (data not shown). The CaM kinase II inhibitor KN-62 at 20 μM also selectively inhibited the stimulatory effects of A23187, suggesting the involvement of calmodulin in its action (data not shown). Paradoxically, the calmodulin inhibitor calmidazolium chloride was also a potent stimulator of EGF-ctF and EGF-hcF release (Fig. 6C). This is similar to its reported effects on 1-selectin release (56). Calmidazolium chloride and A23187 may work through a common calcium-dependent pathway because EGTA or KN-62 could inhibit the action of both (data not shown). The tyrosine phosphatase inhibitor phenylarsilne oxide was also an effective stimulator of both EGF-ctF and EGF-hcF release (Fig. 6D). It probably works through a pathway distinct from either PKC or calcium ionophores since its actions were not inhibited by the presence of either GF109203 or EGTA (data not shown).

We verified that calcium mobilization agents caused an increase in the rate of release of membrane-bound EGF from cells rather than simply increasing ligand secretion. The surface of cells expressing either EGF-ctF or EGF-hcF was biotinylated and then the cells were treated either with or without A23187 for different lengths of time. The cells were then extracted, the ligands were immunoprecipitated and separated by electrophoresis, and the resultant Western blots were probed with strepavidin-horseradish peroxidase. As shown in Fig. 7, the t½ of biotinylated EGF-ctF in untreated cells was greater than 4 h. This decreased to less than 30 min following A23187 addition. Similarly, the t½ of EGF-hcF decreased from 2 h to 30 min following A23187 treatment. These data show that calcium ionophores greatly accelerate the loss of EGF ligands from the cell surface, most likely due to increased ligand proteolysis.

Release of EGF-ctF and EGF-hcF Display Different Sensitivities to Metalloprotease Inhibitors—Previous studies have suggested metalloproteases may be responsible for the release of EGF and HB-EGF (18, 22, 53). We measured the release of EGF from cells expressing either EGF-ctF or EGF-hcF in the absence or presence of the metalloprotease inhibitor, batimastat. The experiments were done in either the presence or ab-

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**Table I**

| Compound               | μM | EGF-ctF | EGF-hcF |
|------------------------|----|---------|---------|
| Phorbol myristate acetate | 1.0 | ++     | +       |
| A23187                 | 1.0 | ++++   | ++++    |
| Calmidazolium          | 25  | +++    | +++     |
| Thapsigargin chloride  | 2.5 | +      | +       |
| Phenylarsilne oxide    | 10  | ++     | ++      |
| Dilutyril cAMP         | 10,000 | -     | -       |
| Forskolin              | 1.0 | -      | -       |
| Cholera toxin          | 0.0012 | -    | -       |
| Isoproterenol          | 10  | -      | -       |
| Insulin                | 1.7 | -      | -       |
| Estrogenα              | 18  | +/-    | +/-     |
| Progesteroneβ          | 16  | +/-    | +/-     |
| Dexamethasone          | 13  | -      | -       |
| TGFα                   | 0.02 | -     | -       |

α Cells were treated overnight with these steroids. The effects on ligand release were variable.
were biotinylated and incubated with or without 1 μM release from EGF-hcF, with a had the most potent inhibitory effect on PMA-induced EGF EGF-ctF or EGF-hcF in a dose-dependent manner. BB-2116 inhibited EGF release from cells expressing either combination with either PMA or A23187. As shown in Fig. 8, tors, we treated cells with different concentrations of the water- hcF than EGF release. This could indicate that metallopro- teases that differ in their sensitivities to batimastat are

sence of stimulators of EGF release. As shown in Table II, we found that batimastat at 5 μM inhibited both constitutive and induced EGF release from cells expressing either EGF-ctF or EGF-hcF. There was little difference in the degree of inhibition observed between different stimulators of EGF release. However, batimastat appeared more effective at inhibiting EGF- hcF than EGF-ctF release. This could indicate that metallopro- teases that differ in their sensitivities to batimastat are responsible for releasing the two ligands.

To further explore whether the release of EGF-ctF and EGF- hcF displayed different sensitivities to metalloprotease inhibi- tors, we treated cells with different concentrations of the water- soluble hydroxylzmate metalloprotease inhibitor, BB-2116, in combination with either PMA or A23187. As shown in Fig. 8, BB-2116 inhibited EGF release from cells expressing either EGF-ctF or EGF-hcF in a dose-dependent manner. BB-2116 had the most potent inhibitory effect on PMA-induced EGF release from EGF-hcF, with a K inhibit value of 0.01 μM. The K inhibit values for A23187-induced EGF release from EGF-hcF and EGF-ctF were about 0.1 and 1 μM, respectively. Inhibition of constitutive EGF release from cells expressing EGF-ctF was less, similar to the effect of batimastat on these cells (Table II). The different dose dependence of BB-2116 on the release of EGF-ctF versus EGF-hcF (>10-fold) suggests that the release of the two artificial ligands are mediated by different metalloproteases.

### DISCUSSION

Many cells, such as epithelial cells, express multiple EGFR ligands, but process each differently (57, 58). For example, in polarized Madin-Darby canine kidney cells, TGFα is specific- cally transported to the basolateral surface and released (40). In contrast, EGF is transported to both cell surfaces but is cleaved rapidly only at the basolateral side (22). The structural basis of specific ligand localization and processing is unknown, but it presumably involves the membrane-anchoring domain. To test this idea, we used a ligand chimera strategy employing two members of the EGFR ligand family, EGF and HB-EGF. The membrane-anchoring domain of this pair of ligands was chosen for our initial studies because these ligands have been reported to display the most disparate behavior and are structural- ly the most dissimilar (12, 22, 35, 59). We found that the behavior of EGF-ctF and EGF-hcF was similar to the reported behavior of the parent ligand contributing the membrane-anchoring domain, but was different between each other. This indicates that differences in transport and processing of EGFR ligands are primarily due to the structure of their membrane-anchoring regions.

We used mammary epithelial cells for these studies because this cell type produces several EGFR ligands in an autocrine fashion (17). Thus they have an active, regulated ligand processing system. Surprisingly, the net rate of ligand processing in the case of either EGF-ctF or EGF-hcF was only sufficient to release a minor fraction of total cell surface ligand. The relatively low release rates were not due to either ligand overex- pression or to the presence of an epitope tag on the ligands (Fig. 2B). In the absence of cell stimulation, the t sub- half of surface-associated EGF-ctF was >4 h. Most of this loss, approximately 10%
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Fig. 8. Inhibition of EGF release as a function of metalloprotease inhibitor concentration. Cells expressing either EGF-hcF (panel A) or EGF-ctF (panel B) were incubated with the indicated concentration of the inhibitor, BB-2116, under constitutive (open circle), PMA-induced (closed circle), or A23187-induced conditions (triangle). Cells were incubated with BB-2116 in the absence or presence of 1 µM PMA or A23187 for 1 h. Medium was collected and EGF concentration was determined by ELISA.

per hour, could be accounted for by constitutive release into the medium. In contrast, EGF-hcF displayed a much lower level of constitutive release (approximately 1–3% per hour), but a more rapid turnover from the cell surface (t½ of approximately 2 h). This was probably due to internalization (and presumed lysosomal degradation) of EGF-hcF. Under conditions of maximal stimulation, however, cell surface EGF-ctF or EGF-hcF was lost much slower than the capacity of the EGFR to internalize the ligand (30 versus 5 min, respectively (52)). Thus it appears that EGFR ligands are released at a sufficiently slow rate to allow efficient capture by cell surface EGFR. Because of this, the degree of receptor occupancy is controlled by the activity of the ligand-releasing enzymes.

We found that a large fraction of EGF-hcF was found in intracellular vesicles, while EGF-ctF was mostly associated with the cell surface. This could be due either to a higher internalization rate for EGF-hcF or a slower rate of recycling. Studies are in progress to distinguish between these two possibilities. Nevertheless, the different cellular distribution of EGF-hcF and EGF-ctF does show that the membrane-anchoring region of EGFR ligands can dictate their trafficking.

We found that EGF-ctF was released constitutively at a higher rate than EGF-hcF. Since the amino acid sequences surrounding the cleavage sites of EGF and HB-EGF are distinct, different proteases may be responsible for releasing the two ligands. Previous studies have shown that when the juxtamembrane domain of TGFA is transferred to non-cleavable membrane proteins, it can render them susceptible to proteolysis, suggesting that sequences in this region can specify proteolysis (60). Alternately, EGF-ctF and EGF-hcF may have access to different proteolytic cleavage systems, due to their specific distribution at the cell surface. In addition, because ligand release depends on both proteolytic activity and substrate availability, the smaller fraction of EGF-hcF on the cell surface may contribute to its lower constitutive release rate.

Phorbol esters, calcium ionophores, calmodulin antagonists, and tyrosine phosphatase inhibitors have all been reported to stimulate the release of several cell surface proteins, including TGFA, HB-EGF, amphiregulin, l-selectin, and HER4 (20, 21, 33, 35, 38, 39). We compared the effects of these agents on EGF-ctF and EGF-hcF release and found that PMA selectively induced EGF release from EGF-hcF. The other agonists, in contrast, stimulated EGF release from cells expressing either EGF-ctF or EGF-hcF. Our results indicate that although the PMA effect is dependent on the membrane-anchoring region of EGF-hcF, the other agonists have a more general effect on ligand release. The mechanisms by which calcium influx and tyrosine phosphatase inhibitors can regulate the release of both EGF-ctF and EGF-hcF are unclear, but they could involve a general stimulation of the cell surface proteolysis system. Calmodulin has been reported to inhibit l-selectin release by directly binding to the cytoplasmic tail of l-selectin (56). However, neither EGF nor HB-EGF contain potential calmodulin-binding sequences in their cytoplasmic domains. In addition, we could detect no specific interactions between calmodulin and EGF-ctF or EGF-hcF in our cells. Although additional studies are required to elucidate how these regulated processes work on a mechanistic level, our data do demonstrate that multiple signal transduction pathways can regulate the release of EGFR ligands. In particular, the PKC pathway appears to be specific for a subset of membrane-anchored EGFR ligands.

Our results confirm that hydroxamic acid-based metalloprotease inhibitors, such as batimastat, can reduce EGF ligand release (21, 22), although they appear more potent in inhibiting EGF-hcF rather than EGF-ctF release. This suggests that different metalloproteases could participate in the release of EGF versus HB-EGF. The selectivity of PMA in stimulating the release of EGF-hcF, but not EGF-ctF, is consistent with this idea. Specificity may be dictated by the predicted cleavage sites as well as the membrane anchoring domains, which may be recognized by different proteases. However, in addition to the cleavage site, other factors can also determine the specificity of ligand release in vivo. For example, the structure of the juxtamembrane domain may influence accessibility to the protease. Binding between other parts of the ligand and the protease may also contribute to the cleavage process. In addition, colocalization of the substrate and protease at the cell surface is necessary for processing. Because inhibitors had a differential effect on constitutive, PMA-induced and A23187-induced ligand release, this indicates that phorbol esters and ionophores are regulating different components in the ligand release pathway. Of particular interest, PMA-induced EGF-hcF release was extremely sensitive to BB-2116, suggesting that inhibitors can be developed to selectively inhibit the proteolytic processing of particular ligands.

Several studies have shown that blocking the release of some EGFR ligands can inhibit EGFR signaling and EGFR-dependent functions (17, 18). In addition, removal of the membrane-anchoring domain of EGFR results in intracrine EGFR signaling (47) whereas the soluble and membrane-bound forms of HB-EGF display distinct activities in regulating cell growth and apoptosis (15, 16). These previous studies show that the activity of the EGFR system can be regulated at the level of ligand distribution and release. Our current study indicates that the release of EGFR ligands from the cell surface is a slow process, and that under normal physiological conditions, behaves as a

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2 J. Dong and H. S. Wiley, unpublished observations.
pseudo-first order reaction. This means that ligand release can be effectively regulated by either altering metalloprotease activity or access to ligands. Since there is little homology in the membrane-anchoring regions among the EGFR ligand precursors, each ligand may be regulated independently. Understanding the differences between EGFR ligands and the conditions under which they are selectively released will be helpful in understanding the roles played by EGFR signaling pathway in physiological and pathological conditions.

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