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Discovery in Human Breast Cancer

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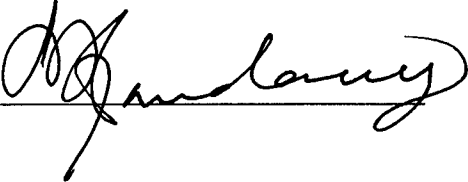
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In humans, the progesterone receptor exists as two isoforms, hPR-A and hPR-B. hPR-B is transcriptionally active, while hPR-A is inactive and acts as a transdominant repressor of estrogen receptor (ER) transcriptional activity. Although, the precise mechanism of hPR-A transrepression is not fully understood, we identified a domain located within the amino terminus of hPR-A, necessary for transrepression. This domain is contained within both PR isoforms, however, its activity is manifested only in hPR-A, suggesting that hPR-A interacts with a set of cofactors that are distinct from those recognized by hPR-B. In support of this hypothesis, we found that the interaction of hPR-A with the corepressor SMRT is stronger than that observed with hPR-B. The importance of such interaction, is demonstrated by using a dominant negative variant of SMRT to partially reverse hPR-A transrepression of ER activity, implicating SMRT in the transrepression by hPR-A. In addition, we show that hPR-B, but not hPR-A, interacts efficiently with the coactivators SRC-1 and GRIP1. Thus, the inability of hPR-A, in contrast to hPR-B, to recruit coactivators, as well as its strong association with corepressor proteins, correlates with the differences in the transcriptional activities of the two PR isoforms.

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Introduction

Analysis of genes uniquely expressed in MCF7 human breast cancer cells in response to estrogen identified NHERF-1. Further analysis of other breast cancer cells, including primary breast tumors, established a close correlation (>90%) between NHERF-1 expression and estrogen-dependent breast cancers. While the role of NHERF-1 in breast cancer remains unclear, our initial data and other recent studies suggest a complex regulatory role for NHERF-1 in estrogen-induced proliferation. NHERF-1 contains two PDZ domains that mediate protein-protein interactions, and three of the proteins NHERF-1 targets have been shown to be involved in breast cancer. These include the NF2 gene product Merlin, which is mutated in a subset of human breast neoplasms. Furthermore, disease-causing mutations in merlin have been linked to loss of NHERF binding. Estrogen also down-regulates the c-Yes non-receptor tyrosine kinase, which requires NHERF-1 for targeting to tight junctions to maintain epithelial cell morphology. Perhaps the most promising NHERF-1 target accounting for estrogen-induced cell proliferation is the PDGFR. NHERF-1 recruits PDGFR via its N-terminal PDZ domain. When combined with the intrinsic capacity of NHERF-1 to dimerize, this promotes PDGFR tyrosine phosphorylation, prolonged MAPK activation, and mitogenic signaling.

With this in mind, we sought to establish breast cancer cell lines, and begin studies to determine NHERF's role in their proliferation.

Body

Using breast cancer cells provided by Dr. Donald McDonnell and the Duke Comprehensive Cancer Center, we have begun characterizing the expression of NHERF mRNA and protein in these cells using available human NHERF-1 cDNA and a polyclonal anti-NHERF-1 antibody respectively as probes in Northern and Westerns. Initial evidence supports the published reports that ER(+) cell lines drastically overexpress NHERF compared to normal epithelial cells, while ER(-) cell lines typically do not. Also consistent with the published data, we have found that this correlation, while very strong (>90%), is not absolute. Therefore, we have procured ER(-) that express NHERF (MDA-MB-453) as well as ones that do not (MDA-MB-231). These cell line, combined with ER(+) lines, should provided critical insight as to NHERF's role in cell proliferation.

To this end, the following experiments are either currently ongoing or being planned:

- 1) Establish the relationship between NHERF-1 expression and cell growth in ER-positive human breast cancer cells
- 2) Establish the requirement for NHERF-1 in ER-mediated cell growth in human breast cancer cells
- 3) Establish the specificity of NHERF-1 as an activator of mitogenic signaling by PDGFR.

Key Accomplishments

- Established several cell lines to study the role of NHERF in growth regulation. Cell lines include:
 - multiple NIH-3T3 cell lines that stably express a tagged NHERF-1 construct
 - MDA-MB-231 breast cancer cells
 - MDA-MB-453 breast cancer cells
 - T47D breast cancer cells
 - ZR-75-1 breast cancer cells
- Begun Characterization of a NHERF-1 null mouse that exhibits a gender specific, growth related phenotype, further implicating both the estrogen activation of NHERF and its role in growth regulation
- Generated several NHERF-1 constructs to facilitate the *in vivo* analysis of its function. Constructs include:
 - N and C terminal GFP constructs
 - Mutants that lack the last four amino acids of NHERF
 - Various phosphorylated serine residues mutated to alanine

Reportable Outcomes

- 1) Attached manuscript of Oncogene review
- 2) Schillace, Robynn V., James W. Voltz, Shirish Shenolikar, and John D. Scott.; (2001) Multiple Interactions Within the AKAP220 Signaling Complex Contribute to the Inhibition of PP1 Activity. J Biol Chem 2001 Apr 13;276(15):12128-34
- 3) Terry-Lorenzo, Ryan T., James W. Voltz, John H. Connor, Shi Li, F. Donelson Smith, Sharon L. Milgram, Roger J. Colbran and Shirish Shenolikar; (2001) Cellular Mechanisms Regulating Protein Phosphatase-1: Molecular Analysis of PP1 Isoform Specificity of Neurabins; manuscript submitted

Conclusions

NHERF-1 is an estrogen regulated gene that regulates growth and development. This is illustrated not only by the evidence presented here, but new evidence that indicates NHERF-1 is a target of cell cycle dependent kinases and shows cell cycle dependant phosphorylation and dephosphorylation. Our goal is to study NHERF in cells that express it endogenously (breast cancer lines), exogenously (NIH3T3 cells), and in animals that lack NHERF-1 (our null mice). We have developed the reagents necessary to begin analyzing NHERF's role in growth regulation.

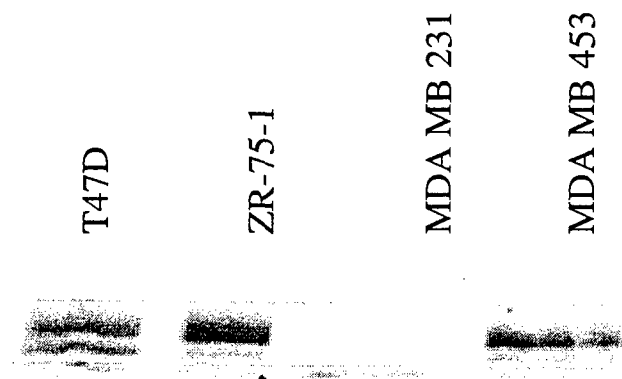


Figure 1 – NHERF-1 is Differentially Expressed in ER(+) and ER(-) Cell Lines – Western Blots of cell lysates from various breast cancer cell lines. Consistent with previous reports, our ER(+) (T47d and ZR75) cell lines over-express NHERF-1, while the ER(-) (231 & 453) lines exhibit variable expression

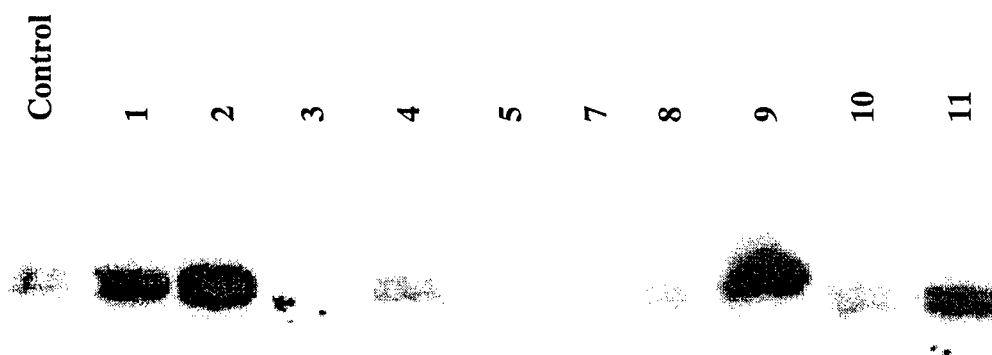


Figure 2– Generation of NIH3T3 Cell Lines Stably Expressing NHERF-1 – Stable cell lines were generated that express NHERF-1 to varying degrees. The NHERF-1 in these cells is tagged with an HA epitope to facilitate study.

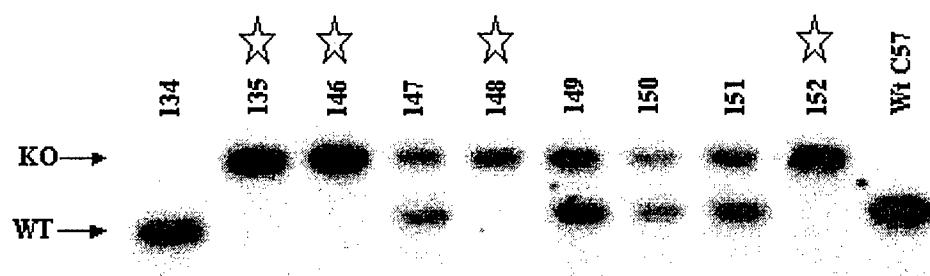
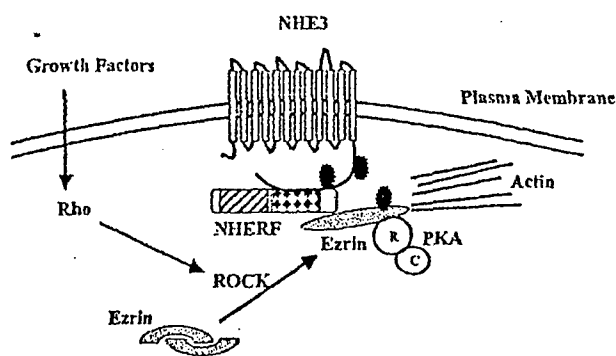


Figure 3 – Generation of NHERF-1 null mice – Genomic DNA was subjected to restriction digest and agarose gel electrophoresis. Following its transfer to nitrocellulose filters, the genomic DNA was probed with a P^{32} labeled NHERF DNA probe, which identified the animals lacking a functional NHERF-1 gene (stars). Initial characterization of these animals has revealed a gender specific growth related phenotype, consistent with NHERF's proposed role in growth regulation.



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Expanding the role of NHERF, a PDZ-domain containing protein adapter, to growth regulation

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NHERF (Na⁺/H⁺ exchanger regulatory factor or NHERF-1) and E3KARP (NHE3 kinase A regulatory protein or NHERF-2) are structurally related protein adapters that are highly expressed in epithelial tissues. NHERF proteins contain two tandem PDZ domains and a C-terminal sequence that binds several members of the ERM (ezrin-radixin-moesin) family of membrane-cytoskeletal adapters. Although identified as a regulator of NHE3, recent evidence points to a broadening role for NHERF in the function, localization and/or turnover of G-protein coupled receptors, platelet-derived growth factor receptor and ion transporters such as CFTR, Na/Pi cotransporter, Na/HCO₃ cotransporter and Trp (calcium) channels. NHERF also recruits non-membrane proteins such as the c-Yes/YAP-65 complex, members of the phospholipase C β family and the GRK6A protein kinase to apical surface of polarized epithelial cells where they regulate or respond to membrane signals. While two distinct models have been proposed for NHERF's role in signal transduction, the common theme is NHERF's ability to bring together membrane and non-membrane proteins to regulate cell metabolism and growth. NHERF overexpression in human breast cancers and mutations in NHERF targets, such as CFTR and merlin, the product of Neurofibromatosis NF2 tumor suppressor gene, that impair NHERF binding suggest that aberrant NHERF function contributes to human disease. *Oncogene* (2001) 00, 000–000.

Keywords: NHERF; PDZ; ERM; epithelium; mitogen; cancer

Introduction

Identified nearly a decade ago as a protein cofactor necessary for cyclic AMP-mediated inhibition of the renal apical Na⁺/H⁺ exchanger isoform 3 (NHE3), the protein adapter, NHERF (Na⁺/H⁺ exchanger regulatory factor, EBP-50 or NHERF-1), recruits a wide variety of cellular proteins (Figure 1; Weinman *et al.*, 1993). Many of these proteins interact with the two tandem PDZ domains (protein-binding domains conserved in the mammalian synaptic protein, PSD-95,

Drosophila Dlg or discs large, and the adherens junction protein, ZO-1) present in both NHERF-1 and its isoform, E3KARP (NHE3 kinase A regulatory protein, TKA-1 or NHERF-2). The two NHERF isoforms are differentially expressed in mammalian tissues, with particularly high levels found in polarized epithelial cells (Weinman *et al.*, 1993). Biochemical and cell biological studies suggest that where they exist together, the NHERF proteins perform overlapping functions as regulators of transmembrane receptors, transporters, and other proteins localized at or near the plasma membrane. Consistent with this model, the ERM (ezrin, radixin, moesin and merlin) family of membrane-cytoskeletal adapters is the most abundant cellular target of NHERF (Murthy *et al.*, 1998; Reczek *et al.*, 1997). ERMs associate with a unique domain C-terminal to the two PDZ domains that is also conserved in both NHERF isoforms. This suggests that ERM proteins are important components of cellular complexes containing NHERF and may cooperate with NHERF to dictate the localization and function of various proteins at or near the actin cytoskeleton. This review will focus on the signaling paradigms established by recent studies of NHERF, specifically those implicated in the growth and transformation of epithelial cells.

Developing role for NHERF in cell physiology: from ion transport to signal transduction

Initial identification of both NHERF-1 and NHERF-2 as essential mediators of hormonal signals that inhibit NHE3 activity in renal (Weinman *et al.*, 1993) and gastrointestinal (Lamprecht *et al.*, 1998) epithelial cells means that it is in this context that we know the most about their mechanism of action. The *in vitro* biochemical studies that isolated the 55 kDa phosphoprotein from solubilized renal brush border membranes suggested that PKA catalyzed NHERF phosphorylation (Weinman *et al.*, 1995). This in turn promoted NHERF's association with the cytoplasmic surface of NHE3 and inhibited Na⁺-H⁺ exchange. Subsequent experiments showed that expression of both NHERF and NHE3 in the NHE- and NHERF-deficient PS120 cells was required to reconstitute cyclic AMP inhibition of NHE3. While several aspects of this model are still

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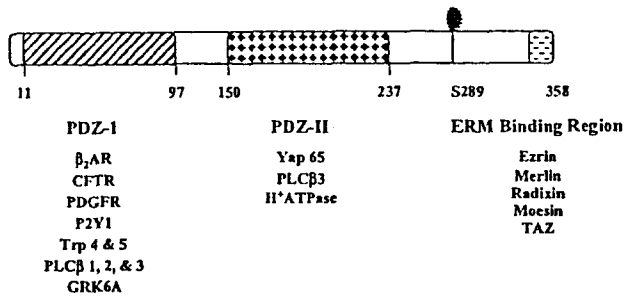


Figure 1 Growing role for NHERF in cell physiology. NHERF contains two tandem and highly homologous PDZ domains (amino acids 11–97 represents PDZ-I—diagonal bars) and amino acids 150–237 constitutes PDZ-II (tiled pattern) as well as C-terminal region of approximately 30 amino acids that associates with members of the ERM family of membrane-cytoskeletal adapters (dashed pattern). While a number of cellular proteins bind PDZ-I, few proteins have been shown to bind PDZ-II. NHE3 binds the region encompassed by PDZ-II and the C-terminus. The precise nature of this association remains unknown. Structure-function relationship for other NHERF targets, such as the type IIA Na/Pi cotransporter and Na/HCO₃ cotransporter, remain to be investigated. Metabolic labeling suggests that NHERF is constitutively phosphorylated on serine-289 (shown as a star)

under investigation, the current data are most consistent with a performed NHE3/NHERF/Ezrin complex that is tethered to the underlying actin cytoskeleton (Weinman *et al.*, 2000). This array of protein-protein interactions is thought to also recruit PKA, which binds ezrin (Dransfield *et al.*, 1997), promoting the phosphorylation of NHE3 on one or more serines within its cytoplasmic tail and inhibiting its transport activity (Figure 2). PKA-mediated phosphorylation also promotes the association of NHE3 with the AP2 adapter and enhances its endocytosis via clathrin-coated pits (Hu *et al.*, 2001). In this manner, NHERF mediates both the acute and chronic regulation of NHE3 by hormones.

The first evidence that NHERF regulated proteins other than NHE3 came from the observation that the β_2 -adrenergic receptor (β_2 AR) associated with proteins other than GTP-binding proteins in an agonist-dependent manner (Hall *et al.*, 1998a). These studies identified NHERF-1 and NHERF-2 as cellular proteins that associated with the C-terminus of the agonist-occupied β_2 AR via their N-terminal PDZ-I domain. The first insights into the structural requirements for NHERF PDZ-I binding also came from studies of the β_2 AR and predicted that other cellular proteins would also bind NHERF. These studies also resolved a long-standing paradox whereby some cyclic AMP-elevating hormones inhibited NHE3 while, like β_2 AR agonists, increased NHE3 activity (Barber *et al.*, 1992). The data suggested two competing mechanisms for NHE3 regulation. The first involved the cAMP-mediated inhibition of Na⁺-H⁺ exchange via PKA phosphorylation of NHE3 discussed above (Figure 2). The second mechanism also required receptor activation but represented direct competition between β_2 AR and NHE3 for NHERF, overwhelming

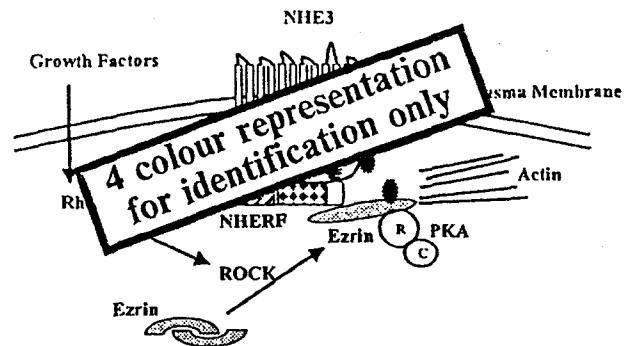


Figure 2 NHERF, a membrane-cytoskeletal adapter. Studies of NHE3 regulation in a number of laboratories point to a preformed complex of NHE3 with NHERF and ezrin that tethers the complex to the actin cytoskeleton underlying apical membranes. Ezrin also positions PKA to phosphorylate one or more sites (shown as stars) in the cytoplasmic tail of NHE3 to regulate transport activity and NHE3 recycling. Growth factor signals mediated by the small GTP-binding protein, Rho, activate the protein kinase, ROCK, which phosphorylates a C-terminal site (shown as a star) in ezrin, converting it from inert soluble dimers to functional monomeric adapters that bind both NHERF and actin to regulate NHE3 and other targets

the effects of cyclic AMP, which required NHERF association and thus increasing NHE3 function. Given the abundance of NHERF in epithelial cells, it is still uncertain under what circumstances each of these mechanisms prevails to up- or down- regulate NHE3 function.

Later studies assigned another function for NHERF association with β_2 AR, namely to define the fate of the internalized receptor during agonist-induced desensitization (Cao *et al.*, 1999). The data suggested that the NHERF-associated β_2 AR was more readily recycled to the cell surface during resensitization. However, NHERF association was disrupted by GRK-5 (an isoform of G-protein-coupled receptor kinase) mediated phosphorylation of β_2 AR within the C-terminal PDZ-binding motif. This led to the rerouting and degradation of the receptor in lysosomes. These studies showed that, as seen with NHE3, NHERF plays multiple roles in regulating signal transduction by the β -adrenergic receptor.

Structure-function studies of NHERF association with the β_2 AR (Hall *et al.*, 1998b) and screening peptide phage display libraries with the NHERF PDZ-I domain (Wang *et al.*, 1998) both predicted that NHERF would bind the C-terminus of CFTR (cystic fibrosis transmembrane conductance regulator), the gene mutated in human cystic fibrosis. Initial studies (Moyer *et al.*, 2000) indicated a critical role for NHERF in apical membrane targeting of CFTR, although the potential for NHERF to link CFTR with ezrin and/or other AKAPs, and by analogy to NHE3 (Figure 2), promote the PKA-activated chloride efflux has also been suggested (Short *et al.*, 1998; Sun *et al.*, 2000a, b). Disease-causing mutations in CFTR have been linked the loss of CFTR/NHERF association, resulting in the mistargeting,

altered recycling, and impaired regulation of the chloride transporter by hormones. Most recent studies suggest that CFTR functions as a homodimeric channel (Raghuram *et al.*, 2001) formed by the recruitment of two CFTR molecules associating with adjacent PDZ domains in a single NHERF molecule, or perhaps by the dimerization of NHERF itself (Fouassier *et al.*, 2000; Shenolikar *et al.*, 2001; Maudsley *et al.*, 2000). This raises the intriguing possibility that NHERF can also bring together two distinct membrane proteins, such as NHE3 and the ROMK channel (Wade *et al.*, 2001) or NHE3 and CFTR (Ahn *et al.*, 2001), to link their functions and regulate ion homeostasis critical for normal epithelial physiology.

NHERF, an amplifier of growth factor signals

Following the molecular cloning of NHERF, its homology with a cDNA encoding TKA-1 (tyrosine kinase activator—Seedorf and Ullrich, Genbank accession number Z50150), was noted. TKA-1 had been identified by its ability to enhance tyrosine phosphorylation by the PDGF (platelet derived growth factor) receptor tyrosine kinase. TKA-1 is in fact NHERF-2/E3KARP. Identification of the consensus motif for PDZ-I had also predicted PDGFR as a potential NHERF target (Hall *et al.*, 1998a). More recent studies have firmly established that the C-terminus of PDGFR associates with PDZ-I of NHERF-1 and NHERF-2 (Maudsley *et al.*, 2000). As PDGFR, like other growth receptors, is activated through ligand-induced dimerization and transphosphorylation of the clustered receptors, NHERF promotes PDGFR dimerization in part due to NHERF's own ability to form dimers (Figure 3). In this manner, NHERF enhances growth factor signaling and activates mitogenic signals transduced by ERKs (extracellular signal-regulated kinases) or MAPKs (mitogen-activated protein kinases). This finding also explained a long-standing puzzle in PDGF signaling, whereby small deletions of the C-terminus of PDGFR attenuated PDGF signaling despite the fact that the receptor bound all the known downstream targets. Several recent studies have established that NHERF dimerizes both *in vitro* and in cells but there is significant disagreement about the precise structural elements required for NHERF dimerization (Maudsley *et al.*, 2000; Fouassier *et al.*, 2000; Shenolikar *et al.*, 2001). Our own studies (Shenolikar *et al.*, 2001) suggested that NHERF dimerization requires PDZ-I. Others have suggested that intervening region between the two PDZ domains (Maudsley *et al.*, 2000) or direct interactions of the two homologous PDZ domains (Fouassier *et al.*, 2000) mediates dimerization. This may not, however, preclude NHERF association with PDGFR and other PDZ-I targets, but clearly, further study is needed to define the role of NHERF in growth factor signaling.

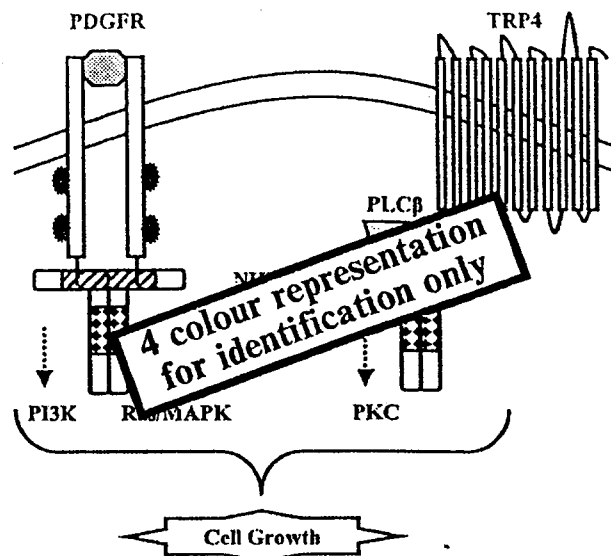


Figure 3 NHERF, a new role in growth factor signaling. The dimerization and activation the PDGF receptor tyrosine kinase is facilitated or stabilized by binding to the NHERF PDZ-I domain. Ability of NHERF to form homodimers may promote PDGFR activation and initiate mitogenic signals via the PI 3-kinase and MAP kinase pathways. In an analogous manner, a dimeric NHERF may also facilitate the association of the Trp4 calcium channel with phospholipase C β isoforms, both of which bind NHERF PDZ-I, to regulate calcium and phosphoinositide metabolism and activate protein kinase C signals required for cell growth

NHERF: targeting non-receptor tyrosine kinases

In contrast to the numerous proteins that bind NHERF PDZ-I (Figure 1), relatively few targets have been identified for PDZ-II. Many of the PDZ-I targets, like GRK6A (Hall *et al.*, 1999), can bind weakly to PDZ-II but a phage display screen using the isolated NHERF PDZ-I and PDZ-II as baits established that distinct consensus motifs were recognized by the two PDZ domains (Wang *et al.*, 1998). This led to the identification of the first PDZ-II target, YAP-65, a protein that also associates with the c-Yes tyrosine kinase (Mohler *et al.*, 1999). Isolation of a protein tyrosine kinase activity on an immobilized NHERF matrix suggests that NHERF recruits the c-Yes kinase via its association with YAP-65 and may localize this tripartite signaling complex at the apical surface in polarized epithelial cells (Mohler *et al.*, 1999). The ability of YAP-65 (via its coiled coil domains) and NHERF to form oligomeric complexes also raises the possibility that larger signaling complexes involving NHERF and YAP65 also exist in cells. A growing paradigm in signal transduction is the role played by protein adapters in restricting the subcellular localization of protein kinases and phosphatases and thereby specifying their cellular functions. By recruiting YAP-65 and c-Yes, NHERF may also dictate the physiological functions of the c-Yes tyrosine kinase in polarized epithelial cells.

During β_2 AR desensitization, the non-receptor tyrosine kinase, c-Src, is recruited to the receptor complex. Both β -arrestin, the phosphoprotein modulator of β_2 AR signaling, and activated c-Src are required for formation of clathrin-coated pits that promote β_2 AR endocytosis (Ahn *et al.*, 1999). Recruitment of c-Src also triggers β_2 -agonist-induced activation of ERK and initiates mitogenic signaling. As NHERF selectively associates with the clustered β_2 AR (ref), there may be crosstalk between NHERF and β -arrestin, which also binds the cytoplasmic tail of β_2 AR in an agonist-dependent manner, to control the internalization and turnover of the β_2 AR and modulate the c-Src-mediated signals that regulate cell growth.

NHERF and phosphoinositide metabolism

Recent observations that PDZ-II in NHERF-2/E3KARP binds phospholipase $C\beta_3$ and potentiates its activity in multiple cell lines points to an important role for NHERF in phosphoinositide signaling (Hwang *et al.*, 2000). Phospholipase $C\beta_3$ cleaves PIP_2 into the two key signaling molecules, diacylglycerol and IP_3 . Subsequent activation of the endoplasmic reticulum-associated IP_3 receptor mobilizes intracellular Ca^{2+} stores, which in conjunction with diacylglycerol activate protein kinase C and facilitate mitogenic signaling. The rise in intracellular calcium or calcium spike in turn initiates an influx of extracellular Ca^{2+} through the store-operated calcium channels (SOCs) on the plasma membrane and refills the depleted internal stores. In this manner, SOCs play an important role in regulating intracellular calcium. A recent report suggests that NHERF-1 PDZ-I associates with two mammalian SOCs, Trp4 and Trp5, as well as the phospholipases $C\beta_1$ and $C\beta_2$ (Tang *et al.*, 2000). As the SOCs and $PLC\beta$ coexist in a complex with NHERF, it suggests that NHERF can link the functions of SOCs to $PLC\beta$ (Figure 3) to coordinate calcium and phosphoinositide metabolism and control cell metabolism and growth.

NHERF, a major target of ERM proteins

Human NHERF was isolated as EBP-50, or ezrin-binding protein of approximate M_r 50 000 (Reczek *et al.*, 1997) and binds most members of the ERM family of cytoskeletal proteins that link membrane proteins to the underlying actin cytoskeleton. ERM proteins also defined plasma membrane structures, such as microvilli, found on the apical surface of polarized epithelial cells. Many cells contain inactive homo- and heterodimers of ezrin bound to other ERM proteins that are incapable of binding either NHERF or F-actin (Nguyen *et al.*, 2001). However, growth factors promote the phosphorylation of a C-terminal residue in ezrin (and moesin) by either $PKC\theta$ or the RhoA-activated protein kinase, ROCK, and activate ezrin, converting it to a monomeric form that readily

associates with NHERF (reviewed in Bretscher, 1999). Thus, it is of interest to note that Rho, but not the closely-related small GTP-binding proteins, Rac1 or Cdc42, regulates NHE3 activity, possibly by increasing the availability of ERM proteins by enhancing their phosphorylation by ROCK (Szasi *et al.*, 2000). (7)

Merlin, a member of the ERM family of adapter proteins and the product of the neurofibromatosis 2 (NF2) gene has been directly linked to growth regulation. NF2 is extensively mutated in a dominantly inherited disorder that predisposes patients to schwannomas and meningiomas (Stokowski and Cox, 2000). Human NF2 mutations are associated with severe morbidity, decreasing life span of the affected individuals to less than 40 years. Merlin, like other ERM proteins, associates with NHERF through its N-terminal ERM domain. More than 80% of the disease-causing mutations located within the ERM domain result in substantially lower affinity of the mutant merlin proteins for NHERF and suggests that NHERF plays a key role in the tumor suppressor activity of merlin.

Ezrin also plays a permissive role in ROCK-mediated transformation of fibroblasts by the Net and Dbl oncogenes (Tran Quang *et al.*, 2000). Net and Dbl are both guanine nucleotide exchange factors that promote GTP loading and activation of Rho. Oncogenic or hyperactivated forms of Net and Dbl result in the accumulation of activated Rho and increase the proliferative signals transduced by Rho. The growth-stimulatory effects of Rho-GTP also require the C-terminal phosphorylation and activation of ezrin and a mutant ezrin lacking the critical phosphorylation site suppresses cell transformation by the Net and Dbl oncogenes.

Ezrin is also overexpressed and highly phosphorylated in cFos-transformed fibroblasts (Lamb *et al.*, 1997). Moreover, ezrin overexpression and phosphorylation has been correlated with altered cell shape and motility, perhaps reflective of the increased invasive properties of malignant cells. Chromophore-assisted laser inactivation (CALI) of ezrin leads to retraction of pseudopodial protrusions, emphasizing its role in cell migration (Jay and Sakurai, 1999). Other ERM proteins, such as radixin and moesin, are down regulated in lung adenocarcinoma compared to normal lung tissue (Tokunou *et al.*, 2000). Finally, a new member of the ERM family, Ehm2, is highly expressed in murine melanoma cells (Shimizu, 2000). These studies suggest that changes in the cellular profile of ERM proteins and their association with NHERF, a major target of ERM proteins in epithelial cells, may promote the transduction of signals that control cell growth and transformation. (6)

NHERF and human cancer

NHERF was recently identified as an mRNA that is highly induced by estrogen in estrogen-receptor (ER)

4) positive breast cancer cells (Edinger *et al.*, 1999; Stemmer-Rachamimov *et al.*, 2001). Estrogen increased NHERF mRNA and protein levels in ER (+) MCF7 cells. Conversely, the expression of estrogen receptor in ER (-) cells restored estrogen's ability to induce NHERF expression in these cells. These and other experiments defined NHERF as an estrogen-inducible gene. However, direct analysis of the mouse NHERF gene has failed to identify estrogen-response element(s) and the mechanism for NHERF regulation by estrogen remains unknown (Weinman *et al.*, 1999).

More extensive analysis of human ER (+) and ER (-) breast cancer cells has further strengthened the link between NHERF expression and estrogen-dependent growth in primary breast carcinomas (Stemmer-Rachamimov *et al.*, 2001). Immunohistochemical studies showed NHERF expression was elevated in breast tumors compared to adjacent normal breast tissue, providing support for a role for NHERF in tumor development. In this regard, PDGF signals are enhanced in human breast carcinomas and have been implicated in tumor progression (Shao *et al.*, 2000). Given the proposed role of NHERF-1 and NHERF-2 in promoting the PDGFR dimerization and activation of mitogenic signals, elevated NHERF expression may be a contributing factor in cell proliferation in the more malignant forms of breast cancer. Finally, the human NHERF gene is located on chromosome 17q25.1, a region that is frequently mutated in human breast and ovarian cancers (GenBank accession # NM_004252; Kalikin *et al.*, 1996, 1997). Thus, deficits in NHERF expression may also result in disturbances in growth signaling and lead to human cancer.

Changes in Na⁺/H⁺ exchange play a role in tumor cell pseudopodial extensions (Lagana *et al.*, 2000) and cell proliferation. NHE3, which represents the predominant NHE isoform in epithelial cells, may act in conjunction with NHERF to regulate proliferation and invasive capacity of breast, ovarian and gastrointestinal cancers.

Concluding remarks

Considerable progress has been made in recent years in expanding the role for NHERF to many aspects of epithelial cell biology. Growing evidence also points to altered NHERF expression and/or function in human disease, including hypertension, acute kidney failure and breast cancer. However, many of the biochemical properties of NHERF, such as those listed below, still poorly understood.

Regulation of NHERF dimerization

NHERF content in renal tissue far exceeds that of its known targets, and while the number of NHERF targets continues to grow, there remain serious questions about the availability of all cellular NHERF to its targets. NHERF's natural propensity to form dimers may provide additional clues to its physiological

regulation. While emerging evidence suggests that a dimeric NHERF acts to regulate some targets, such as CFTR (Ahn *et al.*, 2001) and PDGFR (Maudsley *et al.*, 2000), other functions including NHE3 regulation may utilize a monomeric NHERF. Recent studies suggest that NHERF dimerization may be regulated by protein phosphorylation as treatment of cells with okadaic acid, a protein serine/threonine phosphatase inhibitor and a potent tumor promoter, severely attenuates NHERF dimerization (Shenolikar *et al.*, 2001). Thus, physiological mechanisms that regulate NHERF dimerization may also play an important role in dictating NHERF availability and functions in epithelial cells.

Role of NHERF phosphorylation

Metabolic labeling studies established that NHERF is a phosphoprotein in mammalian cells (Weinman *et al.*, 1995). Hall *et al.* (1999) showed that GRK6A, an isoform of the G-protein-coupled receptor kinase, is the principal NHERF kinase in HEK293 cells. Interestingly, GRK6A binds to NHERF PDZ-I (and PDZ-II) to promote NHERF phosphorylation. However, the cellular signals that modulate GRK6A recruitment and NHERF phosphorylation or the role of the covalent modification at serine-289 (Figure 1) for NHERF function remains unknown.

Competition between NHERF targets

Experimental evidence shows that β_2 AR (Hall *et al.*, 1998b) and CFTR (Ahn *et al.*, 2001) bind PDZ-I but still compete with NHE3, which preferentially associates with PDZ-II and the C-terminus of NHERF and thus enhance Na⁺/H⁺ exchange. As the number of NHERF targets increase, the potential role of competition between NHERF targets, specifically those occupying either PDZ-I or PDZ-II, and the physiological signals that modify NHERF's affinity for certain cellular targets thereby redirecting its functions needs to be explored.

In summary, recent studies suggest that NHERF regulates multiple signaling pathways implicated in growth and function of epithelial cells. Moreover, emerging data points to NHERF as a contributing factor to the development of human cancers. The challenge for future studies will be to define the NHERF-mediated events that are essential for normal cell physiology and elucidate the impact of aberrant NHERF expression and functions in transformation of epithelial cells.

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