Extracellular signal-regulated kinase 8 (ERK8) is the most recently identified member of the ERK subfamily of MAPks. Although other members of the ERK subfamily are established regulators of signaling pathways involved in cell growth and/or differentiation, less is known about ERK8. To understand the cellular function of ERK8, a yeast two-hybrid screen of a human lung library was performed to identify binding partners. One binding partner identified was Hic-5 (also known as ARA55), a multiple LIM domain containing protein implicated in focal adhesion signaling and the regulation of specific nuclear receptors, including the androgen receptor and the glucocorticoid receptor (GR). Co-immunoprecipitation experiments in mammalian cells confirmed the interaction between Hic-5 and both ERK8 and its rodent ortholog ERK7. The C-terminal region of ERK8 was not required for the interaction. Although the LIM3 and LIM4 domains of Hic-5 were sufficient and required for this interaction, the specific zinc finger motifs in these domains were not. Transcriptional activation reporter assays revealed that ERK8 can negatively regulate transcriptional co-activation of androgen receptor and GRα by Hic-5 in a kinase-independent manner. Knockdown of endogenous ERK8 in human airway epithelial cells enhanced dexamethasone-stimulated transcriptional activity of endogenous GR. Transcriptional regulation of GRα and interaction with its ligand binding domain by ERK8 were dependent on the presence of Hic-5. These results provide the first physiological function for human ERK8 as a negative regulator of human GRα, acting through Hic-5, and suggest a broader role for ERK8 in the regulation of nuclear receptors beyond estrogen receptor α.

Since the identification of ERK1 and ERK2, additional ERK family members have been identified and extensively studied. ERK1 and ERK2 are classically activated by growth factors and mediate signals leading to proliferation or differentiation of most cell types (1, 2). These founding members of the ERK subfamily of MAPks have a characteristic threonine-glutamine-tyrosine (TEY) motif that requires dual phosphorylation of the threonine and tyrosine residues for full kinase activity. ERK3 is an atypical member of the ERK/MAPK family having an SEG activation motif. Recently, it has been found to interact with and facilitate MAPK-activated protein kinase 5 (MK5) autoactivation (3, 4). This interaction may play a role in embryonic development. These proteins have overlapping expression patterns in the developing mouse embryo, and there is concurrent reduction of ERK3 in an MK5 knock-out mouse model resulting in embryonic lethality (3, 4). ERK4 was identified by immunologic cross-reactivity with an antibody against ERK1 (5). Little is known about ERK4, including its primary sequence (6). ERK5 has a TEY activation motif and was originally identified as an oxidant-activated ERK (7). Subsequently, it has been found to be activated along a growth factor signaling pathway and to play an important role in angiogenesis and T-cell development. ERK6 has a TGY activation motif and is actually a member of the p38 family of MAPks also known as p38γ (8, 9). ERK7 has a TEY activation motif but is also an atypical member of the ERK family in that it displays considerable constitutive kinase activity independent of a known upstream activator (10). ERK8 is the last member of this MAPK subfamily to be identified (11). Although a relatively low amino acid sequence identity of 69% and initial biochemical characterization of human ERK8 suggested that it might be distinct from rodent ERK7, genome-based analyses revealed that these ERKs are orthologs (12, 13). Their genes are in syntenic regions with erk8 located on human 8q24.3 (11) and erk7 located on mouse 15E1. In addition, their gene structures are similar. The implication of their sequence divergence and distinct biochemical profiles, highly unusual for MAPK orthologs, remains unresolved.

ERK8 and ERK7, like ERK3 and ERK5, are significantly larger than the founding ERK family members, ERK1 and ERK2, primarily because of extended C-terminal regions that have important regulatory functions. Previously, overexpression of ERK7 was found to reduce cell growth independent of kinase activity but dependent on its C-terminal region (15). More recently, overexpression of rat ERK7 was found to increase turnover of the human estrogen receptor α (ERα) via a 26 S proteasome pathway in a kinase-dependent manner (16). Reduced levels of a cross-reactive human protein, presumably ERK8, inversely correlated with ERα levels in human breast cancer cell lines and some advanced human breast carcinomas (16) suggesting a potential regulatory role for ERK7 and possibly ERK8 in ERα signaling.

ERα is a member of the nuclear receptor superfamily of transcription factors. Additional members of this family include the androgen receptor (AR), the glucocorticoid receptor α (GRα), the mineralocorticoid receptor, the progesterone receptor, the thyroid receptor, the retinoic acid receptors, and the peroxisome proliferator-activated receptor-γ (PPARγ). Regulation of gene expression by nuclear receptors is initiated by binding of the specific ligand or hormone, formation of either hetero- or homodimers depending on the individual nuclear receptor, and bind-
ERK8 Regulates GR Transactivation

The yeast two-hybrid system was used to identify Hic-5 as a strong ligand-dependent co-activator of a subset of nuclear receptors and interact with focal adhesion kinase through its N-terminal region. Overexpression of Hic-5 in fibroblasts generated a senescent phenotype. Hic-5 was found to localize to focal adhesions and interact with focal adhesion kinase through its N-terminal region. Competition with paxillin for focal adhesion kinase binding was proposed as the mechanism behind the ability of Hic-5 overexpression to reduce integrin-mediated cell spreading. Hic-5 was also found to be a strong ligand-dependent co-activator of a subset of nuclear receptors in addition to AR(20), including GRα, progesterone receptor, mineralocorticoid receptor, and PPARα but not thyroid receptor or ERα (20–22). In general, the co-activation function of Hic-5 is dependent on one or more of its LIM domains, which are cysteine- and/or histidine-rich regions that resemble zinc fingers.

Here we demonstrate that ERK8 interacts with Hic-5 via its C-terminal LIM3 and LIM4 domains. This interaction is required for the ability of ERK8 to down-regulate transcription of GRα in a kinase-independent manner. These results suggest that one potential function of ERK8 is to negatively regulate nuclear receptor transcription through an interaction with Hic-5.

EXPERIMENTAL PROCEDURES

Materials

Dexamethasone, dihydrotestosterone, bovine insulin, anti-FLAG monoclonal antibody (M2), peroxidase-conjugated anti-FLAG monoclonal antibody (M2), peroxidase-conjugated goat anti-rat IgG, peroxidase-conjugated goat anti-rabbit IgG, and peroxidase-conjugated goat anti-mouse IgG, penicillin, streptomycin, trypsin, and pFLAG-CMV2 were purchased from Sigma. Anti-β-actin IgG was purchased from Abcam (Cambridge, MA). High affinity rat monoclonal antibody (3F10) against the hemagglutinin (HA) epitope and peroxidase-conjugated 3F10 were purchased from Roche Applied Science. Mouse monoclonal antibody (HA.11) against the HA epitope was purchased from Covance Research Products, Inc. (Richmond, CA). Anti-ERK8 antibody has been described previously (11). Anti-Hic-5 monoclonal and polyclonal antibodies were purchased from Pharmingen and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Dulbecco’s modified Eagle’s medium, modified Eagle’s medium, RPMI 1640, and Ham’s F-12 were purchased from Mediatech (Herndon, VA). Fetal bovine serum was purchased from Hyclone (Logan, UT). Protein A-Sepharose was purchased from Repligen (Needham, MA). Protein G-Sepharose and the GST purification system were purchased from GE Healthcare. The QuickChange XL site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). Affinity-purified peroxidase-conjugated goat anti-rabbit IgG was purchased from Jackson ImmunoResearch (West Grove, PA). The adult human lung RNA, SMART cDNA library construction kit, Matchmaker yeast two-hybrid system 3, and Advantage-HF 2 PCR kit were purchased from Clontech. Peroxidase-conjugated rat anti-mouse κ light chain IgG, the eukaryotic TA cloning kit, and Lipofectamine 2000 were purchased from Invitrogen. Enhanced chemiluminescence reagents were purchased from PerkinElmer Life Sciences. The dual-luciferase reporter assay system and pRL-SV40 were purchased from Promega (Madison, WI). RNAqueous RNA extraction kit, Hic-5, and control siRNA were purchased from Ambion (Austin, TX). ERK8 and luciferase siRNA were synthesized by Dharmacon RNA Technologies (Lafayette, CO). TransIT LT-1 was purchased from Mirus Bio Corp. (Madison, WI). TaqMan probes and real time PCR reagents were purchased from Applied Biosystems (Foster City, CA). PCR and sequencing primers were purchased from Integrated DNA Technologies (Coralville, IA). Sequencing was performed by the University of Chicago Cancer Research Center sequencing facility.

Methods

Plasmid Construction and Preparation—Generation of pCR3.1 HA-ERK8, pCR3.1 HA-ERK8 K42R, and pcDNA3 HA-ERK7 has been described previously (10, 11, 15). Site-directed mutagenesis was used to generate the pCR3.1 HA-ERK8 ΔTail mutant and pCR3.1 HA-ERK8 260, which contain amino acids 2–355 and 2–260 of ERK8, respectively. RT-PCR was used to generate the human Hic-5 cDNA corresponding to amino acids 2–461 of the Hic-5 isoform (23, 24) using total RNA isolated from 16HBE14o− cells as a template and primers based on a published full-length human Hic-5 sequence consisting of 461 amino acids (25). The Hic-5 cDNA was cloned into the mammalian expression vector pFLAG-CMV2 in-frame with the FLAG epitope tag to generate the pFLAG-Hic-5 construct. Site-directed mutagenesis was used to generate the following pFLAG-Hic-5 mutants: the ΔLIM4 mutant contains amino acids 2–402; the ΔLIM3 mutant contains amino acids 2–345; the ΔLIM3 mutant contains amino acids 2–345 and 402–461; the LIM3 SS mutant has the amino acid substitutions C369S, C372S; the LIM4 SS mutant has the amino acid substitutions H428S, C432S; and the LIM3 SS mutant has the combined the amino acid substitutions present in the LIM3 SS and LIM4 SS mutants. The mammalian GST expression vector pEBG was generously provided by Dr. Anning Lin. PCR was used to clone the Hic-5 LIM3 (amino acids 343–402), LIM4 (amino acids 402–461), or LIM34 (amino acids 343–461) domains in-frame with GST. The full-length GRIP1 construct pSG5-GRIP1, the full-length human glucocorticoid receptor α construct pSG5-hGRα, and the 3xARE-luciferase reporter construct consisting of three ARE consensus sequences upstream of a minimal c-fos promoter and the firefly luciferase cDNA were generously provided by Dr. Richard Hiipakka. The MMTV-luciferase reporter plasmid was generously provided by Dr. Richard G. Pestell and has been described previously (26). The mammalian two-hybrid system plasmids consisting of GAL4, VP16, and the UAS-luciferase reporter plasmid were generously provided by Dr. Ronald Cohen. The GAL4-hGR LBD construct was generated using PCR to clone the cDNA of hGRa representing amino acids 518–778 into the GAL4 vector in-frame with the GAL4 DNA binding region. PCR was used to clone the full-length HA-ERK8 into the VP16 vector in-frame with the VP16 transactivation domain. All constructs were verified by sequencing. Primer sequences are available upon request. Plasmids were prepared by CsCl-ethidium bromide gradient centrifugation or by purification through columns according to the manufacturer’s instructions (Qiagen, Chatsworth, CA).

Yeast Two-hybrid Screen—Two bait constructs were generated by cloning full-length ERK8 kinase-deficient constructs ERK8 K42R and ERK8 AEF, as described previously (11), into the pGBKT7 vector (Clontech) in-frame with the GAL4 DNA binding domain. A cDNA library was generated using the SMART cDNA library construction kit (Clontech) with adult human lung RNA (Clontech) according to the manufacturer’s instructions. The Saccharomyces cerevisiae strains Y187 and AH109 were transformed with each bait plasmid and the library, respectively. Each bait construct was used independently in a two-hybrid screen of the adult human lung cDNA library in the pGADT7 vector by
yeast mating following the Matchmaker two-hybrid system 3 protocol (Clontech). High stringency selection on SD yeast medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-α-gal), but lacking adenine, histidine, leucine, and tryptophan was performed at 30 °C for 7–10 days. Library plasmids were recovered from α-galactosidase-positive clones, transformed by electroporation into Escherichia coli strain DH5α grown in medium containing ampicillin, and identified by sequencing.

**Immunohistochemistry**—Adult human lung tissue was obtained from the Regional Organ Bank of Illinois under a protocol approved by the University of Chicago Institutional Review Board. Tissue was formalin-fixed and embedded in paraffin. Affinity-purified anti-ERK8 antibody was used at a 1:2000 dilution. Staining was visualized by sequential application of a biotinylated secondary, an avidin-biotinylated horse-radish peroxidase complex (Vectastain), a substrate solution (diaminobenzidine), and counterstaining. Peptide competition was performed using a 5 μM final concentration of the peptide used to generate the antibody. The generation and purification of the ERK8 antibody have been described previously (11). These studies were performed by the University of Chicago Immunohistochemistry Core Facility.

**Cell Culture**—Normal human bronchial epithelial cells were purchased from Cambrex (Walkersville, MD) and cultured in Bronchial Epithelial Growth Medium (Cambrex). COS, MCF-7, T-47D, HeLa, NCI-H292, and A549 cells were obtained from American Type Culture Collection. COS and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin). MCF-7 and T-47D cells were cultured in modified Eagle’s medium + Earle’s salts supplemented with 10% FBS, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 0.01 mg/ml bovine insulin, and antibiotics. A549 cells were cultured in Ham’s F-12 containing 10% FBS, 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, and antibiotics. NCI-H292 cells were cultured in RPMI 1640 containing 10% FBS, 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 10 mM HEPES, and 1.0 mM sodium pyruvate. 16HBE14o— and 1HAEo— cells were generously provided by Dr. Dieter Gruenert (27) and cultured in modified Eagle’s medium supplemented with 10% FBS and antibiotics. All cells were maintained at 37 °C in a 95% air, 5% CO2 atmosphere.

**Transient Transfections and Preparation of Cell Extracts**—COS, 16HBE14o—, and MCF-7 cells were transfected using TransIT LT-1 following the manufacturer’s instructions. For a 100-mm plate of cells, a total of 8 μg of plasmid DNA was used with 16 or 24 μl of TransIT LT-1. Cultured cells were washed twice with ice-cold phosphate-buffered saline and lysed with either 1% Triton-based lysis buffer (TLB), as described previously (11), or with RIPA containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 7.5, 40 mM β-glycerophosphate, 100 mM NaCl, 50 mM NaF, 2 mM EDTA, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotonin, 1 μg/ml leupeptin, and 20 mM p-nitrophenyl phosphate. Protein concentration of the cleared supernatant was estimated as described previously (11).

**Western Analysis**—Cell extracts (10–60 μg of protein per lane) were resolved on an 8 or 10% acrylamide separating gel by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane. Membrane blocking, washing, antibody incubation, and detection by enhanced chemiluminescence were performed as described previously (11).

**Immunoprecipitation**—Immunoprecipitation assays were performed using ectopically expressed epitope-tagged proteins from COS, MCF-7, or 16HBE14o— cells as described previously (11). Proteins were isolated by SDS-PAGE on an 8% acrylamide separating gel and subjected to Western analysis.

**GST Fusion Protein Pulldown**—Lysates from transfected COS cells were adjusted to a final concentration of 1 mg/ml and incubated with glutathione-Sepharose at 4 °C for 90 min. The glutathione-Sepharose beads were collected by brief centrifugation and washed extensively with TLB. Isolated proteins were eluted with 1× Laemmli sample buffer, heated at 95 °C for 5 min, and subsequently processed as described above for Western analysis.

**Luciferase Reporter Assay**—Cells were cultured in medium containing 1% charcoal-stripped FBS and transfected with 1.8 μg of pCR3.1, pCR3.1 HA-ERK8, or pCR3.1 HA-ERK8 K42R; 0.9 μg of pFLAG or pFLAG-Hic-5; 0.3 μg of pSG5-hGRα or pcDNA3-hAR; 1.0 μg of MMTV-Luc or 3xARE-Luc; and 0.25 μg of pRL-SV40 per 6-well plate. COS, MCF-7, and 16HBE14o— cells were used at a density of 1 × 105, 2.5 × 105, and 1.5 × 105 cells/well, respectively. After 16–24 h, the cells were stimulated with either the ligand (10 nM DHT or 100 nM dexamethasone) or vehicle (ethanol). After an additional 16–24 h, the cells were lysed using Passive Lysis Buffer (Promega) and analyzed using the dual-luciferase reporter assay system (Promega) according to the manufacturer’s instructions. Measurements were made using a TD-20/20 luminometer (Turner Designs). Triplicate samples were used for each experiment, and the firefly luciferase measurement was normalized to the Renilla luciferase for each sample. The fold increase was determined by comparing the mean normalized luciferase activity of each treatment group with the vector-transfected, vehicle-treated group within each experiment.

**siRNA Knockdown**—HeLa or 16HBE14o— cells were transfected with siRNA at a final concentration of 20 nm using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). A final siRNA concentration ranging from 10 to 100 nM was used when attempting to knock down Hic-5 in 16HBE14o— cells. For the luciferase reporter assay, co-transfection of 16HBE14o— cells with the MMTV-Luc and pRL-SV40 reporter plasmids was performed with the siRNA transfection using Lipofectamine 2000 as described above. The total plasmid amount was maintained using empty vectors. Cells were harvested 48 h following the co-transfections for RNA isolation, Western analysis, or measurement of luciferase activity.

**RNA Isolation, Reverse Transcription, and Real Time PCR**—Two-step, singleplex real time PCR was performed. Total RNA isolation and RT were performed using the RNAqueous-4PCR kit (Ambion) according to the manufacturer’s protocol. 2 μg of RNase-free DNase I-treated total RNA of each sample were used per RT reaction with the oligo(dT) primer and either MMLV-RT or water. The samples were denatured at 80 °C, annealed at 42 °C for 60 min, heat-inactivated at 92 °C for 10 min, and cooled to 4 °C. Real time PCRs were performed on an Applied Biosystems 7300 Real Time PCR System running sequence detection software version 1.2.3. TaqMan probes for ERK8/MAPK15 (Hs00371187_ml) and β-actin (4352935E) were used following the manufacturer’s instructions. Real time PCR of human αENaC and β-actin was performed using primers described previously (28, 29) at a final concentration of 200 nM and SYBR Green Master Mix (Applied Biosystems). Following a single denaturation step for 10 min at 95 °C, 40 cycles of two-step PCR was performed consisting of 15 s at 95 °C and 1 min at 60 °C. For real time PCR using SYBR Green, melting curve analysis was performed to determine specificity of the PCR products. Data were analyzed using the relative quantification RQ Study software using the comparative C,T method. The amount of ERK8 mRNA or αENaC, normalized to β-actin and relative to the no siRNA, vehicle-treated sample.
ERK8 Regulates GR Transactivation

A

FIGURE 1. Expression of endogenous ERK8 and Hic-5. A, immunohistochemical analysis of adult human lung tissue using either affinity-purified anti-ERK8 antibody without (left) or with (right) blocking peptide reveals expression of ERK8 in airway epithelium. B, Western analysis of lysate from cultured, normal human bronchial epithelial cells using either affinity-purified anti-ERK8 antibody with (1st lane) or without (2nd lane) blocking peptide reveals expression of ERK8 (arrow). A nonspecific protein band is seen in both lanes at a molecular weight slightly greater than that of ERK8. C, Western analysis of cell lysates using either an anti-Hic-5 antibody (top panel) or an anti-β-Actin antibody (bottom panel). Hic-5 is easily detectable in all cell types analyzed except the two breast cancer cell lines, MCF-7 and T-47D. β-Actin is detectable in all cell types analyzed. 1st lane, 16HBE140—human bronchial epithelial cells; 2nd lane, 1HAEo—human airway epithelial cells; 3rd lane, normal human bronchial epithelial cells (NHBEC); 4th lane, NCI-H292 human lung mucoepidermoid carcinoma cells; 5th lane, A549 human lung adenocarcinoma cells; 6th lane, HeLa human cervical adenocarcinoma cells; 7th lane, MCF-7 human breast adenocarcinoma cells; and 8th lane, T-47D human breast ductal carcinoma cells.

calibrator, was calculated using the formula $2^{-\Delta\Delta CT}$. For each sample set, the base line and threshold ($C_T$) were selected automatically with visual verification.

GenBank™ Accession Numbers—The GenBank™ accession numbers for human GRs, human AR, rat ERK7, and human ERK8 are U01351, L29496, AF078798 and AY065978, respectively.

RESULTS

Identification of Hic-5 as a Binding Partner—Previously, we found that ERK8 mRNA is expressed in multiple human tissue types with significantly higher levels in lung (11). Endogenous ERK8 can be detected in the airway epithelium of human lung by immunohistochemistry (Fig. 1A) and at extremely low levels in human bronchial epithelial cells such as 16HBE140—cells (11) and normal human bronchial epithelial cells in culture (Fig. 1B). In order to characterize its cellular function, we sought to identify interacting proteins by using two full-length ERK8 kinase-inactive mutants, ERK8 K42R and ERK8 AEF, as bait in a yeast two-hybrid screen of an adult human lung library. Five independent colonies were identified as the C-terminal LIM domains of Hic-5 or ARAH5. The interaction with one of these Hic-5 clones was verified by a yeast remating assay (data not shown).

Screening for the presence of Hic-5 in cultured cells by Western analysis revealed Hic-5 expression in several human airway epithelial cell types but not in two breast cancer cell lines, MCF-7 and T-47D (Fig. 1C). RT-PCR using total RNA isolated from 16HBE140—cells (27) was used to generate a full-length Hic-5 construct that was cloned in-frame with a FLAG tag in a mammalian expression vector. COS cells were co-transfected with an HA-tagged ERK8 construct and the FLAG-tagged Hic-5, as well as their respective control vectors. After immunoprecipitation with the anti-FLAG antibody, the immunoprecipitated proteins were resolved by SDS-PAGE and analyzed by Western blotting with an anti-HA antibody. HA-ERK8 and pFLAG (3rd lane), pCR3.1 and FLAG-Hic-5 (4th lane), or pCR3.1 and pFLAG (5th lane), Lysates were immunoprecipitated (IP) with an anti-FLAG antibody and separated by SDS-PAGE. Samples were analyzed by immunoblotting (WB) with an anti-HA antibody. 1st lane contains whole cell (WC) lysate from cells transfected with HA-ERK8 alone. B, co-immunoprecipitation of endogenous Hic-5 and exogenous ERK8 in 16HBE140—cells. MCF-7 or 16HBE140—cells (HBE) were transfected with either pCR3.1 (V) or HA-ERK8 (EB). Lysates were immunoprecipitated with an anti-HA antibody and separated by SDS-PAGE. Samples were analyzed by immunoblotting with an anti-HA antibody (top panel) or anti-Hic-5 antibody (middle panel). Whole cell lysates from transfected cells were also resolved by SDS-PAGE and immunoblotted with an anti-HA antibody to verify expression of HA-ERK8 (bottom panel). HA-ERK8 interacts with endogenous Hic-5 in 16HBE140—cells (4th lane, top panel). The IgG heavy chain is visible in the anti-HA and anti-Hic-5 Western (top and middle panels). The data shown are representative of at least three experiments.

To determine whether Hic-5 overexpression is required for this interaction, endogenous Hic-5 was immunoprecipitated from 16HBE140—cells with MCF-7 cells transfected with HA-ERK8. Exogenous HA-ERK8 was detected in Hic-5 immunoprecipitates from 16HBE140—cells (Fig. 2B). However, exogenous HA-ERK8 was not detected in immunoprecipitates from similarly transfected MCF-7 cell lysate using the anti-Hic-5 antibody (Fig. 2B), consistent with the lack of significant endogenous Hic-5 expression in these cells (Fig. 1C). These findings indicate that ERK8 specifically interacts with endogenous Hic-5. Interaction between endogenous ERK8 and Hic-5 in 16HBE140—cells could not be detected by a co-immunoprecipitation assay presumably due to the extremely low levels of endogenous ERK8 (data not shown). Together these co-immunoprecipitation results confirm that ERK8 and Hic-5 are interacting proteins in human airway epithelial cells.

ERK8 Interacts with the LIM3 and LIM4 Domains of Hic-5—The C-terminal LIM domains were the minimal region of Hic-5 that bound to ERK8 in the yeast two-hybrid screen. The four LIM domains of Hic-5, which are cysteine and/or histidine-rich regions that resemble double
ERK8 Regulates GR Transactivation

zinc fingers, have been shown to be important for its function as both a focal adhesion protein and a co-regulator of the transcriptional activity of specific nuclear receptors (21, 24, 30). To determine which LIM domains are required for the ERK8-Hic-5 interaction, Hic-5 LIM deletion mutants were generated that lacked either the third, the fourth, or both the third and the fourth LIM domains (Fig. 3A). COS cells were co-transfected with HA-ERK8 and either the full-length FLAG-Hic-5, the Hic-5 LIM deletion mutants, or the control vector. The FLAG-tagged proteins were immunoprecipitated, resolved by SDS-PAGE, and analyzed by Western blotting with an anti-HA antibody (top panel) or anti-FLAG antibody (bottom panel). Duplicate samples using the FLAG-Hic-5 LIM3 mutant are shown. B, co-immunoprecipitation of ERK8 and Hic-5 LIM4 or LIM34 deletion mutants in COS cells. COS cells were co-transfected with HA-ERK8 (E) and either the full-length FLAG-Hic-5 ( ), or FLAG-Hic-5 ΔLIM4 ( ), or FLAG-Hic-5 ΔLIM34 ( ), lysates were immunoprecipitated with an anti-FLAG antibody and separated by SDS-PAGE along with whole cell lysates from transfected cells. Samples were analyzed by immunoblotting with an anti-HA antibody (top panel) or anti-FLAG antibody (bottom panel). C, co-immunoprecipitation of ERK8 and Hic-5 LIM4 or LIM3 deletion mutants in COS cells. COS cells were co-transfected with HA-ERK8 (E) and either the full-length FLAG-Hic-5 ( ), or FLAG-Hic-5 ΔLIM4 ( ), or FLAG-Hic-5 ΔLIM34 ( ), lysates were immunoprecipitated with an anti-FLAG antibody and separated by SDS-PAGE along with whole cell lysates from transfected cells. Samples were analyzed by immunoblotting with an anti-HA antibody (top panel) or anti-FLAG antibody (bottom panel). Duplicate samples using the FLAG-Hic-5 ΔLIM3 mutant are shown. D, expression of Hic-5 LIM domains as GST fusion proteins in COS cells. COS cells were transfected with plasmids encoding either GST-Hic-5 LIM3 ( ), GST-Hic-5 LIM4 ( ), or GST-Hic-5 LIM34 ( ). Cell lysates were separated by SDS-PAGE and immunoblotted with an anti-GST antibody. E, GST-LIM domain pulldown assay. COS cells were co-transfected with either pCR3.1 ( ) or HA-ERK8 (E) and either GST-Hic-5 LIM3 ( ), GST-LIM4 ( ), or GST-LIM34 ( ). GST fusion proteins were isolated from cell lysates using glutathione-agarose beads. Samples were separated by SDS-PAGE along with whole cell lysates from transfected cells and analyzed by immunoblotting with an anti-HA antibody (top panel) or anti-GST antibody (bottom panel). Duplicate samples using the GST-LIM34 construct are shown. The data shown are representative of at least three experiments.

ERK8 Interacts with the LIM3 and LIM4 Domains of Hic-5

A schematic of the Hic-5 LIM deletion mutants is shown. Site-directed mutagenesis of the full-length FLAG-Hic-5 ( ) was used to generate mutants lacking either LIM domain 4 (Δ4), LIM domain 3 (Δ3), or both LIM domains (Δ34). COS cells were transfected with either FLAG-Hic-5, FLAG-Hic-5 ΔLIM3 (Δ3), or FLAG-Hic-5 ΔLIM34 (Δ34). Lysates were separated by SDS-PAGE and immunoblotted with an anti-FLAG antibody (bottom panel). B, co-immunoprecipitation of ERK8 and Hic-5 LIM4 or LIM34 deletion mutants in COS cells. COS cells were co-transfected with HA-ERK8 (E) and either the full-length FLAG-Hic-5 ( ), or FLAG-Hic-5 ΔLIM4 ( ), or FLAG-Hic-5 ΔLIM34 ( ), lysates were immunoprecipitated with an anti-FLAG antibody and separated by SDS-PAGE along with whole cell lysates from transfected cells. Samples were analyzed by immunoblotting with an anti-HA antibody (top panel) or anti-FLAG antibody (bottom panel). C, co-immunoprecipitation of ERK8 and Hic-5 LIM4 or LIM3 deletion mutants in COS cells. COS cells were co-transfected with HA-ERK8 (E) and either the full-length FLAG-Hic-5 ( ), or FLAG-Hic-5 ΔLIM4 ( ), or FLAG-Hic-5 ΔLIM34 ( ), lysates were immunoprecipitated with an anti-FLAG antibody and separated by SDS-PAGE along with whole cell lysates from transfected cells. Samples were analyzed by immunoblotting with an anti-HA antibody (top panel) or anti-FLAG antibody (bottom panel). D, expression of Hic-5 LIM domains as GST fusion proteins in COS cells. COS cells were transfected with plasmids encoding either GST-LIM3 ( ), GST-LIM4 ( ), or GST-LIM34 ( ). Cell lysates were separated by SDS-PAGE and immunoblotted with an anti-GST antibody. E, GST-LIM domain pulldown assay. COS cells were co-transfected with either pCR3.1 ( ) or HA-ERK8 (E) and either GST ( ), GST-LIM3 ( ), GST-LIM4 ( ), or GST-LIM34 ( ). GST fusion proteins were isolated from cell lysates using glutathione-agarose beads. Samples were separated by SDS-PAGE along with whole cell lysates from transfected cells and analyzed by immunoblotting with an anti-HA antibody (top panel) or anti-GST antibody (bottom panel). Duplicate samples using the GST-LIM34 construct are shown. The data shown are representative of at least three experiments.

The Zinc Finger Motifs of the LIM Domains Are Not Required to Interact with ERK8—LIM domains are classically defined by their zinc finger motifs, which appear to facilitate protein-protein interactions (31). In order to test whether these motifs play a role in the interaction between ERK8 and Hic-5, point mutations were used to disrupt the zinc finger motifs within the LIM3 and LIM4 domains (Fig. 4A). Cysteine-
ERK8 Regulates GR Transactivation

**A**

![Diagram of Hic-5 LIM domain mutants](image)

**B**

![Graph showing MMTV-luciferase/Renilla activity](image)

**C**

![Table showing Anti-FLAG IP and Whole Cell Lysates](image)

**D**

![Western blot analysis](image)

**FIGURE 4.** The zinc finger motifs of the LIM domains are not required to interact with ERK8. **A**, schematic of the Hic-5 LIM domain mutants. Site-directed mutagenesis was used to substitute in two serine residues in the third, fourth, or both of these LIM domains in the full-length FLAG-Hic-5. B, glucocorticoid receptor transcriptional activity assay. COS cells were transfected with an MMTV-luciferase reporter plasmid, a Renilla luciferase plasmid, hGRα, and either pFLAG, pFLAG-Hic-5 (Hic-5), pFLAG-Hic-5 LIM3 SS (Hic-5 LIM3 SS), pFLAG-Hic-5 LIM4 SS (Hic-5 LIM4 SS), or pFLAG-Hic-5 LIM34 SS (Hic-5 LIM34 SS). Cells were treated (+) with 100 nM dexamethasone (Dex) or vehicle (−). For each sample, firefly luciferase activity was measured and normalized to measured Renilla luciferase activity. Normalized luciferase activity is presented as relative to the transactivation seen in the absence of FLAG-Hic-5 and dexamethasone (1st bar). Bars represent the mean ± S.E. of three experiments. C, co-immunoprecipitation of ERK8 and Hic-5 LIM4 and LIM34 zinc finger mutants in COS cells. COS cells were co-transfected with either pCR3.1 (V) or HA-ERK8 (E) and either pFLAG (H), FLAG-Hic-5 (Hic-5), FLAG-Hic-5 LIM3 SS (S3), or FLAG-Hic-5 LIM34 SS (S34). Lysates were immunoprecipitated with an anti-FLAG antibody and separated by SDS-PAGE along with whole cell lysates from transfected samples. Samples were analyzed by immunoblotting with an anti-HA antibody (top panel) or anti-FLAG antibody (bottom panel). D, co-immunoprecipitation of ERK8 C-terminal truncation mutants and Hic-5 in COS cells. COS cells were co-transfected with either pCR3.1 (V), HA-ERK8 (E), HA-ERK8 260 (E′), or HA-ERK8 ΔTail (ΔT) and either pFLAG (H), or FLAG-Hic-5 (H). Lysates were immunoprecipitated with an anti-FLAG antibody (left panels) and separated by SDS-PAGE along with whole cell lysates (right panels) from transfected cells. Samples were analyzed by immunoblotting with an anti-HA antibody (top panels) or anti-FLAG antibody (bottom panels). The positions of HA-ERK7, HA-ERK8, HA-ERK8 ΔT, and HA-ERK8 260 are indicated on the right. The data shown are representative of at least three experiments.

**to-serine substitutions of key residues within the LIM domain consensus sequence (CX₅CX₁₆₋₂₃HX₅CX₅) (31) have been shown to disrupt the zinc finger-binding motif (32). The co-activator function of these mutants was evaluated to determine whether the zinc finger motifs were disrupted. COS cells were co-transfected with wild-type Hic-5 or the mutant forms and hGRα with an MMTV-luciferase reporter construct. A Renilla luciferase construct was used to normalize for transfection efficiency. Transfected cells were treated with dexamethasone or vehicle. Lysates were collected and analyzed for firefly and Renilla luciferase activity. As expected, these mutations were sufficient to reduce the ability of Hic-5 to function as a co-activator of hAR (data not shown) and hGRα (Fig. 4B). However, they did not affect the ability of Hic-5 to interact with ERK8 as determined by a co-immunoprecipitation assay (Fig. 4C). ERK8 interacted with Hic-5 even when the zinc finger motifs of the third and fourth LIM domains were disrupted suggesting that features of the LIM3 and LIM4 domains other than the zinc finger motifs are important for the interaction with ERK8.

**The C-terminal Region of ERK8 Is Not Required to Interact with Hic-5**—Previously, the C-terminal region of ERK7, the rodent ortholog of ERK8, was shown to regulate its cellular localization, constitutive kinase activity, and ability to inhibit growth (10, 15). To determine whether the C-terminal region of ERK8 is required for its ability to interact with Hic-5, two ERK8 mutants were used in co-immunoprecipitation experiments with full-length Hic-5 (Fig. 4D). COS cells were co-transfected with FLAG-Hic-5 and either full-length HA-ERK8, the C-terminal deleted mutant (HA-ERK8 ΔT), or a mutant containing only the N-terminal 260 amino acids (HA-ERK8 260). FLAG-Hic-5 was immunoprecipitated from each lysate, resolved by SDS-PAGE, and analyzed by Western blotting with an anti-HA antibody. The overall expression levels of the C-terminal deleted HA-ERK8 mutants were reduced compared with full-length HA-ERK8 which is consistent with our previous finding for C-terminal deleted HA-ERK7 mutants (10). Comparable amounts of the truncated mutants co-immunoprecipitated with full-length Hic-5, however, relative to their overall expression levels. HA-ERK7 co-immunoprecipitated with FLAG-Hic-5 similar to...**
ERK8 Regulates GR Transactivation

FIGURE 5. ERK8 down-regulates transcriptional activity of AR and GR independent of kinase activity but dependent on Hic-5. A, androgen receptor transcriptional activity assay. COS cells were transfected with a 3xARE-luciferase reporter plasmid, a Renilla luciferase plasmid, and either pFLAG or pFLAG-Hic-5 (Hic-5), and either pCR3.1, pCR3.1 HA-ERK8 (ERK8), or pCR3.1 HA-ERK8 K42R (K42R). Cells were treated with 10 nM DHT (+) or vehicle (−). For each sample, firefly luciferase activity was measured and normalized to measured Renilla luciferase activity. Normalized luciferase activity is presented as relative to the transactivation seen in the absence of FLAG-Hic-5 and DHT (1st lane). Bars represent the mean ± S.E. of three experiments. Lysates were separated by SDS-PAGE and analyzed by immunoblotting. A representative anti-HA Western (top panel) and anti-FLAG Western (bottom panel) are shown. The data shown are representative of three experiments. B, glucocorticoid receptor transcriptional activity assay in COS cells. COS cells were transfected with an MMTV-luciferase reporter plasmid, a Renilla luciferase plasmid, hGRα, either pFLAG or pFLAG-Hic-5 (Hic-5) and either pCR3.1, pCR3.1 HA-ERK8 (ERK8), or pCR3.1 HA-ERK8 K42R (K42R). Cells were treated (+) with 100 nM dexamethasone (Dex) or vehicle (−). For each sample, firefly luciferase activity was measured and normalized to measured Renilla luciferase activity. Normalized luciferase activity is presented as relative to the transactivation seen in the absence of FLAG-Hic-5 and dexamethasone (1st lane). Bars represent the mean ± S.E. of three experiments. Lysates were separated by SDS-PAGE and analyzed by immunoblotting. A representative anti-HA Western (top panel) and anti-FLAG Western (bottom panel) are shown. The data shown are representative of three experiments. C, glucocorticoid receptor transcriptional activity assay in 16HBE14o cells. 16HBE14o cells were transfected with an MMTV-luciferase reporter plasmid, a Renilla luciferase plasmid, hGRα, either pFLAG or pFLAG-Hic-5 (Hic-5) and either pCR3.1, pCR3.1 HA-ERK8 (ERK8), or pCR3.1 HA-ERK8 K42R (K42R). Cells were treated (+) with 100 nM dexamethasone (Dex) or vehicle (−). For each sample, firefly luciferase activity was measured and normalized to measured Renilla luciferase activity. Normalized luciferase activity is presented as relative to the transactivation seen in the absence of FLAG-Hic-5 and dexamethasone (1st lane). Bars represent the mean ± S.E. of three experiments. Lysates were separated by SDS-PAGE and analyzed by immunoblotting. A representative anti-HA Western (top panel) and anti-FLAG Western (bottom panel) are shown. The data shown are representative of three experiments. D, glucocorticoid receptor transcriptional activity assay in MCF-7 cells. MCF-7 cells were transfected with an MMTV-luciferase reporter plasmid, a Renilla luciferase plasmid, hGRα, either pFLAG or pFLAG-Hic-5 (Hic-5), and either pCR3.1, pCR3.1 HA-ERK8 (ERK8), or pCR3.1 HA-ERK8 K42R (K42R). Cells were treated (+) with 100 nM dexamethasone (Dex) or vehicle (−). For each sample, firefly luciferase activity was measured and normalized to measured Renilla luciferase activity. Normalized luciferase activity is presented as relative to the transactivation seen in the absence of FLAG-Hic-5 and dexamethasone (1st lane). Bars represent the mean ± S.E. of three experiments. Lysates were separated by SDS-PAGE and analyzed by immunoblotting. A representative anti-HA Western (top panel) and anti-FLAG Western (bottom panel) are shown. The data shown are representative of three experiments.

In summary, the interaction between Hic-5 and ERK8 is dependent on regions containing the third and fourth LIM domains of Hic-5 and a region within the kinase domain of ERK8. It appears, however, that the specific zinc finger motifs defining a LIM domain and the C-terminal region of ERK8 are not necessary for this interaction.
ERK8 Down-regulates Transcriptional Activity of AR and GR—Because Hic-5 is known to co-regulate the transcriptional activity of a subset of specific nuclear receptors (20–22), it is possible that ERK8 also plays a role in the transcriptional regulation of these nuclear receptors. To explore this possibility, we initially determined the effect of ERK8 on transcriptional co-activation of two of the nuclear receptors regulated by Hic-5, hAR and hGR. Expression of HA-ERK8 or its kinase-deficient mutant in COS cells reduced the transcriptional activity of exogenous hAR and hGRα in response to dihydrotestosterone (DHT) or dexamethasone, respectively (Fig. 5, A and B). Western analysis of the lysates revealed that expression of HA-ERK8, the kinase-deficient HA-ERK8 mutant, and FLAG-Hic-5 was comparable between transfection groups (Fig. 5, A and B, bottom panels). In addition, the kinase-independent suppression of hAR and hGRα transcriptional activity was seen even in the absence of exogenous Hic-5 (Fig. 5B, and data not shown). The ability of ERK8 to down-regulate the transcriptional activity of

**FIGURE 6. Knockdown of endogenous ERK8 in 16HBE14o—cells increases transcriptional activity of endogenous GR.** A, glucocorticoid receptor transcriptional activity assay in 16HBE14o—cells. 16HBE14o—cells were transfected with an MMTV-luciferase reporter plasmid, a Renilla luciferase plasmid, either no siRNA, control siRNA, or ERK8 siRNA. Cells were treated (+) with 100 nM dexamethasone (Dex) or vehicle (−). For each sample, firefly luciferase activity was measured and normalized to measured Renilla luciferase activity. Normalized luciferase activity is presented as relative to the transactivation seen in the absence of siRNA and dexamethasone (1st lane). Bars represent the mean ± S.E. of three experiments. B, knockdown of stably expressed HA-ERK8 in HeLa cells. HeLa cells stably expressing HA-ERK8 (2nd to 4th lanes) following retroviral transduction were transfected without siRNA (2nd lane) or with ERK8 siRNA (3rd lane) or luciferase siRNA (4th lane). Cell lysates, including nontransduced HeLa cells (1st lane), were separated by SDS-PAGE and analyzed by immunoblotting with an anti-HA antibody (top panel) or anti-β-actin antibody (bottom panel). The data shown are representative of three experiments. C, glucocorticoid receptor transcriptional activity assay following knockdown of endogenous ERK8 mRNA in 16HBE14o—cells. 16HBE14o—cells were transfected without siRNA, with control siRNA, or with ERK8 siRNA and treated (+) with 100 nM dexamethasone (Dex) or vehicle (−). Total RNA was extracted and two-step, singleplex real time PCR was performed using ERK8 and β-actin TaqMan probes. Relative quantification was performed using β-actin as the endogenous control and calibrated to the no siRNA, vehicle-treated sample (1st lane). Bars represent the mean ± S.E. of three experiments. D, expression of βENaC mRNA following knockdown of endogenous ERK8 mRNA in 16HBE14o—cells. 16HBE14o—cells were transfected without siRNA, with control siRNA, or with ERK8 siRNA and treated (+) with 100 nM dexamethasone (Dex) or vehicle (−) for 6 h. Total RNA was extracted, and two-step, singleplex real time PCR was performed using specific primers for βENaC and β-actin. Relative quantification was performed using β-actin as the endogenous control and calibrated to the no siRNA, vehicle-treated sample (1st lane). Bars represent the mean ± S.E. of three experiments.
ERK8 Regulates GR Transactivation

First, siRNA constructs were screened by their ability to reduce levels of stably expressed HA-ERK8 in HeLa cells (Fig. 6B). Second, the ability of the siRNA construct to reduce endogenous ERK8 mRNA in 16HBE14o− cells was determined by real time PCR (Fig. 6C). The enhancement of endogenous GR transcriptional activity with ERK8 knockdown is consistent with the ability of overexpressed ERK8 to inhibit GRα transcriptional activity.

To further substantiate the physiologic role of ERK8 in the transcriptional regulation of GR, expression of the glucocorticoid-regulated \( \alpha \) subunit of the epithelial sodium channel (\( \alpha \)ENaC) (34) was evaluated by real time PCR following knockdown of endogenous ERK8 in 16HBE14o− cells. Following 6 h of dexamethasone stimulation, a 1.6-fold increase in \( \alpha \)ENaC mRNA was seen in cells pretreated with ERK8 siRNA compared with cells pretreated with control or no siRNA (Fig. 6D). The additional increase in glucocorticoid-stimulated \( \alpha \)ENaC message seen with ERK8 knockdown is consistent with the increase seen using the MMTV reporter system. Together these data indicate that one physiologic function of ERK8 is to regulate GR transcriptional activity in airway epithelial cells.

ERK8 Interacts with the Ligand Binding Domain of GRα in the Presence of Hic-5 and Hormone Stimulation—Because both hGRα and ERK8 interact with Hic-5, it is possible that ERK8 also interacts directly with hGRα to down-regulate transcription. To test whether ERK8 and hGRα can physically associate, a mammalian two-hybrid system was used. Full-length ERK8 was fused to the activation domain of VP16, and the ligand binding domain (LBD) of hGRα was fused to the DNA binding domain of GAL4. A luciferase reporter construct containing the GAL4 upstream activating sequence (UAS) linked to a minimal promoter was used to determine whether the VP16 fusion protein and GAL4 fusion protein interact in cells. In COS cells transfected with GAL4-hGRα LBD alone, dexamethasone stimulation causes an increase in luciferase activity due to homodimerization and stimulation of the transactivation domain contained in the C-terminal region of hGRα (Fig. 7A). Dexamethasone-stimulated luciferase activity was increased further in the presence of the VP16-ERK8 fusion protein indicating that ERK8 and the LBD of hGRα interact. However, no significant increase in luciferase activity was seen with the identical transfection group in MCF-7 cells that lack significant amounts of Hic-5. On the other hand, increased dexamethasone-stimulated luciferase activity was seen in the presence of co-transfected GRIP1, a known binding partner

FIGURE 7. Binding of ERK8 to the ligand binding domain of hGRα. A, mammalian two-hybrid binding assay in COS cells. COS cells were transfected with a UAS-luciferase reporter plasmid, Renilla luciferase plasmid, either the empty GAL4 vector or GAL4-hGRα LBD, and either VP16 or VP16-HA-ERK8. Cells were treated (+) with either dexamethasone (Dex) or vehicle (−). For each sample, firefly luciferase activity was measured and normalized to measured Renilla luciferase activity. Normalized luciferase activity is presented as relative to the transactivation seen in the absence of GAL4-hGRα LBD, VP16-HA-ERK8, and dexamethasone (1st lane). Bars represent the mean ± S.E. of three experiments. B, mammalian two-hybrid binding assay in MCF-7 cells. MCF-7 cells were transfected with a UAS-luciferase reporter plasmid, Renilla luciferase plasmid, either the empty GAL4 vector or GAL4-hGRα LBD, and either VP16, VP16-HA-ERK8 or GRIP1. Cells were treated (+) with either dexamethasone (Dex) or vehicle (−). For each sample, firefly luciferase activity was measured and normalized to measured Renilla luciferase activity. Normalized luciferase activity is presented as relative to the transactivation seen in the absence of GAL4-hGRα LBD, VP16-HA-ERK8, GRIP1, and dexamethasone (1st lane). Bars represent the mean ± S.E. of three experiments.
and co-activator of hGRα (35) (Fig. 7B). Together, these data suggest that Hic-5 is required for formation of a transcriptional complex between ERK8 and hGRα enabling ERK8 to function as a negative regulator of hGRα transactivation.

**DISCUSSION**

We cloned ERK7 and ERK8 as the last identified members of the ERK subfamily of MAPKs containing a TEY activation motif, but a physiologic role has not been identified to date. In this study we identify the nuclear receptor co-activator Hic-5 as a binding partner of ERK8. This interaction involves both the LIM3 and LIM4 domains of Hic-5 and the kinase domain of ERK8. Functionally, interaction with Hic-5 mediates the ability of ERK8 to down-regulate transcriptional activity of AR and GR in a kinase-independent manner through formation of a transcriptional complex. Knockdown of endogenous ERK8 in human airway epithelial cells using siRNA increased glucocorticoid-stimulated transcriptional activity indicating that ERK8 is a physiologic regulator of GR.

The low level of endogenous protein expression and the unique nature of these ERK orthologs have, perhaps, hindered the identification of their physiologic function until now. Initially, certain aspects of ERK8 suggested that it might not be the human ortholog of rodent ERK7 (11). First, in the context of other ERK family members the shared amino acid identity between ERK7 and ERK8 is remarkably low. Rat ERK1 and ERK2 share 97 and 99% amino acid sequence identity, respectively. Even ERK5 shares an overall amino acid identity of 92% between mouse and human species. ERK7 and ERK8, by contrast, are ~70% identical and diverge even more within their C-terminal domain. Second, ERK7 and ERK8 have distinct biochemical profiles. Human ERK8 is activated along an Src-dependent signaling pathway, whereas rodent ERK7 displays a high degree of constitutive kinase activity in the absence of upstream activators. In addition, ERK8 and ERK7 do not share the same substrate profile, an unusual trait for orthologous protein kinases. Nonetheless, the subsequent availability of genomic data for humans and mice revealed that ERK8 and ERK7 are orthologs that share syntenic genes and gene structures. The explanation for their sequence divergence and distinct biochemical profiles remains enigmatic. These differences also suggest that despite being orthologs, ERK8 and ERK7 may not be functionally interchangeable under all conditions.

Prior information regarding the functional roles of ERK8 and ERK7 has been based on overexpression studies. Previously, we found that exogenous ERK7 can suppress DNA synthesis independent of kinase activity but dependent on its C-terminal region (15). In a more recent study, overexpression of rat ERK7 increased turnover of the human nuclear receptor ERα in a hormone- and kinase-dependent manner through the 26 S proteosome pathway (16). In addition, an inverse correlation between a protein recognized by an antibody generated against rat ERK7 and ERα levels was seen in human breast cancer cell lines and a limited number of advanced human breast carcinoma samples (16). The authors concluded that ERK7 specifically regulates ERα turnover because increased turnover was not seen with 1α,25-dihydroxycholecalciferol, another protein degraded by the 26S proteosome pathway, or other related nuclear receptors, steroiodogenic factor 1, and hAR. Although these studies reveal a possible role for ERK7 and/or ERK8 in the regulation of ERα, given the distinct nature of these ERK orthologs, these results need to be confirmed under physiologic conditions using ERK8 to evaluate human ERα regulation.

The mechanism we have elucidated for ERK8 regulation of AR and GR is distinct from the interaction of ERK7 and/or ERK8 with ERα. Although Hic-5 interacts with both ERK8 and ERK7, it is unlikely that it also participates in a kinase-dependent turnover of ERα. Our results indicate a kinase-independent mechanism is responsible for the transcriptional regulation of AR and GR. Transactivation reporter assays show that ERK8 and its kinase-deficient mutant can down-regulate transcriptional activity of AR and GR in the presence and absence of exogenous Hic-5. In addition, although Hic-5 is a strong co-activator of several nuclear receptors, it is not a significant co-activator of ERα (21). The presence of significant amounts of endogenous Hic-5 in COS (Ref. 33 and data not shown) and 16HBE14o− cells (Fig. 1C) could explain the down-regulation seen in these cells in the absence of exogenous Hic-5 (Fig. 5, B and C). This possibility is supported by the transactivation data in MCF-7 cells that express little or no Hic-5. Unlike in COS and 16HBE14o− cells, ERK8 did not affect the transcriptional activity of hGRα in MCF-7 cells unless exogenous Hic-5 was present (Fig. 5D). Together these findings suggest that Hic-5 mediates the negative regulation of hGRα transcriptional activity by ERK8. The physiologic implications of this transcriptional regulation is seen when endogenous ERK8 is knocked down in human airway cells using specific siRNA. Loss of ERK8 results in a nearly 2-fold increase in dexamethasone-stimulated transcription of an MMTV-driven reporter system and an additional 60% increase in dexamethasone-stimulated αENaC message indicating that ERK8 normally suppresses GR transactivation. Therefore, our findings suggest a broader role for ERK8, and possibly ERK7, in the regulation of nuclear receptors.

The finding that ERK8 can regulate GR through an interaction with Hic-5 is not without precedent. Proline-rich tyrosine kinase-2, Pyk2, a member of the focal adhesion kinase family, is another Hic-5 interacting protein shown previously to regulate transcriptional activity of associated nuclear receptors (33). Pyk2 is able to down-regulate AR transactivation in a kinase-dependent manner by phosphorylating Hic-5 on tyrosine 43. Although not clearly defined, the proposed mechanism is prevention or destabilization of the interaction between Hic-5 and AR following phosphorylation by Pyk2 because wild-type Pyk2, but not a kinase-deficient mutant, blocked the interaction between Hic-5 and AR. In the case of ERK8, however, phosphorylation of Hic-5 does not appear to play a significant role because both the wild-type and kinase-deficient ERK8 mutants have similar effects. It also seems unlikely that the interaction between ERK8 and Hic-5 destabilizes the interaction between Hic-5 and the target nuclear receptor. Our mammalian two-hybrid binding data in COS cells compared with MCF-7 cells suggest that ERK8 only interacts with hGRα in the presence of Hic-5. These findings indicate that the mechanism behind the down-regulation of hGRα and/or AR transcriptional activity by ERK8 differs from that of Pyk2. Rather than phosphorylation-mediated transcriptional complex destabilization, our results suggest a mechanism involving complex formation.

Formation of a complex is consistent with the ability of LIM domain containing proteins to facilitate protein-protein interactions (31). In the case of Hic-5 and GRα, LIM3 and LIM4 have been shown previously to be required for maximal binding of Hic-5 to GRα using a yeast two-hybrid system (30). These LIM domains alone or together with the N-terminal region of Hic-5, however, were not sufficient for GRα binding indicating that the other LIM domains contribute to the binding (30). In the case of Hic-5 and ERK8, both the LIM3 and LIM4 domains are required for maximal binding. Surprisingly, the zinc finger motifs, which essentially define these domains, are not required for this interaction. Unlike in the case of GRα binding, either domain alone is sufficient for binding of ERK8. These slightly different binding requirements imply that the interface between Hic-5 and GRα differs from that of Hic-5 and ERK8 raising the possibility that these three proteins form a complex. Although we did not specifically
ERK8 Regulates GR Transactivation

ERK8 and Hic-5 have a similar role in the differentiation of airway epithelium acting through nuclear receptors.

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