Interaction of Integrin-linked Kinase with the Kidney Chloride/Bicarbonate Exchanger, kAE1*§

Received for publication, March 12, 2007, and in revised form, May 31, 2007. Published, JBC Papers in Press, June 6, 2007, DOI 10.1074/jbc.M702139200

Thitima Keskanokwong†5‡, Haley J. Shandro†‡, Danielle E. Johnson§2, Saranya Kittanakom§4, Gonzalo L. Vilas§3, Paul Thorner***+, Reinhart A. F. Reithmeier§, Varaporn Akkarapatumwong§5, Pa-thai Yenchitsomunan§4, and Joseph R. Casey‡5

From the †Membrane Protein Research Group, Department of Physiology and Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada, the ¶Institute of Molecular Biology and Genetics, Mahidol University, Salaya Campus, Nakhon Pathom 73170, Thailand, the ‡Department of Research and Development, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand, the §Department of Biochemistry, University of Toronto, Toronto, Ontario M5S 1A8, Canada, the **Division of Pathology, Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada, and the ¶¶Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario M5G 1L5, Canada

Kidney anion exchanger 1 (kAE1) mediates chloride/bicarbonate exchange at the basolateral membrane of kidney α-intercalated cells, thereby facilitating bicarbonate reabsorption into the blood. Human kAE1 lacks the N-terminal 65 residues of the erythroid form (AE1, band 3), which are essential for binding of cytoskeletal and cytosolic proteins. Yeast two-hybrid screening identified integrin-linked kinase (ILK), a serine/threonine kinase, and an actin-binding protein as an interacting partner with the N-terminal domain of kAE1. Interaction between kAE1 and ILK was confirmed in co-expression experiments in HEK 293 cells and is mediated by a previously unidentified calponin homology domain in the kAE1 N-terminal region. The calponin homology domain of kAE1 binds the C-terminal catalytic domain of ILK to enhance association of kAE1 with the actin cytoskeleton. Overexpression of ILK increased kAE1 levels at the cell surface as shown by flow cytometry, cell surface biotinylation, and anion transport activity assays. Pulse-chase experiments revealed that ILK associates with kAE1 early in biosynthesis, likely in the endoplasmic reticulum. ILK co-localized with kAE1 at the basolateral membrane of polarized Madin-Darby canine kidney cells and in α-intercalated cells of human kidneys. Taken together these results suggest that ILK and kAE1 traffic together from the endoplasmic reticulum to the basolateral membrane. ILK may provide a linkage between kAE1 and the underlying actin cytoskeleton to stabilize kAE1 at the basolateral membrane, resulting in higher levels of cell surface expression.

* This work was supported in part by Canadian Institutes of Health Research operating grants (to J. R. C. and R. A. F. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3.]
† Supported by a scholarship award from the Royal Golden Jubilee-Ph.D. Program of the Thailand Research Fund.
‡ Supported by a Strategic Training Grant from the Canadian Institutes of Health Research.
§ Supported by a postdoctoral fellowship from Alberta Heritage Foundation for Medical Research.
¶ Supported by Thailand Research Fund Grant BRG4880007.
†5 Scientist of the Alberta Heritage Foundation for Medical Research. To whom correspondence should be addressed. Tel.: 780-492-7203; Fax: 780-492-8915; E-mail: joe.casey@ualberta.ca.

Anion exchanger 1 (AE1,§ band 3, SLC4A1) is a bicarbonate transporter involved in maintaining acid-base homeostasis in the human body (1). There are two human forms of AE1, erythroid (eAE1) and kidney (kAE1). eAE1 or band 3, is the major integral membrane glycoprotein of the erythrocyte, where it serves dual roles of Cl−/HCO3− exchange and cytoskeletal anchorage to red cell membranes (2). kAE1 is the basolateral Cl−/HCO3− exchanger of the acid-secreting α-intercalated cell of the kidney distal tubule (3). Transcription of eAE1 in erythropoiesis is under the control of an erythroid-specific promoter upstream of exon 1, whereas renal transcription arises from a distinct promoter within intron 3 of the AE1 gene (4). Thus, the resultant kidney transcript encodes the kAE1 polypeptide lacking 65 amino acids present at the N terminus of human eAE1 (5). This structural alteration causes major functional differences between eAE1 and kAE1. The N terminus of eAE1 interacts with many proteins, including ankyrin, proteins 4.1, and glycolytic enzymes (3, 6), whereas the N terminus of kAE1 does not bind to these proteins (7, 8).

The three-dimensional structure of the N-terminal 43-kDa cytoplasmic domain of eAE1 revealed a globular structure, composed of 11 β-strands and 10 α-helical segments arranged as an N-terminal interaction domain and a C-terminal dimerization domain (2), but no structure is available for kAE1. Residues 58–68 of eAE1 form the first β-strand in the cytoplasmic domain. Loss of a central strand of β-sheet in kAE1 may thus greatly alter the globular structure of the cytosolic domain, thereby altering its protein interactions. Furthermore, the identity of kAE1-binding protein(s) in α-intercalated cells remains unknown. Previously a protein called kanadaptin (kidney anion exchanger adaptator protein) was reported to interact with mouse N-terminal kAE1 but not to eAE1 (9). Human kanadaptin does not, however, interact with human kAE1 and localizes predominantly to the nucleus (10). This leads to the question:

§ The abbreviations used are: AE1, anion exchanger 1; kAE1, kidney AE1; eAE1, erythroid AE1; CH, calponin homology; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; ILK, integrin-linked kinase; HA, hemagglutinin; BSA, bovine serum albumin; PBS, phosphate-buffered saline; MDCK, Madin-Darby canine kidney cells; dRTA, distal renal tubular acidosis; PH, pleckstrin homology.
what proteins interact with the N-terminal cytoplasmic domain of kAE1 in human kidney cells?

Mutations of the AE1 gene can cause distal renal tubular acidosis (dRTA), the failure of acid excretion in α-intercalated cells (11, 12). To date, AE1 mutations associated with autosomal dominant dRTA are mis-sense mutations encoding residues S89 (R589H, R589S, and R589C), G609R, S613F, A888L/D889X, and an 11-amino acid deletion at the C terminus (R901X). AE1 mutations linked to autosomal recessive dRTA are V488M, G701D, and ΔV850. These mutations cause dRTA either by preventing the movement of mutant and normal proteins to the cell surface (impaired trafficking), causing misfolding of the protein, or sending the mutant proteins to the apical membrane instead of the correct basolateral membrane of epithelial cells (mis-targeting), which results in impaired bicarbonate movement across the basolateral membrane (13). In addition, no mutation in the N-terminal domain of kAE1 has been reported to be associated with dRTA. Deletion of the N terminus of kAE1 and C-terminal R901 has been reported to be associated with dRTA. Deletion of the N terminus of kAE1 and C-terminal R901 resulted in the apical mis-localization of the proteins in Madin-Darby canine kidney cells, type I (MDCKI), suggesting that a determinant within the kAE1 N terminus cooperates with the C terminus for kAE1 basolateral localization (14). Chicken kidney AE1–4 contains a tyrosine-based basolateral localization signal in the N-terminal region (15). Beyond the observations that some mutations of kAE1 cause defects in trafficking to the basolateral membrane, the precise mechanism by which kAE1 is delivered to the basolateral membrane of distal renal tubule cells and is retained there still has not been defined. To understand kAE1 transport, targeting, and regulation, it will be necessary to identify protein(s) that interact with kAE1.

In this study, we used yeast two-hybrid screening of a human kidney cDNA library to identify proteins that interact with the cytoplasmic domain of kAE1. We found that integrin-linked kinase (ILK), a serine/threonine kinase and actin-binding protein, interacts with the N-terminal domain of kAE1. The specific interaction between the two proteins was confirmed by co-immunoprecipitation and affinity co-purification of these proteins co-expressed in transfected human embryonic kidney (HEK 293) cells. The region in the N-terminal cytoplasmic domain of kAE1 that interacts with ILK was also examined. Co-expression of ILK resulted in increased functional expression of kAE1 at the cell surface. ILK interacts with kAE1 early in biosynthesis suggesting that the two proteins traffic together from the endoplasmic reticulum to the cell surface. At the basolateral membrane, ILK may act to link kAE1 to the underlying cytoskeleton, thereby stabilizing kAE1 at the cell surface to increase steady-state level plasma membrane levels of this transport protein.

**EXPERIMENTAL PROCEDURES**

*Yeast Two-hybrid Screening*—The N terminus of human kidney anion exchanger 1 (NkAE1) was used to screen for interacting proteins expressed from a human kidney library using the yeast two-hybrid assay. A cDNA fragment corresponding to amino acids Met66–Pro903 of AE1 was amplified by PCR using primers containing Ncol and SalI restriction sites. Primers used were as follows: 5′-CATGCCATGGACGAAAAGAAC-3′ and 5′-CGCGTCGACTTAGGGGCTGAATGCA-3′. PCR products were inserted in the GAL4-binding domain (GAL4-BD) vector, pGBK7T (Clontech), at the corresponding sites and used as bait (pNkAE1). The resulting constructs were confirmed by automated DNA sequencing. Expression of the bait construct was verified by immunoblotting using GAL4 DNA-BD monoclonal antibody according to the manufacturer’s protocols (Clontech). The bait was transformed into yeast strain AH109 (Clontech) using the lithium acetate method (16). Yeast strain Y187, pre-transformed with a human kidney cDNA library fused to the GAL4 activation domain (GAL4-AD) in the pACT2 vector (Clontech), was mated with the AH109 strain that was pre-transformed with the bait construct. About 1 x 10^8 independent clones were screened on high stringency selective medium lacking adenine, leucine, tryptophan, and histidine (SD/-Ade, -Leu, -Trp, and -His). The tested plates were incubated for 3–20 days at 30 °C. Positive colonies were further screened by α-galactosidase (MEL1 reporter) activity. This MEL1 gene product is actively expressed and secreted to the culture medium when GAL4-BD binds to the MEL upstream-activating sequences (MEL1 UAS) in AH109 strain (18). Isolated positive library clones were re-transformed into strain Y187 and tested for specific interaction with the bait, GAL4-BD vector, human lamin C, and p53 pre-transformed into strain AH109, as described above. cDNA inserts of true positive clones were sequenced and submitted to the data base. A cDNA encoding human integrin-linked kinase (ILK) was identified.

**Plasmid Constructs**—Human kAE1 full-length cDNA was cloned into HindIII and Xhol site of pcDNA 3.1 (Invitrogen) by PCR-based amplification using primers 5′-CCCAAGCTTTAGGACGAAAAGAACGAGGAG-3′ and 5′-CCGCTCGAGT-TAAGCGTAACTCTGGAAAACATGTGGTACACAGGCATTGGCCAC TTC-3′ (underlined sequence represents the hemagglutinin (HA) epitope sequences). The HA tag was introduced at the C terminus. This wild-type kAE1 clone was named pkAE1. cDNAs encoding calponin homology domain (CH) identified in the N terminus of kAE1 with amino acids 27–189 was amplified using pkAE1 as template with specific primers. The PCR fragment with HindIII and Xhol sites at the ends was inserted into pcDNA 3.1 with HA tagged at the C terminus. This construct was named pChkAE1. Human kidney cDNA (a generous gift of Dr. Wanna Thongnoppakhun, Mahidol University) was used as template for amplification of full-length ILK, using primers 5′-CCGGAATTCGAGGACACATTTTCATC-3′ and 5′-CCGCTCGAGTACTTGTCCTGCAT CTCC-3′. The PCR product was cloned into the EcoRI and Xhol sites of pcDNA 3.1/His B vector (Invitrogen), yielding the His-tagged construct pILK. The plasmid pNkILK, containing the coding sequence for ILK with a deletion of amino acids 1–192 at the N terminus, was constructed by PCR-based amplification using pILK as template. The parental mutant clones (generous gift of Dr. Shoukat Dedhar, University of British Columbia) were used as template to make ILK mutants, S343A ILK and E359K ILK, expressing the kinase-dead domain. The PCR fragments of ILK mutants were digested and inserted into EcoRI and Xhol sites of pcDNA 3.1/His B. All constructs were confirmed by automated DNA sequencing.
Cell Culture and Transfection—Plasmid DNA for transfections was purified with the EndoFree™ plasmid kit (Qiagen). Human embryonic kidney (HEK 293) cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% (v/v) fetal bovine serum, 5% (v/v) calf serum, and 1% (v/v) penicillin/streptomycin (Invitrogen) in 5% CO₂ at 37 °C. Cells were plated 4 – 8 h before transfection at a density of 10⁶ per 60-mm dish or ~ 25% confluency. HEK 293 cells were transiently transfected with 1.6 μg of pkAE1 (or pCHkAE1) and 2.0 μg of pILK (or ILK mutants) in combination with empty vector to a total of 3.6 μg of DNA, using the calcium phosphate precipitation method (17).

SDS-PAGE and Immunoblotting—Proteins were electrophoresed on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membrane (18, 19). Immunoblots were probed with either 1:1000 diluted anti-HA polyclonal antibody (Santa Cruz Biotechnology), anti-ILK rabbit polyclonal antibody (Sigma), anti-ILK mouse monoclonal (Upstate), mouse anti-actin monoclonal antibody (Sigma), anti-ILK monoclonal anti-paxillin (BD Transduction Laboratories), or rabbit polyclonal anti-actopaxin antibody (Sigma).

Co-immunoprecipitation—Cells were either co-transfected with HA-tagged kAE1 and His-tagged ILK or kAE1 alone. The transfected cells were washed twice with 5 ml of phosphate-buffered saline (PBS) (150 mM NaCl, 3 mM KCl, 6.5 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) and allowed to detach in 2 ml of PBS for 10 min at room temperature. Cells were collected and lysed with 500 μl of IPB buffer (1 mM EDTA, 0.5% (v/v) Igepal (Nonidet P-40 detergent), 150 mM NaCl, 0.2% (w/v) bovine serum albumin, 10 mM Tris-HCl, pH 7.5, and protease inhibitors) on ice for 15 min. Immunoprecipitations were performed as described previously (18, 19). Immunoprecipitates were analyzed by SDS-PAGE and immunoblotting (18, 19).

Affinity Co-purification—HEK 293 cells co-expressing kAE1 and His-tagged ILK or expressing kAE1 alone were collected and lysed in 1 ml of IPB buffer. Supernatants were incubated with 200 μl of Co³⁺ chelate resin (BD Biosciences) for 16 h at 4 °C with rotation. Resin was collected by centrifugation at 900 × g for 5 min and washed three times with 1× equilibration buffer (150 mM NaCl, 150 mM sodium phosphate buffer, pH 7.0). Bound proteins were eluted with the same buffer, containing 1.5 M imidazole. Eluates were collected, mixed with sample loading buffer, and heated at 65 °C for 5 min. Samples were subjected to SDS-PAGE and immunoblotting.

Triton X-100 Cytoskeleton Extraction—HEK 293 cells co-transfected with kAE1 and ILK or kAE1 alone were washed three times with ice-cold PBS and incubated with 400 μl of Triton X-100 extraction buffer (0.5% (v/v) Triton X-100, 100 mM NaCl, 3 mM MgCl₂, 0.1 mM diethioctetanol, 25 mM KCl, 1.8 mM CaCl₂, 10 mM Tris-HCl, pH 7.5, and protease inhibitors) on ice for 15 min. Cells were collected and sedimented by ultracentrifugation at 30,000 × g for 10 min at 4 °C. The supernatant (detergent-soluble fraction) was taken for immunoblotting. The pellet (detergent-insoluble fraction) was dissolved in 40 μl of SDS buffer (1% (w/v) SDS, 2 mM EDTA, 10 mM Tris-HCl, pH 7.5, and protease inhibitors). IPB buffer (360 μl) was then added to the suspension, and samples were incubated on ice for 15 min. Particulate material was separated by centrifugation at 30,000 × g for 10 min to obtain the supernatant, which now contained insoluble proteins. Soluble and insoluble fractions were resolved on SDS-PAGE and immunoblotting, using anti-HA antibody for kAE1 detection.

Pulse-Chase Assays—The procedure was described previously (20). Briefly, HEK 293 cells were transiently co-transfected with His-tagged ILK and kAE1. After 24 h of growth the cells were radiolabeled for 1 h and were chased before harvesting in lysis buffer (1% CHAPS and 5 mM imidazole) at each time point. The radiolabeled protein was subjected to purification using nickel-nitrilotriacetic acid resin, eluted, and desalted using protein desalting spin columns (Pierce). Immunoprecipitation of associated kAE1 was carried out with 4 μl of rabbit anti-Ct AE1 antibody and followed by protein G-Sepharose. His-tagged ILK was detected using a mouse anti-His₅ antibody. Proteins were resolved by 8% SDS-PAGE and detected by autoradiography.

Cell Surface Biotinylation—Transfected HEK 293 cells were washed with 5 ml of ice-cold PBS and borate buffer (154 mM NaCl, 7.2 mM KCl, 1.8 mM CaCl₂, 10 mM boric acid, pH 9.0). Cells were treated with 4 ml of 0.5 mg/ml Sulfo-NHS-SS-Biotin (Pierce) in 4 °C borate buffer and incubated on ice for 30 min. Unreacted reagent was then quenched by rinsing the cells three times with quenching buffer (192 mM glycine, 25 mM Tris, pH 8.3). Cells were collected and lysed with 500 μl of IPB, on ice for 15 min. Insoluble material was sedimented by centrifugation. Half of the lysate was set aside for immunoblotting (total fraction). The remainder was incubated with ImmunoPure (Pierce) immobilized streptavidin (100 μl) for 12–16 h at 4 °C with rotation to bind the biotinylated proteins. The resin was collected by centrifugation at 7,500 × g, 5 min. The supernatant (unbound fraction) was taken for immunoblotting. The streptavidin resin was washed three times with washing buffer as described above. Sample loading buffer containing 2% (w/v) 2-mercaptoethanol was added to the resin (bound fraction) and heated at 65 °C for 10 min. Samples were analyzed by SDS-PAGE and immunoblotting using anti-HA antibody for kAE1 detection. The blot was stripped and incubated with mouse anti-actin antibody to normalize the protein expression in each fraction.

Measurement of Cell Surface kAE1 Expression by Flow Cytometry—Transiently transfected HEK 293 cells expressing kAE1 HA tagged at position 557 with or without His-tagged ILK were trypsinized for 2 min at 4 °C, centrifuged to collect the cells, and resuspended in Hanks’ balanced salt solution (Invitrogen) with 1% BSA (HBSSB). The cells were incubated with 1:1,000 dilution of mouse anti-HA antibody (Covance Inc.) in HBSSB for 15 min, and then the cells were stained with goat anti-mouse Alexa 488 (1:1,000) (Molecular Probes, Eugene, OR) for 15 min on ice. Samples were washed three times and analyzed using Beckman-Coulter EPICS Elite (BD Biosciences), and the percentage of fluorescence-stained cells was determined to quantify the level of the cell surface expression.

Viral Infection and Expression of kAE1 in MDCK Cells—For virus production, HEK 293 cells, grown in 25-cm² flasks, were co-transfected with 1.5 μg each of the three retroviral plasmids pVpack-GP, pVpack-VSVG, and pFBNeo-kAE1 HA tagged at...
ILK and kAE1 Processing

residue 557 using FuGENE 6 transfection reagent (Roche Diagnostics). Virus supernatant was collected after 2 days of transfection and filtered through 0.45-μm filters to remove all cell debris before use.

Expression of kAE1 was carried out by adding virus supernatant to 30–50% confluent MDCK cells in the presence of 8 μg/ml Polybrene (Sigma). Infected MDCK cells were selected with 1 mg/ml geneticin G418 (Sigma). Polarized MDCK cells were grown on semi-permeable Transwell polycarbonate filters (Corning Glass) for 4–5 days.

Immunofluorescence—Nonpolarized or polarized MDCK cells expressing kAE1 HA557 were grown on glass coverslips or semi-permeable Transwell polycarbonate filters for 4–5 days after confluence. The cells were fixed with 3.7% paraformaldehyde for 10 min, washed with 100 mM glycine, and permeabilized in 0.1% Triton X-100 for 15 min. After blocking nonspecific binding with 1% BSA, the cells were incubated with a mixture of 1:1,000 dilution of a rabbit polyclonal anti-Nt kAE1 antibody and a 1:1,000 dilution of a mouse monoclonal anti-ILK antibody (Upstate Biotechnology, Lake Placid, NY) in PBS, containing 1% BSA for 30 min at room temperature. After several washes, the cells were incubated with a 1:1,000 dilution of Cy3-conjugated anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA) and goat anti-mouse Alexa 488 (1:1,000) (Molecular Probes, Eugene, OR) for 30 min at room temperature.

Human kidney samples were formalin-fixed, and paraffin-embedded tissue sections (5 μm thick) were then mounted on positively charged microscope slides. Tissue sections were baked 12–16 h at 60 °C, dewaxed in xylene, and hydrated in distilled water through decreasing concentrations of alcohol. Paraffin sections were treated with heat-induced epitope retrieval using 0.01 M citrate buffer, pH 6.0, prior to immunostaining. After blocking with 5% BSA, 1:1,000 dilution of rabbit polyclonal anti-Ct AE1 antibody and 1:200 dilution of mouse monoclonal anti-ILK antibody were used for staining of kAE1 and ILK, respectively. Samples were washed with PBS and mounted before observation, using a Zeiss deconvolution fluorescence microscope.

Chloride/Bicarbonate Exchange Assays—Anion exchange assays were performed on transfected HEK 293 cells, using established procedures (21, 22). Two days post-transfection, cells were rinsed with serum-free Dulbecco’s modified Eagle’s medium (Invitrogen) and incubated in 4 ml of serum-free Dulbecco’s modified Eagle’s medium containing 2 μM 2’7’-bis(carboxyethyl)-5,6-carboxyfluorescein-acetoxymethyl ester (37 °C, 15 min). Coverslips were mounted in a fluorescence cuvette and perfused at 3.5 ml/min alternately with Ringer’s buffer (5 mM glucose, 5 mM potassium gluconate, 1 mM calcium gluconate, 1 mM MgSO4, 2.5 mM NaH2PO4, 25 mM NaHCO3, 10 mM HEPES, pH 7.4), containing either 140 mM sodium chloride buffer or 140 mM sodium gluconate (chloride-free buffer). Both buffers were bubbled continuously with air, containing 5% carbon dioxide. Intracellular pH was monitored by measuring fluorescence changes at excitation wavelengths 440 and 502 nm and emission 520 nm, in a Photon Technologies International RCR/Delta Scan spectrofluorometer. Intracellular pH was calibrated, using the nigericin-high potassium method (23), with three pH values between 6.5 and 7.5. Transport rates were determined by linear regression of the initial linear rate of change of pH, using Kaleidagraph software.

kAE1 and ILK Fractionation on Sucrose Gradients—Mice were anesthetized and perfused through the right ventricle with PBS at 37 °C. Kidneys were rapidly dissected and after removal of the renal capsules homogenized at 4 °C in 5 ml of Homogenization buffer (0.32 M sucrose, 0.1 mM EDTA, 1 mM EGTA, 10 mM HEPES, pH 7.5) containing Complete protease inhibitors (Roche Diagnostics) with a Polytron homogenizer (Kinematica GMBH, Switzerland). The resulting homogenate was centrifuged twice at 700 × g for 5 min at 4 °C and the supernatant recovered. Supernatant was centrifuged at 100,000 × g for 1 h at 4 °C. The resulting pellet was incubated in 2 ml of Solubilization buffer (2% Triton X-100, 5 mM EDTA, 0.15 M NaCl, 10 mM Tris-HCl, pH 7.5), containing complete protease inhibitors for 1 h at 4 °C with gentle agitation. After solubilization, an aliquot was taken (input), and the remaining sample was layered on top of a 10-m 5–30% linear sucrose gradient, containing 0.1% (v/v) Triton X-100, 5 mM EDTA, 0.15 M NaCl, 10 mM Tris-HCl, pH 7.5, and Complete protease inhibitors. The gradient was centrifuged at 178,300 × g for 16–18 h at 4 °C in a Beckman SW41 Ti rotor. Six 2-ml fractions were collected from the top of the tube at the end of the run. The presence of kAE1 and ILK in the gradient and input fractions was analyzed by SDS-PAGE and immunoblotting.

RESULTS

Integrin-linked Kinase (ILK) Interacts with kAE1—To identify candidate proteins that interact with kAE1, a yeast two-hybrid screen was performed using the N-terminal cytoplasmic domain of kAE1, NkAE1 (amino acids 66–403 of human eAE1), as bait. The bait was transformed into yeast strain AH109, and expression of GAL4 DNA-BD/NkAE1 fusion protein was verified by immunoblotting using anti-GAL4 DNA-BD monoclonal antibody (data not shown). The AH109 pre-transformed with pNkAE1 bait was used to screen a human kidney library (Clontech) fused to the GAL4-AD vector, pACT2, in Y187 strain using mating assay. Approximately 1 × 106 independent clones were screened on high stringency selective medium, lacking adenine, leucine, tryptophan, and histidine (SD/-Ade, -Leu, -Trp, and -His). Positive blue colonies were further screened using the α-galactosidase (MEL1 reporter) activity. This screening yielded several potential candidate genes that were HIS+, ADE+, and MEL+. To confirm the initial screen, a second binary test was used to test the specificity of candidates from screening with NkAE1 in yeast. cDNAs of candidates were isolated from the library and then re-transformed into the Y187 strain. The re-transformed Y187 yeast were mated with AH109 yeast pre-transformed with the original bait pNkAE1, GAL4-BD vector, and unrelated cDNAs (lamin C and p53). True positive clones were identified on selective medium as indicated by blue colonies (Table 1). The known interaction between p53 and SV40 large T antigen was used as a positive control. No interaction occurred when mating either NkAE1 or GAL4-BD with SV40 large T antigen, which was used as negative control.
Candidate cDNAs were recovered from yeast and then sequenced using primers corresponding to sequences in the vector, flanking the cDNA insertion site. Resulting sequences were identified via genome data base and BLAST searches. ILK (accession number BC001554.1) was identified and chosen for further study because the expression pattern and function of ILK suggested that it might play a role in regulation of kAE1 function and cellular targeting.

**Physical Interaction of kAE1 and ILK in HEK 293 Cells**—To facilitate detection, kAE1 was tagged with an HA epitope at its C terminus, whereas ILK was His<sub>6</sub>-tagged at the N terminus. Expression of the constructs in HEK 293 cells showed robust protein expression at a molecular mass of 96 kDa for kAE1 using the anti-HA antibody (Fig. 1A). The anti-His antibody detected two nonspecific bands in mock-transfected HEK cells and a unique 51-kDa band in ILK-transfected cells. A rabbit polyclonal anti-ILK antibody detected two major bands at ~50 kDa in mock- and ILK-transfected cells. Because the anti-His antibody detected only the lower band (Fig. 1B), we can assign the lower band as ILK and the upper band as a nonspecific band. Comparing the amount of endogenous ILK and transfected ILK over four different transfections, using a mouse monoclonal anti-ILK antibody, we found that the His-tagged ILK was expressed more than 10-fold higher than endogenous ILK (supplemental Fig. 1).

Co-immunoprecipitation experiments were performed using HEK 293 cells either co-expressing HA-tagged kAE1 and ILK or kAE1 alone to confirm the kAE1/ILK interaction identified in the yeast two-hybrid screen. His-tagged ILK was immunoprecipitated from the cell lysate using the anti-His monoclonal antibody, and any associated kAE1 was detected using an anti-HA antibody. kAE1 co-immunoprecipitation was dependent on expression of ILK, indicating the formation of an ILK-kAE1 complex (Fig. 1B, lane 2). In the negative control lane, a faint band of kAE1 was detected, resulting from nonspecific immunoprecipitation by the anti-His antibody (Fig. 1C, lane 3). To verify further the specificity of the protein interaction, the reciprocal isolation, using HEK 293 cells either co-expressing HA-tagged kAE1 and ILK mutants (Fig. 2).

**TABLE 1**

| GAL4-BD fusion | GAL4-AD fusion | Growth on SD/−Trp/−Leu/−His/−Ade | α-Galactosidase activity |
|----------------|----------------|----------------------------------|-------------------------|
| NkAE1          | ILK            | +                                | +                       |
| NkAE1          | SV40 large T antigen | −                  | −                       |
| GAL4-BD       | ILK            | −                                | −                       |
| GAL4-BD       | SV40 large T antigen | −                  | −                       |
| p53            | ILK            | −                                | −                       |
| p53            | SV40 large T antigen | −                  | −                       |
| Lamin C        | ILK            | −                                | −                       |

**FIGURE 1. Interaction of ILK and kAE1 in transfected HEK cells.** A, HEK 293 cells transfected with either HA-tagged kAE1 or His-tagged ILK were lysed with SDS-PAGE sample loading buffer and subjected to SDS-PAGE and immunoblotting. Blots were probed with anti-HA for kAE1 and anti-His or polyclonal anti-ILK antibody. kAE1 was also detected using the anti-His antibody (Fig. 1C, lane 3). These data suggest that ILK and kAE1 can exist in a complex when co-expressed in HEK cells.
ILK and kAE1 Processing

![Diagram of domain structure of human ILK includes ankyn repeats (ANK) and PH at the N terminus (amino acids 1–192) and the C-terminal kinase activity domain (residues 193–452). ∆NtILK is a construct with amino acids 1–192 deleted. S343A and E559K are kinase-inactive point mutants of ILK (locations of mutations indicated by asterisks). B, the interaction of HA-tagged kAE1 with His-tagged ILK mutants was examined by co-immunoprecipitation assays. HEK 293 cells were transfected with kAE1 and the indicated ILK constructs. Cell lysates were prepared and directly analyzed (lanes 1, 3, and 5) or subjected to immunoprecipitation (IP) with anti-His antibody (lanes 2, 4, and 6). Cell lysate expressing kAE1 but not ILK mutants was also subjected to immunoprecipitation with anti-His antibody (lane 7). Samples were immunoblotted and probed with anti-HA antibody (to detect HA-kAE1) or anti-ILK antibody, as indicated. Data are representative of three experiments.

![Identification of the kAE1-binding region in ILK. A, diagram of domain structure of human ILK includes ankyn repeats (ANK) and PH at the N terminus (amino acids 1–192) and the C-terminal kinase activity domain (residues 193–452). ∆NtILK is a construct with amino acids 1–192 deleted. S343A and E559K are kinase-inactive point mutants of ILK (locations of mutations indicated by asterisks). B, the interaction of HA-tagged kAE1 with His-tagged ILK mutants was examined by co-immunoprecipitation assays. HEK 293 cells were transfected with kAE1 and the indicated ILK constructs. Cell lysates were prepared and directly analyzed (lanes 1, 3, and 5) or subjected to immunoprecipitation (IP) with anti-His antibody (lanes 2, 4, and 6). Cell lysate expressing kAE1 but not ILK mutants was also subjected to immunoprecipitation with anti-His antibody (lane 7). Samples were immunoblotted and probed with anti-HA antibody (to detect HA-kAE1) or anti-ILK antibody, as indicated. Data are representative of three experiments.

Incubated with anti-His antibody to precipitate His-tagged ∆NtILK. ∆NtILK bound kAE1 efficiently (Fig. 2B, lane 2), indicating that the C-terminal catalytic domain of ILK is sufficient for kAE1 binding. The upper band (Fig. 2B, lane 1) was nonspecific binding of protein to anti-ILK antibody in cell lysate, but this band was not seen in the immunoprecipitate (lane 2).

The role of ILK kinase activity in ILK biological function is unclear. In at least two cases an active ILK is needed for ILK interaction with another protein; loss of kinase activity of ILK in the S343A mutant compromised ILK interaction with 3-phosphoinositide-dependent kinase (24, 25). Similarly, the E559K ILK kinase-null mutant has impaired binding to paxillin and focal adhesion targets (26). To examine the role of ILK kinase activity in kAE1 binding, we examined binding of kAE1 and the S343A and E559K ILK mutants. Both inactive ILK mutants retained their ability to bind kAE1 (Fig. 2B, lanes 4 and 6). The bands at the position corresponding to a molecular mass smaller than 51 kDa and higher than 29 kDa at the bottom are nonspecific (Fig. 2B, lanes 3 and 5). These bands disappeared in immunoprecipitates (Fig. 2B, lanes 4 and 6). These results show that the C terminus of ILK is sufficient to bind kAE1, and kinase activity of ILK is not necessary for the interaction.

CH Domain in kAE1 Forms a Binding Site for ILK—The C-terminal domain of ILK contains binding sites for β-integrins and the cytoplasmic adaptor proteins, actopaxin (α-par-

In ILK and kAE1 Processing, the authors investigate the interaction between the ILK and kAE1 proteins. They use various experimental techniques, including co-immunoprecipitation assays and immunoblotting, to demonstrate that ILK interacts with kAE1 independently of its kinase activity. The authors also explore the role of the C-terminal domain of ILK in mediating this interaction, showing that it is sufficient for binding to kAE1. This work contributes to our understanding of how these proteins interact and could have implications for the study of cellular adhesion and signaling pathways.
when normalized to the amount of kAE1 at the cell surface when expressed alone, ILK increased the cell surface level of kAE1 by 56% (Fig. 5B). Cytosolic yellow fluorescent protein in transfected cells was used as a control for the spurious biotinylation of cytosolic protein. Yellow fluorescent protein was biotinylated to an extremely low level, indicating that the cell surface biotinylation protocol identified cell surface protein only. Actin was used as an internal control to normalize protein expression.

The effect of ILK on the cell surface expression of kAE1 in HEK 293 cells was also examined by flow cytometry. To reveal the kAE1 at the cell surface, the HA epitope was inserted into the third extracellular loop (position 557) of kAE1 (35). Cells transfected with empty pcDNA vector displayed only low levels of background staining with the anti-HA antibody. HEK 293 cells expressing kAE1 HA557 at their cell surface represented an average of 20 ± 4% (n = 5) of the total cell population, showing heterogeneous levels of staining above the background staining of control pcDNA3 (0.6 ± 0.2%, n = 5). Co-expression of kAE1 HA557 with ILK exhibited an increase in the number of kAE1 HA557-positive cells, to 32 ± 6% (n = 5). These results confirm that overexpression of ILK increased the level of cell surface expression of kAE1 in transfected HEK 293 cells.

Effect of ILK on Cl⁻/HCO₃⁻ Exchange Activity of kAE1—The increase of kAE1 cell surface expression induced by ILK suggested that ILK could alter the level of Cl⁻/HCO₃⁻ exchange activity of ILK/kAE1-expressing cells. In these assays transfected cells were alternately exposed to Cl⁻-containing and Cl⁻-free buffers, to establish transmembrane [Cl⁻] gradients, facilitating kAE1-mediated Cl⁻/HCO₃⁻ exchange. Cl⁻/HCO₃⁻ exchange activity was examined by following changes of intracellular pH associated with AE1-mediated HCO₃⁻ movement (Fig. 6). Transport rates were determined by linear regression of the rate of pHᵢ change in the first 30 s of alkalinization following removal of extracellular chloride. Cells transfected with vector alone displayed a low level of apparent transport activity (Fig. 6). The increase of kAE1 cell surface expression induced by ILK suggested that ILK could alter the level of Cl⁻/HCO₃⁻ exchange activity of ILK/kAE1-expressing cells. In these assays transfected cells were alternately exposed to Cl⁻-containing and Cl⁻-free buffers, to establish transmembrane [Cl⁻] gradients, facilitating kAE1-mediated Cl⁻/HCO₃⁻ exchange. Cl⁻/HCO₃⁻ exchange activity was examined by following changes of intracellular pH associated with AE1-mediated HCO₃⁻ movement (Fig. 6). Transport rates were determined by linear regression of the rate of pHᵢ change in the first 30 s of alkalinization following removal of extracellular chloride. Cells transfected with vector alone displayed a low level of apparent transport activity (Fig. 6).
ILK and kAE1 Processing

FIGURE 4. Identification of the ILK-binding region in kAE1. HEK 293 cells were transiently transfected with HA-tagged kAE1 calponin homology domain (amino acids 27–189 of kAE1) alone or co-transfected with His-tagged ILK. Cell lysates were either directly analyzed (lane 1) or immunoprecipitated (IP) with anti-His antibody (lanes 2 and 3). Blots were probed with anti-HA to detect the CH or with anti-ILK antibody as indicated. Data are representative of three experiments.

6C), which was subtracted from the rate of kAE1-transfected cells during analysis (Fig. 6D). Initial resting pH values of the cell samples were sufficiently similar (7.23 ± 0.01 for kAE1 alone and 7.12 ± 0.06 for kAE1 plus ILK) that pH-dependent changes of buffer capacity of transport activity did not influence the measured rates of transport. From the raw data (Fig. 6, A and B), it is evident that ILK increased the rate of pH change following change of [Cl] in the medium, consistent with a dramatic effect on the Cl⁻/HCO₃⁻ exchange rate. Indeed, analysis revealed that ILK increased kAE1 transport activity by 82 ± 28% (Fig. 6D). The possibility that the effect of ILK was through effects on proteins endogenous to HEK cells was also examined. Cells transfected with ILK alone were subjected to anion exchange assays. We found that the rate of pH change associated with switching from chloride-containing to chloride-free Ringer’s buffer was indistinguishable in ILK and sham-transfected cells (7.23 ± 0.01 for kAE1 alone and 7.12 ± 0.06 for kAE1 plus ILK) that pH-dependent changes of transport activity did not influence the measured rates of transport. From the raw data (Fig. 6, A and B), it is evident that ILK increased the rate of pH change following change of [Cl⁻] in the medium, consistent with a dramatic effect on the Cl⁻/HCO₃⁻ exchange rate. Indeed, analysis revealed that ILK increased kAE1 transport activity by 82 ± 28% (Fig. 6D). The possibility that the effect of ILK was through effects on proteins endogenous to HEK cells was also examined. Cells transfected with ILK alone were subjected to anion exchange assays. We found that the rate of pH change associated with switching from chloride-containing to chloride-free Ringer’s buffer was indistinguishable in ILK and sham-transfected cells (7.23 ± 0.01 for kAE1 alone and 7.12 ± 0.06 for kAE1 plus ILK) that pH-dependent changes of transport activity did not influence the measured rates of transport. From the raw data (Fig. 6, A and B), it is evident that ILK increased the rate of pH change following change of [Cl⁻] in the medium, consistent with a dramatic effect on the Cl⁻/HCO₃⁻ exchange rate. Indeed, analysis revealed that ILK increased kAE1 transport activity by 82 ± 28% (Fig. 6D). The possibility that the effect of ILK was through effects on proteins endogenous to HEK cells was also examined. Cells transfected with ILK alone were subjected to anion exchange assays. We found that the rate of pH change associated with switching from chloride-containing to chloride-free Ringer’s buffer was indistinguishable in ILK and sham-transfected cells (7.23 ± 0.01 for kAE1 alone and 7.12 ± 0.06 for kAE1 plus ILK) that pH-dependent changes of transport activity did not influence the measured rates of transport. From the raw data (Fig. 6, A and B), it is evident that ILK increased the rate of pH change following change of [Cl⁻] in the medium, consistent with a dramatic effect on the Cl⁻/HCO₃⁻ exchange rate. Indeed, analysis revealed that ILK increased kAE1 transport activity by 82 ± 28% (Fig. 6D). The possibility that the effect of ILK was through effects on proteins endogenous to HEK cells was also examined. Cells transfected with ILK alone were subjected to anion exchange assays. We found that the rate of pH change associated with switching from chloride-containing to chloride-free Ringer’s buffer was indistinguishable in ILK and sham-transfected cells (7.23 ± 0.01 for kAE1 alone and 7.12 ± 0.06 for kAE1 plus ILK) that pH-dependent changes of transport activity did not influence the measured rates of transport. From the raw data (Fig. 6, A and B), it is evident that ILK increased the rate of pH change following change of [Cl⁻] in the medium, consistent with a dramatic effect on the Cl⁻/HCO₃⁻ exchange rate. Indeed, analysis revealed that ILK increased kAE1 transport activity by 82 ± 28% (Fig. 6D). The possibility that the effect of ILK was through effects on proteins endogenous to HEK cells was also examined. Cells transfected with ILK alone were subjected to anion exchange assays. We found that the rate of pH change associated with switching from chloride-containing to chloride-free Ringer’s buffer was indistinguishable in ILK and sham-transfected cells (7.23 ± 0.01 for kAE1 alone and 7.12 ± 0.06 for kAE1 plus ILK) that pH-dependent changes of transport activity did not influence the measured rates of transport. From the raw data (Fig. 6, A and B), it is evident that ILK increased the rate of pH change following change of [Cl⁻] in the medium, consistent with a dramatic effect on the Cl⁻/HCO₃⁻ exchange rate. Indeed, analysis revealed that ILK increased kAE1 transport activity by 82 ± 28% (Fig. 6D). The possibility that the effect of ILK was through effects on proteins endogenous to HEK cells was also examined. Cells transfected with ILK alone were subjected to anion exchange assays. We found that the rate of pH change associated with switching from chloride-containing to chloride-free Ringer’s buffer was indistinguishable in ILK and sham-transfected cells (7.23 ± 0.01 for kAE1 alone and 7.12 ± 0.06 for kAE1 plus ILK) that pH-dependent changes of transport activity did not influence the measured rates of transport. From the raw data (Fig. 6, A and B), it is evident that ILK increased the rate of pH change following change of [Cl⁻] in the medium, consistent with a dramatic effect on the Cl⁻/HCO₃⁻ exchange rate. Indeed, analysis revealed that ILK increased kAE1 transport activity by 82 ± 28% (Fig. 6D). The possibility that the effect of ILK was through effects on proteins endogenous to HEK cells was also examined. Cells transfected with ILK alone were subjected to anion exchange assays. We found that the rate of pH change associated with switching from chloride-containing to chloride-free Ringer’s buffer was indistinguishable in ILK and sham-transfected cells (7.23 ± 0.01 for kAE1 alone and 7.12 ± 0.06 for kAE1 plus ILK) that pH-dependent changes of transport activity did not influence the measured rates of transport. From the raw data (Fig. 6, A and B), it is evident that ILK increased the rate of pH change following change of [Cl⁻] in the medium, consistent with a dramatic effect on the Cl⁻/HCO₃⁻ exchange rate. Indeed, analysis revealed that ILK increased kAE1 transport activity by 82 ± 28% (Fig. 6D). The possibility that the effect of ILK was through effects on proteins endogenous to HEK cells was also examined. Cells transfected with ILK alone were subjected to anion exchange assays. We found that the rate of pH change associated with switching from chloride-containing to chloride-free Ringer’s buffer was indistinguishable in ILK and sham-transfected cells (7.23 ± 0.01 for kAE1 alone and 7.12 ± 0.06 for kAE1 plus ILK) that pH-dependent changes of transport activity did not influence the measured rates of transport. From the raw data (Fig. 6, A and B), it is evident that ILK increased the rate of pH change following change of [Cl⁻] in the medium, consistent with a dramatic effect on the Cl⁻/HCO₃⁻ exchange rate. Indeed, analysis revealed that ILK increased kAE1 transport activity by 82 ± 28% (Fig. 6D). The possibility that the effect of ILK was through effects on proteins endogenous to HEK cells was also examined. Cells transfected with ILK alone were subjected to anion exchange assays. We found that the rate of pH change associated with switching from chloride-containing to chloride-free Ringer’s buffer was indistinguishable in ILK and sham-transfected cells (7.23 ± 0.01 for kAE1 alone and 7.12 ± 0.06 for kAE1 plus ILK) that pH-dependent changes of transport activity did not influence the measured rates of transport. From the raw data (Fig. 6, A and B), it is evident that ILK increased the rate of pH change following change of [Cl⁻] in the medium, consistent with a dramatic effect on the Cl⁻/HCO₃⁻ exchange rate. Indeed, analysis revealed that ILK increased kAE1 transport activity by 82 ± 28% (Fig. 6D). The possibility that the effect of ILK was through effects on proteins endogenous to HEK cells was also examined. Cells transfected with ILK alone were subjected to anion exchange assays. We found that the rate of pH change associated with switching from chloride-containing to chloride-free Ringer’s buffer was indistinguishable in ILK and sham-transfected cells (7.23 ± 0.01 for kAE1 alone and 7.12 ± 0.06 for kAE1 plus ILK) that pH-dependent changes of transport activity did not influence the measured rates of transport. From the raw data (Fig. 6, A and B), it is evident that ILK increased the rate of pH change following change of [Cl⁻] in the medium, consistent with a dramatic effect on the Cl⁻/HCO₃⁻ exchange rate. Indeed, analysis revealed that ILK increased kAE1 transport activity by 82 ± 28% (Fig. 6D). The possibility that the effect of ILK was through effects on proteins endogenous to HEK cells was also examined.
polyclonal anti-Ct AE1 antibody to immunoprecipitate kAE1 and with mouse monoclonal anti-ILK to immunoprecipitate ILK. Autoradiographs revealed the interaction of ILK and kAE1 in HEK 293 cells was detected immediately after the 1-h pulse (Fig. 7B). Turnover of kAE1 and ILK in the complex was similar; the kAE1 and a fraction of the His-tagged ILK complex persisted even after the 24-h chase period.

Association of kAE1 with Actin Complex—We found that kAE1 interacts with ILK through the CH domain of kAE1 (Figs. 3 and 4). Because CH domains act as binding sites in intermediaries in complexes with the actin cytoskeleton (27, 29), this led us to test whether ILK promotes interaction of kAE1 with the cytoskeleton. We prepared lysates from cells transfected with kAE1 plus His-tagged ILK and kAE1 alone, and we incubated these with Co2+/H18528 or sham (vector alone) (C, D). Mean anion exchange rate of kAE1. Values are expressed relative to transport rate of cells transiently transfected with kAE1 cDNA alone. Transport rates (n = 3) were corrected for the background activity of HEK 293 cells. *, p < 0.05; unpaired t test. E, HEK 293 cells were harvested from dishes that contained the coverslips used for anion exchange assays. Cell lysates were subjected to SDS-PAGE on 10% acrylamide gels, and transferred to polyvinylidene difluoride membrane. kAE1 was detected with monoclonal anti-AE1 antibody (IVF12).

FIGURE 6. Anion exchange activity of kAE1 in the presence or absence of ILK. Transiently transfected HEK 293 cells were grown on coverslips and then loaded with the pH-sensitive dye 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein-acetoxymethyl ester 2 days post-transfection. Cells were perfused in a fluorescence cuvette alternately with Ringer's buffer containing 140 mM NaCl and L-[35S]methionine-acetoxymethyl ester for 2 days post-transfection. Cells were perfused in 140 mM sodium glucuronate (black bar) or 140 mM sodium gluconate (white bar), as indicated above each panel. Intracellular pH was monitored as detailed under “Experimental Procedures.” Cells were transfected with cDNA encoding kAE1 (A), kAE1 and ILK (B), or sham (vector alone) (C, D), mean anion exchange rate of kAE1. Values are expressed relative to transport rate of cells transiently transfected with kAE1 cDNA alone. Transport rates (n = 3) were corrected for the background activity of HEK 293 cells. *, p < 0.05; unpaired t test. E, HEK 293 cells were harvested from dishes that contained the coverslips used for anion exchange assays. Cell lysates were subjected to SDS-PAGE on 10% acrylamide gels, and transferred to polyvinylidene difluoride membrane. kAE1 was detected with monoclonal anti-AE1 antibody (IVF12).

were eluted from the Co2+/H18528 resin with 1.5 M imidazole. Immunoblot revealed that kAE1 and ILK associated, as expected. The blot was then stripped and re-probed with anti-paxillin and anti-actopaxin antibodies. These immunoblots revealed that endogenous paxillin and actopaxin associated with the complex of ILK-kAE1 (Fig. 8, lane 2). Paxillin or actopaxin were not detected when kAE1 was expressed without ILK (Fig. 8, lane 3). This suggests that kAE1 interacts with the actin cytoskeleton through a complex containing ILK, paxillin, and actopaxin.

Triton X-100 Insoluble Cytoskeleton Extraction—To explore the role of ILK in connecting kAE1 to actin cytoskeleton, the effect of nonionic detergent extraction on solubility of kAE1 was assessed. Treatment of cells with nonionic detergents, such as Triton X-100, solubilizes membrane proteins, while leaving the F-actin cytoskeleton and its associated proteins relatively intact. Cells transfected with kAE1 plus ILK or kAE1 alone were thus treated with extraction buffer containing 0.5% (v/v) Triton X-100. The distribution of kAE1 in insoluble and soluble fractions was examined on immunoblots probed with anti-HA antibody to detect HA-tagged kAE1. The amount of kAE1 in Triton X-100 insoluble fraction significantly increased in the
ILK and kAE1 Processing

FIGURE 8. Co-association of kAE1 with actin complex. HEK 293 cells were transiently transfected with HA-tagged kAE1 alone or co-transfected with His-tagged ILK, as indicated. Cell lysates prepared in detergent were either set aside for later analysis (lane 1) or incubated with Co²⁺ affinity resin at 4 °C (lane 2). Resin was washed and subsequently eluted with imidazole buffer. Samples were subjected to SDS-PAGE and immunoblotting. Blots were probed with anti-HA and anti-His antibodies to detect kAE1 and ILK, respectively and with anti-paxillin and anti-actopaxin antibodies, as indicated. Data are representative of three experiments.

Co-localization of ILK and kAE1—The subcellular location of kAE1 HA557 and endogenous ILK was examined in nonpolarized and polarized MDCK cells. In nonpolarized MDCK (and LLC-PK1 cells) expressing kAE1 HA557, endogenous ILK was primarily dispersed throughout the cell, but it was also found at the plasma membrane of the cells where it was co-stained with kAE1 HA557 (Fig. 10A). Confocal images of stably transfected MDCK cells (Fig. 10B) showed that kAE1 HA557 was at the basolateral membrane, as shown previously (35), whereas endogenous ILK was primarily intracellular. Some overlapping staining between endogenous ILK with kAE1 HA557 was evident at the basolateral membrane, showing that a fraction of ILK co-localized with kAE1 at the basolateral membrane of polarized MDCK cells. Control experiments in which kAE1 HA557-expressing cells were treated with only the fluorescent secondary antibodies revealed a very low level of diffuse non-specific signal (supplemental Fig. 2), indicating that the signals present in Fig. 10 result from kAE1 and ILK.

Co-localization of kAE1 with ILK was also examined in sections of human kidney (Fig. 11). In these experiments kAE1 was localized with a rabbit polyclonal antibody that recognizes the C terminus of AE1, a region shared by both erythroid and kidney variants of AE1. In this regard the small elliptical shapes marked by red staining represent abundant AE1 (band 3) in the erythrocyte membrane and serve as a positive control. In the kidney AE1 localizes exclusively to the basolateral surface of α-intercalated cells (37). In the two sections shown, staining with the anti-AE1 antibody is clearly restricted to erythrocytes and the basolateral surface of a subset of tubular cells. We thus assign the AE1-positive cells as α-intercalated cells, expressing kAE1. ILK staining was indicated by green fluorescence in the sections (Fig. 11). Significant fluorescence, well above background levels (Fig. 11C), is evident in cells of the tubules expressing AE1 but is not restricted to these cells. Interestingly, ILK staining was also evident in the elliptical cells assigned above as erythrocytes. Immunoblots of human erythrocyte lysates probed with anti-ILK antibody revealed a single band with molecular weight consistent with ILK, confirming the presence of ILK in erythrocytes (supplemental Fig. 3). In merged images the presence of yellow staining indicates co-localization of kAE1 (red) and ILK (green) signals in the α-intercalated cells and in erythrocytes (Fig. 11). We conclude that ILK and kAE1 are both expressed in α-intercalated cells, and moreover a fraction of ILK co-localizes at the basolateral surface of these cells with kAE1.
AE1-ILK Complex in Mouse Kidney—The yeast two-hybrid data indicated that ILK and kAE1 can interact. To determine whether such an interaction occurs in vivo, we wanted to examine whether the two proteins formed a complex in the kidney. We thus perfused mice with PBS to remove their blood, which contains abundant amounts of the erythrocyte form of AE1. These kidneys were used to prepare membranes. Detergent lysates of these membranes were analyzed on immunoblots but revealed little ILK (not shown). To examine the localization of ILK in further, we subjected kidney membranes to detergent solubilization ultracentrifugation on 5–30% linear sucrose gradients. Fractions from the gradient were analyzed on immunoblots probed for ILK and kAE1 (Fig. 12). The blot revealed that most ILK migrated to the bottom of the centrifuge tube, consistent with interaction with a large cytoskeletal complex. Only a small amount of ILK was found in lower density fractions in the gradient, consistent with noncytoskeletal associated ILK. Because little ILK could be released with detergent, it is not surprising that low levels of ILK were present in detergent lysates of kidney membranes. It also means that analysis of kAE1/ILK interaction in kidney by co-immunoprecipitation cannot be performed. Consistent with the ILK/kAE1 interac-

FIGURE 10. Localization of endogenous ILK and exogenous kAE1 in nonpolarized and polarized MDCK cells. A, confocal images of nonpolarized MDCK cells expressing kAE1 HA557. Cells were grown on glass coverslips. After fixing and permeabilizing the cells, mouse monoclonal anti-ILK and rabbit polyclonal anti-Nt kAE1 antibodies followed by Alexa 488-conjugated goat anti-mouse IgG and Cy3-conjugated anti-rabbit IgG were used to visualize endogenous ILK and kAE1, respectively. Green shows endogenous ILK expression within the cells. Red shows kAE1 expressed at the plasma membrane and within the cells. Co-localization of kAE1 and endogenous ILK is shown in yellow. B, polarized MDCK cells were grown on polycarbonate filters. Cells were fixed, permeabilized, incubated with mouse anti-ILK and rabbit anti-Nt kAE1 antibodies as above, and observed using a Zeiss LSM510 confocal microscope. x-z cross-section corresponds to side view of the cells, whereas x-y cross-section represents the middle section of the cells. KAE1 is localized to the basolateral membrane of MDCK cells, whereas ILK is localized throughout the cytosol. Yellow indicates ILK co-localization to the basolateral membrane with kAE1.

FIGURE 11. Immunolocalization of ILK in human kidney. Data from two different sections of normal human kidney (A and B) were treated with rabbit polyclonal anti-AE1 C-terminal antibody, followed by goat anti-rabbit Cy3 (top panel, staining in red) and with mouse monoclonal anti-ILK antibody followed by Alexa 488 (middle panel, staining in green). Merged image (bottom panel), with yellow co-localized signal results from kAE1 (red) and ILK (green). C, kidney section stained without primary antibodies showing low background staining. Images were collected with an oil immersion lens, magnification ×40. Scale bar = 20 μm. A = positive for eAE1; B = kAE1 (basolateral membrane); C = ILK (throughout the cell).

FIGURE 12. Association of ILK and kAE1 in detergent lysates of mouse kidney, resolved on sucrose gradient. Mouse kidney membranes and associated cytoskeleton were solubilized with buffer containing 2% Triton X-100 and layered on top of a 5–30% linear sucrose gradient (indicated by the black bar on top of the figure). After 16–18 h of ultracentrifugation, six fractions were collected from the top of the tube. Samples of each fraction and of the original unfracti-
ILK and kAE1 Processing

tion, ILK is found in the sucrose gradient fraction that contains kAE1 (Fig. 12). Little kAE1, however, was found associated with the cytoskeletal fraction of kidney detergent lysates.

DISCUSSION

Yeast two-hybrid screening of a human kidney cDNA library identified proteins that interact with the cytoplasmic N-terminal domain of kAE1. An association between ILK and the N terminus of kAE1 was confirmed in HEK 293 cells, using co-immunoprecipitation and affinity co-purification. We found that kAE1 bound the ILK C-terminal catalytic domain, whereas the N-terminal region of ILK was not required for kAE1 binding. ILK/kAE1 interaction was further supported by co-localization of ILK and kAE1 in α-intercalated cells of the collecting duct. The finding that overexpression of ILK increased the level of kAE1 at the cell surface suggests a role of ILK in cell surface expression of kAE1.

Several lines of evidence converge to show that ILK and kAE1 form a physical complex. A yeast two-hybrid screen identified an interaction of the kAE1 N-terminal domain and ILK. It was not technically possible to verify the interaction by co-immunoprecipitation from kidney cell lysates because ILK associated with an insoluble (presumably cytoskeletal) complex. When co-expressed in transfected HEK 293 cells, full-length kAE1 reciprocally immunoprecipitated with ILK. kAE1 and ILK were both components of a complex with the cytoskeletal proteins, paxillin and actopaxin. Prior investigations found that ILK is expressed in human renal tubules (38), developing mouse collecting duct (39), and broadly in human kidney collecting duct cells (Fig. 11), coincident with the restricted distribution of kAE1 in α-intercalated cells of the collecting duct (3). In this study we confirmed these findings and showed co-localization of ILK and kAE1 at the basolateral surface of human α-intercalated cells. Because we have not yet shown kAE1/ILK interaction using purified proteins, some caution is needed in interpreting these data. It remains possible that an intermediary protein facilitates the kAE1/ILK interaction.

Mapping of the kAE1/ILK interaction showed that amino acids 27–189 of human kAE1 were sufficient to mediate ILK binding. Interestingly, this region corresponds to an amino acid sequence with sequence similarity to regions of calponin and spectrin. The conserved region corresponds to a calponin homology domain, a protein module previously shown to mediate interaction with the actin cytoskeleton (44). Also, kAE1 may interact with ILK at a site different from paxillin and actopaxin. Moreover, we identified the region in kAE1 that mediates ILK interaction. Sequence alignment of the N terminus of kAE1 and proteins containing the CH domain showed that amino acid residues 27–189 of the kAE1 N terminus align convincingly with other CH domains (Fig. 3).

The CH domain is a protein module of about 100 amino acid residues, first identified at the N terminus of calponin, an actin-binding protein of muscle (30, 45). The striking feature shared among CH domains is their tertiary structure; primary sequence identity between CH domains of different proteins ranges from 5 to 20% (31). In this regard the kAE1 CH domain is not exceptional as it displayed 12 and 13% sequence identity with spectrin and calponin CH domains, respectively, over the 152-residue sequence we identified as the CH domain. We examined structural similarities between the N terminus of kAE1 and the CH domain from human β-spectrin (46) by molecular modeling. The globular fold of the CH domain is built of four core helices, three of them forming a loose triple helix bundle and of one to three short helices present in the loops between the core helices (45). The crystal structure for human eAE1 cytoplasmic domain shows that the region corresponding to residues 36–98 of kAE1 has overall similarity to the secondary structure of the spectrin CH domain (Fig. 3, B and C). In particular, three short α-helices are similarly oriented and are separated by sequences of similar length. Moreover, the most highly conserved region corresponds to a surface loop connecting two of the short helices. It is important to note that the model for the N terminus of kAE1 was developed simply by removing the eAE1 N terminus from the eAE1 crystal structure (2). Because the eAE1 N terminus forms the central β-strand of the domain (not shown in the structure of Fig. 3B), the fold of kAE1 will not adopt the illustrated conformation. Indeed, it is likely that the structure will reorient to fill the void formed by loss of the eAE1 N terminus. In so doing we propose that the structure of the kAE1 N-terminal region will resemble the CH domain of β-spectrin more closely (Fig. 3C). Taken together, we propose that the catalytic domain of ILK interacts with the kAE1 CH domain we have uncovered in the N-terminal region of kAE1.

Expression of ILK dramatically increased the Cl−/HCO3 exchange capacity of kAE1-transfected cells. This rise in transport activity corresponded with the increase of kAE1 cell sur-
ILK and kAE1 Processing

The association of kAE1 with the actin cytoskeleton might also have a significant impact on trafficking and targeting of kAE1 to the basolateral membrane of α-intercalated cells in the kidney. Although it remains unclear what mechanism contributes to kAE1 trafficking or targeting to the basolateral membrane, it is possible that several mechanisms are required to ensure the directed movement of kAE1 within the cell and control its delivery to the target membrane. kAE1 has two basolateral targeting determinants, one in the N terminus and another in the C terminus. Deletions at either the N or C terminus resulted in apical localization of the protein, suggesting that the presence of both determinants is essential for correct basolateral localization of kAE1 (14). The nature of the N-terminal basolateral determinant of human kAE1 has yet to be defined.

The enhancement of kAE1 cell surface targeting by ILK is significant to other AE isoforms. AE3 is poorly processed to the cell surface when heterologously expressed and consequently confers a low level of transport function on cells. This led to speculation that tissue-specific factors may be present in cells where AE3 is endogenously expressed but absent from HEK 293 cells (22). ILK may be such a processing-enhancing factor for AE3 as well as for kAE1.

Finally, the observation of ILK immunofluorescence in erythrocytes is interesting. The lack of erythrocyte ILK signal in negative controls processed without primary antibodies combined with the presence of a unique band on immunoblots of human erythrocyte lysates probed with anti-ILK antibody together suggest that ILK is present in human erythrocytes. We could find in the literature no previous data concerning the presence of ILK in erythrocytes. Nonetheless, ILK is viewed as either “widely expressed” or “ubiquitously expressed” (29, 40), so it is not surprising that ILK is found in erythrocytes. Although the localization of eAE1 and ILK in erythrocytes raises the possibility that the proteins form a complex there, further studies will be needed to address the possibility. Because AE1 constitutes 50% of integral membrane protein in the erythrocyte (47), these cells gave an extremely strong
immunofluorescence signal for eAE1, meaning that the eAE1/ILK co-localization data need to be treated with caution.

In conclusion, these data demonstrate for the first time that kAE1 interacts with the C terminus of ILK through the previously undetected calponin homology domain in the kAE1 N-terminal region. ILK facilitates the localization of kAE1 to plasma membrane of HEK 293 cells by mediating kAE1 interaction with the actin cytoskeleton through the paxillin-actopaxin complex. The increased steady-state level of plasma membrane kAE1 enhances cellular Cl⁻/HCO₃⁻ transport capacity.

Acknowledgments—We thank Dr. Michel Jennings (University of Arkansas) for kindly providing the IVF 12 antibody and Dr. Witoon Tiraso-phon and Dr. Shoukat Dedhar (University of British Columbia) for ILK cDNA. We gratefully acknowledge the assistance of Michael Ho for assistance with immunofluorescence of human kidney sections.

REFERENCES

1. Godinich, M. J., and Jennings, M. L. (1995) *Carr. Opin Nephrol. Hypertens.* 4, 398–401
2. Zhang, D., Kiyatkin, A., Bolin, J. T., and Low, P. S. (2000) *Blood* 96, 2925–2933
3. Tanner, M. J. (1997) *Mol. Membr. Biol.* 14, 155–165
4. Schofield, A. E., Martin, P. G., Spillett, D., and Tanner, M. J. (1994) *Blood* 84, 2000–2012
5. Brosius, F. C., III, Alper, S. L., Garcia, A. M., and Lodish, H. F. (1989) *J. Biol. Chem.* 264, 7784–7787
6. Alper, S. L., Stuart-Tilley, A., Simmons, C. F., Brown, D., and Drenckhahn, D. (1994) *J. Clin. Invest.* 93, 1430–1438
7. Ding, Y., Casey, J. R., and Kopito, R. R. (1994) *J. Biol. Chem.* 269, 32201–32208
8. Wang, C.-C., Moriyama, R., Lombardo, C. R., and Low, P. S. (1995) *J. Biol. Chem.* 270, 17892–17897
9. Chen, J., Vijayakumar, S., Li, X., and Al-Awqati, Q. (1998) *J. Biol. Chem.* 273, 1038–1043
10. Kittanakom, S., Keskanokwong, T., Akkarapatumwong, V., Yenchitsomnus, P. T., and Reithmeier, R. A. (2004) *J. Biol. Chem.* 279, 3101–3109
11. Rodriguez-Soriano, J. (2000) *Pediatr. Nephrol.* 14, 1121–1136
12. Bastani, B., and Gluck, S. L. (1996) *Mol. Cell. Biol.* 16, 4099–4109
13. Quilty, J. A., Cordat, E., and Reithmeier, R. A. (2003) *Biochem. J.* 379, 895–903
14. Toye, A. M., Banting, G., and Tanner, M. J. (2004) *J. Cell Sci.* 117, 1399–1410
15. Adair-Kirk, T. L., Dorsey, F. C., and Cox, J. V. (2003) *J. Cell Sci.* 116, 655–663
16. Gietz, R. H., Jean, A. S., Woods, R. A., and Schiestl, R. H. (1992) *Nucleic Acids Res.* 20, 1425
17. Ruetz, S., Lindsay, A. E., Ward, C. L., and Kopito, R. R. (1993) *J. Cell Biol.* 121, 37–48
18. Alvarez, B. V., Vilas, G. L., and Casey, J. R. (2005) *EMBO J.* 24, 2499–2511
19. Zhu, Q., and Casey, J. R. (2004) *J. Biol. Chem.* 279, 23565–23573
20. Cheung, J. C., Li, J., and Reithmeier, R. A. (2005) *Biochem. J.* 390, 137–144
21. Zhu, Q., Lee, D. W. K., and Casey, J. R. (2003) *J. Biol. Chem.* 278, 3112–3120
22. Fujinaga, J., Loiselle, F. B., and Casey, J. R. (2003) *Biochem. J.* 371, 687–696
23. Thomas, J. A., Buchsbaum, R. N., Zimniak, A., and Racker, E. (1979) *Biochemistry* 18, 2210–2218
24. Delcommenne, M., Tan, C., Gray, V., Rue, L., Woodgett, J., and Dedhar, S. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 11211–11216
25. Persad, S., Attwell, S., Gray, V., Mawji, N., Deng, J. T., Leung, D., Yan, J., Sanghera, J., Walsh, M. P., and Dedhar, S. (2001) *J. Biol. Chem.* 276, 27462–27469
26. Nikolopoulos, S. N., and Turner, C. E. (2002) *J. Biol. Chem.* 277, 1568–1575
27. Yu, C., and Dedhar, S. (2001) *J. Cell Biol.* 155, 505–510
28. Yu, C. (2004) *Biochem. Biophys. Acta* 1692, 55–62
29. Yamaji, S., Suzuki, A., Sugiyama, Y., Koide, Y., Yoshida, M., Kanamori, H., Mohri, H., Ohno, S., and Ishigatsubo, Y. (2001) *J. Cell Biol.* 153, 1251–1264
30. Stradal, T., Kranewitter, W., Winder, S. J., and Gimona, M. (1998) *FEBS Lett.* 431, 134–137
31. Banuelos, S., Saraste, M., and Carugo, K. D. (1998) *Structure ( Lond.* 6, 1419–1431
32. Kuhlman, P. A., Hemmings, L., and Critchley, D. R. (1992) *FEBS Lett.* 304, 201–206
33. Corrado, K., Mills, P. L., and Chamberlain, J. S. (1994) *FEBS Lett.* 344, 255–260
34. Matsuda, C., Kameyama, K., Tagawa, K., Ogawa, M., Suzuki, A., Yamaji, S., Okamoto, H., Nishino, I., and Hayashi, Y. K. (2005) *J. Neuropathol. Exp. Neurol.* 64, 334–340
35. Cordat, E., Li, J., and Reithmeier, R. A. (2003) *Traffic* 4, 642–651
36. Quilty, J. A., and Reithmeier, R. A. (2000) *Traffic* 1, 987–998
37. Wagner, S., Vogel, R., Lietzke, R., Koob, R., and Drenckhahn, D. (1987) *Am. J. Physiol.* 253, F213–F221
38. Guo, L., Sanders, P. W., Woods, A., and Wu, C. (2001) *Am. J. Pathol.* 159, 1735–1742
39. Leung-Hagestijn, C., Hu, M. C., Mahendra, A. S., Hartwig, S., Klumt, H. J., Rosenblum, N. D., and Hannigan, G. E. (2005) *Mol. Cell. Biol.* 25, 2210–2218
40. Hannigan, G. E., Leung-Hagestijn, C., Fitz-Gibbon, L., Coppolino, M. G., Radeva, G., Filmus, J., Bell, J. C., and Dedhar, S. (1996) *Nature* 379, 91–96
41. Yu, T., Li, F., Goeicoechea, S., and Wu, C. (1999) *Mol. Cell. Biol.* 19, 2425–2434
42. Nikolopoulos, S. N., and Turner, C. E. (2001) *J. Biol. Chem.* 276, 23499–23505
43. Yu, T., Huang, Y., Zhang, Y., Hua, Y., and Wu, C. (2001) *J. Cell Biol.* 153, 585–598
44. Nikolopoulos, S. N., and Turner, C. E. (2000) *J. Cell Biol.* 151, 1435–1448
45. Bramham, J., Hodgkinson, J. L., Smith, B. O., Uhrin, D., Barlow, P. N., and Winder, S. J. (2002) *Structure ( Lond.* 10, 249–258
46. Carugo, K. D., Banuelos, S., and Saraste, M. (1997) *Nat. Struct. Biol.* 4, 175–179
47. Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971) *Biochemistry* 10, 2606–2617