ERM (ezrin/radixin/moesin) proteins act as cytoplasmic cross-linkers that mediate formation of the membrane-associated cytoskeleton, by simultaneously binding actin filaments and the C-terminal cytoplasmic tails of membrane proteins. ERM proteins also bind neutral endopeptidase 24.11 (NEP), a type II membrane protein, even though the N-terminal cytoplasmic tail of NEP possesses the opposite peptide polarity to that of type I membrane proteins. Here, we determined the crystal structure of the radixin FERM (Four point one and ERM) domain complexed with the N-terminal NEP cytoplasmic peptide. In the FERM-NEP complex, the amphipathic region of the peptide forms a β strand followed by a hairpin that bind to a shallow groove of FERM subdomain C. NEP binding is stabilized by β-β interactions and docking of the NEP hairpin into the hydrophobic pocket of subdomain C. Whereas the binding site of NEP on the FERM domain overlaps with the binding site of intercellular adhesion molecule (ICAM)-2, NEP lacks the Motif-1 sequence conserved in ICAM-2 and related adhesion molecules. The NEP hairpin, although lacking the typical inter-chain hydrogen bond but is stabilized by hydrogen bonds with the main chain and side chains of subdomain C, directs the C-terminal basic region of the NEP peptide away from the groove and toward the membrane. The overlap of the binding sites on subdomain C for NEP and Motif-1 adhesion molecules such as CD44 provides the structural basis for the suppression of cell adhesion through interaction between NEP and ERM proteins.
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L)(V/L/I)XX(A/G) (Motif-1), are found in the C-terminal cytoplasmic tails of adhesion molecules such as CD43 and CD44 that interact with ERM proteins.

Recently, neutral endopeptidase 24.11 (NEP) has been shown to directly bind the FERM domain of ERM proteins (16). NEP was known to inactivate G protein-coupled receptors through cleavage of a variety of physiologically active peptides such as bombesin and neurotensin (17–19). The loss or reduction of NEP expression found in prostate cancer cells suggests that decreased peptidase activity facilitates tumor development by allowing neuropeptides to bind their receptors (20–22). Interestingly, binding of NEP to ERM proteins results in decreased binding of ERM proteins to the hyaluronan receptor CD44, and can affect cell adhesion and migration (16). Furthermore, the NEP cytoplasmic tail directly associates with the tumor suppressor PTEN through electrostatic interactions between the basic region of the NEP tail and the phosphorylation site in the PTEN C-terminal tail (23). Thus, the NEP cytoplasmic tail might play a key role in providing a scaffold for signaling proteins in the regulation of cell proliferation pathways, in addition to organizing the membrane-associated cytoskeleton.

NEP is a type II membrane protein that consists of an N-terminal cytoplasmic tail, a trans-membrane helix, and a C-terminal extracellular domain. The extracellular domain is a metalloproteinase that belongs to the M13 family of zinc peptidases. The N-terminal short cytoplasmic tail of NEP (Fig. 1A) interacts directly with the FERM domain of ERM proteins, even though NEP possesses no apparent Motif-1 (15) or recently determined Motif-2 (4) sequence in the cytoplasmic tail. Moreover, it is uncertain how the FERM domain binds the N-terminal cytoplasmic tail of NEP given that the latter possesses the opposite peptide polarity to the C-terminal tails of adhesion molecules that are representative of type I membrane proteins.

Here, we report on the crystal structure of the radixin FERM domain complexed with the N-terminal cytoplasmic tail of NEP. The structure reveals that the amphipathic region of the NEP peptide forms a β strand and a hairpin that bind to subdomain C of the radixin FERM domain. Mutation studies suggests the FERM-binding motif, Motif-1B, which is distinct from Motif-1. The NEP-binding site overlaps that of the Motif-1-binding site on the FERM domain, suggesting versatility of peptide recognition by the FERM domain. In contrast to adhesion molecules of type I membrane proteins that possess a short basic linker region consisting of about 5 residues between the Motif-1 sequence and the trans-membrane helix, the FERM-binding Motif-1B sequence of the NEP cytoplasmic tail is located at the N-terminal end with a distance of more than 10 residues from the putative trans-membrane helix. The distal location of the FERM-binding site relative to the trans-membrane region of the NEP cytoplasmic tail is shown at the top of the alignment.

FIGURE 1. Comparison of the FERM-binding peptide sequences. A, the peptide sequences of the NEP cytoplasmic tails. Basic and acidic residues are in blue and red, respectively. Of the 22 residues of the NEP peptide, 17 residues (6–22) were defined in the current electron density map. This region displays an N-terminal β-strand structure (7–10) followed by a hairpin consisting of 12 C-terminal residues. Key residues that are involved in binding to the radixin FERM domain are highlighted in yellow. These residues compose the FERM-binding motif (Motif-1β) proposed in this work. B, sequence alignment of the Motif-1 cytoplasmic tails of ERM-binding adhesion molecules such as ICAM-2. The secondary structure of the mouse ICAM-2 cytoplasmic tail is shown at the top of the alignment.
brane helix suggests that the long basic linker could be folded back toward membranes.

EXPERIMENTAL PROCEDURES

Preparation of Crystallization Samples—The FERM domain (residues 1–310) of mouse radixin was expressed in BL21(DE3) RIL cells containing plasmid pGEX4T-3 as a fusion protein with glutathione S-transferase (24). Details of the purification scheme have been previously described (25). The synthesized peptide corresponding to the cytoplasmic tail of mouse NEP (1GRSESQMDITDINAPKPKKKQR22) was purchased from Toray Research Center (Tokyo, Japan). The peptide was dissolved to 7.9 mM concentration in a buffer containing 10 mM MES-sodium (pH 6.8), 50 mM NaCl, and 1 mM dithiothreitol.

Crystallization and Data Collection—The radixin FERM domain and the NEP cytoplasmic peptide were mixed in a 1:5 molar ratio (0.3 mM FERM and 1.5 mM NEP). Crystallization conditions were screened by the sitting-drop vapor diffusion method at 4 °C. The FERM-NEP complex crystals were obtained within 1 week by mixing 0.2 μl of protein solution with 0.2 μl of reservoir solution containing 20% polyethylene glycol 3350 (PEG3350) and 0.2 M DL-maleic acid (pH 7.0). The crystals were stepwise transferred into a cryo-protective solution containing 25% PEG200, 20% PEG3350, and 0.2 M DL-maleic acid (pH 7.0) for flash-cooling. X-ray diffraction data were collected using an ADSC Quantum 315 detector installed on the BL41XU beamline at SPring-8 and processed with the program HKL2000 (26). The crystal data and intensity statistics are summarized in Table 1. The crystallographic analysis of the FERM-NEP complex was refined by restraint least-squares refinement using the program REFMAC (30). Because the resolution was limited, tests of the peptide chain tracing were performed with several peptide models in the reverse direction and in different registers. These models gave higher free R values than that of the correct peptide chain tracing and resulted in poorer electron density for the peptide models. The peptide chain polarity was also supported by results of mutation analyses described below. The crystal contained three FERM-NEP complexes in the asymmetric unit. In one complex, the bound NEP peptide was well defined for 17 residues (residues 6–22) in the electron density map (blue in supplemental Fig. S1), whereas peptides bound to the other complexes gave poor electron density, probably due to crystal contacts with the neighboring molecules. Alanine models composed of five (residues 7–11, cyan in supplemental Fig. S1) and seven (residues 7–13, green in supplemental Fig. S1) residues were built for further refinement. The refinement statistics are summarized in Table 1. The stereochemical quality of the model was checked using the program PROCHECK (31). No residue was found in a disallowed region as defined by the aforementioned program. Figures were prepared using the program PyMOL. Superposition of the FERM domains and peptides were carried out using the program LSQKAB (32).

FIGURE 2. Structure of the radixin-NEP complex. Ribbon representations of the radixin FERM domain complexed with the NEP cytoplasmic peptide (blue). The residue numbers of both NEP peptide ends are indicated. The N-terminal polar residues (1–5) were not defined in the current map. The radixin FERM domain consists of three subdomains: A (82 N-terminal residues in green), B (residues 96–195 in red), and C (residues 204–297 in yellow). Linkers A–B (residues 83–95) and B–C (residues 196–203) are colored gray.

TABLE 1
Crystallographic analysis of the FERM-NEP complex

| Intensity data |                |
|---------------|---------------|
| Space group   | P2_12_1       |
| Cell dimensions; a, b, c (Å) | 108.79, 116.84, 141.88 |
| Resolution (Å) | 3.2           |
| Reflections   |               |
| Measured      | 208,303       |
| Unique        | 30,656        |
| Redundancy     | 6.8 (4.8)     |
| Completeness (%) | 99.7 (97.2)   |
| R cryst (%)   | 16.3 (63.5)   |
| Mean I/σ     | 11.3 (2.0)    |

R cryst (%) = Σ |Fo|−|Fc|/Σ |Fo|, where the free reflections (5.1 % of the total used) were held aside for R cryst throughout refinement.
Peptide Binding Experiment—Based on our crystal structure, the binding affinity for several mutated NEP peptides was examined by using surface plasmon resonance measurements (Biacore 3000, GE Healthcare) to identify determinant residues for the specificity (Fig. 5 and Table 2). Each dissociation constant between the NEP cytoplasmic tail and the radixin FERM domain was determined using equilibrium surface plasmon resonance measurements as previously described (4). The purified radixin FERM domain (0.2–10 μM) was injected into a streptavidin-coated sensor chip (sensor chip SA) to which each peptide was immobilized through the C-terminal biotinylated lysine. All binding experiments were performed at 25 °C with a flow rate of 10 μl min⁻¹ in a buffer consisting of 10 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 0.05% surfactant P20. The kinetic parameters were evaluated using the BIaEvaluation software (GE Healthcare). \( K_D \) values were calculated by averaging more than three independent measurements.

RESULTS

Structural Determination—Previous experiments have shown that a glutathione S-transferase-fused 19-residue peptide of the NEP tail binds the radixin and moesin N-terminal fragments containing the FERM domain (16). The sequence of our NEP peptide was based on the mouse NEP cytoplasmic tail (Fig. 1A). The FERM-N\( \text{E} \) complex crystals were obtained using the 22-residue NEP peptide and the structure was determined by molecular replacement using the free radixin FERM domain as a search
model (11), and subsequently refined to 3.2-Å resolution with an R value of 23.3% (a free R value of 26.7%). The crystallographic statistics are summarized in Table 1.

The asymmetric unit of the crystal contained three FERM-peptide complexes. The three FERM domains have essentially the same structure with an averaged overall deviation of 0.82 Å for 5–296 residues, and with an averaged deviation of 0.56 Å for each pair of subdomains (a detailed structural comparison is given in supplemental Fig. S2). The NEP peptide binds subdomain C of the FERM domain, whereas there were no direct contacts with subdomains A or B (Fig. 2).

The Structure of the FERM Domain in the Radixin-NEP Complex—As previously reported, the radixin FERM domain consists of three subdomains: subdomain A (N-terminal residues 5–82, green) having a typical ubiquitin fold, subdomain B (residues 95–195, red) folded into an α-helix bundle, and subdomain C (residues 204–297, yellow) folded into a standard seven-stranded β-sandwich with a long capping α-helix (Fig. 2).

The overall root mean square deviation between the FERM domain bound to the NEP peptide and the free radixin FERM domain is 0.80 Å for 292 Cα-carbon atoms. Pairwise superposition of each subdomain revealed that subdomain C in the NEP-bound form is similar to that of the ICAM-2-bound form (0.43 Å for 93 Cα-carbon atoms) rather than that on the free form (0.71 Å) (supplemental Fig. S3). Like subdomain C of the ICAM-2-bound form (4, 15), the groove between helix α1C and strand β5C of NEP-bound subdomain C is widened by induced-fit structural changes following peptide binding.

**NEP Peptide Conformation**—As shown in Fig. 1A, the primary structure of the NEP cytoplasmic tail consists of three regions: an N-terminal polar region, a central amphipathic region containing hydrophobic residues, and a C-terminal juxtamembrane basic region. In our complexed structure, the N-terminal polar region is not observed in the electron density map, probably due to its flexibility. The amphipathic region forms a short β strand structure (residues 7–10; Met-Asp-Ile-
A hairpin structure (residues 11–14; Asp-Ile-Asn-Ala), whereas the C-terminal basic region (residues 16–22) forms an extended structure (Fig. 3). The overall peptide exhibits a kink (68°) at the hairpin (Fig. 3A). The extended structure of the C-terminal residues, however, would be induced by crystal contacts with the other two molecules in the asymmetric unit of the crystal. The hairpin resembles a type I reverse turn (Fig. 3B) but lacks the typical N+3 inter-main chain hydrogen bond. The peptide structure was stabilized by van der Waals contacts between Asp11 and Ala14 side chains in addition to interactions with the FERM domain as will be described.

NEP Peptide Binding to the FERM Domain—The amphipathic region in the NEP peptide is essential for the interaction with the FERM domain. Seven residues (Met2–Asn13) of this region dock to a long shallow groove that is formed by nonpolar side chains from strand β5C and helix α1C of subdomain C (Fig. 3C) and bury 1,383 Å² of the total accessible surface area. The β strand of the NEP peptide forms an anti-parallel β-β interaction with strand β5C of subdomain C followed by the hairpin docked into the large pocket PI that is connected to the groove (Fig. 3B). In addition to four regular inter-main chain hydrogen bonds derived from the β-β association, two hydrogen bonds are formed between each main chain amide group of Ile12 and Asn13 of the NEP peptide and the main chain carbonyl group of Arg266 from strand β5C. Furthermore, the hairpin carbonyl groups of NEP interact with side chains Arg266 from β5C and His268 from helix α1C. These additional interactions contribute toward stabilization of the hairpin structure. The FERM-NEP contacts are summarized in Fig. 4A. Using surface plasmon resonance measurements, the wild-type mouse NEP cytoplasmic tail binds the mouse FERM domain with a $K_D$ value of 2.2 μM (Table 1 and Table 2). Mutations of the hairpin residues, Ile12 and Asn13, resulted in significant reduction in binding affinity, less than 20% binding affinity compared with that of the wild type in terms of $K_D$ values (I12A and N13A in Fig. 5), suggesting these residues are major contributors to binding affinity.

The groove of subdomain C creates binding sites, S1–S4, for interacting with the side chains of the NEP β-strand (Fig. 3B). Sites S1 and S3 are formed Asn251, Ser259, and Asn247 from strand β5C, and accommodate the bulky side chains of Met2 and Ile9 with for zipper-like interactions (Fig. 3C). Mutations suggested that these interactions are important for the FERM-NEP binding (Fig. 5). Site S4 formed by three nonpolar residues (Ile248, Leu281, and Met285) is deep and NEP Thr10 docked to this site makes intimate contacts with these residues. In fact, mutations of Thr10 resulted in a serious reduction of binding affinity. Contrary to this, site S2 is shallow and the interactions between NEP Asp8 and this site should be weak, which was supported by mutation. Because the side chain of Asp8 is directed toward the basic cleft between subdomains A and C, the negative charge of the side chain may contribute somewhat to electrostatic interactions. The FERM residues participating in the interactions with NEP are conserved in mammalian species (Fig. 4B). These results suggest the NEP binding motif, MXTTXIN (Motif-Iβ).

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![Figure 5.](Image)

Comparison with the Radixin-ICAM-2 Complex—Previously, we reported the crystal structure of the radixin-ICAM-2 complex in which the ICAM-2 peptide forms a β strand followed by a short $3_10$ helix for binding to the groove between helix α1C and strand β5C of subdomain C (15). This binding site overlaps with the binding site for the NEP peptide of the current structure (Fig. 6A). Superposition of these peptides to the FERM domain showed that two β strands are located at the same binding site, where the NEP hairpin-binding site overlaps the ICAM-2 $3_10$ helix-binding site. The overlapping binding sites for NEP and ICAM-2 imply direct

$^a$ The peptides are for the cytoplasmic tail of mouse NEP (residues 1–22) and its mutation and deletion peptides. Mutated residues in the NEP peptides are shown in bold.

$^b$ Determinant residues in the FERM-binding peptides are boxed. The secondary structure, β-strand of the NEP peptide in our complex structure is indicated by β at the top. The hairpin is indicated.

$^c$ The obtained $K_D$ values with their S.D. All measurements were performed at 25 °C in HBP buffer containing 10 mM Hepes-Na (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 0.05% surfactant P20.

$^d$ Fold reduction in relative affinity based on $K_D$ values ($K_D$ (m)) for the mutant and $K_D$ (w) for the wild-type peptides. ΔΔG = RT ln ($K_D$(m)/$K_D$(w)), with $R = 8.31$ J/mol K and $T = 298$ K.
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competition between NEP and adhesion molecules possessing Motif-1 sequences such as CD44 (16) for ERM binding at a layer beneath the inner plasma membrane (Fig. 6B).

The FERM-binding region of NEP has no apparent similarity with the ICAM-2 signature sequence RXTYPVXXRA or the Motif-1 sequence (Fig. 1B) (15). Structure alignment, however, revealed that some key residues important for FERM binding are replaced with homologous residues in NEP, whereas others are not conserved (Fig. 7A). Some of these nonconserved residues are involved in interactions with different FERM residues. An example of conserved replacement of residues involves replacement of ICAM-2 Val12 with NEP Ile12. The former is located at the N-terminal position of the 3\alpha helix and the latter at the hairpin, and both play a similar role in binding to pocket P1. Another key residue, ICAM-2 Tyr10, which docks into site S4 and forms a hydrogen bond to the conserved radixin His288 (Fig. 7C), is replaced in NEP with a smaller residue, Thr10, which does not form a hydrogen bond to His288 (Fig. 7B). Instead, the main chain carbonyl group of Ile12 of the hairpin forms a hydrogen bond to His288 as described above. ICAM-2 Trp16 is located at the edge of the groove and forms a hydrogen bond to conserved radixin His288 (Fig. 7C), whereas Asn17 at the corresponding position forms a hydrogen bond with the main chains of loop β4C–β5C (Fig. 7B).

DISCUSSION

The FERM domain of ERM proteins mediates the binding to a number of target proteins that are integrated in the plasma membrane or in the cytosol. Most of the target proteins are adhesion molecules represented by type I membrane proteins that have a single transmembrane helix followed by a C-terminal cytoplasmic tail. Generally, these C-terminal cytoplasmic tails possess a short (about 5 residues) juxtamembrane basic region, the FERM-binding region (11 residues in ICAM-2) containing the Motif-1 sequence and an additional C-terminal basic region. The Motif-1 sequence directly binds the groove formed by helix α1C and strand β5C of subdomain C of the FERM domain. Our present study confirmed that the NEP cytoplasmic tail binds to the same groove despite the absence of a Motif-1 sequence and the opposite polypeptide polarity of the tail. The NEP sequence forms a hairpin instead of the 3\alpha helix of ICAM-2 for binding to pocket P1 of the groove. NEP Pro15 might prohibit formation of a 3\alpha helix. In addition to binding to pocket P1, the NEP hairpin plays an additional important role in directing the C-terminal linker region, which contains a juxtamembrane basic region, toward the plasma membrane. We noticed that the C-terminal linker region is longer than those found in adhesion molecules such as ICAM-2, and is long enough to link the transmembrane helix and the FERM-binding region by being folded back toward the membrane.

Several transmembrane-type proteases play important roles in cell-surface proteolysis for the processing of biologically active proteins or peptides that mediate a diverse range of cellular functions. Aberrant expression of these proteases has been implicated in various stages of oncogenesis (33), an example of which involves NEP expression. Loss of NEP expression has been frequently observed in prostate cancer cells, which allows accumulated neuropeptides to bind their receptors and stimulate cell proliferation and migration (20–22). In addition to neuropeptidase activity, NEP is thought to participate in other biological processes that can affect the regulation of cell growth (34). The effects of NEP are mediated by inactivation of substrates such as bombesin and endothelin-1 through its peptidase activity, and through direct protein-protein interactions with ERM proteins and the PTEN tumor suppressor protein (35). Hyaluronan receptor CD44 is co-localized with ERM pro-

![Figure 6. Comparison of FERM-binding modes of NEP and ICAM-2 peptides bound to the FERM domain. A, comparison of NEP and ICAM-2 cytoplasmic peptides bound to the radixin FERM domain. Superposition of the ICAM-2 cytoplasmic tail (magenta) in the FERM-ICAM-2 complex on the NEP (blue)-FERM (gray) complex. The N-terminal (ICAM-2) or C-terminal (NEP) regions that would be linked to the trans-membrane helix is indicated with dotted lines. B, schematic representation of ERM peptides bound to type I and II membrane proteins on the plasma membrane. ERM proteins have the N-terminal FERM (blue triangle) and C-terminal F-actin binding (red block) domains. The FERM domain of membrane-recruited ERM proteins binds the cytoplasmic tails, whereas the C-terminal domain binds actin filaments (cyan). The FERM domain binds the juxtamembrane region (a yellow arrow, Motif-1) of the C-terminal cytoplasmic tail of type I membrane proteins such as adhesion molecules CD44 and ICAM-2. In the case of type II membrane protein NEP, the FERM domain binds the N-terminal cytoplasmic tail at a distal region (a pink arrow, Motif-1) from the membrane. These two binding interactions interfere with one another by direct competition for binding to the same groove of the FERM domain.](image-url)
teins in epithelial cells including prostate cancer cells, and the hyaluronan-CD44-ERM complex has been shown to be involved in the migration of metastatic breast adenocarcinoma cells (36, 37). Linking of the CD44 cytoplasmic tail to cytoskeletal components through ERM proteins is important for CD44-mediated adhesion or invasion. Truncation of the CD44 cytoplasmic tail reduces its capacity to assemble pericellular extracellular matrices and to bind hyaluronan (38). Our previous studies showed a CD44 Motif-1-like sequence (QXXKLXLXXG) was involved in ERM binding (15). Our present study revealed that NEP binding occurs at the Motif-1 binding site on the FERM domain and suggested that NEP binding to ERM proteins suppresses CD44-mediated cell adhesion by direct competition for ERM binding. In normal cells, NEP that is expressed and concentrated at a cell surface in excess relative to CD44 is able to sequester ERM proteins. Thus, our study provides a structural basis for NEP tumor suppression and the role of NEP loss in tumor progression (16).

The C-terminal juxtamembrane region of the NEP cytoplasmic tail contains five basic residues (Lys16, Lys18, Lys19, Lys20, and Arg22), which are projected from the molecular surface of the FERM domain in our FERM-NEP complex. Similarly, the N-terminal basic residues of the ICAM-2 cytoplasmic tail are exposed toward the solvent region and do not participate in interactions with the FERM domain (15). It has been proposed that the juxtamembrane basic region of ICAM-2 interacts with negatively charged PIP2 (15, 39). Like ICAM-2, the basic region of the NEP cytoplasmic tail may play a similar role through interactions with negatively charged phospholipids. Furthermore, the NEP cytoplasmic tail directly associates with the tumor suppressor PTEN through electrostatic interactions between the NEP cytoplasmic tail basic region (residues 16–22) and the phosphorylated C-terminal tail of PTEN (residues 380–385) (23). NEP constitutively recruits and activates PTEN, which antagonizes the activity of phosphatidylinositol 3-phosphate kinase by dephosphorylating phosphatidylinositol 3-phosphate kinase to PIP2, which inhibits the phosphatidylinositol 3-phosphate kinase/Akt oncogenic pathway. Thus, the NEP cytoplasmic tail possesses cooperative activity and seems to play a key role in suppressing both cell adhesion, mediated by the CD44-ERM interaction, and the phosphatidylinositol 3-phosphate kinase/Akt signaling pathway.

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