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Phorbol Ester Induces the Rapid Processing of Cell Surface Heparin-binding EGF-like Growth Factor: Conversion from Juxtacrine to Paracrine Growth Factor Activity

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Vero cell heparin-binding epidermal growth factor–like growth factor (HB-EGF) is synthesized as a 20- to 30-kDa membrane-anchored HB-EGF precursor (proHB-EGF). Localization and processing of proHB-EGF, both constitutive and 12-*O*-tetradecanoylphorbol 13-acetate (TPA)–inducible, was examined in Vero cells overexpressing recombinant HB-EGF (Vero H cells). Flow cytometry and fluorescence immunostaining demonstrated that Vero cell proHB-EGF is cell surface-associated and localized at the interface of cell to cell contact. Cell surface biotinylation and immunoprecipitation detected a 20- to 30-kDa heterogeneous proHB-EGF species. Vero H cell surface proHB-EGF turned over constitutively with a half-life of 1.5 h. Some of the 20- to 30-kDa cell surface-associated proHB-EGF was processed and a 14-kDa species of bioactive HB-EGF was released slowly, but most of the proHB-EGF was internalized, displaying a diffuse immunofluorescent staining pattern and accumulation of proHB-EGF in endosomes. Addition of TPA induced a rapid processing of proHB-EGF at a Pro^{148}–Val^{149} site with a half-life of 7 min. The TPA effect was abrogated by the protein kinase C inhibitors, staurosporine and H7. Kinetic analysis showed that loss of cell surface proHB-EGF is maximal at 30 min after addition of TPA and that proHB-EGF is resynthesized and the initial cell surface levels are regained within 12–24 h. Loss of cell surface proHB-EGF was concomitant with appearance of 14- and 19-kDa soluble HB-EGF species in conditioned medium. Vero H cell–associated proHB-EGF is a juxtacrine growth factor for EP170.7 cells in coculture. Processing of proHB-EGF resulted in loss of juxtacrine activity and a simultaneous increase in soluble HB-EGF paracrine mitogenic activity. It was concluded that processing regulates HB-EGF bioactivity by converting it from a cell-surface juxtacrine growth factor to a processed, released soluble paracrine growth factor.

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INTRODUCTION

Heparin-binding epidermal growth factor–like growth factor (HB-EGF) is a member of the epidermal growth factor (EGF) family that was first identified as a secreted product of macrophages and macrophage-like U-937 cells (Besner *et al.*, 1990; Higashiyama *et al.*, 1991, 1992). HB-EGF purified from the conditioned medium (CM) of U-937 cells is a 20- to 22-kDa glycoprotein found in multiple forms of 76–86 amino acids (Higashiyama *et al.*, 1992). It stimulates proliferation of fibroblasts, smooth muscle cells (SMC), keratinocytes, and hepatocytes, but not of endothelial cells (Besner *et al.*, 1990; Higashiyama *et al.*, 1991, 1992; Marikovsky *et al.*, 1993; Hashimoto *et al.*, 1994; Ito *et al.*, 1994). HB-EGF binds to EGF receptors via its C-terminal region, which has a 40% structural homology to EGF and transforming growth factor-α (TGF-α). A structural feature of HB-EGF (76–86 amino acids) absent in EGF (53 amino acids) and TGF-α (50 amino acids) is the presence of an extended N-terminal region upstream from the EGF-like C-terminal region. Within this N-terminal extension, there is a 21-amino acid very hydrophilic heparin-binding domain (Thompson *et al.*, 1994) that modulates the binding of HB-EGF to EGF receptor and enhances HB-EGF bioactivity for SMC (Higashiyama *et al.*, 1993).

A structural feature common to all members of the EGF superfamily including EGF, TGF-α, amphiregulin, neu differentiation factor/heregulin/glial growth factor/acetylcholine receptor inducing activity, β-cellulin, *C. elegans lin-3*, and *Drosophila spitz* is that they are synthesized as precursor molecules that are tethered to the cell membrane via a transmembrane domain (for review see Massague and Pandiella, 1993). HB-EGF is synthesized as a 208-amino acid precursor (Higashiyama *et al.*, 1991) and hydropathy analysis predicts the presence of a hydrophobic putative transmembrane domain downstream of the EGF-like domain. The membrane-anchored HB-EGF (proHB-EGF) has been shown to be bioactive in at least two ways. One is that proHB-EGF is the receptor for diphtheria toxin (DT) (Naglich *et al.*, 1992), a specific function not shared by other EGF family members (Iwamoto *et al.*, 1994). Second, proHB-EGF stimulates the growth of adjacent cells in a juxtacrine manner (Higashiyama *et al.*, 1995). The processing of proHB-EGF converts it from being a DT receptor to a soluble form of HB-EGF whose mitogenic activity is inhibited by DT (Mitamura *et al.*, 1995) and results in cells becoming DT-resistant (Raab *et al.*, 1994).

Besides the members of the EGF superfamily, other growth factors and cytokines such as tumor necrosis factor-α, colony stimulating factor-1 (CSF-1), and *c-kit* ligand-1, 2 (KL-1, 2) exist as membrane-anchored molecules that are processed to release soluble forms (for review, see Massague and Pandiella, 1993). The processing of the TGF-α precursor has been studied the most intensively and shown to be mediated by a protein kinase C (PKC)-dependent pathway. TGF-α processing can be induced by serum factors, Ca^{2+} ionophores, and phorbol esters (Pandiella and Massague, 1991a, 1991b). Even though cleavage sites have been identified, the precise mechanisms of TGF-α processing are yet to be determined.

The physiological significance of growth factor and cytokine processing is for the most part not well understood. One exception is that aberrant processing of proKL results in abnormal development of melanocytes, germ cells, and hematopoietic stem cells (Flanagan *et al.*, 1991; Huang *et al.*, 1992). These results suggest that membrane-anchored growth factors and cytokines may have unique and significant roles in cell growth and tissue development, and that the processing might be a key event in regulating their activities. Given these considerations, we have analyzed the processing of proHB-EGF. In this study, we demonstrate that in Vero H cells, proHB-EGF is processed constitutively at a relatively low rate and that phorbol ester induces rapid processing. Processing, whether constitutive or induced, converts HB-EGF from being a juxtacrine factor to a paracrine factor and thus could be an important mechanism that regulates HB-EGF growth factor activity.

MATERIALS AND METHODS

Materials

12-*O*-Tetradecanoylphorbol 13-acetate (TPA), bafilomycin A1, and staurosporine were purchased from Wako Pure Chemical Industry (Osaka, Japan). H7 was purchased from Seikagaku (Tokyo, Japan). Sulfo-NHS-biotin and anhydrochymotrypsin-agarose were purchased from Pierce (Rockford, IL). Pronase was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). α-Chymotrypsin (Type VII, tosyl-lysine chloromethyl ketone (TLCK)-treated) was purchased from Sigma (St. Louis, MO). Recombinant human HB-EGF (Thompson *et al.*, 1994), goat anti-HB-EGF antibody #197 (a highly specific neutralizing antibody; Hashimoto *et al.*, 1994), and rabbit anti-HB-EGF antibody #2998 (useful for Western blotting; Dluz *et al.*, 1993), were kind gifts obtained from Drs. Deborah Damm and Judith A. Abraham (Scios Nova, Mountain View, CA). These antibodies were raised against recombinant human HB-EGF. Rabbit anti-human HB-EGF antibody #H6 was raised against a synthetic peptide corresponding to amino acids 54–73 of the HB-EGF precursor, which are in the ectodomain (Iwamoto *et al.*, 1994). Anti-human CD9 monoclonal antibody ALB6 was purchased from Medical and Biological Laboratories (Nagoya, Japan). A DT mutant, CRM 197, was prepared as described previously (Uchida *et al.*, 1973).

Cell Culture

Vero cells (African green monkey kidney cells) were grown in DMEM supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin sulfate (100 μg/ml). EP170.7 cells (Pierce *et al.*, 1988) were grown in RPMI 1640 medium supplemented with 10% FCS, 5% WEHI-3 cell CM (Blotnick *et al.*, 1994), penicillin (100 U/ml), and streptomycin sulfate (100 μg/ml). A

stable transfectant expressing human proHB-EGF, termed Vero H, was prepared by transfecting parental Vero cells with 20 μg of pRcHB-EGF (Iwamoto *et al.*, 1994) using the calcium-phosphate method (Chen and Okayama, 1988), culturing for 48 h, and further culturing for 7 days in selection medium (DMEM/10% FCS/1 mg/ml G418). About 30 G418-resistant colonies were tested for ^{125}I-labeled DT binding as described previously (Mekada and Uchida, 1985). Several of these Vero clones were able to bind ^{125}I-labeled DT better than parental Vero cells and the clone with the highest ability to bind DT (Vero H) was used in these studies.

^{125}I-labeled DT Binding

Purified DT was labeled with Na^{125}I (Amersham, Arlington Heights, IL) using Enzymobeads (Bio-Rad Laboratories, Hercules, CA) as reported previously (Mekada and Uchida, 1985). Two lots of the labeled DT had specific activities of 2.1×10^6 cpm/μg and 2.9×10^7 cpm/μg. ^{125}I-labeled DT binding was analyzed as described previously (Umata *et al.*, 1990). Briefly, Vero and Vero H cells were plated in 12-well plates at 5×10^4 cells/well and incubated for 12 h at 37°C. The cells were incubated with 2 ml of the binding medium (Eagle's minimal essential medium containing nonessential amino acids, 10% FCS, and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.2, with the omission of sodium bicarbonate) on ice for 20 min. Increasing amounts of ^{125}I-labeled DT (2.1×10^6 cpm/μg) were added and the cells were incubated for 10 h at 4°C. The cells were washed, lysed, and cell-associated radioactivity was measured. TPA treatments were carried out in 12-well plates at a cell density of 10^5 cells/well. The cells were cultured with the indicated concentration of TPA in the binding medium for 1 h at 37°C, washed twice with ice-cold PBS, and then incubated in 2 ml of the binding medium without TPA on ice for 20 min. ^{125}I-labeled DT (2.9×10^7 cpm/μg) was added at a final concentration of 100 ng/ml and the cells were incubated at 4°C for 6 h. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and ^{125}I-labeled DT binding was measured as above. Nonspecific binding of ^{125}I-labeled DT was assessed in the presence of a 100-fold excess of unlabeled DT. Specific binding was determined by subtracting the nonspecific binding. The nonspecific binding was less than 10% of the total binding.

Flow Cytometry

Vero H cells were grown in 10-cm culture dishes at a density of 5×10^5 cells/dish and incubated for 12 h. The cells were treated with 64 nM TPA for indicated periods. The cells were washed twice with 2 M NaCl/PBS to remove soluble HB-EGF trapped by cell surface heparan sulfate proteoglycans (HSPG) (Moscatelli, 1987), detached from dishes with PBS containing 0.5 mM EDTA, and recovered by centrifugation at 1000 rpm for 3 min. The cell pellets were washed with ice-cold PBS, and incubated with rabbit anti-HB-EGF antibody #H6 for 30 min on ice. Preimmune rabbit serum was used as a control. After a 30-min incubation, the cells were washed twice with ice-cold PBS, and then fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Cappel, Organon Teknika, Durham, NC) was added. The cells were incubated on ice for 30 min, followed by washing twice with ice-cold PBS. Fluorescence intensity of Vero H cell surface was analyzed using a FACScan (Becton Dickinson, Mountain View, CA). A scatter window was set to eliminate dead cells and cell debris. Cells (10^4) were acquired by list mode, and measurements were performed on a single cell basis and displayed as frequency distribution histograms. Cytometric analyses were measured on a logarithmic scale and mean fluorescence intensity was converted to a linear scale to calculate the relative fluorescence intensity. The relative fluorescence intensity was calculated as the difference in linear mean fluorescence intensity between treated cells and control cells divided by the fluorescence intensity of control cells.

Cell Surface Biotinylation, Immunoprecipitation, and Western Blotting of proHB-EGF

Vero H cells were seeded in 10-cm dishes at a density of 10^6 cells/dish and incubated for 12 h. Cells were washed three times with ice-cold Hanks' buffer and biotinylated with 0.1 mg/ml of sulfo-NHS-biotin in 50 mM HEPES, pH 7.5, 0.15 M NaCl for 15 min on ice. Excess reagent was quenched and removed by washing with ice-cold DMEM/10% FCS. Cells were lysed with a lysis buffer [1% Triton X-100, 3 mM EDTA, 1 mM (*p*-amidinophenyl) methanesulfonyl fluoride HCl, 1 μg/ml aprotinin, 10 μM antipain, 5 μM 3,4-dichloroisocoumarin, and 0.4 M NaCl in 20 mM HEPES, pH 7.2]. After centrifugation at 15000 rpm for 15 min, supernatants were incubated with 10 μg of rabbit anti-HB-EGF antibody #H6 for 2 h at 4°C, followed by incubation with 10 μl of protein A trisacryl (50% suspension) (Pierce) for 2 h at 4°C. The samples were dissolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and fractionated by 15% SDS-PAGE (Laemmli, 1970). Proteins in the gels were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) in 150 mM 3-[cyclohexylamino]-1-propane-sulfonic acid buffer, pH 11.0, containing 20% methanol at 180 mA for 3 h. The nitrocellulose membranes were blocked with 5% skimmed milk in PBS overnight at 4°C. The membranes were incubated for 30 min at room temperature with avidine-conjugated horseradish peroxidase (HRPO; Vector Laboratories, Burlingame, CA). After being washed five times at intervals of 10 min with 0.05% Tween-20 in PBS, the membranes were treated with enhanced chemiluminescence (ECL) Western blotting detection reagents for 1 min (Amersham) at room temperature. The membranes were exposed to Kodak scientific imaging film (Eastman Kodak, Rochester, NY). The densities of bands on the exposed films were analyzed using a Scanning Imager (Molecular Dynamics, Sunnyvale, CA).

Quantitation of Cell Surface proHB-EGF

The proHB-EGF content on the Vero H cell surface was estimated from a standard curve that was established as follows: soluble recombinant HB-EGF (0.2–200 ng/200 μl/well) was immobilized in 96-well immunoplates (Nunc, Rotskilde, Denmark) by an overnight incubation in 50 mM carbonate-bicarbonate buffer, pH 9.5. The efficiency of immobilizing HB-EGF was about 96%, as estimated by mixing cold HB-EGF with ^{125}I-HB-EGF (18,000 cpm/ng; Higashiyama *et al.*, 1992) in a ratio of 9:1, immobilizing the mixture at concentrations of 0.2–200 ng/200 μl/well in 96-well plates, washing three times with PBS, and measuring radioactivity in each well with a gamma counter. Plates containing immobilized HB-EGF were washed three times with PBS and wells were blocked with 0.5% bovine serum albumin in PBS. The plates were washed three times with PBS, and EP170.7 cells, which express EGF receptor and are used for measuring HB-EGF mitogenic activity (Blotnick *et al.*, 1994), were added. Mitogenic activity was measured as described below (see Soluble Growth Factor Activity Measurements). To measure Vero H cell surface proHB-EGF, cells (10^5) were plated in DMEM/10% FCS (500 μl/well, 24-well plates) and incubated for 12 h before washing. The cells were washed twice with DMEM/10% FCS and incubated in DMEM/10% FCS (500 μl/well) with and without 64 nM TPA for 30 min. The CM were harvested and assayed for soluble growth factor activity described below (see Soluble Growth Factor Activity Measurements). For cell surface proHB-EGF activity, cells were washed twice with DMEM/10% FCS/2 M NaCl to remove soluble HB-EGF trapped by cell surface HSPG (Moscatelli, 1987), and assayed for juxtacrine growth factor activity for EP170.7 cells as described below (see Juxtacrine Growth Factor Activity Measurements). Total HB-EGF content in the CM and on the cell surface was calculated from standard curves.

Immunofluorescence Microscopy

Cells plated on coverslips in 6-well culture plates were incubated with 5 μg/ml of rabbit anti-HB-EGF antibody #H6 in DMEM/10% FCS for 1 h on ice, washed three times with and without 0.2 M glycine-HCl buffer, pH 2.0, and fixed with 4% paraformaldehyde in PBS for 10 min at 4°C. Internalization of proHB-EGF was accomplished by a 2-h incubation at 37°C and then washing with 0.2 M glycine-HCl buffer, pH 2.0. Cells were washed three times with PBS, and incubated with FITC-conjugated goat anti-rabbit IgG (1–250 dilution, Cappel) in the dark. The internalized proHB-EGF-#H6 antibody complexes were probed with FITC-conjugated goat anti-rabbit IgG after permeabilization with 0.3% Triton X-100 in PBS for 10 min. The coverslips were washed three times with PBS, rinsed with distilled water, and mounted onto slides with parmafluor aqueous mounting medium (Immunon, Pittsburgh, PA). Cells were viewed on an inverted fluorescence microscope (Zeiss, Oeberkochen, Germany).

Pronase Digestion of Vero H Cell Surface Proteins

Vero H cells plated in 10-cm dishes at a density of 10^6 cells/dish were cultured for 12 h at 37°C. The cells were incubated for 30 min at 37°C in the presence or absence of 500 nM bafilomycin A1 in DMEM/10% FCS, and biotinylated as described above. Biotinylated cells were incubated at 37°C for the indicated times with and without 500 nM bafilomycin A1, washed three times with ice-cold DMEM/20 mM HEPES, pH 7.4, and then treated with 5 mg/ml of pronase in DMEM/20 mM HEPES, pH 7.4, for 7 min at 37°C. At the end of the incubation period, 1 ml of FCS was added to the each dish and the cells were harvested and washed three times with ice-cold DMEM/10% FCS. The cells were subjected to immunoprecipitation and Western blot as described above.

Analysis of HB-EGF Released into Conditioned Medium by Western Blot

Preparation of CM was as follows: Vero H cells were seeded in 10-cm dishes at a density of 1.5×10^6 cells/dish and incubated for 12 h. Cells were washed three times with ice-cold Hanks' buffer, and incubated with 5 ml of DMEM containing 10% FCS depleted of heparin-binding proteins by incubation with an equal volume of heparin-Sepharose CL-6B (Pharmacia LKB Biotechnology, Uppsala, Sweden). The CM was harvested and centrifuged to remove cell debris. Western blotting analysis was performed as follows: Vero H cell CM was applied to a 30 μl heparin-Sepharose CL-6B column. After being washed with 10 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.2, the heparin-Sepharose beads were suspended in 30 μl of SDS-PAGE sample buffer and clarified supernatants were fractionated by 15% SDS-PAGE. Proteins were transferred to nitrocellulose membranes and the nitrocellulose membranes were blocked with 5% skimmed milk as described above. The membranes were incubated with rabbit anti-HB-EGF antibody #2998 in 5% skimmed milk/PBS for 4 h at 4°C and after being washed five times at intervals of 10 min with 0.05% Tween 20 in PBS, the membranes were incubated with HRPO-conjugated goat anti-rabbit IgG (Promega, Madison, WI) for 1 h at room temperature. After washing the membrane five times at intervals of 10 min with 0.05% Tween 20 in PBS, HRPO was detected using an ECL kit. Fifty nanograms of highly purified "peak-1" HB-EGF (Higashiyama *et al.*, 1992) and recombinant HB-EGF (Thompson *et al.*, 1994) were used as standards.

Isolation and Amino Acid Composition Analysis of the Carboxy-Terminal Peptide of the Released HB-EGF

Purified HB-EGF (10 μg) prepared from 10 liters of the CM of TPA-treated Vero H cells was reduced and S-carboxymethylated as previously described (Kerbiriou and Griffin, 1979). This HB-EGF preparation was hydrolyzed with 1% TLCK-α-chymotrypsin in 0.1 M carbonate-bicarbonate buffer, pH 8.1, for 6 h at 37°C. The reaction was stopped by the addition of 1 mM diisopropyl fluorophosphate (Sigma). The reaction mixture was adjusted to pH 5.0 and applied to an immobilized anhydrochymotrypsin-agarose column equilibrated with 50 mM sodium acetate, pH 5.0, containing 10 mM $CaCl_2$. The nonbinding fraction was fractionated by C_{18} (COSMOSIL AR300, 4.6 × 250 mm; Nacalai Tesque, Kyoto, Japan) reversed phase high performance liquid chromatography using a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) with a linear gradient of acetonitrile. The main peak was pooled and applied to Hitachi amino acid analyzer L-8500 (Hitachi, Tokyo, Japan).

Heparin Affinity Chromatography

Chromatography of HB-EGF on immobilized heparin was carried out as previously described (Higashiyama *et al.*, 1992). Briefly, Vero H cell CM was applied to a TSK-heparin 5PW column (8 × 75 mm, Toso, Japan) equilibrated with 0.2 M NaCl, 0.01 M Tris-HCl, pH 7.4, using a fast protein liquid chromatography system (Pharmacia LKB Biotechnology). The column was washed with 20 ml of equilibration buffer and bound proteins were eluted with a 40-ml linear gradient of 0.2–2.0 M NaCl in 0.01 M Tris-HCl, pH 7.4, at a flow rate of 1 ml/min. One milliliter fractions were collected and 10 μl of each fraction was tested for mitogenic activity for EP170.7 cells.

Soluble Growth Factor Activity Measurements

EP170.7 cells were washed with RPMI 1640 medium supplemented with 10% FCS, penicillin (100 U/ml), and streptomycin sulfate (100 μg/ml). The cells (2×10^4) were plated in 96-well plates in a total volume of 200 μl. Appropriate volumes of sample were added to each well and the EP170.7 cells were then incubated for 36 h. Ten microliters of [^{3}H]thymidine (1 μCi/10 μl PBS, ICN Biomedicals, Costa Mesa, CA) were added and after a 4-h incubation, the incorporation of [^{3}H]thymidine into DNA was measured using a 1205 Betaplate system (Pharmacia LKB Biotechnology). Soluble recombinant HB-EGF was used as a standard to estimate the HB-EGF content of the samples.

Inhibition of Growth Factor Activity

Ten microliters of Vero H CM was incubated with 10 μg/ml (final concentration) of goat anti-HB-EGF neutralizing antibody #197, 10 μg/ml (final concentration) of CRM #197 (Mitamura *et al.*, 1995), and 50 μg/ml (final concentration) of mouse anti-CD9 antibody ALB6 or PBS as a control, for 30 min at room temperature. The samples were tested for mitogenic activity for EP170.7 cells. The same concentration of goat anti-HB-EGF neutralizing antibody #197, CRM 197, or mouse anti-CD9 antibody ALB6 was added to neutralize the juxtacrine growth factor activity of Vero H cells simultaneously when EP170.7 cells were added.

Juxtacrine Growth Factor Activity Measurements

A juxtacrine growth factor assay was carried out as described previously (Higashiyama *et al.*, 1995). Briefly, Vero H cells (10^5 cells/well) were plated in DMEM/10% FCS (500 μl/well) in 24-well plates and incubated for 12 h before washing and fixation. The cells were washed twice with DMEM/10% FCS/2 M NaCl to remove soluble HB-EGF trapped by cell surface HSPG (Moscatelli, 1987), and fixed with 5% buffered formalin for 5 min. The formalin-fixed Vero H cells were washed twice with RPMI 1640/10% FCS, and EP170.7 cells (10^5 cells/500 μl/well) were added to the fixed Vero H cells. After 48 h, [^{3}H]thymidine (1 μCi/well; 1 mCi = 37 MBq) was added to the wells and the cells were incubated for 4 h. The EP170.7 cells were harvested and analyzed for incorporation of [^{3}H]thymidine into DNA.

RESULTS

Vero H Cells Overexpress proHB-EGF on the Cell Surface

Vero cells are highly sensitive to the toxic effects of DT, suggesting that they express cell surface proHB-EGF, which is the DT receptor. In addition to parental Vero cells, a stable transfected Vero cell line, Vero H, which overexpresses proHB-EGF, was established. Parental Vero and Vero H cells were analyzed for the ability to bind ^{125}I-labeled DT. Vero H cells bound seven times more DT than did parental Vero cells (Figure 1A).

Phorbol Ester Induces Rapid Processing of Cell Surface proHB-EGF

TPA has been shown to induce processing of membrane-anchored proteins such as proTGF-α (Pandiella and Massague, 1991a, 1991b), L-selectin (Kahn *et al.*, 1994), and CSF-1 (Stein and Rettenmier, 1991). Accordingly, parental Vero and Vero H cells were treated with increasing concentrations of TPA for 1 h at 37°C. Within 1 h, ^{125}I-labeled DT binding was diminished by 75% in both cell types (Figure 1B). Because Vero H cells overexpress proHB-EGF, we used these cells to facilitate analysis of cell surface-associated proHB-EGF for all of the following experiments. Vero H cells were analyzed by immunofluorescence staining using anti-HB-EGF antibody #H6 directed against the ectodomain of proHB-EGF. The cells were washed first with 2 M NaCl to remove any mature HB-EGF that might be bound to cell surface HSPG (Moscatelli, 1987). Addition of anti-HB-EGF antibodies to the cells and incubation with FITC-conjugated goat anti-rabbit IgG demonstrated that Vero H cell surface proHB-EGF was localized at the interface of cell to cell contact (Figure 2, A and C). ProHB-EGF was stained very faintly at the similar site of parental Vero cells (our unpublished observation). The immunofluorescent staining of Vero H cells was totally abrogated by the incubation with 64 nM TPA for 30 min at 37°C (Figure 2, B and D). The CM of Vero H cells was analyzed by heparin affinity column chromatography. In parallel with the loss of the cell surface antigen recognized by the anti-HB-EGF antibodies, there was at least a threefold increased amount of growth factor activity released into CM (Figure 3A). The evidence for this growth factor activity being bioactive HB-EGF was as follows: 1) the mitogenic activity eluted at 1.0–1.2 M NaCl, the elution position of mature HB-EGF (Higashiyama *et al.*, 1992), and 2) the mitogenic activity was inhibited by over 90% with both neutralizing anti-human HB-EGF antibody #197 (Hashimoto *et al.*, 1994) and CRM 197, a specific inhibitor of human HB-EGF mitogenic activity (Mitamura *et al.*, 1995)

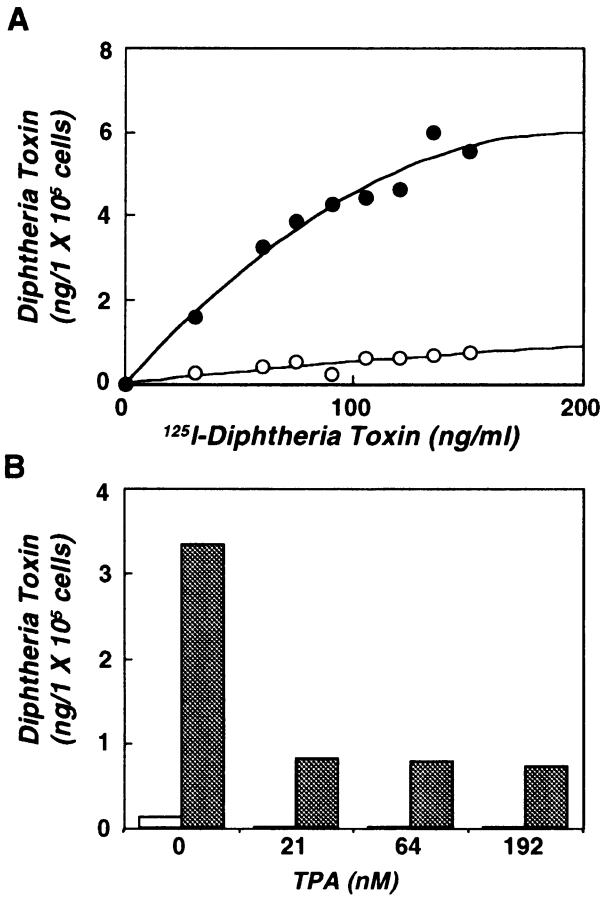

Figure 1. Binding of ^{125}I-labeled DT to Vero and Vero H cells. (A) Increasing amounts of ^{125}I-labeled DT (2.1 × 10^6 cpm/μg) were incubated for 10 h at 4°C in 12-well plates at a cell density of 5 × 10^4 cells/well. Vero (open circles) and Vero H cells (closed circles) were washed and lysed, and the cell-associated radioactivity was measured. (B) Constant amounts of ^{125}I-labeled DT (200 ng, 2.9 × 10^7 cpm/mg) were added to 12-well plates (cell density of 10^5 cells/well), which had been pretreated with increasing concentrations of TPA for 1 h at 37°C. Vero (open bars) and Vero H cells (closed bars) were incubated for 6 h at 4°C and cell associated radioactivity was measured. Under the conditions of these experiments, nonspecific binding of ^{125}I-labeled DT was less than 10% of the total binding.

(Figure 3B). In the absence of TPA, there was some constitutive release of HB-EGF by Vero H cells (Figure 3A), indicating that these cells have an intrinsic mechanism for activation of proHB-EGF processing.

Kinetics of proHB-EGF Processing

The kinetics of Vero H cell surface proHB-EGF processing was analyzed by flow cytometry and a combination of cell surface biotinylation and immunoprecipitation. Within 15 min after addition of TPA, the immunofluorescence intensity of Vero H cells incubated with anti-HB-EGF antibody #H6 and FITC-conjugated goat anti-rabbit IgG was dimin-

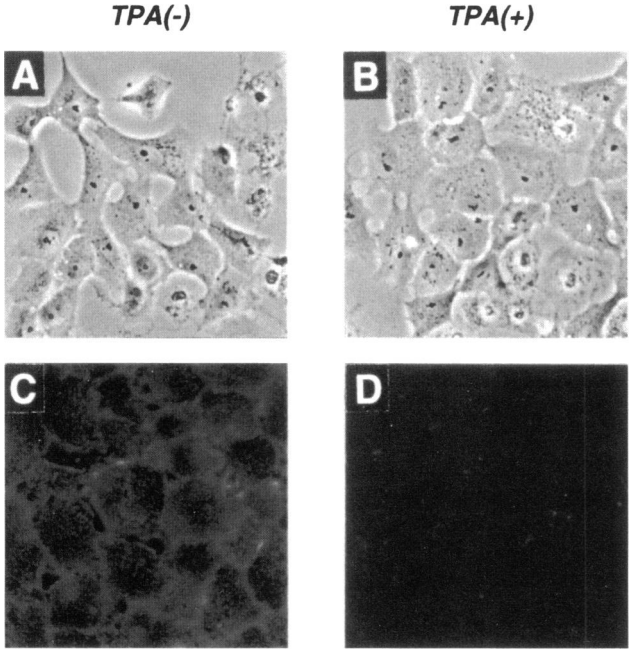

Figure 2. Immunofluorescence staining of cell surface proHB-EGF. Vero H cells were grown for 12 h and incubated with and without 64 nM TPA for 30 min. (A and B) Phase-contrast photographs before and after TPA treatment, respectively. (C and D) Immunofluorescence staining of proHB-EGF before and after TPA treatment, respectively, using anti-HB-EGF antibody #H6.

ished by over 80% (Figure 4, A and B). Loss of cell surface proHB-EGF was optimal at 30 min, began to reappear by 1 h, and increased gradually, reaching the original levels by 24 h after addition of TPA (Figure 4, A and B). Staurosporine and H7 (inhibitors of protein kinase C) at 100 nM and 333 μM, respectively, inhibited TPA-inducible processing (Figure 4C) as well as the constitutive processing of proHB-EGF (our unpublished observation). These results indicate that the TPA-inducible processing is transient and that both inducible and constitutive processing are mediated by PKC.

Molecular Characterization of the Conversion of proHB-EGF to Mature HB-EGF

The molecular masses of cell surface proHB-EGF and mature soluble HB-EGF released by TPA were analyzed. Using cell surface biotinylation and immunoprecipitation, it was determined that cell surface proHB-EGF was heterogeneous with masses of 20–30 kDa (Figure 5A), consistent with previous analysis (Iwamoto *et al.*, 1994). Addition of TPA resulted in the loss of cell surface proHB-EGF within 30 min (Figure 5A). Densitometric analyses of the 20- to 30-kDa bands revealed that the half-life of cell surface proHB-EGF after addition of TPA was 7 min (Figure 5B). On the

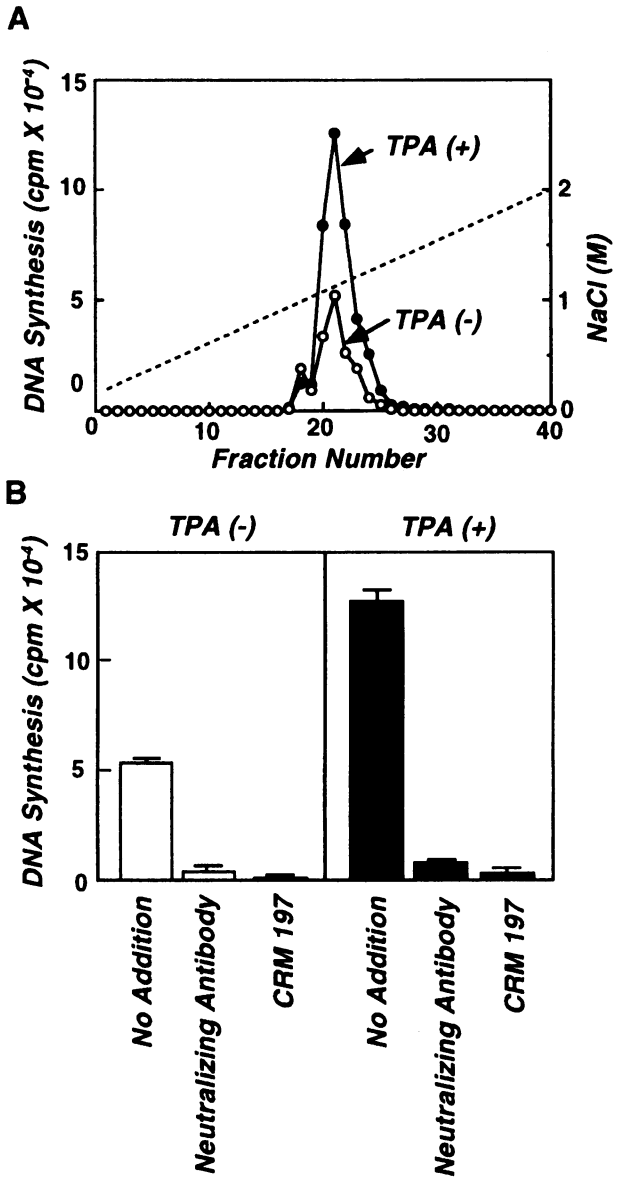

Figure 3. Release of Vero H cell HB-EGF by TPA. (A) CM (100 ml) were prepared from Vero H cells incubated with (closed circles) and without (open circles) 64 nM TPA for 24 h and analyzed by fast protein liquid chromatography TSK-heparin column chromatography. Fractions were collected and tested for mitogenic activity for EP170.7 cells. (B) Aliquots of fraction 21 obtained by heparin affinity chromatography of CM of Vero H cells, treated and not treated with TPA as shown in panel A, were incubated with anti-human-HB-EGF neutralizing antibody #197 or CRM 197 for 30 min at room temperature. The samples were tested for mitogenic activity for EP170.7 cells.

other hand, the half-life of proHB-EGF processed constitutively in non-TPA–treated cells was about 1.5 h (Figure 5, C and D). The appearance of immunoreactive mature HB-EGF in CM after TPA treatment (Figure 5E) coincided well with the disappearance of im-

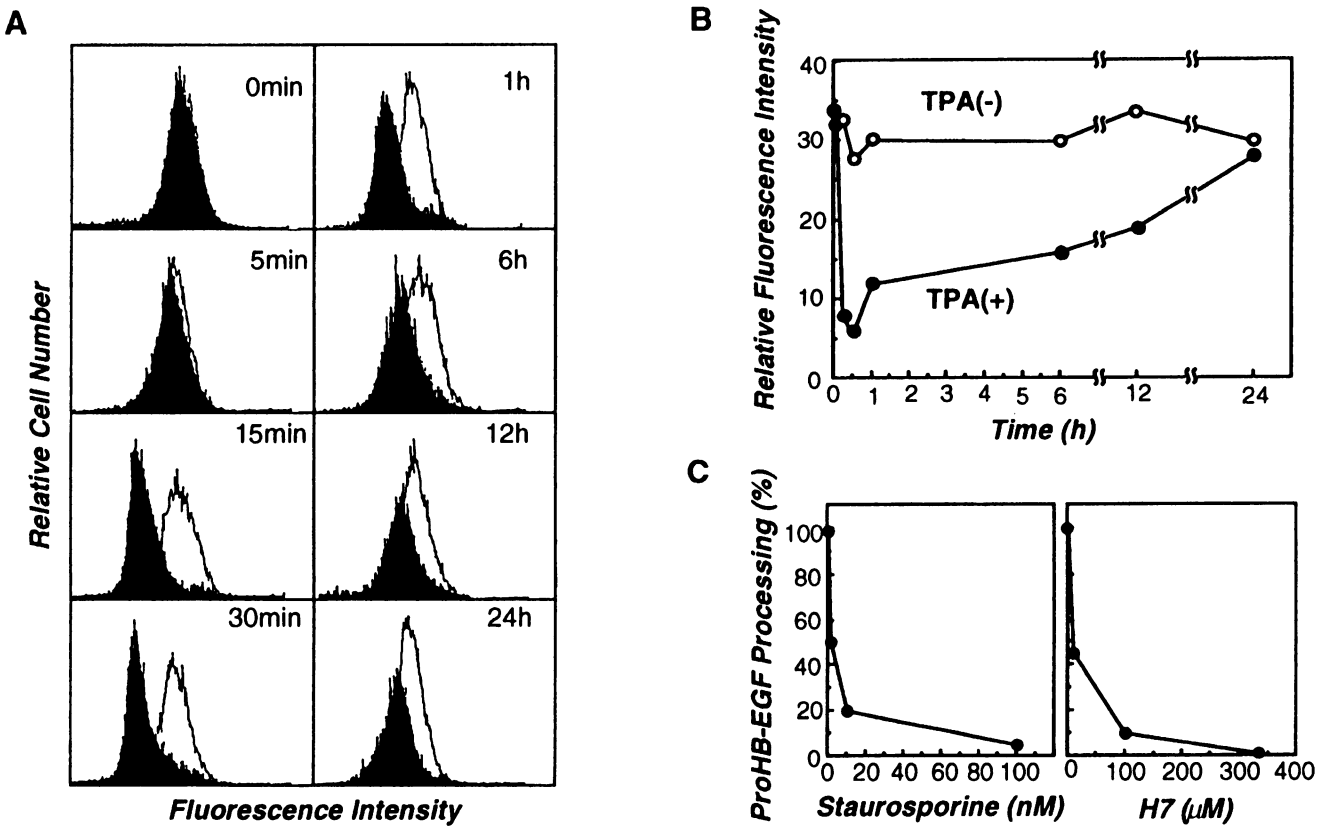

Figure 4. Kinetics of cell surface proHB-EGF processing by TPA. (A and B) Vero H cells were incubated with (closed area, closed circles) or without (open area, open circles) 64 nM TPA for indicated periods and cell surface proHB-EGF levels were measured by flow cytometry at various incubation times of TPA. Each histogram in panel A represents 10^4 cells. In panel B, the absolute fluorescence intensities of the various time points shown in panel A were converted to relative fluorescence intensity as described in MATERIALS AND METHODS. Each point represents the relative fluorescence intensity of 10^4 cells. (C) Vero H cells were incubated with 64 nM TPA and either staurosporine (left panel) or H7 (right panel) at various concentrations and relative fluorescence intensities were calculated.

munoreactive proHB-EGF on the cell surface (Figure 5, A and B). Two species of soluble mature HB-EGF released into the CM of Vero H cells were detected within 5 min after TPA treatment by Western blot following heparin-Sepharose chromatography. One of these, a doublet of about 14 kDa, was also found in the CM of non-TPA–treated cells, but to a lesser extent. A 19-kDa doublet was found exclusively in the CM of TPA-treated cells. This 19-kDa doublet comigrated with native HB-EGF purified from U-937 cell CM (Higashiyama *et al.*, 1992). On the other hand, the 14-kDa doublet bands comigrated with a 75-amino acid recombinant HB-EGF 75 prepared from *Escherichia coli* (Thompson *et al.*, 1994). Taken together, these results suggest that there is constitutive processing of Vero H cell proHB-EGF leading to the release of 14-kDa HB-EGF and that TPA increases the rate of release of this smaller HB-EGF species. TPA, in addition, releases a 19-kDa HB-EGF species.

The kinetics of growth factor activity release into CM was analyzed (Figure 5F). In the absence of TPA, very little bioactive HB-EGF (14 kDa) was released into CM within 12 h. However, in the presence of TPA, substantial bioactive HB-EGF (14 kDa + 19 kDa) was released within 30 min, and maximal release occurred within 1 h. Thus after TPA treatment, the time course of HB-EGF mitogenic activity release into CM correlates well with the loss of immunoreactive cell surface proHB-EGF (Figure 5A). There does, however, seem to be a lag, yet unexplained, of about 30 min in the maximal appearance of soluble bioactive HB-EGF into CM.

Amino acid composition analysis of a carboxy-terminal peptide obtained from a chymotrypsin digest of purified Vero H cell 19-kDa HB-EGF revealed that upon TPA-inducible processing, proHB-EGF is cleaved at Pro^{148}–Val^{149} to release the soluble form (Table 1). This result is also supported by comigration studies of synthetic peptides together with

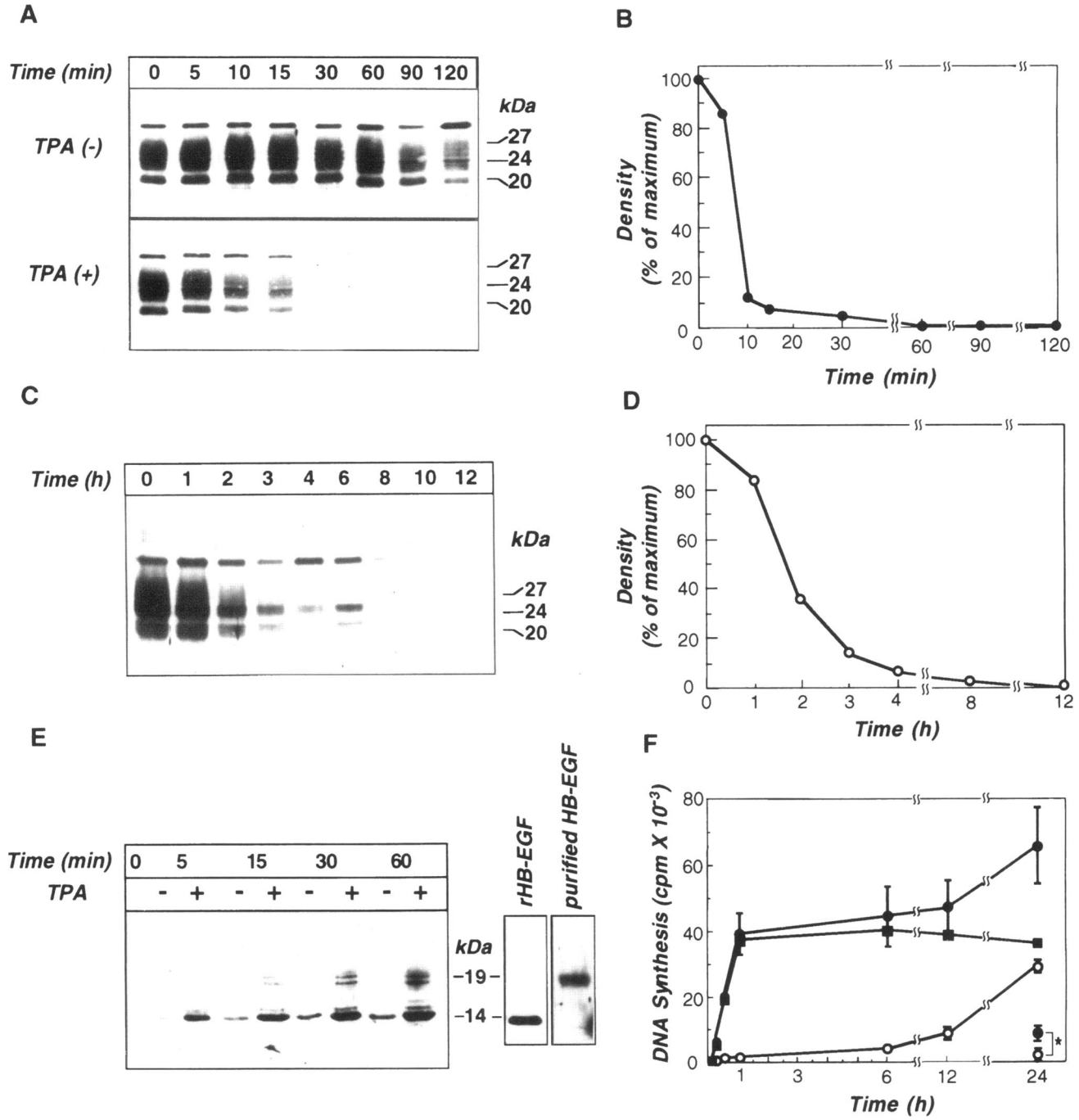

Figure 5. Conversion of cell surface proHB-EGF to soluble released HB-EGF. (A) Vero H cells were biotinylated, incubated without (upper panel) and with (lower panel) TPA at various time intervals up to 2 h, and immunoprecipitated with anti-HB-EGF antibody #H6. The samples were fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, and biotinylated proteins were detected as described in MATERIALS AND METHODS. (B) The total densities of the 20–30 kDa bands shown in the lower panel of panel A (+ TPA) were determined using a densitometer and these densities were plotted against the incubation times of TPA treatment. (C) The same as panel A for non-TPA–treated cells but with time intervals up to 12 h. (D) The total densities of 20- to 30-kDa bands in panel C were analyzed by a densitometer as in panel B. (E) Vero H cells were incubated with and without 64 nM TPA for various incubation intervals. Samples of CM were concentrated on mini heparin-Sepharose columns (30 μl) and fractionated by SDS-PAGE. HB-EGF was detected by Western blot using anti-HB-EGF antibody #2998 followed by HRPO-conjugated anti-rabbit IgG as described in MATERIALS AND METHODS. Recombinant HB-EGF (rHB-EGF) and highly purified U-937 HB-EGF were used as standards. (F) Aliquots of the CM of Vero H cells treated (closed circles) or not treated with 64 nM TPA (open circles) were assayed for their ability to stimulate DNA synthesis in EP170.7 cells at various time

Table 1. Amino acid composition analysis of the HB-EGF carboxy-terminal peptide

Components	Exp. values (pmol)	Relative values[a] (integral no.)	Theoretical values[b]
D (+N)[c]	N.D.[d]	--- (−)	0
T	N.D.	--- (−)	0
S	0.19	0.8 (1)	1
E (+Q)	0.18	0.8 (1)	1
G	0.49	2.2 (2)	2
A	N.D.	--- (−)	0
V	N.D.	--- (−)	0
C	N.D.	--- (−)	1
I	N.D.	--- (−)	0
L	0.46	2.0 (2)	2
Y	N.D.	--- (−)	0
F	N.D.	--- (−)	0
K	N.D.	--- (−)	0
H	0.41	1.8 (2)	2
R	0.21	0.9 (1)	1
P	0.22	1.0 (1)	1

[a] Relative values were calculated based on the contents of leucine residues.

[b] Theoretical values show the amino acid composition of a tentative carboxy-terminal fragment derived from chymotrypsin digests if the soluble HB-EGF were terminated at Pro^{148}.

[c] Amino acids are represented by a single letter.

[d] N.D., not detected.

tryptic fragments (K. Lau and J.A. Abraham, personal communication).

TPA Induces the Loss of proHB-EGF Juxtacrine Activity and the Increase of Soluble HB-EGF Paracrine Growth Factor Activity

It was demonstrated that processing by TPA resulted in the loss of cellular juxtacrine activity. Juxtacrine and paracrine growth factor activities of HB-EGF were analyzed simultaneously before and after TPA treatment. Juxtacrine activity was monitored by measuring the ability of Vero H cells to stimulate DNA synthesis of EP170.7 cells in coculture. Paracrine activity was monitored by measuring the ability of Vero H cell CM to stimulate EP170.7 DNA synthesis. To analyze paracrine and juxtacrine activities quantitatively, we prepared standard curves of both soluble and proHB-EGF mitogenic activities for EP170.7 cells. One nanogram/200 μl/well of soluble HB-EGF is equivalent to about 20 ng/200 μl/well of proHB-EGF in terms of mitogenic activity (Figure 6A).

After a 30-min incubation of Vero H cells with and without TPA, the paracrine activity of soluble HB-EGF in the CM and the juxtacrine activity of proHB-EGF on the cell surface were measured and quantified based on the standard curves. It was calculated that 10^5 Vero H cells expressed approximately the equivalence of 100 ng purified recombinant HB-EGF, more than 99% of which was cell associated proHB-EGF (Figure 6B).

A 30-min incubation of Vero H cells with TPA diminished HB-EGF juxtacrine growth factor activity by 70% and at the same time increased paracrine growth factor activity by 70% (Figure 6B). Anti-HB-EGF neutralizing antibody inhibited both membrane-associated and soluble growth factor activities, demonstrating that both the juxtacrine and paracrine growth factor activities were due to HB-EGF.

CD9 is a transmembrane protein that physically interacts with proHB-EGF/DT receptor and markedly up-regulates DT sensitivity (Iwamoto *et al.*, 1994) and juxtacrine growth factor activity (Higashiyama *et al.*, 1995). Anti-CD9 antibody inhibited juxtacrine proHB-EGF growth factor activity but not paracrine soluble HB-EGF growth factor activity. These results indicate that juxtacrine and paracrine growth factor activities are mediated by membrane associated proHB-EGF and soluble HB-EGF, respectively.

Internalization of proHB-EGF

Although the half-life of constitutive proHB-EGF loss from the cell surface was about 1.5 h (Figure 5, C and D), the constitutive accumulation of soluble HB-EGF into CM was much slower (Figure 5, E and F). These results suggested that substantial proHB-EGF might be internalized without being processed. To examine the internalization of proHB-EGF, Vero H cells were incubated with anti-HB-EGF antibody at 4°C, transferred to 37°C for 2 h, and cell surface immunofluorescent with FITC staining was analyzed. At 4°C, there was intense fluorescent staining at the point of cell to cell contact (Figure 7A), which was diminished markedly by washing with pH 2.0 buffer, which abrogates the total binding of anti-HB-EGF antibody to HB-EGF on the cell surface (Figure 7B). When the Vero H cells incubated with anti-HB-EGF antibodies at 4°C were shifted to 37°C for 2 h, fluorescence intensity decreased markedly (Figure 7C). Washing these cells with pH 2.0 buffer virtually eliminated fluorescent staining (Figure 7D). When cells were permeabilized to allow FITC-conjugated antibody uptake, intense staining of Vero H cells was observed (Figure 7E) even if they were washed with pH 2.0 buffer before permeabilization (Figure 7F). The staining was diffuse throughout the permeabilized cells and there was no

Figure 5 cont. intervals post-TPA treatment. The differences in growth factor activity between paired samples of TPA treated or nontreated cells were calculated (closed squares). Within the asterisk are shown the values of growth factor activity after incubation with anti HB-EGF neutralizing antibody #197, of samples collected after a 24-h incubation with TPA (open circle), or without TPA (closed circle).

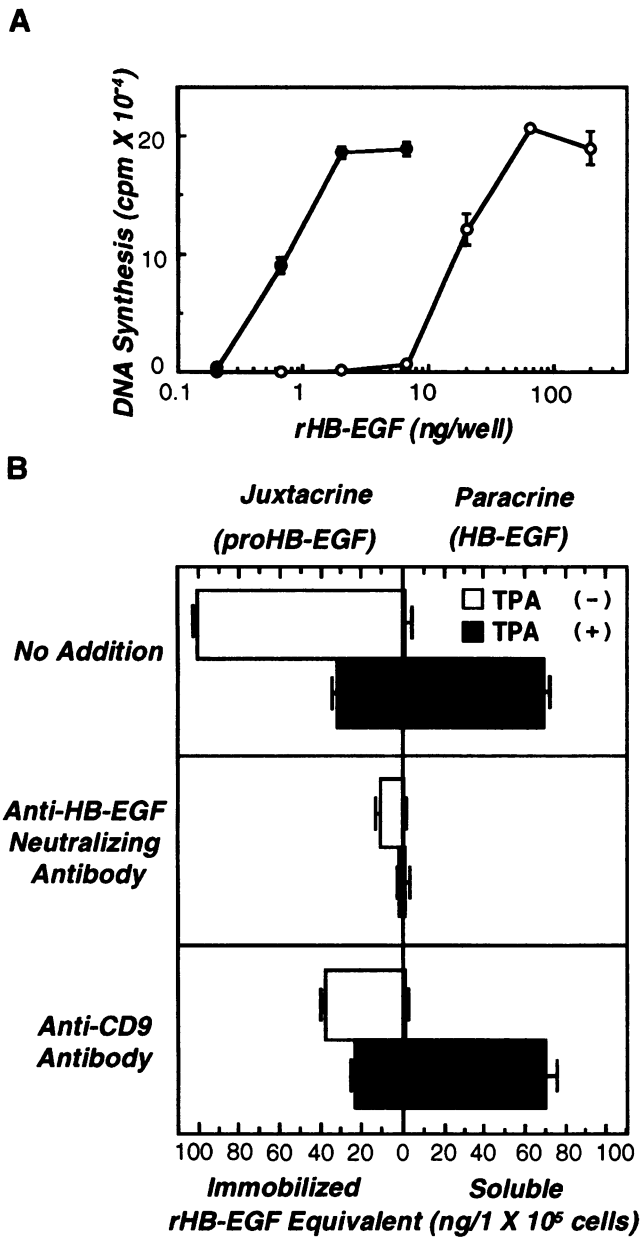

Figure 6. Conversion of HB-EGF juxtacrine activity to paracrine growth factor activity by TPA. (A) Standard curves showing EP170.7 cell growth stimulation were prepared using soluble (closed circle) and immobilized (open circle) recombinant HB-EGF in 96-well plates. (B) Vero H cells (10^5 cells/well) were incubated with (closed bar) and without (open bar) 64 nM TPA for 30 min. Juxtacrine and paracrine growth factor activities for EP170.7 cells were assayed in the absence or the presence of anti-HB-EGF neutralizing antibody #197, and in the presence of anti-CD9 antibody, as described in MATERIALS AND METHODS. HB-EGF protein content on the cell surface and in CM was calculated from standard curves as described in panel A.

staining at the cell-cell contact points. These results demonstrated that cell surface proHB-EGF was internalized constitutively and suggested that the internalization could have been responsible for the low levels of HB-EGF being released into CM under constitutive conditions. However, it is also possible that antibody binding to cell surface–associated proHB-EGF accelerates the internalization of proHB-EGF. Accordingly, to rule out this possibility, we demonstrated constitutive internalization using immunoprecipitation of biotinylated proHB-EGF. Vero H cell surface proteins were biotinylated and incubated for an appropriate period of time at 37°C in the presence or absence of bafilomycin A1, an inhibitor of endosome acidification that causes a low efficiency of protein degradation (Umata *et al.*, 1990). Pronase was used to digest any remaining biotinylated proHB-EGF on the cell surface so that any biotinylated HB-EGF immunoprecipitated by anti-HB-EGF antibody must have been internalized. After a 30-min incubation in the presence of bafilomycin A1, immunoprecipitation detected HB-EGF species in Vero H cells (Figure 8) with the same molecular masses as cell surface proHB-EGF. No HB-EGF species were detected in the absence of bafilomycin A1. These results demonstrated that cell surface proHB-EGF was constitutively internalized in the absence of exogenous stimuli.

DISCUSSION

We have demonstrated that HB-EGF exists in two forms, a cell surface-associated HB-EGF precursor proHB-EGF, and a soluble growth factor released by cells into CM. Conversion of cell-associated proHB-EGF to soluble HB-EGF occurs constitutively at a relatively slow rate but can be induced to occur at a much faster rate by TPA. Concomitant with this processing event, the juxtacrine activity of cell-associated proHB-EGF is abrogated, and soluble mature HB-EGF (a paracrine growth factor) is generated.

ProHB-EGF processing in Vero H cells was monitored in several ways: by flow cytometry, by immunofluorescence staining, by immunoprecipitation of biotinylated HB-EGF, and by Western blot. ProHB-EGF can be localized to the cell surface and is heterogeneous, with molecular masses of about 20–30 kDa. There is constitutive turnover of proHB-EGF with a half-life of about 1.5 h and soluble 14-kDa HB-EGF is released into CM, albeit slowly. Interestingly, most of the cell surface proHB-EGF is internalized rather than released, suggesting that HB-EGF processing is inefficient. The internalization pathway might be used by DT to enter cells (Almond and Eidels, 1994). In contrast with constitutive processing, TPA-inducible processing is rapid with a half-life of 7 min, and highly efficient with over 70% of the proHB-EGF being lost from the cell surface within 15–30 min. The rapid loss of cell surface proHB-EGF is consistent with previous studies showing that phorbol ester diminishes DT sen-

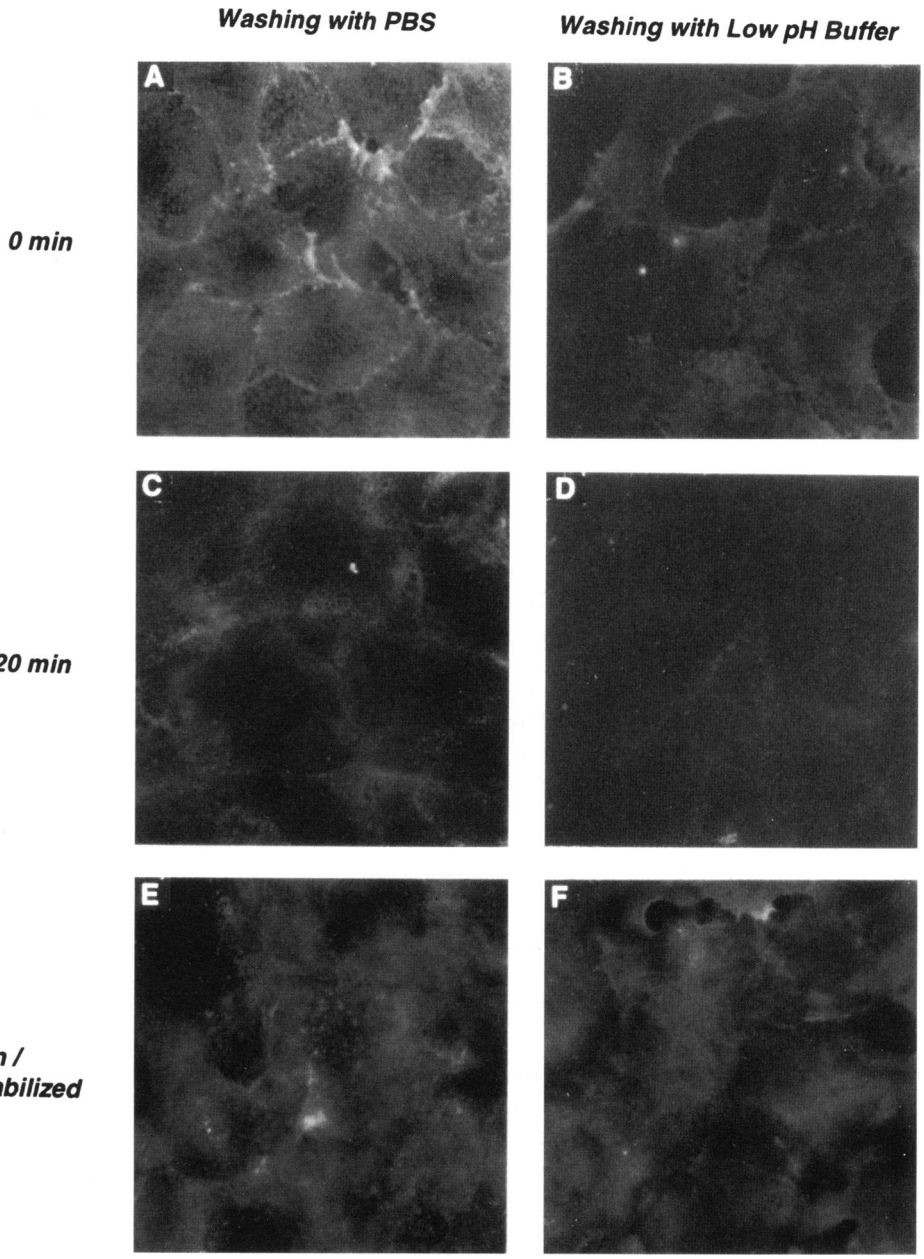

Figure 7. Internalization of proHB-EGF. Vero H cells were incubated with rabbit anti-HB-EGF antibody #H6, followed by washing with PBS (A, C, and E) or with 0.2 M glycine-HCl buffer, pH 2.0 (B, D, and F), and subsequent immunostaining with FITC-conjugated anti-rabbit IgG. (A and B) Cells were incubated with anti-HB-EGF antibody #H6 for 1 h at 4°C and stained with FITC-conjugated antibody. (C and D) Same as panels A and B except that following incubation with anti-HB-EGF antibody #H6, cells were transferred to 37°C for 2 h. (E and F) Same as panels C and D except that cells were permeabilized with 0.3% Triton X-100/PBS after fixation, and then stained with FITC-conjugated antibody.

sitivity (Sandving and Olsnes, 1981; Guillemot *et al.*, 1985; Olsnes *et al.*, 1986). The rate of loss of 20- to 30-kDa cell surface proHB-EGF is correlated with the appearance of soluble bioactive HB-EGF in CM. However, unlike constitutive processing, TPA induces the release of 19-kDa HB-EGF as well as 14-kDa HB-EGF. The significance of these different patterns of processing and release of soluble HB-EGF species is unclear. An important feature of TPA-inducible processing is that after processing is completed, proHB-EGF begins to reemerge on the cell surface. Maximal loss of cell-surface proHB-EGF after addition of TPA occurs within 30–60 min and the original proHB-EGF levels are restored by 12–24 h after addition of TPA. Besides inducing processing, TPA has other traits such as inducing gene transcription. It has been reported that TPA up-regulates the transcriptional level of HB-EGF mRNA in a number of cell types such as endothelial cells, SMC, and monocytes (Temizer *et al.*, 1992; Yoshizumi *et al.*, 1992; Dluz *et al.*, 1993; Ito *et al.*, 1994). However, maximal induction by TPA of elevated HB-EGF mRNA levels occurs within an hour, and induc-

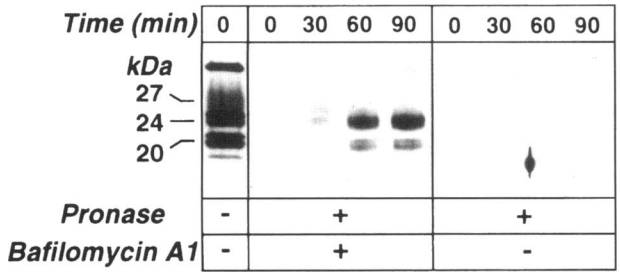

Figure 8. Immunoprecipitation of internalized proHB-EGF. Vero H cells were incubated in the presence or absence of bafilomycin A1. The cells were biotinylated as described in MATERIALS AND METHODS and incubated for the indicated time at 37°C. The cells were immediately treated with pronase and immunoprecipitated using anti-HB-EGF antibody #H6. The immunoprecipitates were analyzed by SDS-PAGE followed by Western blotting as described in MATERIALS AND METHODS. The bands on a nitrocellulose membrane were detected by an ECL kit.

tion of HB-EGF protein takes longer, while processing occurs within 15–30 min. These results suggest that TPA-inducible HB-EGF processing and HB-EGF mRNA and protein induction are independent events.

TPA has previously been demonstrated to induce the processing of a number of membrane-anchored proteins such as TGF-α (Pandiella and Massague, 1991a, 1991b), L-selectin (Kahn *et al.*, 1994), and CSF-1 (Stein and Rettenmier, 1991). HB-EGF has about a 30–40% structural homology to TGF-α (Higashiyama *et al.*, 1991, 1992) and there are some similarities in the processing of these two cell-associated growth factors. For example, both share low basal processing, a rapid induction of processing by phorbol ester at or near the cell surface, and the inhibition of processing by PKC inhibitors such as staurosporine and H7 (Bosenberg *et al.*, 1993). Cleavage of proTGF-α occurs in two steps: a rapid one of about 15 min occurring at the N-terminus, and a rate-limiting step of about 4 h occurring at the C-terminus (Teixido *et al.*, 1990; Pandiella and Massague, 1991b). The second step can be activated by TPA and is thought to be mediated by a PKC-dependent mechanism that activates an elastase-like enzyme. Both N-terminal and C-terminal TGF-α cleavages occur at Ala-Val sites (Derynck *et al.*, 1984; Lee *et al.*, 1985). HB-EGF has no N-terminal Ala-Val sites but does have two Ala-Val sites in the transmembrane domain (Higashiyama *et al.*, 1991). However, these sites don't appear to be involved in HB-EGF processing. N-terminal cleavage of HB-EGF occurs at an Arg^{62}–Asp^{63} site (Ono *et al.*, 1994) whereas C-terminal cleavage of HB-EGF occurs at a Pro^{148}–Val^{149} site. Thus, although there may be similarities in the mechanisms that govern TGF-α and HB-EGF processing, the cleavage sites and the corresponding cleavage enzymes may be different.

The significance of regulated proHB-EGF processing may be as a mechanism for regulating growth factor activity. Essentially, a juxtacrine activity that involves mediating cell to cell contact via the EGF receptor is replaced by a paracrine activity that stimulates cell proliferation, presumably not dependent on cell to cell contact. Many epithelial cells, for example, keratinocytes (Hashimoto *et al.*, 1994), prostate epithelial cells (Freeman and Klagsbrun, unpublished results), uterine epithelium involved in blastocyst adhesion (Das *et al.*, 1994), and breast carcinoma cells (Raab *et al.*, 1994) produce predominantly the cell surface-associated proHB-EGF. Thus, cell surface proHB-EGF might mostly mediate cell to cell interactions in epithelia. On the other hand, inflammatory cells, such as monocytes/macrophages (Besner *et al.*, 1990; Higashiyama *et al.*, 1991) and T lymphocytes (Blotnick *et al.*, 1994) appear to release mature HB-EGF constitutively. These cells circulate and have as a function the ability to deliver cytokines. We speculate that release of soluble paracrine HB-EGF from cell surface proHB-EGF might be an important regulatory mechanism in certain processes such as wound healing (Marikovsky *et al.*, 1993) and inflammation. One could envision, for example that following injury, soluble HB-EGF is rapidly released via a PKC pathway to stimulate proliferation of injured cells. Subsequently, newly synthesized proHB-EGF appears on the cell surface again, acting as a juxtacrine factor involved in maintaining cell to cell contact. In pathological processes such as atherogenesis, released HB-EGF might be responsible for the SMC hyperplasia that occurs (Miyagawa *et al.*, 1995). The cell-associated form of proHB-EGF has some unique functions such as being the receptor for DT (Naglich *et al.*, 1992; Iwamoto *et al.*, 1994). In addition, uterine epithelial cell proHB-EGF might be involved in blastocyst attachment (Das *et al.*, 1994). Given these and other potential physiologically relevant properties of HB-EGF, further studies are needed to elucidate the mechanisms that regulate proHB-EGF processing and release from cells.

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