A metalloprotease–disintegrin, MDC9/meltrin- γ /ADAM9 and PKC δ are involved in TPA-induced ectodomain shedding of membrane-anchored heparin-binding EGF-like growth factor

Yasushi Izumi, Michinari Hirata^{1,2},
Hidetoshi Hasuwa³, Ryo Iwamoto^{1,4},
Toshiyuki Umata², Kenji Miyado³,
Yoko Tamai, Tomohiro Kurisaki⁵,
Atsuko Sehara-Fujisawa⁵, Shigeo Ohno⁶
and Eisuke Mekada^{1,3}

Department of Molecular Biology, Yokohama City University School of Medicine 3-9, Fuku-ura, Kanagawa-ku, Yokohama 236-0004,

¹The Institute of Life Science and ³The Center for Innovative Cancer Research, Kurume University, 2432-3 Aikawa, Kurume, Fukuoka 839-0861, ²Department of Cell Biology, The Tokyo Metropolitan Institute of Medical Science, Honkomagome 3-18-22, Bunkyo-ku, Tokyo 113-8613, ⁴Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka, 812-0054 and ⁵Inheritance and Variation, PRESTO, Japan Science and Technology Corporation, Kyoto, 619-0237, Japan

⁶Corresponding author

e-mail: ohnos@med.yokohama-cu.ac.jp

The ectodomains of many proteins located at the cell surface are shed upon cell stimulation. One such protein is the heparin-binding EGF-like growth factor (HB-EGF) that exists in a membrane-anchored form which is converted to a soluble form upon cell stimulation with TPA, an activator of protein kinase C (PKC). We show that PKC δ binds *in vivo* and *in vitro* to the cytoplasmic domain of MDC9/meltrin- γ /ADAM9, a member of the metalloprotease–disintegrin family. Furthermore, the presence of constitutively active PKC δ or MDC9 results in the shedding of the ectodomain of proHB-EGF, whereas MDC9 mutants lacking the metalloprotease domain, as well as kinase-negative PKC δ , suppress the TPA-induced shedding of the ectodomain. These results suggest that MDC9 and PKC δ are involved in the stimulus-coupled shedding of the proHB-EGF ectodomain.

Keywords: ectodomain shedding/heparin-binding EGF-like growth factor/MDC9/metalloprotease–disintegrin/protein kinase C

Introduction

The extracellular domain of a number of membrane proteins can be cleaved proteolytically causing release into the medium (Massague and Pandiella, 1993; Rose-John and Heinrich, 1994). This proteolytic processing, also referred to as ‘ectodomain shedding’, is observed in growth factors (Massague and Pandiella, 1993), growth-factor receptors (Vecchi *et al.*, 1996), cell-adhesion molecules (Feehan *et al.*, 1996), extracellular matrix proteins (Subramanian *et al.*, 1997; Werb, 1997) and other membrane proteins such as the β -amyloid precursor protein (Esch *et al.*, 1990). Ectodomain shedding of membrane proteins changes the fate, location and mode of action;

thus it affects the biological activities of membrane proteins. For example, the ectodomain shedding of TNF- α (Mohler *et al.*, 1994), FAS-ligand (Tanaka *et al.*, 1998) and the homing receptor L-selectin (Feehan *et al.*, 1996) has been implicated in inflammation, whereas the ectodomain shedding of β APP (Haass and Selkoe, 1993) has been implicated in the pathogenesis of Alzheimer’s disease (Sisodia and Price, 1995). The ectodomain shedding of membrane receptors results in the generation of soluble competitors for their own ligands (Fernandez-Botran, 1991). In the case of membrane-anchored growth factors, ectodomain shedding can convert them into diffusible factors and greatly influence their functions (Massague and Pandiella, 1993). On the one hand, for example, the membrane-anchored form of spitz, a TGF- α -like molecule in *Drosophila*, is inactive biologically and is cleaved proteolytically to yield a soluble active form (Golembo *et al.*, 1996). On the other hand, the membrane-anchored forms of c-kit ligand (Flanagan *et al.*, 1991) and ephrins (ligands of EPH tyrosine kinase receptor, Davis *et al.*, 1994) are fully functional whereas the soluble forms exhibit little or no biological activity. Thus, ectodomain shedding is an important regulatory step in the function of membrane proteins involved in cell–cell communication in development, cell differentiation and tissue maintenance.

Heparin-binding EGF-like growth factor (HB-EGF) is a member of the epidermal growth factor (EGF) family (Higashiyama *et al.*, 1991), which encompasses a number of structurally homologous mitogens including EGF, TGF- α , vaccinia virus growth factor (Carpenter and Wahl, 1990), amphiregulin (Shoyab *et al.*, 1989), β -cellulin (Shing *et al.*, 1993) and epiregulin (Toyoda *et al.*, 1995). Like EGF, TGF- α and amphiregulin, HB-EGF binds to and stimulates the phosphorylation of the EGF receptor. HB-EGF is synthesized as a membrane-anchored precursor protein of 208 amino acids composed of signal peptide, heparin-binding, EGF-like, transmembrane and cytoplasmic domains (Higashiyama *et al.*, 1991). Although the membrane-anchored form of HB-EGF (proHB-EGF) is cleaved on the cell surface to yield a soluble growth factor of 75–86 amino acids, a considerable amount of proHB-EGF remains uncleaved on the cell surface (Goishi *et al.*, 1995). Importantly, proHB-EGF is not only a precursor of the soluble form but is also biologically active in itself; proHB-EGF forms a complex with both CD9 (Mitamura *et al.*, 1992; Iwamoto *et al.*, 1994) and integrin $\alpha 3 \beta 1$ (Nakamura *et al.*, 1995), both localized at cell–cell attachment sites (Nakamura *et al.*, 1995), and transduces biological signals in a nondiffusible manner to neighboring cells (Higashiyama *et al.*, 1995) as has been shown for TGF- α (Brachmann *et al.*, 1989; Wong *et al.*, 1989) and colony-stimulating factor (Stein *et al.*, 1990). Moreover, although secreted mature HB-EGF is a potent

mitogen for a number of cell types (Higashiyama *et al.*, 1991), the membrane-anchored form may act as a negative regulator of cell proliferation (Miyoshi *et al.*, 1997; Takemura *et al.*, 1997). Thus, the processing of the juxtamembrane domain of proHB-EGF to the soluble HB-EGF means the conversion of the mode of action of this growth factor from juxtacrine to paracrine or the switching to the opposite activity for cell growth. ProHB-EGF also acts as the specific receptor for diphtheria toxin (DT) and mediates the endocytosis of the receptor-bound toxin (Naglich *et al.*, 1992; Iwamoto *et al.*, 1994). Interestingly, proHB-EGF is cleaved rapidly to soluble HB-EGF by treatment with TPA, suggesting the involvement of a cellular signaling pathway involving protein kinase C (PKC) (Goishi *et al.*, 1995). The regulated shedding of membrane proteins has also been shown for a number of other membrane proteins including β APP, TGF α , IL-6 and receptor-like protein tyrosine phosphatases (Pandiella and Massague, 1991a,b; Arribas *et al.*, 1996; Aicher *et al.*, 1997). However, the mechanism of the ectodomain shedding of membrane proteins remains totally obscure.

Metalloprotease-disintegrins, or the ADAM family of proteins, make up a recently discovered protein family of membrane-anchored glycoproteins comprising a pro-domain, metalloprotease domain, a disintegrin domain, a cysteine-rich domain, an EGF-like domain, a transmembrane domain and a cytoplasmic domain (Wolfsberg *et al.*, 1995; Blobel, 1997). These proteins have been shown to be involved in cell-cell interactions, cell adhesion and the processing of proteins. More than 20 family members have been identified in a variety of species including mammals, *Xenopus*, *Drosophila* and *Caenorhabditis elegans*. The first identified proteins of the ADAM family were fertilin α and β (also called PH-30 α and β , or ADAM1 and 2), sperm surface proteins involved in fertilization by binding to unknown receptors on the egg (Wolfsberg *et al.*, 1995; Blobel, 1997). Another ADAM protein, meltrin- α (ADAM12), has been implicated in myoblast fusion or myogenesis (Yagami-Hiromasa *et al.*, 1995). The metalloprotease activity of ADAM family members was first revealed for TNF- α converting enzyme (TACE), responsible for the release of TNF- α from the cell surface by proteolytically cleaving the precursor form (Black *et al.*, 1997; Moss *et al.*, 1997). More recently, Kuzbanian (Kuz) in *Drosophila*, whose mammalian homolog is ADAM10 (Howard *et al.*, 1996), has been suggested to be involved in the proteolytic cleavage and activation of Notch receptor (Pan and Rubin, 1997; Chan and Jan, 1998).

To understand the mechanism of the TPA-induced shedding of the proHB-EGF ectodomain, we first examined the role of PKC isotypes and identified PKC δ as the PKC isotype involved in the regulated shedding. Interaction screening of a cDNA library using purified PKC δ as a probe allowed us to identify MDC9 as a specific binding protein for PKC δ . The overexpression of MDC9 resulted in the shedding of proHB-EGF without TPA, and the MDC9 mutants of the metalloprotease domain inhibited TPA-induced shedding. These results provide evidence to suggest that MDC9 is involved in the processing of proHB-EGF and that a direct interaction between PKC δ and MDC9 is involved in the regulated shedding.

Results

Involvement of PKC δ in the TPA-induced shedding of the ectodomain of proHB-EGF

Previous studies on African green monkey kidney Vero cells overexpressing human proHB-EGF (Vero-H cells) have shown that membrane-bound proHB-EGF is processed by a phorbol ester, TPA-dependent mechanism, to yield soluble bioactive HB-EGF (Goishi *et al.*, 1995). Treatment of Vero-H cells with TPA results in a reduction in the cell-surface DT receptor and cell-surface immunoreactivity against anti-HB-EGF antibody, #H6, directed against the ectodomain of proHB-EGF. In parallel with the loss of cell surface immunoreactivity, the amounts of soluble 14 kDa and 19 kDa HB-EGF detected by the anti-HB-EGF antibody #2998 increase in the culture medium. Concomitantly, TPA induces the loss of proHB-EGF juxtacrine activity and an increase in soluble HB-EGF paracrine activity (Goishi *et al.*, 1995). Importantly, these responses occur within 30 min (Goishi *et al.*, 1995; Figure 1A and B). Furthermore, staurosporine and H7 inhibit both the TPA-induced and constitutive shedding of the proHB-EGF ectodomain (Goishi *et al.*, 1995). These results suggest the presence of a protease acting on the cell surface whose activity is regulated by an unknown mechanism through a TPA-responsive protein kinase such as PKC.

As a first step in analyzing the mechanism of the shedding of the proHB-EGF ectodomain, we confirmed the above observation by using immunofluorescence microscopy with #H6, an antibody against the ectodomain of HB-EGF. Vero-H cells were first treated with anti-proHB-EGF antibody #H6 directed against the ectodomain of proHB-EGF, and fixed and visualized using a second antibody, revealing the presence of proHB-EGF immunoreactivity on the outer surface of Vero-H cells. As shown in Figure 1A, staining is concentrated at the cell-cell contact region in addition to the entire cell surface, confirming the previous results (Goishi *et al.*, 1995). The addition of TPA results in the disappearance of the immunoreactivity on the cell surface within 30 min, consistent with the previous report (Figure 1A and B).

In order to obtain direct proof for the involvement of PKC and to obtain information about the PKC isotypes involved in the shedding of proHB-EGF, we next examined the PKC isotypes expressed in Vero-H cells. As shown in Figure 1C, Vero-H cells express at least three TPA-responsive PKC isotypes, PKC α , PKC δ and PKC ϵ . Treatment of the cells with TPA for 12 h results in decreases in the amounts of both PKC δ and PKC ϵ , whereas the amount of PKC α does not change. Although the meaning of this selective down-regulation remains unknown, the results suggest that at least two PKC isotypes, PKC δ and PKC ϵ , are activated in Vero-H cells upon TPA treatment.

In order to clarify the involvement of PKC, we expressed the active mutant of PKC isotypes and examined cell surface immunoreactivity against the ectodomain of proHB-EGF by indirect immunofluorescence. Figure 1D shows examples where Vero-H cells expressing the activated forms of tag-PKC isotypes are visualized using an anti-tag (T7) antibody by indirect immunofluorescence (green). At the same time, the proHB-EGF ectodomain (red) was also visualized as shown in Figure 1A. These

Fig. 1. TPA-induced shedding of proHB-EGF ectodomain. (A) Immunofluorescent localization of the proHB-EGF ectodomain in Vero-H cells. a and b, without TPA; c–j, with TPA (50 ng/ml) for the indicated time. The left-hand panel shows phase contrast images and the right-hand panels show immunofluorescence images. (B) Time course of the TPA-induced shedding of the proHB-EGF ectodomain. The number of proHB-EGF-positive cells was counted based on the data shown in A. (C) Western analysis of PKC isotypes in Vero-H cells. TPA (+) indicates cells treated with 50 ng/ml of TPA for 12 h. (D) Immunofluorescent detection of the shedding of the proHB-EGF ectodomain upon the transient overexpression of constitutive active mutants of PKC isotypes. Vero-H cells were transfected with plasmids encoding constitutive active mutants of PKC α (α R22A/A25E, a and b), PKC δ (DR144/145A, c and d) and PKC ϵ (ϵ K155A/R156A/A159E, e and f). After 48 h of transfection, cells were double-stained with anti-HB-EGF antibody (red) for the proHB-EGF ectodomain and anti-T7-tag antibody (green) for PKC mutants. The anti-T7 antibody shows nonspecific nuclear staining. Arrowheads show cells expressing introduced PKC mutants. (E) Percentage of proHB-EGF-positive cells among transfected cells, determined by immunofluorescence detection as shown in D. The numbers above the bars represent the deviation from the mean of two independent experiments.

PKC mutants show co-factor independent kinase activities *in vitro* and their expression mimics the effect of TPA *in vivo* (Ueda *et al.*, 1996). Interestingly, cells that over-express active PKC δ (tag-DR144/145A) but not active mutants of PKC α (tag- α R22A/A25E) and PKC ϵ (tag- ϵ K155A/R156A/A159E) show a loss of proHB-EGF ecto-

domain staining. Figure 1E shows the quantified results. The PKC mutants used in the present study contain an N-terminal tag sequence, permitting direct comparison of the amounts of PKC mutants expressed in the respective cells. Clearly, the results indicate that the ectopic expression of active PKC δ results in proHB-EGF ectodomain shedding in the absence of TPA.

Next we examined the effect of the ectopic expression of a PKC δ kinase-knockout mutant (DRKA) containing a point mutation at the ATP-binding site that results in greatly diminished kinase activity and the inhibition of PKC δ -dependent AP1 activation (Hirai *et al.*, 1994). To evaluate the expression levels of the respective PKC δ and PKC δ mutant, we monitored cells expressing PKC δ mutants using an anti-PKC δ antibody. As shown in Figure 2A and B, the expression of DRKA results in the inhibition of TPA-induced proHB-EGF ectodomain shedding, whereas the expression of wild-type PKC δ has no effect in the absence of TPA, indicating that the kinase activity of PKC δ is required for the shedding of the proHB-EGF ectodomain. Further, these results support the notion that PKC δ is involved in this process.

Identification of MDC9 as a PKC δ -specific binding protein/substrate

To analyze the mechanism of the involvement of PKC δ in the shedding of the proHB-EGF ectodomain, we searched for binding proteins specific for PKC δ . Our strategy was to screen a mouse fibroblast NIH 3T3 cDNA expression library using purified recombinant autophosphorylated PKC δ as a probe (Izumi *et al.*, 1997). Secondary screening of the cDNA clones for putative PKC δ -binding proteins using other PKC isotypes as probes revealed that most of the cDNA clones encode proteins that bind to multiple PKC isotypes (data not shown). These proteins include MARCKS, a known physiological substrate for PKC, and SRBC, a protein whose mRNA is induced upon serum depletion, both of which bind to PKC α and PKC ϵ as well as to PKC δ *in vitro* (Fujise, *et al.*, 1994; Izumi *et al.*, 1997; data not shown). Among >10 such cDNA clones, clone G1 encoded a protein that showed binding specificity to PKC δ on blot overlay assay (data not shown).

A search of the sequence database revealed that the G1 sequence encodes the C-terminal 127 amino acid residues of MDC9/meltrin- γ /ADAM9. MDC9 is a member of the ADAM family of proteins that share disintegrin and metalloprotease domains in addition to other structural motifs (Figure 3A) (Yagami-Hiromasa *et al.*, 1995; Weskamp *et al.*, 1996). The cloned cDNA encodes the exact C-terminal sequence implicated as the cytoplasmic domain. Considering the structural motif of the proposed extracellular domain of MDC9 and the recent finding that a protein responsible for the processing of TNF α , TACE, is a member of the ADAM family, the present finding of a direct protein–protein interaction between PKC δ and MDC9 suggests a possible functional link between PKC δ and MDC9, and further suggests their involvement in the regulated shedding of the proHB-EGF ectodomain. To examine these possibilities, we next isolated a full-length cDNA for MDC9 and raised an antibody against the C-terminal domain of MDC9.

The initial characterization of the MDC9 mRNA and proteins revealed that MDC9 is a ubiquitously expressed

Fig. 2. A kinase-deficient mutant of PKC δ suppresses the TPA-induced shedding of the proHB-EGF ectodomain in Vero-H cells. (A) Vero-H cells were transfected with plasmids encoding wild-type PKC δ (a–f), the constitutive active mutant DR144/145A (g–i) or the kinase-deficient mutant DRKA (j–o). After 48 h of transfection, cells were double-stained with anti-HB-EGF antibody for the proHB-EGF ectodomain (red) and anti-PKC δ antibody for overexpressed PKC δ mutants (green). Some cells (d–f and m–o) were incubated with TPA (50 ng/ml, for 60 min) prior to staining. Phase contrast images for each field are also shown in the left-hand column. The arrowhead in each image shows the overexpressed cell of wild-type PKC δ or PKC δ mutants. (B) Percentage of proHB-EGF-positive cells among transfected cells shown in A. The numbers above the bars represent the deviation from the mean of two independent experiments.

membrane-spanning protein with a C-terminal cytoplasmic domain containing two SH3-binding motifs (Weskamp *et al.*, 1996). The surface labeling of NIH 3T3 cells also revealed the presence of MDC9 at the cell surface (Weskamp *et al.*, 1996). As shown in Figure 3B, a full-length cDNA for MDC9 introduced into COS cells directed the appearance of two protein bands migrating at ~84 kDa and 120 kDa. The two bands are detected in various cell lines including Vero-H cells and COS cells (Figure 3B), consistent with the earlier observation (Weskamp *et al.*, 1996).

To confirm the interaction between MDC9 and PKC δ *in vivo*, we expressed the tag-MDC9 and PKC isotypes in COS cells, immunoprecipitated MDC9, and examined the immunoprecipitates for the presence of PKC isotypes. Figure 3C shows that PKC δ but not PKC α or PKC ϵ , co-precipitates with tag-MDC9, indicating that the cytoplasmic domain of MDC9 specifically associates with PKC δ *in vivo*. Similar experiments using PKC α/δ and PKC δ/α chimeras, AD335 and DA318, revealed that MDC9 associates with AD335 but not with DA318. AD335 contains the regulatory domain of PKC α linked to the kinase domain of PKC δ , suggesting that the kinase domain of PKC δ is responsible for the interaction with MDC9.

To determine the MDC9 sequence required for the interaction with PKC δ , we constructed a series of glutathione *S*-transferase (GST) fusion proteins containing the cytoplasmic domain of MDC9 as illustrated in Figure 3A. Figure 3D shows the results of a blot overlay assay where GST fusion proteins were fixed to the membrane and probed with autophosphorylated PKC δ , showing that PKC δ binds directly to the cytoplasmic domain of MDC9. Further, except for GST-MDC9-743/827, all the C-terminal deletion mutants associated with PKC δ , indicating that a very short region (amino acid residues 719–745) of MDC9 is sufficient for the interaction with PKC δ . Interestingly, this shortest mutant also interacts with PKC α on blot overlay assay, whereas the whole cytoplasmic domain fails to associate efficiently with PKC α (data not shown). Thus, the 25 amino acid sequence just downstream of the transmembrane domain contains the PKC-binding sequence, whereas the sequence that determines the specificity for PKC δ is not included in this region. This region contains one threonine and two serine residues surrounded by basic residues (Figure 3A), a consensus sequence for PKC phosphorylation. In fact, GST-MDC9 fusion proteins are efficiently phosphorylated by purified PKC δ as shown in Figure 3E. These results clearly show that the cytoplasmic domain of MDC9 is the specific binding protein and substrate for PKC δ *in vitro*.

Overexpression of MDC9 results in the processing of proHB-EGF and the release of bioactive soluble HB-EGF

To test the possibility that MDC9 is involved in the processing of proHB-EGF, we first examined the effect of the ectopic expression of MDC9 on Vero-H cells. Forty-eight hours after transfection with plasmids encoding full-length mouse MDC9 cDNA, the proHB-EGF ectodomain was visualized. As shown in Figure 4C, a and b, in cells expressing MDC9 cDNA (green), the proHB-EGF ectodomain staining (red) is markedly reduced even in the absence of

Fig. 3. Direct interaction between PKC δ and the cytoplasmic domain of MDC9. (A) Schematic structure of MDC9 and GST-fusion proteins containing various regions of the MDC9 cytoplasmic domain. SS, signal sequence; Pro, prodomain; MP, metalloprotease domain; DI, disintegrin domain; Cys, cysteins-rich domain; TM, transmembrane domain. The amino acid sequence of the PKC-binding domain defined in this article is also shown as a one letter symbol. (B) Western analysis of the expression of MDC9 in a variety of cell lines. Cell lysates from various cell lines were subjected to Western blotting and probed with anti-MDC9 antibody alone (upper panel) or with anti-MDC9 antibody and excess amounts of antigenic peptide (lower panel). Arrowheads indicate 84 kDa and 120 kDa bands of MDC9. (C) Co-immunoprecipitation of PKC δ with the cytoplasmic domain of MDC9 in COS cells. COS cells were transfected with plasmids encoding tag-MDC9 alone, single PKC isotypes or their mutants alone, or both. Cell lysates were immunoprecipitated with anti-T7-tag antibody, and the immunoprecipitates were probed with the anti-PKC antibodies shown at the bottom. (D) Direct association of MDC9 and PKC δ *in vitro*. Western blotting of the GST-MDC9 fusion proteins shown in (A) as stained by CBB (left-hand panel) or probed with [³²P]-labeled PKC δ (right-hand panel). The asterisk in each lane indicates the corresponding GST-MDC9 fusion protein. (E) Phosphorylation of GST-MDC9 fusion proteins by purified PKC δ . GST-fusion proteins containing the cytoplasmic domain of MDC9 were incubated with PKC δ and [³²P]-ATP under the conditions described in Materials and Methods. The reaction mixtures were subjected to SDS-PAGE and autoradiography.

TPA treatment. The control transfection with the expression vector (pEF-BOS) gave a result similar to those obtained with nontransfected cells in the presence or absence of TPA (data not shown). The percentage of cells

expressing the proHB-EGF ectodomain was quantitated and is summarized in Figure 4D. In the absence of TPA, all cells express the proHB-EGF ectodomain, whereas the overexpression of MDC9 reduced the expression to 35%.

Fig. 4. Overexpression of MDC9 and MDC9 mutants. (A) Schematic structure of MDC9 and its mutants. Δ PKCBD, Δ MP and Δ PKCMBP are deletion mutants lacking the PKC-binding domain, metalloprotease domain and both domains, respectively. Δ Pro-rich is a deletion mutant lacking the proline-rich domain. In the metalloprotease domain of MDC9, there is a sequence, HEXXH, a putative zinc-binding motif, that is conserved among most corresponding domains of ADAM family proteins. H347,351A are mutants of MDC9 in which the corresponding histidine is replaced by alanine. Cyto is a deletion mutant possessing only the cytoplasmic domain of MDC9, whereas Cyto Δ PKCBD lacks the PKC binding domain of Cyto. (B) Western analysis of MDC9 mutants expressed in COS cells using anti-MDC9 antibody. (C) Immunofluorescent detection of the proHB-EGF ectodomain in Vero-H cells transiently transfected with plasmids encoding MDC9 or its mutants. Vero-H cells were transfected with plasmids encoding wild-type MDC9 (a and b), Δ PKCBD (c and d), Δ MP (e–h), Δ PKCMBP (i–l), H347,351A (m–p), Cyto (q–t) or Cyto Δ PKC (u–x). After 48 h of transfection, the cells were double-stained with anti-HB-EGF antibody for the proHB-EGF ectodomain (red) and anti-MDC9 antibody for overexpressed MDC or its mutants (green). Some cells (g, h, k, l, o, p, s, t, w, x) were incubated with TPA (50 ng/ml, for 60 min) prior to immunofluorescence staining. Arrowheads indicate cells expressing the MDC9 or MDC9 mutants. (D) Percentage of proHB-EGF-positive cells among those expressing MDC9 mutants shown in (C). The numbers above the bars represent the deviation from the mean of two independent experiments, the numbers with asterisks represent the standard deviation from the mean of three independent experiments.

Thus, the ectopic overexpression of MDC9 results in the processing of proHB-EGF in the absence of TPA.

To obtain further biochemical proof that the overexpres-

sion of MDC9 results in the shedding of the proHB-EGF ectodomain, we transfected Vero cells with MDC9 cDNA and isolated stable transformants expressing MDC9. The

Fig. 5. Overexpression of MDC9 results in the release of soluble bioactive HB-EGF. **(A)** Reduced DT-binding activity of MDC9-overexpressing cells. Vero cells (Vero) or Vero-MDC9 cells (V-M) (5×10^5 cells) were cultured for 15 h and then the amounts of proHB-EGF on the cell surface were determined by means of DT binding. TPA (+) indicates cells incubated with TPA (64 nM, for 60 min) prior to the binding assay. The numbers above the bars represent the deviation from the mean of duplicate samples. **(B)** Enhanced shedding of the proHB-EGF ectodomain from MDC9-overexpressing cells under steady-state culture conditions and from TPA-induced cells. Vero cells (Vero) or Vero-MDC9 cells (V-M) (5×10^5 cells) were cultured for 4 days and the amount of HB-EGF secreted into the conditioned medium was determined by means of the DT-binding assay. For TPA treatment, the medium was replaced with fresh culture medium at culture day 4. TPA (64 nM) was then added to the medium and the cells were further cultured for 2 h for 37°C. The conditioned medium was harvested and the amount of secreted HB-EGF was determined. The numbers above the bars represent the deviation from the mean of duplicate samples. **(C)** Western blotting analysis of soluble HB-EGF molecules secreted into the culture medium from Vero cells (V), Vero-MDC9 cells (V-M) and Vero-H cells (V-H). **(D)** Mitogenic activity of soluble HB-EGF secreted into the culture medium. Vero cells were transfected with the indicated amounts of plasmids encoding MDC9 (MDC9) or the vector sequence only (EF). The mitogenic activity of the conditioned medium was determined by measuring the DNA synthesis of co-cultured DER cells as described in Materials and methods. Data are shown as CRM197-inhibitable mitogenic activity, which represents the mitogenic activity attributable to HB-EGF. The numbers above the bars represent the deviation from the mean of duplicate samples.

isolated clone, Vero-MDC9, expresses ~10 times more MDC9 than parental Vero cells but much less MDC9 than transiently expressing cells (data not shown). The effect of MDC9 on the shedding of the proHB-EGF ectodomain was then examined in these cells. We measured both the amount of proHB-EGF on the cell surface and the amount of HB-EGF secreted into the medium by measuring DT-binding activity. The amount of proHB-EGF on the cell surface of Vero-MDC9 was ~60% that of parental Vero cells under steady state culture conditions (Figure 5A), whereas the amount of HB-EGF secreted into the medium from Vero-MDC9 was 2.5–3 times higher than from Vero cells (Figure 5B). When the cells were treated with TPA, most of the proHB-EGF on the cell surface was processed rapidly (Figure 5A) and secreted into the medium (Figure 5B). Western blotting using an anti-HB-EGF neutralizing antibody showed that three species of soluble HB-EGF with molecular masses of ~14 kDa, 18 kDa and 19 kDa are secreted into the conditioned medium from Vero and Vero-MDC9 cells (Figure 5C). That is similar to the HB-EGF forms secreted from Vero-H cells as reported previously (Goishi *et al.*, 1995).

Finally, we tested whether the overexpression of MDC9

results in the release of mitogenically active soluble HB-EGF. To examine this, Vero-H cells were transfected with plasmids encoding MDC9 cDNA or with control vector plasmids and the mitogenic activity of the conditioned media was determined by measuring the rate of DNA synthesis of DER cells as described in Materials and methods. DER cells, similar to EP170.7 cells (Pierce *et al.*, 1988), are a stable transformant of 32D cells expressing EGF receptor, and thus respond only to EGF-receptor ligands in the absence of IL-3. Although DER cells allow the efficient detection of the mitogenic activity of HB-EGF, they also respond to other EGF-receptor ligands. Vero-H cells might secrete other mitogenic factors in addition to HB-EGF. To measure the mitogenic activity attributable to HB-EGF, we used CRM197 as a specific inhibitor of the mitogenic activity of HB-EGF (Mitamura *et al.*, 1995) and determined the CRM197-inhibitable mitogenic activity. As shown in Figure 5D, the conditioned medium of MDC9-transfected Vero-H cells contains 2–3 times more mitogenic activity than that of vector-transfected Vero-H cells. The mitogenic activity attributable to other growth factors was <20% in each experiment. The results, together with those for the transfection of MDC9

to Vero-H cells, led us to conclude that the ectopic expression of MDC9 results in marked enhancement of proHB-EGF processing to yield biologically active soluble HB-EGF.

Mutations in the metalloprotease domain result in the generation of dominant-negative MDC9 mutants that suppress the TPA-induced processing of proHB-EGF

The above results suggest the involvement of both PKC δ and MDC9 in the regulated shedding of proHB-EGF. To investigate whether the MDC9 protease activity is necessary for the processing of proHB-EGF, several mutants of MDC9 were constructed (Figure 4A).

The expressions of these mutants were confirmed in COS cells (Figure 4B). When compared with endogenous MDC9 (Figure 3B), the overexpressed wild-type MDC9 shows a slightly more intensely stained 120 kDa band and a less intense 84 kDa band. Although the nature of the two bands remains to be clarified in future experiments, the lower bands are absent from Δ MP and H347,351A mutants, both of which lack essential sequences in the protease domain. A similar situation occurs for Δ PKCBD, which contains an intact protease domain that is absent from the Δ PKCAMP mutant. It is thus tempting to speculate that the lower bands correspond to the proteolytically processed form and that the protease activity of MDC9 is involved in the processing. In addition to the difference in the lower bands, the upper band of Δ PKCBD and the corresponding bands in Δ MP and Δ PKCAMP show doublets.

Next we examined the effect of the overexpression of these mutants on the ectodomain shedding of proHB-EGF in Vero-H cells. As shown in Figure 4C, none of the mutants examined, except Δ PKCBD, has any significant effect on ectodomain shedding in the absence of TPA, in clear contrast to the results for wild-type MDC9 (Figure 4C, a and b) and Δ PKCBD (Figure 4C, c and d). Importantly, the TPA-induced shedding of proHB-EGF is inhibited by the overexpression of Δ MP (Figure 4C, g and h). H347,351A also suppresses TPA-induced shedding in Vero-H cells (Figure 4C, o and p). In order to compare the effect of mutants more quantitatively, the percentage of proHB-EGF-positive cells was determined, and results are summarized in Figure 4D. These results indicate that ectodomain shedding caused by exogenous MDC9 requires the protease activity of MDC9 and that the deletion of the PKC-binding domain does not affect ectodomain shedding. The dominant-negative effects of MDC9 mutants lacking essential sequences at the metalloprotease domain on TPA-induced shedding support the notion that endogenous MDC9 or its relative is involved in the shedding of the proHB-EGF ectodomain. The Δ PKCAMP mutant, which lacks both the MP- and PKC-binding domains, has dominant negative effects very similar to those of the Δ MP and H347,351A mutants (Figure 4C, k and l); the dominant negative effects of Δ MP are not caused by the titration of PKC δ .

Overexpression of the MDC9 cytoplasmic domain (Cyto) also has a dominant-negative effect on the TPA-induced shedding of the proHB-EGF ectodomain (Figure 4C, s and t, and Figure 4D). This effect was not observed for the Cyto Δ PKCBD mutant, which lacks the PKC-

binding domain (Figure 4C w and x, and Figure 4D), suggesting the involvement of PKC-BD in this effect. The simplest explanation is that the PKC-BD titrates the cellular PKC required for TPA-induced ectodomain shedding, consistent with notion that the TPA-induced ectodomain shedding requires endogenous PKC δ .

Another MDC9 mutant, Δ Pro-rich, which lacks the cytoplasmic proline-rich domain, has a moderate effect on ectodomain shedding in the absence of TPA (Figure 4D). Considering the results for Δ PKCBD, which shows effects very similar to wild-type MDC9, the results with Δ Pro suggest the importance of the proline-rich sequence for the shedding of the proHB-EGF ectodomain.

Discussion

Regulated shedding of the membrane ectodomain

Proteolytic processing of the extracellular domain of a variety of membrane proteins seems to be a rather general strategy for regulating the capacity of cells to respond to extracellular stimuli. However, our knowledge of the molecules involved in this process is quite limited. Although there is no apparent sequence similarity in the cleavage sites, the presence of mutant cell lines defective for the shedding of at least two unrelated molecules, β APP and proTGF- α , suggests that ectodomain shedding of these two molecules may share a common component (Arribas and Massague, 1995). Recent findings on the processing of TNF- α and Notch have revealed that members of the ADAM family of proteases are involved in proteolytic processing (Howard *et al.*, 1996; Black *et al.*, 1997; Moss *et al.*, 1997; Pan and Rubin, 1997). However, the mechanism for their regulation remains unknown. Ectodomain shedding is observed constitutively under normal culture conditions, but can often be stimulated by activators of protein kinase C such as TPA. Thus, a PKC-dependent pathway is thought to be involved in ectodomain shedding (Pandiella and Massague, 1991a; Goishi *et al.*, 1995).

In the present study, we provide evidence that the regulated processing of proHB-EGF requires PKC δ and MDC9, a member of the ADAM family of metalloproteases, and that it involves a direct interaction between PKC δ and the cytoplasmic domain of MDC9. Overexpression of the constitutive active mutants of PKC δ , but not those of PKC α or PKC ϵ , cause the shedding of the proHB-EGF ectodomain in the absence of TPA in Vero-H cells overproducing human proHB-EGF. Furthermore, the TPA-induced ectodomain shedding of proHB-EGF is suppressed by the overexpression of a kinase knockout mutant of PKC δ . These results support the notion that PKC δ is specifically involved in the TPA-induced shedding of the proHB-EGF ectodomain in Vero-H cells. To analyze the mechanism further, we searched for PKC δ -binding proteins and found MDC9 as a PKC δ -specific binding protein. PKC δ , but not PKC α or PKC ϵ , interacts with the whole cytoplasmic domain of MDC9 in COS cells. The interaction between PKC δ and MDC9 requires the kinase domain of PKC δ and a 25 amino acid cytoplasmic sequence of MDC9 is sufficient for the direct interaction with PKC δ *in vitro*. Importantly, overexpression of MDC9 results in the shedding of the proHB-EGF ectodomain in Vero-H cells. MDC9-dependent shedding of the proHB-

EGF ectodomain, as judged by the disappearance of the ectodomain epitope or DT-binding activity from the cell surface, parallels the appearance of DT-binding activity and growth-stimulating activity in the culture medium, confirming the occurrence of physiological processing. Furthermore, MDC9 mutants lacking the protease domain, and those with point mutations in the protease domain, not only fail to induce ectodomain shedding but also suppress TPA-induced shedding, supporting the notion that endogenous MDC9 or related members of the ADAM family are involved in the process. These results are consistent with the hypothesis that MDC9 and its protease activity are involved in TPA-induced ectodomain shedding of proHB-EGF in Vero-H cells.

Proteases required for the shedding of the proHB-EGF ectodomain

Earlier studies have suggested that a metalloprotease(s) is involved in the TPA-induced processing of proHB-EGF (Lanzrein *et al.*, 1995). In Vero-H cells, a hydroxamic-acid-based metalloprotease inhibitor, KB-R8301, inhibits the constitutive and TPA-induced processing of proHB-EGF with an 50% inhibitory concentration (IC₅₀) of ~500 nM (data not shown). Since KB-R8301 and other hydroxamic-acid-based metalloprotease inhibitors have IC₅₀ values of ~1–10 nM for most MMPs (Moss *et al.*, 1997; Yamamoto *et al.*, 1998), the major enzyme involved in the ectodomain shedding of proHB-EGF in Vero and Vero-H cells is not a known member of the MMP family. We also observed that tissue inhibitors of metalloprotease-1 (TIMP-1) do not inhibit the ectodomain shedding of proHB-EGF in Vero-H cells even at concentrations of 10 µg/ml (data not shown), supporting the notion that MMP members are not involved in the process.

In the present study, we show that MDC9 is involved in the TPA-induced shedding of the proHB-EGF ectodomain. Furthermore, mutants with deletions in the metalloprotease domain and point mutations in the zinc-binding motif strongly suggest the requirement of protease activity for the processing of proHB-EGF. However, we have not succeeded in demonstrating the actual proteolytic activity of MDC9 under cell-free conditions in which the soluble form of MDC9 secreted from COS cells is incubated with either solubilized proHB-EGF or with a synthetic peptide containing the juxtamembrane sequence of proHB-EGF. Thus, it remains unclear whether MDC9 cleaves proHB-EGF directly. As shown in blood-clotting systems or in the caspase family system in programmed cell death, related proteases often constitute a protease-cascade reaction. It is, therefore, conceivable that ectodomain shedding is also regulated by such a protease-cascade reaction. If this is the case, the direct interaction with PKCδ supports the notion that MDC9 might be the initial protease in TPA-induced ectodomain shedding of proHB-EGF.

It will be important to determine the cleavage site of HB-EGF, which is released from cells that overexpress MDC9. The cleavage site of proHB-EGF for ectodomain shedding *in vivo* is still uncertain. Previous results, shown by amino-acid-composition analysis of C-terminal chymotryptic peptide of 19 kDa HB-EGF released by TPA treatment of Vero-H cells, suggested that the cleavage site would be at Pro149–Val150 (Goishi *et al.*, 1995). However, it is also possible that after cleavage at C-terminal position

by primary enzyme, additional enzymes may further process secreted HB-EGF by removing Val150 as discussed previously (Suzuki *et al.*, 1997). In any case, a comparison of cleavage sites of HB-EGF released from nontransfected and MDC9-transfected cells will be required for further corroboration of a role of MDC9 in HB-EGF shedding.

Using an *in vitro* assay involving a fusion protein in which alkaline phosphatase (AP) replaced the transmembrane and cytoplasmic domains of HB-EGF, Suzuki *et al.* (1997) reported that MMP-3 is capable of cleaving proHB-EGF at juxtamembrane domains. A more recent report involving an intact cell system suggested the presence of an ionomycin-stimulated pathway for the shedding of the proHB-EGF ectodomain, and that this pathway is not sensitive to PKC inhibitors or to prolonged treatment with TPA, suggesting that this pathway operates independently of the pathway involving PKC (Dethlefsen *et al.*, 1998). Multiple distinct pathways would exist for the regulation of ectodomain shedding of proHB-EGF, as has been suggested in the case of TGF-α (Pandiella and Massague, 1991b). The relationship between these findings and ours remains to be clarified in future experiments.

Regulation of inside-out signaling through PKCδ and MDC9

The sequence and domain structure of MDC9 and other ADAM family metalloproteases suggest that they are synthesized as catalytically inactive zymogens. The inactive state could be maintained through a cysteine switch mechanism and the protein converted to the active forms by unknown mechanisms (Loechel *et al.*, 1998). In the case of meltrin-α (ADAM12), cleavage of the prodomain at a site for furin-like endopeptidases results in the conversion to a proteolytically active protein (Loechel *et al.*, 1998). Most ADAMs, including MDC9, also have furin-cleavage sites at the putative boundary between the prodomain and the metalloprotease domain. Thus, it is possible that TPA treatment may induce the cleavage of MDC9 at the furin site to generate the active form. Furin or a furin-like enzyme has been suggested to be involved not only in N-terminal processing of human proHB-EGF (Nakagawa *et al.*, 1996), but also in the cleavage and activation of proHB-EGF-bound diphtheria toxin (Tsuneoka *et al.*, 1993). However, we think it unlikely that furin is involved in the TPA-induced activation of MDC9 for the following reason. When MDC9 proteins, endogenous or overexpressed, were analyzed by SDS-PAGE and Western blotting, two bands with estimated sizes of 120 kDa and 84 kDa were seen as shown in Figure 3B, and these probably represent the proform and processed form, respectively. TPA treatment does not significantly change the pattern of MDC9 processing (data not shown). Thus, the PKC-dependent activation of MDC9 seems to involve an as yet unidentified mechanism.

The dominant negative effect of Cyto, but not CytoΔPKCBD (Figure 4C and D), suggests that PKCBD is involved in TPA-induced shedding. One explanation is that the endogenous PKCδ required for this process is titrated out by the overexpression of Cyto, supporting our hypothesis for the requirement of PKCδ for PKC-induced shedding. The dominant-negative effects of the MDC9 mutant ΔPKCBDAMP, which lacks both PKCBD and the

metalloprotease domain, suggest the involvement of an additional MDC9 sequence, other than PKCBD, in TPA-induced shedding. Since Cyto Δ PKCBD shows no such effect, some sequence in the extracellular domain must be involved. Interestingly, proHB-EGF forms a complex with integrin $\alpha 3\beta 1$ (Nakamura *et al.*, 1995). Therefore, although the role of the disintegrin domain has not been clarified in processing by ADAM family metalloproteases, it is tempting to speculate that the disintegrin domain of MDC9 plays some role, such as the recognition or binding to proHB-EGF, in TPA-induced shedding. Clarification of the sequence in the extracellular domain of MDC9 required for the dominant negative effect will provide further insight into the mechanism of TPA-induced shedding.

One plausible role for PKC δ in the triggering of proHB-EGF shedding is the recruitment of MDC9 to proHB-EGF. If these proteins localize at different membrane domains under steady-state conditions, the movement of MDC9 to a site that allows interaction with other proteases or with proHB-EGF itself might be essential for shedding. The binding and subsequent phosphorylation of the cytoplasmic domain of MDC9 by PKC δ may trigger this spatial change in MDC9. This model may be able to explain why the overexpression of wild-type MDC9, and even Δ PKCBD, results in shedding in the absence of TPA. Under overexpression conditions, MDC9 and its mutants may be missorted or mislocalized in the cells. The mislocalized MDC9 may act in the processing of the proHB-EGF ectodomain without PKC activation. With regard to the localization and movement of MDC9, the effect of the overexpression of MDC9- Δ Pro-rich is interesting in that it has a moderate effect on ectodomain shedding in the absence of TPA, suggesting the involvement of proline-rich domains. The cytoplasmic tail of MDC9 contains two proline-rich sequences that can bind to the SH3 domains of Src, and may function as a SH3 ligand domain (Weskamp *et al.*, 1996). The identification of molecules that bind to the proline-rich domain of MDC9 might be important.

In conclusion, PKC δ and MDC9, which binds PKC δ at the cytoplasmic domain, are involved in the TPA-induced processing of the proHB-EGF ectodomain. A number of protein ectodomains are known to be processed in response to TPA treatment, suggesting the possible involvement of these proteins in the processing of membrane ectodomains in a variety of biological systems.

Materials and methods

Materials

12-*O*-Tetradecanoylphorbol 13-acetate (TPA) was purchased from Nacalai Tesque Co., Ltd (Kyoto, Japan). KB-R8301 was obtained from Kanebou Co., Ltd (Japan). CRM197, a non-toxic mutant of diphtheria toxin, was prepared as described previously (Uchida *et al.*, 1973).

Plasmids

PKC expression vectors encoding PKC α (YK504), PKC δ (M241), PKC ϵ (YK529), DR144/145A (constitutive active mutant of PKC δ) and DRKA (dominant negative mutant of PKC δ) have been described previously (Hirai *et al.*, 1994; Ohno *et al.*, 1994). Tag- α R22A/A25E (SRHis- α R22A/A25E), tag-DR144/145A (SRHis-DR144/145A) and tag-eK155A/R156A/A159E (SRHis-eK155A/R156A/A159E) encode constitutive active mutants of PKC α , PKC δ and PKC ϵ , respectively (Ueda *et al.*, 1996), which fuse downstream of the six histidine residues and a 12 amino acid sequence from the T7 gene 10 leader sequence. AD335

or DA318 are chimeric mutants that fuse rabbit PKC α (amino acids 1–318) and mouse PKC δ (amino acids 335–674) or mouse PKC δ (amino acids 1–335) and rabbit PKC α (amino acids 318–672), respectively.

Antibodies

Rabbit anti-human proHB-EGF antibody #H6 was raised against a synthetic peptide corresponding to amino acids 54–73 of proHB-EGF (Iwamoto *et al.*, 1994). Goat anti-HB-EGF neutralizing antibody was purchased from R & D Systems. Monoclonal antibodies (mAb) of anti-T7 tag antibody were purchased from Novagen. The monoclonal antibodies (mAb) of anti-PKC α (P16520), anti-PKC δ , (P36520) and PKC ϵ (P14820) were purchased from Transduction Laboratories (TDL). The polyclonal antibody of anti-PKC δ (δ 5) was raised against a synthetic peptide corresponding to amino acids 656–673 of the rat PKC δ (Mizuno *et al.*, 1991), however, mAb of PKC δ (P36520) was raised against N-terminal regulatory region of human PKC δ . Rabbit anti-MDC9 antibody was raised against a synthetic peptide corresponding to amino acids 795–809 of the mouse MDC9. HRP-conjugated sheep anti-rabbit IgG and anti-mouse IgG were purchased from Amersham.

Isolation of a cDNA clone encoding MDC9/meltrin

Mouse PKC δ (400 ng) purified from recombinant baculovirus-infected Sf21 cells was labeled by autophosphorylation with 32 P in 155 μ l of kinase buffer containing 20 mM Tris-HCl, 5 mM MgCl $_2$, 10% glycerol, 0.6 mCi/ml [γ - 32 P]ATP and 40 μ g/ml phosphatidylserine pH 7.5, at 30°C for 2 h. After the phosphorylation reaction, [32 P]-labeled PKC δ was separated from unreacted ATP by gel filtration and used as a probe (10⁶ c.p.m./ml) to screen an NIH 3T3- λ EXlox cDNA expression library by an overlay method as described (Izumi *et al.*, 1997). The initial isolate (clone G1) encoded amino acid residues 719–845 of mouse MDC9. A cDNA fragment that was initially identified as an RT-PCR product encoding a disintegrin domain of MDC9/meltrin (Yagami-Hiromasa *et al.*, 1995) was used as a probe to isolate its full-length cDNAs. They were screened in a cDNA library of a mouse myoblast cell line, C2, made in ZAP II (Stratagene).

Overlay assay using [32 P]-labeled PKC δ as a probe

E. coli lysates containing PKC δ binding proteins were subjected to SDS-PAGE and blotted onto a PVDF membrane. After treatment with a 5% skim milk solution, the PVDF membrane was incubated with [32 P]-labeled PKC (10⁶ c.p.m./ml), diluted in 50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 50 μ g/ml phosphatidylserine and 1% bovine serum albumin (BSA) at room temperature for 5 h. Excess ligand was removed by washing the PVDF membrane with Tris-buffered saline (TBS) and the membrane was subjected to autoradiography.

In vitro phosphorylation of GST-MDC9 proteins by PKC δ

GST-MDC9 proteins were purified on Glutathione-Sephrose 4B (Pharmacia). The reaction mixture contained 20 mM Tris-HCl pH 7.5, 5 mM Mg(OAc) $_2$, 10 μ g/ml leupeptin, 50 ng/ml TPA, 25 μ g/ml phosphatidylserine, 3.74 ng PKC δ and 50 nM of GST-MDC9 proteins in a total volume of 20 μ l. The reaction was started by the addition of 20 μ M ATP and 0.5 mCi [γ - 32 P]ATP and the mixture was incubated at 30°C. After 5 min of incubation, the reaction was stopped by the addition of 5 μ l of 5 $\times$ Laemmli's SDS-sample buffer. Proteins were then separated on SDS-PAGE and subjected to autoradiography.

Immunoprecipitation

COS cells in 10 cm dishes were suspended in 200 μ l of lysis buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM Na $_3$ VO $_4$, 10 μ g/ml leupeptin, 1 mM PMSF, 1.8 μ g/ml aprotinin and 1% Triton X-100. After 30 min incubation on ice, the lysates were clarified by centrifugation at 14 000 r.p.m. for 30 min and incubated with antibodies preabsorbed on Protein G-Sephrose (Pharmacia) for 1 h at 4 °C. The immunocomplexes on Sepharose were washed six times with lysis buffer, after which the proteins were separated by SDS-PAGE, transferred to PVDF membranes and probed with the indicated antibodies.

Construction of MDC9 mutants

An *E. coli* expression vector for GST-MDC9 was constructed using the expression vector pGEX-3X (Pharmacia) and cDNA encoding the MDC9 cytoplasmic region. Deletion mutants of the MDC9 cytoplasmic region were constructed by PCR using clone G1 as a template. The mammalian expression vector for MDC9 was constructed using expression vector pEF-BOS (Mizushima and Nagata, 1990) (pEF-BOS-MDC9). MDC9 Δ PKCBD (pEF-BOS-MDC9 Δ PKCBD), Δ Pro-rich (pEF-BOS-

MDC9 Δ Pro-rich), Δ MP (pEF-BOS-MDC9 Δ MP) and Δ PKCBD Δ MP (pEF-BOS-MDC9 Δ PKCBD Δ MP) were constructed by the deletion of amino acid residues 719–743, 811–845, 256–394, 256–394 and 719–743, respectively. MDC9 H347,351A (pEF-BOS-MDC9 H347,351A) has alanines substituted for the conserved histidines at positions 347 and 351. A *Pst*I–*Hinc*II fragment of MDC9 cDNA was cloned into pBlueScript, and mutations for two histidines were generated by site-directed mutagenesis (Quikchange Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA) using the primers 5'-CCATTGTGCTGCTG-AATTGGGGGCTAACCTTGAATG-3' and 5'-CATTCCAAGGTTA-GCCCCAATTCAGCAGCAACAATGG-3'.

Finally the mutated *Pst*I–*Hinc*II fragment was ligated into the corresponding sites of pEF-BOS-MDC9. MDC9cyto (SRHis–MDC9cyto) was constructed using clone G1 (amino acids 719–845 of mouse MDC9) ligated into SRHis vector (Ueda *et al.*, 1996). MDC9 cyto Δ PKCBD (SRHis–MDC9cyto Δ PKCBD) was constructed using SRHis–MDC9cyto, which encodes amino acid residues 744–845 of mouse MDC9.

Cell culture and transfection

Vero, Vero-H and Vero-MDC9 cells were maintained in modified Eagle's medium (MEM) with nonessential amino acids (MEM-NEAA) supplemented with heat-inactivated 10% fetal calf serum (FCS). Vero-H cells are a stable transformant of Vero cells that overexpress human proHB-EGF (Goishi *et al.*, 1995). Vero-MDC9 cells were isolated transfecting mouse MDC9 cDNA into Vero cells. COS-7 cells from monkey kidney were cultured in Dulbecco's MEM (DMEM) supplemented with 10% FCS. DER cells, 32D cells that stably express EGF receptor, were grown in RPMI 1640 medium containing 10% FCS and 5% WEHI-3 cell-conditioned medium as a source of IL-3. Transfection was carried out with plasmids by electroporation (Bio-Rad, Gene Pulser) according to the manufacturer's instructions.

Western blot analysis

For sample preparation, the cell in culture dishes were directly solubilized by using Laemmli sample buffer (Figures 1C, 3B and 4B) (Laemmli, 1970). Western blot analysis was performed by one-dimensional electrophoresis followed by electrophoretic transfer to PVDF membranes, which were then incubated with various antibodies. Antibodies were detected by chemiluminescence ECL (Amersham).

Immunofluorescence microscopy

Cells plated on coverslips were incubated with 5 μ g/ml of rabbit anti-HB-EGF antibody #H6 in skim milk solution (20 mM Tris–HCl pH 7.2, 0.2 mM CaCl₂, 0.2 mM MgCl₂, 150 mM NaCl and 5% skim milk) for 2 h on ice, washed three times with PBS(+) and fixed with 3.7% formaldehyde in PBS(–) for 1 h on ice. Cells were washed twice with PBS(–), incubated with 0.1 M glycine/50 mM Tris–HCl pH 8.0, for 1 h at 4°C, and overnight with skim milk solution at 4°C. ProHB-EGF on the cell surface was detected by incubating with Cy3-conjugated goat anti-rabbit IgG (Amersham) for 1 h at 4°C and washing three times with PBS(–). In order to detect the expression of PKC isotypes, MDC9 or derived mutants, cells were permeabilized with 0.1% Triton X-100 at 4°C for 5 min and incubated with an appropriate antibody for 1 h at 4°C. After washing three times with TBST (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20), the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (EY Laboratories) for 1 h at room temperature. The cells were washed three times with TBST and observed under a fluorescence microscope. Images were captured with a Princeton Instruments digital camera and manipulated with Adobe Photoshop software.

The percentage of proHB-EGF-positive cells was determined by counting the number of proHB-EGF-positive cells (red fluorescence) among cells concomitantly expressing products of transfected cDNA (green fluorescence). The values were determined based on the results obtained in at least two independent transfections and at least 100 independent cells positive for marker proteins were examined in each experiments for the clear reduction (not complete disappearance) of the staining of proHB-EGF ectodomain. The scoring was performed in a completely blind manner.

DT-binding assay

The binding of ¹²⁵I-labeled DT to cells was measured as described previously (Mekada and Uchida, 1985). Typically, cells transfected with plasmids were cultured with or without 64 nM TPA in binding medium (MEM supplemented with 10% FCS and 20 mM HEPES–NaOH pH 7.2) for 30 min at 37°C. The medium was then replaced with fresh cold binding medium containing 10 μ g/ml heparin and the cells were incubated

with 100 ng/ml ¹²⁵I-labeled DT (10⁷ c.p.m./ μ g) for 8 h at 4°C. The cells were washed and the cell-associated radioactivity was measured. Nonspecific binding of ¹²⁵I-labeled DT was assessed in the presence of a 100-fold excess of unlabeled DT. Specific binding was determined by subtracting the nonspecific binding from the total binding obtained with ¹²⁵I-labeled DT alone. Nonspecific binding accounted for <10% of total binding.

To determine the amount of soluble HB-EGF, conditioned medium was harvested, centrifuged to remove cell debris and incubated with 50 μ l of heparin–Sephacrose CL-6B (Pharmacia LKB Biotechnology, Sweden) for 2 h at 4°C. After washing twice with PBS(+) containing 1mg/ml BSA, the gels were suspended in washing solution and incubated with 100 ng/ml ¹²⁵I-labeled DT for 6 h at 4°C with gentle rotation. The gels were washed and the radioactivity associated with the gels was counted. Specific binding was determined as described above.

Measurement of mitogenic activity

The mitogenic activity of secreted HB-EGF was measured by co-culture assay as follows. Vero-H cells were transfected with control plasmids pEF-BOS or plasmids encoding MDC9 and then seeded into 24-well plates at a density of 5 $\times$ 10⁴ cells/well. After incubation for 24 h at 37°C, the medium was replaced with fresh RPMI 1640 medium supplemented with 10% serum. Then a Transwell chamber (Millipore Corporation, Bedford, MA) was set in each well and DER cells were inoculated into the inside of the Transwell chambers (1 $\times$ 10⁴ cells/well). Cells were cultured for 24 h at 37°C and then incubated with [³H]thymidine (2 μ Ci/ml) for 4 h. The DER cells were then harvested and the radioactivity incorporated into DNA was determined as described previously (Higashiyama *et al.*, 1995). To determine the mitogenic activity attributable to growth factors other than HB-EGF, the rate of DNA synthesis was also determined in the presence of CRM197 (Mitamura *et al.*, 1995). The mitogenic activity derived from HB-EGF was calculated by subtracting the radioactivity obtained in the presence of CRM197 from that obtained without CRM197.

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