Kanadaptin Is a Protein That Interacts with the Kidney but Not the Erythroid Form of Band 3*

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Although epithelial membrane proteins are separately targeted to apical or basolateral domains, some are apically located in one cell type but are basolateral in others. More dramatically, the anion exchanger of a clonal cell line of intercalated cells derived from the kidney can be retargeted from the apical to basolateral domain. This Cl:HCO3\(^{-}\) exchanger, kAE1, is an alternately spliced form of the erythroid anion exchanger (AE1, band 3), but unlike band 3 it does not bind ankyrin. Here we identify a new protein (kanadaptin) that binds to the cytoplasmic domain of kAE1 in vitro and in vivo but not to the erythroid AE1 or to ankyrin.

No significant homologous proteins have been reported so far. Kanadaptin is widely expressed in epithelial (kidney, lung, and liver) and non-epithelial cells (brain and skeletal and cardiac muscle). In kidney, we found by immunocytochemistry that kanadaptin was only expressed in the collecting tubule. In the intercalated cells of this segment, it colocalized with kAE1 in cytoplasmic vesicles but not when the exchanger was in the basolateral membrane. These results raised the possibility that this protein is involved in the targeting of kAE1 vesicles to their final destination.

Epithelial membrane proteins are targeted to apical or basolateral domains using a number of sequence-specific signals (1–3). However, it is becoming increasingly obvious that this process is plastic and flexible in that some proteins, e.g. Na\(^{+}\)K\(^{-}\)-ATPase, glycosylphosphatidylinositol-coupled proteins, the vacuolar H\(^{+}\)-ATPase, and the low density lipoprotein receptor transgene, are targeted to the apical membrane of one epithelial cell type but to the basolateral membrane of another (4–7). In one clonal cell line derived from the intercalated cell of the kidney, a cell specialized for H\(^{+}\) and HCO3\(^{-}\) transport, we found that the same protein, a Cl:HCO3\(^{-}\) exchanger, can be retargeted from the apical to the basolateral domain if this cell is plated at a higher seeding density (8). This anion exchanger is an isoform of the red cell band 3 (AE1)\(^{3}\) (9) that has its first three exons deleted by alternate splicing (we call it here kidney AE1 or kAE1) (10, 11). Band 3 is critical for the maintenance of the biconcave disc shape of red cells where it binds to ankyrin, which in turn binds to spectrin, thereby organizing the submembrane actin cytoskeleton. Ankyrin binds to at least two regions of the large cytoplasmic domain of band 3 (12), one of which is deleted in kAE1. In detailed biochemical studies in vitro, it was found that ankyrin does not bind to the cytoplasmic domain of kAE1 (13, 14). This immediately raised the question of what binds the cytoplasmic domain of kAE1. In this report, we identify a new protein that binds to the cytoplasmic domain of kAE1 but not that of AE1. Although this protein is widely expressed, its expression in the kidney is restricted to the collecting tubule. In the intercalated cells of that segment it colocalizes with kAE1 only when the latter is in intracellular vesicles but not when it had been delivered to the basolateral membrane.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screen**—The cytoplasmic domain of mouse kAE1 was introduced into the bait, pGBD9 (CLONTECH), and transformed into Y190, which was made competent and transformed with a mouse kidney MATCHMAKER cDNA library (CLONTECH). Double transformants were selected on Leu\(^{−}\) Trp \(^{−}\). His\(^{−}\) selection plates and transferred onto nitrocellulose filters. Blue color development was assayed after a freeze-thaw cycle and reaction with 5-bromo-4-chloro-3-indolyl\(\beta\)-galactopyranoside-containing media for 2–12 h at 30 °C. Positive yeast colonies were streaked to isolate single colonies, which were used for plasmid isolation. The plasmids were tested in two yeast strains, Y190 and SPY526. Assays for interaction between various GAL4DNA binding domain (BD) and GAL4 activation domain (AD) fusion proteins were performed in the SPY526 strain.

**In Vitro Binding Assays**—The cytoplasmic domain of kAE1 was cloned into a pBS/ SK vector (Stratagene) downstream of a T7 promoter, transfected, and translated using a TNT T7-coupled reticulocyte lysate system (Promega). In the presence of \(^{35}\)S-methionine, pGEX-kanadaptin was transformed into Escherichia coli to direct the synthesis of a glutathione S-transferase (GST) fusion protein in bacteria. Bacteria were lysed and adsorbed on glutathione-Sepharose beads (Pharmacia Biotech Inc.), and the bound proteins were incubated with \(^{35}\)S-labeled AE1 in the presence of different salt and detergent concentrations. After extensive washing the labeled proteins that bound to the beads were separated on 10% PAGE and analyzed by autoradiography.

**Binding of Intercalated Cell Proteins to Kanadaptin**—Subconfluent monolayers of the intercalated cell were washed three times with phosphate-buffered saline and incubated on ice for 20 min with 50 mM Tris-HCl, pH 7.4, 1 mM MgCl\(_{2}\), 1 mM dithiothreitol, either 0.1 or 1% Nonidet P-40, and the protease inhibitors 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM EGTA, 1 \(\mu\)g/ml pepstatin, and 2 \(\mu\)g/ml each of aprotinin and leupeptin. Cells were then disrupted by shearing with a 25-gauge needle and centrifuged in a microcentrifuge at 15,000 rpm for 20 min. The supernatant was incubated with GST-kanadaptin or GST beads for 2 h at 4 °C. After extensive washing the proteins that bound to the beads were separated on 10% SDS-PAGE and transferred to nitrocellulose paper for immunoblotting with anti-AE1 antibodies.

**Kanadaptin Sequencing and Expression**—The insert in the plasmid pGAD-kanadaptin was extended using 3' and 5' RACE. Mouse kidney "marathon-ready" cDNA (CLONTECH) was used as the template. The T\(_{m}\) of the gene-specific primers was designed to be above 65 °C, and the

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank\(^{TM}\) /EBI Data Bank with accession number(s) AF035526.

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§ The abbreviations used are: AE1, erythroid anion exchanger or band 3; kAE1, the kidney form of AE1; SH3, Src homology domain 3; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; RACE, rapid amplification of cDNA ends; ECM, extracellular matrix.
and 5' RACEs were performed according to the manufacturer's specifications. A mouse multiple tissue blot containing 2 μg of poly(A)^+ RNA (CLONTECH) was hybridized to a ^32P-labeled 900-base pair DNA fragment of kanadaptin under high stringency conditions, and the x-ray film was exposed for 10 h.

Antibodies—The plasmid expressing GST-kanadaptin was generated into the pGEX-2TK vector (Pharmacia), and the expression of the protein was induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside. Bacteria were sonicated in the presence of protease inhibitors, centrifuged, incubated with glutathione-agarose, and washed extensively. Fusion proteins were eluted with 5 mM glutathione; their total amino acids were analyzed to ensure that they coded for authentic kanadaptin and then were used to generate polyclonal antibodies in guinea pigs. Purification of GST-kanadaptin on glutathione columns resulted in a largely degraded protein product despite the presence of a mixture of protease inhibitors. It was only when we included the detergent Sarkosyl (1%) that the proteolysis was reduced, although it was never eliminated. The antisera recognized the fusion proteins whereas preimmune sera did not.

Immunoblot Analysis of Kidney Cytosol—Kidney cortex, medulla, and papilla were cut from frozen rabbit kidneys and homogenized in a buffer containing 250 mM sucrose, 10 mM Tris (pH 8.0), 1 mM phenylmethylsulfonfluoride, and 1 mM EDTA using a Polytron followed by a tissue homogenizer. The homogenate was centrifuged at 1000 × g for 10 min and centrifuged again at 10,000 × g for 20 min (at 4 °C). The resulting supernatant was assayed for protein concentration using the Bradford protein assay (Bio-Rad) and used for immunoblotting with anti-kanadaptin antibody depleted of anti-GST.

Immunocytochemistry of Kanadaptin in the Kidney—Rabbit kidney tissue sections were fixed with methanol at -20 °C for 7 min and stained with kanadaptin antisera with or without a monoclonal mouse anti-AE1 antibody (IVF12; a generous gift of M. Jennings, University of Arkansas). Antibodies were diluted into media containing 10% fetal bovine serum, and the sections were exposed for 2 h at room temperature. After several washes with phosphate-buffered saline, secondary antibodies (donkey anti-guinea pig antibody (fluorescein isothiocyanate) or donkey anti-mouse antibody (rhodamine)) that were diluted into 10% fetal bovine serum and 10% rabbit serum were used. Tissues were analyzed using a Zeiss confocal microscope (model LSM 410; Jena, Germany). Images were saved in TIFF format and analyzed and displayed using the Zeiss LSM 3.95 software.

RESULTS AND DISCUSSION

To identify sequences that interact with the cytoplasmic domain of the kidney AE1, the bait (pGBD9-kAE1) was transformed into strain Y190, which was made competent and transformed with a mouse kidney cDNA library. Approximately 2 × 10⁷ colonies were screened representing 2 × 10⁶ clones. After retesting the blue transformants, cDNA species were recovered.
and tested for specific interaction with kAE1. One cDNA specifically interacted with the cytoplasmic domain of kAE1 but not with vector or with the irrelevant nuclear protein, lamin C (Fig. 1A). The protein (heretofore called kanadaptin, for kidney anion exchanger adaptor protein), interacted again with the cytoplasmic domain of kAE1 even when the two vectors were switched (Fig. 1B) but did not interact with the nuclear protein, SV40 large T antigen, or with itself (not shown). These results demonstrate that kanadaptin interacts specifically with the cytoplasmic domain of kAE1 in yeast.

The cytoplasmic domain of kAE1 differs from that of the erythroid AE1 by deletion of the 79 N-terminal amino acids (the product of the first three exons that are spliced out in the kidney) (9–11). Kanadaptin did not interact with the cytoplasmic domain of AE1 (Fig. 1C). When ankyrin, a well studied binding protein for the cytoplasmic domain of AE1, was used in this assay it interacted with AE1, indicating that the folding of AE1 preserves important aspects of its native conformation (Fig. 1D). The construct we used contained the first 24 ankyrin repeats representing the four important subdomains of ankyrin known to interact with the cytoplasmic domain of AE1 (12, 15). However, ankyrin did not bind to kAE1 (Fig. 1D), confirming the work of others (13, 14), despite the fact that kAE1 colocalizes with ankyrin and fodrin in the basolateral membrane of these cells (16). Ankyrin also did not bind to kanadaptin suggesting that it is not an adaptor that connects KAE1 to ankyrin (Fig. 1D). These results emphasize that the targeting or retention of KAE1 does not follow the same rules as that of AE1 and illustrates the power of alternative splicing in increasing the diversity of interactions of individual proteins.

Kanadaptin was cloned and expressed as a GST fusion protein in bacteria. Purified GST-kanadaptin was loaded onto glutathione-agarose beads and incubated with the expressed [35S]-labeled cytoplasmic domain of kAE1. After extensive washing, it was found that KAE1 interacted with kanadaptin; the interaction was not disrupted by washing with salt concentrations as high as 500 mM, and it was not affected by washing with solutions containing the detergent Nonidet P-40 at concentrations that varied from 0.1 to 1% (Fig. 2A). However, 0.1% Triton X-100 completely disrupted the interaction (Fig. 2A). These results confirm the yeast two-hybrid interaction and further suggest that the interaction is likely due to hydrophobic forces.

GST-kanadaptin beads were incubated with homogenates of the intercalated cell line in the presence of either 0.1% or 1% Nonidet P-40. After extensive washing the proteins bound to the beads were separated on SDS-PAGE and blotted with anti-AE1 antibody. Kanadaptin interacted with in, for instance, brain, testis, or liver?

To examine its function further, we generated a polyclonal antibody by immunizing animals with the GST-kanadaptin fusion protein. Immunoblot studies showed that whereas pre-immune sera did not recognize any proteins in the bacterial lysates, they recognized a new protein of molecular mass of 53 kDa, the molecular mass of 53 kDa; however, there were minor bands of molecular mass of 28 kDa and 50 kDa (Fig. 4A). In rabbit kidney cytosol, the antibody recognized a major protein of apparent molecular mass of 53 kDa; however, there were minor bands of molecular mass of 28 kDa and 50 kDa. Preimmune sera did not recognize any proteins (Fig. 4B). Because the animals were immunized with GST-kanadaptin and the kidney is a rich source of several GST isoforms (many of which have molecular masses around 28 kDa) we depleted the sera of anti-GST antibodies by passing them through agarose beads covalently coupled to GST (Pierce). The results were the same (Fig. 4B). Whether these minor bands are degradation products or the result of alternate splicing will need to be determined. Rabbit kidney cytosol was prepared from cortex, medulla, and papilla, and 50 μg of protein was placed in each lane and blotted with the depleted antisera. Kanadaptin was highly enriched in the papilla compared with the medulla.

In rabbit kidney sections, kanadaptin was expressed in the kidney only in the collecting duct (Fig. 5A), using either the original or GST-depleted antisera. This explains the immunoblot results since the collecting tubule forms a large fraction of...
the tubule segments in the papilla and medulla. There was no specific staining in the glomeruli or in other tubular segments. In the collecting duct, all cell types (principal and intercalated) were stained. The staining pattern was cytosolic with an accentuation in the region of plasma membranes, especially apical membranes. One-third of the cells in this segment are intercalated cells and we (and others) had found that their staining pattern for kAE1 is quite heterogenous (see Ref. 18 for review). In some intercalated cells, kAE1 was localized only in the basolateral membranes, whereas in others it was present in intracellular vesicles. Using simultaneous staining for kanadaptin and kAE1 and thin confocal optical sections, we found remarkable colocalization (Fig. 5, B and C). In a intercalated cells, kAE1 was present in the basolateral membrane and in intracellular vesicles. Kanadaptin was colocalized with kAE1 in these vesicles but not when kAE1 was located in the basal and lateral plasma membranes (Fig. 5, B and C). One possible interpretation of this colocalization is that kanadaptin is an adaptor protein that guides kAE1 vesicles from the cytoplasm to their basal target. Once delivered there, it dissociates from kAE1 to start another cycle. We speculate that kanadaptin is part of a signal transduction machine that is important in targeting these kAE1 vesicles.

Kanadaptin contains a proline-rich sequence (LPPKR-PELPP) that could bind to SH3 domain protein. One proposed function for SH3-containing proteins is to mediate localization of their targets to different cellular compartments including interaction with the cytoskeleton or signal transduction proteins (19). Future studies will aim at identifying the interacting proteins with kanadaptin in the intercalated cells.

The plasticity of epithelial polarity of proton ATPases and anion exchangers was first identified in vivo as a mechanism of regulation of trans-epithelial H⁺ transport in response to an increase in the acid content of the diet (20). Our recent studies...
showed that plasticity is mediated, in vitro at least, by signals from the extracellular matrix (ECM). The factor responsible for the in vitro effect was recently purified and partially sequenced, and this new ECM protein, which we have termed hensin, is restricted in expression to epithelia and in the kidney to the collecting duct (21). Upon localization of this protein to the ECM of intercalated cells in vitro, intracellular signals lead to retargeting of kAE1 from the apical to the basolateral membrane (8, 21). Kanadaptin is likely to be an important mediator of this targeting process. The identification of kanadaptin should facilitate the further purification of the other proteins involved in the retargeting of kAE1-containing vesicles from the apical to basolateral surface. That kanadaptin did not localize to the basolateral membrane raises the possibility that it dissociates from kAE1, perhaps to return and bind to other kAE1 vesicles in the cytoplasm. One interesting issue that is raised by our studies and those of others is what retains kAE1 in the basolateral domain. Fodrin and ankyrin are present in this membrane (16), yet neither binds to kAE1 (Refs. 13 and 14, and this report). If no other proteins are discovered that might anchor it to the basolateral cytoskeleton, it is possible that kAE1-carrying vesicles exist in a mobile pool that can be retracted and inserted into the basolateral membrane under the influence of physiological regulators. That a large fraction of these intercalated cells contains kAE1 only in intracellular vesicles (18) supports this hypothesis. We had previously discovered that the vacuolar proton ATPase in these same cells exists in such a regulated pool (22, 23). An increase in the pCO2...
of the medium acidified the cytoplasm, which led to an increased cell calcium. This in turn triggered the insertion of vesicles enriched in the vacuolar ATPase into the apical plasma membrane, thereby increasing trans-epithelial H+ secretion.

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