Assembly and Trafficking of a Multiprotein ROMK (Kir 1.1) Channel Complex by PDZ Interactions*

Received for publication, October 22, 2003, and in revised form, November 4, 2003
Published, JBC Papers in Press, November 5, 2003, DOI 10.1074/jbc.M311599200

Dana Yoo‡§, Thomas P. Flagg‡, Olav Olsen‡, Viswanathan Raghuram†, J. Kevin Foskett‡, and Paul A. Welling‡¶

From the ‡Department of Physiology, University of Maryland School of Medicine, the §University of Maryland Biotechnology Center, Baltimore, Maryland 21201 and the ¶Department of Physiology, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6985

The ROMK subtypes of inward rectifier K⁺ channels (Kir 1.1, KCNJ1) mediate potassium secretion and regulate NaCl reabsorption in the kidney. In the present study, the role of the PDZ binding motif in ROMK function is explored. Here we identify the Na/H exchange regulatory factors, NHERF-1 and NHERF-2, as PDZ domain interaction partners of the ROMK channel. Characterization of the basis and consequences of NHERF association with ROMK reveals a PDZ interaction-dependent trafficking process and a coupling mechanism for linking ROMK to a channel modifier protein, the cystic fibrosis transmembrane regulator (CFTR). As measured by antibody binding of external epitope-tagged forms of Kir 1.1 in intact cells, NHERF-1 or NHERF-2 coexpression increased cell surface expression of ROMK. Channel interaction with NHERF proteins and effects of NHERF on ROMK localization were dependent on the presence of the PDZ domain binding motif in ROMK. Both NHERF proteins contain two PDZ domains; recombinant protein-protein binding assays and yeast-two-hybrid studies revealed that ROMK preferentially associates with the second PDZ domain of NHERF-1 and with the first PDZ domain of NHERF-2, precisely opposite of what has been reported for CFTR. Consistent with the scaffolding capacity of the NHERF proteins, coexpression of NHERF-2 with ROMK and CFTR dramatically increases the amount of ROMK protein that communopurifies and functionally interacts with CFTR. Thus NHERF facilitates assembly of a ternary complex containing ROMK and CFTR. These observations raise the possibility that PDZ-based interactions may underscore physiological regulation and membrane targeting of ROMK in the kidney.

The ROMK (Kir 1.1 or KCNJ1) subtypes of weakly inward rectifying potassium channels (1) play critical roles in salt and water homeostasis. Chiefly localized on apical membrane of specific epithelial cells in the kidney (2–4), each of the products of the ROMK gene (amino-terminal splice variants are termed

ROMK1 (Kir 1.1a), ROMK2 (Kir 1.1b), ROMK3 (Kir 1.1c) (5) functions as an exquisitely regulated channel for the transport of potassium into the renal tubule lumen. ROMK1, ROMK2, and ROMK3 exhibit nearly identical functional properties but are expressed differentially along the nephron (6) for different physiological duties (7). ROMK2 channels in the thick ascending limb of Henle's loop are responsible for recycling potassium across the apical membrane to maintain avid NaCl reabsorption through the Na⁺/K⁺/2Cl⁻ cotransporter, important for the urinary concentrating mechanism. ROMK1 and ROMK3 channels in the distal nephron, on the other hand, are thought to constitute the final regulated component of the potassium secretory machinery of the kidney, essential for controlling renal potassium excretion and maintaining potassium balance (8). The physiologic significance of these channels is underscored by the link to human disease. Loss-of-function mutations in the ROMK gene cause Bartter's syndrome, a familial salt-wasting nephropathy (9, 10). Mice, lacking the ROMK gene, manifest a similar disorder (11).

Although recombinant ROMK channels share many functional features of the small conductance apical membrane potassium channels in the thick ascending limb and collecting duct, the absence of sensitivity to cytoplasmic ATP has suggested that the native channel might be more complex. Reminiscent of KATP channels in excitable tissues, which are heteromultimeric proteins complexes comprised regulatory sulfonylurea receptor (SUR)1 ATP-binding cassette gene products and pore-forming inward rectifier, Kir 6 subunits (12, 13), present evidence from molecular reconstitution studies indicates that ROMK channels also require a ATP-binding cassette protein cofactor to manifest native channel properties. We and others found that coexpression of CFTR with ROMK in Xenopus oocytes leads to the formation of weakly inward rectifying channels that have acquired sensitivity to sulfonylurea agents (14) and ATP-dependent gating properties (15) like the native channel (16, 17). With observations that the expression patterns of ROMK (2–4) and CFTR (18) overlap along the thick ascending limb and collecting duct apical membrane, it seems plausible that the native ROMK secretory channel may be regulated by CFTR in the kidney. Certainly, the concept has precedent with an ever growing body of data, demonstrating that CFTR not only functions as a Cl⁻ channel but also acts as a “conductance regulator,” modulating a plethora of different epithelial transport proteins (19).
Although the mechanisms by which CFTR modulates ion channels and other transport molecules are poorly understood, observations that CFTR interacts directly with a variety of different PDZ domains (20–23) suggest a potential coupling mechanism. PDZ domains are 90–90-amino acid residue protein interaction modules that generally bind to short motifs (type I PDZ binding motif is recognized by the sequence S/T-X-(A/V/L)M) found at the extreme COOH-terminal tail of certain membrane proteins and cytoplasmic signal transduction molecules to organize multiprotein complex formation on specific membrane domains (24, 25). PDZ proteins that bind to the COOH terminus of CFTR, such as the Na/H antiporter (ROMK) and CFTR into a ternary complex. That ROMK associates directly with NHERF-1 and NHERF-2 in the present study was to test this hypothesis. Here we show the possibility that trafficking of ROMK and interaction with CFTR is required for efficient expression of active ROMK channels on the plasma membrane (32). These observations raise the possibility that trafficking of ROMK and interaction with CFTR are linked by a common PDZ domain-based scaffold. The goal of the present study was to test this hypothesis. Here we show that ROMK associates directly with NHERF-1 and NHERF-2 through a PDZ binding interaction to facilitate expression of ROMK on the plasmalemma and to coordinate the assembly of ROMK and CFTR into a ternary complex.

EXPERIMENTAL PROCEDURES

DNA Constructs—cDNAs encoding ROMK2 cytoplasmic COOH-terminal regions (amino acids 307–372 or 307–389) were amplified by PCR from a full-length template (GenBank NM017023) and cloned in-frame with GST in the fusion expression vector pGEX-5x (Amersham Biosciences) or with LexA in pK262 for GST and yeast two-hybrid studies, respectively. The cDNAs encoding PDZ domains of NHERF-1 (NM042052) and NHERF-2 (AF067371), as indicated under “Results,” were amplified by PCR from full-length templates and cloned in-frame with either a hexahistidine sequence in the pSET plasmid (Invitrogen) or with a trans-activation domain in the pKG4-5 plasmid for generation of recombinant His-tagged NHERF PDZ proteins and prey proteins for yeast two-hybrid studies, respectively. All constructs used for studies in Xenopus oocytes were subcloned between the 5′- and 3′-untranslated region of the Xenopus β-globin gene in the modified pSD64 vector to increase expression efficiency (33). This vector also contains a polyadenylate sequence in the 3′-untranslated region of the dA23dC30. With the exception of EGFP-ROMK, all constructs used for studies in Xenopus oocytes were subcloned between the 5′- and 3′-untranslated region of the Xenopus β-globin gene in the modified pSD64 vector to increase expression efficiency (33). This vector also contains a polyadenylate sequence in the 3′-untranslated region of the Xenopus β-globin gene in the modified pSD64 vector to increase expression efficiency (33). This vector also contains a polyadenylate sequence in the 3′-untranslated region of the Xenopus β-globin gene in the modified pSD64 vector to increase expression efficiency (33). This vector also contains a polyadenylate sequence in the 3′-untranslated region of the Xenopus β-globin gene in the modified pSD64 vector to increase expression efficiency (33). This vector also contains a polyadenylate sequence in the 3′-untranslated region of the Xenopus β-globin gene in the modified pSD64 vector to increase expression efficiency (33).
were transfected with prey, baits, and reporter constructs. In this manner, it was possible to screen for yeast that was transfected with the wild-type and mutant baits and the PDZ domains and then plated onto selection plates to screen for yeast that was transfected with the wild-type and mutant baits and the PDZ domains. Immunoblots using anti-HA antibodies highlighted the cytoplasmic COOH-terminal domain of interest. Silver staining or immunoblotting with anti-NHERF antibodies. Coomassie Brilliant Blue staining. Termination (amino acids 307–369X) was used as bait (in pJK202 containing a His selectible marker). Conditionally expressed NHERF PDZ domains in the NHERF-1 and NHERF-2. In these studies, a LexA action trap system was used according to established methods (38, 39). The interaction trap uses Gal-inducible promoter to express the prey protein conditionally, yeast were grown (Amin) of 0.9–1.2) under conditions that induce (2% galactose and 1% raffinose) or repress (2% glucose) the promoter. Yeast cultures were pelleted (2,000 × g) and resuspended in 750 μl of Z buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, and 50 mM β-mercaptoethanol). For the assay, 100 μl of the Z buffer cell suspension was added to 900 μl of Z buffer in glass culture tubes containing 10 μl of 0.1% SDS and 20 μl of chloroform. Tubes were vortexed for 15 s and equilibrated at 30 °C for 15 min. After equilibration, 200 μl of stock 4 mg/ml ONPG was added to the samples and vortexed for 5 s. Reactions were stopped at 30–4 min by adding 0.5 ml of 1 M Na2CO3, centrifuged to pellet debris, and the reaction product was measured by spectrofluorometry. Miller units were calculated as described previously (40). Results represent the average of at least four independent clones, repeated in duplicate.

cRNA Synthesis—Complementary RNA was transcribed in vitro in the presence of capping analogue G6·(ppp)5 from linearized plasmids containing the cDNA of interest using SP6 RNA polymerase (mMessage Machine, Ambion Inc.). cRNA was purified by phenol-chloroform extraction and precipitated with ammonium acetate/isopropryl alcohol. Yield was quantified spectrophotometrically and confirmed by agarose gel electrophoresis.

Yeast Two-hybrid Interaction Studies—The yeast two-hybrid interaction trap system was used according to established methods (38, 39) to test for interactions between the ROMK2 COOH terminus and the PDZ domains in NHERF-1 and NHERF-2. In these studies, a LexA fusion protein of either a wild-type ROMK COOH terminus (amino acids 307–369X) was used as bait (in pJK202 containing a His selectable marker). Conditionally expressed NHERF PDZ domains in the NHERF-1 and NHERF-2. In these studies, a LexA action trap system was used according to established methods (38, 39). The interaction trap uses Gal-inducible promoter to express the prey protein conditionally, yeast were grown (Amin) of 0.9–1.2) under conditions that induce (2% galactose and 1% raffinose) or repress (2% glucose) the promoter. Yeast cultures were pelleted (2,000 × g) and resuspended in 750 μl of Z buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, and 50 mM β-mercaptoethanol). For the assay, 100 μl of the Z buffer cell suspension was added to 900 μl of Z buffer in glass culture tubes containing 10 μl of 0.1% SDS and 20 μl of chloroform. Tubes were vortexed for 15 s and equilibrated at 30 °C for 15 min. After equilibration, 200 μl of stock 4 mg/ml ONPG was added to the samples and vortexed for 5 s. Reactions were stopped at 30–4 min by adding 0.5 ml of 1 M Na2CO3, centrifuged to pellet debris, and the reaction product was measured by spectrofluorometry. Miller units were calculated as described previously (40). Results represent the average of at least four independent clones, repeated in duplicate.

cRNA Synthesis—Complementary RNA was transcribed in vitro in the presence of capping analogue G6·(ppp)5 from linearized plasmids containing the cDNA of interest using SP6 RNA polymerase (mMessage Machine, Ambion Inc.). cRNA was purified by phenol-chloroform extraction and precipitated with ammonium acetate/isopropryl alcohol. Yield was quantified spectrophotometrically and confirmed by agarose gel electrophoresis.

Oocyte Isolation and Injection—Oocytes from selected female Xenopus laevis (Xenopus Express, Homosassa, FL) were isolated and maintained using the standard procedures as described previously (42). Although the recombinant ROMK channel, as expressed in Xenopus oocytes, has been reported to by some investigators to exhibit sensitiv-
expressed ROMK. Briefly, frogs were anesthetized with 0.15% 3-aminobenzoate and a partial oophorectomy was performed through an abdominal incision. Oocyte aggregates were manually dissected from the ovarian lobes and then incubated in OR-2 medium (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, and 5 mM HEPES, pH 7.5) containing collagenase (type 3, Worthington) for 2 h at room temperature to remove the follicular layer. After extensive washing with collagenase-free OR-2, oocytes were stored at 19 °C in OR-3 medium (50% Leibovit medium, 10 mM HEPES, pH 7.4). After 12–24 h, healthy looking Dumont stage V–VI oocytes were injected pneumatically with 50 nl of diethyl pyrocarbonate-treated water containing ROMK2, CFTR, and/or NHERF-2 cRNA, and then stored in OR-3 medium at 19 °C for 2–6 days.

Electrophysiology—Whole cell currents in Xenopus oocytes were monitored using a two-microelectrode voltage clamp as described previously (42, 45) under conditions in which potassium currents, carried through ROMK, could be isolated. In all studies, we purposely excluded endogenous cAMP analogs or activators to prevent CFTR Cl⁻ channel activation. Briefly, oocytes were bathed in a 5 mM potassium, low Cl⁻ solution (5 mM KCl, 45 mM sodium gluconate, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM HEPES, pH 7.4). Voltage sensing and current injecting microelectrodes had resistances of 0.5–1.5 megohms when back-filled with 3 M KCl. Once a stable membrane potential was attained, oocytes were clamped to a holding potential of −80 mV, and currents were recorded over a continuous 0.2-Hz train of 500-ms voltage steps to 0 mV, permitting a constant monitor of the outward potassium conductance. Periodically, the train was interrupted briefly to determine an IV relationship, monitoring the steady-state current over voltage 500-ms steps ranging from −120 mV to +80 mV in 20-mV increments. Data were collected using an ITC16 analog to digital, digital to analog converter (Instrutech Corp.), filtered at 1 kHz, and digitized on line at 2 kHz using Pulse software (HEKA Electronik) for later analysis. Barium-sensitive outward current (10 mM barium acetate) was measured before and after the addition glibenclamide. The barium-sensitive current in the absence of glibenclamide was considered an estimate of the total ROMK current. The fraction of this current that was glibenclamide-sensitive was considered to represent the fraction of active ROMK channels that functionally interact with CFTR.

RESULTS

Identification of NHERF as a ROMK Binding Partner—As a first step toward testing the hypothesis about ROMK-PDZ protein interactions, we sought to identify PDZ proteins in the kidney which specially interact with the ROMK channel. In these studies GST fusion proteins of either the extreme COOH terminus of ROMK2 (326–372) or a mutant ROMK COOH terminus, lacking the PDZ binding motif (326–369X), were constructed. Binding proteins specific for the GST-ROMK COOH terminus affinity column were purified from rat kidney extracts, resolved by SDS-PAGE, and visualized by silver staining (Fig. 1). The wild-type COOH terminus but not the mutant COOH terminus (369X) uniquely bound several species, indicative of PDZ proteins. The most predominant and consistently observed protein migrated at −50 kDa (n = 3). Based on the molecular mass, we suspected that this protein could be NHERF, a PDZ protein known to interact with CFTR (20, 23). Western blot analysis proved that this was indeed the case; as shown in Fig. 1C, an antibody raised against NHERF (34) specifically detected a ∼50-kDa protein purified on the wild-type COOH terminus but not the mutant COOH terminus (369X).

To validate that the ROMK COOH terminus interacts with NHERF-1 or NHERF-2 rather than an immunologically related protein, GST affinity chromatography studies as above were conducted with extracts from COS-7 cells transfected with either HA-tagged NHERF-1 or HA-tagged NHERF-2. As detected by anti-HA antibodies in Western blot analysis, the wild-type COOH terminus but not the mutant COOH terminus (369X) bound to HA-tagged NHERF-1. Similar observations were made with HA-tagged NHERF-2 (not shown). Thus, the COOH terminus of ROMK, containing a type I PDZ binding motif, is capable of binding directly to NHERF-1 or NHERF-2. To authenticate further ROMK-NHERF interaction, coimmunoprecipitation studies were performed with full-length ROMK and NHERF expressed in a cellular environment. In these studies, COS-7 cells were cotransfected with HA-tagged NHERF (either NHERF-1 or -2) and ROMK or transfected separately with either construct alone. Recovered immunoprecipitates on anti-HA antibody-bound beads were resolved by SDS-PAGE and immunoblotted with an anti-ROMK antibody (35). As shown in Fig. 2, ROMK copurified with NHERF-1 or NHERF-2 in cells transfected with ROMK and NHERF. ROMK could not be immunoprecipitated with an unrelated IgG, and coprecipitation required cotransfection of HA-tagged NHERF and ROMK, providing evidence of specific immunoprecipitation of ROMK and NHERF. Collectively, these studies reveal that ROMK is capable of interacting directly with either NHERF-1 or NHERF-2 in cells.

NHERF Facilitates Cell Surface Expression of ROMK—The ROMK channel is not efficiently expressed on the plasma membrane in mammalian expression systems, accumulating largely within the endoplasmic reticulum (46, 47), and possibility the Golgi apparatus (Fig. 3). Although reminiscent of Kir 6.2 channels, which are retained in the endoplasmic reticulum in the absence of the SUR binding partner (48, 49), ROMK is different in that exit from the endoplasmic reticulum is not facilitated by SUR (43) or CFTR, suggesting a distinct processing mechanism. Because intracellular trafficking of membrane proteins can depend on PDZ interactions, we determined whether NHERF coordinates plasmalemma expression of ROMK. In initial studies, COS-7 cells were cotransfected with GFP-tagged ROMK2 and NHERF-1 or NHERF-2 and visualized by
fluorescent confocal microscopy (Fig. 3). In most cells, NHERF-1 and NHERF-2 colocalized with ROMK2 within the endoplasmic reticulum and Golgi. In some cells, ROMK2 also colocalized with NHERF in dense clusters or patches along segments of the cell periphery (Fig. 3). The redistribution of ROMK to the outer cell border upon NHERF cotransfection is

**Fig. 3.** ROMK2 colocalizes with NHERF-1 and NHERF-2 in a PDZ-dependent manner. Immunolocalization of ROMK2 (green) and either NHERF-1 or NHERF-2 (red) in COS-7 cells transfected with wild-type ROMK2, ROMK2, lacking the PDZ binding motif, 369X, and NHERF-1/2. Comparable results were observed in three separate transfections.
consistent with NHERF-dependent recruitment or retention of the channel on the plasmalemma. To verify that this observation actually represents an authentic increase in cell surface expression, COS-7 cells were cotransfected with NHERF and an extracellular HA-epitope tagged ROMK channel so that channels at the cell surface could be more accurately detected by external HA antibody binding (46). As assessed by immunofluorescent confocal microscopy of nonpermeabilized COS cells, cotransfection of either NHERF-1 or NHERF-2 dramatically increased the number of cells that express the extracellular HA epitope-tagged EGFP-ROMK2 on the cell surface (Fig. 4). Although the majority of cells expressed ROMK on the cell surface in the presence of NHERF, the amount of ROMK on the cell surface was variable. To verify the NHERF-dependent plasmalemma expression of ROMK using an independent biochemical test, we quantified the amount of HA epitope on the cell surface by analytical luminometry. These studies revealed that NHERF expression modestly stimulated plasmalemma expression of ROMK2 above background. Removal of the PDZ binding motif in ROMK2 (369X) prevented colocalization of ROMK2 with NHERF and completely abrogated the NHERF-dependent expression of ROMK2 on the plasma membrane. Thus, NHERF-1 or -2 increases cell surface expression of ROMK2 by a process dependent on a PDZ interaction.

**ROMK Interacts Differentially with the PDZ Domains in NHERF-1 and NHERF-2—** Both NHERF-1 and NHERF-2 have tandem PDZ domains. Present evidence strongly suggests the two domains have different binding specificities (50), providing a potential mechanism to link disparate proteins that preferentially interact with different domains. For instance, the PDZ ligand at the COOH terminus of CFTR binds to the first PDZ domain of NHERF-1 with higher affinity than the second (29), possibility freeing the second PDZ domain to interact with other proteins, such as ROMK. To test this hypothesis, we first measured the capacity of ROMK to interact with each PDZ domain in NHERF-1 using an in vitro protein-protein interaction assay and by yeast two-hybrid.

Fig. 5 illustrates the results of the in vitro interaction assay. In this study, purified GST-ROMK COOH-terminal fusion proteins were resolved by SDS-PAGE, immobilized on nitrocellulose, renatured, and probed for interaction with purified, His-tagged NHERF-1 protein fragments. These included the NH2-terminal two-thirds of NHERF containing the two PDZ domains (PDZ1_PDZ2 amino acids 1–247), the first PDZ domain alone (PDZ1, amino acids 1–102) and the second PDZ domain alone (PDZ2, amino acids 144–247). After hybridization, the GST fusions were washed and blotted with an anti-His antibody to detect bound probe. Significant interaction was only observed between the wild-type GST-ROMK protein and the PDZ 1–2 or PDZ 2 protein fragment, consistent with specific interaction between the ROMK COOH terminus and the second PDZ domain of NHERF-1. Binding was lost when the last three amino acids of ROMK (369X) were removed, consistent with a canonical PDZ interaction.

The binding preference of ROMK for the second PDZ domain in NHERF-1 was corroborated independently using the yeast two-hybrid system. In these studies, yeast were transfected with activation-tagged fusions of the NHERF-1 PDZ domains and LexA fusions of either the wild-type ROMK COOH terminus or the 369X mutant. As assessed by the transcriptional activation of the β-galactosidase gene reporter and quantified using the ONPG solution assay, interaction was observed only between the wild-type ROMK COOH terminus and either PDZ1–2 or PDZ2 (Fig. 6). No reporter activity was detected in yeast grown under conditions that repress expression of the NHERF domain. Furthermore, removal of the last three amino acids of ROMK (369X) completely abrogated the interaction. Thus, ROMK preferentially binds to the second PDZ domain of NHERF-1 through a canonical type-I PDZ interaction.

Although the PDZ domains in NHERF-1 and NHERF-2 are highly conserved, studies on NHERF-1 interaction with CFTR (29) combined with the observation of Sun et al. (23) that CFTR binds to the second PDZ domain better than first PDZ domain of NHERF-2 suggest that the PDZ domains in NHERF-1 and NHERF-2 may exhibit subtle functional differences. Accordingly, we compared the relative strength of interaction between the ROMK COOH terminus and the different PDZ domains in NHERF-1 and NHERF-2 using the yeast two-hybrid system (Fig. 7). As assessed by the transcriptional activation of the β-galactosidase gene reporter and quantified using the ONPG solution assay, the ROMK COOH terminus bound to the second PDZ domain of NHERF-2 with the same affinity as the second PDZ domain of NHERF-1. Surprisingly, however, the first PDZ domain of NHERF-2 also interacted with the COOH terminus of ROMK2, indicating that NHERF-2 may exhibit subtle functional differences. Accord-
of ROMK. In fact, ~4-fold greater reporter activity was induced by the interaction between ROMK and NHERF-2 PDZ1 than with NHERF-2 PDZ2 or NHERF-1 PDZ2.

**NHERF Facilitates Physical and Functional Interaction between ROMK and CFTR**—The studies above, demonstrating that ROMK has a different binding preference for the PDZ domains in NHERF-1 and NHERF-2 than those reported for CFTR, indicate that NHERF has the capacity to act as a scaffolding molecule, which could potentially assemble ROMK and CFTR into a ternary complex. To determine whether this is actually the case, we tested whether NHERF increases the physical interaction between ROMK and CFTR. Because NHERF-2 appears to have a higher binding affinity for ROMK than NHERF-1, and our previous studies indicate that NHERF-2 colocalizes with ROMK2 in the kidney (51), we focused on the effects of NHERF-2. Physical interaction was examined biochemically by coimmunoprecipitation in COS cells transfected with different combinations of ROMK2, CFTR, and NHERF-2 (Fig. 8). In the absence of NHERF-2, a small amount of ROMK2 protein copurified with CFTR, as immunoprecipitated with anti-R CFTR antibodies, consistent with a weak intrinsic interaction between ROMK and CFTR. More importantly, and as predicted by our hypothesis, coexpression of NHERF-2 increased the physical interaction of CFTR with ROMK, as evidenced by the dramatic increase in the amount of ROMK2 that coimmunoprecipitates on anti-

---

**Fig. 5. ROMK interacts differentially with the NHERF-1 PDZ domains in an in vitro protein-protein binding assay.** A, diagram illustrating the domain architecture of NHERF-1 and regions selected for study. B, purified His-tagged fusion proteins of NHERF-1 resolved by SDS-PAGE and visualized by Coomassie Brilliant Blue staining. C, GST and ROMK COOH fusions of GST resolved by SDS-PAGE and visualized by Coomassie Brilliant Blue staining. D, overlay of GST proteins shown in C with the His-tagged PDZ domains of NHERF-1. Bound NHERF PDZ domains were visualized by anti-His binding. Comparable results were observed triplicate experiments.
CFTR-bound beads. Thus, NHERF-2 facilitates physical interaction between ROMK and CFTR.

We and others (14, 15) have observed that ROMK channels expressed in *Xenopus* oocytes develop a low affinity sensitivity to sulfonylurea agents when coexpressed with relatively large amounts of CFTR. Consequently, we quantified the extent to which CFTR modifies ROMK in the presence and absence of NHERF-2 by measuring the fraction of active ROMK channels that acquire glibenclamide sensitivity (Fig. 9). In these studies outward ROMK potassium currents were isolated in *Xenopus* oocytes under a two-microelectrode voltage clamp following the coinjection of combinations of CFTR, ROMK, and NHERF-2 cRNA. Glibenclamide sensitivity was assessed at a dose that is approximately 1 order of magnitude greater than the microscopic $K_i$ (15). Consequently, the fraction of outward potassium current (10 mM barium-sensitive current at 0 mV) that was also glibenclamide-sensitive was taken as an estimate of the portion of the total active ROMK population that interacts at a functional level with CFTR. When equal molar CFTR and ROMK cRNA are expressed in the absence of NHERF-2, only a small fraction of the active ROMK current was found to be glibenclamide-sensitive. Coexpression of NHERF-2 dramatically increased the fraction of ROMK current that is glibenclamide-sensitive, indicating that NHERF-2 facilitates functional coupling between CFTR and ROMK, consistent with a molecular scaffold.

**DISCUSSION**

In the present study, we identify the Na/H exchange regulatory factors, NHERF-1 and NHERF-2, as PDZ domain binding partners of the ROMK channel. Characterization of the biochemical basis and functional consequences of NHERF-ROMK interaction reveals a coupling mechanism for linking ROMK to modifier proteins, expanding the role of the NHERF family.
**Fig. 9. NHERF-2 increases CFTR-dependent modification of ROMK.** Glibenclamide-sensitive outward potassium currents were measured in *Xenopus* oocytes injected with ROMK2 and CFTR and ROMK2, CFTR and NHERF-2. A, currents were measured during a train of continuous 0.2-Hz train of 500-ms voltage steps from −80 to 0 mV. Periodically, the train was interrupted briefly to determine an IV relationship (*), monitoring the steady-state current over voltage 500-ms steps ranging from −120 to +80 mV in 20-mV increments. B, the amount of ROMK current that functionally interacts with CFTR was measured as the total barium-sensitive current that acquires glibenclamide sensitivity.

NHERF-1 (also known as Ezrin-binding protein-50 (52)) and NHERF-2 (also known as E3KARP (53)) were originally discovered as cofactors necessary for cAMP-dependent phosphorylation of NHE3, a brush border Na+/H+ exchanger (54, 55). The coregulatory role appears to be widespread with a growing body of data demonstrating that the NHERF family of proteins facilitate multiprotein signaling complex assembly for efficient phosphorylation and regulation of a variety of transporters, channels, and receptors (for review, see Refs. 26 and 27). The "transducosome" organization function is supported by the domain architecture of these proteins. Both NHERF-1 and NHERF-2 contain two PDZ domains and a COOH-terminal Merlin/Ezrin/Radixin/Moesin (MER) binding domain, allowing recruitment and local organization of MER actin-binding proteins and kinase scaffolds (56, 57), the regulatory subunit of protein kinase A II (23, 58–60) and other kinases (61, 62) with specific PDZ domain-binding protein receptors (31, 63), phosphoacceptors, and other signal transduction molecules (64–66). Recent work has suggested that the tandem PDZ domains in the NHERF proteins might facilitate another scaffolding function, in which regulatory or channel proteins interact with one PDZ domain and modulate the activity of similar proteins that simultaneously bind to the other PDZ domain (67, 68). Our study provides evidence for such a mechanism, revealing that NHERF proteins can coordinate the assembly of a ternary complex, containing the ROMK channel and CFTR.

The heterophilic tethering function is presumably affected by the tandem nature and differential binding properties of the PDZ domains in the NHERF proteins. Characterization of consensus binding sequences of isolated NHERF-1 PDZ domains by phage display, affinity selection techniques revealed that the two PDZ domains have different ligand binding specificities (50) with distinct preferences for residues at the 0, −1, and −3 positions (the COOH-terminal residue is defined as the 0 position) of type 1 PDZ ligands (recognized by the X-S/T-X/I/V/L/M-COOH motif). Our observations that the ROMK COOH terminus, containing a type 1 PDZ binding motif, preferentially binds to the second PDZ domain in NHERF-1 and to the first PDZ domain in NHERF-2 are consistent with this concept and suggest that a PDZ domain might be available for concurrent interaction with a disparate binding partner. Of note, the carboxyl-terminal position in ROMK is occupied by a methionine residue; whereas generally atypical for a type 1 binding sequence, phage display selection studies indicate that second PDZ domain (but not the first PDZ domain) in NHERF-1 prefers peptide binding sequences with a methionine (or leucine) at the zero position. Importantly, the relative binding preference of ROMK is precisely opposite of what has been reported for CFTR. Although the COOH terminus of CFTR is capable of interacting with both PDZ domains in the NHERF proteins (29), it more favorably interacts with PDZ1 in NHERF-1 (29, 69) and PDZ2 in NHERF-2 (23). Collectively these observations indicate that both NHERF forms have the capacity to coordinate ROMK-CFTR assembly.

The PDZ domains in NHERF-1 and NHERF-2 are highly conserved, exhibiting more similarity among equally positioned domains in the two NHERF proteins than with domains in the same NHERF form (75% identity in the first PDZ domains of NHERF-1 and NHERF-2 and 78% identity in the second PDZ domain). Consequently, it is curious that our observations with ROMK along with previous studies of CFTR–NHERF interaction (23, 29) suggest that the like–PDZ domains in the two NHERF proteins have subtle differences in binding preference. Although the precise basis for high affinity binding to each of the NHERF PDZ domains remains largely unknown, the crystal structures of the first NHERF-1 PDZ domain in complex with the COOH-terminal regions of CFTR (70), the β-adrenergic receptor and the platelet-derived growth factor receptor (71) provides some clues. Crystallographic analyses of these structures reveal that the penultimate residues of each ligand are engaged in numerous interactions with residues in PDZ1. Although each different ligand interacts with nearly the identical set of residues, several key interacting residues can undergo large ligand-dependent conformational changes to accommodate the ligand for favorable binding. As a consequence, the ordered water molecules and hydrogen bond networks that stabilize the PDZ1-ligand interactions are different for each ligand (72). Based on these observations, it seems likely that differences in ligand binding preferences among the different NHERF family PDZ domains are determined, at least in part, by the differences in the flexibility of the binding pockets, particularly because the key ligand-interacting residues in NHERF-1 PDZ1 are highly conserved across other NHERF PDZ domains.

Our observation that coexpression of NHERF-2 with ROMK and CFTR dramatically increases the amount of ROMK protein that communopurifies with CFTR is consistent with the scaffolding capacity of NHERF-2 and strongly suggests that NHERF-2 facilitates assembly of a ternary complex containing ROMK and CFTR. In principle, formation of such a complex is determined by the relative abundance of the three components, absolute affinities of ROMK and CFTR for the NHERF PDZ domains, and the number of binding sites in ROMK and CFTR which are available for interaction with NHERF. Like other
Kir channels (73), ROMK channels are produced by the tetrameric arrangement of ROMK subunits, providing four PDZ domain docking sites/functional potassium channel. Thus, the complex could potentially assemble as a dodecamer with up to four CFTR molecules interacting with each functional potassium channel through four NHERF molecules. NHERF proteins can dimerize (74, 75) as well as interact with the actin cytoskeleton through the MERF actin-binding proteins (61), increasing the potential for complexity. On the other hand, because ROMK can interact with both PDZ domains in NHERF-2, there is a possibility, subject to spatial constraints, for NHERF-2 to cross-link ROMK subunits and/or tetramers, and thereby limit the formation of a complex containing all three components. Recent observations by Raghumur et al. (77) that interaction of CFTR with the PDZ2 of NHERF-1 is negatively regulated by phosphorylation of residues in the PDZ2 domain raise the intriguing possibility that the composition and stoichiometry of NHERF complexes are dynamic. Because cross-linking two CFTR molecules through bivalent PDZ domain interactions stimulates CFTR channel gating (22, 29), regulated or competitive replacement of one CFTR with ROMK on the NHERF scaffold could switch the prevailing function of CFTR from a Cl⁻ channel to a conductance regulator.

The involvement of NHERF proteins in CFTR-ROMK assembly might be regarded as a dramatic departure from the mechanism by which the ATP-binding cassette proteins, SUR1 and SUR2A/B, interact with Kir 6.1 and Kir 6.2 to form pancreatic islet β-cell and cardiac KATP channels. In these cases, assembly is solely governed by direct interactions between the transmembrane and cytoplasmic domains of the ATP-binding cassette protein and the potassium Kir channel subunits (48, 49). It should be pointed out, however, that we cannot exclude the possibility that similar direct interactions do not occur between ROMK and CFTR. In fact, previous studies, documenting functional modification of ROMK by CFTR (14, 78, 79) even in the excised patch-clamp configuration (15), coupled with present observations that ROMK communoprecipitates with CFTR in the absence of exogenous NHERF, imply that ROMK is capable of interacting directly with CFTR. Perhaps by simultaneously binding ROMK and CFTR, NHERF proteins simply bring channel components into close proximity to promote low affinity intersubunit interactions.

The requirements for proper trafficking of ROMK channels to the plasma membrane appear to be much different from the prototypical Kᵥ₄₆.2 channels, however. SUR and Kir 6.2 subunits contain endoplasmic reticulum retention/retrieval signals, which are masked upon full assembly of the octameric (4 SUR-4 Kir 6.2) KATP channel complex, providing a quality control mechanism that ensures that only completely assembled channels are delivered to the plasma membrane (48). Although ROMK largely accumulates within the endoplasmic reticulum in mammalian expression systems (46), reminiscent of Kir 6.2 channels, exit from the ER is not facilitated by SUR (80). Instead, our work indicates that either NHERF-1 or NHERF-2, may play an important role in the differential regulation of ROMK channels along the nephron. Importantly, the activity and surface density of functional ROMK channels in the collecting duct, but not the thick ascending limb of Henle’s loop. Cell type-specific expression of ROMK modifier proteins and adaptor molecules, like NHERF-2, may play an important role in the differential regulation of ROMK channels along the nephron. Importantly, the activity and surface density of functional ROMK channels in the collecting duct, but not the thick ascending limb, is exquisitely regulated by aldosterone and other factors to maintain renal potassium secretion in concert with the demands of potassium homeostasis (8). Germane to the observations in the present study with CFTR, studies in CFTR knockout mice indicate that the native ROMK channels in the kidney maintain constitutive gating properties but lose sensitivity to glibenclamide and cyclosporin A (30). It will be interesting to determine whether genetic ablation of NHERF-2 produces a similar phenotype.

In conclusion, we have provided evidence that ROMK binds to NHERF-1 and NHERF-2 through a canonical type 1 PDZ interaction to facilitate association with CFTR and to coordinate cell surface expression of the channel. Our observations raise the possibility that similar PDZ-based interactions may recruit other channel modifiers and accessory proteins into multimeric ROMK complexes for physiological regulation and targeting of the channel on specific plasma membrane domains.

Acknowledgments—We are grateful to Dr. Vijaya Ramesh for providing the HA-tagged NHERF-1 cDNA and anti-NHERF antibodies and to Dr. Randy Hall for providing the NHERF-2 cDNAs.

REFERENCES
1. Ho, K., Nichols, C. G., Lederer, W. J., Lytton, J., Vassilev, P. M., Kazanisika, M. V., and Hebert, S. C. (1993) Nature 362, 31–38
2. Xu, J. Z., Hall, A. E., Peterson, L. N., Bienenkowki, M. J., Eessalu, T. E., and Hebert, S. C. (1997) Am. J. Physiol. 273, F739–F748
3. Mennitt, P. A., Wade, J. B., Eccelberger, C. A., Palmer, L. G., and Frindt, G. (1997) J. Am. Soc. Nephrol. 8, 1823–1830
4. Kehida, Y., Ding, W., Phan, E., Housini, I., Wang, J., Star, R. A., and Huang, C. L. (1998) Kidney Int. 54, 1214–1221
5. Shuck, M. E., Piser, T. M., Bock, J. H., Slightom, J. L., Lee, K. S., and Bienenkowki, M. J. (1997) J. Biol. Chem. 272, 588–593
6. Boim, M. A., Ho, K., Shuck, M. E., Bienenkowki, M. J., Block, J. H., Slightom, J. L., Yang, Y., Brenner, B. M., and Hebert, S. C. (1995) Am. J. Physiol. 268, F1132–F1140
7. Hoh, S. C. (1995) Kidney Int. 48, 1010–1016
8. Giebisch, G. (1998) Am. J. Physiol. 274, F817–F833
9. Simon, D. B., Karet, F. E., Rodriguez-Soriano, J., Hamdan, J. H., DiPietro, A., Stahl, P. E., Slightom, L. W., and Lifton, R. P. (1997) Nat. Genet. 14, 152–156
10. Kusalov, L., International Collaborative Study Group for Bartter-like Syndromes (1997) Hum. Mol. Genet. 6, 17–26
11. Lo, M., Wang, T., Yang, Q., Yang, X., Dong, K., Knepper, M. A., Wang, W., Giebisch, G., Shull, G. E., and Hebert, S. C. (2002) J. Biol. Chem. 277, 37881–37887
12. Aguilar-Bryan, L., Nichols, C. G., Wechsler, S. W., Clement, J. P., Boyd, A. E.,

Assembly and Trafficking of ROMK (Kir 1.1) Channel Complex
Assembly and Trafficking of a Multiprotein ROMK (Kir 1.1) Channel Complex by PDZ Interactions
Dana Yoo, Thomas P. Flagg, Olav Olsen, Viswanathan Raghuram, J. Kevin Foskett and Paul A. Welling

J. Biol. Chem. 2004, 279:6863-6873.
doi: 10.1074/jbc.M311599200 originally published online November 5, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M311599200

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 88 references, 46 of which can be accessed free at
http://www.jbc.org/content/279/8/6863.full.html#ref-list-1