ADAM Binding Protein Eve-1 Is Required for Ectodomain Shedding of Epidermal Growth Factor Receptor Ligands*

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A disintegrin and metalloproteases (ADAMs) are implicated in the ectodomain shedding of epidermal growth factor receptor (EGFR) ligands in EGFR transactivation. However, the activation mechanisms of ADAMs remain elusive. To analyze the regulatory mechanisms of ADAM activation, we performed yeast two-hybrid screening using the cytoplasmic domain of ADAM12 as bait, and identified a protein that we designated Eve-1. Two cDNAs were cloned and characterized. They encode alternatively spliced isoforms of Eve-1, called Eve-1a and Eve-1b, that have four and five tandem Src homology 3 (SH3) domains in the carboxy-terminal region, respectively, and seven proline-rich SH3 domain binding motifs in the amino-terminal region. The short forms of Eve-1, Eve-1c and Eve-1d, translated at Met-371 are human counterparts of mouse Sh3d19. Northern blot analysis demonstrated that Eve-1 is abundantly expressed in skeletal muscle and heart. Western blot analysis revealed the dominant production of Eve-1c in human cancer cell lines. Knockdown of Eve-1 by small interfering RNA in HT1080 cells reduced the shedding of proHB-EGF induced by angiotensin II and 12- O-tetradecanoylphorbol-13-acetate, as well as the shedding of proHB-EGF, membrane-anchored form (proHB-EGF) and proepiregulin by 12- O-tetradeca- nolyphorphol-13-acetate, suggesting that Eve-1 plays a role in positively regulating the activity of ADAMs in the signaling of EGFR-ligand shedding.

Transactivation of the epidermal growth factor receptor (EGFR) has been shown to play a crucial role in the signaling by G-protein-coupled receptors (GPCRs), cytokine receptors, receptor tyrosine kinases, and integrins to a variety of cellular responses (1, 2). The transactivation of EGFR is mediated, in some cases, by EGFR ligand shedding. In particular, the shedding of heparin-binding EGFR-like growth factor (HB-EGF) (3, 4) is known to have a prominent role in regulating the function of this growth factor. HB-EGF plays a physiological role by binding to and stimulating phosphorylation of the EGFR (5, 6). Like other members of the EGFR family, HB-EGF is synthetized as a membrane-anchored form (proHB-EGF) and then processed to a bioactive soluble form (5, 6). This process, called ectodomain shedding of proHB-EGF, is an important post-translational modification that converts a tethered insoluble juxtacrine form into a soluble growth factor with autocrine/paracrine activity (7).

The ectodomain shedding of proHB-EGF is induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) (7) and mitogen-activated protein kinase signaling pathway also contributes to this proteolytic processing (8, 9). In addition, GPCR agonists induce the ectodomain shedding of proHB-EGF, resulting in EGFR transactivation, which is a critical element in GPCR-induced mitogenic signaling (3). We identified a disintegrin and metalloprotease 12 (ADAM12) as a specific enzyme that catalyzes proHB-EGF shedding in EGFR transactivation by GPCR agonists, such as phenylephrine, endothelin-1, and angiotensin II, and results in cardiac hypertrophy (10). Further, ADAM9, ADAM10, ADAM17, matrix metalloproteases 3 and 7 as well as ADAM12 have been reported to be involved in the ectodomain shedding of proHB-EGF (4).

These reports suggest that the ectodomain shedding of EGFR ligands, especially HB-EGF, is key to the molecular mechanism of EGFR transactivation, which would be an important network of GPCR signaling. However, the pathway by which GPCR signaling links to the activation of ADAM enzymes is largely unknown. Elucidation of the regulatory mechanisms of ADAM activation could clarify the signaling pathway of EGFR transactivation by GPCRs.

ADAM12 is a single membrane-spanning protein with a metalloprotease domain in its extracellular portion, and several Src homology 3 (SH3) binding motifs in the cytoplasmic tail. Therefore, it has been anticipated to interact with SH3 domain-containing proteins. Indeed, it has been demonstrated that ADAM12 binds to p85α (11), Src (12, 13), Grb2 (13), and Fish (14). Moreover, the cytoplasmic domain of ADAM12 has been shown to interact with actin cytoskeleton via α-actinin-2 (15). However, it has not been demonstrated that these molecules could affect ADAM12 metalloprotease activity.

In this study, we performed yeast two-hybrid screening to...
identify ADAM12-binding proteins and discovered a protein containing SH3 domains that we designated Eve-1. We showed that Eve-1 binds not only ADAM12 but also ADAM9, ADAM10, ADAM15, and ADAM 17 and is involved in ADAM-mediated ectodomain shedding of several EGFR ligands, including proHB-EGF, proTGF/H9251, proamphiregulin and proepiregulin.

EXPERIMENTAL PROCEDURES

Antibodies—Mouse anti-myc monoclonal antibody (9E10) and anti-FLAG monoclonal antibody (M2) were purchased from Santa Cruz Biotechnology and Sigma-Aldrich, respectively. Rabbit polyclonal antibodies against human ADAM10 and ADAM17 were from BIOMOL Research Laboratories Inc. and R&D Systems Inc., respectively. Rabbit polyclonal antibody against the cysteine-rich region of human ADAM12 was a kind gift from Dr. A. Sehara (Kyoto University, Kyoto, Japan). Rabbit polyclonal antibody against the cytoplasmic region of pro-HB-EGF (H1) has been described previously (16). Rabbit anti-Eve-1 polyclonal antibody was raised against recombinant Eve-1c. Goat alkaline phosphatase (AP) or horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from Promega. Goat rhodamine-conjugated anti-mouse antibody was from Chemicon.

Expression Vectors and Small Interfering RNA (siRNA)—We constructed a yeast expression plasmid encoding GAL4 DNA-binding domain fused with the ADAM12 cytoplasmic domain by inserting ADAM12 cytoplasmic domain cDNA into the EcoRI and SalI multiple cloning sites of the pBTM116 vector (BD Biosciences Clontech). ADAM12 full-length cDNA was cloned into the SacI and SalI sites of the pEYFP-N1 vector (BD Biosciences Clontech). We prepared an adenovirus carrying a gene encoding FLAG epitope-tagged ADAM12 (ADAM12-FLAG) as described previously (10). The cytoplasmic domain of ADAM12 and its truncated regions were cloned into the EcoRI and XhoI sites of the pGEX6P-1 vector (Amersham Biosciences). Eve-1a and Eve-1b full-length cDNAs isolated from a human heart cDNA library by PCR were introduced into the SalI and NotI multiple cloning sites of the modified pME18S vector (pME18S-myc) that possesses an SR/H9251 promoter (17). Eve-1c and Eve-1d cDNAs were amplified using vectors with Eve-1a and Eve-1b inserted as templates, respectively, and cloned into the BglII and NotI sites of the pME18S-myc vector. A series of truncated mutants of the SH3 domains region of Eve-1b were cloned into the EcoRI and NotI sites of the pME18S-myc vector. The pME18S-myc vector was provided by Dr. K. Touhara (University of Tokyo, Tokyo, Japan). Dharmacon Research Inc. chemically synthesized and purified siRNA duplexes. The siRNA sequence targeting Eve-1 corresponded to the coding region 155–175 (5'/AACCUUCUGUAGCUCCCAAAC-3') relative to the first nucleotide of the start codon of Eve-1a (Eve-1b). This sequence had no substantial similarity to any other sequence in the NCBI data base. Scramble-siRNA directed against 5'/GCGCGCUUUGGCGGAUCG-3' was the negative control. No mammalian mRNAs contained this sequence in the NCBI data base.

Fig. 1. Deduced amino acid sequence and domain organization of Eve-1. A, amino acid sequence of Eve-1. A total of 767 amino acids were deduced from the open reading frame of Eve-1a, and a 23-amino acid insert is present in Eve-1b. Predicted SH3 domains are boxed, and seven proline-rich motifs that bind SH3 domains are underlined. B, schematic representation of Eve-1 isoforms.
Yeast Two-hybrid Screening—Yeast strain L40 containing pBTM116-ADAM12 (Cyto) was selected on synthetic complete media lacking tryptophan. A human heart cDNA library in pACT2 was sequentially introduced into the transformant, and yeast cells were plated onto synthetic complete media lacking tryptophan, leucine, and histidine in the presence of 3.5 mM 3-aminotriazole. Transformants grown on the dropout plates at 30 °C for 3–7 days were assayed for β-galactosidase activity. Library plasmid DNA was recovered by transformation into Escherichia coli HB101 cells and sequenced.

Northern Blot Analysis—Positively charged nylon membranes blotted with human poly(A)^+ RNA (human 12-lane multitissue Northern blot, Bd Biosciences Clonetech) was hybridized with a [32P]dCTP-labeled Eve-1 probe covering full-length cDNA. Hybridized membranes were washed in contact with BAS1000 plates (type Bas-H1I) and investigated using the BAS1000 Bio-Image analyzer (Fuji Photo Film, Tokyo, Japan). To confirm the reproducibility of the data, independent experiments were performed twice and human β-actin was the control probe.

Cell Culture and Transfection—HT1080 cells and their stable transfectants that express AP-tagged proHB-EGF (HT1080/HB-EGF-AP cells), both AP-tagged proHB-EGF and angiostatin type I (ATI) receptor (HT1080/HB-EGF-AP/ATI cells), AP-tagged TGF-α (HT1080/TGF-α-AP cells), AP-tagged proamphiregulin (HT1080/AR-AP cells), or AP-tagged proepiregulin (HT1080/EPR-AP cells) were cultured in Eagle's minimum essential medium supplemented with 0.1 mM non-essential amino acids and 10% fetal bovine serum at 37 °C in 5% CO2. Transfection was performed using LipofectAMINE2000 (Invitrogen) by washing with Eagle's minimum essential medium/10% fetal bovine.'°

RESULTS

Identification by Yeast Two-hybrid Screening of Cytoplasmic Proteins That Interact with ADAM12—To identify proteins that interact with the cytoplasmic domain of ADAM12, 3.0 × 10^6 clones of a human heart cDNA library were screened with the use of the yeast two-hybrid system and the ADAM12 cytoplasmic domain as bait. One of the isolated clones, A12-42,
contained a 2.8-kb insert encoding 297 amino acids characterized by four SH3 domains. BLAST searches for the A12-cDNA sequence found that it was identical to the carboxyl-terminal region (amino acid residues 471–767) of a hypothetical protein with an open reading frame of 767 amino acids. We designated the full-length protein Eve-1 because it was the binding “partner” of ADAMs. Two full-length cDNAs that were simultaneously cloned by PCR from a human heart cDNA library, encoded predicted proteins of 767 and 790 amino acids without and with a 23 amino acid insertion, respectively. We designated these proteins Eve-1a and Eve-1b, respectively. Domain structural analysis revealed that Eve-1a and Eve-1b have four and five tandem SH3 domains in the carboxyl-terminal region, respectively. Seven proline-rich SH3 domain binding motifs, (R/K)XXPXX (19, 20), were also found in the amino-terminal region (Fig. 1, A and B). The short forms initially translated at Met-371 of Eve-1a and Eve-1b are human counterparts of mouse Sh3d19, which was recently identified as a novel adaptor protein (21). We designated them Eve-1c and Eve-1d (Fig. 1, A and B). Genomic analysis of the Eve-1 gene revealed that Eve-1a and Eve-1b (Eve-1c and Eve-1d) are alternatively spliced isoforms from a single gene located on chromosome 4q31.

Expression of Eve-1—Northern blotting analysis assessed the expression of Eve-1 mRNA in various human tissues. Hybridization with an Eve-1 probe detected a single 5.8-kb mRNA in most tissues, with relatively abundant expression in skeletal muscle, heart, kidney, and placenta and quite low expression in brain thymus, spleen, and leuocytes (Fig. 2A). To detect Eve-1 protein, human cancer cell lines (breast cancer MDA-MB-231 cells, colon cancer SW480 and WiDr cells, and fibrosarcoma HT1080 cells) were subjected to RT-PCR and Western blotting analyses. Eve-1 gene expression was detected in SW480, WiDr, and HT1080 cells but not in MDA-MB-231 cells (Fig. 2B). Western blotting showed a common band among three cell lines (SW480, WiDr, and HT1080 cells) that completely co-migrated with recombinant Eve-1c (Fig. 2C). Addition of recombinant Eve-1c into HT1080 cell lysate resulted in the increase of the band corresponding to Eve-1c of the positive control and there was no appearance of any additional bands. However, this band was not detected in MDA-MB-231 cells. The bands corresponding to Eve-1a, Eve-1b, and Eve-1d were undetectable in all cell lines tested here. The lower band in SW480 and WiDr cells indicated by an asterisk might be another isoform or a degradation product. These data suggest that Eve-1c is predominantly produced in the tested cancer cell lines.

Human Counterparts of Mouse Sh3d19 Eve-1c and Eve-1d Associate with ADAM12 in Mammalian Cells—Myc epitope-tagged Eve-1a, Eve-1b, Eve-1c, or Eve-1d was transiently expressed in HT1080 cells and detected by Western blotting using an anti-Eve-1 antibody. B, Myc-tagged proteins transiently expressed in HT1080 cells were immunoprecipitated using anti-myc antibody (bottom). Co-immunoprecipitated endogenous ADAM12 was detected by immunoblotting using anti-ADAM12 antibody (top). Asterisk indicates IgG bands. C, ADAM12-FLAG transiently expressed in HT1080 cells was immunoprecipitated using anti-FLAG antibody (top). Co-immunoprecipitated Eve-1c was detected by immunoblotting using anti-Eve-1 antibody (bottom). The mixture of recombinant Eve-1a, Eve-1b, Eve-1c, and Eve-1d expressed in HT1080 cells were used as a positive control.
Eve-1c, and myc-Eve-1d from cell lysates (Fig. 3B, bottom). Myc-Eve-1c and myc-Eve-1d co-immunoprecipitated endogenous ADAM12; 90 kDa corresponded to an active form that was detected by anti-ADAM12 antibody (Fig. 3B, top). However, neither myc-Eve-1a nor myc-Eve-1b co-immunoprecipitated endogenous ADAM12. Furthermore, to confirm that endogenous Eve-1c and Eve-1d can associate with ADAM12, ADAM12-FLAG was transiently expressed in HT1080 cells. Fig. 3C shows that ADAM12-FLAG was immunoprecipitated from cell lysates using anti-FLAG antibody (Fig. 3C, top) and co-immunoprecipitated endogenous Eve-1c was detected by anti-Eve-1 antibody (Fig. 3C, bottom). None of the high molecular mass bands corresponding to Eve-1a, Eve-1b, or Eve-1d was detected by anti-Eve-1 antibody.

Identification of Essential Regions Required for ADAM12-Eve-1 Interaction—To define the binding site of ADAM12 for Eve-1 and vice versa, we performed GST pull-down assay using a series of truncated mutants of cytoplasmic domain of ADAM12 (Fig. 4A) or a series of truncated mutants of SH3 domains region of Eve-1d (Fig. 5A). Myc-Eve-1d bound to GST-P2–4, -P1–2, -P1–3, and -ADAM12 (Cyto) (Fig. 4B). In contrast, myc-Eve-1d did not bind to GST-P4, -P3–4, or -P1 (Fig. 4B). The same results were obtained using myc-Eve-1c (data not shown). These results suggest that P2 region (amino acid residues 829–840) of ADAM12 is required for the binding to Eve-1c or Eve-1d.

On the other hand, GST-ADAM12 (Cyto) bound myc-SH3#1–5, #3–5, #2–4, #3–4, and #3–4 (Fig. 5B). In contrast, GST-ADAM12 (Cyto) did not bind myc-SH3#1–2 and #3–4. In the panel of SH3#3–4, faint band corresponding to the size of myc-SH3#3–4 is background of GST-fusion protein. These results suggest that the region containing SH3–3 and -4 domains of Eve-1d is required for binding to ADAM12.

Colocalization of ADAM12 and Eve-1c—To intracellularly localize ADAM12 and Eve-1c, we transiently co-expressed ADAM12-EYFP and myc-Eve-1c in HT1080 cells. Confocal microscopy revealed that ADAM12-EYFP co-localized with myc-Eve-1c at the leading edge of the cell (arrowheads) (Fig. 6).

Effect of Eve-1 on Ectodomain Shedding of proHB-EGF—We investigated the effect of Eve-1 on ectodomain shedding of proHB-EGF, using siRNA-mediated mRNA destruction. First, to test the effect of Eve-1-siRNA on Eve-1 knockdown, RT-PCR and immunoblot analyses estimated levels of mRNA and protein, respectively. Eve-1 cDNA was amplified by RT-PCR using...
total RNA isolated from HT1080 cells transfected with negative control Scramble-siRNA, but not with Eve-1-siRNA (Fig. 7A). Furthermore, we detected the much lesser amount of endogenous Eve-1c protein in the lysate from HT1080 cells transfected with Eve-1-siRNA than in those transfected with Scramble-siRNA (Fig. 7B). These experiments confirmed that Eve-1-siRNA destroyed Eve-1 mRNA. We further assessed the effect of Eve-1-siRNA on the ectodomain shedding of proHB-EGF. HT1080/HB-EGF-AP/AT1 cells were transfected with Scramble-siRNA or with Eve-1-siRNA, and then we measured AP activity in the conditioned medium of each transfectant after a 30-min incubation with 100 nM angiotensin II. Likewise, proHB-EGF-AP shedding induced by TPA was assayed using HT1080/HB-EGF-AP cells. Although the level of proHB-EGF-AP expression was almost equal (Fig. 7C), Eve-1 knockdown mediated by Eve-1-siRNA attenuated 65 and 70% of angiotensin II- and TPA-induced proHB-EGF-AP shedding, respectively (Fig. 7D).

Because we reported previously that PACSIN3 binds ADAM12 cytoplasmic domain and up-regulated ectodomain

**Fig. 5.** Mapping of ADAM12 binding sites in Eve-1. A, schemes of SH3 domain region deletion mutants of Eve-1. B, ADAM12 binding to the deletion mutants. Extracts prepared from HT1080 cells expressing myc-tagged Eve-1 deletion mutants were incubated with GST or GST-ADAM12 cytoplasmic domain immobilized onto glutathione resin. Bound Eve-1 deletion mutants were detected by immuno-blotting using anti-myc antibody.

**Fig. 6.** Confocal microscopic analyses of the co-localization of Eve-1c and ADAM12. HT1080 cells co-expressing myc-Eve-1c and ADAM12-EYFP were imaged for anti-myc antibody with rhodamine-conjugated anti-mouse antibody (A) and EYFP fluorescence (B). Images are merged on the right in C. ADAM12-EYFP co-localization with myc-Eve-1c in plasma membrane is indicated by arrowheads.
shedding of proHB-EGF (22), we further analyzed the simultaneous requirement of Eve-1 and PACSIN3 for the ectodomain shedding of proHB-EGF. Double knockdown of Eve-1 and PACSIN3 by using their siRNAs did not show the enhanced suppression of proHB-EGF shedding induced by TPA or angiotensin II compared with the effect of the individual knockdown (Fig. 8), suggesting that both Eve-1 and PACSIN3 are equally required for the ADAM-induced proHB-EGF shedding.

Eve-1c and Eve-1d Can Associate with ADAM9, ADAM10, ADAM15, and ADAM17 in Mammalian Cells

Several ADAMs contain proline-rich regions in their cytoplasmic tails, suggesting their possible interaction with Eve-1. To investigate whether Eve-1 can associate with the other ADAM members such as ADAM9, ADAM10, ADAM15, and ADAM17 in Mammalian Cells—Several ADAMS contain proline-rich regions in their cytoplasmic tails, suggesting their possible interaction with Eve-1. To investigate whether Eve-1 can associate with the other ADAM members such as ADAM9, ADAM10, ADAM15, and ADAM17 implicated in the ectodomain shedding of membrane proteins, myc-tagged Eve-1a, Eve-1b, Eve-1c, or Eve-1d was transiently expressed in HT1080 cells and immunoprecipitated using anti-myc antibody. As shown in Fig. 9, endogenous ADAM10 (top) and ADAM17 (top middle) were co-immunoprecipitated with myc-Eve-1c and myc-Eve-1d. Co-expressed FLAG-tagged ADAM9 (bottom middle) and ADAM15 (bottom) were also co-immunoprecipitated with myc-Eve-1c and myc-Eve-1d. However, neither myc-Eve-1a nor myc-Eve-1b co-immunoprecipitated any endogenous or FLAG-tagged ADAM tested here.

Effect of Eve-1 on Ectodomain Shedding of proTGF-α, Proamphiregulin, and Proepiregulin—We assessed the effect of Eve-1-siRNA on the ectodomain shedding of proTGF-α, proamphiregulin, and proepiregulin. As shown in Fig. 10, Eve-1-siRNA down-regulated ~50% of TPA-induced proTGF-α-AP and proepiregulin-AP shedding and ~40% of TPA-induced proamphiregulin-AP shedding, although their expression levels under the conditions of siRNA and scramble RNA treatments were almost equal in each.

DISCUSSION

We searched for clues to help understanding of the regulatory mechanisms of ADAM-mediated proHB-EGF and the other EGFR-ligand shedding induced by GPCR agonists or TPA. We focused on ADAM12, which was recently identified as a shedding enzyme of proHB-EGF. Yeast two-hybrid screening using the cytoplasmic domain of ADAM12 as bait allowed isolation of a protein containing SH3 domains that we called Eve-1.

The Eve-1 gene encodes at least two alternatively spliced isoforms, Eve-1a and Eve-1b. Eve-1a has an incomplete SH3 domain followed by four tandem SH3 domains. On the other hand, Eve-1b has additional 23 amino acids adjacent to the incomplete SH3 domain of Eve-1a, resulting in the production of another complete SH3 domain. That is, Eve-1b contains five SH3 domains, suggesting that these two isoforms are involved in ADAM regulation with functional differences. Our PCR analyses showed that the cloning ratio of Eve-1a and Eve-1b was ~30:1, suggesting that Eve-1a (Eve-1b) and Eve-1c (Eve-1d) are translated from a single mRNA. Western blotting revealed that the exogenous expression of Eve-1a (Eve-1b) did not produce Eve-1c (Eve-1d).
A), and that the Eve-1c was detected dominantly in human cancer cell lines tested here (Fig. 2), indicating the dominant usage of Met 371 as an initiation codon of Eve-1 translation. More precise analyses of the expression profiles of the Eve-1 isoforms in various tissues are required to understand their biological significance. Whereas the usual mechanism of translation in eukaryotes involves the recruitment of the 40 S ribosomal subunit to the 5'-cap structure of the mRNA, a restricted but growing number of viral and cellular mRNA initiate their translation through the recruitment of the mRNA called internal ribosome entry site (23–25). For example, Shc p52 and p46 are translated at the first ATG and the second ATG in exon 2a of the Shc gene, respectively (26), and fibroblast growth factor 2 has five isoforms alternatively translated from a single mRNA (27). It has been reported that the fibroblast growth factor 2 internal ribosome entry site is 176 nucleotides long, is highly structured, and contains two RNA stem loops and a G quartet motif (28). Although the upstream region of the Eve-1 mRNA has no typical G quartet motif, it is predicted to be highly structured, especially in nt sequences 151–651 and 888–1310, by computational structure prediction using Mfold (29) (data not shown), suggesting the existence of an Eve-1 internal ribosome entry site for the alternative translation. Because the usage of alternative initiation codons of fibroblast growth factor 2 is controlled in transformed and stressed cells (30), the translation of Eve-1a (Eve-1b) and Eve-1c (Eve-1d) might be also controlled differently in normal and tumor cells. More precise analyses of Eve-1a (Eve-1b) and Eve-1c (Eve-1d) translation and detection of their proteins are required. On the other hand, analysis of the NCBI database revealed that a transcript of Sh3d19 does not produce mouse counterparts of Eve-1b because of a stop codon located upstream of its coding region. The information retrieved from the database suggests that Eve-1a and Eve-1b have unique functions in humans.

Eve-1 has multiple SH3 domains, so we surmised that Eve-1 interacts with the proline-rich SH3 domain-binding motif(s) in the cytoplasmic tail of ADAM12. A binding site for Eve-1 was mapped in the P2 region of the ADAM12 cytoplasmic tail (Fig. 4). Furthermore, SH3-3 and -4 domains of Eve-1d were shown to be required for binding to the cytoplasmic domain of ADAM12 (Fig. 5). Thus, it is suggested that a subset of SH3 domains 3 and 4 of Eve-1 and P2 of ADAM12 are interactive. ADAM12 also interacts with p85α and Src, and the binding sites for these proteins are located in P2 or P4, and P2 of the ADAM12 cytoplasmic tail, respectively (11, 12). Therefore, it is suggested that several proteins containing SH3 domains share the Eve-1-binding site.

Several members of the ADAM family, such as ADAM9, ADAM10, ADAM15, and ADAM17 as well as ADAM12 have
such regions in their cytoplasmic tails (31). Indeed, some of these ADAM proteins interact with signaling molecules containing an SH3 domain via their cytoplasmic domains (11–14, 32, 33). Thus, several proteins containing SH3 domains are likely to form complexes in the cytoplasmic domains of ADAMs. For example, endophilin I and SH3PX1 form complexes with ADAM9 and ADAM15 (32). Exogenously expressed myc-Eve-1c also co-immunoprecipitated ADAM9, ADAM10, ADAM15, and ADAM17 as well as ADAM12 (Fig. 9). Because ADAM9, ADAM10, ADAM12, and ADAM17 have been shown to be involved multiply in the shedding of EGFR-ligands (4, 34), it is easily speculated that Eve-1 is involved widely in the regulation of ectodomain shedding of EGFR-ligands.

We demonstrated here that the knockdown of Eve-1 by siRNA in HT1080 cells significantly attenuated the shedding of proHB-EGF induced by angiotensin II and TPA (Fig. 9) as well as the shedding of proTGF-α, proamphiregulin, and proepiregulin induced by TPA (Fig. 10), suggesting that Eve-1 widely regulates ADAM-dependent post-translational processing of EGFR ligands. Thus, Eve-1 might be indispensable to the signaling of both angiotensin II and/or TPA-induced EGFR-ligand shedding. To qualify the physiological significance of interactions between Eve-1 and these ADAMs, however, further studies are required for ADAM-mediated ectodomain shedding of other membrane proteins and the other ADAM functions such as cell adhesion.

We have identified very recently another SH3 domain-containing protein, PACSIN3, which can bind to ADAM12 and activate proHB-EGF shedding activity induced by angiotensin II and TPA (22). Because it has not been reported whether ADAM-binding proteins such as endophilin I, SH3PX1, Src, p85α, Grb2, Fish, and α-actinin-2 could affect ADAM metallo-
protease activity, Eve-1 and PACSIN3 are the first molecules that have been characterized as regulators of metalloprotease activity of ADAMs.

Taken together, Eve-1, which was originally identified as an ADAM12-binding protein, is likely to play a physiological role as a binding partner of ADAMs in the signaling pathway of EGFR transactivation by GPCR agonists. Further analyses will clarify the signaling pathway from GPCRs to the shedding of proHB-EGF and the other EGFR ligands leading to EGFR transactivation.

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