Regulation of Expression and Function of Scavenger Receptor Class B, Type I (SR-BI) by Na\(^+\)/H\(^+\) Exchanger Regulatory Factors (NHERFs)*

Received for publication, November 16, 2012, and in revised form, March 4, 2013. Published, JBC Papers in Press, March 12, 2013, DOI 10.1074/jbc.M112.437368

Zhigang Hu\(^{1*}\), Jie Hu\(^{1*}\), Zhonghua Zhang\(^{1*}\), Wen-Jun Shen\(^{1}\), C. Chris Yun\(^{1}\), Catherine H. Berlot\(^{1}\), Fredric B. Kraemer\(^{1}\), and Salman Azhar\(^{1*}\,\!*\)

From the \(^{1}\)Geriatric Research, Education and Clinical Center, Veterans Affairs Palo Alto Health Care System, Palo Alto, California 94304, the \(^{2}\)Division of Endocrinology and \(^{4}\)Division of Gastroenterology and Hepatology, Stanford University, Stanford, California 94305, the \(^{5}\)Division of Digestive Diseases, Department of Medicine, Emory University, Atlanta, Georgia 30322, and the \(^{6}\)Weis Center for Research, Geisinger Clinic, Danville, Pennsylvania 17822-2623

**Background:** SR-BI mediates selective delivery of lipoprotein-CE to the liver, adrenals, and gonads for product formation.

**Results:** NHERFs interact with and down-regulate SR-BI protein levels to inhibit selective CE uptake and steroidogenesis.

**Conclusion:** Two protein domains (PDZ and MERM) are required for NHERF1/2-mediated inhibition of SR-BI expression and function.

**Significance:** This work reveals a novel mechanism of translational/posttranslational regulation of SR-BI.

Scavenger receptor class B, type I (SR-BI) binds HDL and mediates selective delivery of cholesteryl esters (CEs) to the liver, adrenals, and gonads for product formation (bile acids and steroids). Because relatively little is known about SR-BI posttranslational regulation in steroidogenic cells, we examined the roles of Na\(^+\)/H\(^+\) exchanger regulatory factors (NHERFs) in regulating SR-BI expression, SR-BI-mediated selective CE uptake, and steroidogenesis. NHERF1 and NHERF2 mRNA and protein are expressed at varying levels in model steroidogenic cell lines and the adrenal, with only low expression of PDZK1 (NHERF3) and NHERF4. Dibutyryl cyclic AMP decreased NHERF1 and NHERF2 and increased SR-BI mRNA expression in primary rat granulosa cells and MLTC-1 cells, whereas ACTH had no effect on NHERF1 and NHERF2 mRNA levels but decreased their protein levels in rat adrenals. Co-immunoprecipitation, colocalization, bimolecular fluorescence complementation, and mutational analysis indicated that SR-BI associates with NHERF1 and NHERF2. NHERF1 and NHERF2 down-regulated SR-BI protein expression through inhibition of its de novo synthesis. NHERF1 and NHERF2 also inhibited SR-BI-mediated selective CE transport and steroidogenesis, which were markedly attenuated by partial deletions of the PDZ1 or PDZ2 domain of NHERF1, the PDZ2 domain of NHERF2, or the MERM domains of NHERF1/2 or by gene silencing of NHERF1/2. Moreover, an intact COOH-terminal PDZ recognition motif (EAKL) in SR-BI is needed. Transient transfection of hepatic cell lines with NHERF1 or NHERF2 caused a significant reduction in endogenous protein levels of SR-BI. Collectively, these data establish NHERF1 and NHERF2 as SR-BI protein binding partners that play a negative role in the regulation of SR-BI expression, selective CE transport, and steroidogenesis.

Plasma lipoproteins, such as high density lipoprotein (HDL), donate cholesteryl esters (CEs)\(^{2}\) to cells via the selective CE uptake pathway, a process in which CEs are transferred sequentially from HDL particles to the cell surface and then cell interior without the concomitant uptake and degradation of HDL apolipoproteins (1–5). This pathway is a high capacity, physiologically regulated, bulk cholesterol delivery system (6, 7). Steroidogenic cells of the adrenal gland (1, 4, 5, 8, 9) and ovary (3, 10–13) and, under certain physiological conditions, testicular Leydig cells (14) derive the majority of their precursor cholesterol for steroid synthesis and for storage of CEs in the form of lipid droplets through this pathway (15). The selective HDL-CE uptake pathway also plays a major role in plasma cholesterol metabolism by delivering HDL-CE to the liver in the final steps of reverse cholesterol transport for its excretion in bile or bile acid synthesis (2, 4, 16–20). Scavenger receptor class B, type I (SR-BI) is a physiologically relevant cell surface receptor that mediates selective uptake of lipoprotein (HDL)-derived cholesteryl esters (21). SR-BI is a member of the class B scavenger receptor family that also includes CD36, LIMPII, and SR-BII (an isoform of SR-BI with an alternate C-terminal cytoplasmic tail) (6, 7, 15, 22). This receptor, like other family members, contains N- and C-terminal domains as well as an extracellular domain containing cysteine-rich regions and multiple sites for N-linked glycosylation (23–26). Immunochemical and West-

---

* This work was supported, in whole or in part, by National Institutes of Health, NHLBI, Grant R20HL33881. This work was also supported by the Office of Research and Development, Medical Service, Department of Veterans Affairs.

§ To whom correspondence should be addressed: GRECC-182B, Veterans Affairs Palo Alto Health Care System, 3801 Miranda Ave., Palo Alto, CA 94304. Tel.: 650-858-3933; Fax: 650-496-2505; E-mail: salman.azhar@va.gov.

**The abbreviations used are:** CE, cholesteryl ester; hCG, human chorionic gonadotropin; iKEPP, intestinal and kidney-enriched PDZ protein; LH, luteinizing hormone; LPA, lysophosphatidic acid; NHERF, Na\(^+\)/H\(^+\) exchanger regulatory factor; hNHERF, human NHERF; SR-BI, scavenger receptor class B, type I; rSR-BI, rat SR-BI; Bt2cAMP, dibutyryl cyclic AMP; aa, amino acids; qRT-PCR, quantitative RT-PCR; IP, immunoprecipitation; Endo H, endo-β-N-acetylglucosaminidase H; BiFC, bimolecular fluorescence complementation; hHDL3, high density lipoprotein 3.
ern blotting analyses of SR-BI in rodents indicate that it is expressed most abundantly in the liver and in steroidogenic cells of the adrenal gland and ovary, where the selective HDL-CE uptake is greatest (6, 7, 15, 20, 22, 27–30); low levels of basal SR-BI expression are also detected in testicular Leydig cells (14). SR-BI expression is regulated by gonadotropins (LH/FSH and PMSG) and adrenocorticotropic hormone (ACTH) in a tissue-specific manner coordinately with the selective uptake of HDL-CE and steroidogenesis (6, 7, 14, 15, 22, 27–31).

Although in recent years extensive studies have been carried out to delineate the critical events connected with SR-BI-mediated selective delivery of HDL-CE, at present, very little is known about the molecular and cellular mechanisms involved in the regulation of SR-BI expression and its function. However, evidence is now emerging to suggest that hepatic expression of SR-BI can be regulated by both transcriptional and posttranscriptional mechanisms (32, 33). Indeed, recently published work suggests that SR-BI expression is mainly regulated by posttranscriptional mechanisms in the liver (32–35). The posttranscriptional control of hepatic SR-BI protein expression in the liver is primarily dependent on the presence of an adaptor protein, PDZK1 (i.e. this scaffold protein is essential for the normal expression, cell surface localization, and function of hepatic SR-BI) (33–35). Interestingly, steroidogenic tissues express very low levels of PDZK1 (34–38) and normally high levels of SR-BI (14, 27–31), and PDZK1 (NHERF3) deficiency exerts no apparent effect on either SR-BI protein expression or its function (i.e. SR-BI-mediated selective HDL-CE delivery to steroidogenic cells of the adrenal and gonads for CE storage is unaffected by the absence of a functional PDZK1 protein) (34). Currently, there are no known PDZ proteins that can substitute for PDZK1 in modulating the functional expression of steroidogenic SR-BI. Furthermore, with the exception of ACTH and gonadotropins, which transcriptionally regulate SR-BI expression in steroidogenic cells of the adrenal, ovary, and testis, virtually nothing is known about the posttranscriptional regulation of potential posttranscriptional regulators of SR-BI in steroidogenic tissues (6, 7, 14, 15, 22, 27–31), although we have recently reported that microRNAs 125a and 455 posttranscriptionally regulate SR-BI in steroidogenic tissues (39).

PDZK1, also known as Na+/H+ exchanger regulator factor-3 (NHERF3), belongs to a family of scaffolding proteins that also includes NHERF1 (EBP50), NHERF2 (E3KARP), and NHERF4 (IKEPP) (40–42). All of these family members possess tandem PDZ domains; NHERF1 and NHERF2 have two and PDZK1/NHERF3 and NHERF4 have four tandem PDZ domains (40, 42). In addition to PDZ domains, NHERF1 and NHERF2 possess C-terminal MER (merlin-ezrin-radixin-moesin) binding domains, which indirectly tether these proteins to the actin cytoskeleton (43). PDZ domains recognize and bind to a minimum 4-amino acid residue motif that occurs at the C terminus or within the related internal motifs of the target proteins (40, 44, 45). Based on their target or ligand sequences, these PDZ domains can be divided into at least three main classes. The Class I PDZ domain recognizes the motif X(S/T)XΦ (where Φ represents a hydrophobic amino acid, preferably I/V/L/M), Class II PDZ domains recognize the motif XΦXΦ, and Class III domains recognize the motif X(D/E)XΦ (40, 44, 45). There are other PDZ domains that do not fall into any of these classes.

Given that PDZK1 is not expressed at significant levels in steroid-producing tissues, coupled with the demonstration that PDZK1 null mice show normal expression of SR-BI and lipid storage in the adrenal, ovary, and testicular Leydig cells, we considered the possibility that a set of different PDZ domains with specificity for the other three NHERF proteins may be involved in the regulation of SR-BI expression and function in steroidogenic tissues. We searched for and identified additional Class I, II, and III PDZ-interacting domains in the C-terminal cytoplasmic tail of SR-BI using SMART (Simple Modular Architecture Research Tool; available on the EMBL Web site). To a large extent, these PDZ domain sites, like the terminal PDZK1 site (EAKL, Class II PDZ-domain binding site), are well conserved among various mammalian species (i.e. the mouse, rat, hamster, northern tree shrew, rabbit, pig, bovine, and human SR-BI). Using several different approaches, we show that NHERF1 and NHERF2, but not NHERF4, specifically interact with SR-BI and reduce its protein levels. Moreover, we provide evidence that NHERF1/2-induced down-regulation of SR-BI leads to a significant inhibition in both SR-BI-mediated selective HDL-CE uptake and HDL-supported steroid hormone production. These novel findings lead us to conclude that both NHERF1 and NHERF2 act as physiological translational/posttranslational regulators of the functional expression of SR-BI.

**EXPERIMENTAL PROCEDURES**

*Materials—Bt2cAMP, progesterone, insulin, transferrin, hydrocortisone, 17β-estradiol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Thiazolyl Blue), and fatty acid-free bovine serum albumin were supplied by Sigma-Aldrich. Cortrosyn (ACTH) was purchased from Amphastar Pharmaceuticals, Inc. (Rancho Cucamonga, CA). Cholesteroly BODIPY® FLC12 (cholesterol 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoate) was obtained from Molecular Probes (Invitrogen). [1,2-3H]Progesterone (40–60 Ci/mmol; 1.48–2.22 GBq/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). EXPRE35S35S, [35S]-Protein Labeling Mix (73% l-[35S]methionine and 22% l-[35S]cysteine; l-[35S]methionine, 43.5 TBq/mmol or 1175.0 Ci/mmol; l-[35S]cysteine, 39.8 TBq/mmol or 1075.0 Ci/mmol) was obtained from PerkinElmer Life Sciences.

*Animals and Design—All experiments were performed according to procedures approved by the Veterans Affairs Palo Alto Health Care System Institutional Animal Care and Use Committee. Two groups of six, 225–250-g male Sprague-Dawley rats were purchased from Harlan Laboratories (Indianapolis, IN). They were allowed to acclimate to a new controlled environment (25 ± 2 °C, 55 ± 5% relative humidity with a 12-h light/dark cycle) for approximately 1 week. Subsequently, animals were randomly divided into two groups (three rats in each group). Group 1 rats were treated subcutaneously with phosphate-buffered saline (PBS) every 24 h for 4 days, with the last injection on day 4 given 1 h prior to harvesting adrenals; rats in Group 2 were treated subcutaneously with ACTH (Cortrosyn) (10 IU) every 24 h for 4 days, with the ACTH injection on day 4...
given 1 h prior to killing animals. The collected adrenal tissues were stored frozen in liquid nitrogen until used for RNA isolation or Western blotting. Similarly, groups of 50–75-g (22–29-day-old) female Sprague-Dawley rats purchased from Harlan Laboratories were injected subcutaneously with 17β-estradiol (1 mg) for 5 days and subsequently employed for the isolation and culture of granulosa cells as described previously (11, 31).

Rat Ovarian Granulosa Cell Isolation, Culture, and cAMP Treatment—Granulosa cells were isolated from 17β-estradiol-primed ovaries and cultured as described previously (11, 31). In brief, cells were cultured in 35-mm culture dishes that were precoated with 1% fetal bovine serum. Dishes were plated with 1–2 × 10^5 cells in 1.5 ml of basal culture medium (DMEM/F-12-supplemented 15 mM Hepes, bovine serum albumin (1 mg/ml), insulin (2 μg/ml), transferrin (5 μg/ml), hydrocortisone (100 ng/ml), streptomycin (100 μg/ml), and penicillin G (100 units/ml)). After 72 h of culture, the dishes were washed extensively to remove dead and unattached cells. At this stage, the cell viability, as measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation and viability kit (Sigma-Aldrich), averaged >97%. Next, cultured cells were treated either with vehicle alone or with Bt2cAMP (2.5 mM) for 5 days and subsequently employed for the isolation and culture of granulosa cells.

Cell Culture—CHO-K1 (catalogue no. CCL-61), MLTC-1 (catalogue no. CRL-2065™), R2C rat Leydig tumor cells (catalogue no. CCL-1651™), Hep G2 (catalogue no. HB-8065™) and Hepa 1–6 (catalogue no. CRL-1830™) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). CHO-K1 cells were cultured in Ham’s F-12 medium containing 10% fetal bovine serum plus 100 units/ml penicillin and 100 μg/ml streptomycin. MLTC-1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum plus 100 units/ml penicillin and 100 μg/ml streptomycin. R2C cells were cultured in Ham’s F-12 medium supplemented with 2.5% fetal bovine serum, 15% horse serum, and 100 units/ml penicillin and 100 μg/ml streptomycin. COS-7 cells were cultured in DMEM containing 10% fetal bovine serum plus 100 units/ml penicillin and 100 μg/ml streptomycin. Hep G2 cells were cultured in Eagle’s minimum essential medium supplemented with 10% fetal bovine serum plus 100 units/ml penicillin and 100 μg/ml streptomycin. Hepa 1–6 cells were cultured in DMEM containing 4 mM l-glutamine, 4.5 g/liter glucose, 1.5 g/liter sodium bicarbonate, 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. All cell cultures were maintained at 37 °C in a humidified incubator containing 5% CO_2, 95% air. Most of the tissue culture supplies were purchased from Invitrogen through Invitrogen® Cell Culture Media (Grand Island, NY).

Plasmid Constructs—The open reading frame (ORF) of the rat scavenger receptor class B, type I (rSR-BI) was amplified by PCR using a forward primer with a HindIII restriction site (rSR-BI-1) and a reverse primer with an XbaI restriction site (rSR-BI-R) (Table 1). The purified PCR fragment was digested with HindIII and XbaI and subcloned into pcDNA6-V5, and the constructed plasmid was designated as pcDNA6V5-rSR-BI. PCR products of rSR-BI with UTR were used to construct pcDNA6-rSR-BI-stop plasmid without any tag. PCR products of rSR-BI with C-terminal deletion of amino acid residues 465–509 (pcDNA6V5-rSR-BI-464), 495–509 (pcDNA6V5-rSR-BI-494), or 505–509 (pcDNA6V5-rSR-BI-504) were also subcloned into pcDNA6-V5. The mutants of PDZ-interacting domains in the C-terminal cytoplasmic tail of SR-BI were created by site-directed mutagenesis using QuickChange® II site-directed mutagenesis kits (Stratagene, La Jolla, CA). The mutagenic primers are listed in Table 1. Mouse full-length NHERF1 (amino acids (aa) 1–355) (NHERF1) and truncated NHERF1 lacking putative PDZ1 (aa 1–151; NHERF1ΔP1), PDZ2 (aa 104–240; NHERF1ΔP2), or MERM (aa 315–355; NHERF1ΔMERM) were amplified by PCR and subcloned into pcDNA6 with a Myc tag. Likewise, human partial full-length NHERF2 (aa 8–337; NHERF2) and truncated NHERF2 lacking putative PDZ1 (aa 1–105; NHERF2ΔP1), PDZ2 (aa 107–241; NHERF2ΔP2), or MERM (aa 305–337; NHERF2ΔMERM) were also amplified by PCR and cloned into pcDNA6-myc. For other studies, PCR-generated mouse NHERF1, NHERF1 lacking putative PDZ1 (aa 1–151; NHERF1ΔP1); truncated NHERF1 lacking putative PDZ2 (aa 104–240; NHERF1ΔP2) or MERM (aa 315–355; NHERF1ΔMERM); human NHERF2; and truncated NHERF2 lacking putative PDZ1 (aa 1–105; NHERF2ΔP1), PDZ2 (aa 107–241; NHERF2ΔP2), or MERM (aa 305–337; NHERF2ΔMERM) fragments were subcloned into the pGEX-4T3 (Amersham Biosciences) and used to transform Escherichia coli BL-21 cells (Stratagene) in order to express NHERF1, NHERF2, and the various NHERF truncations as GST-tagged fusion proteins (for additional details, see below). To produce pcDNA1-YFP(159–238)-rSR-BI, an rSR-BI ORF fragment was amplified by PCR that also introduced a NotI site at the 5’-end and an XbaI site at the 3’-end, and rSR-BI was cloned into these sites at the C terminus of YFP (YFP(159–238) (46). Likewise, pcDNA1-NHERF1-YFP(1–158)-STOP and pcDNA1-NHERF2-YFP(1–158)-STOP constructs were generated by PCR amplification of human NHERF1 and NHERF2 fragments using NHERF1 and NHERF2 plasmids. The NHERF1 and NHERF2 PCR fragments with HindIII and BamH1 sites were cloned in these sites at the N terminus of YFP(1–158)-STOP, which was produced as follows. YFP(1–158) was amplified by a PCR from EYFP (Clontech) that introduced a substitution of Met for Gln-69 (47). The PCR introduced a BamH1 site at the 5’-end of YFP(1–158) and a stop codon at the 3’-end, followed by a BglII site. A BglII site was introduced into the polylinker of pcDNA/Amp 3’ to the BamH1 site, and YFP(1–158) was subcloned into these sites to produce YFP(1–158)STOPpcDNA1/Amp. The identity of each cDNA was confirmed by sequencing.

Antibodies—A highly specific polyclonal antibody raised against a peptide to the C terminus of mouse SR-BI (amino acids 489–509, AYSELSMPPAKGTVLQEAKL) (31) was used. This antibody detects endogenous levels of total SR-BI with a molecular mass of ~82–84 kDa in rat, mouse, hamster, bovine, and human tissues/cells by Western blotting, immunofluorescence microscopy, and immunoelectron microscopy. Affinity-purified antibodies against NHERF1 (Ab5199) and NHERF2 (Ab2570) were provided by C.C.Y. Ab5199 polyclonal antibody (anti-NHERF1) was raised in rabbits against a full-length NHERF1 expressed as a GST fusion protein (GST-NHERF1) (48, 49). The Ab5199 serum was passed over a DEAE
Affi-Gel Blue column (Bio-Rad) to obtain a purified IgG fraction. This antibody detects endogenous NHERF1, which has a predicted molecular mass of ~50 kDa. The Ab2570 polyclonal antibody (anti-NHERF2) was raised in rabbits against the C-terminal 106 amino acids of NHERF2 expressed as a His-tagged protein (50). Ab2570 was purified by ammonium sulfate precipitation, followed by affinity gel purification through DEAE-Affi-Gel Blue. This antibody detects endogenous NHERF2, which has a predicted molecular mass of 46 kDa.

Quantitative Real-time RT-PCR (qRT-PCR) and RT-PCR—Adrenal glands, liver, ovary, and kidney were removed from the treated rats and snap-frozen in liquid nitrogen. Samples of these frozen tissues were pulverized and converted to a fine powder using a mortar and pestle, which were precooled with liquid nitrogen, and subsequently lysed with the addition of a suitable aliquot of QIAzol lysis reagent (Qiagen, Valencia, CA). Next, total RNA was extracted from tissue samples or cell samples (MLTC-1 or granulosa cells) using an miRNeasy minikit (Qiagen) and reverse-transcribed (2 μg RNA) to cDNA with the Superscript II reverse transcription kit (Invitrogen). For qRT-PCR, cDNA was mixed with 2× SYBR Green PCR Master Mix (Applied Biosystems) and various sets of gene-specific primers and then subjected to RT-PCR quantification using the ABI Prism 7900 HT System (Applied Biosystems). The sequences of the primers used are shown in Table 1. All reactions were performed in triplicate. The relative amounts of mRNA were calculated using the comparative cycle threshold method ($2^{-ΔΔCt}$) (51).

For semiquantitative PCR, the cDNA was mixed with a PCR mixture (Genescript, Piscataway, NJ) and gene-specific primers (SR-BI, NHERF-1, NHERF2, NHERF3 (PDZK1), or NHERF4 (IKEPP)) (Table 1). The PCR conditions were 25 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. The PCR products were subjected to electrophoresis in 2% agarose gels, visualized, and quantified using the Molecular Imager Gel DocTM XRT imaging system (Bio-Rad). Band intensity was expressed as relative absorbance units and normalized using 36B4 as control.

Western Blotting—Adrenals or cells (MLTC-1, R2C, granulosa cells, and Hepa 1–6) were harvested and homogenized in radioimmuneprecipitation assay buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS) supplemented with Thermo Scientific HaltTM protease inhibitor mixture (1 mM 4-(2-aminoethyl)-benzene-sulfonyl fluoride-HCl, 800 μM aprotinin, 50 μM bestatin, 15 μM E-64, 5 mM EDTA, 20 mM leupeptin, and 10 μM pepstatin). Following incubation for 20 min on ice, lysates were cleared by centrifugation at 10,000 × g for 5 min. Supernatants were collected, and protein concentrations were determined by the Bradford assay (Bio-Rad). Suitable aliquots of samples were mixed with equal volumes of 5× Laemmli sample buffer (120 mM Tris-HCl, pH 6.8, 2% SDS (w/v), 10% sucrose (w/v), and 1% 2-mercaptoethanol), and 10–20 μg of total protein of each sample was subjected to 10% SDS-polyacrylamide gel electrophoresis under denaturing conditions. Protein markers were also loaded on the gel. Following electrophoretic separation, the proteins were transferred to Immobilon PVDF membranes (Millipore Corp., Bedford, MA) using standard techniques. Blotted membranes were blocked with Odyssey® blocking buffer (LI-COR Biosciences, Lincoln, NE) for 1 h at room temperature and then incubated with either rabbit anti-SR-BI or mouse anti-β-actin antibodies for 2 h. After three washes with Tris-buffered saline containing 0.1% Tween 20, the membranes were incubated with IRDye® 800CW goat anti-rabbit and IRDye® 680LT goat anti-mouse secondary antibodies (LI-COR Biosciences) for 1 h. Proteins were detected with the Odyssey® Infrared Imaging System (LI-COR Biosciences).

$^{35}$S Labeling and Measurement of de Novo Synthesis of SR-BI—MLTC-1 cells were transiently transfected with the expression plasmid pcDNA6-V5-rSR-BI with or without pcDNA3-hNHERF1 or pcDNA3-hNHERF2 using Lipofectamine™ 2000 transfection reagent. After 24 h, cells were washed with methionine/cysteine-free RPMI 1640 medium (Sigma) containing 2 mM glutamine and 10% dialyzed fetal calf serum (methionine/cysteine-free medium) and incubated with the same medium for 15 min at 37 °C. After this time, the cells were incubated with 1.5 mM of methionine/cysteine-free medium supplemented with EXPRE$^{35}$S (−100 μCi/well) for 10 min at 37 °C. Cells were washed with the regular RPMI 1640 medium, scraped, and transferred to a microfuge tube. Following centrifugation, the cells were then lysed by the addition of 500 μl of TBS (20 mM Tris, pH 7.4, 150 mM NaCl), 1 ml of 1000× protease inhibitor mixture (Sigma), and 500 μl of 2× immunoprecipitation (IP) buffer (50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 2% Nonidet P-40, and 10% glycerol), and cell lysates were incubated at 4 °C for 20 min. After centrifugation, 400-μl aliquots of soluble extracts (adjusted to contain the same amounts of protein) were incubated for 3 h at 4 °C with 1 μg of anti-V5 mAb (or 1 μg of control mouse IgG), 35 μl of Protein G-agarose (50% slurry), and 65 μl of 1× IP buffer. Immunoprecipitated pellets were washed with 1× IP buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40 and 5% glycerol) and subsequently processed for the determination of $^{35}$S radioactivity. Crude cell lysates were also subjected to Western blotting for SR-BI protein expression as described above.

Half-life ($t_{1/2}$) Measurements—MLTC-1 cells were transiently transfected with the expression plasmid pcDNA6-V5-rSR-BI with or without pcDNA3-hNHERF1 or pcDNA3-hNHERF2 and metabolically labeled with ~100 μCi/dish $[^{35}]$S methionine/[^{35}]S cysteine (EXPRE$^{35}$S$^{35}$S) for 1 h at 37 °C in methionine/cysteine-free RPMI 1640 medium, as described above. Following the pulse period, cell monolayers were washed with the regular RPMI 1640 medium before adding chase medium (RPMI 1640 containing 2 mM glutamine, 10% FCS, 500 μg/ml methionine, and 100 μg/ml cysteine) and continuing incubations at 37 °C. After the appropriate chase time, cells were washed, lysed, and processed for the determination of $^{35}$S radioactivity, as described above.

Endo-β-N-acetylglucosaminidase H (Endo H) Treatment—CHO cells were transiently transfected with the expression plasmid pcDNA6-V5-rSR-BI with or without pcDNA3-hNHERF1 or pcDNA3-hNHERF2 using Lipofectamine™ 2000 transfection reagent. After 48 h, cell lysates were prepared in IP buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 5% glycerol) containing 0.5% SDS, 40 mM DTT and heated at 100 °C for 10 min. Next, cell lysates were treated without or with 10 units of Endo H for 3 h at 37 °C in the presence of...
### TABLE 1

Primers used for PCR gene cloning, site-directed mutagenesis, gene deletion, and quantitative real-time PCR

| Gene | Forward (f) and reverse (r) primers | Restriction enzyme sites |
|------|-------------------------------------|--------------------------|
| pcDNA6V5-rSR-BI | 5’-G_AGAACCTTCCACCGGACGTCGAGTTCGACG-3’-r | HindIII/Xbal |
| pcDNA6-rSR-BI-stop | 5’-G_AGAACCTTCCACCGGACGTCGAGTTCGACG-3’-r | HindIII/Xbal |
| pcDNA6V5-rSR-BI-664 | 5’-GTACAGAGAGGTTATGCTCCATCCATG-3’-r | KpnI/Xbal |
| pcDNA6V5-rSR-BI-494 | 5’-GTACAGAGAGGTTATGCTCCATCCATG-3’-r | KpnI/Xbal |
| pcDNA6V5-rSR-BI-504 | 5’-GTACAGAGAGGTTATGCTCCATCCATG-3’-r | KpnI/Xbal |
| pcDNA1-YFP(159–238)-rSR-BI | 5’-CATCGACAGTTATCGCTTCGAGTACCC-3’-r | NotI/Xbal |
| pcDNA1-NHERF1-YFP(1–158)-STOP | 5’-CATCGACAGTTATCGCTTCGAGTACCC-3’-r | HindIII/Xbal |
| pcDNA6-myc-NHERF1 | 5’-CATCGACAGTTATCGCTTCGAGTACCC-3’-r | HindIII/Xbal |
| pcDNA6-myc-NHERF1Δp1 | 5’-CATCGACAGTTATCGCTTCGAGTACCC-3’-r | HindIII/Xbal |
| pcDNA6-myc-NHERF1Δp2 | 5’-CATCGACAGTTATCGCTTCGAGTACCC-3’-r | HindIII/Xbal |
| pcDNA6-myc-NHERF1Δp2a | 5’-CATCGACAGTTATCGCTTCGAGTACCC-3’-r | HindIII/Xbal |
| pcDNA6-myc-NHERF1Δp2a | 5’-CATCGACAGTTATCGCTTCGAGTACCC-3’-r | HindIII/Xbal |
| pcDNA6-myc-NHERF2 | 5’-CATCGACAGTTATCGCTTCGAGTACCC-3’-r | HindIII/Xbal |
| pcDNA6-myc-NHERF2Δp1 | 5’-CATCGACAGTTATCGCTTCGAGTACCC-3’-r | HindIII/Xbal |
| pcDNA6-myc-NHERF2Δp2 | 5’-CATCGACAGTTATCGCTTCGAGTACCC-3’-r | HindIII/Xbal |
| pcDNA6-myc-NHERF2Δp2a | 5’-CATCGACAGTTATCGCTTCGAGTACCC-3’-r | HindIII/Xbal |
| pcDNA6-myc-NHERF2Δp2a | 5’-CATCGACAGTTATCGCTTCGAGTACCC-3’-r | HindIII/Xbal |
| pcDNA6-myc-NHERF2Δp2a | 5’-CATCGACAGTTATCGCTTCGAGTACCC-3’-r | HindIII/Xbal |
| Mouse NHERF1 | 5’-GAAGACGAGAGGTTATGCTCCGAGCCGCGCTC-3’-r | qRT-PCR primers |
| Mouse NHERF2 | 5’-GAAGACGAGAGGTTATGCTCCGAGCCGCGCTC-3’-r | qRT-PCR primers |
| Rat NHERF1 | 5’-GAAGACGAGAGGTTATGCTCCGAGCCGCGCTC-3’-r | qRT-PCR primers |
| Rat NHERF2 | 5’-GAAGACGAGAGGTTATGCTCCGAGCCGCGCTC-3’-r | qRT-PCR primers |
| Mouse 36B4 | 5’-GAGGACGAGAGGTTATGCTCCGAGCCGCGCTC-3’-r | qRT-PCR primers |
| Rat 36B4 | 5’-GAGGACGAGAGGTTATGCTCCGAGCCGCGCTC-3’-r | qRT-PCR primers |
| Rat PDZK1 | 5’-GAGGACGAGAGGTTATGCTCCGAGCCGCGCTC-3’-r | RT-PCR primers |
| Rat NHERF4 | 5’-GAGGACGAGAGGTTATGCTCCGAGCCGCGCTC-3’-r | RT-PCR primers |

- **Mutation of potential LFW PDZ domain binding site in the C-terminal domain of SR-BI**
  - 5’-CATACCAGACGCTGCTCCGAGCCGCGCTC-3’-r
  - Primers for LFW mutation

- **Mutation of potential KEM PDZ domain binding site in the C-terminal domain of SR-BI**
  - 5’-GAGGACGAGAGGTTATGCTCCGAGCCGCGCTC-3’-r
  - Primers for KEAM mutation

- **Mutation of potential AMQA PDZ domain binding site in the C-terminal domain of SR-BI**
  - 5’-GAGGACGAGAGGTTATGCTCCGAGCCGCGCTC-3’-r
  - Primers for AMQA mutation
buffer recommended by the company (New England Biolabs). Samples were loaded onto an SDS-polyacrylamide gel and analyzed by immunoblotting using the anti-V5 monoclonal antibody as described above.

Co-immunoprecipitation—Interactions of SR-BI with NHERF1 and NHERF2 were performed by co-immunoprecipitation followed by immunoblot analysis. CHO cells were transiently co-transfected with the expression plasmid pcDNA6-V5-SR-BI and pcDNA3-hNHERF1 or pcDNA3-hNHERF2 using Lipofectamine™ 2000 transfection reagent (Invitrogen). After 48 h, the cells were lysed with Pierce IP lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 5% glycerol; Pierce) supplemented with Halt™ protease inhibitor mixture (1 mM PMSF, 50 nM bestatin, 15 nM E-64, 5 mM EDTA, 20 nM leupeptin; Pierce) and 10 nM pepstatin), incubated for 15 min on ice, and subsequently homogenized 10 times using a 23-gauge needle. Cellular debris was cleared by centrifugation at 10,000 × g for 5 min, and the supernatant was incubated for 2 h at 4 °C with either normal mouse IgG (control) or mouse anti-V5 IgG. Thirty microliters of A/G-Sepharose were added to each sample, and tubes were incubated for 2 h at 4 °C. Precipitates were collected by gentle centrifugation and washed three times in cold lysis buffer. Proteins were eluted with SDS sample buffer and subjected to SDS-PAGE, and proteins were transferred onto PVDF membranes (Millipore). The blots were incubated with different antibodies (polyclonal rabbit anti-NHERF1, anti-NHERF2, or SR-BI) and protein bands were visualized on an Odyssey infrared imaging system (LI-COR Biosciences) as described above.

GUST Pull-down Assay—Pull-down assays were performed using recombinant proteins fused to GST, R2C cell lysates (containing high levels of endogenous SR-BI), and a Pierce® GST protein interaction pull-down kit (Pierce). GST fusion proteins (GST-NHERF1, GST-NHERF1ΔMERM, GST-NHERF1ΔP1, GST-NHERF1ΔP2, GST-NHERF2, GST-NHERF2ΔMERM, GST-NHERF2ΔP1, and GST-NHERF2ΔP2) and GST (control) were expressed in E. coli BL21 by induction with 0.5 mM isopropyl-β-D-galactopyranoside for 4 h at 27 °C. Bacterial lysates were sonicated, and each protein was purified from soluble fractions on glutathione-agarose (Pierce). For the pull-down assay, suitable amounts of GST or specific GST fusion protein were incubated with glutathione-agarose beads for 1 h at 4 °C. The beads were then washed, centrifuged, and incubated with equal amounts of the SR-BI-rich R2C cell lysates for 1 h at 4 °C. Following incubation, beads were washed three times with wash solution (TBS/ProFound lysis buffer, 1:1). The bound proteins were eluted with elution buffer (100 mM reduced glutathione in wash solution), resolved on SDS-PAGE, and transferred to PVDF membranes. SR-BI protein was detected via Western blotting with rabbit anti-peptide SR-BI polyclonal antibody and IRDye® 800CW goat anti-rabbit antibody, and bands were visualized using the Odyssey® infrared imaging system (LI-COR Biosciences).

Confocal Immunofluorescence Microscopy—CHO cells were grown on poly-d-lysine coated 25-mm round glass coverslips in 6-well tissue culture plates overnight. Subsequently, cells were transfected with 1.0 µg of SR-BI-V5 plasmid DNA with or without NHERF1-c-Myc cDNA construct or with or without NHERF2-c-Myc cDNA construct using Lipofectamine™ 2000 transfection reagent according to the manufacturer’s protocol (Invitrogen). Roughly 36 h after transfection, cells were fixed with 4% paraformaldehyde in PBS at room temperature for 15 min. For immunostaining, cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min and then washed three times in PBS and incubated for 1 h at room temperature in PBS containing 5% normal goat serum and 5% nonfat dry milk (blocking solution). After washing, the cells were incubated overnight at 4 °C or for 2 h at 25 °C with anti-c-Myc mouse monoclonal antibody (for detection of NHERF1-c-Myc and NHERF2-c-Myc) or SR-BI anti-peptide rabbit polyclonal antibody diluted in blocking solution. The coverslips were then washed with PBS, drained, and incubated for 45 min at room temperature with goat anti-mouse IgG Alexa Fluor 488 and goat anti-rabbit IgG Alexa Fluor 568 secondary antibodies in blocking solution. At the end of incubation, the coverslips were incubated with
NHERF1 and NHERF2 Regulation of SR-BI

DAPI in blocking solution for 15 min at room temperature, washed with PBS, drained, and mounted onto glass slides using Fluoromount G (Fisher). Slides were subsequently imaged using a Zeiss LSM 510 Meta confocal microscope (Zeiss, Jena, Germany). Images were processed using LSM510 software.

**Bimolecular Fluorescence Complementation (BiFC) Assay—**
CHO cells were grown in 24-well cell culture plates overnight. Subsequently, cells were transfected with 50 ng of pcDNAI-YFP(159–238)+eSR-BI with or without 50 ng pcDNAI-NHERF1-YFP(1–158)-STOP or with or without 50 ng pcDNAI-NHERF2-YFP(1–158)-STOP construct using LipofectamineTM 2000 transfection reagent according to the manufacturer’s protocol (Invitrogen) (46). As a control, we used YFP159–238 construct. The cells were imaged 36–48 h after transfection using a Zeiss LSM 510 Meta confocal microscope.

**Measurement of SR-BI-mediated Selective HDL-BODIPY-CE Uptake—**
For these studies, apoE-free high density lipoprotein 3 (hHDL3) was used to prepare reconstituted (rec) HDL-BODIPY-cholesterol ester particles (rec-HDL-BODIPY-CE) as described previously (39). MLTC-1 or CHO cells transfected with or without SR-BI, NHERF1, or NHERF2 construct for 40 h were incubated with rec-HDL-BODIPY-CE (50 μg) for 60–180 min at 37 °C. Subsequently, cells were rapidly washed five times in PBS, 0.1% bovine serum albumin at 0–4 °C, and lipids were extracted with hexane/isopropyl alcohol (3:2, v/v) as described previously (39). In each case, a portion of the hexane/isopropyl alcohol extract was transferred to a microplate well, and the fluorescence was measured at an excitation wavelength of 503 nm and emission wavelength of 512 nm using a fluorescence plate reader (Molecular Devices Corp., Sunnyvale, CA). Results are reported as arbitrary units and expressed as relative HDL-BODIPY-CE uptake.

**Measurement of Progestosterone Secretion—**
MLTC-1 cells transfected with or without NHERF1 or NHERF2 for 48 h were incubated with or without Bt2cAMP (2.5 mM), with or without hHDL3 (500 μg of protein/ml), or with or without Bt2cAMP + hHDL3 for 5 h at 37 °C. After incubation, the progesterone concentration in cell plus medium was determined by radioimmunoassay (11).

**Statistical Analysis—**
The results are presented as the means ± S.E. for at least three independent experiments. The data were analyzed by an unpaired Student’s t test. A statistical difference of p < 0.05 was considered significant.

**RESULTS**

**Relative Expression of NHERF Family Members in Rat Liver, Kidney, and Adrenal and R2C Testicular Leydig Tumor Cells—**It has been reported previously that human PDZK1 mRNA is expressed mainly in the liver, kidney, pancreas, and gastrointestinal tract, and only low levels of its expression are detected in steroidogenic tissues, such as the adrenal and testis (36–38). Here, we first used a semiquantitative RT-PCR technique to measure relative mRNA abundance of NHERF1, NHERF2, PDZK1 (NHERF3), and NHERF4 in the rat (as a representative of rodents) liver, kidney, adrenal, and R2C testicular Leydig tumor cells. The results presented in Fig. 1A demonstrate that liver, kidney, adrenal, and R2C cells all express more or less similar levels of NHERF1, whereas adrenal and R2C cells exhibit the highest levels of NHERF2. In contrast, both R2C Leydig cells and adrenal express very low levels of PDZK1 mRNA. Interestingly, all four tissues and cells also express very low levels of NHERF4. Protein levels of NHERF1 and NHERF2 are also high in the ovary and adrenal (Fig. 1B). Because both PDZK1 and NHERF4 are expressed at very low levels in steroid-producing tissues and cells, including ovarian granulosa cells (data not shown) as well as the previously reported lack of effect of PDZK1 on steroidogenic SR-BI, we carried out most of our studies on NHERF1 and NHERF2 only.

**Hormonal Regulation of NHERF1 and NHERF2—**We sought to determine whether NHERF1 and NHERF2 expression is hormonally regulated. RNA was extracted from cultured mouse Leydig tumor cells, MLTC-1 (a representative cell line of Leydig cells), and primary rat ovarian cells pretreated with or without Bt2cAMP (2.5 mM) for 6 and 24 h, respectively, and qRT-PCR was performed. Likewise, groups of rats were treated with ACTH (10 IU every 24 h for 4 days) or vehicle (control), and adrenal samples were subjected to total RNA isolation, followed by qRT-PCR. As expected, the expression of SR-BI mRNA was robustly increased in response to treatment of rat ovarian granulosa and mouse testicular MLTC-1 Leydig tumor cells with Bt2cAMP in vitro (Fig. 2A). Similarly, significant increases in adrenal SR-BI mRNA levels were noted in response to ACTH treatment of rats in vivo (Fig. 2A). As shown in Fig. 2B, NHERF1 mRNA is expressed severalfold (5–10-fold) higher in MLTC-1 cells as compared with ovarian granulosa cells or the adrenal gland. In contrast, NHERF2 is expressed as the predominant form in the adrenal gland, where its mRNA levels are 10–20-fold higher than granulosa or MLTC-1 cells (Fig. 2B). Interestingly, treatment of granulosa and MLTC-1 cells with Bt2cAMP significantly down-regulated the mRNA levels of both NHERF1 and NHERF2. On the other hand, no changes in adrenal mRNA levels of either NHERF1 or NHERF2 were noted following repeated treatment of rats with ACTH (Fig. 2B). These results indicate that NHERF1 and NHERF2 mRNA levels are inversely related to SR-BI levels in granulosa cells and MLTC-1 Leydig cells, but ACTH treatment failed to modulate adrenal mRNA levels of NHERF1 or NHERF2. Western blot analysis, however, indicated that ACTH treatment results in significant reduction in protein levels of both NHERF1 and NHERF2 (Fig. 2C).

**FIGURE 1.** NHERF1, NHERF2, PDZK1 (NHERF3), and NHERF4 expression in rat liver, kidney, adrenal, ovary, and R2C cells. A, RNA was isolated from adrenal, liver, kidney, and R2C samples and reverse-transcribed to cDNA, and cDNAs were amplified with NHERF1, NHERF2, PDZK1 (NHERF3), and NHERF4-specific primers. The PCR products were analyzed by electrophoresis through a 2% agarose gel prestained with ethidium bromide. B, Western blot of NHERF1 and NHERF2 in cell extracts of rat ovary, adrenal, liver, and kidney. β-Actin was used as a loading control.
**NHERF1 and NHERF2 Down-regulate SR-BI Protein Expression and Negatively Regulate SR-BI-mediated Selective HDL-CE Transport and HDL/SR-BI-supported Steroidogenesis**

To determine whether increased expression of NHERF1 or NHERF2 results in alteration in SR-BI expression and selective HDL-CE transport function, we transfected CHO and COS-7 cells with SR-BI cDNA with or without NHERF1 or NHERF2 cDNA constructs. We chose these two cell lines as model systems for the initial studies because CHO cells do not express detectable levels of NHERF2 and express only insignificant amounts of endogenous NHERF1 and SR-BI, and COS-7 cells show very little expression of NHERF-1 and lack endogenous expression of either NHERF2 or SR-BI. Quantitative Western blot data presented in Fig. 3A show that overexpression of either NHERF1 or NHERF2 in CHO cells results in significant down-regulation of SR-BI protein.

Likewise, overexpression of NHERF1 or NHERF2 also leads to attenuation of SR-BI-mediated selective HDL-BODIPY-CE uptake (Fig. 3B). Similar results were obtained when COS-7 cells were transfected with SR-BI with or without NHERF1 or NHERF2 cDNA constructs, demonstrating that NHERF1 and NHERF2 inhibition of SR-BI protein expression (Fig. 3C) and its selective CE transport function (Fig. 3D) is not peculiar to CHO cells. We next determined the effects of NHERF1 and NHERF2 on SR-BI-supported steroidogenesis. Mouse Leydig tumor MLTC-1 cells were transfected with NHERF1, NHERF2, or control DNA, and SR-BI mRNA and protein expression, selective HDL (BODIPY)-CE uptake, and HDL-supported steroidogenesis were measured. MLTC-1 cells are an accepted model for monitoring trophic hormone and SR-BI action on steroidogenesis (39). Quantitative RT-PCR data shown in Fig. 4A demonstrate that overexpression of neither NHERF1 nor
NHERF2 had any significant effect on mRNA levels of SR-BI in MLTC-1 cells. In contrast, Western blot analysis showed a significant reduction in SR-BI protein levels when cells were transfected with NHERF1 or NHERF2, and 48 h after transfection, cell samples were analyzed for SR-BI protein levels by Western blotting (A) or utilized for the measurement of selective HDL-CE uptake using HDL-BODIPY-CE as a tracer (B). C and D, COS-7 cells were co-transfected with SR-BI with or without NHERF1 or NHERF2, and 48 h following transfection, cell samples were employed for the measurement of SR-BI protein levels (C) and selective HDL-CE uptake (D) as described under “Experimental Procedures.” Results are mean ± S.E. (error bars) of three independent measurements. *, p < 0.05.

FIGURE 3. Overexpression of NHERF1 or NHERF2 leads to down-regulation of SR-BI protein expression and inhibition of selective HDL-CE uptake. A and B, CHO cells were co-transfected with SR-BI and either NHERF1 or NHERF2, and 48 h after transfection, cell samples were analyzed for SR-BI protein levels by Western blotting (A) or utilized for the measurement of selective HDL-CE uptake using HDL-BODIPY-CE as a tracer (B). C and D, COS-7 cells were co-transfected with SR-BI with or without NHERF1 or NHERF2, and 48 h following transfection, cell samples were employed for the measurement of SR-BI protein levels (C) and selective HDL-CE uptake (D) as described under “Experimental Procedures.” Results are mean ± S.E. (error bars) of three independent measurements. *, p < 0.05.

FIGURE 4. NHERF1 and NHERF2 negatively regulate SR-BI protein levels, SR-BI-mediated selective HDL-CE uptake, and steroid hormone production in mouse Leydig tumor MLTC-1 cells. A, effects of NHERF1 or NHERF2 on SR-BI mRNA levels. MLTC-1 cells were transfected with control DNA (LacZ), NHERF1 plasmid, or NHERF2 plasmid, and after 48 h of transfection, mRNA expression of SR-BI was measured by qRT-PCR. The expression of SR-BI mRNA was normalized to 36B4. The results are presented as mean ± S.E. (error bars) (n = 3). B, protein expression of SR-BI in MLTC-1 cells in response to overexpression of NHERF1 or NHERF2. Experimental conditions were the same as described in A except after 48 h of transfection, cellular extracts were analyzed for SR-BI protein levels by Western blotting. The SR-BI levels were normalized with β-actin. Data are presented as mean ± S.E. (n = 3). C, effects of NHERF1 or NHERF2 on SR-BI-mediated HDL-CE uptake. Experimental conditions were identical to those described in A except selective HDL-CE uptake was measured fluorometrically at 48 h after transfection using reconstituted HDL-BODIPY-CE as a tracer as described under “Experimental Procedures.” Results are presented as mean ± S.E. (n = 4). D, effects of NHERF1 or NHERF2 on steroid (progesterone) secretion by R2C cells. Experimental conditions were the same as in A except 24 h after transfection, the medium was changed, and dishes were incubated for an additional 24 h in the presence of hHDL3 (500 μg of protein/ml). In each case, the progesterone secreted into the medium was quantified by radioimmunoassay. Data are presented as mean ± S.E. (n = 3). *, p < 0.05.

In an effort to further establish the specificity of the inhibitory action of NHERF1 and NHERF2 on SR-BI protein levels, we transfected mouse hepatoma Hepa 1–6 cells and human hepatocellular carcinoma Hep G2 cells with NHERF1 or NHERF2 cDNA constructs and measured SR-BI protein levels by Western blotting. As shown in Fig. 5A, transient transfection with NHERF1 or NHERF2 cDNA almost completely abolished SR-BI protein expression in Hepa 1–6 cells. Likewise, transfec-
FIGURE 5. Effects of NHERF1 and NHERF2 on endogenous SR-BI protein levels in hepatic Hepa 1–6 and Hep G2 cells (A) and effects of NHERF4 on SR-BI protein levels (B) and selective HDL-CE uptake (C) in CHO cells. A, Hepa 1–6 and Hep G2 cells were transiently transfected with LacZ vector (control) or NHERF1 or NHERF2 plasmid. After 48 h, cell lysates were prepared and subjected to Western blot analysis for SR-BI (or human analog CLA-1) and β-actin. B and C, CHO cells were transiently transfected with SR-BI with or without NHERF4, and after 48 h, cellular extracts were analyzed for SR-BI protein levels by Western blotting (B), or cells were subjected to measurement of selective HDL-CE uptake (C) fluorometrically using HDL-BODIPY-CE as a tracer. Results are mean ± S.E. (error bars) (n = 3), * p < 0.05.

function of Hep G2 cells with NHERF1 or NHERF2 also decreased the expression levels of SR-BI protein as compared with control cells (Fig. 5A). Thus, both NHERF1 and NHERF2 negatively regulate SR-BI protein levels irrespective of cell types. Additional studies demonstrated that another member of the NHERF family, NHERF4, had no effect on either SR-BI protein levels or its selective transport function when CHO cells were transiently co-transfected with SR-BI plus NHERF4 as compared with SR-BI alone (Fig. 5, B and C). In contrast, co-transfection of CHO cells with SR-BI plus PDZK1/NHERF3 up-regulated the expression of SR-BI protein as compared with SR-BI protein levels seen in cells transfected with the SR-BI construct alone (Fig. 6). These results are in agreement with the earlier reports showing that PDZK1/NHERF3 is essential for normal expression of SR-BI in mouse hepatocytes (34–36). Our results further demonstrate that the presentation of PDZK1/NHERF3 can stimulate SR-BI protein expression in even non-hepatic cells expressing SR-BI.

NHERF1 and NHERF2 Effects on the Biosynthesis, Degradation, and Intracellular Processing (Maturation) of SR-BI—Decreased levels of SR-BI in cells co-expressing NHERF1 or NHERF2 could be due to inhibition of SR-BI biosynthesis or accelerated degradation. To address these issues, the rate of biosynthesis of SR-BI was examined by immunoprecipitating SR-BI from [35S]methionine/35S]cysteine pulse-labeled MLTC-1 cells previously transfected with SR-BI with or without NHERF1 or NHERF2 constructs. Quantification of the newly synthesized SR-BI ([35S]-labeled SR-BI) indicated that the expression of NHERF1 or NHERF2 decreased the de novo biosynthesis of SR-BI by 40–50% (Fig. 7A). Western blot analysis further demonstrated that SR-BI protein mass also decreased significantly in cell extracts from cells co-transfected with SR-BI plus NHERF1 or NHERF2 as compared with cells transfected with SR-BI alone (Fig. 7B).

The influence of NHERF1 and NHERF2 expression on the turnover of SR-BI protein was also investigated, and the data are shown in Fig. 8. A semilog plot of the data shows first-order decay of SR-BI in cells transfected with SR-BI alone or co-transfected with SR-BI plus NHERF1 or NHERF2. The calculated half-life ($t_{1/2}$) values are $9.97 ± 1.14$, $12.23 ± 1.44$ ($p = 0.28$ versus control, not significant), and $8.87 ± 0.37$ ($p = 0.41$ versus control, not significant) h, respectively, for cells transfected with SR-BI alone, SR-BI plus NHERF1, and SR-BI plus NHERF2. These studies demonstrate that neither NHERF1 nor NHERF2 apparently exerts any statistically significant effects on the turnover rates of SR-BI.

We next evaluated the impact of NHERF1 and NHERF2 on the intracellular processing and maturation of SR-BI. For these studies, we made use of Endo H (endo-β-N-acetyl glucosaminidase H), which serves as an index of protein processing and maturation through the endoplasmic reticulum and Golgi apparatus. Endo H, a glycosidase, cleaves the chitobiose core of high mannose and some hybrid glycans. When proteins are correctly processed through the endoplasmic reticulum and Golgi, they become resistant to Endo H, which is considered a marker of posttranslational processing and maturation of glycoproteins in the Golgi (52, 53). As shown in Fig. 9, the majority of SR-BI in cell extracts from SR-BI-overexpressing cells was sensitive to Endo H and was converted to the deglycosylated form by Endo H treatment. Thus, the majority of the overexpressed SR-BI did not acquire complex carbohydrates (Endo H resistance) via its further processing through Golgi; these results are in agreement with an earlier report (25). The large portion of SR-BI sensitive to Endo H may be due to the overexpression system, where it is likely that much more SR-BI is synthesized than can be adequately processed through the
endoplasmic reticulum-Golgi pathway. Interestingly, co-expression of either NHERF1 or NHERF2 did not affect the susceptibility of oligosaccharide side chains of SR-BI in the cell extracts to Endo H. These studies lead us to conclude that neither NHERF1 nor NHERF2 exerts any modulatory actions on the posttranslational processing and the cellular maturation of SR-BI.

Depletion of NHERF1 or NHERF2 from MLTC-1 Cells by Specific siRNA Increases SR-BI Protein Levels and Up-regulates Its Selective CE Transport and Steroidogenic Functions—To further establish the inhibitory role of NHERF1 and NHERF2 on SR-BI, we employed siRNA to deplete mRNA and protein levels of NHERF1 and NHERF2 in MLTC-1 cells. Quantitative RT-PCR results demonstrated that transfection of MLTC-1 cells with NHERF1 specific siRNA reduced NHERF1 mRNA levels by 80–85%, whereas transfection with NHERF2 specific siRNA also resulted in significant lowering of NHERF2 mRNA levels (67% of control), although the NHERF2 mRNA level was very low in control MLTC-1 cells (Fig. 10A). Western blot analysis showed that MLTC-1 cells had lower NHERF1 (29% of basal) and NHERF2 (57% of basal) protein levels when transfected.
Effects of siRNAs on NHERF1-, NHERF2-, and SR-BI-selective HDL-CE uptake and steroidogenesis in MLTC-1 cells. A, RNA preparations derived from either control (scrambled) siRNA-, NHERF1 siRNA-, or NHERF2 siRNA-transfected MLTC-1 cells were subjected to qRT-PCR, and changes in mRNA levels of NHERF1, NHERF2, and SR-BI were normalized to 36B4, β-actin. C, MLTC-1 cells were transfected with either control (scrambled) siRNA, NHERF1 siRNA, or NHERF2 siRNA. After 48 h, cell lysates were prepared and subjected to Western blot analysis for SR-BI, NHERF1, and NHERF2, and data were normalized to β-actin. C, MLTC-1 cells were transfected with either control (scrambled) siRNA, NHERF1 siRNA, or NHERF2 siRNA. After 48 h, cells were subjected to measurement of selective HDL-CE uptake fluorometrically using HDL-BODIPY-CE as a tracer as described under “Experimental Procedures.” D, MLTC-1 cells were transfected with either control (scrambled) siRNA, NHERF1 siRNA, or NHERF2 siRNA. After 48 h, hHDL3-stimulated progesterone production was measured as described under “Experimental Procedures.” Results are mean ± S.E. (error bars) (n = 3). *, p < 0.05; **, p < 0.01; ***, p < 0.001.

NHERF1 and NHERF2 Regulation of SR-BI

With their respective siRNAs (Fig. 10B). No changes in SR-BI mRNA levels were noted following transfection of MLTC-1 cells with either NHERF1- or NHERF2-specific siRNA (Fig. 10A). In contrast, transfection of cells with either NHERF1 siRNA or NHERF2 siRNA led to increased SR-BI protein levels (Fig. 10B). These results suggest that transfection of MLTC-1 cells with NHERF1 and NHERF2-specific siRNAs can reduce their endogenous mRNA and protein levels but at the same time increase protein levels of SR-BI without altering SR-BI mRNA levels, consistent with a translational/posttranslational effect of NHERF1/2 on SR-BI expression. We also investigated the effect of siRNA-induced depletion of NHERF1 and NHERF2 on selective HDL-BODIPY-CE uptake. Compared with negative control, transfection of MLTC-1 cells with either NHERF1 siRNA or NHERF2 siRNA significantly increased SR-BI-mediated selective CE uptake (Fig. 10C). To investigate the effects of NHERF1 and NHERF2 specific siRNAs on steroid (progesterone) production, we collected media from the transfected cells that were preincubated with or without hHDL3 or with or without 22(R)-hydroxycholesterol, and assessed progesterone concentrations. Transfection of cells with either NHERF1 or NHERF2 siRNA significantly increased basal and hHDL3-supported progesterone production (Fig. 10D). In contrast, no such stimulatory effect was noted on 22(R)-hydroxycholesterol supported steroidogenesis (Fig. 10D); 22(R)-hydroxycholesterol is a freely diffusible steroid precursor and does not require the participation of SR-BI for its transport to the cell interior.

SR-BI Interacts with NHERF1 and NHERF2—To examine the possibility that there is a direct interaction between SR-BI and NHERF1/NHERF2, we initially transfected CHO cells with SR-BI-V5 plus NHERF1-c-Myc or NHERF2-c-Myc and subsequently examined the colocalization of SR-BI and NHERF1/NHERF2 by confocal microscopy using anti-c-Myc mouse monoclonal antibody (for detection of NHERF1-c-Myc and NHERF2-c-Myc) and an SR-BI anti-peptide rabbit polyclonal antibody. As shown in Fig. 11, the expressed SR-BI-V5 construct was localized both in the plasma membrane and in the intracellular vesicular compartment (Fig. 11, A and E). Likewise, overexpressed NHERF1 or NHERF2, like SR-BI, was also localized throughout the vesicular compartment and at the cell surface (Fig. 11, B and F). Merged images of the cells showed nearly complete co-localization of SR-BI and NHERF1 or NHERF2 proteins (Fig. 11, D and H, yellow), indicating that SR-BI co-localizes with NHERF1 and NHERF2 under conditions of overexpression.

We next performed co-immunoprecipitation experiments using transfected CHO cells to determine whether SR-BI selectively associates with NHERF1 and/or NHERF2 in a cellular context. CHO cells were transiently co-transfected with the
expression plasmid pcDNA6-V5-rSR-BI and pcDNA3-mNHERF1 or pcDNA3-hNHERF2, cell lysates were prepared, and immunoprecipitations were performed with either normal mouse IgG (control) or mouse anti-V5 IgG. Following SDS-PAGE and electrophoretic transfer, membranes were incubated with anti-SR-BI plus anti-NHERF1, or anti-NHERF2, and protein bands were visualized on an Odyssey® infrared imaging system. The results presented in Fig. 12 demonstrate co-immunoprecipitation of a protein complex with anti-V5 monoclonal antibody containing overexpressed SR-BI and NHERF1 or NHERF2. Neither SR-BI nor NHERF1/2 was detectable in a complex with normal mouse IgG, confirming the specificity of co-immunoprecipitation of NHERF1 and NHERF2 with the anti-V5-SR-BI monoclonal antibody (Fig. 12). Collectively, these data provide evidence that SR-BI forms a complex with both NHERF1 and NHERF2 in cells.

We independently assessed the interaction of SR-BI with NHERF1/2 using BiFC (46, 47, 54). This technique involves the production of a fluorescent signal by two nonfluorescent fragments of YFP when they are brought together by interactions between proteins fused to each fragment (45). To perform BiFC, we fused NHERF1 or NHERF2 to the N terminus of residues 1–158 of YFP (pcDNAI-NHERF1- or NHERF2-YFP(1–158)-STOP) and residues 159–238 of YFP were fused to the N terminus of SR-BI (pcDNAI-YFP(159–238)-rSR-BI). Fig. 13 shows that expression of both pcDNAI-YFP(159–238)-rSR-BI and pcDNAI-NHERF1-MERM (Fig. 13A) or NHERF2-YFP(1–158)-STOP (Fig. 13C) resulted in a strong fluorescent signal that was mainly enriched in the vesicular compartment and at the cell surface. In contrast, expression of either pcDNAI-NHERF1- or NHERF2-YFP(1–158)-STOP with pcDNAI-YFP(159–238) did not produce any fluorescent signal (Fig. 13, B and D). These results further confirm a physical interaction between SR-BI and NHERF1/2.

Analysis of NHERF1 and NHERF2 Domains Involved in Protein-Protein Interaction with SR-BI and Inhibition of Its Selective CE Transport Function—As noted above, NHERF1 and NHERF2 each contain two related PDZ domains (40, 42). To determine which of these domains are involved in NHERF1 and NHERF2 interaction with SR-BI, we generated constructs containing deleted PDZ1 (NHERF1ΔP1 and NHERF2ΔP1) or deleted PDZ2 (NHERF1ΔP2 and NHERF2ΔP2) (Fig. 14A). As expected, co-transfection of CHO cells with SR-BI with or without wild-type NHERF1 or NHERF2 decreased SR-BI-mediated selective HDL-BODIPY-CE uptake (Fig. 14B). Disruption of PDZ2 domains in both NHERF1 and NHERF2 blocked the ability of these proteins to inhibit selective CE transport. Interestingly, deletion of the PDZ1 domain in NHERF1, but not in NHERF2, eliminated its ability to inhibit selective transport function of SR-BI, although both mutant proteins were expressed at sufficiently high levels (Fig. 14C). Because both NHERF1 and NHERF2 also possess a C-terminal MERM domain linking them with the actin-associated proteins, merlin, ezrin, radixin, and moesin, we next examined the effects of NHERF1 and NHERF2 lacking the MERM domain (NHERF1ΔMERM and NHERF2ΔMERM) on SR-BI-dependent selective CE transport. Transfection of CHO cells with SR-BI plus NHERF1 deleted of its MERM domain (NHERF1ΔMERM) or NHERF2 deleted of its MERM domain (NHERF2ΔMERM) lacked the ability to inhibit SR-BI protein levels and the selective transport functions of SR-BI (Fig. 14, B and C). Thus, inhibitory actions of NHERF1 on SR-BI-mediated selective CE transport require intact PDZ1, PDZ2, and MERM domains, whereas inhibitory actions of NHERF2 require intact PDZ2 and MERM domains.

To characterize the interaction between PDZ1/2 and MERM domains of NHERF1 and NHERF2 and SR-BI, we constructed GST-tagged NHERF1 and NHERF2 variants in which portions of PDZ1, PDZ2, or the MERM domain (as described above) had been deleted (i.e. pGEX-NHERF1ΔP1, pGEX-NHERF1ΔP2, pGEX-NHERF2ΔP1, pGEX-NHERF2ΔP2, pGEX-NHERF1ΔMERM, and pGEX-NHERF2ΔMERM). These constructs were expressed in E. coli, and glutathione-agarose chromatography purified GST-tagged variants of NHERF1 and NHERF2 proteins were obtained. Equal amounts of these GST fusion proteins, along with GST alone, were absorbed onto glutathione-agarose beads and incubated with cell extracts from R2C Leydig tumor cells. Rat R2C cells express very high endogenous levels of SR-BI (39,
55). GST pull-down Western blot bands are shown in Fig. 14D. GST-NHERF1, GST-NHERF2, GST-NHERF1 lacking PDZ1, GST-NHERF1 lacking PDZ2 domain, GST-NHERF1 lacking MERM domain, GST-NHERF2 lacking PDZ1 domain, and GST-NHERF2 lacking MERM domain proteins all pulled down SR-BI from R2C cell lysate. Only GST-NHERF2 protein lacking PDZ2 failed to pull down SR-BI from the cell lysate under the same conditions. Thus, these studies revealed that the presence of the MERM domain plus one of the PDZ domains with the exception of PDZ1 of NHERF2 are sufficient for NHERF1/2 association with SR-BI. On the other hand, the presence of all of these domains is essential for NHERF1/2-SR-BI association.
mediated inhibition of protein expression and selective transport function of SR-BI.

We next attempted to identify site(s) within the C-terminal domain of SR-BI that interact with NHERF1 and NHERF2. Using SMART, we identified several putative Class I, II, and III PDZ-interacting domains in the C-terminal cytoplasmic tail of SR-BI (Table 2). To a large extent these PDZ domain sites, like the terminal PDZK1 site (EAKL; Class II PDZ domain binding site), are well conserved among various mammalian species (i.e. mouse, rat, hamster, northern tree shrew, rabbit, pig, bovine, and human SR-BI) (Table 2). We generated a series of mutant C-terminal domains of SR-BI with alanine substitutions of the various amino acid residues of the FLFW (underlined), KEAI (shaded), AIQA (underlined), SESL (boldface type), MSPA (boldface type), GTVL (boldface type), and EAKL (underlined) motifs (PDZ domain, Class I, -X(S/T)XH9021 (in boldface type in Table 2); PDZ domain, Class II, -XH9021XH9021 (underlined); PDZ Domain, Class III, -X(D/E)XH9021 (shaded)). As before, CHO cells were co-transfected with NHERF1 or NHERF2 plus wild-type SR-BI or SR-BI bearing mutations in one of the seven putative PDZ recognition motifs (Fig. 15). Western blot analysis and selective CE transport assays indicated that alanine substitution of the FLFW, KEAL, AIQA, SESL, MSPA, GTVL, or EAKL amino acid residues had no effect on the ability of NHERF1 and NHERF2 to cause reduction in SR-BI protein levels (Fig. 15, A and B). However, NHERF1- and NHERF2-mediated lowering of SR-BI protein levels was completely abolished by mutation of the terminal four EAKL residues. These data clearly demonstrate the requirement of the EAKL motif in SR-BI for its association with NHERF1 and NHERF2 to cause reduction in SR-BI protein levels (Fig. 15, A and B). Western blot analysis and selective CE transport assays indicated that alanine substitution of the FLFW, KEAL, AIQA, SESL, MSPA, and GTVL amino acid residues had no effect on the ability of NHERF1 and NHERF2 to cause reduction in SR-BI protein levels (Fig. 15, A and B). However, NHERF1- and NHERF2-mediated lowering of SR-BI protein levels was completely abolished by mutation of the terminal four EAKL residues. These data clearly demonstrate the requirement of the EAKL motif in SR-BI for its association with NHERF1 and NHERF2. We also constructed plasmids containing SR-BI with different C-terminal truncations (pcDNA6V5-rSR-BI-464, pcDNA6V5-rSR-BI-494, and pcDNA6V5-rSR-BI-504). When co-expressed with NHERF1 or NHERF2 in CHO cells, no changes in the expression of these truncated SR-BI proteins were observed (Fig. 15C).

### DISCUSSION

SR-BI, an HDL receptor, mediates the bulk selective delivery of lipoprotein-derived, especially HDL-derived, precursor cho-
lesterol for product formation (bile acids (2, 4, 16–20) and steroids (1, 4, 5–13, 15)). Although most observations on the physiology of SR-BI have been performed using rodent tissues and cells, the recent description of a family having a mutation in SR-BI that is associated with attenuated adrenal steroidogenesis (56) supports the notion that SR-BI functions similarly in humans and rodents. Extensive evidence exists in support of trophic hormone (ACTH and gonadotropins)-mediated transcriptional regulation of steroidogenic SR-BI (14, 27–31), but recent studies from our laboratory suggest that SR-BI expression is also regulated posttranscriptionally by miRNA-125a and miRNA-455 (39). Nonetheless, virtually nothing is known about the possible involvement of accessory protein(s) that may interact with SR-BI in steroidogenic cells and influence its expression and function at the posttranslational level. In the present study, we have described a novel role of NHERF1 and NHERF2 in the regulation of SR-BI and its major functions. This study shows that both NHERF1 and NHERF2 interact with SR-BI and, consequently, inhibit its protein levels, SR-BI-mediated selective HDL-CE uptake, and steroidogenesis in steroidogenic cells. We further demonstrate that both NHERF1 and NHERF2 specifically inhibit the synthesis of SR-BI without apparently impacting its turnover or intracellular processing and maturation. However, at present, we do not know the underlying mechanism(s) by which NHERF1 and NHERF2 inhibit SR-BI biogenesis, and this issue will be explored in future studies.

Although PDZK1 (NHERF3) has previously been shown to be essential for the normal expression, cell surface localization, and function of hepatic, but not steroidogenic, SR-BI (33–35), this is the first report that NHERF1 and NHERF2, expressed endogenously at relatively high but variable levels in steroidogenic cells and liver, serve as negative regulators of SR-BI expression and its associated functions. These two proteins display high homology: 44% identity between NHERF1 and NHERF2 (42). The homology between the two PDZ protein-protein interaction domains is even higher; PDZ1 motifs of NHERF1 and NHERF2 show 72% identity. NHERF1 and NHERF2 are also known to perform similar functions (40–42, 57, 58).

Our studies demonstrate for the first time that NHERF1 and NHERF2 are variably expressed at relatively high levels in model steroidogenic cell lines and rat adrenal. For example, under basal conditions, NHERF1 mRNA is expressed at very high levels in mouse Leydig tumor MLTC-1 cells, at intermediate levels in ovarian granulosa cells, and at very low levels in rat adrenals. In contrast, adrenals and rat Leydig tumor cells R2C exhibit very high levels of NHERF2 mRNA expression, whereas its mRNA levels in MLTC-1 and granulosa cells are very low. Contrary to the current findings, until now it has been widely believed that NHERF1 mRNA has a very restricted pattern of expression, being very highly expressed in the kidney, gastrointestinal tract, placenta, mammary gland, liver, and prostate and expressed at very low levels in many other tissues (42, 59, 60). Similarly, NHERF2 mRNA was shown to have an even more restricted tissue distribution with significant expression noted only in the lung, gastrointestinal tract, kidney, liver, and brain (42, 61). In agreement with previous studies (36–38), only low expression of PDZK1 (NHERF3) mRNA was detected in the steroidogenic MLTC-1, R2C, and granulosa cells and the adrenal gland. Similarly, very low levels of NHERF4 (IKEPP) are observed in the model steroidogenic cell lines and adrenal gland studied; these observations are in line with reports suggesting that NHERF4 displays the most restricted tissue expression among the four NHERFs, with significant expression reported in the gastrointestinal tract and kidney (62, 63). Our studies also demonstrate that ACTH or the second messenger, cAMP, of trophic hormones (ACTH, LH/hCG, or FSH) regulates the expression of NHERF1 and NHERF2 in a tissue- and cell-specific manner. qRT-PCR measurements carried out on RNA samples isolated from primary rat ovarian granulosa cells and mouse Leydig tumor MLTC-1 cells that were pre-exposed with or without β2cAMP showed down-regulation of NHERF1 and NHERF2 mRNAs but up-regulation of mRNA levels of SR-BI. In contrast, adrenals from rats pretreated with ACTH showed no changes in mRNA levels of NHERF1 and NHERF2 but up-regulation of SR-BI mRNA levels, as compared with no ACTH treatment. Interestingly, ACTH treatment, however, decreased the protein levels of both NHERF1 and NHERF2. Thus, the inverse correlation between NHERF1 and NHERF2 and SR-BI expression strengthens our view that these two PDZ scaffolds are physiological regulators of SR-BI. Furthermore, it should be noted that until now, estrogens are the only known hormone regulators of NHERF1 in nonsteroidogenic breast cancer cells, MCF-7 and MDA-MB-231 (42, 64, 65).

We next provide evidence that the two homologous NHERF proteins directly interact with SR-BI and that such protein-protein interactions are necessary for NHERF1/2 inhibition of SR-BI expression, SR-BI-mediated selective HDL-CE uptake, and SR-BI/HDL-supported steroidogenesis. The possibility of an interaction between NHERF1/2 and SR-BI was initially indicated by our use of overexpression and siRNA strategies and was further confirmed by multiple molecular, cellular, and biochemical approaches, including GST pull-down experiments, BiFC, mutational and deletion analyses, and co-immunoprecipitation assays. Furthermore, confocal microscopy of CHO cells co-expressing SR-BI and NHERF1 or NHERF2 demonstrated colocalization of the two proteins, thus further demonstrating a specific association between them.

Our studies also indicate that NHERF1 and NHERF2 can directly repress SR-BI protein expression through protein-protein interactions, thereby negatively regulating SR-BI functions, such as selective HDL-CE transport and HDL-supported steroid production. Initially, we demonstrated that overexpression of NHERF1 and NHERF2 reduces protein levels of SR-BI in two model cell lines, CHO and COS-7 cells, and inhibits its selective cholesteryl transport function. In addition, we showed that a reduction in SR-BI protein levels in response to overexpression of either NHERF1 or NHERF2 also leads to significant inhibition of both SR-BI-mediated selective HDL-CE uptake and progesterone production in MLTC-1 cells. These results were further complemented by demonstration that knockdown of either NHERF1 and NHERF2 by siRNA up-regulates SR-BI protein levels in R2C cells and increases selective HDL-CE transport and steroid production. The NHERF1- and NHERF2-mediated lowering of SR-BI protein levels was also observed following transfection of two hepatoma cell lines, Hepa 1–6.
and Hep G2 cells, demonstrating the actions of NHERF1 and NHERF2 in multiple cell types. The specificity of the inhibitory actions of NHERF1 and NHERF2 was also established by demonstration that co-transfection of CHO cells with SR-BI and NHERF4 constructs had no significant effect on either SR-BI protein expression or its associated function. These various studies lead us to conclude that both NHERF1 and NHERF2 negatively regulate the expression of SR-BI protein levels and, as a result, inhibit SR-BI-linked selective CE transport and steriodogenic processes.

We also analyzed the role of the PDZ and MERM domains of NHERF1/2 and of the putative Class I, II, and III PDZ-interacting domains in the C-terminal cytoplasmic tail of SR-BI in SR-BI-mediated selective HDL-CE uptake. A novel finding of this set of studies is the simultaneous requirement of PDZ1/PDZ2 and MERM domains of NHERF1 and the PDZ2 and MERM domains of NHERF2 in SR-BI-mediated selective HDL-CE uptake. Wild-type NHERF1 or NHERF2 or a mutant construct containing a deleted PDZ1 domain of NHERF2 significantly decreased selective CE uptake when CHO cells were co-transfected with one of these constructs plus SR-BI. When either the PDZ1 or PDZ2 domain of NHERF1 or the PDZ2 domain of NHERF2 was deleted and co-transfected with SR-BI, the inhibitory actions of both NHERF1 and NHERF2 were eliminated. Thus, both PDZ domains of NHERF1 and the PDZ2 domain of NHERF2 are required in mediating the inhibitory actions of these two proteins. In addition, the MERM domain of NHERF1 and NHERF2 is also required for their inhibition of SR-BI-mediated selective CE transport. Deletion of the MERM domain resulted in complete loss of the ability of both NHERF1 and NHERF2 to inhibit SR-BI-mediated selective CE transport. Collectively, these data show a dual requirement of PDZ and MERM domains in NHERF1/2 inhibition of SR-BI selective delivery of HDL-CE. This conclusion is further supported by the demonstration that mutation of the terminal four amino acids (EAKL → AAAA) or partial truncation of the C-terminal domain of SR-BI led to complete loss of the inhibitory actions of both NHERF1 and NHERF2. Interestingly, although we identified several internal putative Class I, II, and III PDZ-interacting domains in the C-terminal cytoplasmic tail of SR-BI, none of the mutations of these putative PDZ-interacting domains resulted in any significant effect on the ability of either NHERF1 or NHERF2 to interfere with the selective transport function of SR-BI.

Both NHERF1 and NHERF2 have previously been shown to play regulatory roles in the cellular trafficking of a large number of membrane proteins, including affecting their apical localization, retention, recycling, or internalization and receptor signaling and desensitization. Some of the most studied common targets of NHERF1 and NHERF2 include Na⁺/H⁺ exchanger NHE3, cystic fibrosis transmembrane conductance regulator, P2Y1, PTEN, β₂-adrenergic receptor, and parathyroid hormone receptor (40, 42, 57, 66–71). In addition, NHERF1 and NHERF2 individually and specifically regulate a large number of target proteins. For example, a number of studies have implicated NHERF1 in the regulation of receptor function, signaling, recycling, or stabilization at the cell surface (42, 66, 67, 72–77), where, in most cases, it inhibits internalization of receptors, such as κ-type opioid neuropeptide receptor and G protein-coupled receptors (42, 67, 75). Interestingly, NHERF1 down-regulation also results in increased expression and function of multidrug resistance protein 4 (78). Likewise, existing literature also supports a modulatory role of NHERF2 in the cellular transport and function of several target proteins, such as adenosine 2b receptor, PMCA2B, phospholipase C-β3, the LPA₂ receptor, mGluR5, CLC-5, SRY, and the LPA₃ receptor (79–87). This differential specificity of NHERF1 and NHERF2 action and target recognition may be necessary to partially overcome the redundant actions of these two NHERFs. Our studies add a new function for both NHERF1 and NHERF2 (i.e. these two proteins via protein–protein interaction inhibit SR-BI protein expression and SR-BI-mediated selective HDL-CE transport function and associated steriodogenesis). This is in contrast to their family member NHERF3 (PDZK1), which is required for normal hepatic SR-BI protein expression (36).

In summary, NHERF1 and NHERF2, but not NHERF4, specifically interact with SR-BI and reduce its protein levels. This effect requires both intact PDZ (PDZ1 and –2 in NHERF1 and PDZ2 in NHERF2) and MERM domains in NHERF1 and NHERF2. In addition, a terminal PDZ domain binding sequence in the C-terminal domain of SR-BI is also needed. Moreover, we show the functional consequence of NHERF1-SR-BI and NHERF2-SR-BI interaction in transfected steriodogenic and nonsteroidogenic cell lines, where SR-BI-mediated selective HDL-CE uptake and HDL-supported steroid hormone production were decreased by co-expression of NHERF1 or NHERF2, and these functions of SR-BI were up-regulated following siRNA-mediated inhibition of NHRF1 and NHERF2 expression. Finally, evidence obtained suggests that trophic hormone (LH/hCG, ACTH) or its second messenger, cAMP, up-regulates SR-BI expression through both increased induction of SR-BI mRNA levels as well as elevation of SR-BI protein levels via the inhibition of NHERF1 and NHERF2. Collectively, these novel findings suggest that both NHERF1 and NHERF2 act as physiological translational/posttranslational regulators of the functional expression of SR-BI. Currently, studies are under way to identify the mechanism(s) involved in NHERF1- and NHERF2-mediated inhibition of SR-BI protein expression.

REFERENCES

1. Gwynne, J. T., and Hess, B. (1980) The role of high density lipoproteins in rat adrenal cholesterol metabolism and steriodogenesis. J. Biol. Chem. 255, 10875–10883

2. Glass, C., Pittman, R. C., Weinstein, D. B., and Steinberg, D. (1983) Dissociation of tissue uptake of cholesterol ester from that of apoprotein A-I of rat plasma high density lipoprotein. Selective delivery of cholesteryl ester to liver, adrenal, and gonad. Proc. Natl. Acad. Sci. U.S.A. 80, 5435–5439

3. Reaven, E., Chen, Y. D., Spicher, M., and Azhar, S. (1984) Morphological evidence that high density lipoproteins are not internalized by steroid producing cells during in situ organ perfusion. J. Clin. Invest. 74, 1384–1397

4. Pittman, R. C., Knecht, T. P., Rosenbaum, M. S., and Taylor, C. A., Jr. (1987) A nonendocytic mechanism for the selective uptake of high density lipoprotein-associated cholesteryl esters. J. Biol. Chem. 262, 2443–2450

5. Azhar, S., Stewart, D., and Reaven, E. (1989) Utilization of cholesterol-rich lipoproteins by perfused rat adrenals. J. Lipid Res. 30, 1799–1810

6. Azhar, S., and Reaven, E. (2002) Scavenger receptor class B1 and selective cholesteryl ester uptake. Partners in the regulation of steriodogenesis. Mol. Cell Endocrinol. 195, 1–26

7. Azhar, S., Leers-Sucheta, S., and Reaven, E. (2003) Cholesterol uptake in...
adrenal and gonadal tissues. The SR-BI and "selective" pathway connection.

Front. Biosci. 8, 998–1029

8. Temel, R. E., Trigatti, B., DeMattos, R. B., Azhar, S., Krieger, M., and Williams, D. L. (1997) Scavenger receptor class B, type I (SR-BI) is the major route for the delivery of high density lipoprotein cholesterol to the steroidogenic pathway in cultured mouse adrenocortical cells. Proc. Natl. Acad. Sci. U.S.A. 94, 13600–13605

9. Cherradi, N., Bideau, M., Arnaudeau, S., Demaurex, N., James, R. W., Azhar, S., and Capponi, A. M. (2001) Angiotensin II promotes selective uptake of high density lipoprotein cholesterol esters in bovine adrenal glomerulosa and human adrenocortical carcinoma cells through induction of scavenger receptor class B type 1. Endocrinology 142, 4540–4549

10. Azhar, S., Tsai, L., Medichera, S., Chandrasekher, Y., Giudice, L., and Reaven, E. (1998) Human granulosa cells use high density lipoprotein cholesterol for steroidogenesis. J. Clin. Endocrinol. Metab. 83, 983–991

11. Reaven, E., Tsai, L., and Azhar, S. (1995) Cholesterol uptake by the "selective" pathway of ovarian granulosa cells. Early intracellular events. J. Lipid Res. 36, 1602–1617

12. Reaven, E., Lue, Y., Nomoto, A., Temel, R., Williams, D. L., van der Westhuizen, D. R., and Azhar, S. (1999) The selective pathway and a high-density lipoprotein receptor (SR-BI) in ovarian granulosa cells of the mouse. Biochim. Biophys. Acta 1436, 565–576

13. Towns, R., Azhar, S., Peegel, H., and Menon, K. M. (2005) LH/hCG-stimulated androgen production and selective HDL-cholesterol transport are inhibited by a dominant-negative CREB construct in primary cultures of rat theca-interstitial cells. Endocrine 27, 269–277

14. Reaven, E., Zhan, L., Nomoto, A., Leers-Sucheta, S., and Azhar, S. (2000) Expression and microvillar localization of scavenger receptor class B, type I (SR-BI) and selective cholesteryl ester uptake in Leydig cells from rat testis. J. Lipid Res. 41, 343–356

15. Hu, J., Zhang, Z., Shen, W.-J., and Azhar, S. (2010) Cellular cholesterol delivery, intracellular processing and utilization for biosynthesis of steroid hormones. Nutr. Metab. 7, 47

16. Rinninger, F., Brundert, M., Jackle, S., Galle, P. R., Busch, C., Izbicki, J. R., Rogiers, X., Henne-Bruns, D., Kremer, B., Broelsch, C. E., and Greten, H. (1994) Selective uptake of high-density lipoprotein-associated cholesteryl esters by human hepatocytes in primary culture. Hepatology 19, 1100–1114

17. Kozarsky, K. F., Donahee, M. H., Rigotti, A., Iqbal, S. N., Edelman, E. R., and Krieger, M. (1997) Overexpression of the HDL receptor SR-BI alters plasma HDL and bile cholesterol levels. Nature 387, 414–417

18. JI, Y., Wang, N., Ramakrishnan, R., Sehaye, E., Huszar, D., Breslow, J. L., and Tall, A. R. (1999) Hepatic scavenger receptor BI promotes rapid clearance of high density lipoprotein free cholesterol and its transport into bile. J. Biol. Chem. 274, 33398–33402

19. Mardones, P., Quiñones, V., Amigo, L., Moreno, M., Miquel, J. F., Wang, S., Yue, H., Derin, R. B., Guggino, W. B., and Li, M. (2000) Accessory protein and NHE3 regulation. J. Biol. Chem. 275, 518–520

20. Van Eck, M., Twisk J., Hoekstra, M., Van Rij, B. T., Van der Lans, C. A., Bos, I. S., Kruit, J. K., Kuipers, F., and Van Berkel, T. J. (2003) Differential effects of scavenger receptor BI deficiency on lipid metabolism in cells of the arterial wall and in the liver. J. Biol. Chem. 278, 23699–23705

21. Acton, S., Rigotti, A., Landschulz, K. T., Xu, S., Hobbs, H. H., and Krieger, M. (1996) Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. Science 274, 518–520

22. Rigotti, A., Miettinen, H. E., and Krieger, M. (2003) The role of the high-density lipoprotein receptor SR-BI in the lipid metabolism of endocrine and other tissues. Endocr. Rev. 24, 357–387

23. Gu, X., Trigatti, B., Xu, S., Acton, S., Babitt, J., and Krieger, M. (1998) The efficient cellular uptake of high density lipoprotein lipids via scavenger receptor class B type I requires not only receptor-mediated surface binding but also receptor-specific lipid transfer mediated by its extracellular domain. J. Biol. Chem. 273, 26338–26348

24. Connelly, M. A., Klein, S. M., Azhar, S., Abumrad, N. A., and Williams, D. L. (1999) Comparison of class B scavenger receptors, CD36 and scavenger receptors mediate high density lipoprotein-cholesterol ester selec-
proteins in the regulation of salt and water transport. *Ann. N.Y. Acad. Sci.* **1165**, 249–260.

42. Ardua, J. A., and Friedman, P. A. (2011) Regulation of G protein-coupled receptor function by Na+/H+ exchange regulatory factors. *Pharmacol. Rev.* **63**, 882–900.

43. Bretscher, A., Edwards, K., and Fehon, R. G. (2002) ERM proteins and Merlin. Integrators at the cell cortex. *Nat. Rev. Mol. Cell Biol.* **3**, 586–599.

44. Hung, A. Y., and Sheng, M. (2002) PDZ domains. Structural modules for protein complex assembly. *J. Biol. Chem.* **277**, 5699–5702.

45. Nourry, C., Grant, S. G., Borg, J.-P. (2003) PDZ domain proteins. Plug and play. *Science’s STKE* **2003**, RE7.

46. Hynes, T. R., Tang, L., Mervine, S. M., Sabo, J. L., Yost, E. A., Deovreets, P. N., and Berlot, C. B. (2004) Visualization of G protein βγ dimers using bimolecular fluorescence complementation demonstrates roles for both β and γ subcellular targeting. *J. Biol. Chem.* **279**, 30279–30286.

47. Griesbeck, O., Baird, G. S., Campbell, R. E., Zacharias, D. A., and Tsien, R. Y. (2001) Reducing the environmental sensitivity of yellow fluorescent protein. *J. Biol. Chem.* **276**, 29189–29196.

48. Hall, R. A., Premont, R. T., Chow, C.-W., Blitzer, J. T., Pitcher, J. A., Clanga, A., Stoffel, R. H., Barak, L. S., Shenolikar, S., Weinman, E. J., Grinstein, S., and Lefkowitz, R. J. (1998) The β2-adrenergic receptor interacts with the Na+/H+ exchanger regulatory factor to control Na+/H+ exchange. *Nature* **392**, 626–630.

49. Lamprecht, G., Weinman, E. J., and Yun, C.-H. (1998) The role of NHERF and E3KARP in the c-AMP-mediated inhibition of NHE3. *J. Biol. Chem.* **273**, 29972–29978.

50. Yun, C.-H., Lamprecht, G., Forster, D. V., and Sidor, A. (1998) NHE3 and E3KARP in the c-AMP-mediated inhibition of Na+/H+ exchange regulatory factor family of PDZ-containing proteins. *Annu. Rev. Physiol.* **60**, 279–314.

51. Singh, A. K., Riederer, B., Krabbenhöft, A., Rausch, B., Bonhagen, J., Lehmann, U., de Jonge, H. R., Donowitz, M., Yun, C., Weinman, E. J., Kocher, O., Hogema, B. M., and Seidler, U. (2009) Differential roles of NHERF1, NHERF2, and PDZK1 in regulating CFTR-mediated intestinal anion secretion in mice. *J. Clin. Invest.* **119**, 540–550.

52. Hall, R. A., Ostedgaard, I. S., Premont, R. T., Blitzer, J. T., Rahman, N., Welsh, M. J., Lefkowitz, R. J. (1998) A C-terminal motif found in the β2-adrenergic receptor, P2Y1 receptor and cystic fibrosis transmembrane conductance regulator determines binding to the Na+/H+ exchanger regulatory factor family of PDZ proteins. *Proct. Natl. Acad. Sci. U.S.A.* **95**, 8496–8501.

53. Takahashi, Y., Morales, F. C., Kreimann, E. L., and Georgescu, M.-M. (2006) P2Y14 receptor interacts with the Na+/H+ exchanger. *J. Biol. Chem.* **281**, 17198–17207.

54. Slattery, C., Jenkin, K. A., Lee, A., Simcocks, A. C., McAinch, A. J., Poronnik, P., and Zhang, J. H. (2006) The association of adaptor proteins with G protein-coupled receptors and receptor tyrosine kinases. *Annu. Rev. Physiol.* **68**, 491–505.

55. Romero, G., von Zastrow, M., and Friedman, P. A. (2011) Role of PDZ proteins in regulating trafficking, signaling, and function of GPCRs. *Methods, means, and opportunity. Adv. Pharmacol.* **62**, 279–314.

56. Nourry, C., Grant, S. G., Borg, J.-P. (2003) PDZ domain proteins. Plug and play. *Science’s STKE* **2003**, RE7.

57. Hammad, M. M., Kuang, Y.-Q., Yan, R., Allen, H., Dupré, D. J. (2010) Dynamic Na+/H+ exchange regulatory factor-1 is involved in chemokine receptor drug resistance. *Cell Physiol. Biochem.* **26**, 114–121.

58. Vergeer, M., Kopperaal, S. J., Franssen, R., Meurs, I., Yost, E., Deovreets, P. N., and Berlot, C. B. (2004) Visualization of G protein βγ dimers using bimolecular fluorescence complementation demonstrates roles for both β and γ subcellular targeting. *J. Biol. Chem.* **279**, 30279–30286.

59. Kerppola, T. K. (2008) Biomolecular fluorescence complementation (BIFC) analysis as a probe of protein interactions in living cells. *Annu. Rev. Biophys.* **37**, 465–487.

60. Bao, R. M., Joy, V., Leers-Sucheta, S., Bose, H. S., Miller, W. L., Azhar, S., and Stocco, D. M. (2011) Dynamic Na+–H+ exchanger regulatory factor-1 and cytoskeleton regulate the traffic and membrane dynamics of G protein-coupled receptors. *J. Biol. Chem.* **282**, 25076–25087.

61. Ardua, J. A., Wang, B., Watkins, S. C., Vilardaga, J.-P., and Friedman, P. A. (2011) Dynamic Na+/H+ exchanger regulatory factor-1 association and dissociation regulate parathyroid hormone receptor trafficking at membrane microdomains. *J. Biol. Chem.* **286**, 35020–35029.

62. Slattery, C., Jenkin, K. A., Lee, A., Simcocks, A. C., McAinch, A. J., Poronnik, P., and Hryciw, D. H. (2011) Na+/H+ exchanger regulatory factor 1 (NHERF1) PDZ scaffold binds an internal binding site in the scavenger receptor megalin. *Cell Physiol. Biochem.* **27**, 171–178.

63. Ritter, S. L., and Hall, R. A. (2009) Fine-tuning of GPCR activity by receptor-interacting proteins. *Nat. Rev. Mol. Cell Biol.* **10**, 819–830.

64. Hammad, M. M., Kuang, Y.-Q., Yan, R., Allen, H., Dupré, D. J. (2010) Na+–H+ exchanger regulatory factor-1 is involved in chemokine receptor homodimer CCR5 internalization and signal transduction but does not affect CXCR4 homodimer or CXCR4–CCR5 heterodimer. *J. Biol. Chem.* **285**, 34653–34664.

65. Nisar, S. P., Cunningham, M., Saxena, K., Pope, R. J., Kelly, E., and Mundell, S. J. (2012) Arrestin scaffolds NHERF1 to the P2Y12 receptor to regulate receptor internalization. *J. Biol. Chem.* **287**, 24505–24515.

66. Hoque, M. T., and Cole, S. P. (2008) Down-regulation of Na+/H+ exchanger regulatory factor 1 increases expression and function of multidrug resistance protein 4. *Cancer Res.* **68**, 4802–4809.

67. Sitaraman, S. V., Wang, L., Wong, M., Bruewer, M., Hobert, M., Yun, C. H., Merlin, D., Madara, J. L. (2002) The adenosine 2b receptor is recruited to the plasma membrane and associates with E3KARP and Ezrin upon agonist stimulation. *J. Biol. Chem.* **277**, 33188–33195.

68. Padányi, R., Xiong, Y., Antalffy, G., Lör, K., Pászty, K., Strehler, E. E., and Eneydi, A. (2010) Apical scaffolding protein NHERF2 modulates the...
calization of alternatively spliced plasma membrane Ca\(^{2+}\) pump 2B variants in polarized epithelial cells. *J. Biol. Chem.* **285**, 31704–31712

81. Hwang, J. I., Heo, K., Shin, K. J., Kim, E., Yun, C., Ryu, S. H., Shin, H. S., and Suh, P. G. (2000) Regulation of phospholipase C-β3 activity by Na\(^+\)/H\(^+\) exchanger regulatory factor 2. *J. Biol. Chem.* **275**, 16632–16637

82. Oh, Y.-S., Jo, N. W., Choi, W., Kim, H. S., Seo, S.-W., Kang, K.-O., Hwang, J.-I., Heo, K., Kim, S.-H., Kim, Y.-H., Kim, I.-H., Kim, J. H., Banno, Y., Ryu, S. O., and Suh, P.-G. (2004) NHERF2 specifically interacts with LPA\(_2\) receptor and defines the specificity and efficiency of receptor-mediated phospholipase C-β3 activation. *Mol. Cell. Biol.* **24**, 5069–5079

83. Paquet, M., Asay, M. J., Fam, S. R., Inuzuka, H., Castleberry, A. M., Oller, H., Smith, Y., Yun, C. C., Traynelis, S. F., and Hall, R. A. (2006) The PDZ scaffold NHERF-2 interacts with mGluR5 and regulates receptor activity. *J. Biol. Chem.* **281**, 29949–29961

84. Hryciw, D. H., Ekberg, J., Ferguson, C., Lee, A., Wang, D., Parton, R. G., Pollock, C. A., Yun, C. C., and Poronnik, P. (2006) Regulation of albumin endocytosis by PSD95/Dlg/ZO-1 (PDZ) scaffolds. Interaction of Na\(^+\)/H\(^+\) exchange regulatory factor-2 with CIC-5. *J. Biol. Chem.* **281**, 16068–16077

85. Poulat, F., de Santa Barbara, P., Desclozeaux, M., Soullier, S., Moniot, B., Bonnaud, N., Boizet, B., and Berta, P. (1997) The human testis determining factor SRY binds a nuclear factor containing PDZ protein interaction domains. *J. Biol. Chem.* **272**, 7167–7172

86. Thevenet, L., Albrecht, K. H., Malki, S., Berta, P., Boizet-Bonhoure, B., and Poulat, F. (2005) NHERF2/SIP-1 interacts with mouse SRY via a different mechanism than human SRY. *J. Biol. Chem.* **280**, 38625–38630

87. Lin, S., Yeruva, S., He, P., Singh, A. K., Zhang, H., Chen, M., Lamprechts, G., de Jonge, H. R., Tse, M., Donowitz, M., Hogema, B. M., Chun, J., Seidler, U., and Yun, C. C. (2010) Lysophosphatidic acid stimulates the intestinal brush border Na\(^+\)/H\(^+\) exchanger 3 and fluid absorption via LPA\(_5\) and NHERF2. *Gastroenterology* **138**, 649–658