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The Shedding of Membrane-anchored Heparin-binding Epidermal-like Growth Factor Is Regulated by the Raf/Mitogen-activated Protein Kinase Cascade and by Cell Adhesion and Spreading*

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Ze'ev Gechtman, José Luis Alonso, Gerhard Raab, Donald E. Ingber, and Michael Klagsbrun‡

From the Departments of Surgical Research and Pathology, Children's Hospital and Harvard Medical School, Boston, Massachusetts 02115

Heparin-binding epidermal-like growth factor (HB-EGF) is synthesized as a transmembrane precursor (HB-EGF_{TM}). The addition of phorbol ester (PMA, phorbol 12-myristate 13-acetate) to cells expressing HB-EGF_{TM} results in the metalloproteinase-dependent release (shedding) of soluble HB-EGF. To analyze mechanisms that regulate HB-EGF shedding, a stable cell line was established expressing HB-EGF_{TM} in which the ectodomain and the cytoplasmic tail were tagged with hemagglutinin (HA) and Myc epitopes, respectively (HB-EGF_{TM}HA/Myc). HB-EGF_{TM}HA/Myc cleavage was followed by the appearance of soluble HB-EGFHA in conditioned medium, the loss of biotinylated cell-surface HB-EGF_{TM}HA/Myc, and the appearance of a Myc-tagged cytoplasmic tail fragment in cell lysates. By using this approach, several novel metalloproteinase-dependent regulators of HB-EGF_{TM} shedding were identified as follows. (i) HB-EGF_{TM}HA/Myc shedding induced by PMA was blocked by the mitogen-activated protein (MAP) kinase kinase inhibitor, PD98059. PMA activated MAP kinase within 5 min, but HB-EGF_{TM}HA/Myc shedding did not occur until 20 min, suggesting that MAP kinase activation was a necessary step in the pathway of PMA-induced HB-EGF_{TM} cleavage. (ii) Activation of an inducible Raf-1 kinase, ΔRaf-1:estrogen receptor, resulted in a rapid MAP kinase activation within 10 min and shedding of HB-EGF_{TM}HA/Myc within 20–40 min. (iii) Serum induced MAP kinase activation and HB-EGF_{TM}HA/Myc shedding that were inhibited by PD98059. (iv) Whereas PMA induced HB-EGF_{TM}HA/Myc shedding in attached cells, no shedding occurred when the cells were placed in suspension. Shedding was fully restored shortly after cells were allowed to spread on fibronectin, and the extent of PMA-induced shedding increased with the extent of cell spreading. PMA induced the same level of MAP kinase activation whether the cells were attached or in suspension suggesting that although MAP kinase activation might be necessary for shedding, it was not sufficient. Taken together, these results suggest that there are two components of cell regulation that contribute to the shedding process, not previously recognized, the Raf-1/MAP kinase signal transduction pathway and cell adhesion and spreading.

The extracellular domains of many membrane-anchored proteins are proteolytically cleaved from the cell surface in a process termed as shedding. Shedding is an irreversible post-translational modification that regulates biological function by releasing growth factors, enzymes, and soluble receptors (1–3). For example, shedding converts a juxtacrine growth factor such as the membrane-anchored TGF-α[1] precursor into a potent paracrine growth factor (4–6). Phorbol esters, such as PMA, are among the best characterized inducers of shedding. PMA treatment of cells results in metalloproteinase-dependent proteolytic cleavage of cell-surface-anchored precursors such as TGF-α, β-APP (6), and TNF-α (7). The PMA-induced shedding of TGF-α has been well characterized (8). It has been suggested that all the components required for TGF-α shedding are located at or close to the cell surface (9). There may be a common mechanism for PMA-induced shedding since a mutant CHO cell line isolated for its inability to cleave TGF-α was unable to cleave β-APP and a variety of other cell-surface molecules in response to PMA (6).

In PMA-induced shedding, the enzymes responsible for proteolytic cleavage and release appear to be metalloproteinases since shedding is blocked by synthetic hydroxamic acid-based compounds that are metalloproteinase inhibitors (10–15). Among the metalloproteinases, the disintegrin metalloproteinases known as ADAMs (A Disintegrin and a Metalloproteinase) have been strongly implicated in shedding (2, 3). ADAM17 had been cloned and identified as the TNF-α-converting enzyme (16, 17). Recent studies suggest that ADAM17/TNF-α-converting enzyme cleaves other cell-surface molecules such as interleukin receptor (18), β-APP (19), L-selectin, and TGF-α (20). Another ADAM family member, MDC9 (ADAM9/Meltrin γ), has been recently shown to be involved in the shedding of HB-EGF_{TM} (21).

Protein phosphorylation may be involved in the regulation of shedding. The PMA-induced shedding of TGF-α, β-APP (8, 14), L-selectin (22), TNF-α and its receptors (7, 23, 24), HER-4/ErbB4 (25), and HB-EGF_{TM} (26, 27) are all inhibited by the relatively nonspecific protein kinase inhibitor staurosporin. Ty-

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‡ To whom correspondence should be addressed: Children's Hospital, 300 Longwood Ave., Boston, MA 02115. Tel.: 617-355-7503; Fax: 617-355-7291; E-mail: klagsbrun@a1.tch.harvard.edu.

[1] The abbreviations used are: TGF-α, transforming growth factor-α; TNF-α, tumor necrosis factor-α; HB-EGF, heparin-binding EGF-like growth factor; EGF, epidermal growth factor; PMA, phorbol 12-myristate 13-acetate; MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MDC9, metalloproteinase/disintegrin/cysteine-rich protein 9; ADAM, a disintegrin and metalloproteinase; β-APP, β-amyloid precursor protein; α-MEM, α-minimal essential medium; CHO, Chinese hamster ovary; HA, hemagglutinin; CM, conditioned medium; MEK, MAP kinase/ERK; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ER, estrogen receptor; CAPS, 3-(cyclohexylamino)propanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid.

rosine phosphorylation (28) and phosphatase inhibitors promote shedding. For example, the shedding of β-APP (29) and TNF-α receptors (30) is induced by okadaic acid, and the shedding of syndecan-1 (31), ErbB4/HER-4 and amphiregulin (32) is induced by pervanadate.

The mechanisms by which PMA induces shedding are still for the most part unknown. To address this question we examined possible mechanisms involved in the PMA-induced shedding of HB-EGF. HB-EGF is a member of the EGF family of growth factors (33) that is synthesized as a membrane-anchored molecule (HB-EGF_{TM}), capable of supporting cell-cell adhesion (34) and juxtacrine stimulation (26, 35). HB-EGF_{TM} is also the receptor for diphtheria toxin (36). PMA treatment induces cleavage of HB-EGF_{TM} within 15 min in a number of cell lines (15, 26, 27, 37). There is a loss of cell-surface associated HB-EGF_{TM}, acquisition of cell resistance to diphtheria toxin (37) and release of the mature soluble form of HB-EGF into conditioned medium (CM) (15, 27, 37). Cleavage of HB-EGF_{TM} is inhibited by metalloproteinase inhibitors (15, 27, 38). Mature soluble HB-EGF is a potent stimulator of cell proliferation and migration, for example of smooth muscle cells (SMC), fibroblasts, and keratinocytes (39–41). HB-EGF may play a role in SMC hyperplasia (39). Its expression is up-regulated in the neointima following balloon injury to rat carotid arteries (42) and in rat models of pulmonary hypertension (43). In addition, it has been detected in medial SMC and in foamy macrophages found in human atherosclerotic plaques (44). It may be that aberrant shedding of HB-EGF may contribute to these pathologies.

Since the conversion of HB-EGF_{TM} to mature soluble HB-EGF has possible physiological and pathological implications, we have further analyzed mechanisms of PMA-induced shedding. In this report we identify several previously unrecognized regulators of HB-EGF_{TM} shedding. These are the Raf-1/MAP kinase cascade and cell adhesion and spreading.

EXPERIMENTAL PROCEDURES

Materials—All cell culture reagents were purchased from Life Technologies, Inc. Anti-phospho-ERK1/2 antibodies and PD98059 were purchased from Calbiochem. Polyclonal goat anti-ERK1/2, polyclonal rabbit anti-Raf-1, and monoclonal anti-Myc antibodies 9E10 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Tamoxifen (4-hydroxy) was purchased from Research Biochemicals International (Natick, MA). Fibronectin was purchased from Becton Dickinson (Franklin Lakes, NJ). Heparin-agarose was purchased from Sigma. EZ-link-sulfo-NHS-Biotin was purchased from Pierce. Gamma bind protein G-Sepharose, was purchased from Amersham Pharmacia Biotech. Horseradish peroxidase-conjugated streptavidin, horseradish peroxidase-conjugated anti-rabbit IgG, and Complete™ mixture of protease inhibitors were purchased from Roche Molecular Biochemicals. Horseradish peroxidase-conjugated anti-mouse IgG was purchased from Promega (Madison, WI). The hydroxamic acid-based metalloproteinase inhibitor, BB3489, was kindly provided by British Biotech (Oxford, UK).

Cell Culture—Chinese hamster ovary (CHO-K1) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in α-minimal essential medium (α-MEM) supplemented with 10% fetal calf serum, 1% glutamine, 1% penicillin and streptomycin, in 5% CO_2. CHO-HB-EGF_{TM}HA/Myc cells were grown to 90–95% confluence in 10-cm dishes (1.8×10^6 cells/dish).

Preparation of Cells Expressing HA, Myc-tagged HB-EGF_{TM}—HB-EGF_{TM}HA/Myc was constructed so that the hemagglutinin (HA) epitope was inserted in the N-terminal extracellular domain between amino acids Leu^{83} and Thr^{85}, and the Myc epitope was placed at the C terminus (Fig. 1*A*). The doubled-tagged construct was prepared as follows. First HB-EGF_{TM}/*myc* was prepared by polymerase chain reaction amplification of the complete open reading frame of HB-EGF cDNA (33) using synthetic DNA oligonucleotide primers: a forward primer, 5′-GCTCTAGAGCATGAAGCTGCTGCCGTCG-3′ corresponding to the 5′ end of the full-length HB-EGF open reading frame, and a reverse primer, 5′-GCTCACAGATCCTCTTCTGAGATGAGTTTTTGTTCGTGGGAATTAGTCATGCC-3′, complementary to the nucleotide sequence of a Myc tag followed by the 3′ end of the full-length HB-EGF. The polymerase chain reaction product was ligated into a pCR3.1 mammalian expression vector using the TA cloning kit (Invitrogen, Carlsbad, CA). HB-EGF_{TM}*ha*/*myc* was prepared using two complementary oligonucleotides 3′-CCTACCCATACGACGTCCCAGACTACG-5′ and 5′-CGTAGTCGTGGACGTCGTATGGGTAGG-3′ encoding the HA epitope flanked by an *Msc*I site. The oligonucleotides were synthesized and annealed to each other and then inserted into a unique *Msc*I site in HB-EGF_{TM} (between amino acids Leu^{83} and Thr^{85}). The correct sequence of HB-EGF_{TM}*ha*/*myc* was confirmed by DNA sequence analysis. For expression, the HB-EGF_{TM}*ha*/*myc* insert was excised from the pCR3.1 vector after an *Eco*RI digest and subcloned into the *Eco*RI site of the pIRES/neo mammalian expression vector (CLONTECH, Palo Alto, CA). The resulting plasmid pIRES/neo-HB-EGF_{TM}*ha*/*myc* was transfected into CHO-K1 cells using LipofectAMINE and opti-MEM transfection medium (Life Technologies, Inc.) according to the manufacturer's instructions. Twenty four hours post-transfection, cells were passaged 1:25 and plated on 10-cm tissue culture dishes. They were grown in α-MEM supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, and 1 mg/ml G418 in 5% CO_2. After 12 days stable clones were selected, expanded, and conditioned media (CM) were collected and tested for the presence of HA-tagged soluble HB-EGF ectodomain by Western blotting with anti-HA antibodies. Five independent clones that overexpressed HB-EGF_{TM}HA/Myc were expanded and characterized.

Expression of of ΔRaf-1:ER cDNA—CHO-HB-EGF_{TM}HA/Myc cells were grown overnight to approximately 65% confluence in 10-cm dishes. They were transfected transiently using LipofectAMINE as above with ΔRaf-1:ER plasmid DNA (16 μg/10-cm dish) alone (provided by Dr. Martin McMahon, University of California, San Francisco/Mt. Zion Cancer Center, San Francisco) (45) or co-transfected with HA-ERK1 cDNA (provided by Dr. John Blenis, Harvard Medical School) (46) and ΔRaf-1:ER cDNA. In co-transfection experiments, the ratio of ΔRaf-1:ER cDNA to HA-ERK1 cDNA was 10-fold. The total amount of cDNA did not exceed 16 μg/10-cm dish. CHO-HB-EGF_{TM}HA/Myc analysis was carried out 22–24 h post-transfection. For stable expression, the ΔRaf-1:ER cDNA construct was transfected into CHO-HB-EGF_{TM}HA/Myc cells as above. Twenty-four hours post-transfection, cells were passaged 1:25 and plated on 10-cm tissue culture dishes. Cells were grown in α-MEM supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, 0.5 mg/ml G418, and 5 μg/ml puromycin (CLONTECH, Palo Alto, CA) in 5% CO_2. After 9 days stable clones were selected, expanded, and assayed for activation of MAP kinase in response to tamoxifen. Five independent clones were chosen for further studies.

Cell-surface Biotinylation—Cells were washed twice with 20 mM Hepes buffer, pH 7.2, 150 mM NaCl (HBS), and incubated on ice with EZ-link-NHS-sulfo-biotin (Pierce, 0.1 mg/ml) in HBS, for 10 min in order to minimize the internalization of cell-surface HB-EGF_{TM}. After aspiration, the cells were washed twice with 20 mM Tris-HCl, pH 7.2, 150 mM NaCl to quench the biotinylation reaction. The cells were washed with HBS, and serum-free α-MEM supplemented with 0.05% BSA (5 ml/plate) was added to cells prior to use.

Cell Fractionation—Cells from a 10-cm dish were harvested by scraping into 1 ml of phospho-homogenization buffer that contained 20 mM sodium phosphate, pH 7.2, 50 mM NaCl, 250 mM sucrose, 2 mM EDTA, 0.5 mM sodium orthovanadate, 10 mM NaF, 5 mM sodium pyrophosphate, and a mixture of protease inhibitors (SPH buffer), and then homogenized by passing six times through a 26.5-gauge needle. The nuclei were pelleted by centrifugation at $400 \times g$. Fractions containing HB-EGF_{TM}HA/Myc were obtained by centrifugation of the post-nuclear supernatants at $15{,}800 \times g$ (P2). HB-EGF_{TM}HA/Myc was solubilized by resuspending the P2 pellets in SPH buffer supplemented with Triton X-100 (1% final concentration) and incubating on ice for 10 min. The Triton X-100-insoluble material was pelleted by brief centrifugation at $15{,}800 \times g$. Biotinylation studies have shown that virtually all cell-surface HB-EGF_{TM}HA/Myc is contained in P2.

Suspension and Re-plating of Cells—Cells grown overnight were washed once with phosphate-buffered saline (PBS) and then detached by incubation with PBS supplemented with 5 mM EDTA for 5 min at 37 °C, 5% CO_2. Cells were washed with Hepes-buffered serum-free α-MEM supplemented with 0.1% BSA and resuspended in serum-free medium containing 0.1% BSA. Cells were either maintained in suspension for 30 min or re-plated after 30 min on bacterial dishes precoated with fibronectin at various densities (0–2500 cm^2), as described previously (47).

SDS-PAGE and Western Blotting—Proteins were resolved on 10 or 15% SDS-PAGE for MAP kinase/ERK or HB-EGF detection, respec-

tively. Proteins were electroblotted for 1.5 h onto a polyvinylidene difluoride membranes (Bio-Rad) in 40 mM CAPS buffer, pH 10.5, in 15% methanol. A constant 24 V was applied. For detection of HB-EGF$_{TM}$HA/Myc and HB-EGFHA, the membranes were blocked with 3% bovine serum albumin in PBS, 0.25% Tween 20 (PBST). The blots were first incubated with anti-HA or anti-Myc monoclonal antibodies (1:5000) and then with anti-mouse IgG conjugated to horseradish peroxidase (1:5000). For detection of phospho-ERK and total ERKs in the blotting, Tris-buffered saline was substituted for PBS. The blots were incubated with anti-phospho-ERK or anti-ERK1/2 antibodies in (1:2000), followed by anti-rabbit or anti-goat IgG, respectively, conjugated to horseradish peroxidase (1:5000). To detect cell-surface biotinylation after immunoprecipitation with anti-HA, biotinylated proteins were detected using horseradish peroxidase-coupled streptavidin (1:5000). The blots were developed using an enhanced chemiluminescence (ECL) kit according to the manufacturer's instructions (NEN Life Science Products).

Immunoprecipitation—Cells from a 10-cm dish were scraped into 1 ml of SPH buffer. Triton X-100 was added to 1% final concentration, and the cells were lysed for 20 min on ice. The insoluble material was pelleted by centrifugation at 14,000 × *g* at 4 °C for 10 min. Supernatants were precleared by incubation with 40 μl of protein G-Sepharose (50% v/v slurry), for 1 h at 4 °C and incubated overnight with 0.2 μg of the appropriate antibody. The immune complexes were collected by incubating the samples with 40 μl of protein G-Sepharose (50% v/v slurry) for 1.5 h at 4 °C, washed four times with lysis buffer, and boiled in 2× Laemmli's sample buffer.

Quantification of HB-EGF$_{TM}$HA/Myc Cleavage and MAP Kinase Activation—The extent of the PMA-induced HB-EGF$_{TM}$HA/Myc cleavage and MAP kinase activation was quantified by densitometric scanning of films obtained after ECL using a UMAX PowerLookII scanner and the NIH Image program. The extent of cleavage was calculated by dividing the amount of the intact HB-EGF$_{TM}$HA/Myc prior to PMA treatment by the amount of intact HB-EGF$_{TM}$HA/Myc after PMA treatment and corrected for loading. The extent of HB-EGF$_{TM}$ cleavage and MAP kinase activation is expressed in arbitrary units.

Differential Interference Contrast Optic Microscopy—Cells were transferred at 37 °C to prewarmed 0.5% glutaraldehyde in PBS. This fixative was replaced with 1% glutaraldehyde in PBS for 10 min. Fixed specimens were washed two times with PBS and once with PBS supplemented with 0.1% bovine serum albumin. The coverslips were mounted in 50 μl of Fluormount-G (Southern Biotechnology Associates, Inc., Birmingham, AL) before being sealed with nail polish. Cells were examined in a Nikon Diaphot 300 inverted microscope, using a Nikon 40× PlanFluor objective and Nomarsky differential interference contrast optic microscopy to visualize the degree of flattening and the cell surface structure of cells. Digital images were captured using a Sensys KAF 1400 cooled output camera (Photometrics, Tuscon, AZ) and acquired with an IPLab image analysis program (Scanalytics, Inc., Fairfax, VA).

RESULTS

An Assay System for Detecting PMA-induced HB-EGF$_{TM}$ Cleavage—To facilitate analysis of the shedding of membrane-anchored HB-EGF (HB-EGF$_{TM}$), the ectodomain and the cytoplasmic tail of human HB-EGF$_{TM}$ were tagged with hemagglutinin and Myc epitopes, respectively, to produce HB-EGF$_{TM}$HA/Myc (Fig. 1*A*). The HA tag was introduced immediately downstream of the propeptide domain in HB-EGF$_{TM}$ since the propeptide is often lost due to proteolytic processing by furin-like enzymes (48). A stable CHO cell line expressing HB-EGF$_{TM}$HA/Myc was prepared. Typically, HB-EGF$_{TM}$HA/Myc was expressed in CHO cells as several species ranging between 25 and 32 kDa (Fig. 1*B, lane 5*). As determined by Western blot analysis, treatment of these cells with 1 μM PMA for 40 min resulted in rapid release of 8–24 kDa HB-EGFHA into the CM (Fig. 1*B, lanes 2 versus 1*), a 7-fold reduction in biotinylated HB-EGF$_{TM}$HA/Myc levels at the cell surface (Fig. 1*B, lane 4 versus 3*), and appearance of a 16-kDa HB-EGFMyc cytoplasmic tail fragment accompanied by a loss of 25–32 kDa HB-EGF$_{TM}$ (Fig. 1*B, lane 5 vesus lane 6*). The HB-EGFHA released into CM was biologically active as demonstrated by its ability to stimulate EGF receptor tyrosine phosphorylation (not shown). Shedding, approximately 80–90%, could be induced at lower PMA concentrations as well, for example by 0.01 μM PMA for 1 h (not shown).

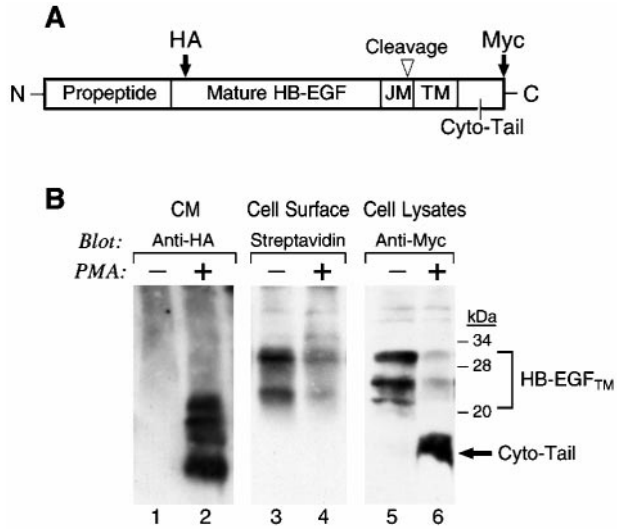

FIG. 1. **Detection of HB-EGF shedding in cells expressing double-tagged membrane-anchored HB-EGF$_{TM}$.** *A,* schematic diagram of epitope-tagged membrane-anchored HB-EGF$_{TM}$HA/Myc. *B,* Western blot. CHO-HB$_{TM}$HA/Myc cells were incubated without (*lanes 1, 3,* and *5*) or with (*lanes 2, 4,* and *6*) PMA (1 μM) for 30 min. *Lanes 1* and *2*, CM were collected and incubated with heparin-Sepharose to precipitate the HB-EGF ectodomain. Western blot was carried out with anti-HA monoclonal antibodies. *Lanes 3* and *4*, the cell surface was labeled by biotinylation prior to PMA treatment. Cell lysates were prepared, and HB-EGF$_{TM}$HA/Myc was precipitated from cell lysates with anti-HA antibodies. Western blot was carried out with streptavidin conjugated to horseradish peroxidase. *Lanes 5* and *6*, cell lysates were analyzed by Western blot with anti-Myc antibodies.

Replacement of the juxtamembrane region of HB-EGF$_{TM}$ with the corresponding region of CD4 abolishes the PMA-induced shedding of HB-EGF$_{TM}$ (15). An HB-EGF$_{TM}$HA/Myc juxtamembrane mutant (HB-EGF$_{TM}$HA/Myc/CD4) was expressed in a stable manner in CHO cells and transiently in COS7 cells. PMA did not induce the cleavage of HB-EGF$_{TM}$HA/Myc/CD4 in either cell line confirming that PMA-induced shedding is due to cleavage of HB-EGF$_{TM}$HA/Myc in the juxtamembrane domain and is not a nonspecific event (not shown). Taken together these results establish the validity and usefulness of analyzing double-tagged HB-EGF$_{TM}$ in shedding studies.

Activation of MAP Kinase by PMA Is Required for the Shedding of HB-EGF$_{TM}$—PMA treatment of CHO cells expressing HB-EGF$_{TM}$HA/Myc resulted in activation of p42 MAP kinase (ERK2), as shown by Western blot analysis using antibodies that recognize only the dually phosphorylated, fully active p42 (ERK2) and p44 (ERK1) MAP kinases (Fig. 2*B, lane 2*), consistent with previous results (49). PMA-induced MAP kinase activation was inhibited by preincubation of these CHO cells with 45 μM PD98059, an inhibitor of MAP kinase kinase (MEK) (Fig. 2*B, lane 4*). Surprisingly, PD98059 inhibited completely PMA-induced HB-EGF$_{TM}$HA/Myc cleavage (Fig. 2*A, lane 4*). In contrast, several other kinase inhibitors such as SB203580, wortmannin, and ML7, which are inhibitors of p38 kinase, phosphatidylinositol 3-kinase, and myosin-light-chain kinase, respectively, did not inhibit PMA-induced HB-EGF$_{TM}$HA/Myc cleavage (not shown). These results suggest that MAP kinase activation is in the pathway that leads to HB-EGF$_{TM}$ shedding.

A time course analysis showed that p42 MAP kinase (ERK2) was fully activated within 5 min after addition of PMA (Fig. 3*C, lane 2*). However, HB-EGF$_{TM}$HA/Myc cleavage did not occur readily until about 20 min after PMA addition, as detected by the appearance of a cleaved Myc-tagged cytoplasmic-tail fragment in cell lysates (Fig. 3*A, lane 4*) and the appearance of released HB-EGFHA in CM (Fig. 3*B, lane 4*). These results

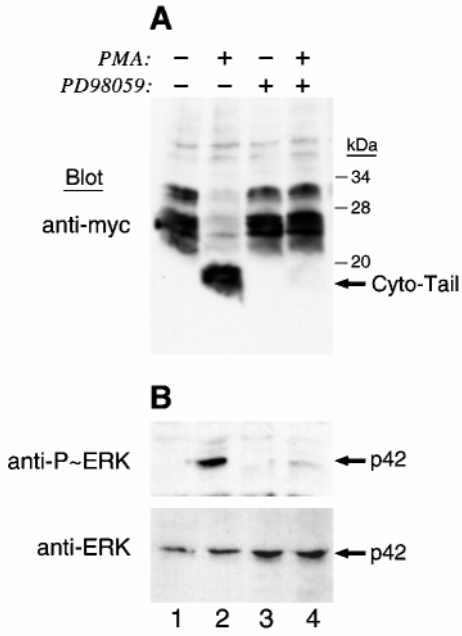

FIG. 2. **The MEK inhibitor, PD98059, blocks PMA-activated shedding of HB-EGF$_{TM}$.** CHO-HB$_{TM}$HA/Myc cells were preincubated with (*lanes 3* and *4*) or without (*lanes 1* and *2*) 25 μM PD98059 for 45 min and then with (*lanes 2* and *4*) or without (*lanes 1* and *3*) 1 μM PMA for an additional 30 min. Cell lysates were prepared and analyzed by Western blot. *A,* Western blot of HB-EGF$_{TM}$HA/Myc in cell lysates with anti-Myc antibodies as in Fig. 1*B*. *B,* Western blot of activated MAP kinase with antibodies (*upper panel*) that recognize dually phosphorylated MAP kinase only. The blot was stripped and re-probed with anti-ERK1 antibodies (*lower panel*).

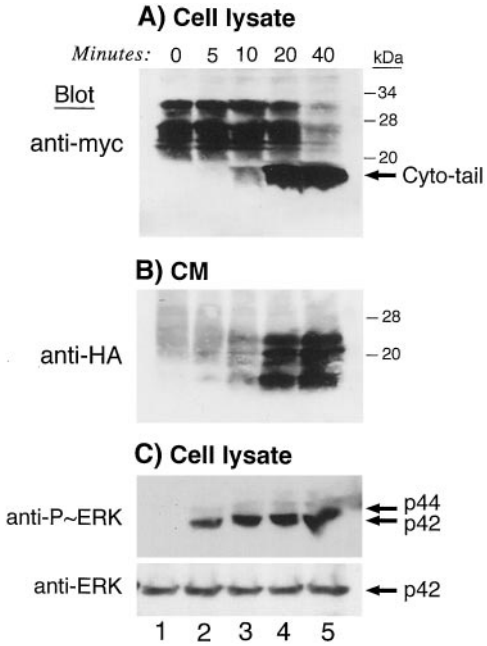

FIG. 3. **Time course of HB-EGF$_{TM}$ cleavage and MAP kinase activation.** CHO cells expressing HB-EGF$_{TM}$HA/Myc were incubated with 1 μM PMA for 0–40 min. CM was collected, and cell extracts were prepared. *A,* Western blot of cell lysates with anti-Myc antibodies as in Fig. 1*B*, *3rd panel*. *B,* Western blot of CM with anti-HA antibodies as in Fig. 1*B, 1st panel*. *C,* Western blot with anti-phospho-ERK antibodies as in Fig. 2*B*.

indicate that PMA-induced MAP kinase activation precedes HB-EGF shedding.

Activation of HB-EGF$_{TM}$ Shedding by an Inducible Raf-1 Kinase—The involvement of the MAP kinase cascade in regulating HB-EGF$_{TM}$ shedding was explored further using an inducible Raf-1 kinase (45). This fusion protein, designated ΔRaf-1:ER, consists of an estradiol-binding domain of the estrogen receptor (hbER) fused to the kinase domain of the Raf-1 kinase (CR3). Treatment of cells expressing ΔRaf-1:ER with the estradiol analogue, tamoxifen, activates the kinase domain of ΔRaf-1:ER and causes rapid activation of the MAP kinase cascade (45). Transient expression of ΔRaf-1:ER in CHO cells expressing HB-EGF$_{TM}$HA/Myc resulted in a rapid induction of HB-EGF$_{TM}$HA/Myc cleavage after addition of 1 μM tamoxifen, as detected by appearance of cleaved Myc-tagged cytoplasmic tail fragment and reduction in the amount of an intact HB-EGF$_{TM}$HA/Myc (Fig. 4*A, top, lane 2*). Tamoxifen treatment also activated HA-tagged ERK1 that was co-expressed in these cells (Fig. 4*A, bottom, lane 2*). In contrast, the kinase-inactive mutant ΔRaf-1:ER did not induce HB-EGF$_{TM}$HA/Myc shedding (Fig. 4*A, top, lane 4*) nor ERK1 activation (Fig. 4*A, bottom, lane 4*) in response to tamoxifen. A stable cell line expressing both HB-EGF$_{TM}$HA/Myc and ΔRaf-1:ER was prepared. MAP kinase activation in response to ΔRaf-1:ER stimulation with 1 μM tamoxifen could be detected by 5 min (Fig. 4*B, bottom, lane 2*) and maximally by 10 min of ΔRaf-1:ER stimulation (Fig. 4*B, bottom, lane 3*). HB-EGF$_{TM}$HA/Myc cleavage was detected initially by 20 min (Fig. 4*B, top, lane 4*), and little if any intact HB-EGF$_{TM}$HA/Myc was found after 40 min (Fig. 4*B, top, lane 5*). Lower concentrations of tamoxifen were also effective, and about 80–90% shedding was induced by treatment with 0.01 μM tamoxifen for 1 h (not shown). The MEK inhibitor PD98059 almost completely inhibited (85–90%) the ΔRaf-1:ER-induced shedding of HB-EGF$_{TM}$HA/Myc in this cell line (not shown), suggesting the MAP kinase cascade is the major signaling pathway leading to HB-EGF$_{TM}$HA/Myc shedding in response to ΔRaf-1:ER activation. Together these results suggest that the Raf-1/MEK/ERK signaling pathway regulates HB-EGF$_{TM}$HA/Myc shedding.

HB-EGF$_{TM}$ Shedding Is Metalloproteinase-dependent—Preincubation of cells with the hydroxamic acid-based metalloproteinase inhibitor BB3489 completely blocked the cleavage of HB-EGF$_{TM}$HA/Myc in response to PMA (Fig. 5*A, lane 4* compared with *lane 2*) and in response to ΔRaf-1:ER activation (Fig. 5*B, lane 4* compared with *lane 2*). These results show that HB-EGF$_{TM}$HA/Myc cleavage is dependent on metalloproteinase activity and is consistent with previous reports showing the involvement of metalloproteinases in PMA-induced shedding of membrane-anchored HB-EGF (15, 38).

Activation of HB-EGF$_{TM}$ Shedding by Serum—In order to analyze the regulation of shedding in response to more physiologically relevant stimuli than PMA and Raf-1, CHO cells expressing HB-EGF$_{TM}$HA/Myc were incubated with fresh serum, a rich source of growth factors (Fig. 6). Treatment of serum-starved cells with 10 or 20% serum for 1 h activated MAP kinase (Fig. 6*B, lanes 2* and *3*) and induced HB-EGF cleavage as determined by a 30–40% reduction in the amount of cell-associated membrane-anchored 25–32 kDa HB-EGF$_{TM}$ and by the appearance of the cytoplasmic tail fragment (Fig. 6*A, lanes 2* and *3*). Preincubation with the MEK inhibitor, PD98059, inhibited 10 and 20% serum-induced MAP kinase activity (Fig. 6*B, lanes 6* and *7*), loss of cell-associated 25–32 kDa HB-EGF$_{TM}$ (Fig. 6*A, lanes 6* and *7*), and appearance of the cytoplasmic tail fragment (Fig. 6*A, lanes 6* and *7*). A time course analysis indicated that MAP kinase was activated by 5 min and shedding occurred within 20 min (not shown). Pretreatment of cells with the metalloproteinase inhibitor, BB3489, blocked serum-induced HB-EGF shedding (Fig. 6*A, lane 8*) but, as expected, not MAP kinase activation (Fig. 6*B, lane 8*). Taken together, these results suggest that serum-

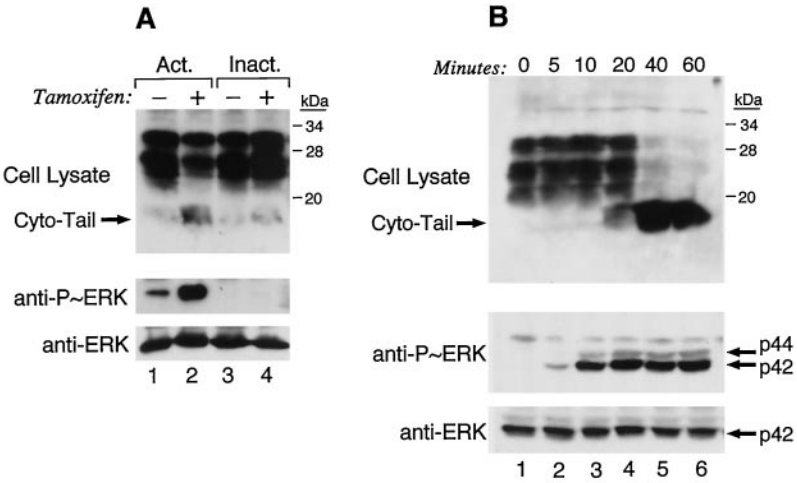

FIG. 4. **Induction of the shedding of HB-EGF$_{TM}$HA/Myc by hormone-responsive Raf-1 kinase.** *A,* CHO-HB$_{TM}$HA/Myc cells were co-transfected either with kinase-active (*Act.*) ΔRaf-1:ER (*lanes 1* and *2*) or the kinase-inactive (*Inact.*) mutant of ΔRaf-1:ER (*lanes 3* and *4*) and in both cases with HA-tagged ERK1. After 24 h, an estradiol analogue, tamoxifen (1 μM), was added (*lanes 2* and *4*) for 45 min or not added (*lanes 1* and *3*). At the end of the incubation period cell lysates were prepared. *Top,* lysates were analyzed by Western blot with anti-Myc antibodies as in Fig. 1*B*. *Bottom,* Western blot with anti-phospho-ERK antibodies as in Fig. 2*B*. *B,* a stable CHO-HB$_{TM}$HA/Myc cell-line co-expressing ΔRaf-1:ER was treated with tamoxifen (1 μM) for 0–60 min. At the end of the incubation the cells lysates were prepared and analyzed by Western blot with anti-Myc antibodies (*top*) or Western blot with anti-phospho-ERK antibodies (*bottom*).

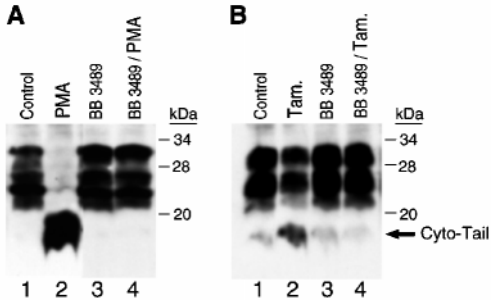

FIG. 5. **PMA- and ΔRaf-1:ER-mediated cleavages of HB-EGF$_{TM}$HA/Myc are dependent on metalloproteinase activity.** *A,* CHO cells expressing HB-EGF$_{TM}$HA/Myc were preincubated with or without 10 μM BB3489 for 60 min and subsequently with or without 1 μM PMA for 40 min. Cells lysates were analyzed by Western blot with anti-Myc antibodies. *Lane 1*, no addition; *lane 2*, PMA; *lane 3*, BB3489; *lane 4,* BB3489 followed by PMA. *B,* CHO cells expressing HB$_{TM}$HA/Myc cells were transiently transfected with ΔRaf-1:ER cDNA. After 24 h cells were incubated without (*lanes 1* and *2*) or with BB3489 (*lanes 3* and *4*) for 1 h followed by induction (*lanes 2* and *4*) or no induction (*lanes 1* and *3*) of ΔRaf-1:ER kinase by 1 μM tamoxifen (*Tam*) for 45 min. Western blot was carried out using anti-Myc antibodies as above.

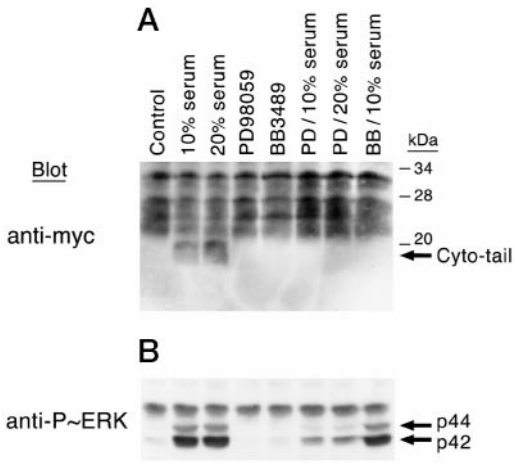

FIG. 6. **Serum induces MAP kinase- and metalloproteinase-dependent shedding of HB-EGF$_{TM}$HA/Myc.** CHO cells expressing HB-EGF$_{TM}$HA/Myc were grown to 80–90% confluence and serum-starved for 4 h. *Lane 1*, no addition; *lane 2*, addition of 10% serum for 1 h; *lane 3*, addition of 20% serum for 1 h; *lane 4*, 45 min incubation with the MEK inhibitor, PD98059 (45 μM); *lane 5*, 45 min incubation with the metalloproteinase inhibitor, BB3489 (20 μM); *lane 6*, 45 min preincubation with PD98059 prior to 1 h incubation with 10% serum; *lane 7*, 45 min preincubation with PD98059 prior to 1 h incubation with 20% serum; *lane 8*, 45 min preincubation with BB3489 prior to 1 h incubation with 10% serum. *A,* Western blot of cell lysates with anti-Myc antibodies as in Fig. 1*B, 3rd panel*. *B,* Western blot of lysates with anti-phospho-ERK antibodies as in Fig. 2*B*.

derived factors can induce HB-EGF shedding via MAP kinase- and metalloproteinase-dependent mechanisms as is the case with PMA and with Raf-1.

PMA Does Not Induce Shedding of HB-EGF$_{TM}$ in Suspended Cells—The experiments reported so far showing that PMA induces shedding of HB-EGF$_{TM}$HA/Myc were carried out with attached CHO cells (Fig. 7*A, lanes 1* and *2*; Fig. 7*B*, *lanes 1* and *2*). However, when the cells were placed into suspension PMA failed to induce shedding (Fig. 7*A, lanes 3* and *4*; *B, lanes 3* and *4*). This effect was reversible, and the ability of PMA to induce HB-EGF$_{TM}$HA/Myc shedding was fully restored within 1 h after plating suspended cells on fibronectin (Fig. 7*A, lanes 5* and *6*; *B, lanes 5* and *6*). On the other hand, PMA was still able to induce MAP kinase activation in suspended cells (Fig. 7*C, lane 4*) in the same manner as in cells grown on tissue culture plastic (Fig. 7*C, lane 2*) or in cells reattached by plating on fibronectin (Fig. 7*C, lane 6*). The level of cell-surface biotinylated HB-EGF$_{TM}$HA/Myc was unaffected by PMA in suspended cells (Fig. 7*B, lane 4*), suggesting that the lack of shedding in suspension is not due to unavailability of cell-surface HB-EGF$_{TM}$HA/Myc caused by internalization. Taken together, these results indicate that the inability of PMA to induce the cleavage of HB-EGF$_{TM}$HA/Myc in suspended cells is not due to an impaired activation of the MAP kinase cascade in the absence of cell adhesion. Thus, MAP kinase activity may be necessary but not sufficient to promote shedding.

To analyze further the affect of cell spreading on HB-EGF$_{TM}$HA/Myc shedding, cells were cultured on dishes coated with increasing densities of fibronectin from 18 to 2500 ng/cm^2 and treated with PMA (Fig. 8). Increased cell spreading (Fig. 8*A*) resulted in a direct proportional enhancement of PMA-induced HB-EGF$_{TM}$HA/Myc shedding (Fig. 8*B*). On the other hand, MAP kinase activity was independent of the degree of cell spreading (Fig. 8*B*). Thus the increased shedding due to

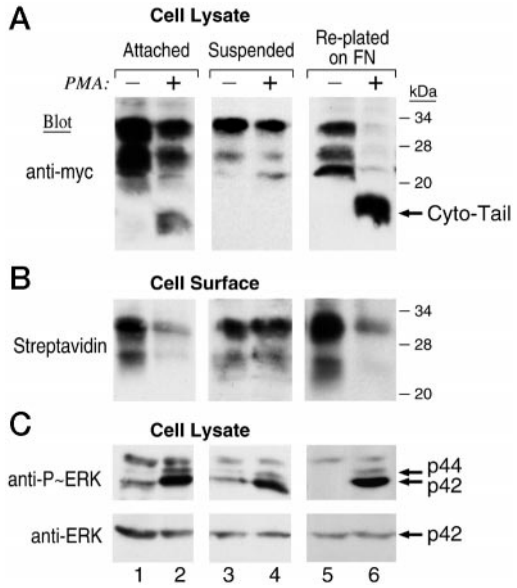

FIG. 7. **PMA does not induce HB-EGF$_{TM}$HA/Myc shedding in suspended cells.** CHO cells expressing HB$_{TM}$HA/Myc were grown overnight on tissue culture plastic dishes (*lanes 1* and *2*). They were detached and either resuspended in serum-free medium for 15 min (*lanes 3* and *4*) or re-plated on Petri dishes precoated with fibronectin (2500 ng/cm^2) for 1.5 h (*lanes 5* and *6*). Cells under these three conditions were incubated in the absence (*lanes 1, 3,* and *5*) or the presence (*lanes 2, 4,* and *6*) of PMA (1 μM) for 30 min. *A.* HB-EGF$_{TM}$HA/Myc and its cleaved cytoplasmic tail fragment were detected by Western blot with anti-Myc antibodies as in Fig. 1*B, 3rd panel*. *B,* biotinylated cell-surface HB-EGF$_{TM}$HA/Myc was detected by Western blot with streptavidin as in Fig. 1*B*, *2nd panel*. *C,* active MAP kinase was detected by Western blot as in Fig. 2*B*.

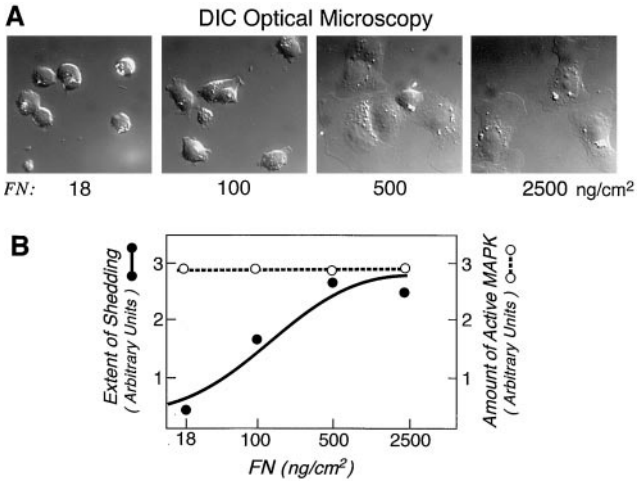

FIG. 8. **The shedding of HB-EGF$_{TM}$HA/Myc is proportional to the degree of spreading.** *C,* cells were grown overnight, detached for 15–20 min, and re-plated on glass coverslips or Petri dishes that were precoated with various densities of fibronectin; 18, 100, 500, and 2500 ng/cm^2, and treated with PMA (1 μM) for 20 min. Cells were allowed to spread for 1 h on fibronectin-coated glass coverslips and were treated with PMA and fixed. *A,* cell morphology was visualized using differential interference contrast (*DIC*) optics microscopy at × 400. Cells were detached and plated on fibronectin-coated Petri dishes and allowed to spread for 1 h, followed by addition or no addition of PMA. *B,* cell extracts were prepared, and HB-EGF$_{TM}$HA/Myc cleavage and MAP kinase activation were detected by Western blot with anti-Myc and anti-phospho-ERK antibodies, respectively, as in Fig. 1*B, 3rd panel* and Fig. 2*B,* respectively. The extent of the PMA-induced HB-EGF$_{TM}$HA/Myc cleavage and MAP kinase activation were quantified as described under "Experimental Procedures" and are expressed in arbitrary units.

spreading was not a result of increased MAP kinase activity but to some other variables associated with cell shape changes.

DISCUSSION

Previous work from our laboratory and others (26, 37) have shown that PMA induces the shedding of soluble HB-EGF from its transmembrane precursor HB-EGF$_{TM}$. We have now identified, for the first time, two regulators of PMA-induced shedding of HB-EGF, the Raf/MAP kinase cascade and cell adhesion and spreading. In order to monitor shedding, a double-tagged CHO cell line (CHO-HB-EGF$_{TM}$HA/Myc) was established with HA epitope placed N-terminal to the mature HB-EGF domain and Myc epitope placed at the C terminus of HB-EGF$_{TM}$. Shedding was monitored by measuring (i) the release of soluble 8–24-kDa HB-EGFHA into CM, (ii) the loss of transmembrane 25–32-kDa HB-EGF$_{TM}$HA/Myc in cell lysates, (iii) the loss of biotinylated cell surface 25–32-kDa HB-EGF, and (iv) the appearance in lysates of a 16-kDa HB-EGFMyc C-terminal fragment. The soluble HB-EGFHA released into the CM was active as measured by its ability to stimulate the tyrosine phosphorylation of EGF receptors. Monitoring the loss of the full-length HB-EGF$_{TM}$ and the appearance of a cleaved cytoplasmic tail fragment obviated problems that might arise from measuring the conditioned medium alone such as the possible immobilization of soluble active HB-EGF on the cell surface.

PMA treatment of cells induces a wide variety of cellular responses including MAP kinase activation (49). The activation of MAP kinase by PMA can be blocked by pretreating cells with the specific MAP kinase kinase (MEK) inhibitor PD98059 as demonstrated by the inhibition of ERK1 and ERK2 dual phosphorylation. Surprisingly, preincubating the cells with PD98059 also completely blocked the PMA-induced cleavage of HB-EGF$_{TM}$. A time course analysis indicated that the activation of MAP kinase occurred within 5 min and preceded soluble HB-EGF release, which required 10–20 min. Thus, MAP kinase activation appears to be upstream of shedding. It is not known which events downstream of MAP kinase activation lead to proteolytic processing of HB-EGF$_{TM}$. However, the rapidity of the induction of MAP kinase activation and of HB-EGF$_{TM}$ shedding in response to PMA suggests that new gene expression or protein synthesis is not required for these activities. MAP kinase activation is a response to growth factor stimulation of cells that results in enhanced cellular proliferation (50, 51) and motility (52). As an inducer of HB-EGF$_{TM}$ shedding, MAP kinase may be a mediator of sustained and amplified growth factor activity. In this model, growth factors such as EGF, TGF-α, and HB-EGF bind to their receptor and activate MAP kinase which leads to proliferation but also to the release of more growth factor from the membrane-anchored precursor resulting in an autocrine amplification loop. EGF and TGF-α could participate in such a loop since it has been shown that they enhance the shedding of membrane-anchored TGF-α (53). However, it was not demonstrated whether MAP kinase activation is a necessary step in the release of membrane-anchored TGF-α.

Activation of the MAP kinase cascade by PMA is in part due to the activation of certain PMA-responsive protein kinase C isoforms that activate Raf-1 (54, 55). We demonstrate here that Raf-1 kinase-mediated activation of MAP kinase also leads to HB-EGF shedding. This was shown by using a hormone-inducible fusion Raf-1 chimeric protein (ΔRaf-1:ER) that consists of the protein kinase domain of Raf-1 fused to the estradiol binding domain of the estrogen receptor (45) and that is activated by estradiol or its analogue tamoxifen. Hormone treatment of a stable cell line expressing both HB-EGF$_{TM}$ and ΔRaf-1:ER resulted in a rapid activation of MAP kinase within 5–10 min and shedding of HB-EGF$_{TM}$HA/Myc within 20–40 min. Thus, as before, MAP kinase activation preceded HB-EGF$_{TM}$ shedding; however, the MAP kinase activation and induction of

shedding in response to Raf-1 was slightly slower than in response to PMA. The MEK inhibitor, PD98059, inhibited the shedding of HB-EGF$_{TM}$ by 85–90% and ERK2 dual phosphorylation by 60–70% in response to ΔRaf-1:ER activation suggesting that the Raf-1-induced shedding of HB-EGF$_{TM}$ occurs mostly via the MAP kinase cascade. Previously, it was shown using differential display that HB-EGF mRNA was one of the four mRNAs induced by transient activation of ΔRaf-1:ER in 3T3 fibroblasts and that soluble HB-EGF appeared in the CM (56). Thus, it is possible that Raf-1 activation results in both HB-EGF synthesis and MAP kinase-dependent HB-EGF$_{TM}$ release leading to autocrine HB-EGF growth factor activity which may contribute to the oncogenic properties of Raf-1.

Since phorbol esters and Raf-1 may be considered as nonphysiological stimuli of HB-EGF shedding, a more physiological approach was attempted using serum, a rich source of growth factors such as PDGF. Serum has been previously demonstrated to induce the shedding of proTGF-α (8). Addition of 10–20% fresh fetal calf serum to serum-starved CHO cells expressing HB-EGF$_{TM}$HA/Myc resulted in the rapid activation of p42 and p44 MAP kinases (ERKs), and the shedding of HB-EGF$_{TM}$ within an hour as monitored by the loss of cell-surface HB-EGF$_{TM}$ and the appearance of the cytoplasmic tail fragment in cell lysates. The extent of serum-induced shedding, 30–40%, was not as great as that induced by PMA and Raf-1. This result could be due to the relatively low concentration of growth factors in serum and/or the down-regulation of growth factor receptors which does not occur with PMA and Raf-1. Shedding was blocked by PD98059 and BB3489 indicating that serum-induced shedding of HB-EGF$_{TM}$ was MAP kinase- and metalloproteinase-dependent. These results suggest that PMA-, Raf-1-, and serum-induced shedding are regulated by common mechanisms.

Another novel regulator of HB-EGF$_{TM}$ shedding is the degree of cell adhesion and spreading. PMA is not able to induce shedding of HB-EGF$_{TM}$ in suspended cells. This inability of PMA to induce shedding is not due to cell death since HB-EGF$_{TM}$ shedding was fully reversible upon re-plating of cells nor is it due to internalization of HB-EGF$_{TM}$ since biotinylated HB-EGF$_{TM}$ was found to remain present on the cell surface of suspended cells. Furthermore, the inability of PMA to induce shedding of HB-EGF$_{TM}$ in suspended cells is not due to lack of MAP kinase activation since PMA activated MAP kinase in suspended cells as efficiently as in attached cells. These results are consistent with previous studies showing that growth factors can stimulate MAP kinase activity in cells that are kept in suspension for short periods (57, 58). The degree of cell spreading appears to regulate HB-EGF$_{TM}$ shedding. When cells were plated on increasing fibronectin densities, the extent of PMA-induced shedding of HB-EGF$_{TM}$ increased in proportion to the degree of cell spreading. On the other hand, PMA-induced MAP kinase activation was independent of the degree of spreading on fibronectin. Taken together, it appears that MAP kinase activation is necessary for HB-EGF$_{TM}$ shedding but not sufficient since cell adhesion is also required. How cell-spreading contributes to HB-EGF$_{TM}$ shedding is not known. However, recent studies demonstrate that the progression of growth factor-stimulated cells through late G_1 phase of the cell cycle can be controlled by modulating the cell shape or cytoskeleton tension (59, 60). Apparently, cell shape also controls the growth amplification loop that is mediated by MAP kinase activation and associated HB-EGF$_{TM}$ release.

The proteinase involved in cleaving HB-EGF$_{TM}$ is a metalloproteinase since the hydroxamic acid-based metalloproteinase inhibitor, BB3489, blocked the shedding of HB-EGF$_{TM}$ completely in response to PMA, activation of ΔRaf-1:ER, and serum. These results are consistent with previous studies implicating a metalloproteinase in HB-EGF$_{TM}$ shedding (15, 21, 38). A recent report has implicated an ADAM family member MDC9/Meltrin γ in the PMA-induced processing of HB-EGF$_{TM}$ (21). Soluble MDC9/Meltrin γ could not cleave soluble HB-EGF$_{TM}$ *in vitro* (21) suggesting its HB-EGF$_{TM}$ cleaving activity was dependent on being associated with intact membrane. In our experiments, the metalloproteinase-dependent cleavage of HB-EGF$_{TM}$ in a cell-free system was abolished upon the addition of mild detergents (such as CHAPS or octyl glucoside) at concentrations that do not inhibit matrix metalloproteinase activity. Together, these results indicate that both HB-EGF$_{TM}$ and the metalloproteinase need to be membrane-anchored for the cleavage to take place, as has been proposed previously for other shedding events (3, 61).

The mechanisms described here that regulate HB-EGF$_{TM}$ shedding might have a broader role. For example, it has been recently suggested that MAP kinase is involved in the shedding of β-APP (62). Thus delineating the mechanisms that regulate HB-EGF$_{TM}$ shedding might lead to new strategies aimed at inhibiting shedding of membrane-anchored precursors such as β-APP and TNF-α which have pathological consequences.

In conclusion, the results of this study suggest that there are previously unrecognized regulatory elements of HB-EGF$_{TM}$ shedding, including the Raf-1/MAP kinase pathway and cell adhesion and spreading. Additional studies will be required in order to identify the components downstream of MAP kinase that link the growth factor-activated cascade to HB-EGF$_{TM}$ shedding.

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