Peptide binding consensus of the NHE-RF-PDZ1 domain matches the C-terminal sequence of cystic fibrosis transmembrane conductance regulator (CFTR)

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Abstract The Na^+-H^+ exchanger regulatory factor (NHE-RF) is a cytoplasmic phosphoprotein that was first found to be involved in protein kinase A mediated regulation of ion transport. NHE-RF contains two distinct protein interaction PDZ domains: NHE-RF-PDZ1 and NHE-RF-PDZ2. However, their binding partners are currently unknown. Because PDZ domains usually bind to specific short linear C-terminal sequences, we have carried out affinity selection of random peptides for specific sequences that interact with the NHE-RF PDZ domains and found that NHE-RF-PDZ1 is capable of binding to the CFTR C-terminus. The specific and tight association suggests a potential regulatory role of NHE-RF in cystic fibrosis transmembrane conductance regulator (CFTR) function.

Key words: Cystic fibrosis; PDZ domain; Peptide display; PKA

1. Introduction

The Na^+-H^+ exchanger regulatory factor (NHE-RF) was first identified as a cofactor necessary for the protein kinase A mediated inhibition of Na^+-H^+ exchanger activity in renal brush border membranes [1]. Such activity was recently confirmed in tissue culture cells using a cloned cDNA [2]. This 42 kDa phosphoprotein contains two tandem protein interaction modules known as PDZ domains, which usually bind to short linear C-terminal sequences [3,4]. In addition to kidney brush border membrane where the NHE-RF protein was first isolated, both the messenger RNA and the protein of NHE-RF have been found in a wide variety of other tissues, suggesting that its functional roles may extend to other membrane signalling systems. Supporting this notion, recent studies of interacting proteins for members of the ezrin-radixin-moesin (ERM) family have identified an ERM binding phosphoprotein 50 (EBP50) from human placenta. Molecular cloning of EBP50, which binds tightly to ezrin, has revealed that EBP50 is a human counterpart of NHE-RF [5]. Thus, NHE-RF is associated with cytoskeletal structures.

Although the specific mechanistic role of NHE-RF in the PKA mediated regulation remains unknown, the phosphorylation appears to be essential for the regulatory function of NHE-RF. Since NHE-RF contains two homologous PDZ domains, it was thought that they might interact with the C-termini of the Na^+-H^+ exchanger and/or ezrin. Interestingly, recent evidence suggests that the C-terminus of the Na^+-H^+ exchanger 3 (NHE3) is not required for the binding of E3KARP (also known as tyrosine kinase activator-1, TKA-1, GenBank accession number: Z50150), a homologous protein of NHE-RF with similar modulatory activity to NHE3 in cell culture [2]. The interaction between NHE-RF (or EBP50) and ERM protein was shown to be mediated by the N-terminal domain of ezrin [5]. Thus, neither the C-terminus of NHE3 nor that of ezrin is required for the interaction with NHE-RF or EBP50. Therefore, the PDZ domains of NHE-RF may interact with other additional proteins that may be essential for the NHE-RF function in vivo.

In an effort to identify binding partners of NHE-RF, we have determined the peptide binding sequences for individual NHE-RF PDZ domains using random peptide display techniques [6]. We found that the two PDZ domains possess distinct ligand specificity. Based on the resultant binding consensus of the PDZ domains, a number of candidate interacting proteins for NHE-RF have been identified. Among them is the cystic fibrosis transmembrane conductance regulator (CFTR), whose C-terminus (TRL-COOH) matches the binding consensus of NHE-RF-PDZ1 and binds tightly to NHE-RF.

2. Methods and materials

2.1. Vector construction and fusion protein expression

NHE-RF cDNA was amplified from mouse total RNA by the reverse transcriptase (RT)-PCR method and confirmed by DNA sequencing. The PCR fragments corresponding to both the full-length coding sequence and the individual PDZ domains of NHE-RF were subcloned into the pGEX4T2 vector in order to produce glutathione S transferase (GST)-PDZ fusion proteins. The PCR fragments corresponding to the human CFTR C-terminal regions were subcloned into pGEX4T3 and pML80 vectors in order to produce GST-CFTR and *Lac*I repressor fusion of CFTR (*Lac*I-CFTR). The PCR fragments corresponding to the full-length and truncated NHE-RF were subcloned into the pCGN vector in order to produce the 12CA5-tagged fusion proteins in mammalian cells [7]. A series of oligonucleotides were synthesized for high fidelity PCR to amplify corresponding coding fragments. The sequences of the oligonucleotides are available on request. The GST fusion production and purification were carried out according to the standard protocol provided by the manufacturer (Pharmacia Biotech, Uppsala, Sweden). The expression of the HA-tagged fusion proteins was carried out by transient transfection of HEK293 cells [7].

2.2. Affinity panning

GST fusion proteins corresponding to NHE-RF-PDZ1 and NHE-RF-PDZ2 were used in affinity panning of the random peptide li-

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brary. The procedures for PDZ domain panning and ELISA detection have been described previously [6].

2.3. Pull-down experiments

At 48 h post-transfection, the HEK293 cells were washed twice with PBS and lysed in PBS supplemented with 1% Triton X-100, 5 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride (PMSF), 10 mM benzamidine-HCl, 10 μg/ml phenanthroline, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A. The supernatant was collected after centrifugation of the lysate at 27 000×*g* for 15 min at 4°C and incubated with a GST or GST-CFTR fusion protein bound to glutathione-agarose beads. After gentle rotation for 1 h at 4°C, the beads were washed once with lysis buffer, twice with lysis buffer supplemented with 0.5 M NaCl and three times with PBS at 4°C. Prior to SDS-polyacrylamide gel electrophoresis (PAGE), the bound material was eluted with SDS sample buffer. After SDS-PAGE separation the NHE-RF protein was detected by immunoblot with the mAb12CA5 antibody to the HA tag.

3. Results

3.1. Distinct binding specificity of the NHE-RF PDZ domains

A general feature of the PDZ domain mediated protein interactions is their ability to recognize a short carboxylated peptide at the C-terminus of a target protein. The crystal structure of the third PDZ domain in PSD-95 (PSD-95 PDZ3) has revealed that the binding pocket of the PDZ domain recognizes the last three residues and the C-terminal carboxyl group. Based on the cocrystal structure, amino acid positions that are involved in determining the peptide binding specificity have been inferred [8]. Comparison of primary sequences of different PDZ domains has shown a significant degree of divergence in some of the corresponding positions, suggestive of a distinct peptide binding specificity of the NHE-RF PDZ domains (Fig. 1).

To determine the peptide binding specificity for the PDZ domains of NHE-RF, two GST fusion proteins corresponding to PDZ1 (a.a. 1 to 107, NHE-RF-PDZ1) and PDZ2 (a.a. 133 to 244, NHE-RF-PDZ2) were constructed (see Section 2). Using the purified individual fusion proteins as targets, we carried out an affinity panning selection from a library of 15-mer random peptides. This technique is based on the strong protein-DNA interaction between the *Lac*I repressor and the lac operator sequence [9] and has previously been used to decode the binding specificity of various PDZ domains including an orphan PDZ domain of neuronal nitric oxide synthase (nNOS) [6] and PDZ domains of *Inactivation-no-afterpotential* D (INAD) ([10] and also see Section 2). After three rounds of affinity panning, the binding specificity of the isolated clones was examined by ELISA. The clones that specifically interacted with the NHE-RF PDZ domains, but not with the control GST fusion protein, were selected for DNA sequencing, which allows for the deduction of amino acid sequence of the binding peptides.

Fig. 2A shows an amino acid sequence alignment of the 34 independent peptide sequences recognized by NHE-RF-PDZ1. Among them, 20 clones end with TRL, five end with TRF, five end with SRL, two end with TYL, one ends with TRI, and one ends with ARL. There is no strong amino acid preference at the −3 position. The shortest peptide is the RFBP-I7 clone, where TRL alone is sufficient for binding to NHE-RF-PDZ1. Based on the amino acid abundance at the last three positions, the consensus sequence for NHE-RF-PDZ1 is S/T- R/Y-L-COOH (Fig. 3A). In the 16 independent peptide sequences recognized by NHE-RF-PDZ2, leucine again is strongly preferred (81%) at the 0 position. At the −1 position, tryptophan is also strongly preferred (93%). Interestingly, the −3 position of the NHE-RF-PDZ2 binding peptides is strongly preferred by serine or threonine (81%). Based on the amino acid abundance at each position, the consensus sequence for NHE-RF-PDZ2 is S-S/T-W-L-COOH (Fig. 3B). Consistent with the binding preference, we failed to detect any of the identified peptides capable of binding to both NHE-RF PDZ domains (Wang and Li, unpublished data). Thus, the NHE-RF-PDZ1 and NHE-RF-PDZ2 exhibit distinct peptide binding specificity.

3.2. Biochemical association between CFTR and NHE-RF

Identification of the ligand binding consensus of PDZ domains 1 and 2 of NHE-RF allows for a search of potential NHE-RF interacting proteins present in protein sequence databases. Interestingly, the human CFTR protein C-terminus ends with TRL-COOH, a perfect match for the PDZ1 binding consensus. This region is highly conserved and found in CFTR proteins from various species (Fig. 4), consistent with the idea that it may serve an important physiological function. To test the possible interaction between CFTR and NHE-RF, we generated three *Lac*I fusion proteins corresponding to the C-terminus of human CFTR (Fig. 5A). The CFTR-C1 contains the whole putative C-terminal cytoplasmic region of CFTR (a.a. 1211 to 1481) including the predicted nucleotide binding domain 2 (NBD2). The CFTR-C2 contains the last 94 amino acids immediately downstream from the NBD2. The

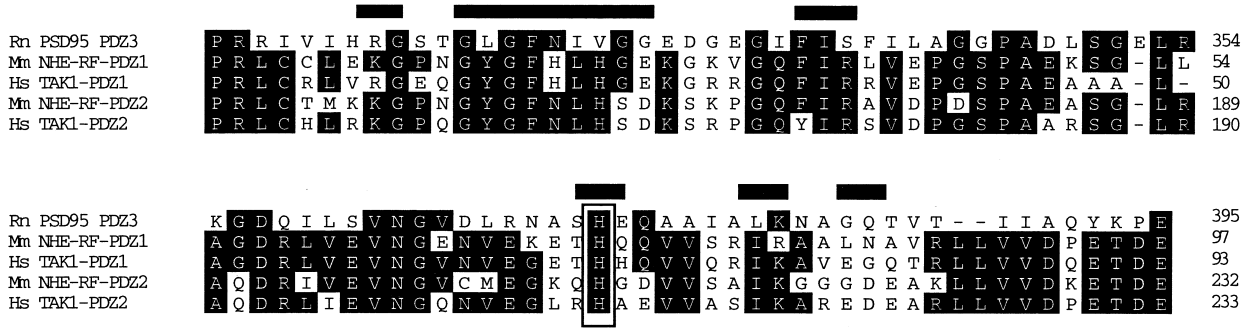


Fig. 1. Amino acid sequence alignment of the PDZ domains of NHE-RF and TKA-1 (Z50150) against the sequence of PDZ3 of PSD-95. Highlighted residues are conserved among most of the aligned PDZ domains. The black bars above the PSD-95 sequence indicate regions or residues that are putatively involved in determining the binding specificity of peptides [8]. The boxed residue is thought to determine the side chain preference at the −2 position of the bound peptide [6,8]. The numbers indicate the amino acid positions in the corresponding proteins.

A

NHE-RF-PDZ1 Binding Peptides

RFBP-I1	.....*GGG*VLEI**TRL***
RFBP-I2	*GGG*WVCRHTRWEEDC**TRL***
RFBP-I3	.....*GGG*WD**TRL***
RFBP-I4	*GGG*MTPSWVVVTSGS**TRL***
RFBP-I5	*GGG*SLVIPAIVPGTW**TRL***
RFBP-I6	*GGGG*SCALDVRVGEW**TRL***
RFBP-I7	.....*GGG***TRL***
RFBP-I8	*GGG*ELGGGPAMVEWM**TRL***
RFBP-I9	*GGG*VPSIDGDGLGDD**TRL***
RFBP-I10	.....*GGG*VVVEE**TRL***
RFBP-I11	*GGG*RGKLSWDNVNEW**TRL***
RFBP-I12	*GGGF*CIRGDGMGVMG**TRL***
RFBP-I13	*GGGC*LAGSGAGTFEW**TRL***
RFBP-I14	*GGGG*SGNKVVYDVEW**TRL***
RFBP-I15	*GGG*RKVQSDVRWSLG**TRL***
RFBP-I16	*GGGT*EGKWAIFDTLT**TRL***
RFBP-I17	*GGGP*TCALTDTGCED**TRL***
RFBP-I18	*GGGG*KSKWCSRGVEN**TRL***
RFBP-I19	....*GGG*TWDPLPMC**TRL***
RFBP-I20	*GGGY*ATGLDSVEPEE**TRL***
RFBP-I21	*GGGV*LELASCHMSQT**TRF***
RFBP-I22	.....*GGGC*E**TRF***
RFBP-I23	.....*GGG*MLIYEV**TRF***
RFBP-I24	*GGGT*GCARDTEEWDV**TRF***
RFBP-I25	.....*GGGY*L**TRF***
RFBP-I26	.....*GGGT*FMWD**TRI***
RFBP-I27	*GGGV*AWEGIGGHEEE**SRL***
RFBP-I28	.....*GGG*EMEN**SRL***
RFBP-I29	*GGGC*QLCVVGNGRQD**SRL***
RFBP-I30	*GGGV*WFDTDDRGPQD**SRL***
RFBP-I31	.....*GGGW***ARL***
RFBP-I32	.....*GGGF*DAEVC**TYL***
RFBP-I33	*GGGV*VFGRDNSWPEC**TYL***

B

NHE-RF-PDZ2 Binding Peptides

RFBP-II1	.....*GGGP*L**NAWL***
RFBP-II2	*GGGA*VTRGERGHME**SEWL***
RFBP-II3	*GGGK*RGPDTRLEDM**SSWL***
RFBP-II4	...*GGGV*DGYDCCA**SHWL***
RFBP-II5	.....*GGGT*TS**SSWL***
RFBP-II6	.....*GGG***STWL***
RFBP-II7	*GGGA*TATDRCGASK**STWM***
RFBP-II8	*GGGA*LVCGRTGPLD**STWM***
RFBP-II9	.....*GGGL***NSWL***
RFBP-II10	.....*GGGE*EAVGE**SSWL***
RFBP-II11	*GGGV*CQLLECGGTT**STWM***
RFBP-II12	.....*GGGV***SIWL***
RFBP-II13	.....*GGGE***SCWL***
RFBP-II14	*GGGV*RKHRTYSMGE**NTFL***
RFBP-II15	.....*GGGR*HT**SEWL***
RFBP-II16	*GGGD*VEPGGSVCQL**TSWL***

Fig. 2. Amino acid sequence alignment of the binding peptides of the NHE-RF PDZ domains. A group of 48 colonies was randomly selected after three rounds of affinity panning against either NHE-RF-PDZ1 or NHE-RF-PDZ2 and their binding specificity was tested individually by the *Lac*I ELISA [6]. The NHE-RF-PDZ-specific clones were sequenced. Amino acid sequences of independent clones are aligned. The asterisk indicates a stop codon. The three italic 'GGG's are part of the linker sequence which separates *Lac*I from random peptides in the vector.

CFTR-C3 contains only the last 15 amino acids. The three fusion proteins produced in *Escherichia coli* were incubated with purified NHE-RF-PDZ1 and NHE-RF-PDZ2. All three CFTR-*Lac*I fusion proteins are capable of binding to NHE-RF-PDZ1 but not to NHE-RF-PDZ2 as determined by ELISA. The binding signal of the CFTR C-terminal fusions to NHE-RF is comparable with that of a peptide (RFBP-I31) that is isolated from the previous affinity panning experiment (Fig. 5B).

To assess the binding affinity between NHE-RF and CFTR, we performed surface plasmon resonance (SPR) measurements (BIACore) using the immobilized CFTR peptide. By perfusing various concentrations of the purified NHE-RF-PDZ1, we found that the dissociation constant of NHE-RF-PDZ1 with the CFTR C-terminal peptide is 48 nM.

The C-terminal region of NHE-RF contains several phosphorylation sites including a putative kinase A site (Ser-338) and two potential cdc2 sites (Ser-279 and Ser-301). Because it was thought that phosphorylation may play a key role in the NHE-RF function, we tested whether the presence of the C-terminal region of NHE-RF would affect the interaction between NHE-RF-PDZ1 and the C-terminus of CFTR. The full-length NHE-RF as well as the truncated NHE-RF (with the deletion of the C-terminal region which contains the phosphorylation sites) were transfected in HEK293 cells. At 48 h after transfection, cells were harvested and lysed in the presence of 1% Triton X-100 (see Section 2). The expression of NHE-RF can be detected by immunoblot analysis. The full-length NHE-RF from transfected cells are two closely migrating bands (Fig. 6, lane 4), characteristic of the NHE-RF protein in native tissues (Wang and Li, unpublished data). The lysates were incubated with either GST alone or the GST-CFTR-C3 fusion protein. Fig. 6 shows that GST-CFTR-C3, containing only the last 15 amino acids of CFTR, is capable of pulling down both the truncated NHE-RF, containing only the two PDZ domains, and the full-length NHE-RF from HEK293 cells (Fig. 6, lanes 3 and 6).

4. Discussion

Affinity selection of specific binding sequences from a random peptide library has revealed that PDZ1 and PDZ2 of NHE-RF have distinct ligand binding specificity. Interestingly, the binding consensus of NHE-RF-PDZ1 is in good agreement with the C-terminal sequence of CFTR. The results of binding studies demonstrate that NHE-RF is capable of interacting directly with CFTR.

Mutagenesis and structural studies have shown that histidine-372 of PSD-95 is essential for determining the amino acid specificity at the −2 position of the T/S-X-V binding consensus [6,8]. The strong preference of serine or threonine at the −2 position for NHE-RF-PDZ1 and NHE-RF-PDZ2 is in good agreement with the existing evidence, since histidine residues are found in both PDZ domains at the corresponding positions (see boxed residue in Fig. 1). The PSD-95 PDZ3 binds to a consensus of E-(S/T)-X-V-COOH [6]. Consistent with the weak binding preference at the −1 position, a co-crystal of PSD-95 PDZ3 with a bound peptide shows that the side chain of the −1 residue points away from the PDZ binding pocket [8]. In contrast to a typical PDZ domain, such as PDZ3 of PSD-95, both NHE-RF-PDZ1 and NHE-RF-PDZ2 have strong preference for residues at the −1 position (Fig. 3), supporting the notion that NHE-RF-PDZ1 and NHE-RF-PDZ2 have distinct substrate specificity.

Although it is well documented that NHE-RF serves as a cofactor in the cAMP mediated inhibition of NHE3 [1,11], it remains unknown whether NHE-RF participates in the PKA

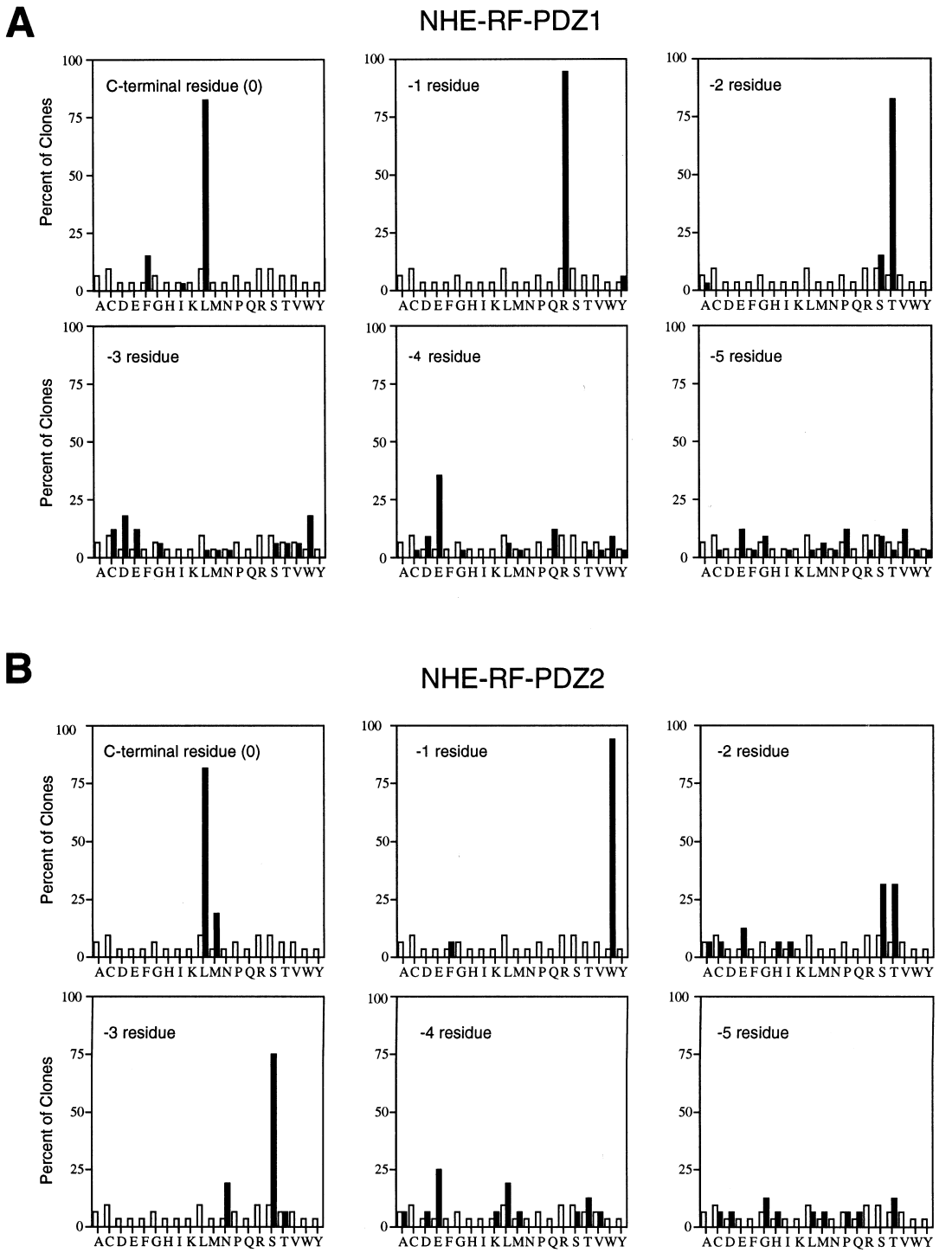


Fig. 3. Amino acid abundance at each position for the NHE-RF-PDZ1 (panel A) and NHE-RF-PDZ2 (panel B) binding peptides was determined and is shown in histogram format. The horizontal axis indicates the amino acid in single-letter codes. The filled bars show actual abundance in percentage; empty bars indicate expected abundance in the starting library.

modulation through a more general mechanism which is not restricted to Na^+-H^+ exchangers. It is clear, however, that the NHE-RF protein has been found in many tissues including, but not limited to, tissues where NHE3 is expressed [1,5]. Most importantly, in addition to renal brush border membrane, we found that the NHE-RF protein was also present in lung and pancreas, which are the sites where the function of CFTR is essential ([12] and Wang and Li, unpublished results). It is, therefore, conceivable that NHE-RF may play an important regulatory role in membrane signalling systems in addition to Na^+-H^+ exchangers.

Besides activation by protein kinase A, the CFTR chloride channel activity is also regulated by a number of other mechanisms. In particular, it is known that actin filaments are somehow involved in channel gating of CFTR [13–15]. For example, cytochalasin D significantly attenuates both $^{36}Cl^-$ effluxes and whole-cell current in bronchial epithelial cells after activation by typical CFTR agonists. What would be

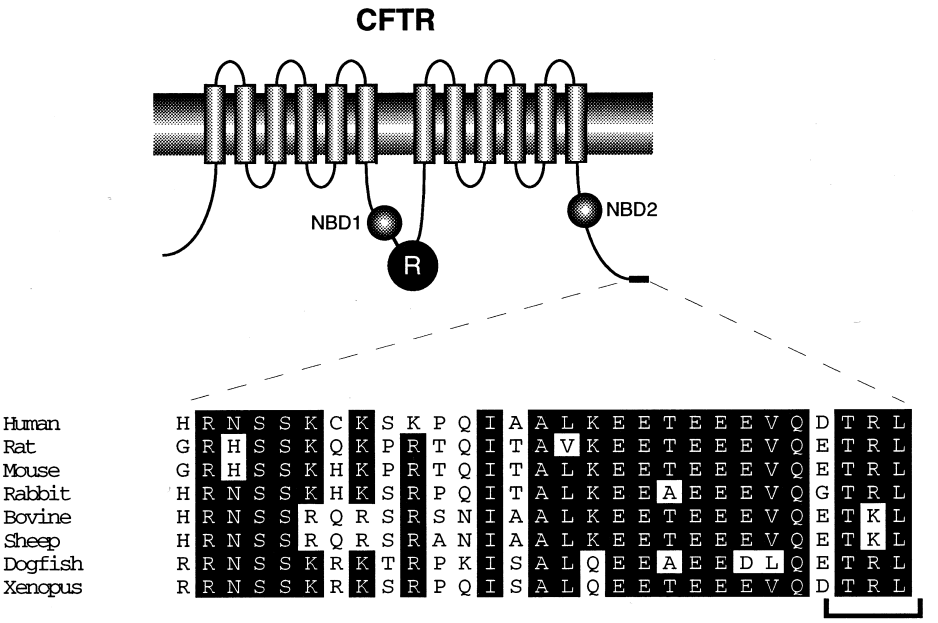
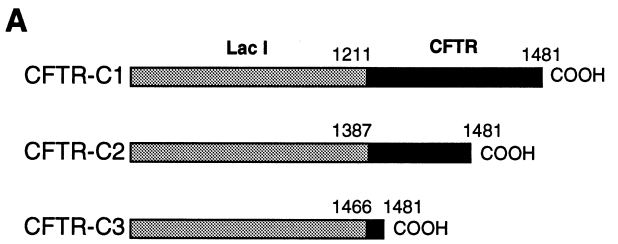


Fig. 4. Amino acid sequence alignment of C-termini of CFTR from human (P13569), rat, mouse (M60439), rabbit (Q00554), bovine (P35071), sheep (U20418), dogfish (P26362), and *Xenopus* (U60209). The bracketed last three residues are in good agreement with the binding consensus of NHE-RF-PDZ1.



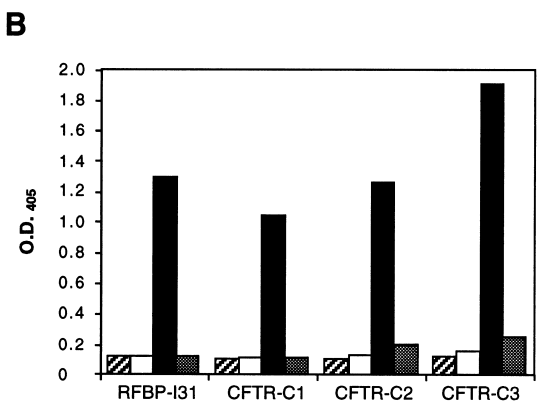


Fig. 5. Direct binding of the CFTR C-terminus to NHE-RF-PDZ1. A: Schematic diagram of three *Lac*I fusion constructs of CFTR C-terminal fragments. The shaded boxes represent the *Lac*I portion; the filled boxes represent the CFTR coding segment. The numbers above each construct indicate the corresponding amino acid positions in the human CFTR protein. B: Binding of CFTR to the NHE-RF PDZ domains. The association of *Lac*I fusions with various target proteins was tested by ELISA. Microtiter wells were coated with BSA only (dashed box), GST-NAB (a control GST fusion protein, open box), GST-NHE-RF-PDZ1 (filled box), or GST-NHE-RF-PDZ2 (gray box). Binding signal is shown in optical density at a wavelength of 405 nm, and individual *Lac*I fusions are as indicated. The corresponding amino acid sequence of RFBP-I31 can be found in Fig. 2A.

the functional role of the NHE-RF and CFTR interaction? The ability of NHE-RF to bind both CFTR and ezrin suggests a working model whereby NHE-RF serves as an anchor linking CFTR to ezrin. Because ezrin normally binds to actin, the linkage mediated by NHE-RF would allow for a physical connection of membrane-bound CFTR to cytoskeletal components.

The PKA mediated activation is essential for CFTR function in vivo [16]. However, the mechanistic details of the PKA stimulation remain unknown. In addition to direct phospho-

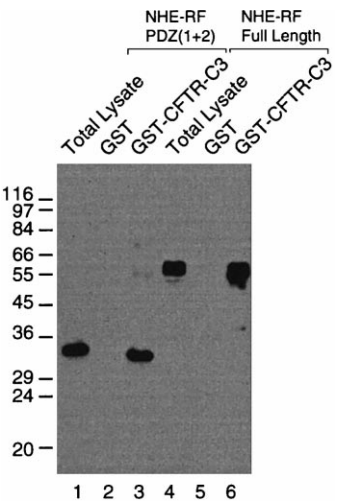


Fig. 6. Binding of CFTR to NHE-RF. Full-length and truncated NHE-RF were expressed transiently in HEK293 cells as 12CA5-tagged fusions. At 48 h after transfection, soluble cell lysates were prepared (lanes 1 and 4). After incubation with GST only (lanes 2 and 5) or GST-CFTR-C3 (lanes 3 and 6), the bound proteins were separated by SDS-PAGE. The full-length or truncated NHE-RF were detected by probing the filter with the monoclonal antibody specific for the 12CA5 tag. The protein molecular weight standards are marked on the left in kDa.

rylation of the CFTR protein, existing evidence also supports a model of the PKA-stimulated vesicle fusion [17]. Recent work reported that ezrin, a member of the ERM family, is capable of binding to PKA regulatory subunit II (RII) [18]. Thus, NHE-RF may also function by binding to members of the ERM family, thereby recruiting PKA machinery into the proximity of the acting site.

Cystic fibrosis is a common genetic disorder in the Caucasian population. Although the major mutation of ΔF508 accounts for 70% of the disease alleles, more than 550 additional mutant alleles of different forms have been identified. Interestingly, there are at least three nonsense mutations mapped at the C-terminus after NBD2 (see a recent review by Zielenski and Tsui [12]). These genetic data link the CFTR function to the integrity of the C-terminus, which may function by interacting with cytoplasmic proteins, such as NHE-RF. With the identification of the peptide binding preference of the NHE-RF PDZ domains and biochemical confirmation of the physical association between NHE-RF and CFTR in vitro, it is now possible to design experiments to test directly whether and how NHE-RF may participate in CFTR function in vivo.

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