Characterization of cytoskeletal protein 4.1R interaction with NHE1 (Na\(^+\)/H\(^+\) exchanger isoform 1)

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NHE1 (Na\(^+\)/H\(^+\) exchanger isoform 1) has been reported to be hyperactive in 4.1R-null erythrocytes [Rivera, De Franceschi, Peters, Gascard, Mohandas and Brugnara (2006) Am. J. Physiol. Cell Physiol. 291, C880–C886], supporting a functional interaction between NHE1 and 4.1R. In the present paper we demonstrate that 4.1R binds directly to the NHE1cd (cytoplasmic domain of NHE1) through the interaction of an EED motif in the 4.1R FERM (4.1/ezrin/radixin/moesin) domain with two clusters of basic amino acids in the NHE1cd, K\(^{519}\)R and R\(^{556}\)FNKKYYK, previously shown to mediate PIP\(_2\) (phosphatidylinositol 4,5-bisphosphate) binding [Aharonovitz, Zaun, Balla, York, Orlowski and Grinstein (2000) J. Cell. Biol. 150, 213–224]. The affinity of this interaction \((K_a = 100–200 \text{ nM})\) is reduced in hypertonic and acidic conditions, demonstrating that this interaction is of an electrostatic nature. The binding affinity is also reduced upon binding of Ca\(^{2+}\)/CaM (Ca\(^{2+}\)-saturated calmodulin) to the 4.1R FERM domain. We propose that 4.1R regulates NHE1 activity through a direct protein–protein interaction that can be modulated by intracellular pH and Na\(^+\) and Ca\(^{2+}\) concentrations.

Key words: calmodulin, cytoskeleton, Na\(^+\)/H\(^+\) exchanger isoform 1 (NHE1), protein 4.1R, sodium/proton exchange.

INTRODUCTION

NHE (Na\(^+\)/H\(^+\) exchanger) 1 is the most ubiquitously expressed member of the NHE family and is therefore often considered the ‘housekeeping’ NHE. NHE1 participates in the regulation of intracellular volume and pH in all cell types, including erythrocytes [1]. It is also the major isoform expressed in mammalian erythrocytes [1–6]. Highly relevant to the present study is that NHE1 is the only resident plasma membrane NHE isoform in erythrocytes. There is now a large body of evidence for the membrane skeleton being involved in regulating the basal rate of NHE1-mediated ion transport and in the transmission of signals that modulate NHE1 activity in response to physical stimuli [1–7]. Additionally, a recent study showed that Add2 (\(\beta\)-adducin)-null mouse erythrocytes, which adopt a spherocytic shape, lack \(\alpha\)-adducin and SLC9A1/NHE1 [8]. Protein 4.1R-null mouse erythrocytes exhibit a dramatic NHE1 phenotype characterized by cell dehydration and high intracellular Na\(^+\) resulting from hyperactivity of NHE1 [9,10]. This latter observation demonstrates unequivocally a role for 4.1R in modulating NHE1 activity. The aim of the present study was to extend these findings by performing a detailed characterization of 4.1R\(^{80}\)-NHE1 interaction and of its regulation.

Protein 4.1R is a key membrane skeletal protein in erythrocytes. The erythrocyte isoform, 4.1R\(^{80}\), is composed of four major chymotryptic domains, an N-terminal 30 kDa domain referred to as the ‘FERM’ (4.1/ezrin/radixin/moesin) domain, a 16 kDa domain, a 10 kDa domain and a C-terminal 24 kDa domain [11,12]. The FERM domain mediates interaction with various transmembrane proteins, including the anion exchanger band 3 [13–15], GPC (glycophorin C) [16], CD44 [17] and with the membrane-associated protein p55 [18,19]. The 10 kDa domain of 4.1R\(^{80}\) binds to spectrin and actin filaments [11,12]. Through these multiple and dynamic interactions, 4.1R\(^{80}\) participates in the maintenance of the mechanical stability of human erythrocytes.

CaM (calmodulin), in association with Ca\(^{2+}\), regulates 4.1R\(^{80}\) binding to membrane proteins [12,20,21] and to the spectrin–actin complex [20] in human erythrocytes. As a result, it destabilizes the erythrocyte membrane mechanical stability [12,22]. We have previously shown that the binding affinity of the 4.1R FERM domain for band 3 and GPC decreases when Ca\(^{2+}\)/CaM (Ca\(^{2+}\)-saturated calmodulin) binds simultaneously to two regions of the 4.1R FERM domain: the A\(^{290}\)KKLWKVCEHHTFFRL peptide (pep 11) that mediates Ca\(^{2+}\)-independent CaM binding and the A\(^{185}\)KKLMSYGVDDLHKAKDL peptide that is responsible for Ca\(^{2+}\)-sensitive CaM binding [23]. Ser\(^{185}\) in the second peptide sequence is critical for the Ca\(^{2+}\)-dependent regulation of the 4.1R FERM domain binding to membrane proteins by CaM, and mutation of Ser\(^{185}\) results in a loss of Ca\(^{2+}\)/CaM regulatory activity [23,24].

On the basis of sequence homology with the motif in the cd (cytoplasmic domain) of AE1 (anion exchanger isoform 1 or band 3), referred to as band 3cd, which we have previously shown mediates the interaction of band 3 with 4.1R [15], we identified positively charged candidate binding sequences in the juxta-membrane region of NHE1 cytoplasmic domain (NHE1cd) that may mediate NHE1 interaction with 4.1R [Figure 1A]. Strikingly, these basic amino acids sequences in the NHE1cd,
to assess non-specific binding) were incubated with \[35S\]methionine-labelled in vitro-translated NHE1cd lacking amino acids 501–637 in its juxta-membrane region (lane 2) or GST alone (lane 3; proteins encoding the C-terminal NHE1cds (amino acids 501–815; lane 1) or of a variant of the charged residues further downstream being referred to as the M2 motif (R 556FNKKYVKK) present in the juxta-membrane region of the NHE1cd (shown in bold and larger font). This interaction with PIP2 (phosphatidylinositol 4,5-bisphosphate) acid cluster KKK, very similar to the RRR band 3 motif involved in the 4.1R80 interaction, is domain exon 5-encoded peptide (underlined characters). Strikingly, a positively charged amino acid cluster interacts primarily with a negatively charged EED motif located in the 4.1R FERM (shown in bold and larger font) in the juxta-membrane region of the cd [4]. This critical for the 4.1R80–NHE1 interaction. The interaction between domain and two clusters of basic amino acids in the NHE1cd as 4.1R80 to NHE1 differed from that previously described because Ca\(^{2+}/CaM\) reduces the affinity of 4.1R80 binding to NHE1, thus favouring PIP2 binding to NHE1, and that sustained binding of PIP2 to NHE1 may account for the hyperactivation of NHE1 observed in 4.1R-null mouse erythrocytes [25]. The present study provides novel insights into the complexity and the versatility of the structural and functional interactions between the cytoskeleton and ion exchangers.

**EXPERIMENTAL**

**Cloning of cytoplasmic domain of transmembrane proteins**

cDNAs encoding full-length (P501–Q815) or truncated (N638–Q815) cds of wild-type rat (Rattus norvegicus) NHE1 (NHE1cd, NCBI accession number NP_036784), or mutant M1 (K501KKQETKR→AAAQETAA), mutant M2 (R506FNKKYVK→AAFNAYVAA) and the double mutant M1 + M2 rat NHE1 cd N-terminal region (L506–Q575) were cloned into the GST (glutathione transferase)-fusion protein vector pGEX-2T (GE Healthcare) (Figures 1A and 1B). A less extensively mutated version of the M1 mutant (K501KK→AAA), referred to as the M3 mutant, was generated from the wild-type construct by site-directed mutagenesis following the manufacturer’s recommendations (Stratagene). Preparation of the 43 kDa cytoplasmic domain of human band 3cd has been described previously [23].

**Preparation of recombinant full-length 4.1R80 and its polypeptide fragments**

cDNAs encoding full-length human 4.1R80 (NCBI accession number P11171, expressing the exon 16-encoded region, but lacking the exon 17b-encoded region) was cloned 5’-NsiI-Xhol-3’ into the His-tagged pET31b(+) vector (Novagen). His-tagged 4.1R80 was expressed in BL21 Gold (DE3) Escherichia coli cells (Stratagene) and purified according to the manufacturer’s instructions (Novagen). Recombinant 4.1R FERM domain was cloned into the pGEX-KG vector and expressed as a GST-fusion protein in BL21 bacteria [27]. Following sonication [10 kHz, (50 % power out) for 5 min on ice using an ultrasonic homogenizer (Smut NR-50 M, Microtec Nition)] the bacterial lysate was loaded on to a glutathione-affinity purification column and the recombinant GST-fusion protein was eluted from the column after cleavage of the GST tag with thrombin as described previously [27]. After desalting, the protein was further purified on a heparin–Sepharose column to remove contaminants and breakdown products. Finally, the 4.1R FERM domain was loaded on to a Sepharyl S-100 size-exclusion chromatography column equilibrated with 50 mM Tris/HCl (pH 7.5), 0.5 M NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM benzamidine, 1 % glycerol and 2 mM NaF. Preparation of the exon 5 region-deleted FERM domain (FERM ΔExon5) and E9ED mutagenesis to A96AA (FERM exon 5 mut.) have been described previously [28]. A cDNA encoding exon 5-encoded human 4.1R80 polypeptide was cloned using 5’-EcoRI-Xhol-3’ into the GST-fusion protein vector pGEX4T-2 vector (GE Healthcare). The domains of 4.1R80, including the 16 kDa, 10 kDa and 22 kDa C-terminal domains were also expressed as GST-fusion proteins as described previously [23]. All of the constructs were checked by DNA sequencing (Elim BioPharmaceuticals) prior to expression in bacteria. After affinity purification, recombinant proteins were dialysed extensively against 10 mM Na2HPO4/NaH2PO4 (pH 7.4) containing 0.15 M NaCl (PBS) prior to use in IAsys® binding assays. Protein purity was assessed by

![Figure 1 Alignment of AE1 (band 3) and the NHE1cd juxta-membrane regions and of 4.1R exons 5-encoded wild-type and EED mutant peptides and pull-down assay showing interaction of 4.1R80 with the cd of the sodium/proton exchanger NHE1cd.](image-url)

(A) The motif responsible for anion exchanger AE1 (also known as band 3, NCBI accession number P23562) interaction with 4.1R80 has been identified as a positively charged RRH cluster (shown in bold and larger font) in the juxta-membrane region of the cd [4]. This cluster interacts primarily with a negatively charged EED motif located in the 4.1R FERM domain exon 5-encoded peptide (underlined characters). Strikingly, a positively charged amino acid cluster KKK, very similar to the RRH band 3 motif involved in the 4.1R80 interaction, is present in the juxta-membrane region of the NHE1cd (shown in bold and larger font). This cluster is part of the previously defined M1 motif (V501KKQETKR), another cluster of positively charged residues further downstream being referred to as the M2 motif (R506FNKKYVK→AAFNAYVAA) and the double mutant M1 + M2 rat NHE1cd N-terminal region (L506–Q575) were cloned into the GST (glutathione transferase)-fusion protein vector pGEX-2T (GE Healthcare) (Figures 1A and 1B). A less extensively mutated version of the M1 mutant (K501KK→AAA), referred to as the M3 mutant, was generated from the wild-type construct by site-directed mutagenesis following the manufacturer’s recommendations (Stratagene). Preparation of the 43 kDa cytoplasmic domain of human band 3cd has been described previously [23].

K501KKQETKR and R506FNKKYVK→AAFNAYVAA, were previously reported to play a key role in increasing NHE1 activity through their interaction with PIP2 (phosphatidylinositol 4,5-bisphosphate) [25]. We identified the E9ED sequence in the 4.1R80 FERM domain and two clusters of basic amino acids in the NHE1cd as critical for the 4.1R80–NHE1 interaction. The interaction between 4.1R80 and NHE1 differed from that previously described between 4.1R80 and band 3, not only because of the type and number of motifs it involved, but also because the binding of 4.1R80 to NHE1 was more sensitive to tonicity and pH than 4.1R80 binding to band 3. Furthermore, the interaction between 4.1R80 and NHE1cd differed from that previously described for ERM (ezrin/radixin/moesin) proteins and NHE1cd [26] because the binding of 4.1R80 required both basic sequences. In addition, because Ca\(^{2+}/CaM\) reduced the binding affinity of the 4.1R80 FERM domain for NHE1cd, we hypothesize that Ca\(^{2+}/CaM\) reduces the affinity of 4.1R80 binding to NHE1, thus favouring PIP2 binding to NHE1, and that sustained binding of PIP2 to NHE1 may account for the hyperactivation of NHE1 observed in 4.1R-null mouse erythrocytes [25]. The present study provides novel insights into the complexity and the versatility of the structural and functional interactions between the cytoskeleton and ion exchangers.
SDS/PAGE. Proteins were separated on a 12.5% polyacrylamide gel and stained with Gelcode Blue® (Pierce). The FERM domain concentration was determined by absorbance at 280 nm, the ε<sub>1%</sub> corresponding to 14 at molar concentration for tyrosine (ε = 1340), tryptophan (ε = 5550) and cysteine (ε = 200) [29].

**Preparation of recombinant CaM**

A cDNA encoding recombinant CaM with four extra N-terminal amino acids, Gly-Ser-His-Met, was a gift from Ms Marilyn Parra (Lawrence Berkeley National Laboratory, University of California at Berkeley, CA, U.S.A.). Human CaM was cloned into the His-tag pET15b (+) vector after restriction of the NdeI cloning site. Recombinant CaM was purified sequentially on a Ni<sup>2+</sup> column, a phenyl Sepharose column and a Sephacryl S-100 column equilibrated with 50 mM Tris/HCl (pH 7.5) containing 500 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 2 mM NaF and 1% glycerol. Protein retention time was recorded on an Akta Prime Plus™ system (GE Healthcare). The purity of recombinant CaM was assessed by SDS/PAGE (15% gel) and Western blot analysis. The CaM concentration was calculated based on the absorbance at 280 nm and an ε<sub>1%</sub> of 1.6 for CaM [27].

**Preparation of PC (phosphatidylcholine) and PIP<sub>2</sub>/PC liposomes and liposome binding to NHE1<sub>cd</sub>**

PC liposomes containing 20% PIP<sub>2</sub> (PIP<sub>2</sub>/PC) or liposomes containing only PC (used as a control) were prepared as described previously [30,31]. Liposomes were resuspended at 1 mg/ml in 20 mM Mes (pH 6.0 or 6.8), or 50 mM Tris/HCl (pH 7.5), 0.1 M NaCl and 1 mM EDTA with or without 1.5 mM CaCl<sub>2</sub>. PIP<sub>2</sub>/PC or PC liposome binding to NHE1<sub>cd</sub> immobilized on an aminosilane cuvette was evaluated by measurement of maximal binding as described below and in our previous study [32].

**Pull-down assays**

Pull-down assays were performed as described previously [26,33]. Briefly, GST or GST-fusion proteins encoding full-length or truncated cDNA of NHE1 (P<sup>501–Q815</sup> and N<sup>638–Q815</sup>) were purified from BL21 cells induced at 28°C with 1.500 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 2 mM NaF and 1% glycerol. Protein retention time was recorded on an Akta Prime Plus™ system (GE Healthcare). The purity of recombinant CaM was assessed by SDS/PAGE (15% gel) and Western blot analysis. The CaM concentration was calculated based on the absorbance at 280 nm and an ε<sub>1%</sub> of 1.6 for CaM [27].

**Resonant mirror detection binding assays**

Kinetic analysis

Interactions of the 4.1R FERM domain with NHE1<sub>cd</sub> were examined using the IAsys<sup>®</sup> resonant mirror detection system following the manufacturer’s instructions (Affinity Sensors) [34]. In the following, the protein immobilized on the cuvette is referred to as the ‘ligand’, whereas the protein added in solution to the cuvette is referred to as the ‘analyte’. The GST-fusion protein of NHE1<sub>cd</sub> was immobilized on aminosilane cuvettes as described previously [17,24]. The 4.1R FERM domain, dissolved in 50 mM Tris/HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA and 1 mM 2-mercaptoethanol (buffer A), was added at concentrations ranging from 50 nM to 1 μM. All of the binding assays were conducted at 25°C with constant stirring. Kinetic analysis of analyte binding to ligand was conducted as described previously [12,17,23]. Dissociation constants at equilibrium (K<sub>d</sub>) were calculated using eqn (1):

\[
K_d = \frac{k_a}{k_d}
\]

where k<sub>a</sub> is the association rate constant and k<sub>d</sub> is the dissociation rate constant. The K<sub>d</sub> value was obtained from the means of three to five measurements for k<sub>a</sub> and k<sub>d</sub> and was confirmed by Scatchard plotting using maximum binding (B<sub>max</sub>) and molar concentrations of analyte [23,24,35]. The B<sub>max</sub> value was calculated from the binding characteristics using the software package FAST-fit<sup>®</sup> version 2.1.

**Maximum binding**

The maximum response detected upon addition of the 4.1R FERM domain to GST–NHE1<sub>cd</sub>, B<sub>max</sub> (expressed in arc seconds), was estimated from the binding profile using the software package FAST-fit<sup>®</sup> version 2.1. The stoichiometry of the 4.1R FERM domain binding to GST–NHE1<sub>cd</sub> was calculated from the equation: (B<sub>max</sub> 4.1R FERM domain/32428):(GST–NHE1<sub>cd</sub>/30381), where 32428 and 30381 are the apparent molecular masses in Da for the 4.1R FERM domain and GST–NHE1<sub>cd</sub> respectively) following the manufacturer’s protocols. The cuvettes were reused after cleaning with 20 mM HCl. The original binding curves could be replicated after HCl washing, indicating that the washing had not denatured the bound ligands.

**NaCl-dependent FERM domain binding to NHE1<sub>cd</sub> and band 3cd**

4.1R FERM domain binding to NHE1<sub>cd</sub> was evaluated in the presence of increasing NaCl concentrations (~0.1–0.5 M) in 50 mM Tris/HCl (pH 7.5), 1 mM EDTA and 1 mM 2-mercaptoethanol, using B<sub>max</sub>, as described above.

**pH-dependent 4.1R FERM domain binding to NHE1<sub>cd</sub> and band 3cd**

The 4.1R FERM domain was dissolved in various buffers: 0.1 M NaCl, 1 mM EDTA and 1 mM 2-mercaptoethanol; 50 mM CH<sub>3</sub>COOH/CH<sub>3</sub>COONa buffered at pH 4.0, 5.0, 5.5 or 6.0; 25 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> buffered at pH 6.0, 6.5, 6.8, 7.0, 7.2, 7.5 or 8.0; 50 mM Tris/HCl, buffered at pH 7.5, 8.0 or 8.5; and 50 mM NaHCO<sub>3</sub>/Na<sub>2</sub>CO<sub>3</sub> buffered at pH 9.0. Kinetic binding analysis of NHE1<sub>cd</sub> with the band 3cd was then performed.

**Binding ratio determination using the quartz crystal microbalance**

Single-Q<sup>®</sup> (Scinics) based on quartz crystal microbalance was used to determine the stoichiometry of the 4.1R FERM domain binding with NHE1<sub>cd</sub>. NHE1<sub>cd</sub> was immobilized on the sensor chip according to SingleQ instructions and changes in its mass following the addition (binding) of the 4.1R FERM domain were recorded [36]. The maximal binding value (B<sub>max</sub>) of the 4.1R FERM domain with NHE1<sub>cd</sub> immobilized on the sensor chip was determined.
Table 1  *In vitro* interactions of 4.1R<sup>80</sup> with NHE1<sub>cd</sub>

The *K<sub>d</sub>* values for interactions of the 4.1R<sup>80</sup>, several versions of the 4.1R FERM domain or the 4.1R FERM domain exon 5-encoded peptide with immobilized NHE1<sub>cd</sub> are shown. Wild-type 4.1R FERM domain (FERM), or a FERM domain either lacking exon 5 (FERM/A<sub>Exon5</sub>) or bearing a mutation of the EED motif (FERM EED mut.) were probed. The sequences for wild-type NHE1<sub>cd</sub> (Wild), or M1, M2, M3 and M1 + M2 NHE1<sub>cd</sub> mutants are shown in Figure 1. Each analyte (50 nM–1 μM) was incubated with the immobilized ligand as described in the Experimental section. The *K<sub>d</sub>* values were determined from the binding curves obtained by the resonant mirror detection method using the software package FAST-FM<sup>®</sup>.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>NHE1&lt;sub&gt;cd&lt;/sub&gt;</th>
<th>*k&lt;sub&gt;s&lt;/sub&gt; (M&lt;sup&gt;−1&lt;/sup&gt;s&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>*k&lt;sub&gt;0&lt;/sub&gt; (s&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>*K&lt;sub&gt;d&lt;/sub&gt; (nM)</th>
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<tr>
<td>4.1R&lt;sup&gt;80&lt;/sup&gt; Wild</td>
<td>(1.0 ± 0.20)×10&lt;sup&gt;−3&lt;/sup&gt;</td>
<td>(9.6 ± 0.11)×10&lt;sup&gt;−3&lt;/sup&gt;</td>
<td>99 ± 23</td>
<td></td>
</tr>
<tr>
<td>4.1R&lt;sup&gt;80&lt;/sup&gt; FERM</td>
<td>(4.3 ± 0.19)×10&lt;sup&gt;−3&lt;/sup&gt;</td>
<td>(9.5 ± 0.18)×10&lt;sup&gt;−3&lt;/sup&gt;</td>
<td>214 ± 17</td>
<td></td>
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<tr>
<td>4.1R&lt;sup&gt;80&lt;/sup&gt; Exon5</td>
<td>(4.8 ± 0.25)×10&lt;sup&gt;−3&lt;/sup&gt;</td>
<td>(5.1 ± 0.30)×10&lt;sup&gt;−3&lt;/sup&gt;</td>
<td>106 ± 6</td>
<td></td>
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<tr>
<td>4.1R&lt;sup&gt;80&lt;/sup&gt; FERM/A&lt;sub&gt;Exon5&lt;/sub&gt; Wild</td>
<td>No binding</td>
<td>No binding</td>
<td>No binding</td>
<td></td>
</tr>
<tr>
<td>4.1R&lt;sup&gt;80&lt;/sup&gt; FERM EED mut. Wild</td>
<td>(1.2 ± 0.14)×10&lt;sup&gt;−4&lt;/sup&gt;</td>
<td>(1.7 ± 0.15)×10&lt;sup&gt;−4&lt;/sup&gt;</td>
<td>1442 ± 211</td>
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<tr>
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<td>No binding</td>
<td>No binding</td>
<td></td>
</tr>
<tr>
<td>4.1R&lt;sup&gt;80&lt;/sup&gt; FERM M2</td>
<td>No binding</td>
<td>No binding</td>
<td>No binding</td>
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<tr>
<td>4.1R&lt;sup&gt;80&lt;/sup&gt; Exon5 M1</td>
<td>(2.5 ± 0.25)×10&lt;sup&gt;−2&lt;/sup&gt;</td>
<td>(1.2 ± 0.30)×10&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>4791 ± 652</td>
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<tr>
<td>4.1R&lt;sup&gt;80&lt;/sup&gt; Exon5 M2</td>
<td>(2.4 ± 0.25)×10&lt;sup&gt;−3&lt;/sup&gt;</td>
<td>(6.2 ± 0.30)×10&lt;sup&gt;−3&lt;/sup&gt;</td>
<td>26 060 ± 2465</td>
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<tr>
<td>4.1R&lt;sup&gt;80&lt;/sup&gt; FERM M3</td>
<td>(1.2 ± 0.10)×10&lt;sup&gt;−3&lt;/sup&gt;</td>
<td>(6.5 ± 0.36)×10&lt;sup&gt;−2&lt;/sup&gt;</td>
<td>545 ± 47</td>
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<td>No binding</td>
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**RESULTS**

Direct interaction of 4.1R<sup>80</sup> with NHE1 *in vitro*

Our previous finding that NHE1 is hyperactive in 4.1R-null mouse erythrocytes [10] suggested a functional interaction between 4.1R and NHE1. In testing this prediction, we now show that this phenotype reveals an actual physical interaction between 4.1R<sup>80</sup> and NHE1. A GST-fusion protein encoding the cytoplasmic domain of NHE1 (NHE1<sub>cd</sub>; amino acids 501–815) was able and NHE1. A GST-fusion protein encoding the cytoplasmic domain of NHE1 (NHE1<sub>cd</sub>) was able to pull down *in vitro*-translated human 4.1R<sup>80</sup> (Figure 1C, lane 1). In contrast, a GST–NHE1<sub>cd</sub> construct lacking the juxta-membrane region of NHE1<sub>cd</sub> (amino acids 501–637; Figure 1C, lane 2), or GST alone (Figure 1C, lane 3), showed a markedly reduced interaction with 4.1R<sup>80</sup>. This observation documented that the NHE1 peptide encompassing P<sup>99</sup>–N<sup>55</sup> mediated NHE1 interaction with 4.1R<sup>80</sup> (Figure 1B). IAsys®-based *in vitro*-binding assays enabled us to further identify the minimal region in NHE1<sub>cd</sub> interacting with 4.1R<sup>80</sup> to the L<sup>260</sup>–Q<sup>272</sup> peptide (Table 1). Of particular note, the binding affinity of 4.1R<sup>80</sup> for NHE1 was very similar (*K<sub>d</sub>* ~100–200 nM; Table 1) to those previously reported for 4.1R<sup>80</sup> binding to its two major transmembrane binding partners in erythrocytes, GPC and band 3 [19,23].

Mapping of the motifs in the 4.1R FERM domain and NHE1<sub>cd</sub> responsible for their interaction

Having confirmed a direct interaction of 4.1R<sup>80</sup> with NHE1, we mapped the critical motifs in both 4.1R<sup>80</sup> and NHE1 responsible for this interaction. The use of various 4.1R<sup>80</sup> recombinant proteins enabled us to show that the 4.1R FERM domain, and more specifically a 35-amino-acid peptide encoded by alternative exon 5 within this domain, mediated the 4.1R<sup>80</sup> interaction with NHE1 (Table). Although the 4.1R FERM domain bound to NHE1<sub>cd</sub> with a similar affinity as full-length 4.1R<sup>80</sup>, a variant 4.1R FERM domain lacking the exon 5-encoded peptide failed to interact with NHE1 (Table 1). Mutation of the EED motif within the exon 5-encoded peptide, a motif previously reported to participate in 4.1R<sup>80</sup> interaction with band 3 (Figure 1A) [28], resulted in a significant decrease in 4.1R<sup>80</sup> binding affinity for NHE1<sub>cd</sub> (*K<sub>d</sub>* ~1400 nM; Table 1). The other domains of 4.1R<sup>80</sup>, 16 kDa, 10 kDa spectrin/actin binding domain and C-terminal 24 kDa domain did not bind to NHE1<sub>cd</sub> (results not shown).

Alignment of the amino acid sequences of rat AE1/band 3 and rat NHE1 juxta-membrane regions of their cds revealed the presence of a common cluster of positively charged residues, i.e. RRR and KKK respectively (Figure 1A). Such a positively charged cluster in NHE1 was an obvious candidate for interaction with the negatively charged EED motif in 4.1R<sup>80</sup>. However, mutation of this K<sup>513</sup>KK→AAA cluster (referred to as the M3 motif) induced only a slightly decreased affinity for NHE1 and 4.1R<sup>80</sup> (FERM domain) binding (*K<sub>d</sub>* = 545 nM; Table 1). In contrast, a more extensive mutation of this region K<sup>513</sup>KKQETKR→AAAQETAA, previously characterized as the M1 motif (Figure 1A), mutation of another more distal cluster of positively charged residues, R<sup>513</sup>FKNYKVVK→AFNAYVAA, previously characterized as the M2 motif (Figure 1A) [25], and simultaneous mutation of both the M1 and M2 motifs resulted in a dramatic decrease in 4.1R<sup>80</sup> binding affinity for NHE1 (*K<sub>d</sub>* = 4791 nM, 26 060 nM and no binding respectively; Table 1). These data led us to conclude that both M1 and M2 sequences are necessary to mediate NHE1 interaction with 4.1R<sup>80</sup>. Of particular note, these two motifs are absent in the same region of band 3cd (Figure 1A), which strongly suggests a different type of interaction between 4.1R<sup>80</sup> and band 3 and 4.1R<sup>80</sup> and NHE1.

IAsys® binding analysis estimated the binding ratio of the 4.1R FERM domain to NHE1 as approximately 1:1 (Figure 2). Binding affinity was calculated by Scatchard plot analysis (Figure 2, insert), with maximal binding being observed in the 10<sup>−7</sup> M range. Another method, based upon the use of a quartz crystal microbalance biosensor, confirmed the binding ratio and binding affinity values (results not shown). These results indicate that one molecule of the 4.1R FERM domain interacts simultaneously with the two NHE1<sub>cd</sub> binding sites described above.
Influence of pH on the 4.1R FERM domain–NHE1cd interaction

Because NHE1 plays a key role in intracellular pH homeostasis, we investigated whether the 4.1R FERM domain binding to NHE1cd is regulated by pH. As shown in Figure 3(A), the 4.1R FERM domain binding to NHE1cd as a function of pH adopted a deep parabola shape, the maximum binding (minimum $K_d$ $\sim$ 150 nM) being observed at pH 6.5. The affinity of the 4.1R FERM domain binding to NHE1cd was similar between pH 6.0 and 7.2. In contrast, binding affinities decreased dramatically at more basic ($\sim$ pH 8.0) or acidic pH values ($\sim$ pH 5.5); the dissociation constants ($K_d$) increased more than 20-fold at pH 5.5 and 8.5 (Supplementary Figure S1 at http://www.BiochemJ.org/bj/446/bj4460427add.htm). In contrast, the $K_d$ values for 4.1R FERM domain binding to band 3 were not significantly different over a large range of pH (5.0–8.5). In the more physiological range of pH 6.5–7.5, the $K_d$ value of 4.1R FERM domain binding to NHE1cd was significantly higher than the affinity for binding to the band 3cd (Figure 3B). Furthermore, maximum binding affinity ($K_d$ $\sim$ 50 nM) for 4.1R FERM domain interaction with band 3 was observed at pH 7.0, which further supports different characteristics for 4.1R$^{\text{cd}}$ interaction with NHE1 and band 3.

Influence of Na$^+$ concentration on the 4.1R FERM domain–NHE1cd interaction

In addition to the pH-dependent differences between 4.1R$^{\text{cd}}$ binding to NHE1cd and the band 3cd, we also found that the 4.1R FERM domain interaction with NHE1cd, but not the band 3cd, is more sensitive to Na$^+$. The binding of the 4.1R FERM domain to NHE1cd at pH 7.5 was already reduced by 50% at NaCl concentrations as low as 0.2 M, the binding being totally inhibited at 0.3 M NaCl (Figure 3B). In contrast, 4.1R FERM domain binding to band 3 was unaffected up to 0.5 M NaCl, the binding being ablated at 1.0 M NaCl. 4.1R FERM domain binding to NHE1cd was therefore much more sensitive to variations in NaCl concentration than that to the band 3cd, in particular in the physiological range of 0.1–0.2 M NaCl. These observations supported once again the different characteristics for 4.1R$^{\text{cd}}$ interaction with NHE1 and band 3.

Regulation of the 4.1R FERM domain–NHE1cd interaction by Ca$^{2+}$/CaM

We have previously shown that CaM binds to the 4.1R FERM domain in the absence of Ca$^{2+}$ and decreases binding affinity of the 4.1R FERM domain for its binding partners in a Ca$^{2+}$-dependent manner [12,23,37]. We therefore asked whether there is a Ca$^{2+}$/CaM-dependent regulation of 4.1R FERM domain binding to a NHE1cd construct lacking the two CaM-binding sites, i.e. R$^{306}$-A$^{309}$ and N$^{64}$-L$^{68}$ [38,39]. In the absence of CaM, the $K_d$ value observed for the 4.1R$^{\text{cd}}$–NHE1 interaction at a physiological pH of 7.5 was similar to that of previously characterized binding partners (Table 2). At pH 6.0, the 4.1R FERM domain could still bind to NHE1cd (Table 2) and to Ca$^{2+}$/CaM with a $K_d$ value of $\sim$ 100 nM ($k_1$ $= 6.7 \times 10^{-2}$ M$^{-1}$ s$^{-1}$ and $k_2$ $= 7.0 \times 10^5$ s$^{-1}$). Ca$^{2+}$/CaM binding to 4.1R FERM domain reduced the binding affinity of the 4.1R FERM domain for NHE1cd (Table 2), the inhibitory effect of Ca$^{2+}$/CaM being more pronounced at pH 7.5 than at pH 6.0. These results indicate that the 4.1R FERM domain–NHE1 interaction is regulated by CaM in a Ca$^{2+}$-dependent manner, and that this regulation is likely most dramatic at physiological pH values.

![Figure 3](http://www.BiochemJ.org/bj/446/bj4460427add.htm) Relative pH- and Na$^+$-dependence of 4.1R FERM domain binding to NHE1cd and the band 3cd

(A) 4.1R FERM domain binding to NHE1cd or the band 3cd immobilized on aminosilane cuvette was measured at various pH values using the IAsys$^\text{TM}$ system. Small and large circles represent raw data from three independent determinations and mean values respectively. (B) A magnification of the data shown in (A) in the pH 6.5–7.5 range is shown. Circles and diamonds indicate 4.1R FERM domain binding to NHE1cd and band 3 respectively. Open and closed symbols correspond to individual and averaged raw data respectively. (C) Circles and diamonds represent binding data for NHE1cd and the band 3cd respectively. Small circles and diamonds and large circles and diamonds represent individual and averaged raw data respectively. The binding (%) at each concentration of NaCl is relative to the maximum (100%) binding detected at equilibrium in 0.1 M NaCl.

We then investigated the Ca$^{2+}$-dependence of the CaM-modulated interaction of the 4.1R FERM domain with NHE1cd at pH 7.4 (Figure 4). At Ca$^{2+}$ concentrations greater than 0.01 μM (pCa = 8), the extent of 4.1R binding to NHE1 started to decline, the maximal inhibition being reached at Ca$^{2+}$ concentrations...
Table 2 Ca\textsuperscript{2+}/CaM-mediated down-regulation of the 4.1R FERM domain interaction with NHE1cd

The $K_d$ values for the 4.1R FERM domain interaction with immobilized NHE1cd either in the presence (Ca\textsuperscript{2+}) or absence of Ca\textsuperscript{2+} (EDTA) and CaM are shown. Each analyte (50 nM–1 μM) was incubated with the identified immobilized ligand as described in the Experimental section. The $K_d$ values (means ± S.D.) were determined from the binding curves obtained by the resonant mirror detection method in three to five independent experiments using the software package FAST-Fit\textsuperscript{TM}.

(a) pH 7.5

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<th>$K_d$ (M\textsuperscript{-1}•s\textsuperscript{-1})</th>
<th>$k_d$ (s\textsuperscript{-1})</th>
<th>$K_d$ (nM)</th>
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<td>(1.8 ± 0.12) × 10\textsuperscript{4}</td>
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<tr>
<td>−</td>
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<td>(1.7 ± 0.23) × 10\textsuperscript{4}</td>
<td>(7.1 ± 0.17) × 10\textsuperscript{-3}</td>
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<tr>
<td>+</td>
<td>−</td>
<td>(1.3 ± 0.11) × 10\textsuperscript{4}</td>
<td>(5.4 ± 0.18) × 10\textsuperscript{-3}</td>
<td>396 ± 9</td>
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<tr>
<td>+</td>
<td>+</td>
<td>(1.4 ± 0.13) × 10\textsuperscript{4}</td>
<td>(9.3 ± 0.20) × 10\textsuperscript{-3}</td>
<td>6362 ± 634</td>
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</table>

(b) pH 6.0

<table>
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<th>$K_d$ (M\textsuperscript{-1}•s\textsuperscript{-1})</th>
<th>$k_d$ (s\textsuperscript{-1})</th>
<th>$K_d$ (nM)</th>
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<tr>
<td>−</td>
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<tr>
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<td>(1.6 ± 0.20) × 10\textsuperscript{-2}</td>
<td>1413 ± 58</td>
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</tbody>
</table>

![Figure 4](image4.png)

**Figure 4** Ca\textsuperscript{2+}-dependence of the 4.1R FERM domain binding to NHE1cd

4.1R FERM domain binding to NHE1cd was measured at various concentrations of Ca\textsuperscript{2+} either in the presence (circles) or absence (diamonds) of 5 μM CaM. Small and large circles represent individual and averaged raw data respectively. Ca\textsuperscript{2+} concentrations (pCa) were adjusted with a Ca\textsuperscript{2+}/EGTA buffer system. 4.1R FERM domain binding to NHE1cd was plotted as a function of the pCa concentration. The maximal extent of binding under different experimental conditions was quantified as described in the Experimental section. Maximal binding in the presence of EGTA was used to normalize the extent of binding under different experimental conditions. In the absence of CaM, there was no change in the binding of the 4.1R FERM domain to NHE1cd regardless of the Ca\textsuperscript{2+} concentration (diamonds).

of 100 μM and higher (pCa = 4). Half-maximal inhibition was observed at a Ca\textsuperscript{2+} concentration of ~0.1 μM (pCa = 7). In the absence of CaM, Ca\textsuperscript{2+} had no effect on the 4.1R–NHE1 binding affinity (Figure 4). At pH 6.0, binding of the 4.1R FERM domain to NHE1cd was no longer dependent on Ca\textsuperscript{2+} (results not shown).

**Discussion**

Mice with engineered deletions of the four members of the protein 4.1 family (4.1R, 4.1G, 4.1N and 4.1B) have been the subject of an increasing number of studies over the last 10 years [11,21]. Relevant to the present study, erythrocytes from 4.1R-null mice exhibit hyperactivity of NHE1 compared with erythrocytes from wild-type mice [10]. We now show that 4.1R directly binds to NHE1 in vitro and identify the motifs in 4.1R and NHE1 mediating this interaction. We also reveal that the 4.1R-null–NHE1 interaction is modulated by changes in pH and by concentrations of Na\textsuperscript{+} and Ca\textsuperscript{2+}/CaM. Our in vitro data clearly demonstrate that at acidic pH, 4.1R dissociates from NHE1cd, but binding of PIP2 to NHE1cd is increased. This distinct behaviour may be heightened by variations in intracellular CaM and Ca\textsuperscript{2+} concentrations as the regulatory effect of Ca\textsuperscript{2+}/CaM on the 4.1R-null–NHE1 interaction contrasts dramatically with its inability to regulate the PIP2–NHE1 interaction. We hypothesize that the antagonistic effects of 4.1R and PIP2 on NHE1 activity [10,25] play an important role in the regulation of NHE1 activity and that, in absence of 4.1R, sustained binding of PIP2 to NHE1cd facilitates increased NHE1 activity. This phenotype may be heightened in erythrocytes because their PIP2 content is higher than in other cells [40].

The L\textsuperscript{2}EEDY sequence that is shown in the present study to mediate 4.1R\textsuperscript{L20} interaction with NHE1 has been reported to enable 4.1R\textsuperscript{L20} interaction with band 3 [14]. Analysis of the three-dimensional structure of the 4.1R FERM domain reveals that the EED motif is located in a loop structure [41] (Figure 6A). The side chains of each amino acid of the EED motif adopt completely different directions, conferring upon this motif a "T-like" shape (Figure 6A). Another important finding of the present study is that NHE1 interaction with 4.1R\textsuperscript{L20} requires simultaneously the M1 and M2 motifs in the NHE1cd and, as a correlate, that two motifs in the FERM domain are likely involved in this interaction.
Protein 4.1R interaction with NHE1

Figure 6 Three-dimensional schematic representation of the 4.1R80 binding site for NHE1cd and a proposed model for regulation of NHE1 activity by 4.1R80 in erythrocytes

(A) A representation of the 4.1R FERM domain (PDB code 1GG3; [41]) three-dimensional structure was obtained using NCBI three-dimensional structure viewer (see the Experimental section). Key residues responsible for predicted interactions are displayed in both NHE1cd and 4.1R FERM domain. These interactions include electrostatic interactions between 4.1R80 E38 and NHE1 K513, 4.1R80 D40 and NHE1 K515, and 4.1R80 R29 and NHE1 E517. A hydrophobic interaction between 4.1R80 L36 and NHE1 A511 is also predicted. 4.1R80 E39 is not predicted to play a role in the 4.1R80 interaction with NHE1. (B) Schematic representation of the lipid bilayer and NHE1–4.1R80 interaction, involving the motifs M1 and M2 in NHE1cd and the EED motif in the 4.1R FERM domain. Competition between 4.1R and PIP2 for binding to NHE1 as a function of the Ca2+/CaM concentration is also shown.

as well. Our predicted model is supported by our finding that 4.1R FERM domain binds to NHE1cd with a 1:1 stoichiometry. The observation that the 4.1R FERM domain cannot bind to a double (M1 + M2) mutant NHE1cd supports the idea that 4.1R FERM domain binds simultaneously to the M1 and M2 motifs in NHE1cd.

The three-dimensional structure of the NHE1cd has not been resolved, except for the CaM-binding sites and the CHP (calcineurin B homologous protein) domain [42,43]. A recent study reported that the distal part of the C-terminal NHE1cd is intrinsically disordered, but that it contains conserved regions of transient α-helicity that play an important role in NHE1 trafficking [44]. We predict that the spacing of the two basic residue clusters in NHE1cd, K515KKQETKR and R556FNKKVVKK, would enable wrapping of the 4.1R EED motif, despite the presence of phenylalanine and valine hydrophobic residues. In that respect, the 4.1R FERM domain–NHE1 interaction may resemble a key–keyhole structure. Crystal structure studies will enable further characterization of these interactions at the atomic level.

The 4.1R80-NHE1 interaction differs from the 4.1R80–band 3 interaction in which only one motif in the band 3cd has been shown to interact with 4.1R80 [14]. The distinction is not only structural as we also show that, whereas the 4.1R FERM domain interaction with NHE1cd is highly sensitive to variations in Na+ concentration and to a lesser extent to variations in pH, that of the 4.1R FERM domain with band 3cd is not. We hypothesize that these differences may result from a different type of interaction, with the 4.1R80 interaction with band 3 probably involving hydrophobic residues, such as Leu37 and Tyr40. These striking observations lead us to propose that upon changes in intracellular pH and Na+ concentration 4.1R80 would rapidly dissociate from NHE1, but would still be bound to band 3. We predict that the high sensitivity of the 4.1R–NHE1 interaction to serum NaCl concentrations could be highly relevant in pathological conditions of hyponatraemia (<135 mEq/l) or hypernatraemia (>145 mEq/l) that result primarily from an excess and loss of free water respectively, and that have both been associated with seizures due to either brain swelling or shrinkage. Indeed, an
increase in 4.1R binding to NHE1 in hyponatraemic conditions (Figure 3C) would result in a decrease in NHE1 activity [10] and therefore in a decrease in influx of Na⁺ in cells in an attempt to avoid a further decrease in serum Na⁺ levels, whereas the opposite would be predicted to occur in hypernatraemic conditions.

We have shown that the functional 4.1R FERM domain–NHE1 complex is potentially regulated by Ca²⁺ through CaM binding to the 4.1R FERM domain in erythrocytes, the inhibitory effect of Ca²⁺/CaM on the 4.1R⁰⁻NHE1 interaction being maximal around physiological pH (pH 7.5). Under physiological conditions, CaM is mostly unsaturated (Ca²⁺⁻free) in erythrocytes [27]. Given that there are an estimated 3 × 10⁵ molecules of CaM and 2 × 10⁷ molecules of 4.1R⁰ per erythrocyte [27], one can predict that all of the 4.1R⁰ molecules are potentially complexed with CaM in erythrocytes. We have shown that the recruitment of CaM may confer stability on the 4.1R⁰ structure [27]. Upon an increase in intracellular Ca²⁺, Ca²⁺/CaM complex formation would result in the dissociation of 4.1R⁰ from NHE1, thus promoting NHE1 activity. This hypothesis is consistent with previous reports which show that an increase in intracellular Ca²⁺ is accompanied by an increase in NHE1 activity [45, 46], this effect requiring CaM binding to NHE1 [38, 39].

On the basis of the results of the present study, we propose a model for NHE1 regulation by protein 4.1R⁰ in erythrocytes (Figure 6B), in which an increase in intracellular [Na⁺] is mediated by NHE1 activity, resulting in a rapid dissociation of 4.1R⁰ from NHE1. In contrast, the 4.1R⁻⁻ interaction with band 3cd is not affected by such changes in pH or [Na⁺], possibly because of the hydrophobic nature of 4.1R⁻⁻–band 3 interaction.

The functional consequences of these structural differences are also true for cytoskeletal proteins. Thus ezrin, a member of the protein 4.1 superfamily of cytoskeletal proteins that is not expressed in erythrocytes, also binds to NHE1cd, but only through the M2 motif [4, 6, 47]. The poor conservation of 4.1R⁰ from NHE1. In contrast, the 4.1R⁻⁻ interaction with band 3cd is not affected by such changes in pH or [Na⁺], possibly because of the hydrophobic nature of 4.1R⁻⁻–band 3 interaction.

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Protein 4.1R interaction with NHE1


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SUPPLEMENTARY ONLINE DATA

Characterization of cytoskeletal protein 4.1R interaction with NHE1 (Na\(^{+}/\)H\(^{+}\) exchanger isoform 1)

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Figure S1 Changes in dissociation rate constant (k\(_{d}\)) and association rate constant (k\(_{a}\)) for 4.1R FERM domain binding to NHE1cd as a function of pH

Changes in the K\(_{d}\) value resulted primarily from changes in k\(_{a}\) value, although both the k\(_{d}\) and k\(_{a}\) values changed significantly between pH 7.0 and 7.5.

Figure S2 Three-dimensional representation of the EED motif in the 4.1R FERM domain and of the corresponding motif in the ezrin FERM domain

The residues E\(_{38}\)ED in human 4.1R FERM domain and the corresponding residues, R\(_{40}\)EWV, in human ezrin are depicted using a stick and ball model. The PDB accession code is 1GG3 and 1NI2 for the human 4.1R and ezrin FERM domains respectively.

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