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**Analysis of Stress- And
Inflammatory Cytokine-Induced
Ectodomain Shedding of HB-EGF**

**Hisanori Takenobu
2003**

Osaka University

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**A Dissertation Submitted to
The Graduate School of
Osaka University
for The Degree of
Doctor of Philosophy**

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Abstract

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 (proHB-EGF), and proHB-EGF is cleaved at the cell surface to yield soluble HB-EGF by a mechanism called "ectodomain shedding." I show here that the ectodomain shedding of proHB-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-1 β also stimulated the ectodomain shedding of proHB-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 proHB-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 proHB-EGF shedding in Vero cells. Stress-induced proHB-EGF shedding was not inhibited by the inhibitors of TPA- or LPA-induced proHB-EGF shedding or by dn forms of molecules involved in the TPA- or LPA-induced proHB-EGF shedding pathway. Reciprocally, SB203580 or dnp38 MAPK did not inhibit TPA- or LPA-induced proHB-EGF shedding. These results indicate that stress-induced proHB-EGF shedding is mediated by p38 MAPK and that the signaling pathway induced by stress is distinct from the TPA- or

LPA-induced proHB-EGF shedding pathway. Stress-induced shedding was inhibited by metalloprotease inhibitor, but putative dominant negative form of ADAM9, ADAM10, ADAM12 and ADAM17 did not block the stress-induced shedding. These results suggest that p38 MAPK-mediated shedding pathway required metalloproteases, but ADAM9, 10, 12 and 17 are not involved in this process.

Abbreviations

ADAM, a disintegrin a metalloprotease (a disintegrin and metalloprotease).

ca, constitutively active.

CSF, colony stimulation factor.

CMV, cytomegalovirus.

dn, dominant-negative.

DTR, diphtheria toxin receptor.

DTT, dithiothreitol.

EDTA, ethylene diamine tetra-acetic acid.

EF-2, elongation factor-2.

EGF, epidermal growth factor.

EGFP, enhanced GFP.

EGFR, epidermal growth factor receptor.

ErbB, epidermal growth factor receptor.

ERK, extracellular-regulated protein kinase

GFP, green fluorescent protein.

GPCR, G protein-coupled receptor.

HA, hemagglutinin.

HB-EGF, heparin-binding EGF-like growth factor.

IL, interleukin.

JNK, c-Jun N-terminal kinase.

KL, Kit ligand.

LPA, lysophosphatidic acid.

MAPK, mitogen-activated protein kinase.

MDC, metalloprotease disintegrin.

MEK, MAPK/extracellular signal-regulated kinase kinase.

MKK, MAPK kinase.

MMP, matrix metalloprotease

PBS, phosphate-buffered saline.

PKC, protein kinase C.

proHB-EGF, a membrane-anchored precursor form of HB-EGF.

PVDF, polyvinylidene fluoride.

SAPK, stress-activated protein kinase.

SDS, sodium dodecyl sulfate.

SDS-PAGE, polyacrylamide gel electrophoresis.

sHB-EGF, soluble form of HB-EGF.

TACE, tumor necrosis factor- α converting enzyme.

TNF- α , tumor necrosis factor- α .

TPA, 12-O-tetradecanoylphorbol-13-acetate.

wt, wild type.

Introduction

1. Identification of HB-EGF

Heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF); (for reviews, Davis-Fleischer and Besner, 1998; Iwamoto and Mekada, 2000) was the first identified in 1990 in a culture medium of human macrophages (Besner et al., 1990). The conditioned media was analyzed by heparin-sepharose affinity chromatography, and eluted proteins tested for growth factor activity using DNA synthesis assay on BALB/c 3T3 cells. A peak of activity eluted from heparin-sepharose columns by 1.0 M NaCl, was detected. Subsequently, this macrophage-derived heparin-binding growth factor was found to be a member of the EGF family of growth factors (Higashiyama et al., 1991).

2. Diphtheria Toxin Receptor

Diphtheria toxin, secreted by *Corynebacterium diphtheriae*, is a cytotoxic protein that inhibits cellular protein synthesis by inactivating elongation factor-2 (EF-2) through the ADP-ribosylation (for review, Pappenheimer, 1977). This toxin specifically recognizes cell surface protein (diphtheria toxin receptor) and it, or at least a fragment of it, enter to the cytosol where it exerts toxicity. Diphtheria toxin receptor was identified and purified from the membrane fraction of monkey kidney-derived Vero cells with an affinity chromatography of diphtheria toxin or diphtheria toxin mutant protein CRM197 (Mekada et al., 1988; Mekada et al., 1991). Then, diphtheria toxin receptor was shown to be identical to the membrane-anchored form of HB-EGF by expression cloning method on

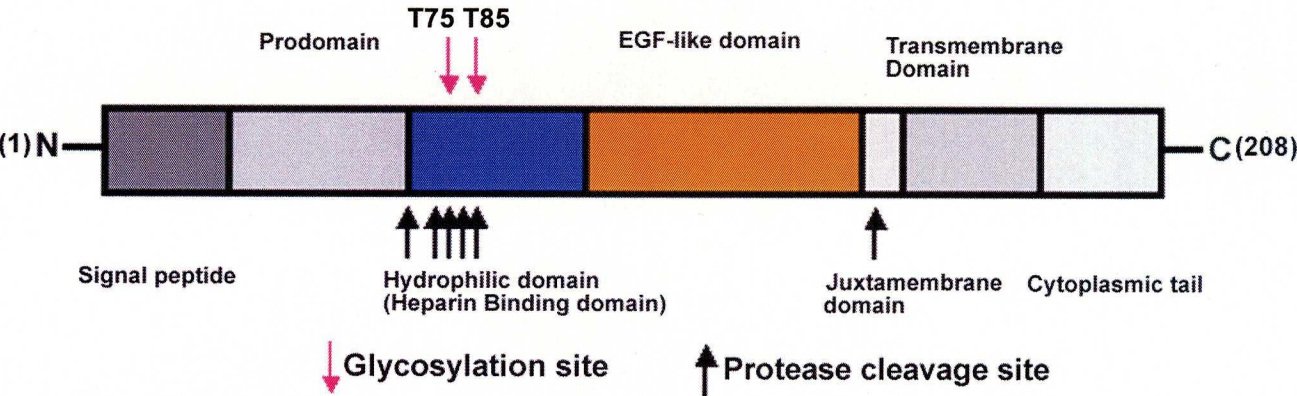
mouse cell, which expressed the diphtheria toxin sensitivity gene (Naglich et al., 1992) and by protein analysis (Iwamoto et al., 1994). HB-EGF is expressed in a wide variety of mammalian species, including human, monkey, rabbit, guinea pig, rat and mouse. However, rat and mouse are not sensitive to diphtheria toxin, due to amino acid substitutions in their HB-EGF molecules (Mitamura et al., 1995).

3. Structure and function of HB-EGF molecule

Human HB-EGF was purified to homogeneity from a conditioned medium of U-937 cells, a human macrophage-like cell line (Higashiyama et al., 1991). Based on the N-terminal amino acid sequence from purified proteins, human HB-EGF gene was cloned in 1991 from a U-937 cDNA library. The cDNA sequence analysis revealed that human HB-EGF precursor (proHB-EGF) is comprised of 208 amino acid residues with a putative signal sequence, prodomain, hydrophilic domain, EGF-like domain, juxtamembrane domain, transmembrane domain and cytoplasmic domain (Fig. 1A). The hydrophilic domain (also referred to as the heparin-binding domain) is a target of processing. HB-EGF is cleaved at at least 5 sites within this domain. This domain also includes 2 O-glycosylation sites, which position 75 and 85 threonines of human HB-EGF (Fig. 1A). HB-EGF shows strong heparin-binding properties that are mainly due to the highly charged amino acids within this domain. The heparin-binding property of HB-EGF is a unique characteristic, which might cause the differences in its biological activity from other EGF family proteins. EGF-like domain contains the conservative six cysteine residues that form three

Fig. 1

A



B

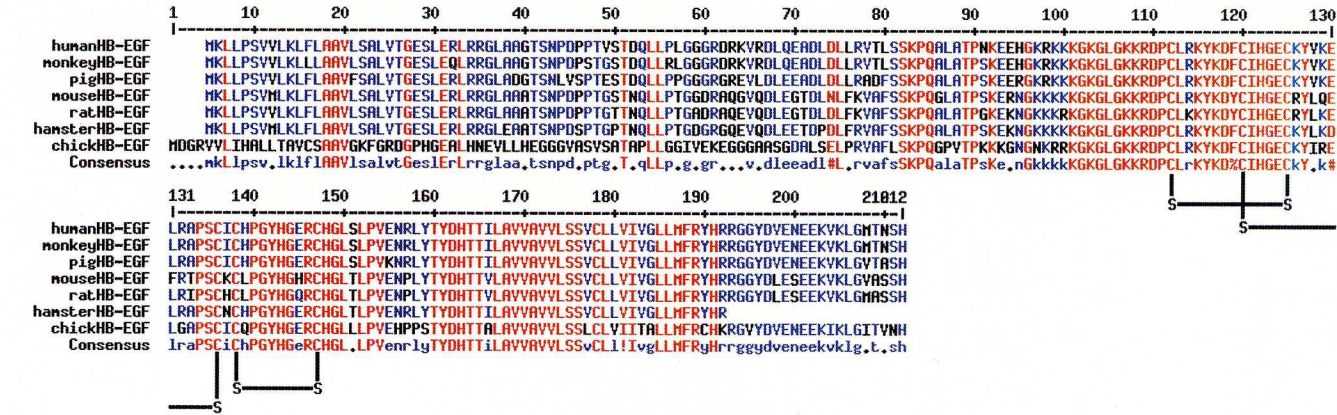


Fig. 1. Structure of proHB-EGF.

A, Predicted domain structure of the primary translation structure of human proHB-EGF. Black Arrows indicate cleavage sites of proHB-EGF. Predictable cleavage sites of human proHB-EGF are Arg62 | Asp63, Leu72 | Arg73, Arg73 | Val74, Leu76 | Ser77, Gln81 | Ala82 or Leu152 | Pro153. B, Comparison of HB-EGF amino acid sequences from various species. Red characters indicate completely identical amino acids among 7 sequences. Blue characters are identical in at least 6 of 7 species. S-S of below of sequences indicate that disulfide bonds between 6 cysteines of EGF like domain.

disulfide bridges, like other EGF family proteins. The juxtamembrane domain between the EGF-like and transmembrane domains is the second target for processing of proHB-EGF by proteases. A putative C-terminal cleavage site of human proHB-EGF is between leucine 148 and proline 149 in the juxtamembrane domain. This processing, called “ectodomain shedding”, of proHB-EGF makes a soluble 75–86-amino acid growth factor (sHB-EGF) (Goishi et al., 1995). Although the regulating process of proHB-EGF ectodomain shedding yields substantial sHB-EGF, a considerable amount remains uncleaved at the cell surface. Diphtheria toxin binds to the uncleaved form of HB-EGF, *i.e.* proHB-EGF. The transmembrane and cytoplasmic domains are 47 amino acid residues; and the particular activities of these domains are unknown.

HB-EGF genes were cloned from several species. These amino acid sequences are highly conserved (Fig .1B). Genbank IDs of HB-EGFs are AY164533 (human) (Fen et al., 1993), AH006744 (mouse) (Abraham et al., 1993), M93012 (monkey) (Naglich et al., 1992), NM_012945 (Rat) (Abraham et al., 1993), AF069753 (Chinese Hamster) (Cha et al., 1998), AF131224 (chick) (Fu et al., 1999) and Y15731 (pig) (Pascall, 1997). The primary structure of HB-EGF is similar to the EGF family protein, amphiregulin, which also has prodomain, heparin-binding domain, EGF-like domain and transmembrane domain (Masague´ and Pandiella, 1993).

HB-EGF binds to the EGF receptor (ErbB1) and the related receptor tyrosine kinase (ErbB4), and activates them (Elenius et al., 1997). In addition to being a precursor of sHB-EGF, proHB-EGF is a biologically active molecule,

forming complexes with both CD9 (Mitamura et al., 1992) and integrin $\alpha 3\beta 1$ (Nakamura et al., 1995) to transduce biological signals to neighboring cells in a non-diffusible manner (Higashiyama et al., 1995).

4. Unique biological activities of HB-EGF

sHB-EGF is a potent mitogen like other EGF family growth factors. SHB-EGF highly accelerates the mitosis of DER cells, which are mouse 32D cells expressing human EGF receptor (EGFR). In contrast, DER cells undergo apoptosis when DER cells are co-cultured on Vero-H cells producing proHB-EGF; suggesting that proHB-EGF negatively regulates cell proliferation (Iwamoto et al., 1999). This result suggests that two forms of HB-EGFs, sHB-EGF and proHB-EGF, have opposite effects on cell proliferation. These unique characters of HB-EGF indicate that cleavage of proHB-EGF (ectodomain shedding) could result in the conversion of the function, or to inhibition or promotion of cell proliferation.

5. Physiological roles of HB-EGF

Northern blotting experiments using mouse, rat and human cDNA clones as probes revealed HB-EGF transcript expression in multiple tissues including brain, lung, heart and skeletal muscle (Abraham et al., 1993). HB-EGF mRNA distribution was also analyzed by RT-PCR in a variety of porcine tissues (Vaughan et al., 1992). The principal sites of HB-EGF synthesis were skin, kidney, lung, heart, brain and male reproductive tissues. Expression was also detected in various regions of the adult central nervous system. Lower but

detectable signals were found in spleen, lymph node and thymus. Taken together, these results indicate that HB-EGF mRNA is present in a wide range of body tissues. In tissue, HB-EGF, especially the secreted form (sHB-EGF), is a potent mitogen and chemoattractant for a number of cell types, including vascular smooth muscle cells, fibroblasts, and keratinocytes (Higashiyama et al., 1992). HB-EGF is implicated in a number of physiological and pathological processes in the body (Raab and Klagsbrun, 1997), including wound healing (Marikovsiky et al., 1993; Tokumaru et al., 2000), kidney collecting duct morphogenesis (Takemura et al., 2001), blastocyst implantation (Das et al., 1994), cardiac hypertrophy (Asakura et al., 2002; Fujino et al., 1998; Perrella et al., 1994; Iwamoto et al., 2003), smooth muscle cell hyperplasia (Miyagawa et al., 1995), pulmonary hypertension (Lemjabbar and Basbaum, 2002), and oncogenic transformation (Fu et al., 1999).

Recently, proHB-EGF deficient mice were produced (Iwamoto et al., 2003). HB-EGF-null mice (HB-EGF(del/del) mice) show severe defects in heart function, including an enlarged heart chamber, and thick valves; most deficient mice dying within a week from heart failure. HB-EGF null mice exhibit the phenotypes shown in EGFR-null mice and ErbB2 conditional-null mice. These results suggest that in addition to EGFR, HB-EGF signals by means of activation of ErbB2 and ErbB4 in the heart.

More recently, mice producing uncleavable HB-EGF or sHB-EGF were generated. Those with uncleavable HB-EGF were almost similar in phenotype to HB-EGF defective mice, while mice producing sHB-EGF showed severe hypertrophy in the heart and thick skin (Iwamoto and Mekada, unpublished

work). These results suggest that strict control of proHB-EGF cleavage (ectodomain shedding) is critical for the proper regulation of the activity of this growth factor.

6. Ectodomain shedding of HB-EGF

Ectodomain shedding of membrane binding proteins were noticed in the early 1980s'. (Plesser et al., 1980; Thomopolulos et al., 1982). The release of an extracellular domain through limited proteolysis was recognized as a general mechanism to regulate the function of transmembrane proteins. Ectodomain shedding occurs at or near the cell surface and is a regulated process; it can be activated by several stimuli. For example, with phorbol esters, it is well-characterized that nonphysiological compounds with the ability to activate protein kinase C (PKC), are the most common way to activate ectodomain shedding. Typically, soon after phorbol ester addition, cells shed the ectodomains of a considerable fraction of the cell surface molecules (Arribas and Borroto, 2002).

Ectodomain shedding of EGF family proteins was reported in 1987 (Brigman et al., 1987). Accumulation on the cell surface of proTGF- α has been demonstrated by staining with antibodies, and by the quantities of cell-associated proTGF- α to digestion by added proteases (Brachmann et al., 1989; Wong et al., 1989; Teixido et al., 1990; Anklesaria et al., 1990; Mueller et al., 1990). Cleavage of proTGF- α , proCSF-1, proKL, and proHB-EGF occurs after they reach the cell surface (Flanagan et al., 1991; Perez et al., 1990; Stein et al., 1990; Goishi et al., 1995). This proteolytic system (involved in

proTGF- α cleavage) differs from general protein maturation or degradation systems, including the N-terminus of cleavage for maturation of EGF family proteins, such as those in the Golgi or lysosomal component (Bosenberg et al., 1992). ProTGF- α or proHB-EGF retained in pre-Golgi compartments by the action of brefeldin A are not susceptible to cleavage (Bonsenberg et al., 1992; data not shown).

Cleavage of proHB-EGF was also confirmed with phorbol esters stimuli (Lanzrein et al, 1995). Ectodomain shedding of proHB-EGF is induced by both physiological and pharmacological agonists such as 12-O-tetradecanoylphorbol-13-acetate (TPA). In monkey kidney Vero cells, the presence of a constitutively active form of PKC δ results in the ectodomain shedding of proHB-EGF, and dominant-negative (dn) PKC δ suppresses TPA-induced shedding, suggesting that the PKC δ isoform contributes to the ectodomain shedding of proHB-EGF. PKC δ binds to the cytoplasmic domain of ADAM9/MDC9/meltrin- γ (Izumi et al., 1998), a metalloprotease belonging to the ADAM (a disintegrin a metalloprotease) family (Weskamp et al., 1996). Overexpression of ADAM9 induces proHB-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 PKC δ in the proHB-EGF shedding pathway (Izumi et al., 1998), although the role of this protease in the direct cleavage of proHB-EGF remains unclear.

Lysophosphatidic acid (LPA) and other ligands of the seven-transmembrane G protein-coupled receptors (GPCR), such as Angiotensin II, Bombesin or Endothelin, also stimulate proHB-EGF shedding

(Prenzel et al., 1999; Hirata et al., 2001; Umata et al., 2001). The activation of shedding by such ligands is crucial for the transactivation of the EGFR by G protein-coupled receptor ligands (Prenzel et al., 1999; Eguchi et al., 2001; Fujiyama et al., 2001; Kalmes et al., 2001). EGFR transactivation is an important HB-EGF mechanism for cell mitosis in vascular smooth muscle cells or the other cells (Andreas et al., 2001). The mechanism of transactivation includes 2 steps. In the first, GPCR or the other stimuli activate metalloproteases and should begin to shed EGFR ligands. In the second step, EGFR ligands would be binding to and activating EGFR. Activation of EGFR would occur not only in an autocrine mode but also in a paracrine mode in adjacent cells. EGFR transactivation is instigated by GPCR ligands, PDGF, growth factors, phorbol esters, cytokines or cell damage. Some transactivation studies have suggested that heparin interrupted EGFR phosphorylation. Among the EGF family of growth factors, amphiregulin and HB-EGF stand out because they possess a heparin-binding domain adjacent to the EGF-like unit. Not only heparin, but also CRM197 or HB-EGF neutralizing antibody inhibited GPCR ligand-induced EGFR phosphorylation, indicating that HB-EGF is a critical molecule within the EGF-family of growth factors in the transactivation of EGFR.

In Vero cells, the Ras-Raf-MEK and Rac signaling pathways are activated in LPA-induced proHB-EGF shedding (Umata et al., 2001). LPA-induced shedding is inhibited by dnHa-Ras, dnRac1, and the MEK inhibitor PD98059, but not by either dnPKC δ or dnADAM9. Conversely, although TPA-induced shedding is inhibited by dnPKC δ 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 proHB-EGF ectodomain shedding, here designated the LPA- and TPA-induced pathways. ProHB-EGF expression is enhanced in various tissues upon injury and inflammation (Blotnick et al., 1994; Koh et al., 2001; Fang et al., 2001; Miyazaki et al., 2001; Sakai et al., 2001; Nguyen et al., 2000; Morita et al., 1993; Yoshizumi et al., 1992). 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 in pathological processes, including atherosclerosis, that follow from inappropriate inflammation.

7. Does Stress Induce HB-EGF Shedding?

Cellular stress and inflammation may function as triggers of ectodomain shedding. Because, most stimuli which cause HB-EGF ectodomain shedding or EGFR transactivation are also factors inducing the activation of HB-EGF transcription. Some stress-inducing stimuli, such as hypertonic osmotic pressure or oxidative stress, are reported to activate HB-EGF transcription in rat smooth muscle cells. On the other hand, oxidative or osmotic stresses induced EGFR transactivation in vascular smooth muscle cells. In this work, I investigated whether stimuli inducing increased transcription of HB-EGF cause ectodomain shedding of HB-EGF. I demonstrate in this work that various stress-inducing stimuli and inflammatory cytokines strongly induce ectodomain shedding, acting through a new p38 MAPK-mediated shedding pathway, distinct from both the TPA- and LPA-induced signaling pathways. Results of this work

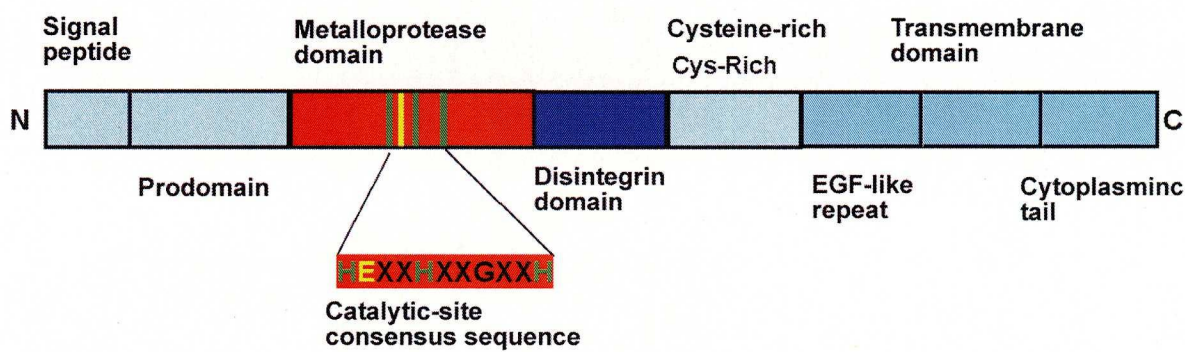
suggest that HB-EGF activity is regulated at the transcriptional level and by ectodomain shedding responding to stress and inflammation. HB-EGF may cause cardiac hypertrophy, atherosclerosis, kidney injury, and so on. Especially, as HB-EGF is required for vascular smooth muscle cells to proliferate in atherosclerosis. Vascular smooth muscle cells require ERK, p38 and JNK activation for proliferation *in vitro* (Kavurma and Khachigain, 2003). However, in actual atherosclerosis, it does not yet understood what stimulation is the trigger. Various stresses and cytokines are possible candidates for the trigger for HB-EGF shedding and EGFR transactivation in the vascular system or in the heart. In fact, p38 MAPK was shown to be activated before vascular hyperplasia by vascular balloon-injury (Ohashi et al., 2000). Cardiac specific dominant negative p38 MAPK transgenic mice were resistant to cardiac fibroblast cells proliferation but developed hypertrophy (Zhang et al., 2003). Such evidence suggests that p38 MAPK mediated vascular smooth muscle cells proliferation or cardiac fibrosis were able to mediate the HB-EGF shedding cascades in this work.

8. ADAM family proteases

A disintegrin metalloproteases (ADAMs) are considered to be strong candidates as proteases causing ectodomain shedding of many EGF superfamily growth factors (Schlondorff and Blobel, 1999; Seals and Courtneidge, 2003). ADAMs are a family of transmembrane glycoproteins having a role in cell-cell interactions and in the processing of ectodomains of proteins (Fig. 2). They are characterized by a conserved domain structure,

Fig. 2

A



B

mouse ADAM9	335-	QITVETFASIVAH	ELGHN	LG	MN	DDGRECF	-364
bovine ADAM10	371-	HVPPKVSHITFA	EVGHN	NFG	SP	DSGTECT	-400
human ADAM12	338-	SDNPLGAAVTLA	ELGHN	NFG	MN	DTLDRGC	-367
mouse ADAM17	393-	TILTKEADLVTT	HELGN	NFG	AE	DPGLAE	-422

Fig. 2. Structure of ADAM proteases.

A, Domain organization of ADAM proteases. Red bar indicates predicted metalloprotease domain. Green and yellow characters are conserved amino acids (HEXXHXXGXXH) of proteases catalytic-site. B, Comparison of a conserved protease catalytic sites of mouseADAM9 (Yagami-Hiromasa et al., 1995), bovineADAM10 (Howard et al., 1996), humanADAM12 (Gilpin et al., 1998) and mADAM17 (Black et al., 1997).

consisting of an N-terminal signal sequence followed by prodomain, metalloprotease and disintegrin domains, a cysteine-rich region, usually containing an EGF-like repeat, and finally a transmembrane domain and cytoplasmic tail (Fig. 2A) (Blobel and White, 1992; Wolfsberg and White 1996; Blobel, 1997; Black and White, 1998). The adamalysins subfamily also contains the class III snake venom metalloprotease and the ADAM-TS (-a thrombospondin like) family, which although similar to ADAMs, can be distinguished structurally. Most ADAMs have a relatively well-conserved metalloprotease domain, which contain a zinc binding catalytic-site consensus sequence (HEXXHXXGXXH) (Fig. 2B). But, half of the known ADAMs are predicted to be catalytically inactive, because of incomplete consensus catalytic sequences. The cytoplasmic tails of ADAMs are highly variable both in length and in sequence. This domain contains specialized motifs that have been postulated to be involved in the inside-out regulation of metalloprotease activity. The most common motifs are poly-proline sites for SH3 domain-containing proteins. These SH3-binding sites are present in human ADAM7, 8, 9, 12, 15, 17, 19, 22, 29, and 33. ADAM9 and 15 have an extensive array of protein-protein interaction sites, including SH-3 binding domains and potential sites for tyrosine, threonine or serine phosphorylation / SH-2-association. It is possible that ADAMs (9, 15 and the others) associate with a number of different proteins including adaptors, such as endophilin, SH3PX1, and Grb2, and three Src family tyrosine kinases (Howard et al., 1999; Nelson et al., 1999; Poghsyan et al., 2001). Some ADAMs proteases have conserved RGD motifs in a disintegrin domain. Human ADAM15 also has a RGD consensus sequence, and

associates with integrins $\alpha v\beta 3$ and $\alpha 5\beta 1$ in an RGD-dependent manner (Blobel et al., 1992). Most ADAMs, however, do not have RGD sequences in their disintegrin domain. The functions of the Cysteine-rich and EGF-like domains are still not identified. Perhaps the most of compelling piece of data concerning a cysteine-rich domain-specific function is that it acts as a ligand for the cell-adhesion molecule syndecan. ADAMs are also found in *Caenorhabditis elegans* and *Drosophila*.

In humans, 19 ADAM genes are identified. ADAM17/TACE (TNF- α converting enzyme), ADAM9/MDC9/meltrin- γ and ADAM10/Kuz (Kuzbanian) were also studied in the ADAMs family regarding proteases activity. It was reported that ADAM17/TACE cleaves TNF- α , TGF- α , APP, HB-EGF or many kinds of membrane proteins in vitro and in vivo. Actually, TNF- α peptide was directly cleaved by recombinant ADAM17, and TNF- α processing was significantly inhibited by targeted disruption of the adam17 gene (Black et al., 1997; Moss et al., 1997). However, it is not know whether ADAM17 cleaves TNF- α directly, because processing of TNF- α can still occur in cells derived from ADAM17 deficient mice. ADAM10 is considered an active protease for Notch and Delta. Kuz (*Drosophila* ADAM10) deficient or carrying loss of function *kuz* flies show an increase in the proportion of cells adopting a neural fate; the phenotypes were the same as Notch defect *Drosophila*. ADAM10 deficient mice die by day 9.5 of embryogenesis with pronounced defects in the neural and cardiovascular systems. ADAM9 is involved in ectodomain shedding of HB-EGF, which is mediated by PKC δ activation, in Vero-H cells (Izumi et al., 1998). However, ADAM9 deficient mice normally are viable and fertile, and do

not show any major pathologies (Weskamp et al., 2002). ADAM10 and ADAM9 were also shown to cleave APP and the other membrane proteins.

HB-EGF is reported to be shed by ADAM9, ADAM10, ADAM12 and ADAM17 (Izumi et al., 1998; Lemjabbar and Basbaum, 2002; Yan et al., 2002; Asakura et al., 2002; Sunnarborg et al., 2002). In Vero-H cells, overexpression of ADAM9 shows ectodomain shedding of HB-EGF. However, ADAM10, ADAM12 and ADAM17 did not induce HB-EGF shedding by overexpression of wild types of these ADAMs (Yamazaki et al, unpublished works).

In this work, I tried to identify ADAMs for stress-induced HB-EGF shedding cascades. But, the dominant negative forms of ADAM9, ADAM10, ADAM12 or ADAM17 were not seen to inhibit stress-induced proHB-EGF shedding.

Experimental Procedures

Reagents

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

Antibodies

The goat antibody specific for the C terminus of human proHB-EGF (C-18) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-HB-EGF neutralizing antibody was obtained from R&D Systems (Minneapolis, MN). A rabbit antiserum against the N terminus of human proHB-EGF (H6) was raised against a peptide of the human HB-EGF N-terminal region (Iwamoto et al., 1994). Rabbit anti-mouse ADAM9 antibody was raised against a glutathione S-transferase fusion protein containing the mouse ADAM9 C-terminal region (amino acids 718–845). Horseradish peroxidase-conjugated goat anti-rabbit and unconjugated anti-FLAG (M2) monoclonal antibodies were purchased from Zymed Laboratories Inc. (San Francisco, CA) and Sigma-Aldrich Co. (St. Louis, MO), respectively. Horseradish peroxidase- and

Cy3-conjugated donkey anti-goat antibodies were obtained from Chemicon International, Inc. (Temecula, CA). Rabbit anti-phospho-JNK1/2, anti-JNK, anti-phospho-p38 MAPK and anti-p38 MAPK antibody were purchased from Cell Signaling Technology (Beverly, MA).

Plasmids

pEGFP-c1, a plasmid encoding GFP, was purchased from Becton Dickinson Biosciences Clontech (Palo Alto, CA). The mouse dnPKC δ (pSRD-kinase knockout mouse PKC δ , R144A, R145A, K376R) (Hirai et al., 1994; Ohno et al., 1994) and the mouse dnADAM9 H347A, H351A mutant (pEF-BOS-ADAM9, H347A, H351A (Izumi et al., 1998)) were kindly provided by S. Ohno (School of Medicine, Yokohama City University). The FLAG-tagged mouse dnADAM9 E348A mutant (pDisplay/HA/FLAG-ADAM9 E348A), bovine dnADAM10 E382A mutant (pCX4/FLAG-ADAM10 E382A), human dnADAM12 E346A mutant (pDisplay/HA/FLAG-ADAM12 E348A) and mouse dnADAM17 E406A mutant (pDisplay/HA/FLAG-ADAM17 E406A) were generated by site-directed PCR-based mutagenesis. Primer sequences used are:

A9-glu-fwd, 5'-CAACAATGGATGCAAATGTCTCCACAG-3';

A9-glu-rev, 5'-CTCATGCTTTGGGGCATAACCTTGG-3';

A10-glu-fwd, 5'-TGGACATAACTTTGGATCTCCGCATG -3';

A10-glu-rev, 5'-ACTGCATGAGCAAACGTAATGTGAGAGAC-3';

A12-glu-fwd, 5'-GCACATGCTCTGGGCCACAATTTTC-3';

A12-glu-rev, 5'-CAGGGTCACGGCTGCACCAAGG-3';

A17-glu-fwd, 5'-CTTTGGGACATAATTTTGGAGC-3';

A17-glu-rev, 5'-CATGAGTTGTAACCAGGTCAG-3'.

FLAG-tagged mouse dnRac1N17 (pEF-BOS-Rac1, T17N) were gift from K. Kaibuchi (Nara Institute of Science and Technology) (Umata et al., 2001). FLAG-tagged rat dnp38 MAPK (pcDNA3-rp38a, T180A, Y182F) was kindly provided by H. Hatanaka and Y. Ishikawa (Institute of Protein Research, Osaka University) (Ishikawa et al., 2000; Yamagishi et al., 2001). FLAG-tagged mouse dnJNK1 (pCMV5-JNK1, T183A, Y185F) (Derijard et al., 1994), and a constitutively active form of human MKK6 (caMKK6, pcDNA3.1-MKK6, S207E, T211E) (Fujishiro et al., 2001) were gift from R. J. Davis (University of Massachusetts Medical School) and Y. Gotoh (University of Tokyo), respectively.

Cell Culture and Transfection

Vero-H cells (Goishi et al., 1995) were maintained in modified Eagle's medium with nonessential amino acids (MEM-NEAA) supplemented with 10% fetal calf serum. Transfections into Vero-H cells were performed using the calcium phosphate technique (Chen and Okayama, 1987). Overnight cultured Vero-H cells (5×10^5 cells / 10-cm dish) were incubated in 10 ml of MEM-NEAA with 10 % fetal calf serum, for 30 min at 2 % CO₂, 35 °C. Plasmids (total 20µg each) were mixed with 0.5 ml of 0.25 M CaCl₂ and 0.5 ml of 2 x BBS (280 mM NaCl, 50 mM BES, 2 1.5 mM Na₂HPO₄ 50 mM BES pH 6.95), and was added into medium. 24 h after of plasmid solution was added cells were washed PBS twice, and cells were cultured for 24 h in MEM-NEAA with 10 % fatal calf serum.

In Fig. 7, transfection into Vero-H cells were performed by

electroporation (Bio Lad Gene Pulser), according to the manufacturer's instruction. Vero-H cells (about $5 \times 10^7 \sim 1 \times 10^8$ cells) were recovered in centrifuge tubes. Cells were suspended with KPBS (120.7 mM KCl, 31 mM NaCl, 10 mM MgCl_2 , 8.1 mM NaHPO_4 , 1.46 mM KH_2PO_4), and the concentration of cells were re-suspended in KPBS $1.5 \times 10^7 \sim 2 \times 10^7$ cells. 300 μl (5×10^6) of cell-suspension were mixed with plasmids (total 32 μg each), and were recovered into cuvettes. The suspension mixes were pulsed by Gene Pulser, at 950mF, 250V. Transfected cells were cultured for 24 h in MEM-NEAA with 10 % fetal calf serum.

Shedding Analysis by Western Blot

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 $\mu\text{g}/\text{ml}$), IL-1 β (4 ng/ml), anisomycin (10 $\mu\text{g}/\text{ml}$), sorbitol (0.4 M), or H_2O_2 (0.5 mM). When indicated, cells were also treated 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 SpectrolinkerTM (Spectronics Corp., Westbury, NY) (Galibert et al., 2001). 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 MgCl_2 , 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 20 $\mu\text{g}/\text{ml}$ antipain, and 10 $\mu\text{g}/\text{ml}$ chymostatin, pH 7.4). After 5 min on ice, the lysates were clarified by centrifugation at 15,000 rpm for 5min. Recovered supernatant incubated with Laemmli SDS gel

sample buffer at 96 °C for 2 min (Laemmli, 1970). Lysates were analyzed by 15 % SDS-PAGE. After electrophoresis, the proteins were transferred onto PVDF membrane. The membrane was blocked with TBS (20 mM Tris-HCl, 100 mM NaCl, pH7.5) containing 1 % skim milk. Western blot was performed using a goat antibody specific for the human proHB-EGF C terminus, in blocking solution with 0.05 % Tween 20. The antibody was visualized with horseradish peroxidase-conjugated donkey anti-goat IgG using ECL Plus (Amersham Biosciences, Piscataway, NJ). To detect sHB-EGF in the culture medium, sHB-EGF was trapped with heparin-Sepharose beads and then eluted with Laemmli SDS gel sample buffer at 96 °C for 2 min. The eluted materials were subjected to by 15 % SDS-PAGE and 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 ProHB-EGF Ectodomain

Cells Plated on cover-slips which were incubate with 10 µg / ml of heparin for wash out of HSPG binding sHB-EGF. The cover-slips were incubate with 1 µg / ml of goat anti-HB-EGF neutralizing antibody in PBS containing 1 % skim milk solution at 4 °C for 2h, washed thee times with PBS and fixed 3.7 % formaldehyde in PBS for 15 min at room temperature. Cells were washed twice with PBS, and 30 min with PBS containing 1 % skim milk. ProHB-EGF on cell surface was detected by incubating with Cy3-conjugated anti-goat IgG for 30 min at room temperature (Izumi et al., 1998). To detect the

expression of ADAM9 or FLAG-tagged transfected proteins, after fixed cells were permeabilized with 0.1 % Triton X-100 for 3 min at room temperature and wash three times. The cells were incubated with 1 % skim milk / PBS for 30 min at room temperature for blocking. Then stained with 1 μ g / ml of anti-ADAM9 or anti-FLAG antibody indicated, followed by fluoresce in isothiocyanate-conjugated second antibody in 1 % skim milk / PBS for 30 min at room temperature. To detect cells transfected with dnPKC δ , caMKK6 or vector (pEF-BOS) alone as a control, EGFP (pEGFP-c1) was cotransfected. Images were captured with Olympus microscope BX50 (Tokyo, Japan), Chilled CCD Camera System (Hamamatsu Photonics Corp., Shizuoka, Japan) FISH imager TM system (Carl Zeiss, Inc., Germany). The percentage of proHB-EGF-positive cells was determined by counting the number of proHB-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 positive for either the transfected or marker proteins in each experiment. Scoring was performed in a completely blind manner.

MAPK Phosphorylation Analysis by Western Blot Analysis

Vero-H cells (1×10^6 cells/ 10-cm dish) were cultured for 30 h in serum-free modified Eagle's medium with nonessential amino acids and then treated with stimulus. Following incubation for 30 min with stimuli, cells were collected and re-suspended in 200 μ l of Laemmli SDS gel sample buffer containing inhibitors of proteases and phosphatases (50 mM Na₄P₂O₇, 10 mM

NaF, 2 mM Na₃VO₄, 1 mM DTT, 0.5 mM Phenylarsineoxide, 0.2 mM PMSF, 20 µg/ml antipain, and 10 µg/ml chymostatin, pH 7.4). Lysates incubated with Laemmli SDS gel sample buffer at 96 °C for 2 min (Laemmli, 1970) and sonication. Lysates were analyzed by 12 % SDS-PAGE. After electrophoresis, the proteins were transferred onto PVDF membrane. The membrane was blocked with TBS (20 mM Tris-HCl, 100 mM NaCl, pH7.5) containing 1 % skim milk. Western blot was performed using a goat antibody specific for the human proHB-EGF C terminus, in TBS containing 1 % skim milk (anti-JNK or anti-phospho-JNK) or containing 1 % skim milk and 0.05 % Tween 20 (anti-p38 MAPK or anti-phospho-p38 MAPK). The antibody was visualized with horseradish peroxidase-conjugated donkey anti-rabbit IgG using ECL Plus.

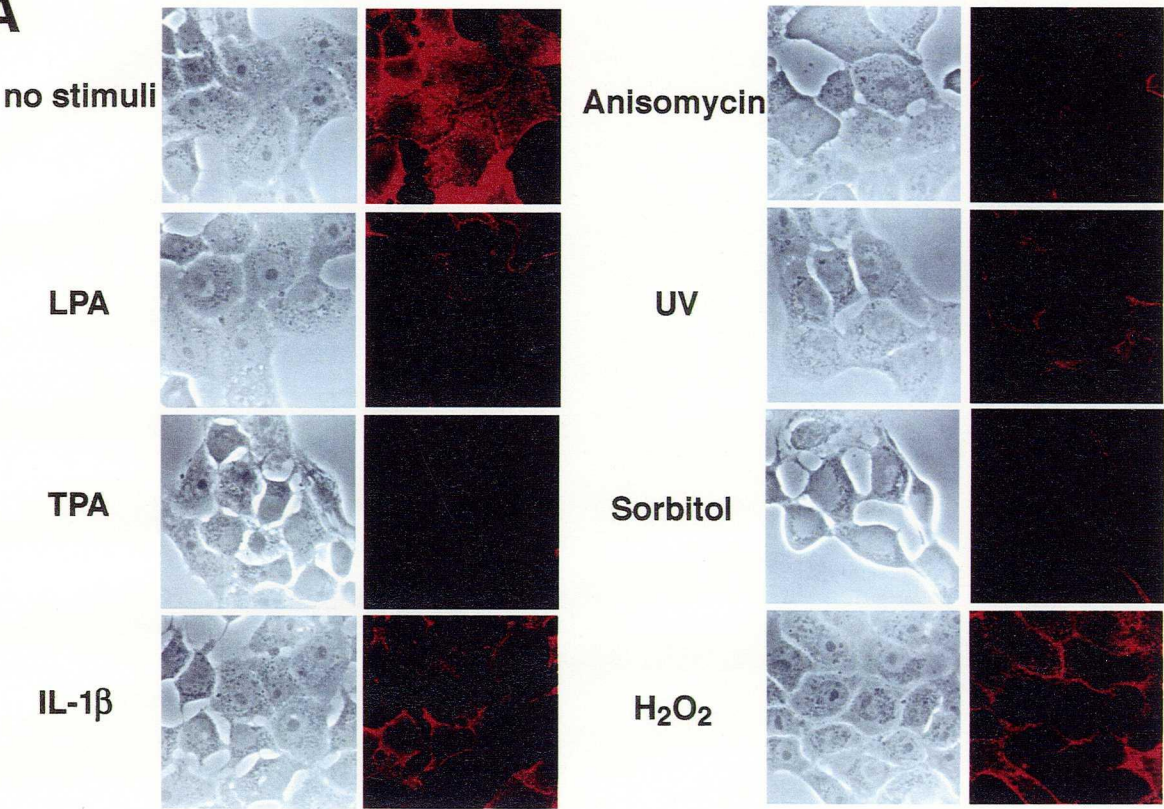
Results

Stimuli Inducing Various Stresses and IL-1 β Lead to ProHB-EGF Shedding

Vero-H cells are stable Vero transfectants overexpressing human proHB-EGF (Goishi et al., 1995). This cell line consistently exhibits responses to TPA or LPA stimuli inducing proHB-EGF ectodomain shedding (Izumi et al., 1998; Hirata et al., 2001; Umata et al., 2001). Using Vero-H cells, I examined the effect of pro-inflammatory cytokines and stress-inducing stimuli on the induction of proHB-EGF ectodomain shedding. First, I examined the ectodomain shedding of proHB-EGF by immunofluorescence microscopy using an antibody specific for the proHB-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. ProHB-EGF fluorescence at the cell surface disappeared following addition of either LPA (20 μ g/ml) or TPA (64 nM) to the culture medium (Fig. 3A) (Izumi et al., 1998; Hirata et al., 2001; Umata et al., 2001). In addition, the pro-inflammatory cytokine IL-1 β (4 μ g/ml) also led to the disappearance of proHB-EGF surface immunofluorescence. Moreover, stimuli well known to induce a cellular stress response (Kyriakis and Avruch, 2001), such as translation inhibitors (anisomycin, 10 μ g/ml) (Cano et al., 1994), exposure to UV light (40 mJ/cm²) (Galibert et al., 2001), hypertonic osmotic pressure (sorbitol, 0.4 M) (Galibert et al., 2001), or oxidative stress (H₂O₂, 0.5 mM), also induced the disappearance of proHB-EGF immunofluorescence from the cell surface. Western blot analysis confirmed the ectodomain shedding of

Fig. 3

A



B

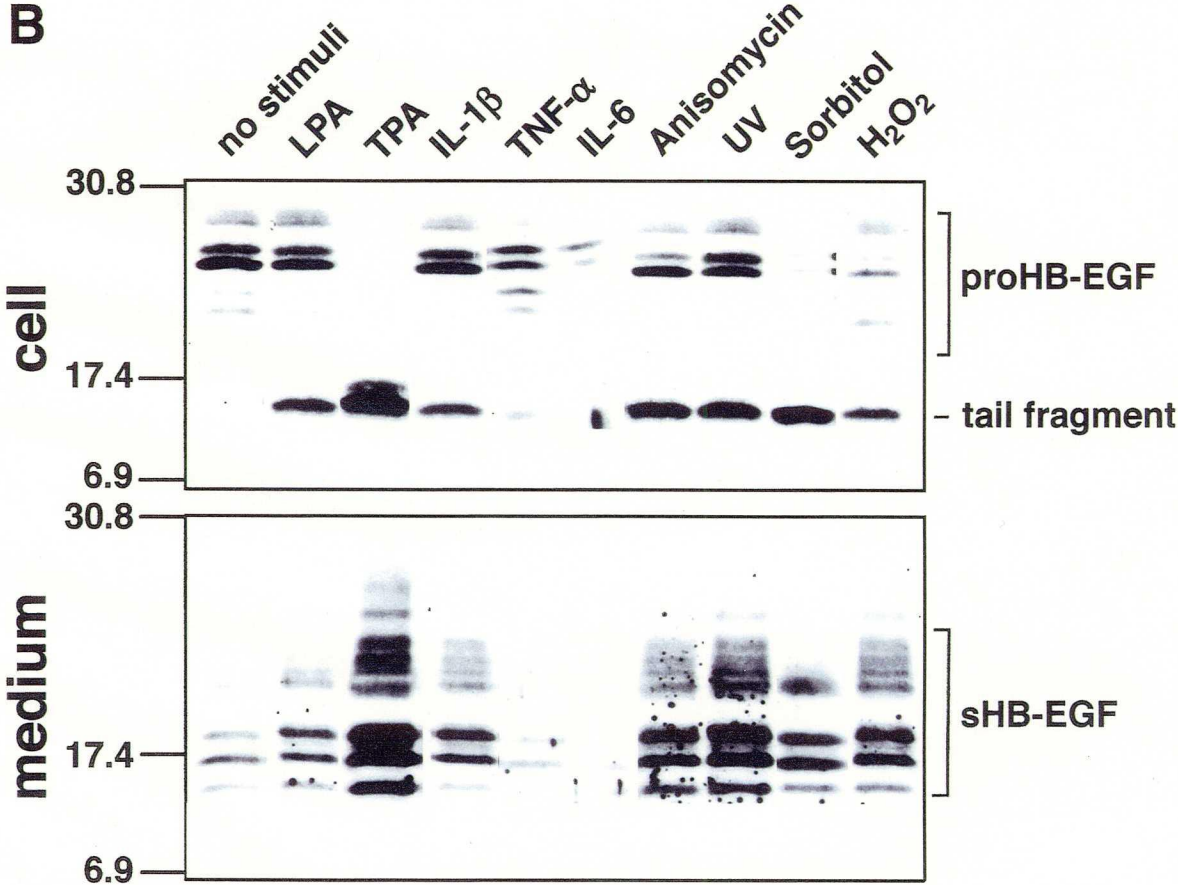


FIG. 3. Stress- and inflammatory cytokine-induced ectodomain shedding of proHB-EGF.

Vero-H cells were treated with LPA (20 µg/ml), TPA (64 nM), IL-1β (4 ng/ml), TNF-α (50 ng/ml), IL-6 (20 ng/ml), anisomycin (10 µg/ml), UV light (40 mJ/cm²), sorbitol (0.4 M), or H₂O₂ (0.5 mM) for 30 min, and then ectodomain shedding was detected by immunostaining of proHB-EGF and Western blot analysis. *A*, immunofluorescence detection of proHB-EGF at the cell surface. Cells were stained with anti-proHB-EGF neutralizing antibody, followed by Cy3-conjugated anti-goat IgG. The representative images for the samples treated with LPA, TPA, IL-1β, anisomycin, UV light, sorbitol, and H₂O₂ are shown. The *left panels* are phase-contrast images, whereas the *right panels* are immunofluorescence images. *B*, Western blot analysis. The *upper panel* illustrates full-length proHB-EGF and the tail fragment within cell lysates, detected using an antibody raised against the proHB-EGF C terminus. The *lower panel* shows sHB-EGF appearing in the culture medium, detected using an antibody raised against the proHB-EGF N terminus.

proHB-EGF in Vero-H cells (Fig. 3B). 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 proHB-EGF and the shed soluble EGF-like domain, respectively. In cell lysates, bands ranging from 20 to 30 kDa corresponded to proHB-EGF, whereas bands between 17.4 and 6.9 kDa were proteolytic fragments composed of the cytoplasmic and transmembrane domains of proHB-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-1 β , anisomycin, UV light, and sorbitol, in addition to LPA and TPA, induce proHB-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 proHB-EGF protein present through an unknown mechanism. While culturing Vero-H cells, centrifuging on a swing-rotor at 1000 rpm for 1 minute, and incubation as a mimic of shearing stress also induced proHB-EGF ectodomain shedding (Rijken et al., 1992; data not shown). In contrast, the pro-inflammatory cytokines TNF- α and IL-6 did not affect shedding. According to signal-transduction experiments, TNF- α and IL-6 were not able to activate for SAPKs in Vero-H cells. From these results, one possibility is that TNF- α and IL-6 receptors do not exist in Vero-H cells.

I also examined the time course of proHB-EGF shedding induced by IL-1 β , 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

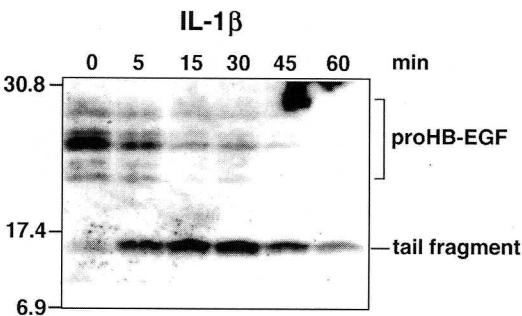
cell lysates (Fig. 3B), I utilized Western blot analysis for the cell lysates and cultured medium to examine this event (data of cultured medium are not shown). As shown in Fig. 4, ectodomain shedding was observed following treatment with each of these stress stimuli over a time course similar to that observed for TPA- and LPA-induced shedding, on immunofluorescence and cell lysates (Goishi et al., 1995; Izumi et al., 1998; Umata et al., unpublished works).

Stress-induced ProHB-EGF Shedding Involves p38 MAPK

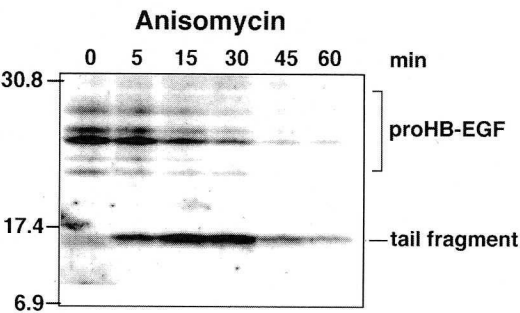
Signals from pro-inflammatory cytokines and cellular responses to stress are generally mediated by SAPKs (Kyriakis and Avruch, 2001). Therefore, I hypothesized that stress-induced proHB-EGF shedding is mediated by a SAPK family kinase. I examined the activation of p38 MAPK and the various JNKs, major members of the SAPK family, following stress-inducing stimulation of Vero-H cells. After treatment with LPA, TPA, IL-1 β , anisomycin, UV irradiation, or sorbitol, Vero-H cell lysates were analyzed by Western blotting with either an anti-phospho-p38 MAPK or an anti-phospho-JNKs 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 following treatment with either TPA or IL-1 β . SB203580, an inhibitor of p38 MAPK (Lee et al., 1994), significantly reduced p38 MAPK activity at a concentration of 2 μ M (Fig. 5A). JNK1/2 and p38 MAPK were activated by treatment with LPA, TPA, IL-1 β , anisomycin, UV light, and sorbitol, although 2 μ M SB203580 could not inhibit JNK1/2 phosphorylation (Fig. 5B). The results confirm that SB203580 specifically inhibits p38 MAPK activation. TNF- α and

Fig. 4

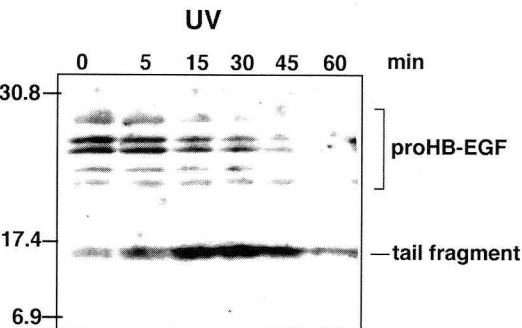
A



B



C



D

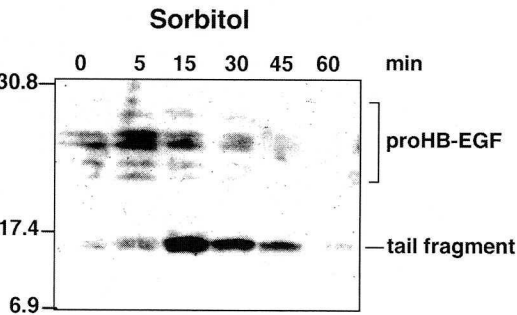
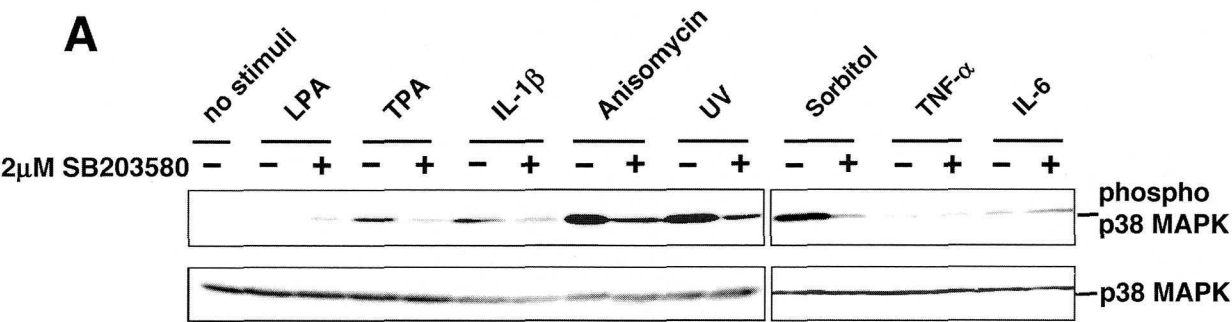


FIG. 4. Time course of stress- and IL-1 β -induced ectodomain shedding.

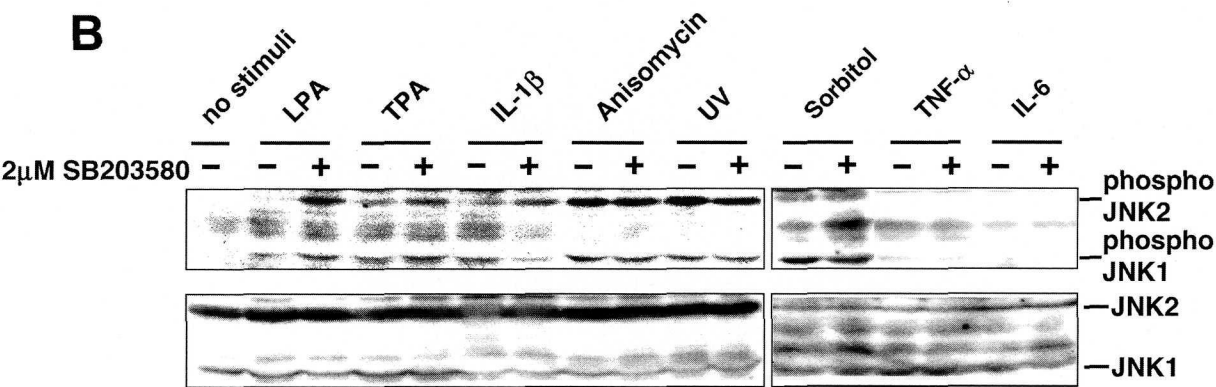
Vero-H cells were treated with 4 ng/ml IL-1 β (A), 10 μ g/ml anisomycin (B), 40 mJ/cm² UV light (C), or 0.4 M sorbitol (D). After the times indicated, the cells were harvested, and the cell lysates were analyzed by Western blotting with an antibody raised against the proHB-EGF C terminus.

Fig. 5

A



B



C

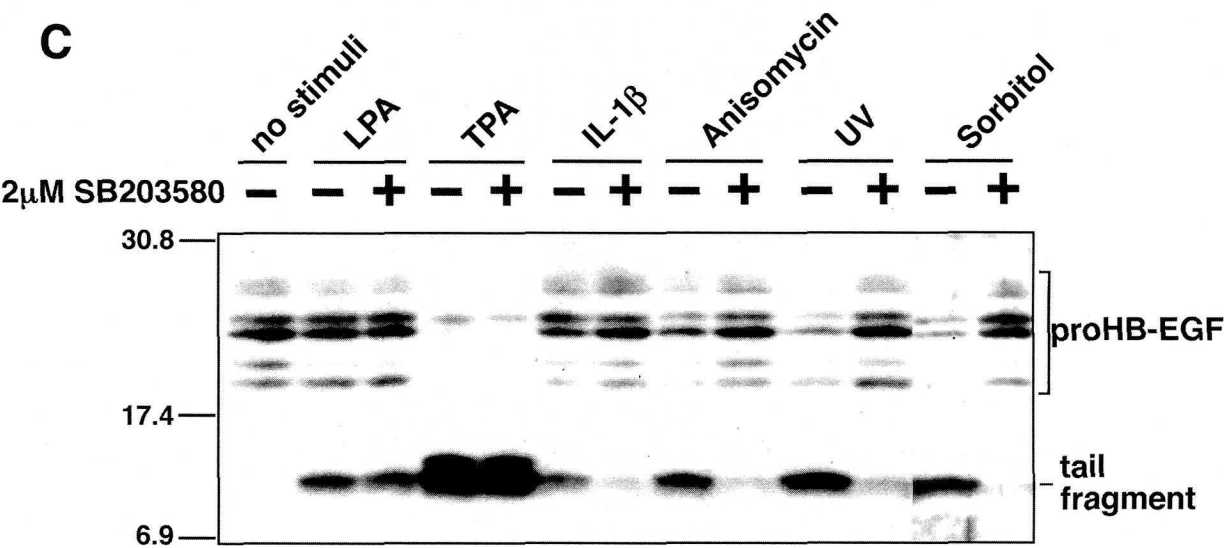


FIG. 5. Effects of SB203580 on the activation of p38 MAPK and JNK1/2 or on proHB-EGF ectodomain shedding.

Serum-starved Vero-H cells were pretreated with or without 2 μ M SB203580 for 1 h. Cells were then treated with IL-1 β 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 proHB-EGF, detected by Western blotting using an antibody raised against the proHB-EGF C terminus.

IL-6 could not activate either p38 MAPK (Fig. 5A) or JNK (Fig. 5B) in Vero-H cells, concurring with their lack of proHB-EGF cleavage (Fig. 3B). I next inspected SB203580 inhibition of proHB-EGF ectodomain shedding induction by IL-1 β , anisomycin, UV light, or sorbitol to verify the involvement of p38 MAPK. 2 μ M SB203580 drastically inhibited the stress-induced shedding caused by IL-1 β , anisomycin, UV light, or sorbitol (Fig. 5C), whereas TPA- and LPA-induced shedding was not affected. Even concentrations of SB203580 in excess of 10 μ M could not inhibit TPA- and LPA-induced shedding (data not shown). These results suggest that proHB-EGF ectodomain shedding induced by IL-1 β , anisomycin, UV light, or sorbitol requires p38 MAPK activation.

I examined the requirement for p38 MAPK in stress-induced proHB-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-1 β , anisomycin, UV light, or sorbitol enhanced proHB-EGF shedding in vector-transfected cells (Fig. 6B), shedding was inhibited in FLAG-positive cells expressing dnp38 MAPK (Fig. 6, A and B). This confirmed that LPA- and TPA-induced proHB-EGF ectodomain shedding was not inhibited by dnp38 MAPK (Fig. 6B). However, transfection experiments of wild type p38 MAPK, which might constitutively have a weak activation effect, inhibited stress- and IL-1 β induced shedding (data not shown). It is difficult to ascertain a mechanism from only this result; but it might need only a specific position of p38 MAPK molecules.

MKK6 is a specific activator of p38 MAPK but not JNKs or ERKs (Han et al., 1996). Consistent with the role of the p38 MAPK pathway in shedding,

Fig. 6
A

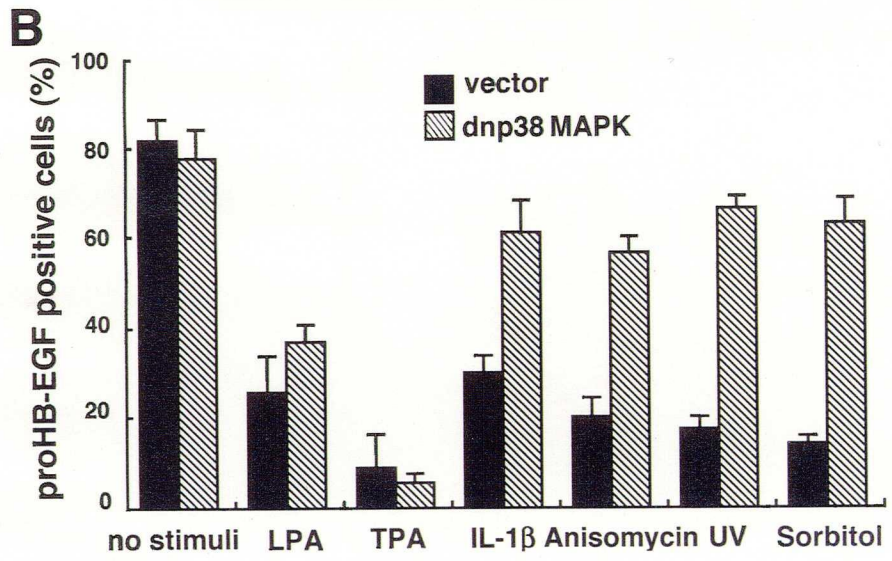
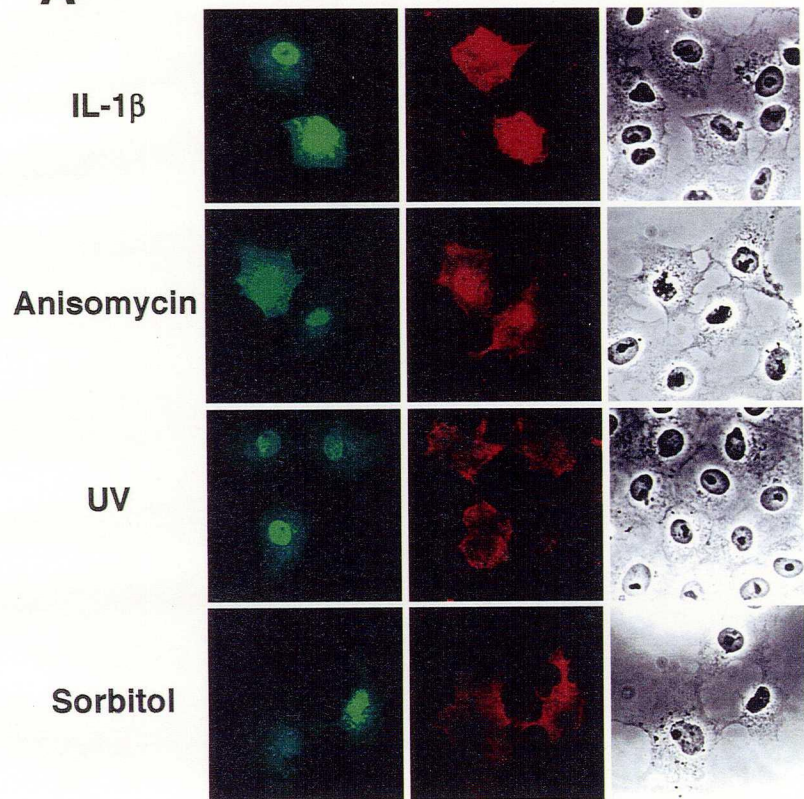


FIG. 6. Inhibition of stress- and IL-1 β -induced ectodomain shedding by dnp38 MAPK.

A, Vero-H cells were transfected with a plasmid encoding FLAG-tagged dnp38 MAPK (20 μ g) or with vector (18 μ g) plus EGFP (2 μ g) as a control. After 48 h of transfection, the cells were incubated with LPA, TPA, IL-1 β , anisomycin, UV light, or sorbitol and then double-stained with anti-HB-EGF antibody for the detection of the proHB-EGF ectodomain (*red*) and with anti-FLAG antibody for the detection of dnp38 MAPK expression (*green*). The *right panels* are phase-contrast images. Only the representative images of dnp38 MAPK-transfected cells are shown. B, shown is the percentage of proHB-EGF-positive cells among transfected cells, determined by immunofluorescence detection as shown in A. For vector-transfected cells, the percentage of proHB-EGF-positive cells among GFP-positive cells was determined.

transfection of a constitutively active form of MKK6 resulted in proHB-EGF ectodomain shedding in the absence of stress-inducing stimuli in a dose-dependent manner (Fig. 7). These results indicate that p38 MAPK activation is required for stress-induced proHB-EGF shedding in Vero-H cells.

SB203580 (2 μ M) reduced the activity of p38 MAPK, but not that of JNK1/2, as shown in Fig. 5 (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, I tested whether SP600125, an inhibitor of JNK (Bennet et al., 2001), abrogates stress-induced proHB-EGF shedding in Vero-H cells. SP600125 (5 μ M) significantly reduced JNK1/2 phosphorylation caused by TPA, IL-1 β , anisomycin, and sorbitol treatment (Fig. 8A), whereas the same concentration of SP600125 could not inhibit p38 MAPK phosphorylation caused by IL-1 β , anisomycin, and sorbitol treatment (Fig. 8B). Based on these results, I tested whether SP600125 inhibits stress-induced ectodomain shedding in Vero-H cells. SP600125 (5 μ M) did not inhibit proHB-EGF ectodomain shedding induced by any stimulus (Fig. 8C). In UV light-irradiated cells, JNK1/2 phosphorylation was strong and was not significantly reduced with 5 μ M SP600125. SP600125 (20 μ M) 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, I used dnJNK1 to examine the involvement of the JNK pathway in stress-induced proHB-EGF shedding. Following the transfection of FLAG-tagged dnJNK1, cells were treated with various stimuli as in Fig. 6B and then double-stained for proHB-EGF and

Fig. 7

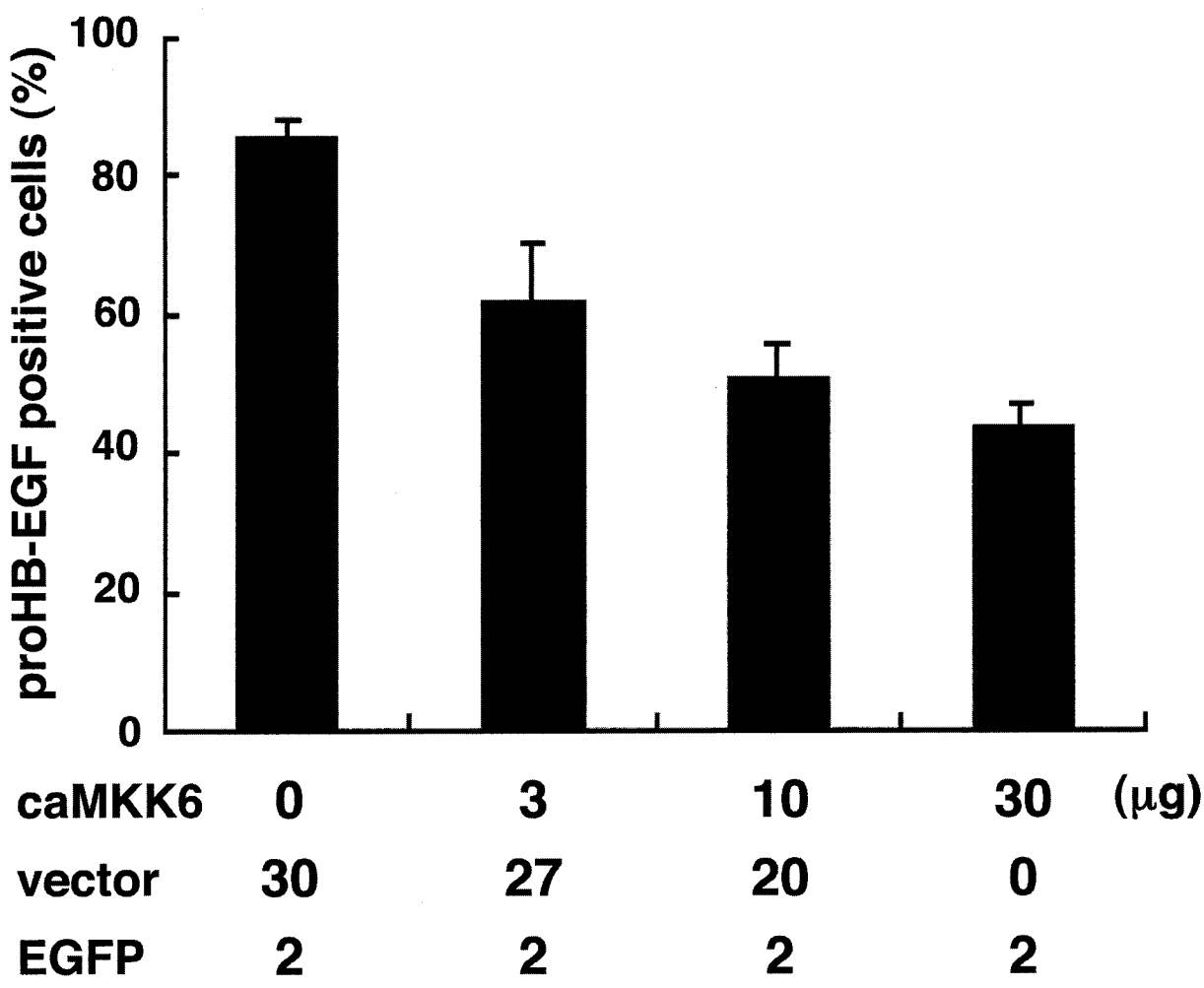


FIG. 7. Enhanced ectodomain shedding by a constitutively active form of MKK6.

Vero-H cells were transfected with a plasmid encoding constitutively active (*ca*) MKK6 by electroporation (Bio-Rad, Gene Pulser) according to the manufacturer's instruction. The empty vector (pEF-BOS) and a plasmid encoding EGFP were also cotransfected at the amounts indicated. The percentage of proHB-EGF-positive cells among GFP-positive cells was determined.

Fig. 8

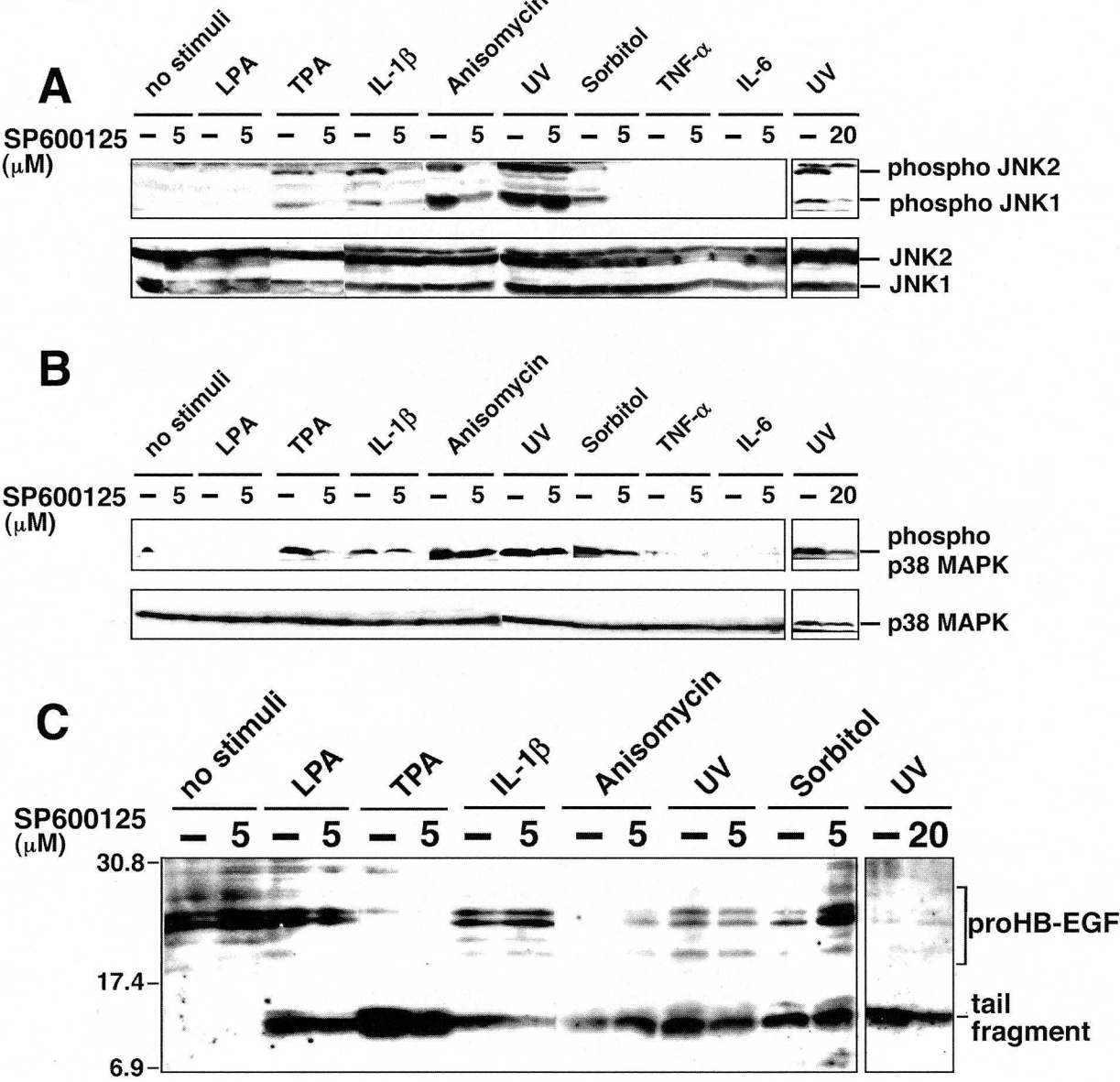


FIG. 8. Effects of the JNK inhibitor SP600125 on stress-induced ectodomain shedding.

Serum-starved Vero-H cells were pretreated with or without SP600125 at the indicated concentrations for 1 h and then treated with IL-1 β or stress-inducing or other stimuli for 30 min. Cell lysates were analyzed by Western blotting to detect activation of JNK1/2 and p38 MAPK and ectodomain shedding. *A* and *B*, activation levels of JNK1/2 and p38 MAPK, respectively. *C*, ectodomain shedding of proHB-EGF.

dnJNK1. ProHB-EGF ectodomain shedding induced by each stimulus was not inhibited in FLAG-positive cells expressing dnJNK1 (Fig. 9). From these results, I conclude that the JNK pathway is not involved in stress-induced proHB-EGF ectodomain shedding in Vero-H cells.

p38 MAPK-mediated Shedding Cascades Are Independent of the Other Shedding Cascades

Using Vero-H cells, I demonstrated that the LPA-induced shedding cascade involves the Ras-Raf-MEK and small GTPase Rac pathways, whereas TPA-induced proHB-EGF shedding involves PKC δ and ADAM9. IL-1 β , anisomycin, and additional stresses induce shedding through a pathway involving the activation of p38 MAPK. I next analyzed the connection of the p38 MAPK-mediated pathway with either the LPA- or TPA-induced pathways. I tested the effect of the MEK inhibitor PD98059 and the PKCs inhibitor Ro-31-8220 on p38 MAPK-mediated proHB-EGF ectodomain shedding (Dudley et al., 1995; Bradshaw et al., 1993). PD98059 inhibited LPA-induced, but not TPA-induced, shedding (Fig. 10A); whereas Ro-31-8220 inhibited TPA-induced, but not LPA-induced, shedding (Fig. 10B), confirming previous results (Umata et al., 2001). p38 MAPK-mediated stress-induced shedding was not inhibited by either PD98059 or Ro-31-8220. I also transfected dnPKC δ and dnRac1. Neither dnPKC δ nor dnRac1 inhibited p38 MAPK-mediated shedding, with the exception of partial inhibition observed for sorbitol-induced shedding by dnRac1 (Fig. 11). These data indicate that the stress-induced shedding cascade mediated by p38 MAPK activation functions independently of the LPA- and

Fig. 9

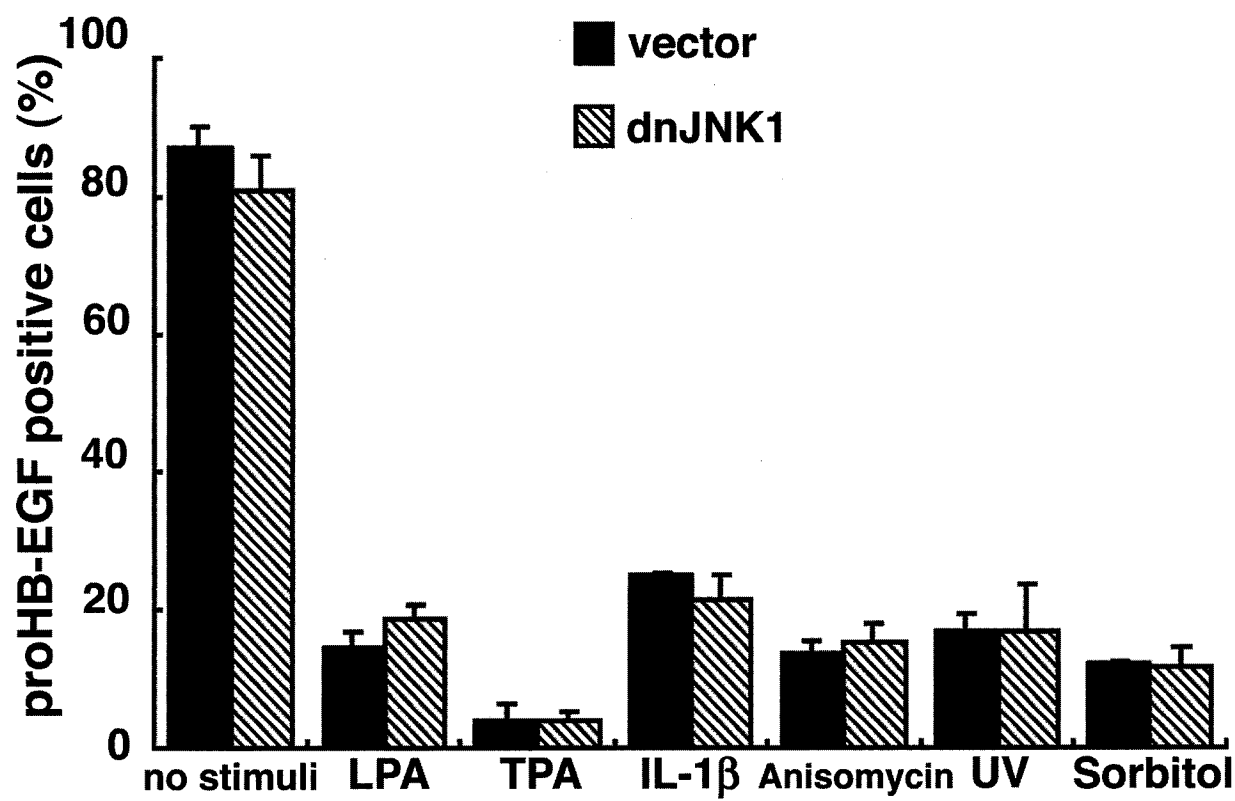


FIG. 9. Effects of dnJNK1 on stress-induced ectodomain shedding.

Vero-H cells were transfected with a plasmid encoding FLAGtagged dnJNK1 (20 µg) or with vector (pEF-BOS; 18 µg) plus EGFP (2 µg). After 48 h of transfection, the cells were incubated with LPA, TPA, IL-1 β , anisomycin, UV light, or sorbitol and then stained with anti-HB-EGF antibody for the detection of the proHB-EGF ectodomain. The expression of dnJNK1 was detected by anti-FLAG antibody. The percentage of proHB-EGF-positive cells among FLAG-positive cells was determined. For cells transfected with the empty vector, the percentage of proHB-EGF-positive cells among GFP-positive cells was determined.

Fig.10

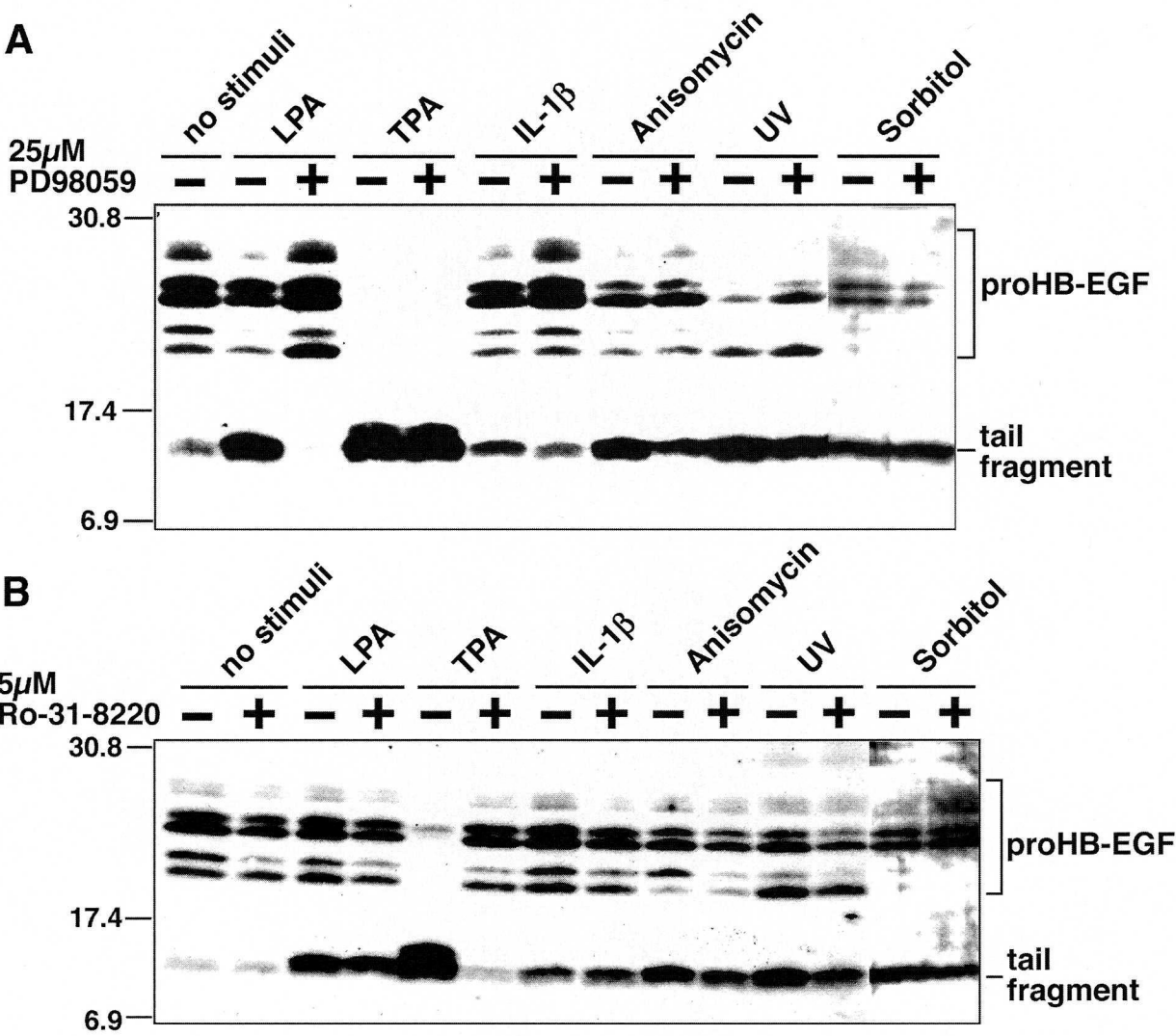


FIG. 10. Effects of the MEK inhibitor PD98059 and the PKC inhibitor Ro-31-8220 on the ectodomain shedding of proHB-EGF.

Serum-starved Vero-H cells were pretreated with 25 μ M PD98059 (A) or 5 μ M Ro-31-8220 (B) for 1 h. As a control, 0.1% Me₂SO, 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 proHB-EGF C terminus.

Fig. 11

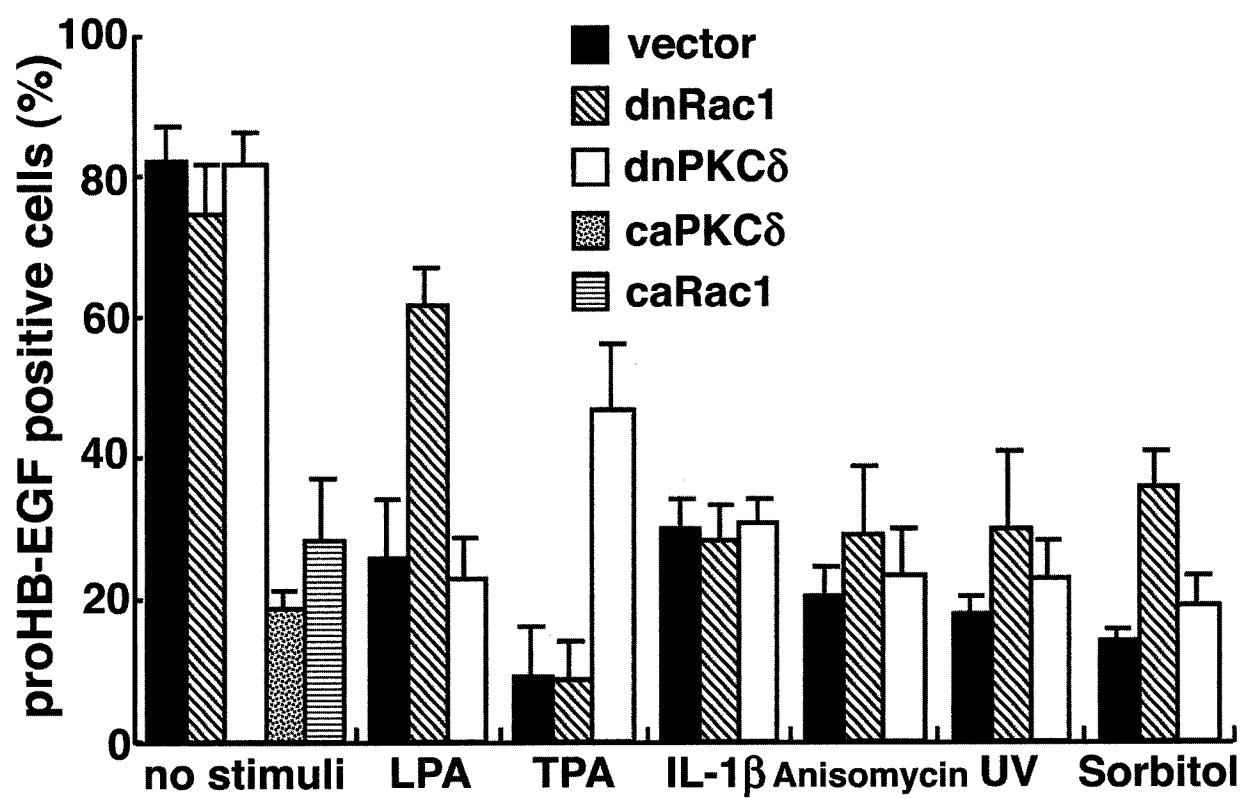


FIG. 11. Effects of dnRac1 and dnPKC δ on the ectodomain shedding of proHB-EGF.

Vero-H cells were transfected with plasmids encoding FLAG-tagged dnRac1 (20 μ g) and dnPKC δ (18 μ g) plus EGFP (2 μ g) or with vector (18 μ g) plus EGFP (2 μ g) as a control. After 48 h of transfection, the cells were incubated with LPA, TPA, IL-1 β , anisomycin, UV light, or sorbitol and then stained with anti-HB-EGF antibody for the detection of the proHB-EGF ectodomain. The expression of dnRac1 was detected by anti-FLAG antibody. The percentage of proHB-EGF-positive cells among FLAG-positive cells was determined. For cells transfected with either dnPKC δ or the empty vector, the percentage of proHB-EGF-positive cells among GFP-positive cells was determined. *ca*, constitutively active.

TPA-induced shedding cascades

Metalloproteases Are Involved in Stress-induced Shedding

Metalloprotease inhibitors inhibit both TPA- and LPA-induced proHB-EGF shedding. I treated cells with a hydroxamic acid-based metalloprotease inhibitor, KB-R8301, to examine the role of metalloproteases in stress-induced shedding. KB-R8301 (10 μ M) inhibited the proHB-EGF shedding induced by IL-1 β , anisomycin, UV light, H₂O₂, and sorbitol (Fig. 12A), indicating that metalloproteases are required for p38 MAPK-mediated proHB-EGF shedding as well.

Stress-induced Shedding Is Not Mediated by ADAM9, 10, 12 or 17

ADAM family metalloproteases are involved in ectodomain shedding of a variety of proteins such as TNF- α and Delta (Blobel, 2000, Black and White, 1998). ADAM9 is known to be involved in TPA-induced proHB-EGF shedding in Vero-H cells (Izumi et al., 1998). To examine the role of ADAM9 in p38 MAPK-mediated shedding, I examined the effect of overexpression of dnADAM9 on shedding. I used two ADAM9 mutants, one carrying the modification of the conserved histidine (Fig. 2) 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); and tested these mutant's behavior in Vero-H cells. ADAM9 (H347A, H351A) were not detected on cell surfaces (data not shown). However, there was inhibition of shedding activity induced by TPA. I investigated the influence of these mutants on stress- and IL-1 β

Fig. 12

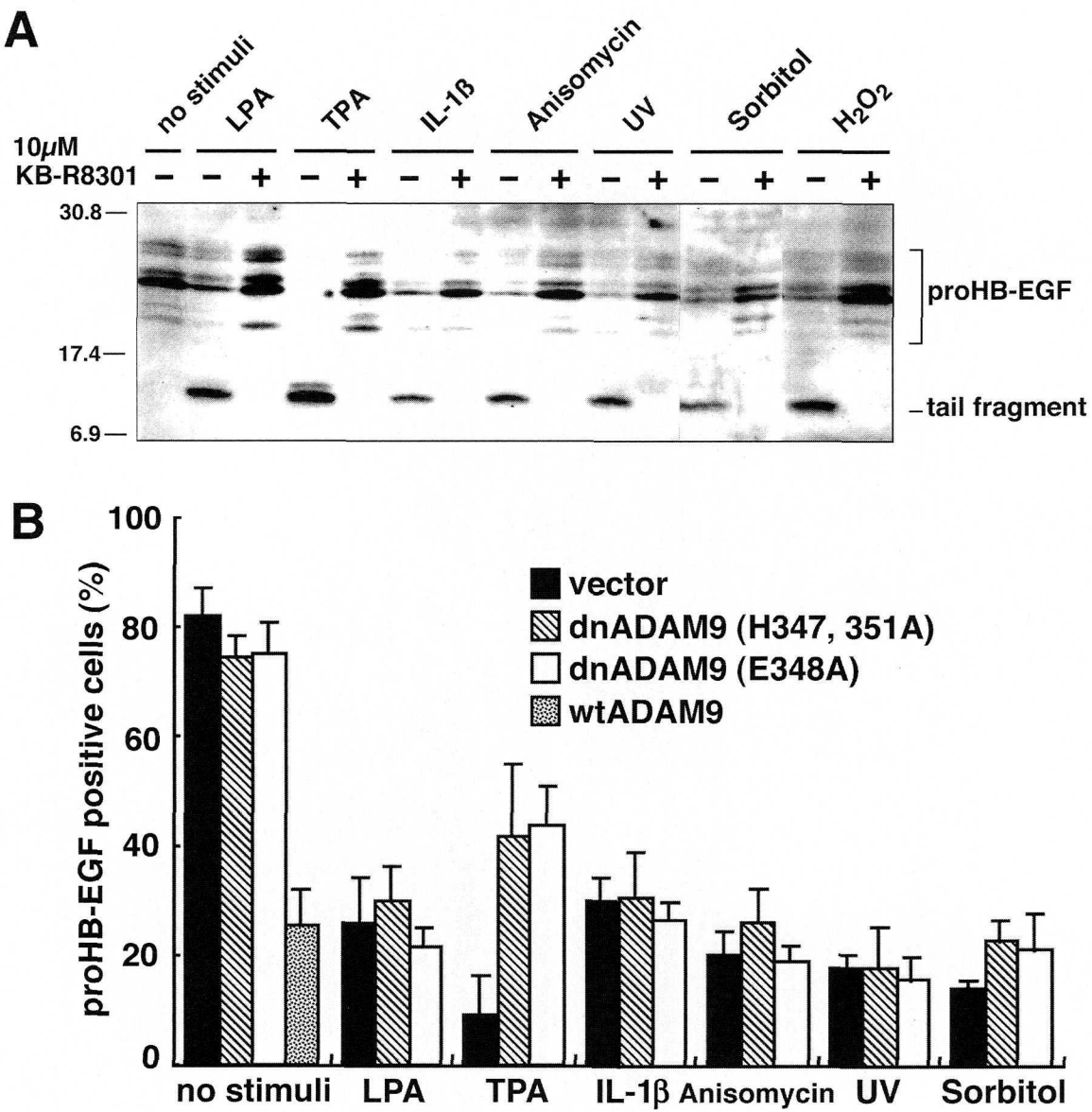


FIG. 12. Inhibition of stress- and IL-1 β -induced proHB-EGF shedding by the metalloprotease inhibitor KB-R8301, but not dnADAM9.

A, effect of the metalloprotease inhibitor KB-R8301. Vero-H cells were pretreated with 10 μ M KB-R8301 or 0.1% Me₂SO 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 proHB-EGF C terminus. *B*, effect of dnADAM9. Vero-H cells were transfected with plasmids encoding dnADAM9 (H347A, H351A) (20 μ g) and FLAG-tagged-dnADAM9 (E348A) (20 μ g) or with vector alone (18 μ g) plus EGFP (2 μ g) as a control. After 48 h of transfection, cells were incubated with LPA, TPA, IL-1 β , UV light, or sorbitol and then double-stained with either anti-HB-EGF, anti-FLAG or anti-ADAM9 antibody. The percentage of proHB-EGF-positive cells among FLAG or ADAM9-positive cells was determined.

-induced shedding; both inhibited TPA-induced shedding, but did not affect LPA-, stress-, and IL-1 β -induced shedding (Fig. 12B). These results indicate that ADAM9 is not required for stress- and IL-1 β -induced proHB-EGF shedding in Vero-H cells. Then, I generated glutamic acid to alanine mutant in the catalytic domain of ADAM10, 12 and 17, which are reported to cleave HB-EGF (Yan et al., 2002; Asakura et al., 2002; Kurisaki et al., 2002; Sunnarborg et al., 2002). However, these mutants did not prohibit stress- and IL-1 β -induced, and LPA HB-EGF shedding (Fig. 13). Only dnADAM17 (E406A) inhibited TPA-induced proHB-EGF shedding, which might have seen activated PKC δ depleted by dnADAM17.

Fig. 13

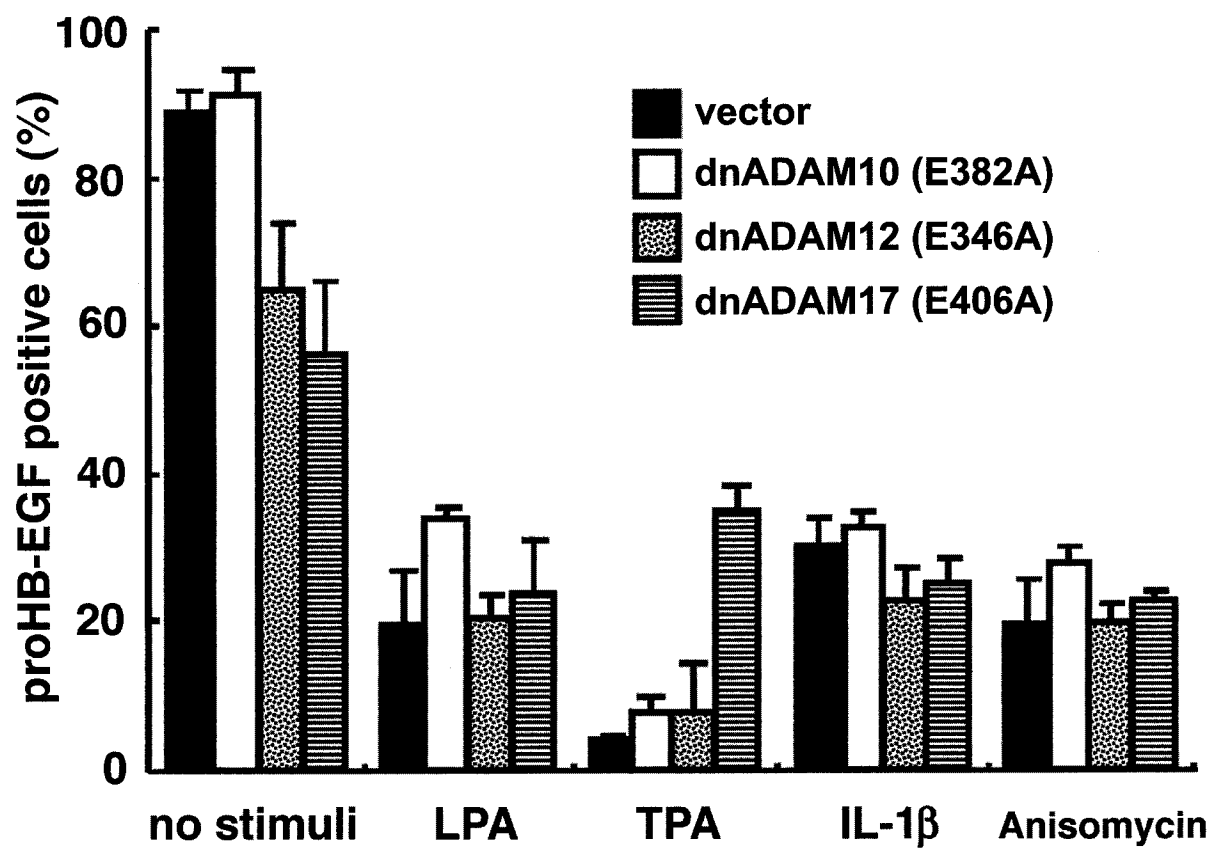


FIG. 13. Effects of dnADAMs on the ectodomain shedding of proHB-EGF.

Effect of dnADAM10, dnADAM12 and dnADAM17. Vero-H cells were transfected with plasmids encoding FLAG-tagged-dnADAM10 (E382A) (20 µg), FLAG-tagged-dnADAM12 (E346A) (20 µg) and FLAG-tagged-dnADAM17 (E406A) (20 µg) or with vector alone (18 µg) plus EGFP (2 µg) as a control. After 48 h of transfection, cells were incubated with LPA, TPA, IL-1 β , UV light, or sorbitol and then double-stained with either anti-HB-EGF or anti-FLAG antibody. The percentage of proHB-EGF-positive cells among FLAG-positive cells was determined.

Discussion

HB-EGF contributes to tissue repair processes, acting in response to various injuries (Marikovski et al., 1993; Tokumaru et al., 2000; Fang et al., 2001; Michalsky et al., 2001). HB-EGF also participates in pathological processes, including smooth muscle cell hyperplasia (Miyagawa et al., 1995), restenosis following balloon injury (Igura et al., 1996), and cardiac hypertrophy (Asakura et al., 2002; Fujino et al., 1998; Perrella et al., 1994). Ectodomain shedding is critical for the biological activity of this growth factor (Iwamoto and Mekada, 2000). In addition to regulation of growth factor activity, proHB-EGF ectodomain shedding contributes to the transactivation of EGF receptors following ligation by G protein-coupled receptors and other ligands (Prenzel et al., 1999). Recent studies suggest that proHB-EGF ectodomain shedding and transactivation are involved in the pathological processes of cardiac hypertrophy and pulmonary hypertension (Asakura et al., 2002; Lemjabbar and Basbaum, 2002). Although functions for HB-EGF in physiological and pathological processes have been increasingly reported, information about shedding-inducing stimuli and the downstream signaling processes involved is limited (Gechtman et al., 1999).

Third Cascades for HB-EGF Ectodomain Shedding

Two distinct signaling pathways contribute to proHB-EGF shedding in Vero-H cells, the TPA-induced PKC δ - and ADAM9-mediated pathway and the LPA-induced MEK- and Rac-mediated pathway. Using Vero-H cells, I

determined that various stress-inducing and inflammatory stimuli also trigger ectodomain shedding in a p38 MAPK-mediated manner. As this cascade does not require the activation of MEK, Rac, PKC δ or ADAM9, it appears to function independently of the TPA- and LPA-induced pathways. Thus, the p38 MAPK-mediated pathway is a third signal cascade that can induce proHB-EGF shedding in these cells. In addition, HB-EGF overexpressed SKOV-3 cells, which are human ovary cancer cells, or mouse HB-EGF overexpressed NIH-3T3 cells were induced to shedding by stress stimuli. Stress-induced shedding of SKOV-3 or NIH-3T3 cells were inhibited not only by a p38 inhibitor, but also by a MEK inhibitor. These results suggest that cells other than Vero cells also had a proHB-EGF shedding cascades property; though these cascades might be more complicated.

Signal Cascades Inducing HB-EGF Shedding.

Stress-induced ectodomain shedding is not specific to proHB-EGF. Osmotic stress induces L-selectin shedding in neutrophils (Rizoli et al., 1999). UV light and osmotic pressure promote the shedding of pro-transforming growth factor- α and proneuregulin in Chinese hamster ovary cells (Montero et al., 2002). Although stress activates both p38 MAPK and JNK, this and previous studies (Rizoli et al., 1999; Montero et al., 2002; Fan et al., 1999) suggest that p38 MAPK, rather than JNK, is the primary contributor to the stress-induced ectodomain shedding of a number of membrane proteins. Inflammatory cytokines, including TNF- α , IL-1 β , and IL-6, also stimulate membrane protein shedding (Franzke et al., 2002; Levine et al., 1996; Yabkowitz et al., 1999). In

Vero-H cells, however, neither ectodomain shedding nor p38 MAPK activation was observed following treatment with either TNF- α or IL-6 (Figs. 3B and 5A). Although it is possible that Vero-H cells do not bare the receptors ligating TNF- α and IL-6, it appears that signaling pathways induced by TNF- α and IL-6 do not contribute to proHB-EGF shedding.

MKK6 and MKK3 are upstream molecules for p38 MAPK, but not JNKs. In contrast, MKK4 and MKK7 specifically phosphorylate JNKs. Fig. 8 shows that caMKK6 activated proHB-EGF shedding. Contrarily, wild type MKK4 or 7 overexpressing cells did not exhibit shedding (data not shown). Although the present data indicates that proHB-EGF shedding is mediated by activation of p38 MAPK, it does not indicate whether MKK6 participates in these shedding cascades since there are a number of upstream molecules for p38 MAPK. Stress- and IL-1 β -induced sheddings were inhibited by dnRas and wtPTEN. However, there is little reporting indicating Ras activates p38 MAPK, and the relationship between PTEN and p38 MAPK is also unknown (Pruitt et al., 2002). Although Ras would have participated in the upstream of this pathway, it is difficult to clarify any further with overexpression experiments.

Which Proteases Are Involved in This Shedding Pathway?

I have demonstrated that stress- and IL-1 β -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 (Hooper et al., 1997). Therefore, a metalloprotease such as matrix metalloprotease or ADAM that

works in close proximity to the membrane may participate in proHB-EGF shedding. The ADAM family metalloproteases contain a conserved sequence (HEXXHXXGXXH) that is a putative zinc-binding motif within the catalytic domain. Our laboratory 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 proHB-EGF shedding (Izumi et al., 1998). Thus, ADAM9 is involved in proHB-EGF shedding induced by treatment of Vero-H cells with TPA. In this study, I constructed another dnADAM9 mutant (E348A) to examine its effect on TPA-induced proHB-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 dnADAM9 (H347A, H351) nor dnADAM9 (E348A) inhibited LPA-, stress-, or IL-1 β -induced proHB-EGF shedding in Vero-H cells. From these results, I conclude that ADAM9 does not participate in LPA-, stress-, or IL-1 β -induced proHB-EGF shedding. Recent studies suggest that, in addition to ADAM9, ADAM10/Kuzbanian (Lemjabbar and Basbaum, 2002; Yan et al., 2002), ADAM12/meltrin- γ (Asakura et al., 2002), and ADAM17/TACE (Sunnarborg et al., 2002) function in proHB-EGF shedding in various cell systems. I therefore constructed putative dnADAM10, dnADAM12, and dnADAM17 mutants by substituting the conserved glutamic acid within the catalytic domain with an alanine (Glu mutants). These mutants were tested for their ability to inhibit

stress- and IL-1 β -induced shedding in Vero-H cells; and did not prevent stress- and IL-1 β -induced proHB-EGF shedding. Although I have not confirmed the loss of catalytic activity by these constructed Glu mutants, these results suggest that ADAM10, ADAM12, and ADAM17 are not sheddases for stress- and IL-1 β -induced proHB-EGF shedding in Vero-H cells. Thus, the protease responsible for p38 MAPK-mediated proHB-EGF shedding in Vero-H cells remains to be identified.

There are a number of proteases on cell surfaces. Even if only for metalloproteases, human cells have about 20 types of ADAMs and about 20 types of MMPs. It is impossible to try all the dominant negatives of metalloproteases; moreover, a shedding pathway which was induced by only one kind of stimulus might be using several proteases. In the upper panel of Fig. 3B, TPA-induced shedding produced multiple tail fragments. When these tail fragments were analyzed by Tris-Tricine PAGE, they divided into at least three bands (data not shown). It was reported that human proHB-EGF of juxtamembrane cleaves at only one site, which is between leucine 148 and proline 149. But multiple tail fragments showed that other cleavage sites should exist in the juxtamembrane domain of HB-EGF. Thus, cleavage sites could be identified by collecting tail fragments and analyzing the sequence of N-terminus.

Biological Significance of Stress-induced Shedding Cascades

What is the biological significance of stress-induced proHB-EGF shedding? A number of reports indicate that the transcription of proHB-EGF is

up-regulated in response to oxidative, ischemic, osmotic, and mechanical stresses (Koh et al., 2001; Fang et al., 2001; Miyazaki et al., 2001; Sakai et al., 2001; Nguyen et al., 2000; Morita et al., 1993). The inflammatory cytokines IL-1 β and TNF- α also markedly increase proHB-EGF gene expression (Yoshizumi et al., 1992). The release of sHB-EGF from membrane by ectodomain shedding is a prerequisite for mitogenic activity as a paracrine or autocrine growth factor. Therefore, stresses and inflammatory cytokines may also induce proHB-EGF shedding, in addition to their transcriptional up-regulation. In response to stresses and inflammations, HB-EGF activity might be regulated at both 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.

Possible relation of pathological process and stress-induced ectodomain shedding of HB-EGF

i. Heart

Both HB-EGF knock-out and knock-in mice have a heart phenotype (Iwamoto et al., 2003; Iwamoto and Mekada, unpublished work). Probably, abnormal regulation of proHB-EGF shedding may cause heart failure or hypertrophy. Shear stress has been reported as one of the causes of cardiac hypertrophy; the expression of HB-EGF mRNA was induced in rat vascular injury of balloon catheter (Igura et al., 1996). In these operations, blood vessels are forcibly extended by balloon; after balloon operations for arteriosclerosis, patients often suffer cardiac hypertrophy and arteriosclerosis again. Vessel

injury would cause the induction of cytokines and GPCR ligand production. Cytokines, such as IL-1 β , and GPCR ligands, would induce proHB-EGF ectodomain shedding in cardiomyocytes, as has been shown by LPA and angiotensin,. Balloon pressure will directly induce proHB-EGF shedding at vascular smooth muscle cells by shear or pressure stress. Transgenic mice with cardiac-specific expression of dominant negative forms of p38 MAPK have been reported; Dn-p38 mice developed cardiac hypertrophy but were resistant to cardiac fibrosis (Zhang et al., 2003). This result suggests that increasing fibroblasts in the heart requires p38 MAPK activation. However, p38 MAPK activation might not be the main candidate causing the development of cardiac hypertrophy.

ii. Vascular Systems

Vascular endothelial as well as aortic smooth muscle cells and platelets are highly expressing of HB-EGF. sHB-EGF is considered one of the main causes of arteriosclerosis, which is caused by a mitogen of smooth muscle cells. Arteriosclerosis occurs from many factors, including growth factors, cytokines, osmotic stress, oxidative stress, and GPCR ligands. Several endogenous growth factors synthesized by smooth muscle cells are believed to be important in initiating smooth muscle cell migration and proliferation following arterial injury (Majesky et al., 1990). HB-EGF ectodomain shedding could occur from mechanisms almost similar to those of cardiac hyperplasia (Ross, 1986; Klagsbrun and Edelman, 1989).

To directly examine the role of HB-EGF in the pathogenesis of

atherosclerosis, immunohistochemical localization was performed on human aortic walls and atherosclerotic plaques (Miyagawa et al., 1995). HB-EGF was expressed in the smooth muscle cells and macrophages of normal human aortic walls, and individuals with varying stages of atherosclerosis showed significantly higher levels of protein. *In vitro* culture of smooth muscle cells needs ERK, p38 and JNK phosphorylation (Kavurma and Khachigain, 2003). In contrast, balloon-injury stimulation which induced vascular hyperplasia, activated p38 MAPK phosphorylation *in vivo* (Ohashi et al., 2003). An assumption can be made that balloon-injury might induce HB-EGF shedding *via* p38 MAPK activation. To explain that, that HB-EGF was shedding in actual vessels needs to be shown; at present it has not been.

iii. Renal Systems and Urinary Tract

The renal, bladder, urethra, and renal pelvis are parts from which osmotic stress may occur, other than blood vessels. In immunohistochemical and in situ hybridization studies of adult rat kidneys, or human bladder, have shown that tubular epithelial cells of the S3 segment of the outer stripe in the outer modulla were the major renal sources of HB-EGF. HB-EGF expression has not been detected in the glomeruli or some parts of the kidney; however, increased expression of HB-EGF mRNA in kidneys of diabetic rats suggests that it may be involved in the development of diabetic nephropathy (Nakagawa et al., 1997; Freeman et al., 1997; Lee et al., 1995). In addition, northern blotting and in situ hybridization demonstrated that HB-EGF mRNA is induced in the kidney *in vivo* after acute tubular injury (Homma et al., 1995). In renal systems,

activation of Fc receptors or anti-glomerular basement membrane antibody (anti-GBM) induced anti-GBM glomerulonephritis (Suzuki et al., 1998; Feng et al., 2000). These anti-GBM glomerulonephritis were blocked by angiotensin-II receptor antagonist or anti-HB-EGF neutralizing antibody. Stress-induced proHB-EGF shedding in renal and urinary systems would cause kidney inflammation; simultaneously, inflammations would lead to induction of proHB-EGF synthesis by positive feedback.

iv. Wound Healing

HB-EGF is a component in wound fluid, as shown by analysis from porcine partial-thickness excisional wounds (Marikovsky et al., 1993). Human burn wound fluid also contains a 10-15 kDa form of HB-EGF. Immunohistochemical analysis of basal epithelium of normal skin, determined that it was uniformly distributed throughout surface epithelium of partial thickness burn wounds (McCarthy et al., 1996). The soluble form of HB-EGF is a potent mitogen for fibroblasts and keratinocytes, as well as being an inducer of cell migration (Higashiyama et al., 1993; Tokumaru et al., 2000); it has been suggested that sHB-EGF might be involved in the induction of granulation tissue and re-epithelization (Marikovsky et al., 1993). Roles of HB-EGF in wound healing are not the only mitogenic activity of sHB-EGF; growth-arrest and maintain activity of proHB-EGF for contacting cells are also (Iwamoto et al., 1999). When epithelia of tissues are damaged, surrounding cells such as platelets, monocytes, macrophages, and other cells in the damaged tissues, release a number of factors including LPA and cytokines. GPCR or

SAPK-inducible cytokine receptors induce ectodomain shedding of proHB-EGF (Prenzel et al., 1999; Umata et al., 2001). As well, shedding of proHB-EGF might take place in the wounded tissues themselves, which depending on the damage such as from UV.

Conclusion

Ectodomain sheddings of proHB-EGF are induced by various stresses or by IL-1 β in Vero-H cells. The stress-induced shedding cascades are mediated by activation of p38 MAPK; these cascades being independent from TPA- and LPA-induced proHB-EGF shedding. Metalloproteases are involved in p38 MAPK-mediated proHB-EGF shedding cascades, as well as PKC- and MEK- mediated cascades. However, ADAM9, ADAM10, ADAM12 and ADAM17 are not b proteases directly cleaving proHB-EGF in p38 MAPK-mediated cascades. This report also suggests that excess release of sHB-EGF, from the continual exposure of tissues to stress and inflammation, might result in pathological hyperplasia or inflammation of cardiac, smooth muscle and renal cells.

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