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# Extracellular Calcium Influx Stimulates Metalloproteinase Cleavage and Secretion of Heparin-Binding EGF-Like Growth Factor Independently of Protein Kinase C

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**Abstract** The phorbol ester, tetradecanoyl-phorbol 13-acetate (TPA), stimulates rapid proteolytic processing of the transmembrane, pro- form of heparin-binding epidermal growth factor-like growth factor (HB-EGF) at cell surfaces, suggesting the involvement of protein kinase C (PKC) isoforms in the HB-EGF secretion mechanism. To test this possibility, we expressed a chimeric protein, consisting of proHB-EGF fused to placental alkaline phosphatase (AP) near the amino terminus of processed HB-EGF, in NbMC-2 prostate epithelial cells. The proHB-EGF-AP chimera localized to plasma membranes and functioned as a diphtheria toxin receptor. Secreted HB-EGF-AP bound to heparin and exhibited potent growth factor activity. The presence of the AP moiety allowed highly quantitative measurements of cleavage-secretion responses of proHB-EGF to extracellular stimuli. As expected, rapid secretion of HB-EGF-AP was induced in a time- and dose-dependent manner by TPA. However, this was also observed with the $Ca^{2+}$ ionophore, ionomycin, suggesting the involvement of extracellular $Ca^{2+}$ ions in the secretion mechanism. Ionomycin-induced secretion was inhibited by extracellular calcium chelation but not by the PKC inhibitors, GF109203X, staurosporine, or chelerythrine. The TPA-mediated secretion effect was inhibited by staurosporine, GF109203X, and by pretreatment with TPA, but not by calcium chelation. A small secretion response was induced by thapsigargin, which releases $Ca^{2+}$ from intracellular stores, but this was completely eliminated by extracellular calcium chelation. Ionomycin- and TPA-induced HB-EGF-AP secretion was not dependent on the presence of the proHB-EGF cytoplasmic domain and was specifically inhibited by the metalloproteinase inhibitors 1,10-phenanthroline and tissue inhibitor of metalloproteinase-1 (TIMP-1). These data demonstrate that extracellular $Ca^{2+}$ influx activates a membrane-associated metalloproteinase to process proHB-EGF by a pathway that does not require PKC. J. Cell. Biochem. 69:143–153, 1998. © 1998 Wiley-Liss, Inc.

**Key words:** HB-EGF; cleavage-secretion; PKC; ErbB1; EGF receptor; matrix metalloproteinase

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is an activating ErbB1/EGF receptor ligand and a potent fibroblast and smooth muscle cell mitogen, originally purified from macrophage-like cells [Higashiyama et al., 1991, 1992]. In recent studies, HB-EGF has been shown to be synthesized by a wide variety of cell types, including T lymphocytes [Blotnick et al., 1994], smooth muscle cells [Dluz et al., 1993; Freeman et al., 1998], uroepithelial cells [Freeman et al., 1997], and carcinoma cells originating from breast [Raab et al., 1994], liver [Inui et al., 1994], and pancreas [Kobrin et al., 1994]. HB-EGF is initially expressed as a membrane-anchored protein (proHB-EGF) from which the mature, secreted growth factor is derived by proteolytic processing [Raab et al., 1994; Goishi et al., 1995]. Like

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the other ErbB1 ligands (EGF, TGF$\alpha$, amphiregulin, betacellulin, and epiregulin), HB-EGF may be involved in tumor growth. The secreted form of HB-EGF is a potent mitogen for fibroblasts and some normal and transformed epithelial cells. In addition, enhanced expression of HB-EGF on the surface of human hepatocellular [Inui et al., 1994] and pancreatic cancer cells [Kobrin et al., 1994] suggests an autocrine, paracrine, or juxtacrine role for HB-EGF in neoplastic progression. ProHB-EGF contains a single membrane-anchoring domain and a small cytoplasmic tail domain and can associate with other cell surface proteins, including DRAP27/CD9 and the $\alpha_3\beta_1$ integrin [Nakamura et al., 1995]. ProHB-EGF is of additional interest because it has been identified as the cell-surface receptor for diphtheria toxin (DT) in DT-sensitive cells [Naglich et al., 1992]. DT mediates cell killing by binding of the holotoxin at a discrete site on the proHB-EGF ectodomain, after which the toxin-proHB-EGF complex is internalized by receptor-mediated endocytosis. Internalization allows the catalytic moiety of the toxin access to the protein synthesis machinery.

Several groups have demonstrated that treatment of adherent cells expressing proHB-EGF with the phorbol ester, tetradecanoyl-phorbol 13-acetate (TPA), results in the rapid release of biologically active HB-EGF into the surrounding medium [Temizer et al., 1992; Raab et al., 1994; Goishi et al., 1995; Freeman et al., 1997]. Proteolytic processing of the membrane-anchored form of a growth factor is potentially an important regulatory mechanism for conversion from predominantly a juxtacrine form of activity to paracrine or autocrine activity. TPA is a potent protein kinase C activator, and the fact that pharmacologic inhibition of PKC diminishes TPA-stimulated HB-EGF secretion is evidence for the involvement of one or more PKC isoforms in the proHB-EGF cleavage-secretion mechanism [Raab et al., 1994; Goishi et al., 1995]. In this study we have expressed in adherent cells a proHB-EGF-alkaline phosphatase fusion protein, and deletion mutants of this chimera, to better understand the mechanism of HB-EGF cleavage-secretion. Although we have found additional evidence for involvement of PKC in this process, we also report the first identification of a PKC-independent pathway for HB-EGF processing. This second pathway is activated by influx of extracellular $Ca^{2+}$ and, like the PKC-mediated pathway, activates a membrane-associated metalloproteinase for cleavage-secretion of membrane proHB-EGF.

## EXPERIMENTAL PROCEDURES

### Materials

12-*O*-tetradecanoyl-phorbol 13-acetate (TPA), EGTA, ionomycin, p-nitrophenyl phosphate disodium, diphtheria toxin, and AEBSF were purchased from Sigma (St. Louis, MO). Staurosporine, GF109203X, thapsigargin, phosphatidylcholine-specific phospholipase C (from *Bacillus cereus*), and phospholipase D (from *Streptomyces chromofuscus*) were purchased from Biomol (Plymouth Meeting, PA). Highly purified TIMP-1 was isolated from bovine scapular cartilage as previously described [Moses et al., 1990]. Chelerythrine chloride was purchased from LC Laboratories (Woburn, MA). Aprotinin, leupeptin, and PMSF were from Boehringer Mannheim (Indianapolis, IN). In all cases, inhibitors and activators were tested across dose ranges based on concentration values previously reported in the literature.

### Cell Culture

NbMC-2 cells [Freeman et al., 1994] and transfected NbMC-2 clonal sublines were maintained in T-medium: 4:1 DMEM and Ham's F12K supplemented with 5% fetal bovine serum (FBS; BioWhittaker, Walkersville, MD), 0.25 μg/mL, adenine, 0.24 μg/mL biotin, 5 μg/mL insulin, 13.6 pg/mL triiodothyronine, 100 μg/mL streptomycin, and 100 U/mL penicillin. Transfected cells demonstrating neomycin resistance were maintained in T-medium supplemented with 300 μg/mL active G418 (geneticin; Gibco, Grand Island, NY).

### HB-EGF-AP Fusion Proteins and Expression in NbMC-2 Cells

Details of construction of the full-length and cytoplasmic tail/membrane anchor deletion HB-EGF-AP chimeric proteins used in this study have been described [Chen et al., 1995]. In these constructs, human placental alkaline phosphatase (AP) cDNA was inserted within the hydrophilic amino-terminal extension domain of human proHB-EGF, between positions encoding Leu83 and Thr85. The cytoplasmic tail/membrane-anchor deletion construct terminates at Pro149. The tail-less fusion protein, containing the membrane anchor segment, was

derived from the full-length HB-EGF-AP construct by replacing the codon for histidine 187 of the human HB-EGF cDNA sequence with a stop codon at that position by oligo-directed mutagenesis. This resulted in a fusion protein in which the terminal amino acid is Tyr (human HB-EGF position 186). Absence of the proHB-EGF tail domain in the expressed protein was confirmed in transfected cells by immunostaining with an antibody (3100 [Chen et al., 1995]), raised against the 16 C-terminal amino acids of human proHB-EGF and shown to be specific for the proHB-EGF cytoplasmic tail. HB-EGF-AP cDNAs were cloned into the pRc/CMV plasmid (Invitrogen, Carlsbad, CA), which confers neomycin resistance, and were transfected into NbMC-2 cells by lipofection using the lipofectamine system (Gibco/BRL, Gaithersburg, MD) according to the manufacturer's directions. Stable transfectants were selected by growth in G418. Cellular expression of the fusion proteins was verified by immunoprecipitation, using an anti-HB-EGF antibody (197) [Blotnick et al., 1994] and anti-AP antibody (1801) (Medix Biotech, Custer City, CA). Secretion of intact HB-EGF-AP was further verified by precipitation of protein in conditioned medium with heparin-Separose (CL6B, Pharmacia, Piscataway, NJ), followed by Western blot analysis with a second anti-AP antibody (18-099) (Zymed, San Francisco, CA).

### Verification of Cell Surface Expression of HB-EGF-AP

Detection of HB-EGF-AP at the cell surface in transfected NbMC-2 cells was performed in two ways: (1) analysis of diphtheria toxin (DT) sensitivity by determining the effect of DT on protein synthesis rate as described [Freeman et al., 1997] (NbMC-2 cells are of rat origin and are therefore DT-insensitive); and (2) immunofluorescent localization by confocal imaging, using an anti-AP antibody (1801, Medix Biotech) as described previously [Chen et al., 1995].

### Alkaline Phosphatase (AP) Reporter Assays

Cells were plated into 24-well plates and, after reaching confluency (approximately 50,000 cells/well), were treated with the various agents in triplicate, in serum-free medium, at the concentrations and time periods indicated. Twenty-microliter samples were removed from each test well and were added to individual wells of a 96-well culture plate containing 100 μl of AP substrate solution. The final AP reaction mixture was 5 mM p-nitrophenyl phosphate disodium, 1 mM diethanolamine (pH 9.5), and 50 μM $MgCl_2$, 0.15M NaCl, 0.5M Tris, and 5 mM EDTA (pH 9.7). After incubation overnight, AP activity was measured with a microplate reader (Molecular Devices Corp., Palo Alto, CA) at 405 nm. Error bars in all figures refer to standard deviation.

### Heparin Chromatography and Determination of Mitogenic Activity

Media (200 ml) from cells transfected with HB-EGF-AP expression constructs were applied at 4°C to a heparin-Sepharose mini-column (Pharmacia LKB code: 17-0467-01), equilibrated with 0.2 M NaCl, 10 mM Tris-HCl, pH 7.4. The column was washed extensively (10 col. vol.) with this buffer and bound proteins were subsequently eluted with 5 ml 2M NaCl, 10 mM Tris-HCl, pH 7.4. The eluate was diluted 1/10 with 10 mM Tris-HCl, pH 7.4, and applied to a TSK-heparin 5PW FPLC (fast protein liquid chromatography) column (8 x 75 mm, Tosohaas, Montgomeryville, PA). Bound protein was eluted with a 40-ml linear gradient of 0.2–2 M NaCl in 10 mM Tris-HCl, pH 7.4, at a flow rate of 1 ml/min. Mitogenic activity was tested using Balb/c3T3 fibroblasts as described [Higashiyama et al., 1991, 1992].

## RESULTS

To study agonist-induced secretion of soluble HB-EGF from adherent cells, we used a series of chimeric proteins in which placental alkaline phosphatase (AP) was fused to the ectodomain of proHB-EGF (Fig. 1). The chimeras consist of the human HB-EGF precursor, with the catalytic moiety of AP fused to the hydrophilic region of the mature HB-EGF peptide. A full-length HB-EGF-AP fusion protein, an HB-EGF-AP fusion protein with the cytoplasmic tail segment deleted, and a fusion protein with both the cytoplasmic tail segment and the membrane anchor deleted were used in these experiments. All three proteins were stably expressed in NbMC-2 rat prostate epithelial cells under the control of the CMV immediate-early promoter to facilitate constitutive, high-level expression. Presence of a heat-stable AP enzyme activity in the medium was used in the selection of HB-EGF-AP-expressing clones. In AP-secreting cells chosen for further study, expression of the fusion proteins was verified by immunoprecipitation with anti-HB-EGF and anti-AP antibodies and visualization by SDS-

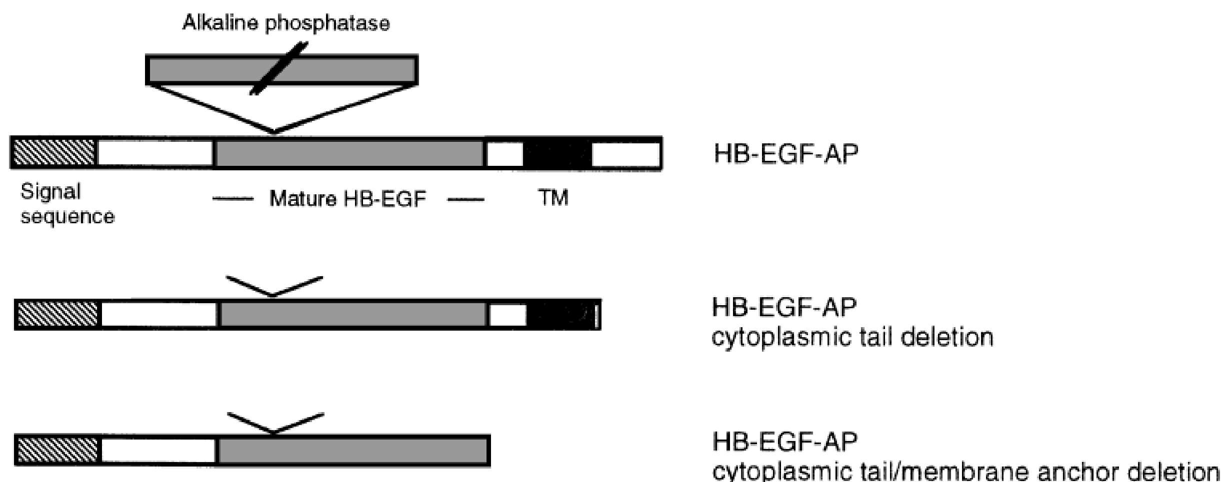


**Fig. 1.** HB-EGF-AP fusion proteins used in this study. Human placental alkaline phosphatase (AP) cDNA was inserted between positions encoding Leu83 and Thr85 of human proHB-EGF cDNA. The tail-less construct terminates at Tyr186. The membrane-anchor/cytoplasmic tail deletion construct terminates at Pro149.

PAGE (not shown). Further analysis demonstrated that HB-EGF-AP expressed in transfected cells localized to plasma membranes and conferred DT-sensitivity (not shown), indicating a subcellular localization similar to the native protein and an orientation toward the extracellular space of the DT-binding region, which resides within the EGF-like motif [Mitamura et al., 1995]. The DT-sensitivity results also indicated that the full-length and cytoplasmic tail-deleted HB-EGF-AP chimeras were capable of functioning as DT receptors similarly to the native protein, in that they were able to mediate DT internalization. In transfected cells, AP activity slowly accumulated in the medium under serum-free conditions, indicating that the secretory mechanism is cell-associated and does not involve serum proteins. Fractionation by heparin-affinity FPLC of medium conditioned by cells transfected with HB-EGF-AP demonstrated that the secreted protein bound to immobilized heparin and was eluted with 0.8 M–1 M NaCl, demonstrating an affinity for heparin similar to native HB-EGF (Fig. 2). Secreted HB-EGF-AP demonstrated potent mitogenic activity (Fig. 2A) and migrated as a single species in SDS-PAGE gels (Fig. 2A, inset). These data strongly suggest that an authentic cleavage-secretion mechanism is functioning in this system.

### Secretion of HB-EGF by PKC Activation

The enzymatic activity of the AP tag was used in subsequent experiments as a quantitative measure of the secretion of HB-EGF into the medium following the challenge of transfected cells with potential secretion agonists and inhibitors. Protein kinase C (PKC) phosphorylates a large group of proteins involved in intracellular signaling pathways and has been implicated in regulated cell-surface shedding of HB-EGF. To verify that the full-length HB-EGF-AP chimera behaved similarly to the native protein with respect to induced secretion by PKC activation, HB-EGF-AP-expressing cells were treated with varying concentrations of the phorbol ester, tetradecanoyl-phorbol 13-acetate (TPA), a PKC activator. TPA treatment induced a dose- and time-dependent increase in AP activity in the medium, with a corresponding loss of AP activity in the cell lysate (Fig. 3A–C). This observation is consistent with previous demonstrations of HB-EGF secretion evoked by TPA treatment, and it suggests the involvement of PKC in this process. To verify this interpretation, we examined the effects of the non-specific PKC inhibitors staurosporine, and the specific PKC inhibitors GF109203X and chelerythrine, on TPA-induced HB-EGF-AP secretion. In the presence of staurosporine and GF109203X, TPA-stimulated secretion of AP activity was reduced nearly to baseline levels (Fig. 3D). In contrast, at concentrations reported to be specific for PKC (0.66 μM to 4 μM [Herbert et al., 1990]), chelerythrine had no inhibitory effect.

The inhibitory activity of chelerythrine for all PKC isoforms has not yet been documented, and it is possible that chelerythrine may not inhibit all PKCs that respond to phorbol ester

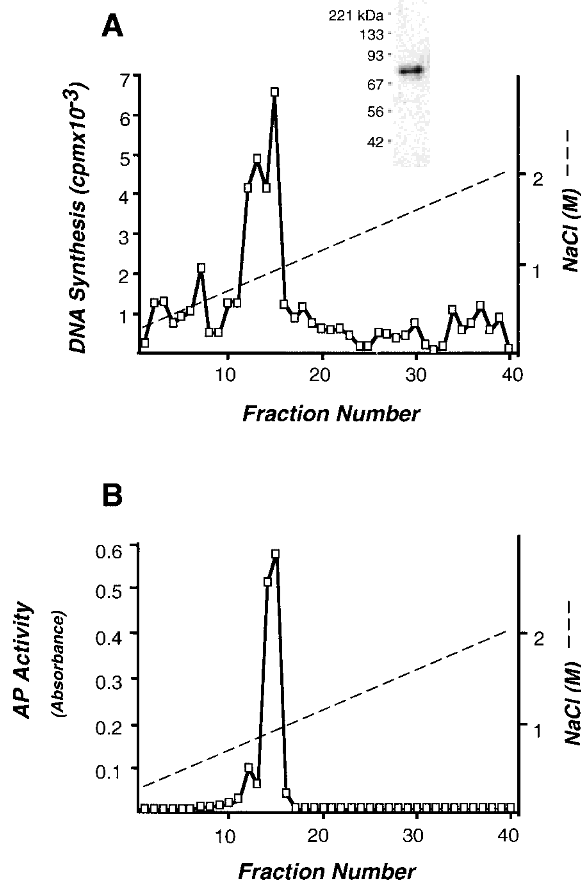


**Fig. 2.** Elution profile from heparin-affinity FPLC of medium conditioned by cells expressing the full-length HB-EGF-AP fusion protein. **A:** Secreted HB-EGF-AP is a mitogen for Balb/c3T3 cells. **B:** Functionally active alkaline phosphatase (AP). The inset in A shows the processed HB-EGF-AP protein, precipitated with heparin-Sepharose and identified by Western blot using an anti-AP antibody. These data indicate that processed HB-EGF-AP is a functional growth factor and exhibits an affinity for heparin that is similar to the native protein.

activation. Therefore, two additional tests were performed to explore the role of PKC in regulated HB-EGF secretion. Prolonged exposure of cells to TPA results in the down-regulation of cellular PKC activity; thus, a loss of a response after TPA pretreatment has been used as a basis to argue for the involvement of PKC in a variety of cellular processes. TPA-inducible AP activity released into the medium was reduced by over 50% in cells pre-treated for 24 h with TPA (Fig. 3D). Since both phorbol esters and DAG bind and activate PKC in virtually the same manner, we also investigated the effects of exogenous phospholipase C (PLC) and phospholipase D (PLD), as a source for DAG, on secretion of HB-EGF. HB-EGF-AP-expressing cells were treated with PLC and PLD for 6 and 24 h. Treatment of cells with both enzymes for 6 h had no effect on release of HB-EGF-AP but cells treated for 24 h with PLC showed significant release of AP activity into the medium (Fig. 3E). The stimulatory effects of PLC were over sevenfold greater than for PLD. During receptor-mediated signaling, hydrolysis of membrane phosphatidylcholine by PLC leads directly to the generation of DAG and subsequent PKC activation while hydrolysis of phosphatidylcholine by PLD results in the formation of DAG by an indirect mechanism [Thompson et al., 1991]. These two experiments further support the involvement of PKC in the secretion mechanism; however, the failure of chelerythrine to inhibit TPA-mediated secretion, in combination with the inability of TPA pretreatment to completely inhibit secretion induced by TPA, also suggest the existence of alternative mechanisms of regulated HB-EGF secretion that do not involve the cPKC and nPKC isoforms (PKCs activatable by phorbol esters and DAG).

### Secretion of HB-EGF Mediated by Extracellular $Ca^{2+}$

Additional experiments with the calcium ionophore, ionomycin, indicated that this agent was also an HB-EGF secretion agonist of similar or greater potency than TPA. Therefore, we set out to determine the relationship of this secretory mechanism to the one stimulated by TPA. Ionomycin induced release of alkaline phosphatase activity from HB-EGF-AP-expressing cells in a dose-dependent manner (Fig. 4A). The ionomycin effect was completely blocked in medium containing the $Ca^{2+}$ chelator EGTA. These data indicate that HB-EGF secretion can be induced by the influx of $Ca^{2+}$ ions across the plasma membrane. To determine whether PKC plays a role in ionomycin-induced secretion of HB-EGF, cells were pretreated cells with the PKC inhibitors staurosporine, GF109203X, and chelerythrine prior to the addition of ionomycin. As shown in Figure 4B, the presence of these agents had no significant inhibitory effect on ionomycin-induced release of AP activity, indicating that the ionomycin secretion mechanism is PKC-independent.

We subsequently tested the effect of increased intracellular $Ca^{2+}$ alone on secretion of HB-EGF-AP. For these experiments we used thapsigargin, a specific inhibitor of the $Ca^{2+}$-

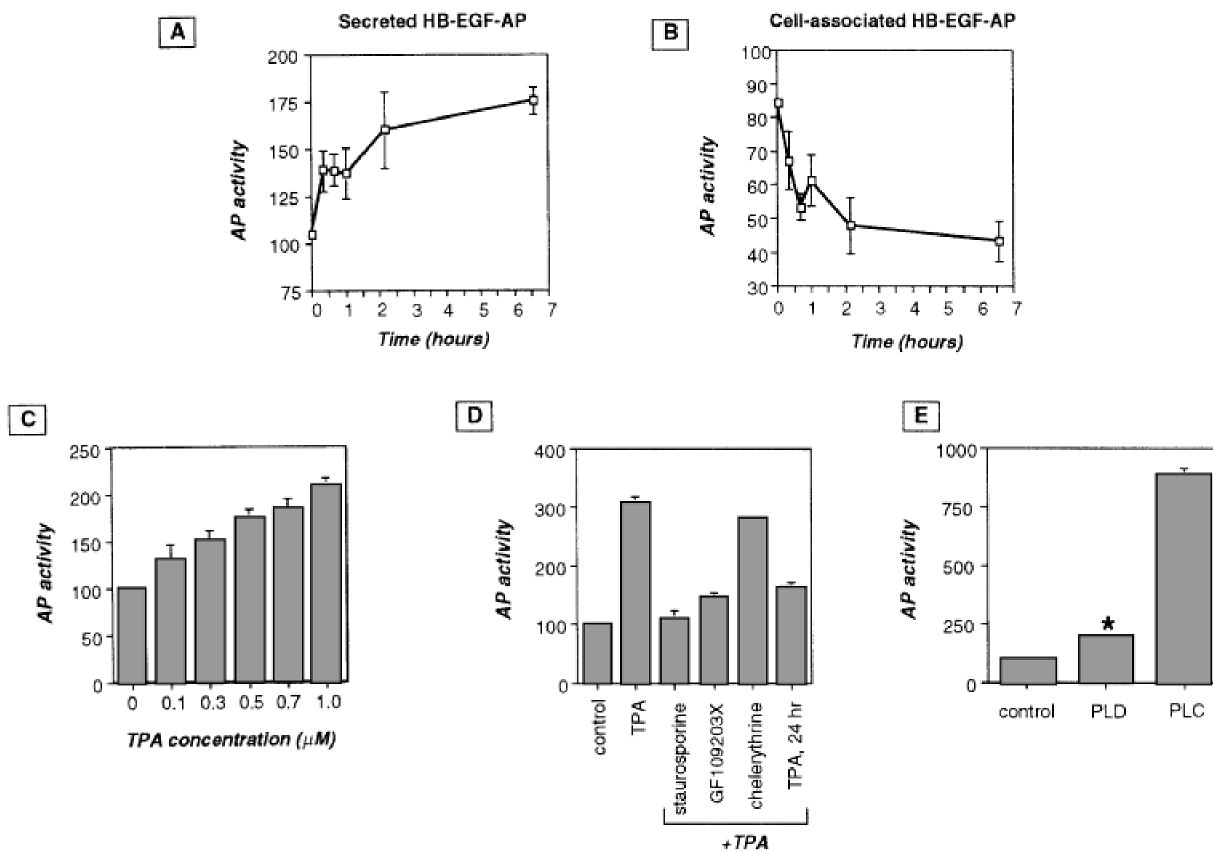


**Fig. 3.** Cleavage-secretion of HB-EGF-AP induced by activation of PKC. **A,B:** Time-dependent secretion of AP activity by HB-EGF-AP-expressing cells in response to 300 nM TPA. A indicates AP activity in the medium; B indicates AP activity in the cell lysate. All data are expressed as percent of control (unstimulated cells). **C:** Dose-dependent secretion of AP activity in response to TPA. Cells were treated with TPA in serum-free medium for 30 min prior to measurement of secreted AP activity. **D:** Effect of PKC inhibition on TPA-stimulated release of HB-EGF-AP. Treatment was with 1 μM TPA in the presence or absence of the PKC inhibitors, staurosporine (100 ng/ml), GF109203X (1 μM), and chelerythrine (0.66 μM) for 30 min. PKC activity was also down-regulated by pretreatment with 1 μM TPA for 24 h; the medium was then removed and replaced with fresh medium containing 1 μM TPA. Sampling of medium was at 1 h. **E:** Effect of exogenous PLC (1.4 U/ml) and PLD (60 U/ml) on secretion of HB-EGF-AP. Cells were treated for 24 h before medium was sampled for AP measurement. $^*P < .001$ (n = 4) vs. non-treated controls.

ATPase pump that sequesters intracellular $Ca^{2+}$. Thapsigargin induced a small but statistically significant increase in AP activity in $Ca^{2+}$-containing medium; however, this effect was not seen in medium containing EGTA (Fig. 4C). The raising of intracellular $Ca^{2+}$ levels with thapsigargin has the ancillary effect of activating $Ca^{2+}$ channels in the plasma membrane [Zitt et al., 1996]. The fact that chelation of $Ca^{2+}$ in the medium abolished the thapsigargin effect suggests the exclusive involvement of extracellular $Ca^{2+}$ in the secretory mechanism.

To determine whether extracellular $Ca^{2+}$ influx is required if TPA is used as a secretion agonist, cells were challenged with TPA in medium containing EGTA. No significant inhibitory effect on secretion was observed (Fig. 4D), indicating that TPA-induced secretion acts through an alternative mechanism that does not require $Ca^{2+}$ influx.

## Agonist-Induced Secretion of HB-EGF-AP Does Not Require the Cytoplasmic Domain of the HB-EGF Precursor

Cells expressing HB-EGF-AP chimeras with deletions of the cytoplasmic tail segment and the cytoplasmic tail plus the membrane anchor (Fig. 1) were challenged in a similar fashion with TPA and ionomycin. As anticipated, cells expressing the cytoplasmic tail/membrane-anchor deletion constitutively exported AP activity into the medium, as assayed over a 12-h period (not shown). Cells expressing only the cytoplasmic tail deletion demonstrated a simi-

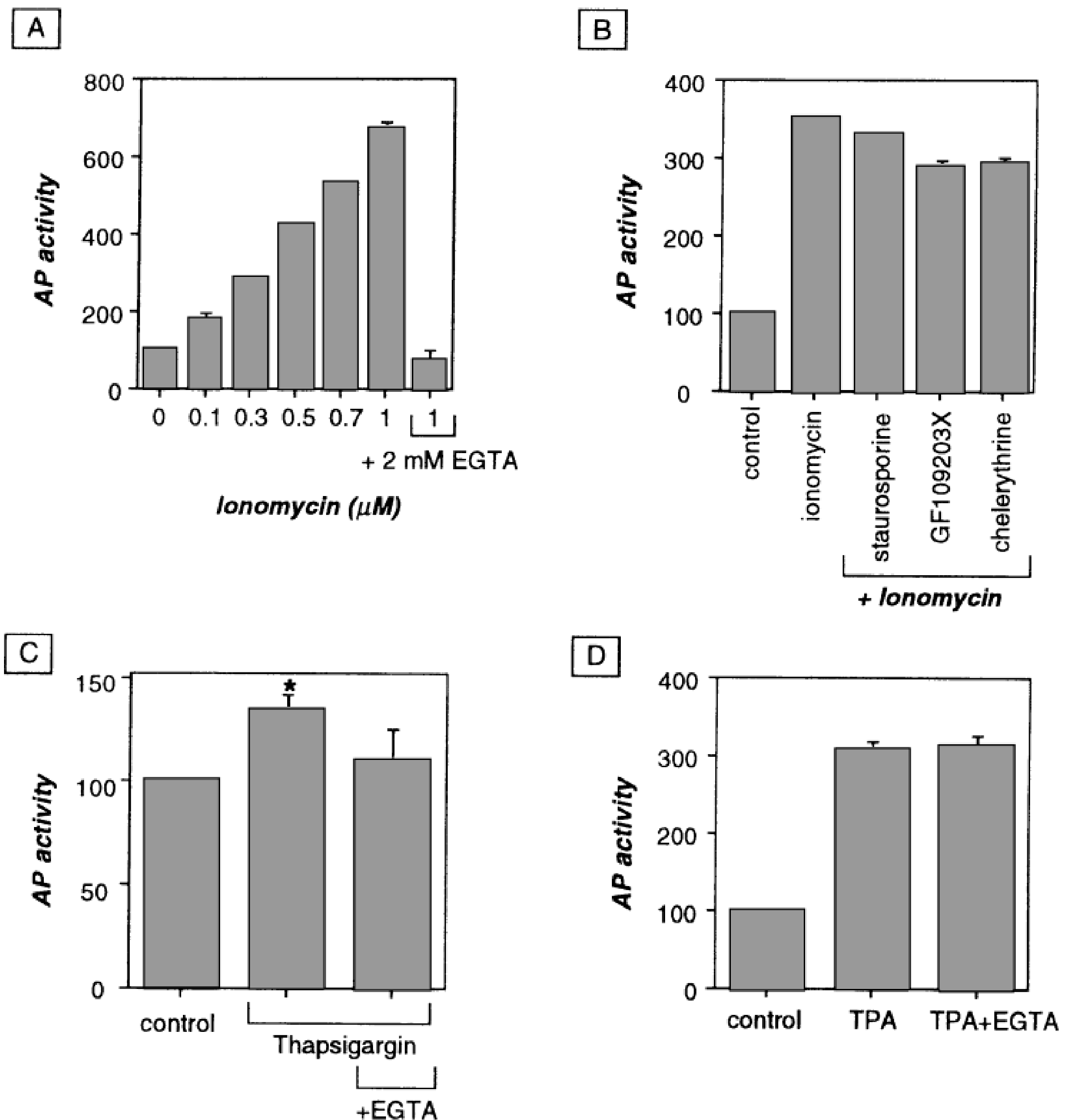


**Fig. 4.** Cleavage-secretion of HB-EGF-AP induced by influx of extracellular $Ca^{2+}$. **A:** Dose-dependent secretion of HB-EGF-AP in response to ionomycin and inhibition of secretion with the $Ca^{2+}$ chelator, EGTA (2 mM). In A,B, and C, medium was taken for AP measurement after 30 min. All data are expressed as percent of control (unstimulated cells). **B:** Cells were treated with 1 μM ionomycin in the presence of staurosporine (100 ng/ml), GF109203X (1 μM), and chelerythrine (0.66 μM). **C:** Effect of increase in intracellular calcium. Cells were treated with 10 μM thapsigargin in the presence and absence of media containing 2 mM EGTA. $^*P < .05$ (n = 4) vs. non-treated control. **D:** Effect of extracellular $Ca^{2+}$ chelation on TPA-stimulated HB-EGF-AP secretion. Medium was taken for AP measurement at 1 h after treatment with 1 mM TPA in normal medium or in medium containing 2 mM EGTA.

lar response to TPA and ionomycin as shown above using cells expressing the full-length form of the protein (Fig. 5); this indicates that the cytoplasmic tail is not required for agonist-induced secretion of HB-EGF. In contrast, ionomycin and TPA had no effect on alkaline phosphatase secretion in cells expressing the mutant containing both the cytoplasmic tail/membrane-anchor deletion. This is consistent with the interpretation that agonist-mediated stimula-

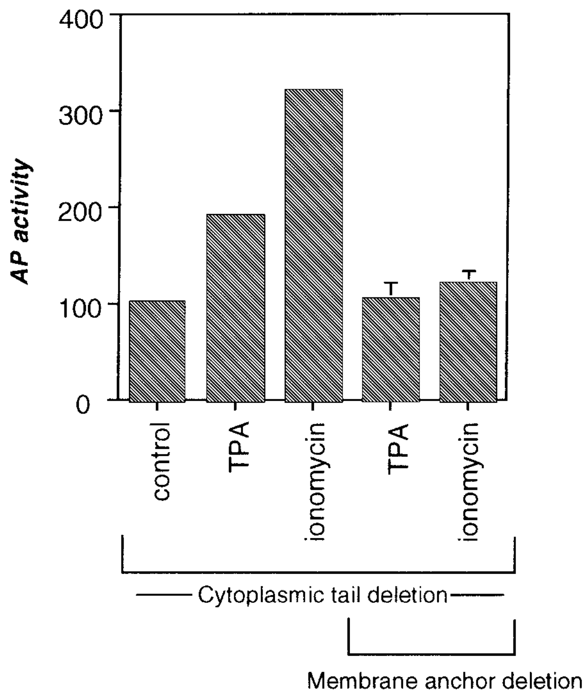


**Fig. 5.** Effect of deletion of the HB-EGF cytoplasmic tail and membrane anchor on secretion of HB-EGF-AP stimulated by 1 μM TPA and 1 μM ionomycin. Medium was sampled after 30 min. Data are expressed as percent of control (unstimulated cells).

tion of HB-EGF-AP secretion is the result of proteolytic processing of the membrane-anchored precursor.

### Inhibition of Induced HB-EGF Secretion by Metalloproteinase Inhibitors

To obtain additional evidence that this secretion mechanism involves a proteolytic cleavage event, and to attempt to obtain information on the identity of the activatable proteinase(s), we tested a panel of protease inhibitors with varying specificities for their ability to inhibit agonist-stimulated release of AP activity from HB-EGF-AP-expressing cells. Addition of the serine protease inhibitors, aprotinin and leupeptin, and the aspartic acid protease inhibitor pepstatin to the cultures had minimal or no inhibitory effect on ionomycin- and TPA-stimulated secretion of AP activity (Fig. 6). In contrast, the metalloproteinase inhibitors 1,10-phenanthroline, and purified TIMP-1 [Moses et al., 1990], significantly inhibited AP secretion induced by both ionomycin and TPA. These results strongly suggest the involvement of one or more metalloproteinases in the proteolytic cleavage of cell-surface proHB-EGF under conditions where the secretion signal originates from activation of the $Ca^{2+}$-dependent pathway or the PKC pathway.

## DISCUSSION

With the exception of TGFα, there has been very little study of mechanisms controlling the regulated cleavage-secretion of the ErbB1 ligands, all of which are initially expressed as membrane-anchored precursors. It is not known, for example, to what extent information accumulated on the mechanisms involved in TGFα cell surface processing applies to the other ErbB1 ligands expressed by mammalian cells. Both PKC-dependent and PKC-independent pathways have been proposed to regulate TGFα processing [Pandiella and Massaguè, 1991a,b; Pandiella et al., 1992].

In this study we have used chimeric proteins, in which membrane-anchored proHB-EGF was fused to a reporter enzyme, placental alkaline phosphatase (AP), to study regulated cleavage-secretion of HB-EGF. This report demonstrates

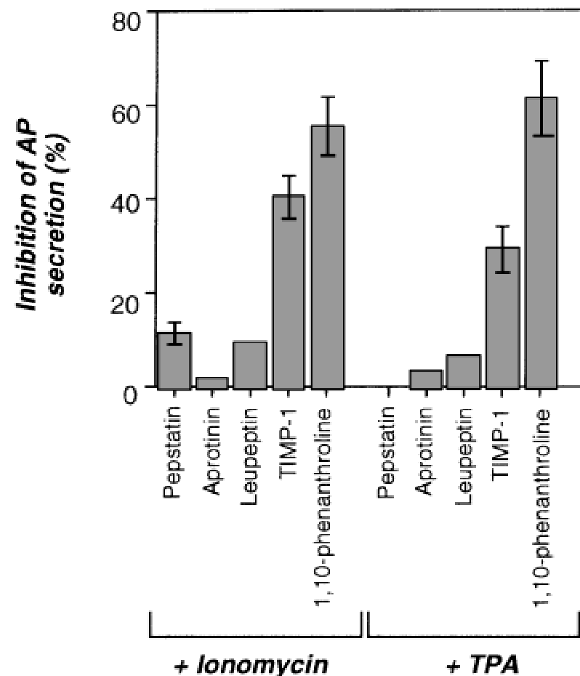


**Fig. 6.** Effect of proteinase inhibitors on the ionomycin- and TPA-stimulated secretion of HB-EGF-AP. Cells were incubated with 1 μM ionomycin or 1 μM TPA in the presence of the aspartic acid inhibitor, pepstatin (10 μM), the serine proteinase inhibitors aprotinin (6 μg/ml) and leupeptin (10 μg/ml). For the metalloproteinase inhibitors, (1) data for 0.25 mM 1,10 phenanthroline and 20 μg/ml TIMP-1 are shown for ionomycin and (2) data for 0.25 mM 1,10 phenanthroline and 50 μg/ml TIMP-1 are shown for TPA. Medium was sampled at 30 min after challenge with the secretion agonists.

the feasibility and highly quantitative nature of this approach as a means of examining mechanisms of regulated secretion of an ErbB1/EGF receptor ligand. Previous studies of TGF$\alpha$ cleavage-secretion have monitored cell surface shedding of the growth factor ectodomain in genetically manipulated cells by immunoprecipitation of processed growth factor and/or a cell-associated tail fragment . In comparison to these more traditional methods, the use of an enzyme activity to quantitatively assess the magnitude, duration, and kinetics of the secretion response allows unambiguous confirmation of relatively small effects (e.g., as little as 0.3-fold deviation can be demonstrated to be statistically significant) (Fig. 3C). With this strategy we have identified two independent pathways of regulated secretion of HB-EGF from adherent cells. One is activatable by extracellular $Ca^{2+}$ influx into the cell and is PKC-independent. The second pathway is TPA-activatable and is partly PKC-dependent and extracellular $Ca^{2+}$-independent.

The first pathway was activated by the $Ca^{2+}$ ionophore ionomycin and was inhibited completely by chelation of extracellular $Ca^{2+}$. Three potent PKC inhibitors did not block the ionomycin-induced secretion of the AP reporter activity, indicating that secretion induced by $Ca^{2+}$ influx can occur in the absence of functional PKC. Increases in intracellular free $Ca^{2+}$ concentrations above normal baseline levels (10–100 nM), to concentrations in the μM range, can activate PKC [Bootman and Berridge, 1995]. However, the lack of an effect of PKC inhibition on $Ca^{2+}$-stimulated HB-EGF-AP secretion indicates that possible downstream PKC activation is not required for functional activation of this pathway.

The second HB-EGF cleavage-secretion pathway identified was activatable by the phorbol ester, TPA. The involvement of PKC in HB-EGF release from adherent cells has been suggested previously by results demonstrating rapid conversion of cell surface proHB-EGF to the soluble form after phorbol ester treatment. Although TPA is a potent PKC agonist, stimulation by TPA is insufficient to demonstrate conclusively the involvement of PKC-dependent regulation. For example, the TPA-induced cleavage of membrane-bound angiotensin converting enzyme (ACE) from mouse ACE89 cells has been reported not to be inhibitable by several potent PKC inhibitors, including staurosporine, indicating that phorbol ester-mediated cleavage-secretion of cell surface proteins can also result from activation of PKC-independent pathways [Ramchandran et al., 1994]. We conclude here that PKC is involved in the phorbol ester-stimulated pathway regulating HB-EGF secretion. The TPA-mediated secretion response was significantly attenuated in the presence of the PKC inhibitors staurosporine and GF109203X. GF109203X is reported to be highly specific for inhibition of PKC, and both staurosporine and GF109203X are known to inhibit both $Ca^{2+}$-dependent and $Ca^{2+}$-independent PKC isoforms [Simpson et al., 1993; Martiny-Baron et al., 1993]. We were also able to attenuate the TPA response by pretreatment of the cells with TPA, consistent with the fact that prolonged phorbol ester treatment down-regulates PKC activity. Interestingly, however, a third PKC inhibitor, chelerythrine, did not significantly inhibit the secretion response at doses shown previously to be specific for several PKC isoforms. This finding, which was highly reproducible with several independent lots of the reagent and multiple clonal populations of cells, cannot presently be explained; however, differential effects between staurosporine and chelerythrine have been reported by others [Herbert et al., 1993; Zachow and Terranova, 1993; Maurer et al., 1996]. In addition, the ability of chelerythrine to inhibit PKC activity has not yet been tested for all known PKC isoforms. One possibility is that chelerythrine-insensitive PKC isoforms can mediate HB-EGF secretion and these are expressed in NbMC-2 cells. Another line of evidence consistent with the involvement of PKC is the observed effect of exogenous PLC, which was substantially more potent than exogenous PLD at stimulating HB-EGF-AP shedding. This difference is likely to be due to the fact that activation of PLC results in the direct release of DAG and subsequent PKC activation, while PLD activation results primarily in the liberation of phosphatidic acid, only some of which is converted to DAG [Fukami and Takenawa, 1992].

Our data also suggest that mobilization of $Ca^{2+}$ from intracellular stores by a PLC-mediated mechanism is insufficient for regulated HB-EGF cleavage-secretion. Treatment of HB-EGF-AP-transfected cells with thapsigargin, a sequiterpene lactone that selectively elevates intracellular $Ca^{2+}$ levels by inhibiting the $Ca^{2+}$-ATPase pumps in the endoplasmic reticulum [Thastrup et al., 1990; Ghosh et al., 1991], induced a small release of AP reporter activity. This minimal effect was blocked under condi-

tions of $Ca^{2+}$ chelation, suggesting that extracellular $Ca^{2+}$, exclusively, mediates HB-EGF cell surface shedding. Importantly, the phorbol ester-stimulated secretion pathway is not attenuated by chelation of extracellular $Ca^{2+}$, indicating that the $Ca^{2+}$-mediated mechanism is a distinct pathway.

TPA and ionomycin both induced rapid HB-EGF-AP secretion from cells expressing an HB-EGF-AP mutant with a cytoplasmic tail deletion, but not from a mutant where both the proHB-EGF cytoplasmic tail and membrane anchor were deleted. These data indicate that, unlike TGFα, where regulated secretion induced by phorbol esters apparently requires a valine residue at the carboxyl terminus [Bosenberg et al., 1992], the HB-EGF cleavage-secretion pathways activated by ionomycin and TPA do not require amino acid residues within the tail domain. This is an important example of an apparent difference in the mechanisms of cell surface processing of HB-EGF and TGFα, illustrating how conclusions from data obtained with one ErbB1 ligand might not necessarily apply to other ligands capable of activating the same receptor. It is interesting to note that the proHB-EGF tail domain is one of the most highly conserved regions of the HB-EGF protein [Abraham et al., 1993]. Further, there is significant amino acid sequence conservation between the proHB-EGF and the pro-amphiregulin tail domains, suggesting that the cytosolic region of these growth factor precursors provides an important, albeit currently unknown, function despite our data showing an absence of measurable inhibitory effects on secretion with deletion of the proHB-EGF tail segment.

The HB-EGF secretion response was significantly attenuated with the metalloproteinase inhibitor, 1,10-phenanthroline, and also by the physiologic metalloproteinase inhibitor, TIMP-1. In contrast, the serine/cysteine protease inhibitors, aprotinin and leupeptin, and the aspartic acid protease inhibitor, pepstatin, had little or no inhibitory effect when either ionomycin or TPA was used as the secretion agonist. The relative potency of the various inhibitors was similar with both agonists. Lanzrein et al. [1995] concluded that one or more metalloproteinases regulate loss of diphtheria toxin (DT) receptors from DT-sensitive Vero cells. Consistent with this interpretation, MMP-3 (stromelysin-1) has recently been identified as a metalloproteinase capable of cleaving membrane-anchored HB-EGF at a specific juxtamembrane sequence [Suzuki et al., 1997]. Our results indicate that both the extracellular $Ca^{2+}$-mediated pathway and the PKC-mediated pathway activate one or more metalloproteinases at the cell surface to process proHB-EGF to the secreted form of the growth factor.

In conclusion, we present the first direct evidence for multiple independent signals mediating cleavage-secretion of HB-EGF from adherent cells. Our observations indicate that distinct secretory pathways are likely to regulate bioavailability of the soluble forms of HB-EGF, and by extension, other ErbB ligands, in diverse physiologic settings.

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## REFERENCES

Abraham JA, Damm D, Bajardi A, Miller J, Klagsbrun M, Ezekowitz RAB (1993): Heparin-binding EGF-like growth factor: Characterization of rat and mouse cDNA clones, protein domain conservation across species, and transcript expression in tissues. Biochem Biophys Res Commun 190:125–133.

Blotnick S, Peoples GE, Freeman MR, Eberlein TJ, Klagsbrun M (1994): T lymphocytes synthesize and export heparin-binding epidermal growth factor-like grows factor and basic fibroblast growth factor mitogens for vascular cells and fibroblasts: Differential production and release by CD4+ and CD8+ T cells. Proc Natl Acad Sci USA 91:2890–2894.

Bootman MD, Berridge MJ (1995): The elemental principles of calcium signaling. Cell 83:675–678.

Bosenberg MW, Pandiella A, Massaguè J (1992): The cytoplasmic carboxy-terminal amino acid specifies cleavage of membrane TGFa into soluble growth factor. Cell 71: 1157–1165.

Chen X, Raab G, Deutsch U, Zhang J, Ezzell RM, Klagsbrun M (1995): Induction of heparin-binding EGF-like growth factor expression during myogenesis. Activation of the gene by MyoD and localization of the transmembrane form of the protein on the myotube surface. J Biol Chem 270:18285–18294.

Dluz SM, Higashiyama S, Damm D, Abraham JA, Klagsbrun M (1993): Heparin binding epidermal growth factor-like growth factor expression in cultured fetal human vascular smooth muscle cells. Induction of mRNA levels and secretion of active mitogen. J Biol Chem 268:18330–18334.

Freeman MR, Bagli DJ, Lamb CC, Guthrie PD, Uchida T, Slavin RE, Chung LWK (1994): Culture of a prostatic cell line in basement membrane gels results in an enhancement of malignant properties and constitutive alterations in gene expression. J Cell Physiol 158:325–336.

Freeman MR, Yoo JJ, Raab G, Soker S, Adam RM, Schneck FX, Renshaw A, Klagsbrun M, Atala A (1997): Heparin-binding EGF-like growth factor is an autocrine growth factor for human urothelial cells and is synthesized by epithelial and smooth muscle cells in the human bladder. J Clin Invest 99:1028–1036.

Freeman MR, Paul S, Kaefer M, Ishikawa M, Adam RM, Renshaw AA, Elenius K, Klagsbrun M (1998): Heparin-binding EGF-like growth factor in the human prostate: Synthesis predominantly by interstitial and vascular smooth muscle cells and action as a carcinoma cell mitogen. J Cell Biochem 68:328–338.

Fukami K, Takenawa T (1992): Phosphatidic acid that accumulates in platelet-derived growth factor-stimulated Balk/c 3T3 cells is a potential mitogenic signal. J Biol Chem. 267:10988–10993.

Ghosh TK, Bian J, Short AD, Rybak SL, Gill DL (1991): Persistent intracellular calcium pooly depletion by thapsigargin and its influence on cell growth. J Biol Chem 266:24690–24697.

Goishi K, Higashiyama S, Klagsbrun M, Nakano N, Umata T, Ishikawa M, Mekada E, Taniguchi N (1995): Phorbol ester induces the rapid processing of cell surface heparin-binding EGF-like growth factor: conversion from juxtacrine to paracrine growth factor activity. Mol Biol Cell 6:967–980.

Herbert JM, Augereau JM, Gleye J, Maffrand JP (1990): Chelerythrine is a potent and specific inhibitor of protein kinase C. Biochem Biophys Res Commun 172:993–999.

Higashiyama S, Abraham J, Miller J, Fiddes JC, Klagsbrun M (1991): A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. Science 251:936–-939.

Higashiyama S, Lau K, Besner GE, Abraham JA, Klagsbrun M (1992): Structure of heparin-binding EGF-like growth factor. Multiple forms, primary structure, and glycosylation of the mature protein. J Biol Chem 267: 6205–6212.

Inui Y, Higashiyama S, Kawata S, Tamura S, Miyagawa J-I, Taniguchi N, Matsuzawa Y (1994): Expression of heparin-binding epidermal growth factor in human hepatocellular carcinoma. Gastroenterology 107:1799–1804.

Kobrin MS, Funatomi H, Friess H, Buchler MW, Stathis P, Korc M (1994): Induction and expression of heparin-binding EGF-like growth factor in human pancreatic cancer. Biochem Biophys Res Commun 202:1705–1709.

Lanzrein M, Garred Ø, Olsnes S, Sandvig K (1995): Diphtheria toxin endocytosis and membrane translocation are dependent on the intact membrane-anchored receptor (HB-EGF precursor): Studies on the cell-associated receptor cleaved by a metalloprotease in phorbol-ester-treated cells. Biochem J 310:285–289.

Martiny-Baron G, Kazanietz MG, Mischak H, Blumberg PM, Kochs G, Hug H, Marmé D, Schächtele C (1993): Selective inhibition of protein kinase C isozymes by the indolocarbazole Gö 6976. J Biol Chem 268:9194–9197.

Maurer JA, Wenger BW, McKay DB (1996): Effects of protein kinase inhibitors on morphology and function of cultured bovine adrenal chromaffin cells: KN-62 inhibits secretory function by blocking stimulated $Ca^{2+}$ entry. J Neurochem 66:105–113.

Mitamura T, Higashiyama S, Taniguchi N, Klagsbrun M, Mekada E (1995): Diphtheria toxin binds to the epidermal growth factor (EGF)-like domain of human heparin-binding EGF-like growth factor/diptheria toxin receptor and inhibits specifically its mitogenic activity. J Biol Chem 270:1015–1019.

Moses MA, Sudhalter J, Langer R (1990): Identification of an inhibitor of neovascularization from cartilage. Science 248:1408–1410.

Naglich JG, Metherall JE, Russell DW, Eidels L (1992): Expression cloning of a diphtheria toxin receptor: Identity with a heparin-binding EGF-like growth factor precursor. Cell 69:1051–1061.

Nakamura K, Iwamoto R, Mekada E (1995): Membrane-anchored heparin-binding EGF-like growth factor (HB-EGF) and diphtheria toxin receptor-associated protein (DRAP27)/CD9 form a complex with integrin $\alpha 3\beta 1$ at cell-cell contact sites. J Cell Biol 129:1691–1705.

Pandiella A, Massagué J (1991a): Multiple signals activate cleavage of the membrane transforming growth factor: A precursor. J Biol Chem 266:5769–5773.

Pandiella A, Massagué J (1991b): Cleavage of the membrane precursor for transforming growth factor is a regulated process. Proc Natl Acad Sci USA 88:1726–1730.

Pandiella A, Bosenberg MW, Huang EJ, Besmer P, Massagué J (1992): Cleavage of membrane-anchored growth factors involves distinct protease activities regulated through common mechanisms. J Biol Chem 267:24028–24033.

Raab G, Higashiyama S, Hetelekidis S, Abraham JA, Damm D, Ono M, Klagsbrun M (1994): Biosynthesis and processing by phorbol ester of the cell surface-associated precursor form of heparin-binding EGF-like growth factor. Biochem Biophys Res Commun 204:592–597.

Ramchandran R, Sen GC, Misono K, Sen I (1994): Regulated cleavage-secretion of the membrane-bound angiotensin-converting enzyme. J Biol Chem 269:2125–2130.

Simpson J, Johnson MS, Mitchell R. (1993) H7-resistant protein kinase C substrates in two-dimensional gels of proestrous rat anterior pituitary gland. Biochim Biophys Acta 1220:69–75.

Suzuki M, Raab G, Moses MA, Fernandez CA, Klagsbrun M (1997): Matrix metalloproteinase-3 releases active heparin-binding EGF-like growth factor by cleavage at a specific juxtamembrane site. J Biol Chem 272:31730–31737.

Temizer DH, Yoshizumi M, Perrella MA, Susanni EE, Quertermous T, Lee ME (1992): Induction of heparin-binding epidermal growth factor-like growth factor mRNA by phorbol ester and antiotensin II in rat aortic smooth muscle cells. J Biol Chem 267:24892–24896.

Thastrup O, Cullen PJ, Drøbak BK, Hanley MR, Dawson AP (1990): Thapsigargin, a tumor promoter, discharges intracellular $Ca^{2+}$ stores by specific inhibition of the endoplasmic reticulum $Ca^{2+}$-ATPase. Proc Natl Acad Sci USA 87:2466–2470.

Thompson NT, Bonser RW, Garland LG (1991): Receptor-coupled phospholipase D and its inhibition. TIPS 12:404–408.

Zachow RJ, Terranova PF (1993): Involvement of protein kinase C and protein tyrosine kinase pathways in tumor necrosis factor-alpha-induced clustering of ovarian theca-interstitial cells. Mol Cell Endocrinol 97:37–49.

Zitt C, Zobel A, Obukhov AG, Harteneck C, Kalkbrenner F, Lückhoff A, Schultz G (1996): Cloning and functional expression of a human $Ca^{2+}$-permeable cation channel activated by calcium store depletion. Neuron 16:1189–1196.