Direct Binding of Neutral Endopeptidase 24.11 to Ezrin/Radixin/Moesin (ERM) Proteins Competes with the Interaction of CD44 with ERM Proteins*

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Akira Iwase, Ruqian Shen, Daniel Navarro, and David M. Nanus

Neutral endopeptidase 24.11 (NEP) is a cell surface peptidase expressed by numerous tissues including prostatic epithelial cells. We reported that NEP inhibits prostate cancer cell proliferation and cell migration by enzymatic inactivation of neuropeptide substrates and through protein-protein interaction independent of catalytic function. The cytoplasmic domain of NEP contains a positively charged amino acid cluster, previously identified as a binding site for ezrin/radixin/moesin (ERM) proteins. We report here that NEP co-immunoprecipitates with ERM proteins in NEP-expressing LN-CaP prostate cancer cells and MeWo melanoma cells. Co-immunoprecipitation showed that ERM proteins associate with wild-type NEP protein but not NEP protein containing a truncated cytoplasmic domain or point mutations replacing the positively charged amino acid cluster. In vitro binding assays showed that NEP binds directly to recombinant N terminus fragments of ERM proteins at the positively charged amino acid cluster within the NEP cytoplasmic domain. Binding of ERM proteins to NEP results in decreased binding of ERM proteins to the hyaluronan receptor CD44, a main binding partner of ERM proteins. Moreover, cells expressing wild-type NEP demonstrate decreased adhesion to hyaluronic acid and cell migration. These data suggest that NEP can affect cell adhesion and migration through direct binding to ERM proteins.

Neutral endopeptidase 24.11 (NEP) is a cell surface peptidase expressed by various tissues including prostate, kidney, intestine, endometrium, and lung. NEP is a type II integral membrane protein consisting of three domains: an N-terminal short cytoplasmic domain, a hydrophobic transmembrane domain and a large extracellular domain that is responsible for catalytic activity. NEP cleaves peptide bonds on the amino side of hydrophobic amino acids and inactivates a variety of physiologically active neuropeptides such as neurotensin, bombesin, and endothelin-1 (ET-1) (2–4). Consequently, loss or decreases in NEP expression may promote peptide-mediated proliferation by allowing an accumulation of higher peptide concentrations at the cell surface and facilitate the development or progression of neoplasia. In this regard, we showed that NEP expression is decreased in prostate cancer (PC) cells, resulting in bombesin- and ET-1-mediated stimulation of cell proliferation and migration (6, 7) and ligand-independent activation of the insulin-like growth factor-1 receptor, leading to Akt phosphorylation (8).

Using a catalytically inactive mutated NEP protein, we recently showed that NEP associates with tyrosine-phosphorylated Lyn kinase, which then binds the p85 subunit of phosphatidylinositol 3-kinase, resulting in an NEP-Lyn-phosphatidylinositol 3-kinase protein complex. This complex competitively blocks the interaction between focal adhesion kinase and phosphatidylinositol 3-kinase, indicating that the NEP protein inhibits cell migration via a protein-protein interaction independent of its catalytic function (7). That study did not elucidate which portion of the NEP protein, intracellular or transmembrane, was involved in NEP action. To identify the region of NEP involved in protein-protein interactions, we analyzed the intracellular cytoplasmic tail of the NEP protein and identified a positively charged amino acid cluster, similar to a previously identified region located in the intracellular portion of the hyaluronan receptor, CD44. In contrast to NEP, cell surface CD44 can promote cell migration and invasiveness by binding and localizing matrix metalloproteinases at the cell surface (9, 10) and by interacting with and binding to ezrin/radixin/moesin (ERM) proteins, which interact with the cytoskeleton (11, 12). We report here that NEP directly interacts with ERM proteins in vitro and in vivo via a positively charged amino acid cluster in the cytoplasmic domain of NEP. Moreover, the interaction of NEP with ERM proteins competes with the interaction of ERM proteins with CD44. These data suggest that NEP can affect cell adhesion and migration through competitive binding with CD44 to ERM proteins.

EXPERIMENTAL PROCEDURES

Cells and Antibodies—LN-CaP, PC-3, TSU-Pr1 cells, and their derivatives were maintained in RPMI 1640 medium supplemented with 2 mM glutamine, 1% nonessential amino acids, 100 units/ml streptomycin, and penicillin containing 10% fetal calf serum. MeWo melanoma cells were maintained in modified Eagle’s medium containing 10% fetal calf serum. Antibodies used include goat anti-ERM polyclonal antibody C-15 and mouse anti-NEP monoclonal antibody FR4D11 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); mouse anti-radixin and moesin mAb Ab-1 (Lab Vision Corp.); mouse anti-NEP mAb NCL (Novocastra Laboratories Ltd.); mouse anti-NEP mAb J5 (Beckman Coulter, Inc.); rabbit anti-moesin polyclonal antibody and rabbit anti-ezrin polyclonal...
antibody (Upstate Biotechnology, Inc., Lake Placid, NY); and mouse anti-CD44 mAb 2C5 (R&D Systems, Inc.).

**Site-directed Mutagenesis, Vector Construction, and Transfection—**

Full-length NEP cDNA was subcloned into the tetracycline-repressible transactivator protein-responsive plasmid, pTRE (pTRE/NEP) as previously described (6). NEP cDNA with a cytoplasmic domain deletion was constructed by site-directed mutagenesis (QuikChange, Stratagene) and subcloned into pTRE (pTRE/NEP/Δcyt). To substitute positively charged amino acids in the cytoplasmic domain of NEP, K79K94, with Q79N94, PCR-based site-directed mutagenesis was performed using a set of primers including point mutations (underlined) as follows: 5′-CTG GAT ACT AAT CCA CAA CAG CAA AAG CCA CAG CAA TGG ACT CCA CTG GAG-3′ and 5′-CTC GAG TGT AAT CCA CAA CAG CAA AAG CCA CAG CAA TGG ACT CCA CTG GAG-3′. The autoradiography of the transcription products were incubated with 1–2 μg of GST fusion proteins immobilized on glutathione-Sepharose beads and washed with binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40) for 4°C with rocking. Complexes were pelleted at 10,000 × g for 2 min, washed three times in 0.5 ml of binding buffer, and subjected to SDS-PAGE. The gels were fixed, dried, and subjected to autoradiography.

**Immunofluorescent Microscopy—**

Cells after fixation with 3.7% formaldehyde in PBS were treated with 0.2% Triton X-100 in PBS for 10 min and soaked in PBS containing 1% BSA (BSA-PBS) and 5% skim milk for 30 min. Cells were then incubated with anti-NEP mAb H9262 and anti-ERM polyclonal antibody C-15 diluted with BSA-PBS for 1 h, washed three times for 5 min with BSA-PBS, and incubated with secondary antibodies rhodamine-conjugated anti-goat IgG (Jackson Immunoresearch Laboratories, Inc.) and fluorescein isothiocyanate-conjugated anti-mouse IgG (Santa Cruz Biotechnology, Inc.) for 1 h. Cells were examined using an Axiovert 35 fluorescence microscope (Carl Zeiss Microimaging, Inc.).

**Hyaluronic Acid-Binding Assay—**

An adhesion assay for cells to hyaluronic acid was performed using methods described previously (19–21) with modifications. Flat bottom 96-well plates were coated with 100 μl of hyaluronic acid (1 mg/ml in PBS) for 1 h at 37°C. Wells were rinsed with PBS, and remaining nonspecific binding sites were blocked by incubating the plates with 1 ml of PBSA dissolved in RPMI 1640. Immune complexes were treated with BSA only. 1 × 10⁴ WT-5 and QN-12 cells cultured with or without tetracycline for 72 h were plated in 100 μl of RPMI 1640 with 1% BSA and incubated for 1 h at 37°C. The total number of cells (TC) was obtained by counting cells in three different areas from each sample using a 1 × 1-mm grid before nonadherent cells were removed. Nonadherent cells were removed by washing, and the number of attached cells (AC) in the AC area was counted. The percentage of adherent cells (AC/TC × 100) was calculated. Statistical analysis (Student’s t test) was performed using SigmaStat (SPSS Science).

**Cell Migration Assay—**

Migration assays were performed as described (7) using 24-well Transwell cell culture chambers (Costar). Following culture for 48 h in medium with or without tetracycline, TN-2, WT-5, and QN-12 cells were harvested, washed, and resuspended in serum-free medium containing 10 ml ET-1 and added to the upper chambers at a concentrations of 10 × 10⁴ cells/well. Cells were allowed to migrate for 12 h at 37°C in a humidified atmosphere containing 5% CO₂. Membranes were fixed and stained with May-Grünwald staining. Nonmigrated cells on the upper side of the membrane were removed with a cotton swab. Migrated cells attached to the lower side of the membrane were enumerated using a light microscope at ×20 magnification. Each data point represents the average cell number of six independent microscopic fields from a single experiment. Statistical analyses were performed using an unpaired t test. All migration assays were performed on three separate occasions with similar results.

**RESULTS—**

**Co-immunoprecipitation of ERM Proteins with Endogenous NEP—**

ERM proteins share 75–80% sequence homology and are predominantly co-expressed and localized just beneath specialized domains of plasma membranes, such as microvilli and cell-cell or cell-substrate adhesion sites, where actin filaments are densely associated (22, 23). ERM proteins contain an N terminus membrane-binding domain and a C terminus actin-binding domain acting as cross-linkers between the cell membrane and the actin cytoskeleton (22, 23). A number of proteins including CD44, CD43, intercellular adhesion molecule (ICAM)-1, -2, -5, and syndecan-2 have been identified as ERM-binding membrane proteins (11, 24–27). A positively charged...
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Amino Acid Cluster in the Cytoplasmic Domain of NEP

To confirm that NEP interacts with ERM proteins via positively charged amino acids contained within its cytoplasmic domain, we constructed cytoplasmic mutants of NEP: a cytoplasmic domain-truncated NEP expression vector (pTRE/NEPdcyto) and a cytoplasmic domain-mutated NEP expression vector (pTRE/NEPΔcyto) in which positively charged amino acids were replaced with neutral amino acids. We then established stable cell lines using parental TSU-Pr1 cell containing the tetracycline-repressible (tet-off) NEP expression system (empty vector control TN-12, WT-5, CM-2, and QN-12) (Fig. 2, A and B). As shown in Fig. 2C, Western blotting of NEP immunoprecipitates indicated that NEP and ERM proteins associated in cell lysates of WT-5 cells that express wild-type NEP, but not in cell lysates from CM-2 or QN-12 cells that express cytoplasmic domain-truncated or cytoplasmic domain-mutated NEP, respectively. These results were confirmed by Western blotting using an anti-NEP antibody of ERM immunoprecipitates, which showed wild-type NEP, not truncated or mutated NEP, co-immunoprecipitated with radixin and moesin (Fig. 2D). These data suggest that the interaction between NEP and ERM proteins occurs via the positively charged amino acids within the cytoplasmic domain of NEP.

Interaction of ERM Proteins with the Positively Charged Amino Acid Cluster in the Cytoplasmic Domain of NEP

Previously studies show that individual ERM proteins co-express and co-localize in cells, but different cell types may contain varying amounts of each protein (28). Therefore, we first examined expression levels of ezrin, radixin, and moesin in PC cell lines and MeWo melanoma cells by immunoblotting (Fig. 1B). NEP-negative TSU-Pr1, DU-145, and PC-3 cells possessed similar amounts of radixin and moesin, whereas LNCaP and MeWo cells (which express NEP) contained lower levels of moesin compared with radixin. LNCaP cells expressed less ezrin in comparison with the other cell lines. Western analysis of immunoprecipitates of the NEP protein with antibodies to moesin/radixin and ezrin showed that NEP co-immunoprecipitated with ERM proteins in LNCaP and MeWo cells, but not TSU-Pr1, DU-145, or PC-3 cells (Fig. 1C). This association was confirmed by Western blotting of ERM immunoprecipitates using anti-NEP antibody (Fig. 1D). These results indicate that endogenous NEP in LNCaP and MeWo cells associates with ERM proteins in vivo.

**Fig. 1.** Association of endogenous NEP and ERM proteins in PC cells and MeWo cells. A, amino acid sequences of the juxtamembrane cytoplasmic domains of mouse CD44, mouse ICAM-2, and human NEP. The numbers correspond to amino acid positions from the transmembrane domain. Positively charged amino acid residues are presented in boldface letters. Accession numbers are BC051388 (mouse CD44), BC038970 (mouse ICAM-2), and NM_000902 (human NEP). B, cells were lysed with radiimmune precipitation assay buffer, and the samples (10 μg of protein/lane) were subjected to 10% SDS-PAGE followed by immunoblotting using anti-NEP mouse monoclonal antibody (top panel), anti-ezrin rabbit polyclonal antibody (second panel), anti-radixin/moesin rabbit monoclonal antibody (third panel), or anti-moesin rabbit polyclonal antibody (bottom panel). C, the lysates were immunoprecipitated with anti-NEP mouse monoclonal antibody (top panel), or normal mouse IgG as control (Cont.). The immunoprecipitates were separated by SDS-PAGE followed by immunoblotting with antisera to NEP mouse monoclonal antibody (Blot: NEP), anti-ezrin rabbit polyclonal antibody (Blot: ezrin), or anti-radixin/moesin mouse monoclonal antibody (Blot: Rad/Moe). Note that ezrin and radixin co-immunoprecipitated with endogenous NEP in LNCaP and MeWo cells, but not in NEP-negative TSU-Pr1, DU-145, or PC-3 cells. The band at 55 kDa is IgG heavy chain. D, immunoblotting using anti-NEP mouse monoclonal antibody (Blot: NEP), anti-ezrin rabbit polyclonal antibody (Blot: ezrin) or anti-radixin/moesin mouse monoclonal antibody (Blot: Rad/Moe) of immunoprecipitates with anti-ERM goat polyclonal antibody (ERM) or normal goat IgG (Cont.). The numbers on the left indicate the positions of the molecular size markers. The positions of NEP, ezrin, radixin, and moesin are indicated by arrows on the right.
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Direct Binding of ERM Proteins to NEP Cytoplasmic Domain

GST Fusion Proteins—Previous reports suggest that there are two mechanisms by which ERM proteins bind to integral membrane proteins. The brush border Na+/H+ exchanger (NHE3) binds to ezrin indirectly via NHE3 kinase A regulatory protein (E3KAPR), whereas most ERM binding partners including CD44, CD43, ICAM-2, and NHE1 bind to ERM proteins directly (29, 30). To confirm a direct interaction of NEP with ERM proteins, we carried out an in vitro binding assay using in vitro translated ERM proteins and the GST fusion cytoplasmic domain of NEP (Fig. 3A). GST itself possessed no binding affinity for recombinant C-terminal, N-terminal, or full-length radixin (Fig. 3B, upper panel, lanes 2, 6, and 10). N-terminal radixin bound to GST fusion protein of the cytoplasmic domain of NEP (GST-NEP1–19), whereas C-terminal or full-length radixin did not (Fig. 3B, upper panel, lanes 3, 7, and 11). In contrast, the NEP-GST fusion protein containing amino acid substitutions for positively charged amino acids (Lys12–Lys13–Lys14 → Gln-Asn-Ile) did not bind either C-terminal, N-terminal, or full-length radixin (Fig. 3B, upper panel, lanes 4, 8, and 12). Similar results were obtained for moesin (Fig. 3B, lower panel) and ezrin (data not shown). The switch between active membrane-bound or dormant cytoplasmic ERM proteins depends on protein conformation, which is regulated by intramolecular and intermolecular interactions between the N and C terminus (31). ERM activation occurs through disruption of the head (N terminus) to tail (C terminus) interaction, allowing binding of the N and C terminus to the cell membrane and actin, respectively (32, 33). Our results suggest that NEP can only bind to the active, unmasked N-terminal region of ERM proteins when the N and C terminus interaction is disrupted.

Localization of ERM Proteins and Endogenous, Wild Type, or Mutated NEP—We next performed immunofluorescence studies to assess the distribution of NEP and ERM proteins in LNCaP cells, which express both of these proteins endogenously. Double staining of NEP and ERM proteins revealed an overlapping distribution at the cell surface, which was enriched at cell-cell adhesion sites (Fig. 4, right panel, arrowheads) and microvilli-like structures (Fig. 4, left panel, arrows). We next performed immunofluorescence analysis of WT-5 cells expressing wild-type NEP and QN-12 cells expressing NEP containing amino acid substitutions of Lys12–Lys13–Lys14 → Gln-Asn-Ile. In WT-5 cells, NEP was detected in the plasma membrane along the smooth edge of the cells, and NEP and ERM proteins co-localized and co-concentrated in areas along the plasma membrane (Fig. 5, left panel). In contrast, in QN-12 cells, co-localization of NEP and ERM proteins was impaired with the mutation of the cytoplasmic domain of NEP, although...
most of ERM proteins still showed concentration at the cell-cell adhesion site (Fig. 5, right panel). These results suggest that the positively charged amino acids in the cytoplasmic domain of NEP are essential for co-localization of NEP and ERM proteins in vivo.

Competitive Binding of ERM Proteins to NEP and CD44—ERM proteins have been reported to bind to numerous proteins including CD44, CD43, ICAM-1, -2, -3, syndecan-2, and NHE1. This fact suggests that the expression status of these binding partners of ERM proteins may modify the binding of ERM proteins. The hyaluronan receptor, CD44, which is one of the main binding partners of ERM proteins, can mediate interaction between cells and the extracellular matrix, and therefore CD44 is believed to play an important role in cell migration and invasion (10, 34). In contrast, we have demonstrated that NEP inhibits cell migration (7). To determine whether the binding of ERM proteins to NEP affects the binding of ERM proteins to CD44, we assayed for CD-44/ERM co-immunoprecipitates in control TN-12 cells, WT-5 cells, and QN-12 cells, all of which possess endogenous CD44. Induced expression of NEP following tetracycline removal did not alter CD44 expression in TN12, WT-5, or QN-12 cells (data not shown). However, the association of CD44 with ERM proteins significantly decreased in WT-5 cells expressing NEP following tetracycline removal (Fig. 6, upper panel, lane 4) compared with WT-5 cells lacking NEP expression cultured in the presence of tetracycline (Fig. 6, upper panel, lane 3). In contrast, expression of a mutated NEP protein in QN-12 cells (that do not associate with ERM proteins) did not significantly alter the association of CD44 with ERM proteins (Fig. 6, upper panel, lanes 5 and 6). These data suggest that the binding of ERM proteins to NEP competes...
with the binding of ERM proteins with CD44.

Expression of Wild-type but Not Mutated NEP Inhibits Cell Adhesion to Hyaluronic Acid and Cell Migration—The association of CD44 with ERM proteins is reported to be involved in cell-cell adhesion and cell motility of neoplastic cells (35, 36). In addition, binding of CD44 to hyaluronic acid may be a critical step in CD44-enhanced tumor-promoting effects (20). We assayed whether the NEP-induced inhibition of the association of CD44 with ERM proteins interfered with the ability of cells to bind to hyaluronic acid. WT-5 and QN-12 cells (cultured in the presence and absence of tetracycline) did not adhere to BSA-treated control plates without hyaluronic acid coating (data not shown). As illustrated in Fig. 7, similar adhesive capacities to hyaluronic acid were observed in WT-5 and QN-12 cells cultured in the presence of tetracycline, which suppresses NEP expression, and in QN-12 cells expressing mutated NEP (right panel). In contrast, in QN-12 cells, co-localization of NEP and ERM proteins was impaired with the mutation of the cytoplasmic domain of NEP (left panel).

We next performed a cell migration assay to assess the effect of wild-type NEP and mutated NEP. We previously reported that expression of wild-type NEP in WT-5 cells results in >80% inhibition of cell migration compared with control (WT-5 cells cultured with tetracycline) and that this inhibition results from both catalytically dependent and independent factors (7). Cell migration assays using WT-5 and QN-12 cells cultured with and without tetracycline demonstrated a 75% decrease in migration in WT-5 cells expressing NEP (p < 0.001), whereas QN-12 cells expressing mutated NEP resulted in 25% decrease of migration (p = 0.009) compared with control cells in which NEP was expressed. Partial inhibition of cell migration in

Fig. 5. Immunofluorescence localization of wild-type NEP and site-directed mutant of NEP. WT-5 cells stably expressing wild-type NEP and QN-12 cells expressing the site-directed mutant of NEP were cultured without tetracycline and doubly stained with anti-NEP antibody (upper panel) and anti-ERM antibody (middle panel). Merged photographs are shown in the lower panel. In WT-5 cells, wild-type NEP co-localized with ERM proteins (left panel). In contrast, in QN-12 cells, co-localization of NEP and ERM proteins was impaired with the mutation of the cytoplasmic domain of NEP (right panel).

Fig. 6. Inhibition of binding of ERM to CD44 by interaction of NEP and ERM. ERM immunoprecipitates (ERM) from control TN-12, WT-5, and QN-12 cells cultured with (+) or without (−) 1 μg of tetracycline (Tet), control immunoprecipitates using normal goat IgG (Cont.), and total cell lysates (C. L.) from WT-5 cells were separated by SDS-PAGE followed by immunoblotting using anti-CD44 mouse monoclonal antibody (Blot: CD44), anti-NEP mouse monoclonal antibody (Blot: NEP), or anti-radixin/moesin mouse monoclonal antibody (Blot: Rad/Moe). Note that co-immunoprecipitated CD44 in WT-5 without tetracycline showed weak binding (upper panel) compared with wild-type NEP co-immunoprecipitated with ERM proteins (middle panel). Total CD44 in cell lysates of WT-5 cells showed a similar amount with or without tetracycline. IP, immunoprecipitation.

Fig. 7. Adhesion of WT-5 and QN-12 cells to hyaluronic acid. WT-5 and QN-12 cells cultured with or without 1 μg/ml tetracycline were plated in hyaluronic acid-coated wells of a 96-well plate. Following a 1-h incubation at 37°C, TC and AC after removal of nonadherent cells were counted in the designated area. The percentage of adhesion cells to hyaluronic acid (HA) was calculated by AC/TC × 100. Each value represents the mean ± S.D. from three independent experiments. The difference between tetracycline-untreated and tetracycline-treated cells was statistically significant (Student’s t test, p = 0.003) only in WT-5 cells expressing wild-type NEP.
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QN-12 cells most likely results from the remaining enzymatic activity of mutated NEP that can inactivate ET-1 that stimulates cell migration. These results suggest that the mutation in the cytoplasmic domain of NEP impairs the inhibitory effect of NEP on cell migration.

**DISCUSSION**

Recent studies indicate that the biological and regulatory function of cell surface peptidases extend beyond catalytic inactivation of neuropeptide substrates to include signal transduction (37) and protein-protein interactions (38, 39). Our extensive studies on NEP in PC indicate that the NEP protein has numerous biological effects. NEP expression is diminished in most PCs, and reexpression of NEP inhibits PC cell growth, cell migration, and tumorigenicity (6, 14). Enzymatic inactivation of neuropeptides such as ET-1 and bombesin can account for some of the biologic effects of NEP; however, we showed that NEP also inhibits focal adhesion kinase phosphorylation and PC cell migration by interacting with Lyn kinase and phosphatidylinositol 3-kinase independent of the catalytic activity of NEP (7). It is likely that NEP possesses other undiscovered functions independent of its catalytic activity that may contribute to tumor suppression, since several unknown proteins associate with NEP in LNCaP cells as determined by surface-enhanced laser desorption/ionization mass spectrometry (40).

In the current study, we found that NEP binds to ERM proteins through its cytoplasmic domain. ERM proteins play an important role in regulating the organization of the actin cytoskeleton through their role as cross-linkers between actin filaments and membrane proteins (12). ERM proteins and their binding partners have been extensively studied in fibroblasts, whereas binding partners of ERM proteins in epithelial cells are not as well defined. Yonemura et al. (11) showed that CD44, CD43, and ICAM-2 bind to moesin at the positively charged amino acid cluster within their cytoplasmic domains. Our data indicate that NEP, which possesses a shorter cytoplasmic domain in comparison with CD44 and CD43, also binds ERM proteins and that a similar positively charged amino acid cluster in the cytoplasmic domain is required. Both CD44 and NEP, another ERM binding partner, possess two ERM binding motifs (17), which may increase their affinity for ERM proteins.

There are few reports investigating the relationship of ERM proteins with their binding partners in tumor cells. Reorganization of the cytoskeleton is critical to cell adhesion, cell motility, and cell migration. Suppression of ERM proteins by antisense oligonucleotides resulted in the destruction of both cell-cell and cell-substrate adhesion in mouse mammary tumor MTD-1A cells (41), and suppression of ezrin in colorectal cancer cells resulted in a reduced cell-cell adhesiveness and a gain in cell motility and invasive capacity (42). The interaction of ERM proteins with membrane proteins is likely to be involved in tumor cell morphology and motility, processes that contribute to specific characteristics of the malignant phenotype such as migration and/or invasion.

The significance of the varying levels of ezrin, radixin, and moesin detected in the cell lines in this study is unknown. We did detect an inverse relationship between expression of another ERM binding partner, CD44, and NEP expression. Induced expression of NEP (resulting in an NEP-ERM association) led to a significant decrease in the interaction between CD44 and ERM. The function of CD44 appears to relate to the isoform pattern of CD44 expression and is likely to be cell type-specific (34, 43). Although the amount of CD44 including variant isoforms often increases in cancer (44–46), there is disagreement between studies on whether CD44 expression is correlated with cancer development (47). In PC, expression of CD44 standard (CD44s) is reported to be higher in neoplastic prostate as compared with benign prostate (48). Other studies show that CD44s is reduced in metastatic PC (49) or does not correlate with metastases or PC differentiation (50). In PC cell lines, CD44 is expressed in DU-145 and PC-3 cells (51, 52), and a neutralizing antibody to CD44 inhibited cell proliferation and invasive activity to basement membrane in PC-3 and TSU-P1 cells (52). Harrison et al. (53) recently reported that CD44 co-localizes with ezrin in DU-145 and PC-3 cells and that the interaction of CD44 with ezrin is increased during tumor cells–endothelial cell interaction under HGF stimulation. Similarly, Zohar et al. (36) reported that hyaluronan-CD44-ERM complex is involved in the migration of metastatic breast adenocarcinoma cells. CD44 has also been implicated in promoting migration in melanoma (53). In addition, Jiang et al. (54) recently showed, using truncated mutants of CD44, that the binding of CD44 to hyaluronic acid and retention of the pericellular matrix by CD44 require the cytoplasmic domain of CD44, which suggests that the binding of the cytoplasmic domain of CD44 to cytoskeletal components like ERM proteins may be important for adhesion and/or invasion mediated by CD44. Taken together, these studies suggest that in PC, CD44 promotes tumor cell adhesion and/or invasion and that this effect involves ERM proteins. Thus, NEP could suppress the interaction of ERM with CD44, thereby inhibiting tumor cell adhesion and cell migration by recruiting ERM proteins to the cytoplasmic domain of NEP from CD44 (Fig. 8).

In summary, our findings indicate that NEP-mediated inhibition of cell adhesion occurs in part through binding with ERM proteins and that the interaction between NEP and ERM proteins may result from the ability of NEP to suppress cell adhesion through recruitment of ERM proteins from CD44. Further studies will help define the influence of this complex, including signaling pathways, on cancer cell proliferation, adhesion, and migration.

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