PACSIN3 Binds ADAM12/Meltrin α and Up-regulates Ectodomain Shedding of Heparin-binding Epidermal Growth Factor-like Growth Factor*

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The transactivation of epidermal growth factor receptor (EGFR) by G-protein-coupled receptor (GPCR) agonists is a critical element in various responses of diverse cell types including fibroblasts, keratinocytes, astrocytes, and smooth muscle cells (1, 2). A novel mechanistic concept of the EGFR transactivation-signaling pathway involves the proteolytic release of heparin-binding EGFR-like growth factor (HB-EGF) at the surface of cells stimulated with GPCR agonists (3). HB-EGF is a member of the EGFR family that directly binds EGFR and thereby enhances its phosphorylation, resulting in cell growth and differentiation (4). Like other members of the EGFR family, HB-EGF is synthesized as a membrane-anchored form (proHB-EGF) and then proteolytically processed to become a bioactive soluble form, a process that is called ectodomain shedding. The ectodomain shedding of proHB-EGF is an important post-translational modification that converts a tethered insoluble juxtaacellular growth factor into a soluble ligand leading to the autocrine or paracrine activation of EGFR.

Studies of GPCR mitogenic signaling have proven that EGFR transactivation is dependent on HB-EGF in smooth muscle cells (2), cardiac endothelial cells (5), and cardiomyocytes (6), as well as in various pathological processes such as cardiac hypertrophy (6), chronic active gastritis associated with Helicobacter pylori (7), and cystic fibrosis (8).

Growing evidence points to a disintegrin and metalloproteinases (ADAMs) as key enzymes of proHB-EGF shedding in EGFR transactivation signaling. All ADAMs have an extracellular portion with a metalloprotease domain, a transmembrane region, and a cytoplasmic tail, and several ADAMs have metalloprotease activity. Lemjabbar and Basbaum (8) described that stimulation of platelet-activating factor receptor transactivated EGFR through the shedding of proHB-EGF by ADAM10 in the human epithelial cell line HM3. Yan et al. (9) demonstrated that stimulation with the bombesin receptor transactivated EGFRs via the ADAM10-dependent cleavage of proHB-EGF in COS-7 cells. We also identified ADAM12 as a specific enzyme that catalyzes proHB-EGF shedding in EGFR transactivation by GPCR agonists, such as phenylephrine, endothelin-1, and angiotensin II causing cardiac hypertrophy (6). Izumi et al. (10) showed that ADAM9 is involved in TPA-induced shedding of HB-EGF in Vero-H cells when protein kinase Cδ is activated. However, Weskamp et al. (11) found that TPA-stimulated shedding of HB-EGF is unaffected in embryonic fibroblasts derived from mice lacking ADAM9. Moreover, Kurisaki et al. (12) discovered that TPA-induced proHB-EGF shedding is completely abrogated in embryonic fibroblasts derived from mice lacking ADAM12, arguing against an essential role for ADAM9 in proHB-EGF shedding.

These reports indicate that the ectodomain shedding of EGFR ligands, especially of proHB-EGF, is central to GPCR and EGFR communication. However, the underlying mechanisms of the ligand shedding-dependent EGFR transactivation...
pathway are largely unknown. Thus, elucidation of the regulatory mechanisms of ADAMs is essential to understand the ligand shedding-dependent EGFR transactivation pathway. The present study focuses on ADAM12, which is involved in the proHB-EGF shedding induced by TPA and angiotensin II.

EXPERIMENTAL PROCEDURES

Expression Vectors and Small Interfering RNA (siRNA)—The yeast expression plasmid encoding the GAL4 DNA-binding domain fused to the human ADAM12 cytoplasmic domain was constructed by inserting ADAM12 cytoplasmic domain complementary DNA (cDNA) into EcoRI and SalI sites in the multiple cloning sites of the pBTM116 vector (Clontech). We similarly constructed yeast expression plasmids encoding GAL4 DNA-binding domain fused to human ADAM9, ADAM10, ADAM15, ADAM17, and ADAM19 cytoplasmic domains. We prepared an adenovirus carrying a gene encoding FLAG epitope-tagged ADAM12 as described (6). ADAM12 full-length was cloned into the pEGFP-N1 expression vector (Clontech). We similarly constructed yeast expression plasmids encoding the GAL4 DNA-binding domain fused to human ADAM9, ADAM10, ADAM15, ADAM17, and ADAM19 cytoplasmic domains. We prepared an adenovirus carrying a gene encoding FLAG epitope-tagged ADAM12 as described (6).

Yeast Two-hybrid Screening—Yeast strain L40 containing pBTM116-ADAM12-Cyto was selected on synthetic complete medium lacking tryptophan. A human heart cDNA library in pH2T2 was introduced into the transformant, and then yeast cells were plated on synthetic complete medium lacking tryptophan, leucine, and histidine in the presence of 35 µCi/ml 3-aminoisobutylazide. We assayed the β-galactosidase activity of transformants grown on the dropout plates at 30 °C for 3–7 days. Library plasmid DNA was recovered by transformation into Escherichia coli HB101 cells and sequenced.

Cell Culture and Transfection—HT1080 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.

Preparation of Cell Extracts—Cells were washed with phosphate-buffered saline and lysed in 50 mM Tris-HCl (pH 7.4) containing 120 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM leupeptin, 1.5 mM pepstatin, 1 mM aprotinin, and 50 mM...
sodium fluoride (lysis buffer). After centrifugation for 15 min at 12,000 rpm, the supernatant was collected as cell extract.

**Pull-down Assay with Immobilized Glutathione S-Transferase (GST) Fusion Proteins**—We separated GST and GST-fusion proteins using SDS-PAGE and stained them with Coomassie Brilliant Blue. The proteins were immobilized onto glutathione-Sepharose and exposed to pull-down FLAG-tagged ADAM12 (FLAG-ADAM12) from cell lysates. Cell lysates were incubated with either GST or GST fusion proteins on the resin in 500 μl of the lysis buffer described above with gentle agitation at 4 °C for 4 h. After extensive washing with lysis buffer, bound proteins were released by boiling in SDS-sample buffer, separated by SDS-PAGE, and then immunoblotted.

**Immunoprecipitation**—The HT1080/HA-PACSIN3 cell lysates were incubated for 2 h with an anti-HA polyclonal antibody (Y11) (Santa Cruz Biotechnology). Protein G-Sepharose (Amersham Biosciences) was added, and the mixture was incubated for 4 h. The immunoprecipitates were collected by centrifugation, washed five times with lysis buffer, separated by SDS-PAGE, and then immunoblotted.

**Immunoblot Analysis**—Resolved proteins were transferred onto an Immobilon-P membrane (Millipore) and immunoblotted against the following primary antibodies: anti-HA monoclonal antibody (12CA5) (Roche Applied Science), anti-FLAG monoclonal antibody (M2) (Sigma), or anti-PACSIN3 polyclonal antibody raised against GST fusion protein. The secondary antibody was either horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody (Promega). The immunoreactive proteins were visualized using the ECL detection system (Amersham Biosciences).

**Confocal Microscopy**—HT1080/HA-PACSIN3 and HT1080/HA-PACSIN3-ΔSH3 cells were transiently transfected with pEGFP-ADAM12.
Thereafter, the cells were fixed with 4% paraformaldehyde, permeabilized using 0.1% Nonidet P-40, and stained with anti-HA monoclonal antibody followed by goat Cy3-conjugated anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Fluorescent images were acquired at the resolution of 512 × 512 using a Bio-Rad Radiance 2000 confocal device fitted on a Nikon Eclipse E-600 microscope with a 100 x/1.4 PlanAPO oil-immersion objective. Red and green signals were collected sequentially to avoid bleed through.

**Assay of ProHB-EGF-AP Shedding**—Cells at 80–90% confluency were transfected with 0.8 μg of siRNA (per well of 24-well plates) and incubated for 40 h. The cells were then incubated for 60 min at 37 °C with 50 nM TPA or 100 nM angiotensin II. Aliquots (100 μl) of conditioned media were transferred to 96-well plates, and AP activity was measured as described previously (14).

**RESULTS**

**Identification of Binding Proteins That Interact with ADAM12 by Yeast Two-hybrid Screening**—To identify proteins that interact with the cytoplasmic domain of ADAM12, 3.0 × 10⁶ clones of a human heart cDNA library were screened using the yeast two-hybrid system and the ADAM12 cytoplasmic domain as bait. Thirteen positive clones were obtained and sequenced. A homology search in GenBank cDNA data bases using the BLAST program revealed that two of the isolated clones overlap with the carboxy-terminal region of PACSIN3 cDNA. PACSIN3 full-length cDNA was cloned by PCR from a human heart cDNA library, and interaction between PACSIN3 and the cytoplasmic domain of ADAM12 in yeast cells was confirmed by growth under nutritional selection and by β-galactosidase production (Fig. 1A). The domain structure of PACSIN3 has a Fes/CIP4 homology domain in the amino-terminal region, followed by a coiled coil domain and a SH3 domain at the carboxy-terminal region (Fig. 1B) (15).

**PACSIN3 Associates with ADAM12 in Vitro and in Vivo**—We performed in vitro binding assays to confirm that PACSIN3 and ADAM12 interact. FLAG-ADAM12 bound to GST-PACSIN3 fusion protein but not to GST alone in extracts from HT1080 cells expressing FLAG-ADAM12 (Fig. 2A). To further confirm that these two proteins can associate in mammalian cells, FLAG-ADAM12 was transiently expressed in HT1080/HB-AP/PACSIN3 cells. Fig. 2B shows that anti-HA antibody immunoprecipitated HA-PACSIN3 from cell lysates of HT1080 cells transfected with HA-PACSIN3, but not from mock-transfected cell lysates (middle panel), and co-immunoprecipitated FLAG-ADAM12, which was detected by the anti-FLAG antibody (upper panel). FLAG-ADAM12 expression was detected by anti-FLAG antibody in the lysates of HT1080 cells transfected with FLAG-ADAM12 but not in lysates from mock-transfected HT1080 cells (lower panel).

**Determination of Cytoplasmic Domain of ADAM12 Required for PACSIN3 Binding**—To define the binding site of ADAM12 for PACSIN3, we assayed binding in vitro using a series of truncated mutants of the cytoplasmic domain of ADAM12 (Fig. 3, A and C). HA-PACSIN bound to GST-ADAM12-Cyto-1, -2, and -3 equally and to GST-ADAM12-Cyto-4 and -5 to a lesser extent in extracts from HT1080 cells expressing HA-PACSIN3 (Fig. 3B). In contrast, HA-PACSIN3 did not bind to GST-ADAM12-Cyto-6 and -7 (Fig. 3B). These results suggest that P1 (amino acid residues 754–759) and P2 (amino acid residues 829–840) are required for binding to PACSIN3.

**Co-localization of ADAM12 and PACSIN3**—To determine the intracellular localization of ADAM12 and PACSIN3, EGF-fused ADAM12 (ADAM12-EGFP) was transiently expressed in HT1080/HB-PACSIN3 or HT1080/HB-PACSIN3-ΔSH3 cells. Fluorescence microscopy revealed that ADAM12-EGFP and HA-PACSIN3 co-localized at intracellular vesicles and at the leading edge of the cell (Fig. 4, A–C). On the other hand, the combination of ADAM12-EGFP and HA-PACSIN3-ΔSH3 (Fig. 4, D–F) did not co-localize.

**Effects of PACSIN3 on Ectodomain Shedding of ProHB-EGF Induced by TPA and Angiotensin II**—To investigate the effect of PACSIN3 on the ectodomain shedding of proHB-EGF induced by TPA, PACSIN3 or PACSIN3-ΔSH3 was overexpressed in HT1080/HB-EGF-AP cells. The AP activity in the conditioned medium of each transfectant was then measured after a 60-min incubation with 50 nM TPA. The overexpression of PACSIN3 enhanced TPA-induced proHB-EGF-AP shedding ~5-fold compared with the control, whereas the effect of PACSIN3-ΔSH3 overexpression was quite minimal (Fig. 5A). However, PACSIN3 overexpression did not enhance proHB-EGF-AP shedding induced by angiotensin II to a statistically significant extent (Fig. 5B). To confirm that PACSIN3 functions as an up-regulator of proHB-EGF ectodomain shedding, we destroyed the mRNA using siRNA mediation. To test the effect of PACSIN3-siRNA on knockdown, we estimated the level of endogenous PACSIN3 protein by immunoblotting. We detected less endogenous PACSIN3 protein in the lysate from HT1080 cells transfected with PACSIN3-siRNA than in those transfected with negative control scramble siRNA (Fig. 6A). This result confirmed that PACSIN3-siRNA reduced endogenous PACSIN3 protein levels. We then assessed the effect of PACSIN3-siRNA on the ectodomain shedding of proHB-EGF induced by TPA. HT1080/HB-EGF-AP cells were transfected with Scramble-siRNA or with PACSIN3-siRNA, and AP activity was measured in the same manner as described above.

In addition, proHB-EGF-AP shedding induced by angiotensin II was assayed using HT1080/HB-EGF-AP/AT1 cells. PACSIN3 knockdown mediated by PACSIN3-siRNA attenuated 50
and 45% of the control levels of TPA- and angiotensin II-induced proHB-EGF-AP shedding, respectively (Fig. 6, B and C).

**Specificity of PACSIN3 Binding to ADAMs**—We studied the specificity of ADAMs for PACSIN3 association. Fig. 7 shows the binding affinity of the cytoplasmic domains of various ADAM proteins for PACSIN3 determined by the yeast two-hybrid system. The cytoplasmic domains of ADAMs 9, 10, 12, 15, and 19 interacted with PACSIN3 but not those of ADAM17 both in yeast growth (Fig. 7A) and β-galactosidase assays (Fig. 7B).

**DISCUSSION**

The current study investigated the regulatory mechanisms of ADAM-dependent proHB-EGF shedding induced by TPA or GPCR agonists. We then focused on ADAM12 that has recently been identified as a shedding enzyme involved in proHB-EGF. Since the cytoplasmic tail of human ADAM12 has four class I (R/K)XXPXXP and three class II PXXPXR/K SH3 domain-binding motifs grouped into four proline-rich regions (Fig. 3A), ADAM12 probably interacts with several signaling molecules containing SH3 domains. Yeast two-hybrid screening using the cytoplasmic domain of ADAM12 as bait resulted in the isolation of PACSIN3 characterized by Fes/CIP4 homology and SH3 domains in the amino- and carboxyl-terminal regions, respectively. PACSIN3 is supposed to be a cytoplasmic molecule involved in endocytosis. For example, PACSIN3 binds endocytic proteins such as dynamin, synaptojanin 1, and N-WASP in vitro, and PACSIN3 overexpression blocks endocytosis (16).

ADAM12 interacts with signaling molecules containing an SH3 domain such as Src and the p85 regulatory subunit of phosphatidylinositol 3-kinase, and the binding sites for these proteins are located in P2, and in P2 or P4 of the ADAM12 cytoplasmic tail, respectively (17–19). Binding assays using a series of truncated mutants mapped the PACSIN3-binding sites to P1 and P2 in ADAM12 cytoplasmic tail. These findings suggest that the PACSIN3-binding sites are shared by several proteins containing SH3 domains, although whether the binding of these proteins to ADAM12 cytoplasmic tail is exclusive remains unknown.

Microscopy revealed that HA-PACSIN3 was diffused throughout the cytoplasm and partially localized in the plasma membrane where some endogenous ADAM12 also resides. On the other hand, ADAM12-EGFP was intracellularly distributed in a vesicle-like manner, consistent with other reports indicating that exogenous ADAM12 is localized mainly in the endoplasmic reticulum, trans-Golgi networks, and partially in the plasma membrane (20, 21). HA-PACSIN3 co-localized with ADAM12-EGFP in the plasma membrane, suggesting that PACSIN3 associates with correctly transported ADAM12 in the plasma membrane. Our findings are also supported by the notion that mutant PACSIN3-ASH3 do not co-localize with wild type ADAM12, and previously reported ADAM13 binds to and co-localize with PACSIN2 (22).

We demonstrated that PACSIN3 knockdown by siRNA in HT1080 cells partially attenuated the shedding of proHB-EGF induced by TPA and angiotensin II. Thus, PACSIN3 seems necessary, but alone is insufficient, to regulate the signaling pathway of the ectodomain shedding of HB-EGF induced by
both TPA and by angiotensin II. In addition, PACSIN-like molecules might be involved in regulating the ectodomain shedding of proHB-EGF induced by TPA and angiotensin II. We also question whether PACSIN3 regulates the ectodomain shedding of proHB-EGF induced by other GPCR.

PACSIN3 can interact with the cytoplasmic tails of ADAM9, ADAM10, ADAM15, and ADAM19 as well as with those of ADAM12 (Fig. 7), suggesting that PACSIN3 widely regulates ADAM-dependent post-translational modification. The physiological significance of interactions between PACSIN3 and these ADAMs, however, remains obscure.

We postulated that PACSIN3 plays a physiological role as a binding partner of ADAM12 in the signaling pathway of proHB-EGF shedding induced by GPCR agonists. Further analyses will clarify the signaling pathway from GPCRs to the processing of proHB-EGF leading to EGFR transactivation.

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