The Stress- and Inflammatory Cytokine-induced Ectodomain Shedding of Heparin-binding Epidermal Growth Factor-like Growth Factor Is Mediated by p38 MAPK,Distinct from the 12-O-Tetradecanoylphorbol-13-acetate- and Lysophosphatidic Acid-induced Signaling Cascades*

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Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a critical growth factor for a number of physiological and pathological processes. HB-EGF is synthesized as a membrane-anchored form (pro-HB-EGF), and pro-HB-EGF is cleaved at the cell surface to yield soluble HB-EGF by a mechanism called “ectodomain shedding.” We show here that the ectodomain shedding of pro-HB-EGF in Vero cells is induced by various stress-inducing stimuli, including UV light, osmotic pressure, hyperoxidation, and translation inhibitors. The pro-inflammatory cytokine interleukin-1β also stimulates the ectodomain shedding of pro-HB-EGF. An inhibitor of p38 MAPK (SB203580) or the expression of a dominant-negative (dn) form of p38 MAPK inhibited the stress-induced ectodomain shedding of pro-HB-EGF, whereas an inhibitor of JNK (SP600125) or the expression of dnJNK1 did not. 12-O-Tetradecanoylphorbol-13-acetate (TPA) and lysophosphatidic acid (LPA) are also potent inducers of pro-HB-EGF shedding in Vero cells. Stress-induced pro-HB-EGF shedding was not inhibited by the inhibitors of TPA- or LPA-induced pro-HB-EGF shedding or by dn forms of molecules involved in the TPA- or LPA-induced pro-HB-EGF shedding pathway. Reciprocally, SB203580 or dn p38 MAPK did not inhibit TPA- or LPA-induced pro-HB-EGF shedding. These results indicate that stress-induced pro-HB-EGF shedding is mediated by p38 MAPK and that the signaling pathway induced by stress is distinct from the TPA- or LPA-induced pro-HB-EGF shedding pathway.

Heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) (1), a member of the EGF family, binds to the EGF receptor (ErbB1) and the related receptor tyrosine kinase (ErbB4) and activates them (2). HB-EGF, especially the secreted form (sHB-EGF), is a potent mitogen and chemottractant for a number of cell types, including vascular smooth muscle cells, fibroblasts, and keratinocytes (3). HB-EGF is implicated in a number of physiological and pathological processes in the body (4), which include wound healing (5, 6), kidney collecting duct morphogenesis (7), blastocyst implantation (8), cardiac hypertrophy (9–11), smooth muscle cell hyperplasia (12), pulmonary hypertension (13), and oncogenic transformation (14).

HB-EGF, synthesized as a membrane-anchored precursor protein (pro-HB-EGF) of 208 amino acids, is composed of a signal peptide, a heparin-binding domain, an EGF-like region, a juxtamembrane domain, a transmembrane domain, and a cytoplasmic tail (3). Pro-HB-EGF is cleaved at the cell surface within the juxtamembrane domain through a process called ectodomain shedding, which results in secretion of a soluble 75–86-amino acid growth factor (sHB-EGF) (15). Although the regulated process of pro-HB-EGF ectodomain shedding yields significant sHB-EGF, a considerable amount remains uncleaved at the cell surface. In addition to being a precursor of sHB-EGF, pro-HB-EGF is a biologically active molecule, forming a complex with both CD9 (16) and integrin αβ3 (17) to transduce biological signals to neighboring cells in a non-diffusible manner (18). In contrast to the mitogenic effect of sHB-EGF (4), pro-HB-EGF negatively regulates cell proliferation (19). These results suggest that strict control of pro-HB-EGF ectodomain shedding is critical for the proper regulation of the activity of this growth factor.

Pro-HB-EGF ectodomain shedding is induced by both physiological and pharmacological agonists such as 12-O-tetradecanoylphorbol-13-acetate (TPA). TPA, an activator of protein kinase C (PKC), potently induces the ectodomain shedding of pro-HB-EGF (15) and of other membrane proteins (20). In monkey kidney Vero cells, the presence of a constitutively active form of PKCδ results in the ectodomain shedding of pro-HB-EGF, and dominant-negative (dn) PKCδ suppresses TPA-induced shedding, suggesting that the PKCδ isoform contributes to the ectodomain shedding of pro-HB-EGF. PKCδ binds to the cytoplasmic domain of ADAM9/MDC9/meltrin-γ kinase; MKK, MAPK kinase; IL, interleukin; TNF-α, tumor necrosis factor-α; GFP, green fluorescent protein; EGF, enhanced GFP; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase.
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(21), a metalloprotease belonging to the ADAM (a disintegrin and metalloprotease) family (22). Overexpression of ADAM9 induces pro-HB-EGF shedding, which can be inhibited by the expression of the dnADAM9 mutant H347A,H351A in Vero-H cells. Thus, ADAM9 is thought to act downstream of PKCδ in the pro-HB-EGF shedding pathway (21), although the role of this protease in the direct cleavage of pro-HB-EGF remains unclear.

Lysophosphatidic acid (LPA) and other ligands of the seven-transmembrane G protein-coupled receptors also stimulate pro-HB-EGF shedding (23, 24). The activation of shedding by such ligands is crucial for the transactivation of the EGF receptor by G protein-coupled receptor ligands (23, 25, 26). In Vero cells, the Ras-Raf-MEK and Rac signaling pathways are activated in LPA-induced shedding (27). LPA-induced shedding is inhibited by dnHa-Ras, dnRac1, and the MEK inhibitor PD98059, but not by either dnPKCδ or dnADAM9. Conversely, although TPA-induced shedding is inhibited by dnPKCδ and dnADAM9, such shedding is not affected by dnHa-Ras, dnRac1, and the MEK inhibitor. These results indicate that two distinct signaling pathways function to regulate pro-HB-EGF ectodomain shedding, here designated the LPA- and TPA-induced pathways.

Pro-HB-EGF expression is enhanced in various tissues upon injury and inflammation (28–35). The induction of this cellular response under these conditions suggests that HB-EGF is involved in both physiological tissue repair, including processes such as wound healing, and pathological processes, including atherosclerosis, that follow from inappropriate inflammation. Cellular stress and inflammation may function as triggers of ectodomain shedding. It remains unclear, however, if inflammatory cytokines and stimuli inducing cellular stress responses can trigger pro-HB-EGF ectodomain shedding. We demonstrate here that various stress-inducing stimuli and inflammatory cytokines strongly induce ectodomain shedding, acting through a p38 MAPK-mediated pathway, distinct from both the TPA- and LPA-induced signaling pathways. Our results suggest that HB-EGF activity is regulated at the transcriptional level and by ectodomain shedding, which responds to stress and inflammation.

EXPERIMENTAL PROCEDURES

Reagents—TPA, Ro-31-8220, and anisomycin were purchased from Nacalai Tesque Co., Ltd. (Kyoto, Japan). LPA was obtained from Funa-koshi Co., Ltd. (Tokyo, Japan). Recombinant human IL-1β, human TNF-α, and mouse IL-6 were acquired from PeproTech (Rocky Hill, NJ). PD98059 and SB203580 were purchased from Calbiochem. SP600125 (17256) was obtained from BIOMOL Research Labs Inc. (Plymouth Meeting, PA). H2O2 was acquired from Santoku Chemical Industries Co., Ltd. (Tokyo). KB-R8301 was obtained from Nippon Organon K. K. (Osaka, Japan).

Antibodies—The goat antibody specific for the C terminus of human pro-HB-EGF (C-18) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-HB-EGF neutralizing antibody was obtained from R&D Systems. A rabbit antiserum against the N terminus of human pro-HB-EGF (H6) was derived as described (36). Rabbit anti-mouse ADAM9 antibody was raised against a glutathione S-transferase fusion protein containing the mouse ADAM9 C-terminal region (aromatic acids 718–845). Horseradish peroxidase-conjugated goat anti-rabbit and unconjugated anti-FLAG (M2) monoclonal antibodies were purchased from Sigma Chemical Co., Ltd. Rabbit anti-FLAG antibody and Sigma, respectively, were conjugated with horseradish peroxidase- and Cy3-conjugated donkey anti-goat antibodies were obtained from Chemicon International, Inc. (Temecula, CA).

Plasmids—pEGFP-c1, a plasmid encoding GFP, was purchased from Clontech. dnPKCs (R144A, R145A, K576R) (37, 38) and the mouse dnADAM9 mutant H347A,H351A (21) have been described previously. The FLAG-tagged mouse ADAM9 mutant E248A was generated by site-directed PCR-based mutagenesis. FLAG-tagged dnRac1 (N17Rac1) has been described previously (27). FLAG-tagged dpn38 MAPK (T180A,Y182F) was kindly provided by H. Hatanaka and Y. Ishikawa (Institute of Protein Research, Osaka University). pCMV5-JNK1(1,AFP), a plasmid encoding FLAG-tagged dnJNK1 (39), and a constitutively active form of MKK6 (EE-MKK6) (40) were kindly provided by R. J. Davis (University of Massachusetts Medical School) and Y. Gotoh (University of Tokyo), respectively.

Cell Culture and Transfection—Vero-H cells (15) were maintained in modified Eagle’s medium with nonessential amino acids supplemented with 10% fetal calf serum. Transfections into Vero-H cells were performed using the calcium phosphate technique (41) unless otherwise stated.

Shedding Analysis by Western Blotting—Vero-H cells (5 × 10^5 cells/6-cm dish) were cultured for 6–12 h in serum-free modified Eagle’s medium with nonessential amino acids and then treated with TPA (64 nM), LPA (20 μg/ml), IL-1β (1 ng/ml), anisomycin (10 μg/ml), sorbitol (5% w/v), or indicated, cells were washed with either TNF-α (50 ng/ml) or IL-6 (20 ng/ml). For UV irradiation, Vero-H cells in 6-cm dishes in 3 ml of modified Eagle’s medium with nonessential amino acids were exposed to 40 mJ/cm^2 UV radiation (254 nm) using a Spectrolinker (Spectronics Corp.). Following incubation for 30 min with stimuli, cells were collected and resuspended in 140 μl of lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl, 0.1 M NaCl, 10 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 20 μg/ml antipain, and 10 μg/ml chymostatin, pH 7.4). After 5 min on ice, the lysates were clarified by centrifugation at 15,000 rpm. Western blot analysis was performed using a goat antibody specific for the human pro-HB-EGF C terminus. The antibody was visualized with horseradish peroxidase-conjugated anti-goat IgG using ECL Plus (Amersham Biosciences). To detect sHB-EGF in the culture medium, sHB-EGF was trapped with heparin-Sepharose beads and then eluted with SDS gel sample buffer. The eluted materials were subjected to Western blot analysis and detected with rabbit anti-human HB-EGF antibody H6. The antibody was visualized with horseradish peroxidase-conjugated anti-rabbit IgG using ECL Plus.

Immunofluorescence Detection of the Shedding of the Pro-HB-EGF Ectodomain—Pro-HB-EGF was detected on cells using goat anti-HB-EGF neutralizing antibody and Cy3-conjugated anti-goat IgG as described (21). To detect the expression of transfected proteins, fixed cells were permeabilized with 0.1% Triton X-100 for 3 min and then stained with anti-FLAG antibody or the antibodies indicated, followed by fluorescein isothiocyanate-conjugated second antibody. To detect cells transfected with dnPKCs or vector (pEF-BOS) alone as a control, EGFP (pEGFP-c1) was cotransfected. Images were captured with FISH Imager™ system (Carl Zeiss, Inc.). The percentage of pro-HB-EGF-positive cells was determined by counting the number of pro-HB-EGF-positive cells among the total cells concomitantly expressing the products of the transfected cDNA. Values were determined based on the results obtained in at least two independent transfections from at least 100 independent cells per field for the transfected or marker proteins in each experiment. Scoring was performed in a completely blind manner.

RESULTS

Stimuli Inducing Various Stresses and IL-1β Lead to Pro-HB-EGF Shedding—Vero-H cells are stable Vero transfectants overexpressing human pro-HB-EGF (15). This cell line exhibits consistent responses to stimuli inducing pro-HB-EGF ectodomain shedding (21, 24, 27). Using Vero-H cells, we examined the effect of pro-inflammatory cytokines and stress-inducing stimuli on the induction of pro-HB-EGF ectodomain shedding. First, we examined the ectodomain shedding of pro-HB-EGF by immunofluorescence microscopy using an antibody specific for the pro-HB-EGF ectodomain. Vero-H cells were pretreated with the indicated stimuli and then cultured for 30 min. Cells were treated with anti-HB-EGF neutralizing antibody and then fixed and visualized using a secondary antibody. Pro-HB-EGF fluorescence at the cell surface disappeared following addition of either LPA (20 μg/ml) or TPA (64 nM) to the culture medium (Fig. 1A) (21, 24, 27). In addition, the pro-inflammatory cytokine IL-1β (4 μg/ml) also led to the disappearance of pro-HB-EGF surface immunofluorescence. Moreover, stimuli well known to induce a cellular stress response (42), such as translation inhibitors (anisomycin, 10 μg/ml) (43), exposure to UV light (40 mJ/cm^2), hypertonic osmotic pressure (sorbitol, 0.4 M), or oxidative stress (H₂O₂, 0.5 mM), also induced the disappearance of pro-HB-EGF immunofluorescence from the cell surface.
Western blot analysis confirmed the ectodomain shedding of pro-HB-EGF in Vero-H cells (Fig. 1B). Immunoblot analysis of Vero-H cell lysates (upper panel) and the culture medium (lower panel) following incubation with the indicated stimuli detected the cytoplasmic domain of pro-HB-EGF and the shed soluble EGF-like domain, respectively. In cell lysates, bands ranging from 20 to 30 kDa correspond to pro-HB-EGF, whereas bands between 17.4 and 6.9 kDa are proteolytic fragments composed of the cytoplasmic and transmembrane domains of pro-HB-EGF (referred to as the “tail fragment”). Consistent with the appearance of the tail fragment, sHB-EGF appeared concurrently in the culture medium. These results indicate that IL-1β, anisomycin, UV light, and sorbitol, in addition to LPA and TPA, induce pro-HB-EGF cleavage to generate both the tail fragment in cell lysates and sHB-EGF in the culture medium. Although H₂O₂ generated the tail fragment in cell lysates and sHB-EGF in the culture medium, this stimulus also reduced the total pro-HB-EGF protein present through an unknown mechanism. Centrifuging as a mimic of shearing stress also induced pro-HB-EGF ectodomain shedding (data not shown). In contrast, the pro-inflammatory cytokines TNF-α and IL-6 did not affect shedding. Rather, these cytokines reduced the total pro-HB-EGF protein, although the reduced level varied depending on the experiments.

We also examined the time course of pro-HB-EGF shedding induced by IL-1β, anisomycin, UV light, and sorbitol. As the appearance of sHB-EGF in the culture medium correlated well with the appearance of the tail fragment in the cell lysate (Fig. 1B) (27), we utilized Western blot analysis only for the cell lysates to examine this event. As shown in Fig. 2, ectodomain shedding was observed following treatment with each of these stress stimuli at a time course similar to that observed for TPA-induced shedding (15, 21).

**Stress-induced Pro-HB-EGF Shedding Involves p38 MAPK**—Signals from pro-inflammatory cytokines and cellular responses to stress are generally mediated by SAPKs (42). Therefore, we hypothesized that stress-induced pro-HB-EGF shedding is mediated by a SAPK family kinase. We examined the activation of p38 MAPK and the various JNKs, major members of the SAPK family, following stress-inducing stimulation of Vero-H cells. After treatment with LPA, TPA, IL-1β, anisomycin, UV irradiation, or sorbitol, Vero-H cell lysates were analyzed by Western blotting with either anti-phospho-p38 MAPK or anti-phospho-JNK antibody to monitor kinase phosphorylation status. Phosphorylated p38 MAPK was strongly observed after treatment with anisomycin, UV light, or sorbitol, with moderate increases in phosphorylation follow-
HB-EGF and dnp38 MAPK. Although treatment with IL-1 

Cells were then treated with IL-1β or stress-inducing or other stimuli for 30 min. Cell lysates were analyzed by Western blotting to detect activation of p38 MAPK and JNK1/2 and ectodomain shedding. A, activation level of p38 MAPK, detected with anti-phospho-p38 MAPK antibody (upper panels) and anti-p38 MAPK antibody (lower panels). B, activation level of JNK1/2, detected with anti-phospho-JNK1/2 antibody (upper panels) and anti-JNK1/2 antibody (lower panels). C, ectodomain shedding of pro-HB-EGF, detected by Western blotting using an antibody raised against the pro-HB-EGF C terminus.

We examined the requirement for p38 MAPK in stress-induced pro-HB-EGF shedding using dnp38 MAPK. Following the transfection of FLAG-tagged dnp38 MAPK, cells were treated with various stimuli and then double-stained for pro-HB-EGF and dnp38 MAPK. Although treatment with IL-1β, anisomycin, UV light, or sorbitol enhanced pro-HB-EGF shedding in vector-transfected cells (Fig. 4B), shedding was not inhibited by dnp38 MAPK (Fig. 4B). MKK6 is a specific activator of p38 MAPK (45). Consistent

with the role of the p38 MAPK pathway in shedding, transfection of a constitutively active form of MKK6 resulted in pro-HB-EGF ectodomain shedding in the absence of stress-inducing stimuli in a dose-dependent manner (Fig. 5). These results indicate that p38 MAPK activation is required for stress-induced pro-HB-EGF shedding in Vero-H cells. SB203580 (2 μM) reduced the activity of p38 MAPK, but not that of JNK1/2, as shown in Fig. 3 (A and B). At the same concentration, SB203580 inhibited stress-induced shedding, suggesting that JNK is not involved in stress-induced shedding in Vero-H cells. To examine the involvement of JNK in stress-induced shedding directly, we tested whether SP600125, an inhibitor of JNK (46), abrogates stress-induced pro-HB-EGF shedding in Vero-H cells. SP600125 (5 μM) significantly reduced JNK1/2 phosphorylation caused by TPA, IL-1β, anisomycin, and sorbitol treatment (Fig. 6A), whereas the same concentration of SP600125 could not inhibit UV light-irradiated cells, JNK1/2 phosphorylation was strong and was not significantly reduced with 5 μM SP600125. SP600125 (20 μM) inhibited JNK1/2 phosphorylation caused by UV light, but SP600125 scarcely inhibited UV light-induced shedding even at this concentration.
In addition to the JNK inhibitor, we used dnJNK1 to examine the involvement of the JNK pathway in stress-induced pro-HB-EGF shedding. Following the transfection of FLAG-tagged dnJNK1, cells were treated with various stimuli as Fig. 4B and then double-stained for pro-HB-EGF and dnJNK1. Pro-HB-EGF ectodomain shedding induced by each stimulus was not inhibited in FLAG-positive cells expressing dnJNK1 (Fig. 7). From these results, we conclude that the JNK pathway is not involved in stress-induced pro-HB-EGF ectodomain shedding in Vero-H cells.

p38 MAPK-mediated Shedding Cascades Are Independent of

the Other Shedding Cascades—Using Vero-H cells, we demonstrated that the LPA-induced shedding cascade involves the Ras-Raf-MEK and small GTPase Rac pathways, whereas TPA-induced pro-HB-EGF shedding involves PKC and ADAM9. IL-1β, anisomycin, and additional stresses induce shedding through a pathway involving the activation of p38 MAPK. We next analyzed the connection of the p38 MAPK-mediated pathway with either the LPA- or TPA-induced pathway. We tested the effect of the MEK inhibitor PD98059 (47) and the PKC inhibitor Ro-31-8220 (48) on p38 MAPK-mediated pro-HB-EGF ectodomain shedding. PD98059 inhibited LPA-induced, but not TPA-induced, shedding (Fig. 8A), whereas Ro-31-8220 inhibited TPA-induced, but not LPA-induced, shedding (Fig. 8B), confirming previous results (27). p38 MAPK-mediated stress-induced shedding was not inhibited by either PD98059 or Ro-31-8220. We also transfected dnPKC θ or dnRac1. Neither dnPKC θ nor dnRac1 inhibited p38 MAPK-mediated shedding, with the exception of partial inhibition observed for sorbitol-induced shedding by dnRac1 (Fig. 9). These data indicate that the stress-induced shedding cascade mediated by p38 MAPK activation functions independently of the LPA- and TPA-induced shedding cascades.

Metalloproteases Are Involved in Stress-induced Shedding—Metalloprotease inhibitors inhibit both TPA- and LPA-induced pro-HB-EGF shedding. We treated cells with a hydroxamic acid-based metalloprotease inhibitor, KB-R8301, to examine the role of metalloproteases in stress-induced shedding. KB-R8301 (10 μM) inhibited the pro-HB-EGF shedding induced by IL-1β, anisomycin, UV light, H2O2, and sorbitol (Fig. 10A), indicating that metalloproteases are required for p38 MAPK-mediated pro-HB-EGF shedding as well.

ADAM family metalloproteases are involved in the ectodomain shedding of a variety of proteins such as TNF-α and Delta (49, 50). ADAM9 is known to be involved in TPA-induced pro-HB-EGF shedding in Vero-H cells (21). To examine the role of ADAM9 in p38 MAPK-mediated shedding, we examined the effect of overexpression of dnADAM9 on shedding. Two ADAM9 mutants were used, one carrying the modification of the conserved histidine residues in the catalytic domain to alanines (H347A,H351A) and the other carrying a modification of the conserved glutamic acid in the catalytic domain to alanine (E348A). We investigated the influence of these mutants on stress- and IL-1β-induced shedding. Both mutants inhibited
EGF shedding in Vero-H cells. Vero-H cells were transfected with plasmids encoding pro-HB-EGF, dnRac1 (20\(^{\text{g}}\)o r5) or wt\(\text{ADAM9}\) encoding FLAG-tagged dnRac1 (20\(^{\text{g}}\)). As a control, 0.1% Me\(_2\)SO, the solvent of these inhibitors, was added. The cells were treated with each stimulus for 30 min. Ectodomain shedding was detected by Western blot analysis of cell lysates using an antibody raised against the pro-HB-EGF C terminus.

**FIG.8.** Effects of the MEK inhibitor PD98059 and the PKC inhibitor Ro-31-8220 on the ectodomain shedding of pro-HB-EGF. Serum-starved Vero-H cells were pretreated with 25 \(\mu\text{M}\) PD98059 (A) or 5 \(\mu\text{M}\) Ro-31-8220 (B) for 1 h. As a control, 0.1% Me\(_2\)SO, the solvent of these inhibitors, was added. The cells were treated with each stimulus for 30 min. Ectodomain shedding was detected by Western blot analysis of cell lysates using an antibody raised against the pro-HB-EGF C terminus.

**DISCUSSION**

HB-EGF contributes to tissue repair processes acting in response to various injuries (5, 6, 30, 51). HB-EGF also participates in pathological processes, including smooth muscle cell hyperplasia (12), restenosis following balloon injury (52), and cardiac hypertrophy (9–11). Ectodomain shedding is critical for the biological activity of this growth factor (53). In addition to regulation of growth factor activity, pro-HB-EGF ectodomain shedding contributes to the transactivation of the EGF receptor following ligation by G protein-coupled receptors and other ligands (23). Recent studies suggest that pro-HB-EGF ectodomain shedding and transactivation are involved in the pathological processes of cardiac hypertrophy and pulmonary hypertension (9, 13).

Although a function for HB-EGF in physiological and pathological processes has been increasingly reported, information about shedding-inducing stimuli and the downstream signaling processes involved is limited (54). Two distinct signaling pathways contribute to pro-HB-EGF shedding in Vero-H cells, the TPA-induced PKC\(_\varepsilon\)- and ADAM9-mediated pathway and the LPA-induced MEK- and Rac-mediated pathway. Using Vero-H cells, we determined that various stress-inducing and inflammatory stimuli also trigger ectodomain shedding in these cells.

FIG. 9. Effects of dnRac1 and dnPKC\(_\varepsilon\) on the ectodomain shedding of pro-HB-EGF. Vero-H cells were transfected with plasmids encoding FLAG-tagged dnRac1 (20 \(\mu\text{g}\)) and dnPKC\(_\varepsilon\) (18 \(\mu\text{g}\)) plus EGFP (2 \(\mu\text{g}\)) or with vector (18 \(\mu\text{g}\)) plus EGFP (2 \(\mu\text{g}\)) as a control. After 48 h of transfection, the cells were incubated with LPA, TPA, IL-1\(\beta\), anisomycin, UV light, or sorbitol and then stained with anti-HB-EGF antibody for the detection of the pro-HB-EGF ectodomain. The expression of dnRac1 was detected by anti-FLAG antibody. The percentage of pro-HB-EGF-positive cells among GFP-positive cells was determined. ca, constitutively active.

FIG. 10. Inhibition of stress- and IL-1\(\beta\)-induced pro-HB-EGF shedding by the metalloprotease inhibitor KB-R8301, but not wt\(\text{ADAM9}\). A, effect of the metalloprotease inhibitor KB-R8301. Vero-H cells were pretreated with 10 \(\mu\text{M}\) KB-R8301 or 0.1% Me\(_2\)SO for 1 h and then treated with each stimulus for 30 min. Ectodomain shedding was detected by Western blot analysis of cell lysates using an antibody raised against the pro-HB-EGF C terminus. B, effect of wt\(\text{ADAM9}\). Vero-H cells were transfected with plasmids encoding the dominant-negative mutants H347A, H351A (20 \(\mu\text{g}\)) and E348A (20 \(\mu\text{g}\)) or with vector alone (18 \(\mu\text{g}\)) plus EGFP (2 \(\mu\text{g}\)) as a control. After 48 h of transfection, the cells were incubated with LPA, TPA, IL-1\(\beta\), UV light, or sorbitol and then double-stained with either anti-HB-EGF or anti-ADAM9 antibody. The percentage of pro-HB-EGF-positive cells among ADAM9-positive cells was determined. \(\text{wt}\), wild-type.
ectodomain shedding nor p38 MAPK activation was observed following treatment with either TNF-α or IL-6 (Figs. 1B and 3A). Although it is possible that Vero-H cells do not bear the receptors ligating TNF-α and IL-6, it appears that signaling pathways induced by TNF-α and IL-6 do not contribute to pro-HB-EGF shedding.

We have demonstrated that stress- and IL-1β-induced shedding requires protease(s) abrogated by metalloprotease inhibitors. This result is in agreement with data obtained for TPA- and LPA-induced shedding and for additional shedding cascades of transmembrane molecules (61). Therefore, a metalloprotease such as matrix metalloprotease or ADAM that works in close proximity to the membrane may participate in pro-HB-EGF shedding. The ADAM family metalloproteases contains a conserved sequence (HEXXH) that is a putative zinc-binding motif within the catalytic domain. We previously constructed a mutant form of ADAM9 (H347A,H351A, in which the conserved histidines are replaced with alanines) that was found to inhibit TPA-induced pro-HB-EGF shedding (21).

Thus, ADAM9 is involved in pro-HB-EGF shedding induced by treatment of Vero-H cells with TPA. In this study, we constructed another dnADAM9 mutant (E348A) to examine its effect on TPA-induced pro-HB-EGF shedding. As E348A exhibited a subcellular localization similar to that of native ADAM9 when expressed in Vero-H cells, E348A may be a more suitable dnADAM9 mutant than the H347A,H351A mutant, which exhibited an altered localization pattern. Both dominant-negative mutants inhibited TPA-induced shedding, confirming the role of ADAM9 in TPA-induced shedding. Neither H347A, H351A nor E348A inhibited LPA-, stress-, or IL-1β-induced pro-HB-EGF shedding in Vero-H cells. From these results, we conclude that ADAM9 does not participate in LPA-, stress-, or IL-1β-induced pro-HB-EGF shedding.

Recent studies suggest that, in addition to ADAM9, ADAM10/Kuzbanian (13, 62), ADAM12/meltrin-β (9), and ADAM17/TACE (TNF-α converting enzyme) (63) function in pro-HB-EGF shedding in Vero-H cells. Thus, the protease responsible for p38 MAPK-mediated pro-HB-EGF shedding in Vero-H cells remained to be identified.

What is the biological significance of stress-induced pro-HB-EGF shedding? A number of reports indicate that the transcription of pro-HB-EGF is up-regulated in response to oxidative, ischemic, osmotic, and mechanical stresses (29–34). The inflammatory cytokines IL-1β and TNF-α also markedly increase pro-HB-EGF gene expression (35). The release of sHB-EGF from the membrane by ectodomain shedding is a prerequisite for the mitogenic activity as a paracrine and autocrine growth factor. Therefore, stress and inflammatory cytokines may also induce pro-HB-EGF shedding in addition to their transcriptional up-regulation. In response to stress and inflammation, HB-EGF activity may be regulated at both the transcriptional and ectodomain shedding levels. Considering the role of HB-EGF in tissue repair, the rapid release of sHB-EGF following stress-inducing stimuli may facilitate the repair of wounded tissues. These results also suggest that excess release of sHB-EGF by continual exposure of tissues to stress and inflammation may result in the pathological hyperplasia of cells such as smooth muscle and cardiac cells.

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