Nardilysin Enhances Ectodomain Shedding of Heparin-binding Epidermal Growth Factor-like Growth Factor through Activation of Tumor Necrosis Factor-α-converting Enzyme*

Received for publication, February 10, 2006, and in revised form, August 4, 2006 Published, JBC Papers in Press, August 21, 2006, DOI 10.1074/jbc.M601316200

Eiichiro Nishi†1, Yoshinori Hiraoka§, Kazuhiro Yoshida‡, Katsuya Okawa‡, and Toru Kita§

From the †Molecular Pathology Unit, Horizontal Medical Research Organization, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan

‡1 The abbreviations used are: HB-EGF, heparin-binding epidermal growth factor-like growth factor (HB-EGF) is synthesized as a transmembrane protein that can be shed enzymatically to release a soluble growth factor. Ectodomain shedding is essential to the biological functions of HB-EGF and is strictly regulated. However, the mechanism that induces shedding remains unclear (1–4). Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is synthesized as a transmembrane protein (pro-HB-EGF) that can be shed proteolytically to release a soluble growth factor (5–8). Metalloproteinases have been implicated as sheddases of pro-HB-EGF because various metalloproteinase inhibitors inhibit HB-EGF ectodomain shedding efficiently. MMP3 (matrix metalloproteinase-3), MMP7, ADAM9 (a disintegrin and metalloproteinase-9), ADAM10, ADAM12, and ADAM17 (tumor necrosis factor-α-converting enzyme (TACE)) have all been suggested as the proteases responsible (9–14). Ectodomain shedding of pro-HB-EGF is induced by various stimuli, including phorbol esters, calcium ionophore, and lyso-phosphatidic acid (6, 15, 16). With respect to the mechanism of the induction, however, very little is known.

Ectodomain shedding of HB-EGF is indispensable for G-protein-coupled receptor-induced epidermal growth factor receptor (EGFR) transactivation, which plays critical roles in biological consequences of G-protein-coupled receptor activation (17). The physiological significance of HB-EGF ectodomain shedding was further underscored by the findings that knock-in mice harboring an uncleavable mutant construct of HB-EGF show phenotypes very similar to those of HB-EGF knock-out mice (e.g. hypertrophied cardiac valve and dilated heart) and that knock-in mice with the soluble mutant have even more severe phenotypes (18–20). Notably, a similarly defective valvulogenesis was observed in both EGFR- and TACE-deficient mice, suggesting that HB-EGF-induced EGFR activation and TACE-induced HB-EGF shedding are required for valvulogenesis (19, 21). In other words, ectodomain shedding of pro-HB-EGF is required for the activation of EGFR by HB-EGF. The same conclusion was obtained in a study that showed that most of the biological effects of EGFR ligands in cell culture are blocked by metalloprotease inhibitors (22).

Like other members of the epidermal growth factor family, heparin-binding epidermal growth factor-like growth factor (HB-EGF) is synthesized as a transmembrane protein that can be shed enzymatically to release a soluble growth factor. Ectodomain shedding is essential to the biological functions of HB-EGF and is strictly regulated. However, the mechanism that induces shedding remains unclear. We have recently identified nardilysin (N-arginine dibasic convertase (NRDc)), a metalloendoprotease of the M16 family, as a protein that specifically binds HB-EGF (Nishi, E., Prat, A., Hospital, V., Elenius, K., and Klagsbrun, M. (2001) EMBO J. 20, 3342–3350). Here, we show that NRDc enhances ectodomain shedding of HB-EGF. When expressed in cells, NRDc enhanced the shedding in cooperation with tumor necrosis factor-α-converting enzyme (TACE; ADAM17). NRDc formed a complex with TACE, a process promoted by phorbol esters, general activators of ectodomain shedding. NRDc enhanced TACE-induced HB-EGF cleavage in a peptide cleavage assay, indicating that the interaction with NRDc potentiates the catalytic activity of TACE. The metalloendoprotease activity of NRDc was not required for the enhancement of HB-EGF shedding. Notably, a reduction in the expression of NRDc caused by RNA interference was accompanied by a decrease in ectodomain shedding of HB-EGF. These results indicate the essential role of NRDc in HB-EGF ectodomain shedding and reveal how the shedding is regulated by the modulation of sheddase activity.

Ectodomain shedding is an irreversible post-translational modification that releases the extracellular domain of membrane-anchored proteins through proteolysis. A broad spectrum of membrane proteins are susceptible to ectodomain shedding. Ectodomain shedding of most proteins occurs constitutively in resting cells, but can be rapidly and markedly induced by cell activation. However, the mechanism that induces shedding remains unclear (1–4). Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is synthesized as a transmembrane protein (pro-HB-EGF) that can be shed proteolytically to release a soluble growth factor (5–8). Metalloproteinases have been implicated as sheddases of pro-HB-EGF because various metalloproteinase inhibitors inhibit HB-EGF ectodomain shedding efficiently. MMP3 (matrix metalloproteinase-3), MMP7, ADAM9 (a disintegrin and metalloproteinase-9), ADAM10, ADAM12, and ADAM17 (tumor necrosis factor-α-converting enzyme (TACE)) have all been suggested as the proteases responsible (9–14). Ectodomain shedding of pro-HB-EGF is induced by various stimuli, including phorbol esters, calcium ionophore, and lysophosphatidic acid (6, 15, 16). With respect to the mechanism of the induction, however, very little is known.

Ectodomain shedding of HB-EGF is indispensable for G-protein-coupled receptor-induced epidermal growth factor receptor (EGFR) transactivation, which plays critical roles in biological consequences of G-protein-coupled receptor activation (17). The physiological significance of HB-EGF ectodomain shedding was further underscored by the findings that knock-in mice harboring an uncleavable mutant construct of HB-EGF show phenotypes very similar to those of HB-EGF knock-out mice (e.g. hypertrophied cardiac valve and dilated heart) and that knock-in mice with the soluble mutant have even more severe phenotypes (18–20). Notably, a similarly defective valvulogenesis was observed in both EGFR- and TACE-deficient mice, suggesting that HB-EGF-induced EGFR activation and TACE-induced HB-EGF shedding are required for valvulogenesis (19, 21). In other words, ectodomain shedding of pro-HB-EGF is required for the activation of EGFR by HB-EGF. The same conclusion was obtained in a study that showed that most of the biological effects of EGFR ligands in cell culture are blocked by metalloprotease inhibitors (22).
Nardiylisin Enhances HB-EGF Shedding through TACE

We have previously shown that nardiylisin (N-arginine dibasic convertase (NRDc)) binds specifically to HB-EGF among EGF family members (23). NRDc was originally identified as a dibasic selective metalloendopeptidase of the M16 family (24, 25). Expression of the enzyme is widespread and especially high in testis, heart, and skeletal muscle (26). NRDc is expressed mainly in the cytoplasm, but interestingly, it is also exported out of cells and distributed on the cell surface, although it has no apparent signal peptide sequence (27–29). While examining the biological significance of the binding of NRDc to pro-HB-EGF, we found that NRDc enhances ectodomain shedding of the growth factor. Here, we demonstrate that a metalloendopeptidase, NRDc, potentiates the catalytic activity of TACE and enhances ectodomain shedding of HB-EGF.

EXPERIMENTAL PROCEDURES

Plasmids—cDNA encoding human NRDc (Met50–Lys1150, completely matching GenBank™ accession number BC008775) was cloned into pcDNA3.1/V5-His (Invitrogen) to generate pcDNA3.1-hNRDc-V5. The cDNA of human NRDc C-terminally tagged with FLAG was cloned into pcDNA3 and pFastBac1 (Invitrogen) to generate pcDNA3-hNRDc-FLAG and pFastBac1-hNRDc-FLAG, respectively. A cDNA encoding an enzymatically inactive mutant of human NRDc was obtained by substituting the Glu235 codon (GAG) with an Ala codon (GCG) using the PCR technique. The full-length human TACE cDNA was cloned into pME18S to generate pME18S-TACE. The expression plasmid for hemagglutinin (HA)-tagged HB-EGF was described previously (30). The expression plasmid for alkaline phosphatase (AP)-tagged HB-EGF (31) was a gift from S. Higashiyama.

Small Interfering RNAs (siRNAs)—The sequences for siRNA duplexes were as follows: NRDc-1, 5′-GGGUAGUGAAUAUGCCUACACGUAU-3′; NRDc-2, 5′-GACUAUUGACCGUGUUAUCCAGC3′; Control-2, 5′-ACGUAACGGUGGAGAAA-3′. NRDc-1 and NRDc-2 are Stealth™ siRNA duplex oligoribonucleotides synthesized by Invitrogen. Control-2 is a control (nonsilencing) siRNA purchased from Qiagen Inc. Control-1 is a Stealth RNAi negative control duplex with medium GC content purchased from Invitrogen, the sequence of which is not available to the public.

Antibodies and Reagents—The antibodies were from the following sources: anti-HA tag antibody HA11, Covance; anti-V5 tag antibody, Invitrogen; anti-FLAG tag antibody M2, Sigma; anti-TACE antibodies (C-15 and H-300), Santa Cruz Biotechnology, Inc.; anti-TACE antibody (clone 111633), R&D Systems; anti-HB-EGF antibody, Calbiochem; and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody, Research Diagnostics, Inc. Anti-TACE antibody 41 (used for immunoprecipitation) was a gift from M. E. Milla. Mouse anti-NRDc monoclonal antibody 23 was raised against recombinant human NRDc in our laboratory. Recombinant NRDc was synthesized using the Bac-to-Bac baculovirus expression system (Invitrogen). Briefly, pFastBac1-hNRDc-FLAG was introduced into DH10Bac competent cells to isolate recombinant bacmid DNA. Sf9 cells were transfected with the bacmid DNA, and the recombinant baculovirus-containing supernatant was harvested. Sf9 cells inoculated with the recombinant virus were lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 μg/ml leupeptin, 0.2 mg/ml Pefabloc SCPLUS (Roche Applied Science), and 1% Nonidet P-40, and the total cell lysate was loaded onto an anti-FLAG antibody M2 affinity gel column (Sigma) and eluted with FLAG peptide (100 μg/ml). Recombinant TACE was purchased from R&D Systems.

Cells and Transfections—COS-7 and 293T cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. MKN45 cells were grown in RPMI 1640 medium containing 10% fetal calf serum. Transfections were carried out using FuGENE 6 (Roche Applied Science) for plasmids and siFECTOR (B-Bridge International Inc.) for siRNA according to the manufacturers’ instructions. CHO-K1 cells stably expressing HA-HB-EGF (CHO-K1-HA-HB-EGF cells) and AP-HB-EGF (CHO-K1-AP-HB-EGF cells) were selected in nutrient mixture F-12 containing 10% fetal calf serum and 500 mg/ml G418, respectively. Clones of CHO-K1-HA-HB-EGF cells were tested for HA-HB-EGF expression by Western blotting with anti-HA antibody. CHO-K1-AP-HB-EGF cells were sorted by the expression of AP-HB-EGF (using anti-AP antibody) using a FACSAria flow cytometer (BD Biosciences).

Western Blot Analysis—Cells were lysed in lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, and protease inhibitor mixture (Roche Applied Science). Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose filters. After blocking with 5% nonfat milk in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.05% Tween 20, filters were incubated with primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies. The immobilized peroxidase activity was detected with the enhanced chemiluminescence system (ECL, Amersham Biosciences).

Immunoprecipitation—Cells lysates were incubated with the appropriate antibodies, and immune complexes were collected with protein G-Sepharose beads.

Pulldown Assay—Recombinant NRDc (FLAG-tagged at the C terminus; 11 μg/ml), TACE (10 μg/ml), and HB-EGF (2.8 μg/ml) were mixed in 50 mM Tris-HCl (pH 8.9), 1 mM CaCl₂, and 15 mM ZnCl₂. The mixture was incubated with anti-FLAG antibody-conjugated beads for 4 h, followed by extensive washes, and the precipitates were subjected to SDS-PAGE and blotted with anti-TACE antibody (clone 111633).

Peptide Cleavage and Molecular Mass Analysis—The fluorescence-quenching peptide substrate corresponding to the cleavage site of HB-EGF (2-(N-methylamino)benzoyl-LPVENRLKYK(2,4-dinitrophenyl)d-Arg-amide) was synthesized by Peptide Institute, Inc. (Osaka, Japan). Peptide substrate for the HB-EGF cleavage site (GLSLPVENLRTYD) used for molecular mass analysis was synthesized by Hokkaido System Science Co., Ltd. (Hokkaido, Japan). In vitro peptide cleavage assays were performed in 25 mM Tris-HCl (pH 9.0) and 2.5 μM ZnCl₂. Molecular mass analyses of peptides were performed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) using an Ultraflex TOF/TOF system (Bruker Daltonics Inc.).

Immunocytochemistry—Cells were fixed with 50% acetone and 50% methanol. Nonspecific staining was blocked with blocking solution (5% donkey and 1% sheep sera in phosphate-
buffered saline. The cells were incubated overnight at 4 °C with a proper dilution of primary antibodies in blocking solution, followed by the respective secondary antibodies for 1 h at room temperature. The stained cells were observed with a Zeiss LSM 510 META laser scanning confocal microscope or a Zeiss Axioskop 2 fluorescence microscope. Collected data were exported as eight-bit TIFF files and processed using ImageJ Version 1.30 (available at rsb.info.nih.gov/ij/).

RESULTS
NRDc Induces Ectodomain Shedding of HB-EGF—To define the biological significance of the binding of NRDc to pro-HB-EGF, we examined the effects of NRDc on HB-EGF ectodomain shedding. To facilitate detection of shed soluble HB-EGF in the conditioned medium, two N-terminally tagged pro-HB-EGFs, HA-HB-EGF (30) and AP-HB-EGF (31), were used. Recombinant NRDc tagged with FLAG at the C terminus was produced in a baculovirus-insect cell system and purified by immunoaffinity chromatography with anti-FLAG antibody. Coomassie Blue staining of eluted fractions showed very high purity of the recombinant NRDc (Fig. 1A), and the enzymatic activity of NRDc was confirmed by conducting a cleavage assay with a fluorescence-quenching peptide substrate (2-((N-methylamino)benzoyl)-GGFLRRVGK(2,4-dinitrophenyl)-amide; fluorescence intensity at 340/430 nm of 38,740 arbitrary units at 177 μM recombinant NRDc and of 1581 arbitrary units at the same concentration of the enzymatically inactive mutant of NRDc). Treatment of CHO-K1-HA-HB-EGF cells with recombinant

FIGURE 1. Recombinant NRDc induces ectodomain shedding of HB-EGF. A, recombinant NRDc was synthesized as described under “Experimental Procedures.” The total cell lysate of Sf9 cells inoculated with a recombinant baculovirus (starting material (ST)) was loaded onto an anti-FLAG antibody-conjugated gel column and eluted with FLAG peptide. The starting material, the flow-through (FT), and three fractions of the eluate (1, 2, and 3) were separated by SDS-PAGE and subjected to either Coomassie Blue staining (upper panel) or Western blotting with anti-FLAG antibody (lower panel). B, CHO-K1-HA-HB-EGF cells were incubated with the indicated concentrations of recombinant NRDc or 100 nM PMA for 4 h. The soluble HB-EGF released in the conditioned medium (CM) was partially purified with heparin-Sepharose beads and separated by SDS-PAGE, followed by Western blotting with anti-HA antibody. Molecular masses are indicated to the left. Comparable data were obtained in three independent experiments, and a representative gel is shown. AU, arbitrary units.

Nardilysin Enhances HB-EGF Shedding through TACE

FIGURE 2. Expression of NRDc induces ectodomain shedding of HB-EGF, and NRDc enzymatic activity is not required for the induction. A, an expression vector encoding AP-HB-EGF was cotransfected into COS-7 cells with a control vector, an expression vector for wild-type NRDc-V5 (WT), or an enzymatically inactive mutant of NRDc-V5 (E235A (E > A)). The culture medium was changed to Dulbecco’s modified Eagle’s medium with 0.1% bovine serum albumin 20 h post-transfection. The AP activity in the conditioned medium was measured after an additional 4 h of incubation. Data represent the means ± S.E. for five independent experiments. *, p < 0.04 (Student’s t test). AU, arbitrary units. B, total cell lysates of transfected cells were collected, and NRDc expression was analyzed by Western blotting with anti-V5 antibody.

NRDc Induces Ectodomain Shedding of HB-EGF—To define the biological significance of the binding of NRDc to pro-HB-EGF, we examined the effects of NRDc on HB-EGF ectodomain shedding. To facilitate detection of shed soluble HB-EGF in the conditioned medium, two N-terminally tagged pro-HB-EGFs, HA-HB-EGF (30) and AP-HB-EGF (31), were used. Recombinant NRDc tagged with FLAG at the C terminus was produced in a baculovirus-insect cell system and purified by immunoaffinity chromatography with anti-FLAG antibody. Coomassie Blue staining of eluted fractions showed very high purity of the recombinant NRDc (Fig. 1A), and the enzymatic activity of NRDc was confirmed by conducting a cleavage assay with a fluorescence-quenching peptide substrate (2-((N-methylamino)benzoyl)-GGFLRRVGK(2,4-dinitrophenyl)-amide; fluorescence intensity at 340/430 nm of 38,740 arbitrary units at 177 μM recombinant NRDc and of 1581 arbitrary units at the same concentration of the enzymatically inactive mutant of NRDc). Treatment of CHO-K1-HA-HB-EGF cells with recombinant
NRDc induced ectodomain shedding of HB-EGF in a dose-dependent manner (Fig. 1B). Recombinant NRDc also enhanced HB-EGF shedding in CHO-K1-AP-HB-EGF cells in a similar manner (Fig. 1C). Interestingly, NRDc induced HB-EGF shedding more rapidly compared with phorbol 12-myristate 13-acetate (PMA) in the early time course.

Although NRDc is found in conditioned media, it is found primarily in the cytosol and on the cell surface (27, 29). Thus, we examined the effect of coexpression of NRDc and AP-HB-EGF on HB-EGF shedding. Cells expressing NRDc released significantly more AP activity in the conditioned medium compared with cells transfected with an empty vector (Fig. 2A). The enzymatically inactive mutant of NRDc (E235A) also enhanced HB-EGF shedding (Fig. 2A), indicating that the metalloendopeptidase activity of NRDc is not required for the enhancement of HB-EGF shedding. Western blot analysis showed a comparable expression level of NRDc and the inactive mutant of NRDc (Fig. 2B). These results indicate that NRDc does not directly cleave HB-EGF, suggesting that it modulates shedding by regulating other pathways of the mechanism.

**NRDc and TACE Cooperate to Enhance Ectodomain Shedding of HB-EGF**—Several lines of evidence indicate that TACE/ADAM17, which was originally identified as a sheddase for tumor necrosis factor-α (32, 33), plays an essential role in ectodomain shedding of HB-EGF. 1) Recombinant TACE cleaves the peptide corresponding to the processing site of pro-HB-EGF (14); 2) PMA-induced HB-EGF shedding is significantly reduced in TACE−/− cells (34); 3) restoration of TACE expression in TACE−/− cells results in a significant increase in HB-EGF shedding (14); and 4) HB-EGF-null mice, knock-in mice with an uncleavable mutant of HB-EGF, and TACE-null mice show a similar heart valve phenotype (18–20). To determine whether NRDc has a cooperative effect on TACE activity, cotransfection experiments were performed in COS-7 cells, and HB-EGF ectodomain shedding was evaluated by Western blotting for HB-EGF released into the conditioned medium. The transfection of only NRDc had a significant but relatively small effect on HB-EGF shedding (2.3-fold increase) (Fig. 3, A, compare the first and second bars; and B, compare the first and second bars), compatible with the result in Fig. 2A. However, coexpression of NRDc and TACE dramatically enhanced HB-EGF ectodomain shedding compared with expression of TACE alone (Fig. 3, A, compare the third and fourth lanes; and B, compare the third and fourth bars). A quantitative analysis of shed HB-EGF in the five independent experiments clearly showed that the effect of the combination was not additive, but was synergistic (2.3-fold increase by NRDc, 4.5-fold increase by TACE, and 11.3-fold increase by NRDc and TACE) (Fig. 3B). We performed the same cotransfection experiments in a different cell line (293T cells) and obtained very similar results (Fig. 3C). The synergistic effect of the enzymatically inactive mutant of NRDc on TACE-induced
NRDc and TACE form a complex, a process enhanced by PMA. A, 293T cells were transfected with combinations of expression vectors for NRDc-V5, TACE, and HA-HB-EGF as indicated. The conditioned medium (CM) and total cell lysates (TCL) were collected as described in the legend to Fig. 3A. Immunoprecipitation (IP) with anti-TACE antibody 41 was done for the total cell lysates, and precipitates were blotted with anti-V5 (NRDc), anti-HA (HB-EGF), and anti-TACE (C-15) antibodies. The soluble HB-EGF released in the conditioned medium was detected as described in the legend to Fig. 1B. Comparable data were obtained in three independent experiments, and a representative gel is shown. B, 293T cells were transiently transfected with expression vectors for NRDc-V5 and TACE, fixed, and stained with anti-TACE (H-300; green) and anti-V5 (red) antibodies. A mixture of Alexa Fluor 594-conjugated donkey anti-mouse IgG and Alexa Fluor 488-conjugated donkey anti-rabbit IgG was used as secondary antibodies. Stained cells were visualized by confocal microscopy. Arrowheads show the co-localization of NRDc and TACE. C, 293T cells transfected with expression vectors for NRDc-V5 and TACE (left panel) or NRDc-FLAG and TACE (right panel) were incubated in the absence or presence of PMA for 2 h. Total cell lysates were collected, and immunoprecipitation with anti-TACE antibody 41 (left panel) or anti-FLAG antibody M2 (right panel) was carried out. The precipitates and total cell lysates were separated by SDS-PAGE and blotted with anti-NRDc antibody 23 or anti-TACE antibody (C-15). A quantitative analysis of the coprecipitated proteins (upper panels) by densitometry is shown (fourth left and right panels). The ratio was arbitrarily set at 1 in the cells treated without PMA (first lane) and shown in the bars. Similar results were obtained in three independent experiments, and a representative result is shown. D, MKN45 cells (a gastric cancer cell line) were incubated in the absence or presence of PMA for 2 h. The total cell lysates were collected and immunoprecipitated with anti-TACE antibody 41, and Western blotting was conducted as described for C. Comparable results were obtained in three independent experiments, and a representative result is shown. E, MKN45 cells were incubated in the absence (left panel) or presence (right panel) of PMA for 2 h, fixed, and stained with anti-NRDc antibody 23. Alexa Fluor 594-conjugated donkey anti-mouse IgG was used as secondary antibody, and the nucleus was subsequently stained with 4′,6-diamidino-2-phenylindole. Stained cells were visualized by fluorescence microscopy.
HB-EGF shedding was also examined in 293T cells. The inactive NRDC mutant showed a similar enhancing effect on HB-EGF shedding compared with wild-type NRDC (Fig. 3, C and D), indicating that the metalloendopeptidase activity of NRDC is not required for the shedding-enhancing effect of NRDC. HB-EGF has multiple forms because of N-terminal heterogeneity and O-glycosylation as reported previously (35) and shows different patterns in different cell lines (Fig. 3, A and C).

NRDC Forms a Complex with TACE—To investigate how NRDC potentiates TACE-induced HB-EGF shedding, the formation of a complex between TACE and NRDC was examined by conducting coprecipitation experiments. Immunoprecipitation of cotransfected cell lysates with anti-TACE antibody confirmed the formation of such a complex (Fig. 4A, third and fourth lanes). When HB-EGF was expressed, anti-TACE antibody also coprecipitated HB-EGF (Fig. 4A, fourth lane), indicating that NRDC, TACE, and HB-EGF form a complex. Immunocytochemical staining of cotransfected cells showed a partial co-localization of TACE and NRDC on the plasma membrane (Fig. 4B), supporting the results of coprecipitation.

PMA Treatment Enhances the Formation of a Complex between NRDC and TACE—Ectodomain shedding of most proteins occurs constitutively in resting cells, but can be dramatically induced by cell activation (1, 36). However, TACE processing and expression levels are not increased by cell activation (32, 37). To examine whether the interaction of NRDC and TACE is involved in the regulation of induced HB-EGF shedding, the effect of PMA, a general activator of ectodomain shedding, on NRDC-TACE complex formation was examined. In 293T cells transfected with NRDC and TACE, immunoprecipitation with anti-TACE antibody demonstrated that PMA stimulation increased the engagement of NRDC with TACE (by 1.6-fold as determined by densitometry), whereas PMA did not affect the total amount of TACE and NRDC (Fig. 4C, left panels). To confirm the effect of PMA through a reciprocal immunoprecipitation, cells were transfected with NRDC-FLAG and TACE, followed by immunoprecipitation with anti-FLAG antibody. In this experimental setting, PMA also enhanced the formation of a complex between NRDC and TACE (by 2.2-fold) (Fig. 4C, right panels). Furthermore, PMA accelerated the process of endogenous proteins in MKN45 cells (by 2.8-fold), a gastric cancer cell line (Fig. 4D). NRDC is expressed primarily in the cytoplasm, but it is also found on the cell surface (27–29).

Immunocytochemistry of transfected cells suggested that...
NRDc and TACE form a complex mainly on the plasma membrane (Fig. 4B). Therefore, we examined the effect of PMA on the distribution of endogenous NRDc in MKN45 cells. Immunofluorescence staining with anti-NRDc antibody showed that PMA increased NRDc expression on the plasma membrane (Fig. 4E). These results indicate that NRDc plays a central role in the regulation of induced ectodomain shedding through the translocation and formation of a complex with TACE.

**NRDc Enhances HB-EGF Shedding through TACE**

**Inhibition of NRDc Expression Results in Decreased Shedding of HB-EGF**—To confirm that NRDc is essential for ectodomain shedding of HB-EGF, RNA-mediated interference was used to inhibit its expression. The effect of two distinct siRNA duplexes against NRDc and two control siRNAs on HB-EGF shedding was examined in COS-7 cells. Transfection of the siRNAs against NRDc resulted in a reduction of endogenous NRDc protein expression by 80.9 ± 3.1% (NRDc-1) and 80.4 ± 8.3% (NRDc-2), respectively, whereas the siRNAs did not affect the expression levels of TACE, HB-EGF, and GAPDH (Fig. 6, A and B). Constitutive HB-EGF shedding in siRNA-treated cells was significantly decreased (27.3 ± 4.0% by NRDc-1 (p = 0.003) and 22.3 ± 13.5% by NRDc-2 (p = 0.02)) compared with that in control siRNA (Control-1)-treated cells (Fig. 6C). PMA-induced HB-EGF shedding was also reduced to
a greater extent in those cells (34.0 ± 7.6% by NRDr1 [p = 0.003] and 27.3 ± 11.3% by NRDr2 [p = 0.006]). These results further establish that NRDr plays a critical role in both the constitutive and inducible ectodomain shedding of HB-EGF.

DISCUSSION

Our findings have identified NRDr, a metalloendopeptidase of the M16 family, as a potent activator of HB-EGF ectodomain shedding. The effect of NRDr appears not to be direct, but to be mediated by TACE because 1) an enzymatically inactive mutant of NRDr also enhanced the shedding, 2) NRDr and TACE cooperated to enhance the shedding in a cell-based assay, and 3) NRDr enhanced the catalytic activity of TACE in an in vitro peptide cleavage assay using a HB-EGF cleavage site peptide. We also demonstrated that NRDr and TACE form a complex in cells and direct interaction of the recombinant proteins. These results suggest that NRDr potentiates TACE activities via direct interaction. We examined TACE first because several lines of evidence have indicated that it is a specific sheddase for HB-EGF (14, 19, 34). Moreover, TACE is involved in ectodomain shedding of numerous membrane proteins and is recognized as the prototypical sheddase among ADAM proteins (1, 36). Of course, we cannot exclude the possibility that NRDr enhances the catalytic activities of other ADAM proteins or matrix metalloproteinases, which is an important issue to be resolved.

Phorbol esters (e.g. PMA) have been used as general activators of ectodomain shedding in many studies (1, 2, 36). However, the mechanism that underlies the activation of shedding was unclear. Here, we have demonstrated that NRDr forms a complex with TACE and that the process is potentiated by PMA, although PMA does not affect the total amounts of these proteins. Pulldown assays and in vitro peptide cleavage assays revealed that NRDr bound to the extracellular domain and potentiated the activity of TACE. These results indicate that the PMA-induced formation of a complex with NRDr at least partially explains how PMA activates TACE in HB-EGF shedding. However, the precise mechanism by which the formation of the complex is enhanced has not been clarified. NRDr is a cytosolic enzyme with no signal peptide; however, it is exported out of the cell by unknown mechanisms (27–29). The translocation of NRDr from the cytosol to the cell surface might nicely explain how the formation of a complex with TACE is enhanced by PMA without any significant change in the total expression level of the protein. Our staining data support this idea because PMA increased NRDr expression on plasma membranes, where NRDr and TACE mainly co-localized. Further study (for example, time-lapse observations of NRDr and TACE by confocal microscopy or a cell-surface biotinylation study) will be required to understand the regulation of NRDr translocation and the PMA-inducible interaction of NRDr with TACE.

Our findings indicate that NRDr is required for ectodomain shedding of HB-EGF because inhibition of its expression by RNA interference was accompanied by a reduction in constitutive and PMA-induced shedding. Several reasons for the relatively small effect of siRNA on the shedding (NRDr-1 siRNA, 27.3% for constitutive shedding and 34.0% for stimulated shedding) can be proposed. 1) The remnant of NRDr (20% of total expression) might be enough to enhance HB-EGF shedding. 2) There are unidentified proteins that have redundancy for NRDr in the shedding-enhancing effect. 3) Cell-surface NRDr might be less affected by gene knockdown because of the difference in stability between cell-surface and cytosolic NRDr. These possibilities are under investigation.

We identified NRDr as a protein that specifically binds HB-EGF (23) and demonstrated that the heparin-binding domain of HB-EGF is critically involved in the binding (29). Here, an in vitro peptide cleavage assay showed that NRDr enhanced the catalytic activity of TACE. The peptide used in the assay has only 8 amino acids corresponding to the juxtamembrane domain of HB-EGF. These results indicate that the effect of NRDr on the activity of TACE for ectodomain shedding of HB-EGF is not dependent on the capacity of NRDr to bind HB-EGF. In other words, the effect of NRDr might not be specific to HB-EGF, but might apply to a broader range of membrane substrates of TACE. Our pilot study suggested that NRDr enhances ectodomain shedding of multiple TACE substrates, including other EGFR ligands, tumor necrosis factor-α, and amyloid-β precursor protein. The similar pattern of distribution of TACE and NRDr mRNAs found in most tissues and the especially high levels in heart, skeletal muscle, and testis (26, 32) support the idea that NRDr acts as a general activator of TACE in a physiological context. When NRDr-deficient animals become available, studies with them should show that NRDr is essential in the modulation of TACE activity and ectodomain shedding in vivo.

NRDr is a metalloendopeptidase that belongs to the M16 family. An enzymatically inactive NRDr mutant containing an altered active site retained the ability to enhance the catalytic activity of TACE and ectodomain shedding of HB-EGF. We have also demonstrated that the enzymatic activity of NRDr is not required for binding HB-EGF or the enhancement of cell migration (23). A metalloprotease activity-independent function of a M16 family member has been indicated for the Saccharomyces cerevisiae AX11 gene product, Ax11p, which is essential for the processing of pheromones and the selection of bud sites in yeast. The enzymatic activity is required for the former, but not the latter (38, 39). Although no substrates for NRDr in vivo have been identified, NRDr may act as a multifunctioning protein like Ax11p.

These findings imply that the activation of HB-EGF shedding can be blocked by inhibiting the interaction between NRDr and TACE. If true, NRDr could be an attractive therapeutic target for cancer and cardiovascular diseases because the EGFR signaling pathway is already an approved therapeutic target for cancer and because HB-EGF ectodomain shedding has been implicated in those diseases (8, 13, 40). Our findings have revealed a novel mechanism by which ectodomain shedding of HB-EGF is regulated. More studies on NRDr-TACE interactions will shed light on potential anti-shedding therapies.

3 Y. Hiraoka, K. Yoshida, M. Ohno, K. Okawa, T. Kita, and E. Nishi, manuscript in preparation.
Nardilysin Enhances HB-EGF Shedding through TACE

Acknowledgments—We thank N. Nishimoto for excellent technical assistance; K. Yoshino, M. Tanaka, S. Higashiyama, K. Matsumura, and M. E. Milla for materials; and P. W. Park and R. A. Black for critically reading the manuscript.

REFERENCES

1. Arribas, J., and Borroto, A. (2002) Chem. Rev. 102, 4627–4638
2. Blobel, C. P. (2005) Nat. Rev. Mol. Cell Biol. 6, 32–43
3. Schlondorff, J., and Blobel, C. P. (1999) J. Cell Sci. 112, 3603–3617
4. Kheradmand, F., and Werb, Z. (2000) BioEssays 22, 8–12
5. Iwamoto, R., Yamazaki, S., Asakura, M., Takashima, S., Hasuwa, H., and Mekada, E. (1997) J. Biol. Chem. 272, 31730–31737
6. Doedens, J. R., and Black, R. A. (2000) Biochem. Biophys. Res. Commun. 277, 143–153
7. Gechtman, Z., Alonso, J. L., Raab, G., Ingber, D. E., and Klagsbrun, M. (1999) J. Biol. Chem. 274, 28828–28835
8. Nishi, E., and Klagsbrun, M. (2004) Biochim. Biophys. Acta 163, 229–238
9. Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Srinivasan, S. S., Boiani, N., Schooler, K. A., Gerhart, M., Davis, R., Fitzer, J. N., Johnson, R. S., Paxton, R. J., March, C. J., and Cerretti, D. P. (1997) Nature 385, 729–733
10. Moss, M. L., Jin, S. L., Milla, M. E., Bickett, D. M., Burkhart, W. Carter, H. L., Chen, W. J., Clay, W. C., Didsbury, J. R., Hassler, D., Hoffman, C. R., Cost, T. A., Lambert, M. H., Leenstetter, M. A., McCauley, P., McGehee, G., Mitchell, J., Moyer, M., Pahel, G., Rocque, W., Overton, L. K., Schoenen, F., Seaton, T., Su, J. L., Warner, J., Willard, D., and Becherer, J. D. (1997) Nature 385, 733–736
11. Sahin, U., Weskamp, G., Kelly, K., Zhou, H. M., Higashiyama, S., Peschon, J., Hartmann, D., Saftig, P., and Blobel, C. P. (2004) J. Cell Biol. 164, 769–779
12. Higashiyama, S., Kau, K., Besner, G. E., Abraham, J. A., and Klagsbrun, M. (1992) J. Biol. Chem. 267, 6205–6212
13. Fujita, A., Oka, C., Arikawa, Y., Katagai, T., Tonouchi, A., Kuhara, S., and Misumi, Y. (1994) Nature 372, 567–570
14. Adam, N., Blondell, K., Ashby, M. N., and Boone, C. (1995) Science 270, 464–467
15. Tanaka, Y., Miyamoto, S., Suzuki, S. O., Oki, E., Yagi, H., Sonoda, K., Yamazaki, A., Mizushima, H., Maehara, Y., Mekada, E., and Nakano, H. (2005) Clin. Cancer Res. 11, 4783–4792
16. Fujita, A., Oka, C., Arikawa, Y., Katagai, T., Tonouchi, A., Kuhara, S., and Misumi, Y. (1994) Nature 372, 567–570
17. Nishi, E., and Klagsbrun, M. (2003) J. Biol. Chem. 278, 32260–32267
18. Dong, J., Opresko, L. K., Dempsey, P. I., Lauffenburger, D. A., Coffey, R. J., and Wiley, H. S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6235–6240
19. Dethlefsen, S. M., Raab, G., Moses, M. A., Adam, R. M., Klagsbrun, M., and Prat, A. (2002) Biochem. J. 367, 229–238
20. Green, P. J., Magnuson, T., Douglas, P. S., Morgan, J. P., and Neel, B. G. (2000) Nature 402, 884–888
21. Chen, B., Bronson, R. T., Klaman, L. D., Hampton, T. G., Wang, J. F., Green, P. J., Magnussen, T., Douglas, P. S., Morgan, J. P., and Neel, B. G. (2000) Nat. Genet. 24, 296–299
22. Chen, B., Bronson, R. T., Klaman, L. D., Hampton, T. G., Wang, J. F., Green, P. J., Magnussen, T., Douglas, P. S., Morgan, J. P., and Neel, B. G. (2000) J. Biol. Chem. 275, 14598–14607
23. Fujita, A., Oka, C., Arikawa, Y., Katagai, T., Tonouchi, A., Kuhara, S., and Misumi, Y. (1994) Nature 372, 567–570
24. Adam, N., Blondell, K., Ashby, M. N., and Boone, C. (1995) Science 270, 464–467
25. Tanaka, Y., Miyamoto, S., Suzuki, S. O., Oki, E., Yagi, H., Sonoda, K., Yamazaki, A., Mizushima, H., Maehara, Y., Mekada, E., and Nakano, H. (2005) Clin. Cancer Res. 11, 4783–4792