Oligomerization of NHERF-1 and NHERF-2 PDZ Domains: Differential Regulation by Association with Receptor Carboxyl-Termini and by Phosphorylation †

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Oligomerization of NHERF-1 and NHERF-2 PDZ Domains: Differential Regulation by Association with Receptor Carboxyl-Termini and by Phosphorylation†

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ABSTRACT: PDZ domains bind to the carboxyl-termini of target proteins, and some PDZ domains are capable of oligomerization to facilitate the formation of intracellular signaling complexes. The Na^+/H^+ exchanger regulatory factor (NHERF-1; also called "EBP50") and its relative NHERF-2 (also called "E3KARP", "SIP-1", and "TKA-1") both have two PDZ domains. We report here that the PDZ domains of NHERF-1 and NHERF-2 bind specifically to each other but not to other PDZ domains. Purified NHERF-2 PDZ domains associate with each other robustly in the absence of any associated proteins, but purified NHERF-1 PDZ domains associate with each other only weakly when examined alone. The oligomerization of the NHERF-1 PDZ domains is greatly facilitated when they are bound with carboxyl-terminal ligands, such as the carboxyl-termini of the β_2-adrenergic receptor or the platelet-derived growth factor receptor. Oligomerization of full-length NHERF-1 is also enhanced by mutation of serine 289 to aspartate (S289D), which mimics the phosphorylated form of NHERF-1. Co-immunoprecipitation experiments with differentially tagged versions of the NHERF proteins reveal that NHERF-1 and NHERF-2 form homo- and hetero-oligomers in a cellular context. A point-mutated version of NHERF-1 (S289A), which cannot be phosphorylated on serine 289, exhibits a reduced capacity for co-immunoprecipitation from cells. These studies reveal that both NHERF-1 and NHERF-2 can oligomerize, which may facilitate NHERF-mediated formation of cellular signaling complexes. These studies furthermore reveal that oligomerization of NHERF-1, but not NHERF-2, is highly regulated by association with other proteins and by phosphorylation.

Many transmembrane receptors and channels associate with PDZ[1] domain-containing proteins (*1*). Such interactions may influence the function of receptors and channels in several ways: (i) direct modulation of the activity of the receptors and channels, (ii) alteration of the subcellular localization of the receptors and channels, and/or (iii) facilitation of the formation of intracellular signaling complexes between receptors, channels and other signaling proteins such as enzymes and cytoskeletal elements. The function of PDZ proteins as scaffolding proteins in the formation of intracellular signaling complexes may be greatly promoted by oligomerization of PDZ domains; examples of both homo-oligomerization (*2*−*4*) and hetero-oligomerization (*5*−*9*) have been reported for various PDZ domains.

The Na^+/H^+ exchanger regulatory factors, NHERF-1 and NHERF-2, are PDZ domain-containing proteins originally identified on the basis of their ability to regulate Na^+/H^+ exchange (*10*, *11*). The PDZ domains of NHERF-1 specifically recognize the carboxyl-terminal motif S/T-x-L (*12*, *13*) and are known to associate with a small number of transmembrane proteins other than Na^+/H^+ exchangers, including the β_2-adrenergic receptor (β_2AR) (*13*−*15*), cystic fibrosis transmembrane conductance regulator (CFTR) (*12*, *13*, *16*), the sodium-bicarbonate cotransporter (*17*), the platelet-derived growth factor receptor (PDGFR) (*18*) and the B1 subunit of the H^+ ATPase (*19*). Both NHERF-1 and NHERF-2 also bind via their carboxyl-terminal regions to the actin-associated FERM proteins ezrin, radixin, moesin and merlin (*20*, *21*). For this reason, a commonly used alternative name for NHERF-1 is "EBP50" (for ezrin-binding phosphoprotein of 50 kDa) (*20*). NHERF-2 has been alternatively called "E3KARP" (*11*) and "SIP-1" (*22*); this protein has been less well-studied than NHERF-1 and few if any functional differences are known between the two proteins. One difference is that NHERF-1 is known to be a

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[1] Abbreviations: PDZ, PSD-95/Discs-large/ZO-1 homology; NHERF, Na^+/H^+ exchanger regulatory factor; β_2AR, β_2-adrenergic receptor; CFTR, cystic fibrosis transmembrane conductance regulator; PDGFR, platelet-derived growth factor receptor; FERM, band 4.1/ezrin/radixin/moesin/merlin homology; EBP50, ezrin-binding phosphoprotein of 50 kDa; E3KARP, NHE3 kinase A regulatory protein; SIP-1, SRY-interacting protein; PCR, polymerase chain reaction; GST, glutathione-S-transferase; CT, carboxyl-terminus; PSD-95, postsynaptic density protein of 95 kDa; MAGI, membrane-associated guanylate kinase-like protein with an inverted domain structure; RGS, regulator of G protein signaling; His, hexahistidine-tagged; HRP, horseradish peroxidase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DSP, dithiobis[succinimidyl propionate]; EDTA, ethylenediamine tetraacetic acid; WT, wild-type; HA, hemagglutinin; HEK, human embryonic kidney.

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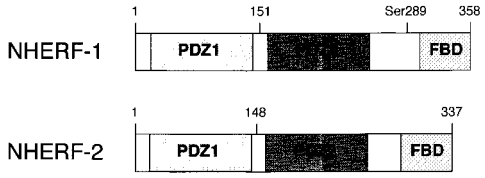

FIGURE 1: Schematic diagram of NHERF-1 and NHERF-2 domain organization. Both NHERF-1 and NHERF-2 possess two PDZ domains and a carboxyl-terminal FERM-binding domain (FBD). The amino acid positions for the "PDZ1" and "PDZ2" fusion proteins used in the studies reported here are as follows: NHERF-1 PDZ1 = 1−151, NHERF-1 PDZ2 = 152−358, NHERF-2 PDZ1 = 1−148, NHERF-2 PDZ2 = 149−337. The position of Ser289 in NHERF-1, which has been mutated to both alanine and aspartate for the studies reported here, is also shown in the diagram.

cellular phosphoprotein while NHERF-2 is not. NHERF-1 is phosphorylated in various cell types on serine 289 (*23*, *24*) and perhaps other sites, but the functional significance of this phosphorylation is unknown.

It has recently been reported that the PDZ domains of NHERF-1 can oligomerize (*18*, *25*, *26*). This oligomerization is physiologically important in that it facilitates the ability of NHERF-1 to potentiate PDGFR activity (*18*). Many questions remain, however, regarding NHERF oligomerization. It is not known, for example, whether the PDZ domains of NHERF-2 can oligomerize like those of NHERF-1. Furthermore, the specificity of NHERF PDZ domain oligomerization has not been explored; it is not known if the NHERF PDZ domains associate with many other types of PDZ domains or only with each other. Finally, while it has been shown that NHERF-1 can oligomerize in cells in a physiologically relevant fashion, it is not known if this process is regulated in any way. In the experiments described in the present report, we have examined these issues of specificity and regulation for NHERF-1 and NHERF-2 PDZ domain oligomerization.

MATERIALS AND METHODS

Preparation of Plasmids and Fusion Proteins. Hexahistidine- and S-tagged NHERF fusion proteins, for both full-length NHERF-1 and NHERF-2 and for various NHERF truncations, were created via insertion of PCR products derived from a rabbit NHERF cDNA into pET-30A (Novagen), followed by expression and purification, as previously described (*13*, *23*). The domain structure of NHERF-1 and NHERF-2 is shown in Figure 1. The "PDZ1" constructs for NHERF-1 and NHERF-2 correspond to amino acids 1−151 and 1−148 of the two proteins, respectively, while the "PDZ2" constructs correspond to amino acids 152−358 and 149−337 of NHERF-1 and NHERF-2, respectively. These inserts were also shuttled into the pGEX-4T1 vector (Amersham Pharmacia Biotech) and used to transform BL-21 cells (Stratagene) in order to express NHERF-1, NHERF-2 and the various NHERF truncations as GST-tagged fusion proteins.

A number of other PDZ domains were also expressed as GST-tagged fusion proteins for the studies described here. The PSD-95 "PDZ1+2" and "PDZ3" constructs were prepared via PCR from a rat PSD-95 cDNA kindly provided by Morgan Sheng (Harvard Medical School). The PDZ1+2 (corresponding to amino acids 59−303 of rat PSD-95) and PDZ3 (corresponding to amino acids 307−446) were inserted into the pGEX-4T1 vector using *Eco*R1 and *Xho*1 sites introduced during PCR. The MAGI-2 PDZ1, PDZ2, and PDZ3 constructs were prepared via PCR from a human MAGI-2/AIP1 cDNA kindly supplied by Christopher Ross (Johns Hopkins University School of Medicine). The MAGI-2 PDZ1 (corresponding to amino acids 420−559 of human MAGI-2), PDZ2 (corresponding to amino acids 599−743), and PDZ3 (corresponding to amino acids 772−911), like the PSD-95 PDZ domain inserts, were inserted into pGEX-4T1 at the *Eco*R1 and *Xho*1 sites. The lone PDZ domain of Shank (corresponding to amino acids 587−686 of rat Shank1a) was similarly inserted into pGEX-4T1 using *Eco*R1 and *Sal*1 sites introduced via PCR from a cDNA kindly supplied by Morgan Sheng. The sequences of all constructs prepared by PCR were verified by ABI sequencing. The PDZ domain of RGS12 (corresponding to amino acids 1−94 of rat RGS12) was also expressed using a previously described construct (*27*) kindly provided by David Siderovski (University of North Carolina, Chapel Hill).

The β_2AR carboxyl terminus (the last 80 amino acids of the human β_2-adrenergic receptor) as well as the PDGFR-β receptor carboxyl terminus (the last 45 amino acids of the human PDGFR-β) were expressed as GST fusion proteins using the pGEX-2TK vector (Pharmacia) as previously described (*18*). A mutant β_2AR-CT, with the last six amino acids changed to VQDTRL, was prepared via PCR using the same 5′ oligo as for the wild-type β_2AR-CT and the following 3′ oligo: 5′-GTGACGCTCGAGTTACAGCCGT-GTGTCTTGTACACTACAATTCCTCCCTTGTGAATC-3′. The resultant PCR product was digested with *Eco*R1 and *Xho*1 and inserted into pGEX-4T1 for expression as a GST fusion protein. The S289A NHERF-1 mutant in pET30A has been described previously (*24*). A novel NHERF-1 point mutant, S289D, was created by PCR amplification from the native rabbit NHERF cDNA using mutant sequence oligonucleotides; the point mutation at position 289 was confirmed by ABI sequencing.

Blot Overlay Assays. NHERF-1 and NHERF-2 binding to various GST-PDZ domain fusion proteins was assayed via a blot overlay technique, as previously described (*13*). The GST fusion proteins (2 μg/lane) were run on 4−20% SDS−PAGE gels (Novex), blotted and overlaid with His-NHERF-1 or His-NHERF-2 in "blot buffer" (2% nonfat dry milk, 0.1% Tween-20, 50 mM NaCl, 10 mM Hepes, pH 7.4) for 1 h at room temperature. The blots were washed three times with 10 mL of blot buffer and then incubated for 1 h at room temperature with S-protein horseradish peroxidase conjugate (Novagen) diluted 1:4000 in blot buffer to detect the S-tag on the overlaid His-NHERF fusion proteins. Finally, the blots were washed three more times with 10 mL of blot buffer and visualized via enzyme-linked chemiluminescence using the ECL kit from Amersham Pharmacia Biotech. His-NHERF binding to the GST-PDZ domain panel was studied alone as well as in the presence of either control GST or a carboxyl-terminal fusion protein (such as β_2AR-CT-GST). The experiments in the presence of control GST yielded identical results to experiments where no GST was added, indicating that GST alone does not have any effect on the overlay assay.

Plate Assays. GST-NHERF-1 and GST-NHERF-2 fusion proteins, as well as GST alone, were expressed as described above and purified on glutathione agarose beads (Sigma).

The GST fusion proteins were then eluted from the beads using 250 mM reduced glutathione (Sigma) in "harvest buffer" (10 mM HEPES, 50 mM NaCl, 5 mM EDTA, and 1 mM benzamidine). The excess glutathione was removed via multiple rounds of concentration and dilution with fresh harvest buffer. The eluted GST fusion proteins were used to coat the wells of 96-well high affinity binding dishes (Fisher). Each well was coated overnight at 4 °C with 1 μg of fusion protein in 100 μL final volume of harvest buffer. After the coating solution was removed, the wells were washed twice with harvest buffer and then blocked with blot buffer for 30 min at room temperature. For experiments where NHERF binding was assessed in the presence of β_2AR-CT-GST, the wells were preincubated with the β_2AR-CT-GST for 30 min. His-tagged NHERF fusion proteins diluted in blot buffer (either in the presence or absence of β_2AR-CT-GST) were added to the precoated wells at the indicated concentrations and incubated for 1 h at room temperature. The wells were then washed five times with 200 μL of blot buffer. For detection, S-protein alkaline phosphatase conjugate (Novagen) diluted 1:4000 in blot buffer was added to the wells (100 μL/well) and incubated for 1 h at room temperature. The wells were then washed four times with blot buffer and twice with harvest buffer. Following the final wash, each well was incubated with 100 μL of the alkaline phosphatase substrate (Bio-Rad), and the absorbance at 405 nm was determined using a Thermomax microplate reader (Molecular Devices) in order to provide quantitative values for the amount of His-NHERF fusion protein bound to each well. Specific binding was defined as the binding observed to wells coated with a PDZ-GST fusion protein minus the binding observed to wells coated with GST alone on the same plate. For experiments where the binding of His-NHERF-1 was studied in the presence of β_2AR-CT-GST, nonspecific binding of His-NHERF-1 to wells coated with GST alone was also assessed in the presence of β_2AR-CT-GST; the nonspecific binding in the presence of β_2AR-CT-GST did not significantly differ from nonspecific binding in the absence of β_2AR-CT-GST.

Pull-Down Assays. GST-NHERF-1 and GST-NHERF-2 fusion proteins were purified on glutathione agarose beads as described above. Aliquots of the fusion protein/bead mixture in 1.5 mL microcentrifuge tubes were blocked for 1 h with 1 mL of a 3% BSA buffer (also containing 10 mM HEPES, 50 mM NaCl, and 0.1% Tween 20). Equal concentrations of various His-tagged fusion proteins were then incubated with the beads in 1 mL of the 3% BSA blocking buffer at 4 °C with end-over-end rotation for 1 h. The beads were washed five times with ice-cold 3% BSA blocking buffer and washed twice with harvest buffer. The proteins were eluted from the beads with 1× SDS−PAGE sample buffer, resolved via SDS−PAGE and transferred to nitrocellulose. The His-tagged fusion proteins were detected via Western blotting with S-protein HRP conjugate from Novagen (1:4000), and bands were visualized via chemiluminescence as described above.

Cell Culture and Transfection. All tissue culture media and related reagents were purchased from Gibco/Life Technologies. HEK-293 cells were maintained in complete medium (minimal essential medium plus 10% fetal bovine serum and 1% penicillin/streptomycin) in a 37 °C/5% CO_2 incubator. To express Flag- and/or HA-tagged versions of NHERF-1 and NHERF-2, 4 μg total DNA (NHERF-1 and/or NHERF-2 cDNAs in modified versions of the vector pBK-CMV from Stratagene) was mixed with Lipofectamine (15 μL) and added to 5 mL of minimal essential medium in 10 cm tissue cultures plates containing HEK-293 cells at approximately 50−80% confluency. Following a 3 h incubation, 6 mL of complete medium was added. Twenty-four hours later, the medium was replaced with fresh complete medium, and the cells were incubated for an additional 24−48 h before harvesting. The expression of NHERF-1 in transfected cells was roughly double the expression of endogenous NHERF-1, as assessed by semiquantitative Western blotting of control versus transfected cell lysates with an anti-NHERF-1 antibody (BD Transduction Laboratories).

Immunoprecipitation. For immunoprecipitation experiments performed in the presence of DSP cross-linking, a 25 mM stock of DSP (from Pierce) was prepared in DMSO, and 400 μL was added to 10 mL of PBS covering plates of transfected HEK-293 cells (yielding a final concentration of 1 mM DSP for cross-linking). The plates were incubated for 30 min at room temperature and then washed twice with PBS containing 50 mM Tris to remove and inactivate any residual DSP. Cells were harvested and lysed in 500 μL of ice-cold lysis buffer (10 mM Hepes, 50 mM NaCl, 5 mM EDTA, 1 mM benzamidine, 0.5% Triton X-100). The lysate was solubilized via end-over-end rotation at 4 °C for 30 min and clarified via centrifugation at 14 000 rpm for 15 min. A small fraction of the supernatant was taken at this point and incubated with SDS−PAGE sample buffer in order to examine expression of proteins in the whole cell extract. The remaining supernatant was incubated with 30 μL of anti-Flag M2 affinity gel (Sigma) for 2 h with end-over-end rotation at 4 °C. After five washes with 1.0 mL of lysis buffer, the immunoprecipitated proteins were eluted from the beads with 1× SDS−PAGE sample buffer, resolved by SDS−PAGE and subjected to Western blot analysis with antibodies to the Flag epitope (anti-Flag monoclonal Ab from Sigma) and/or antibodies to the HA epitope (12CA5 monoclonal Ab from Roche). For studies where the amount of co-immunoprecipitated HA-NHERF protein was semiquantitatively compared between different conditions, autoradiograms were scanned with an Epson 1200U flatbed scanner and the relative intensities of immunoprecipitated bands were quantified using the NIH Image program (version 1.62). To assess immunoprecipitation specificity, immunoprecipitated samples were not only probed with anti-Flag and anti-HA antibodies, they also were probed with antibodies detecting several irrelevant proteins, such as RGS1 and 14-3-3, which are highly expressed in HEK-293 cells and easily detectable with commercially available antibodies (Santa Cruz Biotechnology). No immunostaining for these proteins was detectable in the Flag-NHERF immunoprecipitates, either in the absence or the presence of DSP cross-linking, indicating that the immunoprecipitations were specific.

RESULTS

Specificity of NHERF PDZ Domain Oligomerization. The potential association of NHERF-1 and NHERF-2 PDZ domains with each other and with non-NHERF PDZ domains was examined in blot overlay experiments. For these studies, hexahistidine-tagged NHERF-1 ("His-NHERF-1") was over-

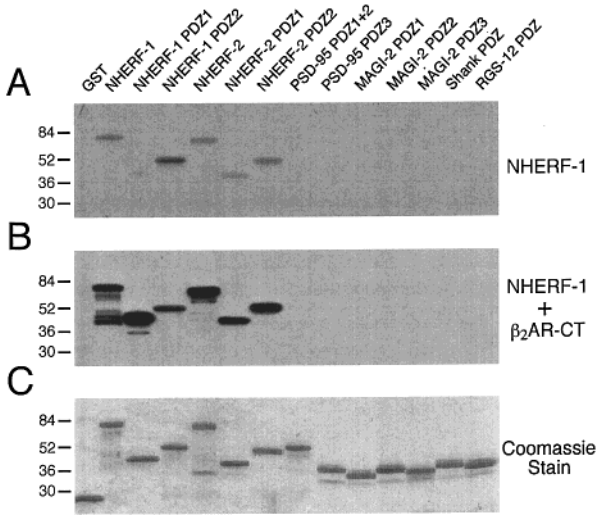

FIGURE 2: NHERF-1 binds specifically to NHERF-1 and NHERF-2 PDZ domains but not to other PDZ domains. (A) Overlay of His-NHERF-1 onto GST-tagged PDZ domain fusion proteins. His-NHERF-1 (100 nM) binds weakly to full-length NHERF-1 and NHERF-2, as well as to the two halves of each of these proteins, but exhibits no evident binding to the other PDZ domains examined. Each lane contains 2 μg of purified GST-PDZ fusion protein, which was transferred to nitrocellulose and probed with the His-NHERF-1 fusion protein. (B) Association of His-NHERF-1 with the β_2AR-CT (200 nM) enhances NHERF-1 binding to NHERF PDZ domains in the overlay assay. When the experiment shown in panel A is repeated in the presence of GST-β_2AR-CT in the overlay solution, binding of His-NHERF-1 to the GST-tagged NHERF PDZ fusion proteins is markedly enhanced. Binding of His-NHERF-1 to non-NHERF PDZ domains, however, is still undetectable. The data shown are representative of four independent experiments. (C) Coomassie stain showing the relative size and loading of the various GST fusion proteins. The positions of molecular mass standards (kDa) are shown on the left.

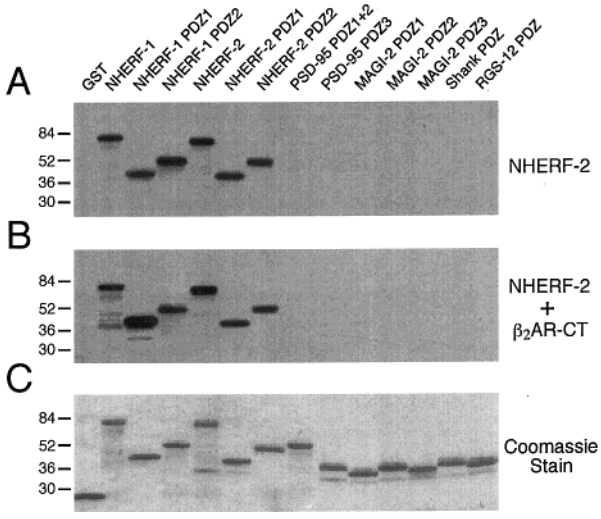

FIGURE 3: NHERF-2 binds specifically to NHERF-1 and NHERF-2 PDZ domains but not to other PDZ domains. (A) Overlay of His-NHERF-2 onto GST-tagged PDZ domain fusion proteins. His-NHERF-2 (100 nM) binds robustly to full-length NHERF-1 and NHERF-2, as well as the two halves of each of these proteins, but exhibits no evident binding to the other PDZ domains examined. As in the experiments illustrated in Figure 2, each lane contains 2 μg of purified GST-PDZ fusion protein, which was transferred to nitrocellulose and probed with the His-NHERF-2 fusion protein. (B) Association of His-NHERF-2 with the β_2AR-CT (200 nM) has only a marginal effect on NHERF-2 binding to NHERF PDZ domains in the overlay assay. When the experiment shown in panel A is repeated in the presence of GST-β_2AR-CT in the overlay solution, binding of His-NHERF-2 to the GST-tagged NHERF PDZ fusion proteins is only slightly enhanced. These results contrast sharply with the marked β_2AR-CT-induced potentiation of NHERF-1 binding to the NHERF PDZ domains observed in the experiments illustrated in Figure 2. Binding of His-NHERF-2 to non-NHERF PDZ domains is not detectable under any of the conditions examined. The data shown are representative of four independent experiments. (C) Coomassie stain showing the relative size and loading of the various GST fusion proteins. The positions of molecular mass standards (kDa) are shown on the left.

laid onto various GST-tagged PDZ domains and detected via a far Western blot approach. As shown in Figure 2A, His-NHERF-1 bound weakly to GST-NHERF-1 and GST-NHERF-2 as well as to the individual GST-NHERF PDZ domains, but did not detectably bind to any of the non-NHERF PDZ domains examined (including the three PDZ domains of PSD-95, the first three PDZ of MAGI-2, and the individual PDZ domains of Shank and RGS-12). It has previously been reported that NHERF-1 exhibits enhanced oligomerization in the presence of a PDZ domain "ligand" such as the PDGFR carboxyl-terminus (*18*), and therefore, we examined the binding of His-NHERF-1 to the GST-PDZ domain blot panel in the presence of the β_2AR carboxyl-terminus. As the data shown in Figure 2B reveal, binding of His-NHERF-1 to the GST-NHERF-1 and GST-NHERF-2 PDZ domains was markedly enhanced by the presence of the β_2AR-CT. However, the presence of the β_2AR-CT did not lead to any detectable association of His-NHERF-1 with the various non-NHERF PDZ domains.

The overlay experiments onto the GST-PDZ domain panel were repeated using His-NHERF-2 instead of His-NHERF-1. Like His-NHERF-1, overlaid His-NHERF-2 exhibited binding to GST-NHERF-1 and GST-NHERF-2 PDZ domains but not to any of the other PDZ domains examined (Figure 3A). However, the binding of His-NHERF-2 alone to the GST-NHERF PDZ domains was substantially more robust than that observed with His-NHERF-1 alone. Moreover, when the His-NHERF-2 overlay experiments were repeated in the presence of the β_2AR-CT (Figure 3B), only a slight increase in binding was observed, in contrast to the large increase in binding observed in the His-NHERF-1 overlay experiments.

Quantitation of NHERF Oligomerization in Plate Binding Assays. To quantify the differential effect of the β_2AR-CT on NHERF-1 versus NHERF-2 oligomerization, saturation binding studies using a plate assay were performed. Samples of GST-NHERF-1, GST-NHERF-2, or control GST were immobilized in the wells of a 96-well plate, and the binding of increasing amounts of either His-NHERF-1 or His-NHERF-2 was measured. These experiments were performed in both the absence and presence of the β_2AR-CT. As shown in Figure 4A, the binding of His-NHERF-1 to the immobilized GST-NHERF-1 was saturable, with an apparent K_D of 850 nM. The presence of the β_2AR-CT in the binding assay did not alter the maximum amount of His-NHERF-1 binding, but did result in a significant leftward shift of the His-NHERF-1 binding curve and an improvement of the apparent K_D of NHERF-1/NHERF-1 oligomerization to 78 nM.

Binding of His-NHERF-2 in the plate assay was saturable, with an apparent K_D of 126 nM (Figure 4B). The observation that the affinity of His-NHERF-2 binding in the plate assay was more than 5-fold higher than that for His-NHERF-1

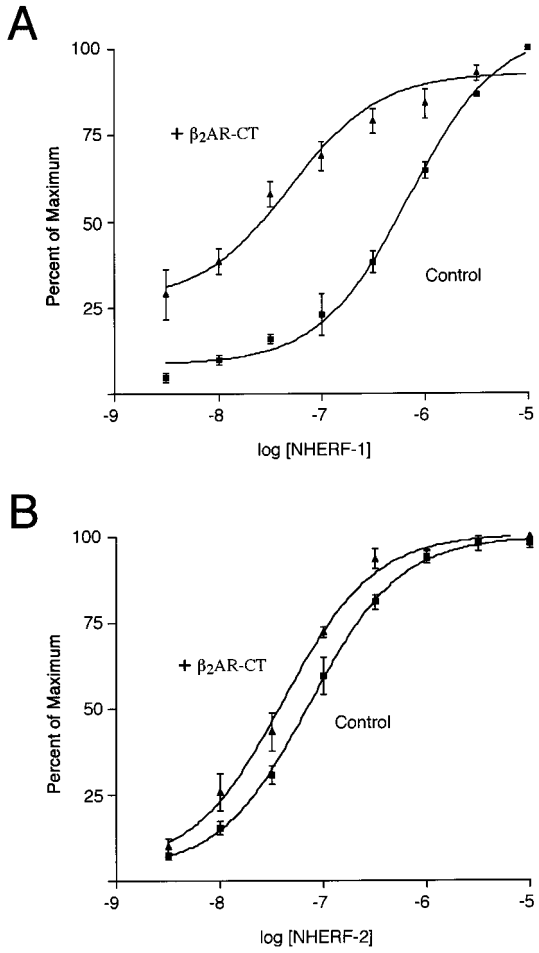

FIGURE 4: Estimations of the binding affinity for NHERF-1/NHERF-1 and NHERF-2/NHERF-2 associations. (A) The apparent binding affinity of His-NHERF-1 for GST-NHERF-1 is enhanced more than 10-fold by the presence of the β_2AR-CT. A plate binding assay was used to quantitate the binding of His-NHERF-1 (at eight concentrations ranging from 3 nM to 10 μM) to 1 μg to GST-NHERF-1 adsorbed to wells of a 96-well plate. The experiments were performed in the absence and presence of 200 nM β_2AR-CT-GST. In the presence of the β_2AR-CT, the apparent binding affinity of His-NHERF-1 for GST-NHERF-1 was improved from 850 nM to 78 nM. (B) The apparent binding affinity of His-NHERF-2 for GST-NHERF-2 is enhanced less than 2-fold by the presence of the β_2AR-CT. As in the experiments illustrated in panel A, plate binding assays were performed to quantitate the binding of His-NHERF-2 (at eight concentrations ranging from 3 nM to 10 μM) to 1 μg GST-NHERF-2 adsorbed to wells of a 96-well plate. The experiments were performed in the absence and presence of 200 nM β_2AR-CT-GST. In the presence of the β_2AR-CT, the apparent binding affinity of His-NHERF-2 for GST-NHERF-2 improved only slightly, from 126 nM to 68 nM. The data shown in both panels are derived from three to four independent experiments for each condition, with each experiment being performed in triplicate.

binding was consistent with the findings described above for the blot overlay experiments. Also consistent with the blot overlay results was the finding that His-NHERF-2 binding in the plate assay experiments was only slightly enhanced by the presence of the β_2AR-CT (K_D = 68 nM). Thus, the affinity of NHERF-2 oligomerization in the plate assay was enhanced less than 2-fold, as compared to the more than 10-fold enhancement observed for NHERF-1 oligomerization.

As noted above, it has previously been reported that association with the PDGFR-CT enhances NHERF-1/NHERF-1 oligomerization (*18*). The findings described here, that the β_2AR-CT can also profoundly enhance NHERF-1/NHERF-1 oligomerization, demonstrate that this effect is not specific to the PDGFR-CT but rather is likely to be a general effect of engagement of the NHERF-1 PDZ domains by carboxyl-terminal ligands. To further explore this issue, we compared the ability of several carboxyl-terminal ligands for NHERF-1 PDZ1 to enhance NHERF-1 oligomerization. As shown in Figure 5, equal concentrations of the β_2AR-CT and PDGFR-CT enhanced NHERF-1/NHERF-1 oligomerization by nearly equal amounts. A mutant version of the β_2AR-CT-GST, with the last six amino acids mutated to conform to the optimal NHERF-1 PDZ1-binding motif of VQDTRL (*12*, *13*), also markedly enhanced NHERF-1 oligomerization. In contrast, a high concentration (200 μM) of a six amino acid peptide, VQDTRL, which represents the ideal NHERF-1 PDZ1 binding motif isolated from the context of a larger polypeptide, did not have any apparent effect on NHERF-1 oligomerization. The VQDTRL peptide did, however, compete for binding of the β_2AR-CT-GST to NHERF-1 PDZ1 with an affinity of approximately 20 μM (data not shown) and also blocked the ability of the β_2AR-CT to enhance NHERF-1 oligomerization, indicating that the peptide bound to NHERF-1 but simply did not improve oligomerization like the longer receptor carboxyl-terminus fusion proteins.

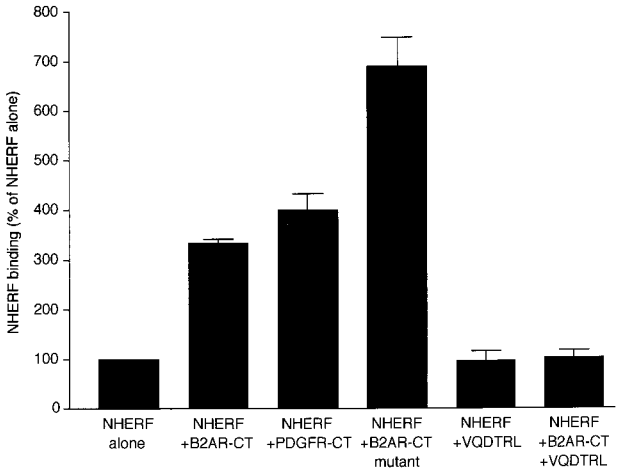

FIGURE 5: β_2AR-CT-GST, PDGFR-CT-GST, and a β_2AR-CT-GST mutant enhance NHERF-1 oligomerization equally, but a short NHERF-1-binding peptide does not promote NHERF-1 oligomerization. The plate binding assay was employed to quantitate the abilities of β_2AR-CT-GST (200 nM), PDGFR-CT-GST (200 nM), and a β_2AR-CT-GST mutant terminating in the ideal NHERF-1 PDZ1-binding motif of VQDTRL (200 nM) to enhance the binding of His-NHERF-1 (100 nM) to wells coated with 1 μg of GST-NHERF-1. Both β_2AR-CT-GST and PDGFR-CT-GST enhanced NHERF-1/NHERF-1 association by roughly 4-fold at the concentrations examined, while the β_2AR-CT-GST mutant was slightly better at enhancing NHERF-1 oligomerization. These findings are consistent with observations that the β_2AR-CT and PDGFR-CT have roughly comparable affinities for binding to NHERF-1, while the β_2AR-CT mutant binds NHERF-1 with a slightly higher affinity. In contrast to the three CT-GST fusion proteins, a short NHERF-1-binding peptide, VQDTRL (200 μM), did not promote NHERF-1/NHERF-1 association at all. The VQDTRL peptide did, however, block the effect of the β_2AR-CT-GST in this assay when the two were incubated together, suggesting that the peptide does bind to NHERF-1 but simply does not promote NHERF-1 oligomerization. The data shown are derived from three independent experiments, each one performed in triplicate.

Mutation of Ser289 Alters NHERF-1 Oligomerization. Cellular NHERF-1 is known to be a phosphoprotein (*20*, *23*, *24*), and Ser289 has been identified as a major site of cellular NHERF-1 phosphorylation (*23*, *24*). The functional significance of NHERF-1 phosphorylation on Ser289, however, is completely unknown. To address this issue, we created a mutant version of NHERF-1 in which Ser289 is mutated to aspartate (S289D), to potentially mimic the phosphorylated form of NHERF-1. Phosphorylation of NHERF-1 on Ser289 in cells is known to retard the mobility of NHERF-1 on SDS−PAGE gels, such that the protein appears slightly larger following phosphorylation (*24*). The mobility of the S289D NHERF-1 mutant on SDS−PAGE gels is also retarded relative to the mobility of wild-type NHERF-1 and the previously described S289A mutant NHERF-1 (Figure 6A). This change in mobility of the S289D mutant lends support to the idea that replacing the serine at this position with aspartate mimics conformational changes induced by phosphorylation of NHERF-1 on Ser289.

We next examined the ability of the S289D mutant NHERF-1 to bind β_2AR-CT and to oligomerize. Overlay and pull-down experiments revealed that both the S289D mutant and the previously described S289A mutant (*24*) bind β_2AR-CT with an affinity comparable to that of wild-type NHERF-1 (data not shown). In contrast, the S289D mutation had a marked effect on NHERF-1 oligomerization: pull-down experiments with GST- and His-tagged fusion proteins revealed that His-S289D NHERF-1 was pulled down much more efficiently than wild-type His-NHERF-1 by wild-type GST-NHERF-1 (Figure 6B). Plate binding assays also revealed that the S289D mutant displays a markedly enhanced capacity for oligomerization. Binding of His-S289D to wells coated with GST-S289D was more than 15-fold higher than binding of an equal concentration of wild-type His-NHERF-1 to wells coated with wild-type GST-NHERF-1 (Figure 6C). Oligomerization of the S289D mutant was enhanced by association with the β_2AR-CT, although the fold increase in binding induced by the β_2AR-CT was somewhat reduced relative to that observed for wild-type NHERF-1.

Cellular Co-immunoprecipitation Studies. To examine whether NHERF-1 and NHERF-2 might form oligomeric complexes in a cellular environment, co-immunoprecipitation studies were performed. For these experiments, HA-tagged and Flag-tagged versions of NHERF-1 and NHERF-2 were expressed either individually or in combinations in HEK-293 cells. Following immunoprecipitation of Flag-tagged proteins, co-immunoprecipitation of HA-tagged proteins was assessed via Western blot. As shown in Figure 7A, HA-NHERF-2 robustly co-immunoprecipitated with Flag-NHERF-2, while co-immunoprecipitation of HA-NHERF-1 with Flag-NHERF-1 or Flag-NHERF-2 was detected either very faintly or not at all. To improve detection of cellular NHERF-1/NHERF-1 homo-oligomeric complexes, the co-immunoprecipitation studies were repeated following treatment of the cells with DSP, which can covalently cross-link associated proteins together. Under these conditions, co-immunoprecipitation of HA-NHERF-1 with Flag-NHERF-1 and Flag-NHERF-2 was now easily detectable (Figure 7B). Co-immunoprecipitation of HA-NHERF-2 with Flag-NHERF-2, which was easily detectable in the absence of cross-linking, was not significantly enhanced following treatment of the cells with DSP. These data suggest that cellular NHERF-1/NHERF-1 and NHERF-1/NHERF-2 complexes are stabilized by amine-reactive protein cross-linking, while cellular NHERF-2/NHERF-2 complexes form more readily under non-cross-linked conditions and are not stabilized by treatment with the cross-linking reagent.

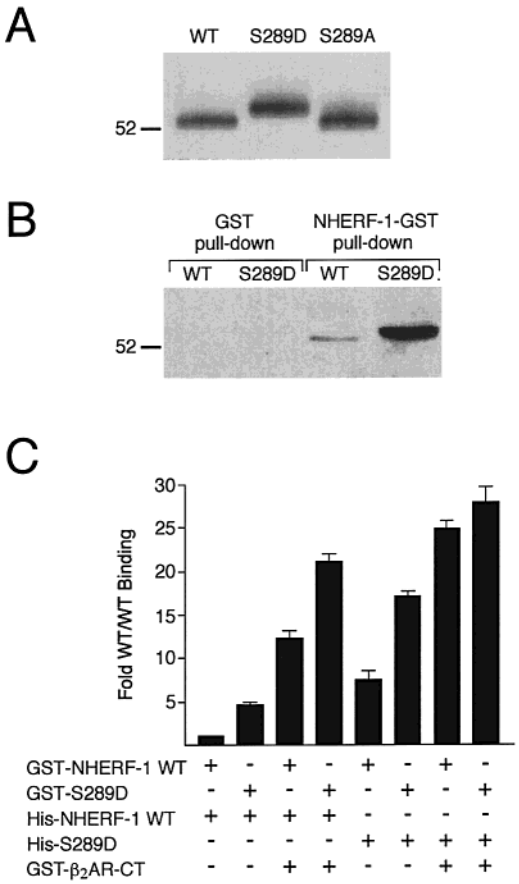

FIGURE 6: NHERF-1 S289D mimics phosphorylation of NHERF on Ser289 and enhances NHERF-1 oligomerization. (A) The S289D mutation shifts the mobility of NHERF-1 on SDS−PAGE. Wild-type His-NHERF-1, His-NHERF-1 S289D, and His-NHERF-1 S289A fusion proteins were run on a 12% SDS−PAGE gel and examined via Western blot using S-protein HRP conjugate for detection. The S289D mutation induces a mobility shift in NHERF-1 similar to that observed for cellular NHERF-1 phosphorylated on Ser289. (B) The S289D mutation enhances NHERF-1/NHERF-1 oligomerization in a pull-down assay. Equal concentrations (100 nM) of wild-type His-NHERF-1 and the His-NHERF-1 S289D mutant were incubated with glutathione agarose beads loaded with either control GST or GST-NHERF-1. NHERF-1/NHERF-1 oligomerization was then assessed via a pull-down assay. No binding of His-NHERF-1 or His-NHERF-1 S289D to control GST was detectable under these conditions. GST-NHERF-1 beads, in contrast, consistently pulled down both His-NHERF-1 and His-NHERF-1 S289D, with pull-down of the mutant fusion protein being markedly enhanced relative to that of the wild-type. These data are representative of three independent experiments. (C) The S289D mutation enhances NHERF-1/NHERF-1 oligomerization in a plate binding assay. The binding of His-NHERF-1 (100 nM) or His-NHERF-1 S289D (100 nM) to wells coated with 1 μg of either GST-NHERF-1 or GST-NHERF-1 S289D was quantitated in the absence and presence of β_2AR-CT-GST (200 nM). The S289D mutant demonstrated an enhanced ability to oligomerize under all conditions examined, including in the presence of the β_2AR-CT-GST. The data for each condition are expressed as a fold increase in binding over that observed for His-NHERF-1 wild-type binding to GST-NHERF-1 wild-type on the same plate. These data are derived from three independent experiments, each one performed in triplicate.

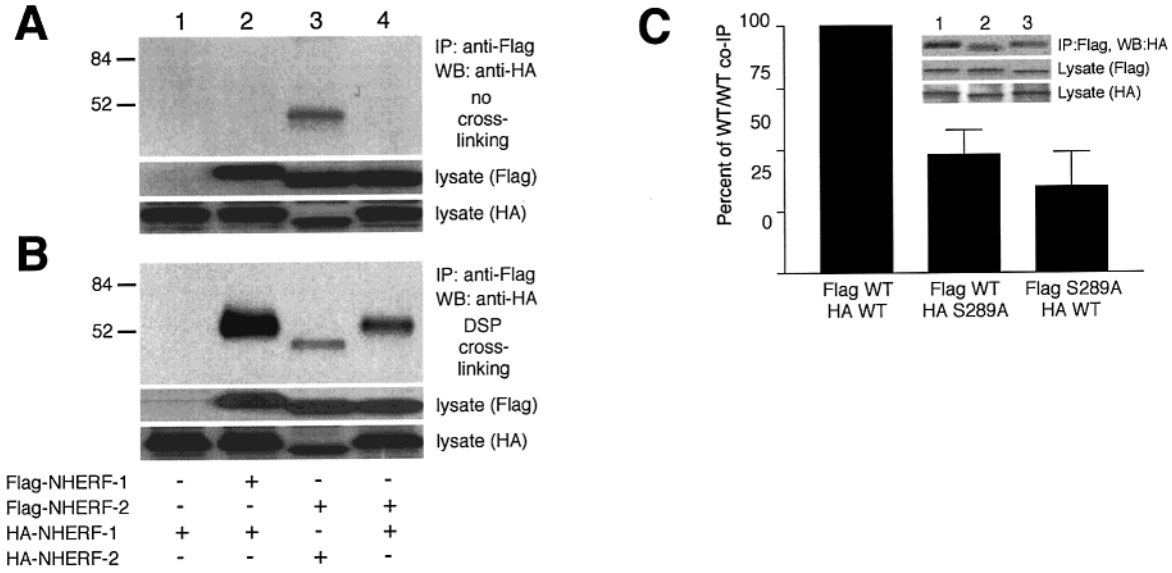

FIGURE 7: NHERF-1 and NHERF-2 form homo-oligomers and hetero-oligomers in a cellular environment. (A) Co-immunoprecipitation of HA-NHERF-2 with Flag-NHERF-2 (lane 3) is detectable in the absence of cross-linking. Co-immunoprecipitation of HA-NHERF-1 with Flag-NHERF-1 (lane 2) or Flag-NHERF-2 (lane 4) was not consistently observed under non-cross-linked conditions. The relative expression levels of all the expressed proteins were determined by Western blots of the cell lysates, as shown in the lower half of this panel. The data shown are representative of three to four independent experiments. (B) Co-immunoprecipitation of NHERF-1, but not NHERF-2, is enhanced by cross-linking. Cells expressing various combinations of epitope-tagged NHERF-1 and NHERF-2 were treated with the membrane-permeable cross-linker DSP (1 mM for 30 min), solubilized and incubated with anti-Flag agarose beads for immunoprecipitation. Co-immunoprecipitation of HA-NHERF-1 with Flag-NHERF-1 (lane 2) or Flag-NHERF-2 (lane 4), which had been difficult to detect in the absence of cross-linking, was easily observable following DSP cross-linking. Co-immunoprecipitation of HA-NHERF-2 with Flag-NHERF-2 (lane 3), in contrast, was not enhanced by DSP cross-linking. The relative expression levels of all the expressed proteins were determined by Western blots of the cell lysates, as shown in the lower half of this panel. The data shown are representative of four independent experiments. (C) The S289A NHERF-1 mutant exhibits a reduced capacity for oligomerization in cells. HEK-293 cells coexpressing Flag-NHERF-1 wild-type (WT) plus HA-NHERF-1 S289A, or Flag-S289A plus HA-NHERF-1 WT, were cross-linked with DSP, harvested, and incubated with anti-Flag agarose beads to immunoprecipitate the Flag-tagged proteins. Co-immunoprecipitation of the coexpressed HA-tagged proteins was assessed via Western blot and expressed as a percentage of the amount of co-immunoprecipitation observed in matched plates of cells expressing Flag-NHERF-1 WT plus HA-NHERF-1 WT. Expression levels of NHERF-1 WT and NHERF-1 S289A were comparable across all of these experiments, as shown in the inset to the graph, but co-immunoprecipitation of the HA-S289A mutant (as well as co-immunoprecipitation of wild-type HA-NHERF-1 with the Flag-S289A mutant) was consistently reduced relative to co-immunoprecipitation of HA-tagged wild-type NHERF-1 with Flag-tagged wild-type NHERF-1. The bars represent mean ± SEM for three independent experiments. The data from a representative experiment are shown in the inset. The top blot strip shows the amount of HA-tagged NHERF-1 co-immunoprecipitated by equal levels of Flag-tagged NHERF-1, with the lane order as follows: (1) Flag-WT/HA-WT, (2) Flag-WT/HA-S289A, (3) Flag-S289A/HA-WT. The lower two blot strips show the expression levels of the various epitope-tagged proteins in the starting cell lysates.

Most NHERF-1 expressed in HEK-293 cells is constitutively phosphorylated on Ser289 (*24*). We therefore examined whether the NHERF-1 S289A mutant, which cannot be phosphorylated on Ser289, exhibits an altered capacity for oligomerization in cells. As shown in Figure 7C, cotransfected combinations of Flag-NHERF-1 WT/HA-S289A and Flag-S289A/HA-NHERF-1 WT exhibited markedly reduced levels of co-immunoprecipitation relative to the co-immunoprecipitation observed from matched plates of cells co-expressing Flag- and HA-tagged wild-type NHERF-1. These data suggest that phosphorylation of NHERF-1 on Ser289 in cells promotes NHERF-1 oligomerization.

DISCUSSION

The findings reported here demonstrate that the PDZ domains of NHERF-1 and NHERF-2 can form oligomers with each other but not with any of the other PDZ domains that were examined. The panel of proteins studied here includes the PDZ domains of PSD-95, which have been previously shown to oligomerize with other PDZ domains such as that of nNOS (*5*−*7*, *9*). However, the PSD-95 PDZ domains exhibit no detectable association with the NHERF PDZ domains. While a variety of PDZ domains have been reported to oligomerize (*2*−*9*), the generality of this phenomenon has not been widely explored. Our data indicate that oligomerization of the NHERF PDZ domains is quite specific, much like the association of the NHERF PDZ domains with their carboxyl-terminal ligands (*12*, *13*).

While NHERF-1 and NHERF-2 are alike in that their PDZ domains can oligomerize with each other, the two NHERF proteins are distinct in the manner in which this oligomerization is regulated by carboxyl-terminal PDZ ligands. In the case of NHERF-2, oligomerization is a constitutive property of the protein, with the NHERF-2 PDZ domains exhibiting robust homo- and hetero-oligomerization in the absence of any other associated proteins. The PDZ domains of NHERF-1, in contrast, oligomerize with a fairly low affinity, as has previously been reported (*18*, *25*, *26*). The affinity for oligomerization of NHERF-1 PDZ1 is enhanced by more than 10-fold when the PDZ domain is associated with a ligand such as the β_2AR-CT or PDGFR-CT. This finding is striking, since oligomerization of other PDZ domains has been reported to be either unaffected (*3*) or inhibited (*5*−*7*) by association of the PDZ domains with carboxyl-terminal ligands. Our data demonstrate that PDZ

domain oligomerization can be regulated not only negatively but also positively by carboxyl-terminal PDZ domain ligands.

There are at least two conceivable mechanisms by which the β_2AR-CT might enhance NHERF-1 oligomerization: (i) β_2AR-CT might act as a "bridge" by simultaneously binding two NHERF-1 proteins and stabilizing their interaction, or (ii) β_2AR-CT might bind a single NHERF-1 protein and induce conformational changes in NHERF-1 that enhance association of the NHERF-1 PDZ domains with other NHERF PDZ domains. The latter hypothesis is supported by our data. NHERF-1 binding to immobilized NHERF-2 was strongly promoted by the presence of β_2AR-CT (as shown in Figure 2), but NHERF-2 binding to immobilized NHERF-1 was enhanced only slightly by β_2AR-CT (as shown in Figure 3). These results suggest that for β_2AR-CT to promote oligomerization, it must bind to NHERF-1 that is not immobilized (i.e., NHERF-1 that is free in solution and able to undergo conformational changes). Moreover, previous work has demonstrated that it is possible to make truncated versions of NHERF-1 PDZ1 that bind β_2AR-CT but do not oligomerize (*18*); these findings also argue against the possibility that the β_2AR-CT acts as a bridge to link together two NHERF-1 proteins.

NHERF-1 oligomerization is strongly promoted by association of the NHERF-1 PDZ domains with a variety of different carboxyl-terminal ligands: β_2AR-CT, PDGFR-CT, and a mutant β_2AR-CT terminating in the ideal NHERF-1 PDZ1 binding motif of VQDTRL. However, a short peptide corresponding to the VQDTRL motif alone does not alter the ability of NHERF-1 to oligomerize, despite the fact that this peptide is capable of binding to the NHERF-1 PDZ domains and inhibiting binding of the β_2AR-CT. The reason for the difference between the short peptide and the longer carboxyl-terminal proteins is not clear, but one potential explanation is that the fairly large size of the β_2AR-CT and PDGFR-CT may allow these proteins to allosterically induce conformational changes in the NHERF-1 PDZ1 domain that are not induced by the small peptide. Like other short peptides that bind to PDZ domains (*6*, *28*), the VQDTRL peptide exhibits a fairly low affinity for PDZ domain-binding relative to larger fusion proteins containing the same PDZ-binding motifs. Nonetheless, such peptides may prove to be useful research tools, especially those peptides (like the VQDTRL peptide) that have differential effects on PDZ/PDZ interactions versus PDZ/carboxyl-terminal ligand interactions.

NHERF-1 oligomerization is promoted in our studies not only by association with the β_2AR-CT or PDGFR-CT but also by mutation of Ser289 to aspartate to mimic the phosphorylated form of NHERF-1. This residue can be phosphorylated in vitro by several kinases (*23*, *24*), but a large proportion of cellular NHERF is constitutively phosphorylated on Ser289 due to a high-affinity interaction between NHERF and the G protein-coupled receptor kinase 6A (GRK6A) (*24*). The functional significance of this phosphorylation has been unknown, as it does not appear to alter the ability of NHERF-1 to regulate Na^+/H^+ exchange (*29*). Our data suggest that phosphorylation at this position potentiates the ability of NHERF-1 to oligomerize, indicating that NHERF-1 phosphorylation may alter aspects of cellular NHERF function that are dependent on NHERF-1 oligomerization. Oligomerization of the S289D mutant NHERF-1 in our studies was still potentiated by association with the β_2AR-CT-GST, and thus it is likely that these two means of promoting NHERF-1 oligomerization are mechanistically distinct. Since Ser289 is far removed from the first NHERF-1 PDZ domain, phosphorylation of this residue (or mutation to aspartate) most likely induces a global conformational change in the NHERF-1 protein that alters the accessibility of the PDZ domains. Association of carboxyl-terminal ligands with the NHERF-1 PDZ domains, in contrast, probably enhances oligomerization by inducing more local conformational changes directly in the PDZ domain region. This idea is supported by the finding that the β_2AR-CT can still enhance the oligomerization of NHERF-1 PDZ1 even when NHERF-1 PDZ1 is examined alone, out of the context of the full-length NHERF protein (*18*).

At present, few if any functional differences are known between NHERF-1 and NHERF-2. Both proteins are known to regulate Na^+/H^+ exchange (*10*, *11*, *29*), both are known to bind to similar carboxyl-terminal motifs on target proteins (*12*, *13*), and both are known to associate via their own carboxyl-termini with the FERM family of proteins (band 4.1, ezrin, radixin, moesin and merlin) (*20*, *21*, *28*). One difference between these two closely related gene products is that they have distinct tissue distributions: NHERF-2 is widely expressed in many cell types (*11*, *22*), whereas NHERF-1 shows a more restricted pattern of expression, being very highly expressed in the kidney, intestine, prostate and mammary tissue and expressed at lower levels elsewhere (*10*, *20*). Moreover, NHERF-1 expression is known to be regulated by estrogen (*31*), while this has not been shown for NHERF-2. The differing tissue distributions of these two proteins suggests that they may play distinct cell-specific roles in the regulation of cellular signaling. Our data demonstrate a key functional difference between NHERF-1 and NHERF-2 in that they exhibit differential regulation of their oligomerization. Oligomerization is a constitutive property of NHERF-2, whereas oligomerization of NHERF-1 is highly regulated, both by association with other proteins and by phosphorylation. This elucidation of differences in the oligomerization of the two NHERF proteins represents a key step toward understanding the different roles that these two proteins may play in various cellular signaling pathways.

What is the physiological significance of NHERF PDZ domain oligomerization? It has previously been shown that the ability of NHERF-1 to potentiate PDGFR signaling is dependent on the ability of NHERF-1 to oligomerize (*18*). Moreover, many cellular functions of NHERF-1 and NHERF-2 may depend on the ability of these proteins to act as scaffolds aiding in the formation of intracellular signaling complexes (*32*, *33*). Oligomerization of the NHERF proteins might be expected to play a key role in such scaffolding functions. For example, it has recently been reported that the mammalian Trp4 and Trp5 calcium channels, as well as isoforms of phospholipase Cβ known to regulate the Trp channels, all bind to the first PDZ domain of NHERF-1 and can be physically linked in cells via association with NHERF-1 (*34*). However, since all of these proteins associate with the same domain of NHERF-1, it is not clear how NHERF-1 might bridge these proteins together without oligomerization of NHERF-1 itself. Our data demonstrate that the PDZ domains of NHERF-1 and NHERF-2 can specifically oligomerize with each other but not with other PDZ domains. The studies

described here also reveal that there are profound differences between NHERF-1 and NHERF-2 in terms of the regulation of their oligomerization. These findings shed light on how the NHERF proteins may differentially influence the various intracellular signaling pathways in which they are involved.

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