Evidence of a Structural and Functional Ammonium Transporter RhBG-Anion Exchanger 1-Ankyrin-G Complex in Kidney Epithelial Cells*

Received for publication, September 8, 2014, and in revised form, January 12, 2015: Published, JBC Papers in Press, January 23, 2015, DOI 10.1074/jbc.M114.610048

Sandrine Genetet1,5,4, Pierre Ripoche1,5,4, Caroline Le Van Kim1,5,4, Yves Colin1,5,4, and Claude Lopez1,5,1,2

From 1INSERM U1134, 75739 Paris, France, the 5Université Paris Diderot, Sorbonne Paris Cité, UMR_S1134, 75739 Paris, France, the 4Institut National de la Transfusion Sanguine, 75739 Paris, France, and the 1Laboratoire d’Excellence GR-Ex, 75238 Paris, France.

Background: Up to now, there was no evidence for an interaction of the kidney anion exchanger 1 (kAE1) with ankyrin.

Results: kAE1 actually associates with epithelial ankyrin-G and renal ammonium transporter RhBG, which also binds ankyrin-G.

Conclusion: RhBG, kAE1 and ankyrin-G form a structural/functional complex in kidney epithelial cell lines.

Significance: This complex could participate in the regulation of acid-base homeostasis.

The renal ammonium transporter RhBG and anion exchanger 1 kAE1 colocalize in the basolateral domain of α-intercalated cells in the distal nephron. Although we have previously shown that RhBG is linked to the spectrin-based skeleton through ankyrin-G and that its NH3 transport activity is dependent on this association, there is no evidence for an interaction of kAE1 with this adaptor protein. We report here that the kAE1 cytoplasmic N terminus actually binds to ankyrin-G, both in yeast two-hybrid analysis and by coimmunoprecipitation in situ in HEK293 cells expressing recombinant kAE1. A site-directed mutagenesis study allowed the identification of three dispersed regions on kAE1 molecule linking the third and fourth repeat domains of ankyrin-G. One secondary docking site corresponds to a major interacting loop of the erythroid anion exchanger 1 (eAE1) with ankyrin-R, whereas the main binding region of kAE1 does not encompass any eAE1 determinant. Stopped flow spectrofluorometry analysis of recombinant HEK293 cells revealed that the Cl−/HCO3− exchange activity of a kAE1 protein mutated on the ankyrin-G binding site was abolished. This disruption impaired plasma membrane expression of kAE1 leading to total retention on cytoplasmic structures in polarized epithelial Madin-Darby canine kidney cell transfectants. kAE1 also directly interacts with RhBG without affecting its surface expression and NH3 transport function. This is the first description of a structural and functional RhBG-kAE1 ankyrin-G complex at the plasma membrane of kidney epithelial cells, comparable with the well known RhAE1-ankyrin-R complex in the red blood cell membrane. This renal complex could participate in the regulation of acid-base homeostasis.

Erythroid anion exchanger 1 (eAE1),2 also known as Band 3, is the major integral membrane protein of the red blood cell (RBC). This glycoprotein of 911 amino acids consists of three distinct regions: (i) an N-terminal cytoplasmic domain (404 residues) that binds various glycolytic enzymes, protein tyrosine kinases and hemoglobin, as well as cytoskeletal proteins such as bands 4.1 and 4.2, and ankyrin-R; (ii) a membrane multi-spanning domain (467 residues) mediating the electroneutral chloride/bicarbonate (Cl−/HCO3−) exchange function; and (iii) a short C-terminal cytoplasmic domain (40 residues) that binds carbonic anhydrase II to form a functional metabolon (3). By its association with proteins Rh, the carrier of the Rhesus blood group antigens, and RhAG, which define the core of the Rh membrane complex, eAE1 modulates their expression level (4). RhAG belongs to the ammonium transporters/methyammonium-ammonium permease/Rh superfamil of ammonium transporters and allows the facilitated transport of NH3 across the RBC membrane (5). Ankyrin-R mediates skeleton attachment of Rh and RhAG (6); therefore an RhAE1 macrocomplex model has been proposed (4) in which the Rh complex and eAE1 are associated with each other and the spectrin-based skeleton through their common direct interaction with ankyrin-R (6). The RhAE1-ankyrin-R macrocomplex could constitute a metabolon allowing transport of gas and/or ions across the RBC membrane (4).

The kidney isoform of AE1 (kAE1) is identical to eAE1, except that it lacks the N-terminal 65 amino acids because of an alternate transcription start site (7). kAE1 is located in the basolateral domain of the acid-secreting α-intercalated cells of the distal tubule (8), where it plays an important role in bicarbonate reabsorption into the blood and in urinary acidification. Mutations of kAE1 are responsible for dominant or recessive distal renal tubular acidosis, which results in impaired acid secretion.

* This work was supported by institutional funding to the INSERM U1134 and Institut National de la Transfusion Sanguine. This study was supported by Laboratory of Excellence GR-Ex Grant ANR-11-LABX-0051. The labex GR-Ex is funded by the program Investissements d’Avenir of the French National Research Agency through Grant ANR-11-IDEX-0005-02.

1 These authors contributed equally to this work.

2 To whom correspondence should be addressed: INSERM, UMR_S1134, INTS, 6 Rue Alexandre Cabanel, 75015 Paris, France. Tel.: 33-1-44-49-30-95; Fax: 33-1-43-06-50-19; E-mail: claude.lopez@inserm.fr.

3 The abbreviations used are: AE1, type 1 anion exchanger; eAE1, erythroid AE1; kAE1, kidney AE1; Rh, Rhesus; Nter, N terminus; RBC, red blood cell; MDCK, Madin-Darby canine kidney; PLA, proximity ligation assay; BCECF-AM, 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxyethyl ester; DIDS, 4,4′-diisothiocyanostilbene-2,2'-disulfonic acid, disodium salt; ni, noninduced; i, induced.
The Renal RhBG-kAE1-Ankyrin-G Complex

In α-intercalated cells, kAE1 is colocalized with the basolateral ammonium transporter RhBG (9, 10), a renal homologue of the erythroid RhAG protein (11). We have previously shown that RhBG is tethered to the membrane skeleton through its interaction with ankyrin-G (1) and that this linkage is crucial for its NH₄ transport activity (2). Ankyrins constitute a family of membrane adaptor proteins that link various membrane-associated proteins (ion channels and cell adhesion molecules) to the spectrin-based membrane skeleton. In vertebrates, three different genes Ank1, Ank2, and Ank3 encode ankyrin-R, ankyrin-B, and ankyrin-G, respectively. Ankyrin-R expression is restricted to erythrocytes, neurons, and striated muscle; ankyrin-B is broadly expressed and represents the major form in the nervous system; ankyrin-G is the most widely distributed species and is predominant in epithelial cells. These last two forms are not found in red cells (12, 13). Two past studies from 1994 (14) and 1995 (15) reported that the kAE1 isoform does not bind ankyrin in vitro; however, ankyrin-G was still not characterized at that time, and these experiments were achieved using ankyrin-R, which is not found in kidney epithelial cells. The crystal structure of the N-terminal cytoplasmic domain (Nter) of eAE1, although not resolving the first 54 amino acids, showed that the absence of residues 1–65 in kAE1 results in a deletion of a central strand in the major β-sheet. The authors assumed that this deletion might cause the loss of interaction between kAE1-Nter and ankyrin, protein 4.1, and glycolytic enzymes (16). A comparative biophysical analysis of cytoplasmic domains of eAE1 and kAE1 concluded that the absence of the central β-strand in the latter results in a less stable and more open structure than that of eAE1 and may account in part for the altered protein binding properties of kAE1-Nter (17).

Despite these presumptions, there is so far no experimental evidence of the absence of interaction between kAE1 and the epithelial form of ankyrin, ankyrin-G. We made the hypothesis that, similarly to erythroid RhetAE1-ankyrin-R, a RhBG-kAE1-ankyrin-G complex might be present at the plasma membrane of kidney epithelial cells. In this study, we demonstrate the existence of a physical interaction between kAE1 and ankyrin-G and also between kAE1 and RhBG. Moreover, ankyrin-G binding might be crucial for the activities of the membrane partners in this complex: RhBG and kAE1. This newly characterized RhBG-kAE1-ankyrin-G complex could participate in the regulation of acid/base homeostasis by excreting ammonium and protons in urine.

**EXPERIMENTAL PROCEDURES**

**Materials**—Primers used in PCR and mutagenesis experiments were from Eurofins MWG/Operon (Ebersberg, Germany). The QuikChange II XL site-directed mutagenesis kit was provided by Stratagene (La Jolla, CA). The pGBK7T and pGADT7 vectors were from the Matchmaker Gal4 two-hybrid System 3 (Clontech). The pcDNA4/TO-pcDNA6/TR (T-REX™ System) vectors were purchased from Invitrogen. BCECF-AM fluorescent probe and AE1 inhibitor DIDS were obtained from Sigma-Aldrich. Mouse BRIC 6 and rat BRAC 17 monoclonal antibodies to extracellular epitopes of human AE1 were provided by the International Blood Group Reference Laboratory (Bristol, UK). Rabbit polyclonal antiserum against the cytoplasmic N terminus of AE1 was a gift from Dr. Carsten Wagner (University of Zürich, Zürich, Switzerland). Rabbit and A-08 mouse polyclonal antibodies raised against, respectively, the cytoplasmic C terminus and extracellular loops of human RhBG have been described previously (1, 2, 18). Mouse anti-human ankyrin-G and antiZO-1 (Alexa Fluor 488-conjugated) monoclonal antibodies were purchased from Invitrogen and Molecular Probes (Saint-Aubin, France), respectively. Complete protease inhibitor mixture was supplied by Roche Applied Science.

**Yeast Two-hybrid Studies**—The pGADT7 constructs containing the four individual D1, D2, D3, and D4 sequences coding for the repeat subdomains of the membrane binding domain of ankyrin-G, fused to GAL4 activation domain cDNA, were previously described (1). A cDNA fragment encoding the cytoplasmic N-terminal domain of kAE1 (kAE1-Nter, residues 1–339, starting from the kAE1-specific ATG codon) was amplified by PCR from the plasmid pUC18-eAE1 containing the erythroid AE1 coding sequence and cloned in the pGBK7T vector in-frame with the DNA binding domain of GAL4. Mutant forms of kAE1-Nter were obtained by site-directed mutagenesis according to the supplier’s instructions (Stratagene) or PCR amplification from pGBK7T-kAE1-Nter recombinant DNA or by gene synthesis by Eurofins MWG/Operon. All the inserts were sequenced by GATC Biotech (Konstanz, Germany). Cotransformation of the AH109 yeast strain with pGBK7T and pGADT7 constructs and analysis of protein interactions were carried out as described previously (1).

**Construction of kAE1 Mammalian Expression Vectors**—Full-length cDNA encoding kAE1 was amplified by PCR from the plasmid pUC18-eAE1 with the addition of a Kozak sequence at its 5’ end and subcloned in the pcDNA4/TO vector. The kAE1 cDNA lacking coding sequences necessary for interaction with ankyrin-G was synthesized by Eurofins MWG/Operon and then subcloned in the pcDNA4/TO vector. All the inserts were sequenced by GATC Biotech.

**Cell Culture, Transfection, Flow Cytometry, and Confocal Microscopy**—HEK293 cells and Madin-Darby canine kidney (MDCK) cells were supplied by the American Type Culture Collection (Manassas, VA). HEK293 cells were grown in Dulbecco’s modified Eagle’s medium/F12/Glutamax I (Invitrogen) supplemented with 10% (v/v) fetal calf serum tetracycline-free (Dutscher, Brumath, France), 1× nonessential amino acids (Invitrogen), 1× antibiotic-antimycotic (Invitrogen), 25 mM NaHCO₃, and 25 mM Hepes, and MDCK cells were cultured in Iscove’s modified Dulbecco’s medium/GlutaMAX I (Invitrogen) supplemented with 10% fetal calf serum and antibiotic-antimycotic. T-Rex (Invitrogen) is a Tet-regulated mammalian expression system based on the binding of tetracycline to a Tet repressor and derepression of the promoter controlling the expression of the gene of interest. The establishment of a HEK293 cell line inducible for expression of recombinant kAE1 protein was achieved as described previously for recombinant eAE1 expression (19). In kAE1-RhBG coexpression experiments, HEK293 stable transfectants (pcDNA6/TR + pcDNA4/TO-kAE1) were cotransfected with pcDNA3-RhBG vector (1) and selected using 0.8 mg/ml neomycin (Geneticin, Invitrogen). Surface expression of kAE1 and RhBG on HEK293 cells was detected by flow cytometry, using a FACSCantio II flow cytometer (BD Bio-
fluency on poly-L-lysine coverslips were transiently transfected
with primary and Alexa Fluor secondary antibodies, and ana-
immunostained with appropriate primary and Alexa Fluor sec-
ondary antibodies as described previously (19), and examined
by confocal microscopy using a Zeiss LSM700 inverted confo-
cal microscope equipped with a ×100 oil immersion objective
with a numerical aperture of 1.4. MDCK cells grown to subcon-
fluence on poly-L-lysine coverslips were transiently transfected
with pcDNA4/TO-kAE1 vector using TurboFect transfection
reagent (Thermo Scientific, St. Leon-Rot, Germany), cultured
for 4 days to allow polarization, immunostained with appropri-
ate primary and Alexa Fluor secondary antibodies, and ana-
yzed by confocal microscopy.

Proximity Ligation Assay—Proximity ligation assay (PLA) was used for in situ detection of protein interactions. HEK293 cells expressing recombinant proteins were cultured on coverslips, induced or not for kAE1 expression as above, fixed with 4% paraformaldehyde (w/v), permeabilized in 1% SDS (w/v), and blocked with background reducing buffer (Dako, Copenhagen, Denmark), as described previously (1). Then the samples were incubated for 1 h at room temperature with the following pairs of antibodies, according to the interaction studied: rabbit anti-AE1 (1:4000) and mouse anti-ankyrin-G (10 µg/ml) for kAE1-ankyrin-G, rabbit anti-RhBG (1:500), and mouse anti-
ankyrin-G (10 µg/ml) for RhBG-ankyrin-G. PLA was per-
fomed in a humid chamber at 37 °C according to the manufac-
turer's instructions (Olink Bioscience, Uppsala, Sweden).
Briefly, after three washings in buffer A, the samples were incu-
bated with mouse plus and rabbit minus PLA probes and
washed again, and ligation mixture was then added for 30 min.
Washings were repeated, and the samples were finally incu-
bated for 100 min with the amplification mixture containing the fluorescently labeled DNA probe, washed in buffer B,
mounted in ProLong Gold antifade reagent with DAPI (Invit-
rogen), and analyzed by confocal microscopy. For kAE1-RhBG
interaction, samples (not permeabilized) were incubated with mouse A-08 anti-RhBG (1:3000) and rat BRAC17 anti-AE1 (4
µg/ml) conjugated with minus PLA probe (according to Olink's
instructions for Probemaker kit), followed by the mouse plus
PLA probe and then treated as above. A positive signal indicat-
ing an interaction is produced when the two tested proteins are
30 nm apart.

Protein Extraction, Electrophoresis and Western Blot
Analysis—HEK293 cell lines were lysed for 1 h at 4 °C in lysis
buffer (150 mM NaCl, 20 mM Tris-HCl, pH 8, 5 mM EDTA)
containing complete protease inhibitor mixture and 1% Triton
X-100. Lysates were centrifuged at 15,000 × g for 15 min at 4 °C.
Aliquots of lysate supernatants were mixed with 5× loading
buffer (1.25 mM sucrose, 20% SDS, 250 mM Tris-HCl, pH 6.8,
25% β-mercaptoethanol, 1% bromphenol blue) before elec-
rophoresis. SDS-PAGE was performed using 4–12% gradient
polyacrylamide gels according to Laemmli (20), using MOPS
SDS running buffer (Novex, Saint-Aubin, France). Western
blots were performed on nitrocellulose membranes which then
were incubated with rabbit anti-AE1 antibody (1:10,000) fol-
lowed with anti-rabbit IgG peroxidase-conjugated secondary
antibody (1:1,000) (P.A.R.I.S., Compiegne, France). Immunob-
blots were visualized using the ECL Plus Western blotting
detection system (Amersham Biosciences).

Stopped Flow Analysis—Ammonium transport (RhBG) and
Cl−/HCO3− exchange (kAE1) functions were determined by
stopped flow spectrofluorometry analysis using the fluorescent
pH-sensitive probe BCECF-AM (10 µM) at pH 7.2, essentially as
described previously (10, 19). Briefly, parental and transfected
HEK293 cells were exposed either to an outwardly directed 20
mEq NH4+ at 15 °C (ammonium transport) or to inwardly
directed 10 meq HCO3−/CO2 and outwardly directed 67.5 meq
Cl− gradients at 30 °C (Cl−/HCO3− exchange). When indi-
cated, the anion transport inhibitor DIDS was added to the cells
at 10 µM 30 min before analysis. The pH-dependent fluores-
cence changes of BCECF were monitored at a 485-nm excita-
tion wavelength, and the emitted light was filtered with a
520-nm cutoff filter. Data from three to four time courses were
averaged and fitted to a monoeponential function using the
simplex procedure of the Biokine software package (Bio-Logic).
Determined alkalinization rate constants could be compared in
the different samples because cell sizes were identical in all
preparations. They represent a reliable indication of transport
functions because in each case, NH4 or HCO3− movements
result in pH modification with always the same buffer power.
Over the pH range used (7.0–7.8), the relative fluorescence of
the dye was proportional to the pH variation ($R^2 = 0.993$), as
determined by titration of HEK293 cells incubated in 2 ml of
isoionic buffer (130 mM NaCl, 5 mM KCl, 10 mM Hepes/NaOH,
pH 7.2) containing 1 µM valinomycin and 1 µM carbonyl cyanid
4-(trifluoromethoxy phenylhydrazone) (Sigma-Aldrich). Titra-
tions were performed using a classical spectrofluorimeter by
stepwise additions of 2 µl of 1 N KOH.

RESULTS

Interaction of kAE1 with Ankyrin-G in Yeast Two-hybrid
Analysis—kAE1 interacts with ankyrin-R through its cytoplas-
ic N terminus (3). To investigate whether the nonerythroid
and epithelial homologue ankyrin-G could be a partner of
kAE1, the N-terminal cytoplasmic tail of kAE1 (kAE1-Nter)
was fused in-frame to the GAL4 DNA binding domain of the
yeast two-hybrid pGBK7T vector. The four repeat domains
of ankyrin-G (D1, D2, D3, and D4) individually fused in-frame
to the GAL4 activation domain of the pGBD7T vector were
already available (1). The recombinant vectors were cotrans-
fomed in the AH109 yeast strain, and their ability to grow in
good stringency selective medium lacking adenine, histidine,
leucine, and tryptophan was analyzed (Fig. 1A). These experi-
ments, confirmed by β-galactosidase activity assays (not
shown), indicated that kAE1-Nter interacts with the D3 and D4
repeat domains of ankyrin-G. No association between kAE1-
Nter and the D1 and D2 repeat domains could be detected.

Mapping of the Ankyrin-G-Binding Site in the Cytoplasmic N
Terminus of kAE1—We next performed site-directed mutagen-
esis on the pGBK7T-kAE1-Nter vector to localize the site of

The Renal RhBG·kAE1·Ankyrin-G Complex

MARCH 13, 2015 • VOLUME 290 • NUMBER 11

JOURNAL OF BIOLOGICAL CHEMISTRY 6927
interaction with the D3 and D4 repeat domains of ankyrin-G, using the two-hybrid system. First, the deletion of the D3-D4 ankyrin-R binding site in eAE1 protein (amino acids 175–185; Ref. 21) was achieved (amino acids 110–120 in kAE1 numbering). The deleted 11 residues were substituted with a bridging Gly-Gly dipeptide, as originally done by Chang and Low (21) on eAE1. Analysis of growth on selective medium and β-galactosidase activity of cotransformed yeast showed a strong interaction of kAE1-Nter d110–120 mutant with D3 and D4 repeat domains of ankyrin-G, apparently equivalent to that of wild-type N terminus of kAE1 (kAE1-Nter) (Fig. 1B), indicating that ankyrin-G binding site in kAE1-Nter might be different from ankyrin-R binding site in eAE1-Nter. We then generated a series of mutants with progressive deletions from the C-terminal end of the N terminus (N21, N41, N51, N81, N109, N120, N240, N275, N301, and N319) were performed by PCR-amplifying kAE1-Nter fragments starting at codons 22, 42, 52, 82, 110, 121, 241, 276, 302, and 320, respectively (Fig. 1B). No modification of interaction with ankyrin-G was observed from N21 to N240 mutants; this suggests that the full interaction still detected with the C-terminal mutants (from C120 to C23) was most probably artifactual and caused by a modified conformation of these short peptides. From N275 to N319 mutants, interaction with ankyrin-G was progressively reduced but not completely inhibited (Fig. 1B). Alanine scanning on the N319 mutant was then performed, by stretches of four amino acids (D320A/I321A/R322A/R323A, R324A/Y325A/P326A/Y327A, Y328A/L329A/S330A/D331A, N332A, I332A/T333A/D334A/A335, and F336A/S337A/P338A/Q339A) were generated from kAE1-Nter in the pGBKT7 vector, as described under “Results.” kAE1-Nter S1 and S2 were created by synthesis of mutant kAE1-Nter N319a, N319b, N319d, and N319e mutants were not further affected in their capacity to bind ankyrin-G, compared with N319, whereas the interaction of N319c mutant with ankyrin-G was completely abolished. However, this short mutant lacks the 240 first amino acids of full-length kAE1-Nter, spatial conformation of which might be important for the interaction. A synthetic N terminus was therefore created that contained both a deletion between amino acids 240 and 320 and the four substitutions Y328A/L329A/S330A/D331A, Y328A/L329A/S330A/D331A as N319c, I332A/T333A/D334A/A335 = N319d, and F336A/S337A/P338A/Q339A = N319e (Fig. 1B). N319a, N319b, N319d, and N319e mutants contribute to interaction with ankyrin-G only as a second-
ary domain. The ankyrin-G binding site is thus dispersed on three distinct regions in the N terminus of kAE1: amino acids 110–120, 241–319, and 328–331.

Expression of Recombinant Wild-type and Mutant kAE1 in HEK293 Cells—We constructed a synthetic cDNA encoding a whole kAE1 protein lacking in its cytoplasmic N terminus the two peptidic regions (amino acids 110–120 and 241–319) and with alanine substitutions for residues 328–331, named kAE1-delABS (deletion of ankyrin-G binding site) and thus identical to kAE1-Nter S2 mutant in its cytoplasmic N terminus. HEK293 cells, lacking endogenous expression of kAE1 (22), were stably transfected with wild-type or mutant kAE1 cDNA using the tetracycline-inducible expression system previously described (19) to circumvent the loss of long term expression of AE1 protein (23, 24). Flow cytometry experiments using BRIC6 monoclonal antibody revealed wild-type kAE1 at the surface of tetracycline-induced transfected cells (Fig. 2A, kAE1, white peak). More than 99% of the transfected cells expressed kAE1 protein after Zeocin selection and cell sorting. As expected, no labeling could be detected either on parental HEK293 cells (not shown) or on noninduced kAE1-transfected cells (Fig. 2A, kAE1, gray peak). Likewise, the delABS mutant was not revealed at the cell surface (Fig. 2A, delABS, superimposed gray and white peaks). Immunofluorescence confocal microscopy on intact cells using BRIC6 confirmed these results (Fig. 2B, middle and lower left panels): wild-type kAE1 was localized on the cell surface, whereas kAE1-delABS was not detectable. The same result was obtained when using BRAC17, another exofacial epitope-recognizing antibody (not shown). After cell permeabilization, wild-type kAE1 was located almost exclusively at the cell membrane, whereas kAE1-delABS mainly accumulated on cytoplasmic structures and was partly detectable at the plasma membrane, using anti-Nter AE1 antibody (Fig. 2B, middle and lower right panels). kAE1 was not expressed in parental (not shown) or noninduced transfected cells (kAE1 ni; Fig. 2B, top panels). Western blot analysis of Triton X-100 solubilized HEK293 cell proteins revealed that wild-type kAE1 and kAE1-delABS migrate in SDS-PAGE at an estimated apparent molecular mass of 93 and 83 kDa, respectively, as expected (Fig. 2C), which rules out an altered synthesis or stability of the mutant protein. kAE1-delABS therefore is not efficiently trafficked to the plasma membrane and might also display a defect of folding that would explain failure to detect the protein at the surface of intact cells.

Interaction of kAE1 and RhBG with Ankyrin-G in Proximity Ligation Assay—To strengthen the data of kAE1 interaction with ankyrin-G from two-hybrid experiments, we performed PLA in recombinant HEK293 cells. This ex vivo coimmunoprecipitation allows the detection of protein-protein interactions...
in situ without altering the cellular environment, with a distance resolution of \( \leq 30 \) nm (25). Preliminary immunofluorescence experiments showed that wild-type kAE1 colocalized with endogenous ankyrin-G (Fig. 3A). The delABS mutant partially colocalized with this adaptor protein (Fig. 3A, delABS i, Merged) and showed an essentially submembranous and internal pattern of expression (by comparison with ankyrin-G expression). This observation attested that at least some protein could reach and tether to the plasma membrane. PLA signal (Fig. 3B) was clearly revealed in HEK293 cells expressing recombinant kAE1 (tetracycline-induced) and endogenous ankyrin-G (kAE1 i), whereas no signal was visible either in non-induced transfected cells (kAE1 ni) or in induced cells transfected with eAE1 cDNA (not shown). Thus kAE1 does interact with ankyrin-G, and as expected, eAE1 (which binds erythroid ankyrin-R) does not. Although kAE1-delABS partially colocalized with ankyrin-G, no signal could be detected by PLA (Fig. 3B, delABS i), indicating an absence of interaction, as found in two-hybrid experiments. We have previously demonstrated by yeast two-hybrid analysis, immunohistochemistry on kidney epithelial tissue, and immunofluorescence on polarized MDCK cells that RhBG interacts with ankyrin-G (1). Colocalization of both proteins was checked in HEK293 cells expressing either wild-type RhBG or RhBG-F419A/L420A/D421A mutant (binding or not binding, respectively, to ankyrin-G; Ref. 1) (Fig. 4A). PLA performed on these cells showed a bright signal with wild-type RhBG, whereas none was visible with the mutant RhBG-F419A/L420A/D421A protein (Fig. 4B), which confirmed the binding of wild-type RhBG, but not of RhBG-F419A/L420A/D421A, to ankyrin-G.

Interaction of RhBG with kAE1 in Proximity Ligation Assay—To search for a potential direct interaction between RhBG and kAE1, HEK293 cells expressing inducible wild-type kAE1 or delABS mutant were stably cotransfected with wild-type or F419A/L420A/D421A RhBG cDNA. Confocal microscopy revealed that RhBG colocalized with kAE1 at cell surface, whether wild-type proteins (Fig. 5A) or the different combinations of wild-type/mutant proteins (kAE1-F419A/L420A/D421A, delABS-RhBG, delABS-F419A/L420A/D421A; not shown) were analyzed. Using PLA, we revealed a clear interaction between the two proteins, in every configurations (kAE1-RhBG and delABS-F419A/L420A/D421A, Fig. 5B, right panels; kAE1-F419A/L420A/D421A and delABS-RhBG, not shown). As a control, no signal was detected in noninduced (ni) cells for kAE1 expression (Fig. 5B, left panels). Moreover, kAE1-ankyrin-G interaction was kept in the kAE1-RhBG cotransfectants, with (wild type) or without (F419A/L420A/D421A) RhBG binding to ankyrin-G (not shown). Likewise, RhBG was still interacting with ankyrin-G in the presence of wild-type kAE1 or kAE1-delABS (not shown). Altogether, these data

![Image](https://example.com/image.png)

**FIGURE 3.** Analysis of the interaction between kAE1 and ankyrin-G by coimmunoprecipitation in situ. A, colocalization of recombinant kAE1 with endogenous ankyrin-G. HEK293 transfected cells grown on poly-L-lysine coverslips and tetracycline-induced for wild-type (kAE1 i) or delABS (delABS i) kAE1 expression were fixed and permeabilized as in Fig. 2, stained with rabbit anti-AE1 (kAE1, red) and mouse anti-ankyrin-G (Ankyrin-G, green), then stained with Alexa Fluor 568 anti-rabbit and Alexa Fluor 488 anti-mouse IgG, respectively, and analyzed by confocal microscopy. Merged, overlay of kAE1 and ankyrin-G immunofluorescence (IF) signals. B, interaction of kAE1 with ankyrin-G. The same cells grown on poly-L-lysine coverslips were either tetracycline-induced (i) or noninduced (ni), treated, and stained with primary antibodies as in A and then subjected to PLA analysis. Samples were mounted in ProLong Gold antifade reagent with DAPI, allowing cell nuclei staining. The red PLA signal was clearly visible in cells expressing wild-type kAE1 (kAE1 i) and absent in those expressing kAE1-delABS (delABS i) or noninduced for kAE1 expression (kAE1 ni). Inset, magnification (\( \times 16 \)) of a selected region of PLA image. Scale bars, 15 \( \mu m \).
show that RhBG and kAE1 are associated not only through their common binding to ankyrin-G but also by a direct interaction between them. RhBG-kAE1 association is independent of their ankyrin-G binding, and the interaction of one transmembrane protein with ankyrin-G is not affected by expression and ankyrin-G binding of the other.

Impact of kAE1 Expression on RhBG Expression and Activity in HEK293 Cells—Considering the above results, expression and NH$_3$ transport function of recombinant RhBG were tested in our inducible kAE1 expression system. Flow cytometry analysis of HEK293 cells cotransfected with kAE1 and RhBG cDNAs, wild-type or mutated on their ankyrin-G binding site, showed no main difference of wild-type RhBG or F419A/L420A/D421A mutant expression, when comparing each cell line in induced and noninduced kAE1 expression conditions (Table 1). Surface expression of RhBG-F419A/L420A/D421A protein was lower than that of wild-type RhBG, most probably because of the disruption of its anchorage to the membrane skeleton. Then NH$_3$ transport activity of RhBG was measured by stopped flow spectrofluorometry, using BCECF-AM as an intracellular fluorescent pH-sensitive probe in the presence of an inwardly directed 20 meq NH$_4^+$ gradient at 15 °C and pH 7.2, as described previously (2, 10). This temperature (instead of the more physiological 37 °C) was used because kinetics at 37 °C are too rapid (much <1 s) to precisely measure NH$_3$ transport. The time course of the fluorescence increase (proportional to intracellular pH increase as determined by a titration on HEK293 cells; see “Experimental Procedures”) was recorded (Fig. 6) and allowed the calculation of alkalinization rate constants $k$ (s$^{-1}$) (Table 1). Comparison of $k$ values between noninduced (ni) and induced (i) conditions for each cell line revealed no significant difference (Mann-Whitney U test). These constants were then corrected taking into account the relative surface expression level of RhBG in each cell line as compared with cells expressing wild-type RhBG and noninduced for kAE1 expression ($k_{AE1 \text{ ni-RhBG}}$, 100%), and by subtracting the passive diffusion constant of cells expressing kAE1 alone ($k_{AE1 \text{i}}$) which was identical to that of noninduced ($k_{AE1 \text{ ni}}$) or nontransfected cells (Table 1, $k = 0.18 \pm 0.02$ s$^{-1}$). Thus, transport efficiencies were comparable between HEK293 cells expressing RhBG without (ni) or with (i) simultaneous expression of wild-type kAE1 or delABS mutant (between 100 and 129%). Moreover, inhibition of NH$_3$ transport in cells expressing an RhBG protein deficient in ankyrin-G binding (RhBG-F419A/L420A/D421A) was not modified by kAE1 expression (between 8 and 13% of residual activity).

Cl$^-$/HCO$_3^-$ Exchange Activity of Recombinant kAE1 in HEK293 Cells—Recently, we described the measurement of the rapid and specific Cl$^-$/HCO$_3^-$ exchange by stably expressed eAE1 in HEK293 cells, using stopped flow spectrofluorometry (19). Likewise, we determined here the Cl$^-$/HCO$_3^-$ exchange capacity of recombinant wild-type kAE1 or delABS mutant by

![FIGURE 4. Analysis of the interaction between RhBG and ankyrin-G by coimmunoprecipitation in situ. A, colocalization of recombinant RhBG with endogenous ankyrin-G. HEK293 cells transfected with wild-type (RhBG) or mutant (F419A/L420A/D421A) RhBG cDNA were grown on poly-L-lysine coverslips, fixed, and permeabilized as in Fig. 2, stained with rabbit anti-RhBG (RhBG, red) and mouse anti-ankyrin-G (Ankyrin-G, green), then stained with Alexa Fluor 568 anti-rabbit and Alexa Fluor 488 anti-mouse IgG, respectively, and analyzed by confocal microscopy. Merged, overlay of RhBG and ankyrin-G immunofluorescence (IF) signals. B, interaction of RhBG with ankyrin-G. The same cells were treated and stained with primary antibodies as in A and then subjected to PLA analysis. Samples were mounted in ProLong Gold antifade reagent with DAPI, allowing cell nuclei staining. The red PLA signal was clearly visible in cells expressing wild-type RhBG and absent in those expressing RhBG-F419A/L420A/D421A. Inset, magnification (×16) of a selected region of PLA image. Scale bars, 15 μm.](https://example.com/Figure4.png)
The methodology, using BCECF-AM and in the presence of inwardly directed 10 meq HCO\(_3^-\)/H\(_2\)CO\(_3\) and outwardly directed 67.5 meq Cl\(^-\)/H\(_2\)CO\(_3\) gradients at 30 °C and pH 7.2. As mentioned above for RhBG activity, experiments were not performed at 37 °C, because anion exchange lasts <1 s at this temperature, precluding an accurate determination of alkalinization rate constants. Using these optimal thermal conditions (30 °C), we observed a fast intracellular alkalinization for cells expressing kAE1 (kAE1, k = 0.25 ± 0.03 s\(^{-1}\)) compared with parental or noninduced cells lacking kAE1 expression (nt and kAE1 ni, k = 0.05 ± 0.02 and 0.04 ± 0.02 s\(^{-1}\), respectively) (Fig. 7, A and B).

After incubation with the anion exchanger inhibitor DIDS, Cl\(^-\)/HCO\(_3^-\) exchange by kAE1 was reduced at the parental HEK293 cell level (Fig. 7, A and B, k = 0.06 ± 0.05 s\(^{-1}\)). HEK293 cells expressing kAE1-delABS (not binding to ankyrin-G) exhibited an alkalinization similar to noninduced or DIDS-treated wild-type kAE1 cells (Fig. 7, A and B, delABS i, k = 0.06 ± 0.02 s\(^{-1}\)), indicating that this mutant protein is not functional. Of note, a kAE1 mutant protein still bearing one of the three ankyrin-G binding sites, i.e., the amino acids 110–120 (major ankyrin-R binding site in eAE1 protein), exhibited the same characteristics as the delABS mutant: no detectable surface expression, no interaction with ankyrin-G in PLA, and no residual Cl\(^-\)/HCO\(_3^-\) exchange activity in HEK293 cells (not

### TABLE 1

| Cell lines | Expression | Alkalization rate constants (k) | Transport efficiency |
|------------|------------|---------------------------------|---------------------|
| nt         | 0          | 0.18 ± 0.02 (3)                | 0                   |
| kAE1 ni    | 0          | 0.18 ± 0.02 (5)                | 0                   |
| kAE1 i     | 0          | 0.18 ± 0.02 (3)                | 0                   |
| kAE1 ni-RhBG | 100          | 0.90 ± 0.10 (7)                | 100                 |
| kAE1 i-RhBG | 98          | 0.93 ± 0.12 (8)                | 106                 |
| delABS ni-RhBG | 95              | 1.06 ± 0.07 (4)               | 129                 |
| delABS i-RhBG | 110          | 0.98 ± 0.09 (5)                | 101                 |
| kAE1 ni-F419A/L420A/D421A | 85          | 0.26 ± 0.05 (5)                | 13                  |
| kAE1 i-F419A/L420A/D421A | 71          | 0.22 ± 0.04 (3)                | 8                   |
| delABS ni-F419A/L420A/D421A | 35          | 0.21 ± 0.01 (3)                | 12                  |
| delABS i-F419A/L420A/D421A | 55          | 0.22 ± 0.02 (3)                | 10                  |

* Relative values to mean fluorescence intensity for kAE1 ni-RhBG.

* The values are means ± S.D.; n values are in parentheses.

* Relative values of alkalization rate constants corrected for membrane expression of RhBG in kAE1 ni-RhBG cell line and after subtraction of the passive diffusion constant (kAE1 ni).
shown). This result confirmed that these 11 residues constitute only a secondary ankyrin-G binding site in the kAE1 protein, as deduced from our yeast two-hybrid data.

Expression of Recombinant Wild-type and Mutant kAE1 in Polarized MDCK Cells—To validate the data from experiments in HEK293 cells, kAE1 expression was investigated in polarized epithelial MDCK cells. Because several attempts to obtain tetracycline-inducible expression of kAE1 after stable transfection have been unsuccessful, subconfluent MDCK cells were transiently transfected with wild-type or delABS mutant kAE1 cDNA, allowed to polarize for 4 days, labeled with rabbit anti-

AE1 and mouse anti-ankyrin-G, and analyzed by immunofluorescence confocal microscopy (Fig. 8). Wild-type kAE1 (red, left panels) was properly expressed at the basolateral membrane (XY horizontal and XZ sections) as compared with ZO-1 staining (green, bottom panel) representative of the tight junctions and colocalized with endogenous basolateral ankyrin-G (merged, second left panel). In contrast, the delABS mutant exhibited an exclusive intracellular labeling indicating a total retention on cytoplasmic membranes (red, right panels) and did not colocalize with ankyrin-G (merged, second right panel). In polarized epithelial cells, the delABS protein is thus unable to anchor to the plasma membrane. These results, together with PLA data in HEK293 cells, constitute a strong evidence of a physical RhBG-kAE1-ankyrin-G complex in kidney epithelial cells.

DISCUSSION

Up to now, the erythroid Rh-eAE1-ankyrin-R complex was considered not to have a counterpart in epithelial kidney cells. Indeed, although we have previously shown that RhBG interacts with ankyrin-G (1), it was generally assumed that kAE1 was not able to bind to this membrane adaptor protein because of a predicted altered structure of its truncated N terminus. In this study, we provided evidence that kAE1 actually binds not only to ankyrin-G but also to RhBG. This structural complex is also functional because interaction with ankyrin-G appears to be essential for the activity of its two transmembrane partners.

By yeast two-hybrid studies, we showed a direct interaction in vitro between the cytoplasmic N terminus of kAE1 and the D3-D4 repeat subdomains of the membrane binding domain of ankyrin-G. The D3-D4 domains of ankyrins associate with a number of membrane proteins, for example: Na⁺,K⁺-ATPase (26), voltage-gated sodium channels (27), nervous system cell adhesion molecules (28)), and particularly RhBG (1) and eAE1 (29). Of note, eAE1 has been shown to also interact with the D2 domain of ankyrin-R, and the region involved in this binding was located in the extreme N terminus of eAE1, which is actually lacking in kAE1 protein (i.e. amino acids 1–65) (29, 30).

Mapping of ankyrin-G binding determinants in the N terminus
Moreover, the three ankyrin species (R, B, and G) display 17% redundancy of ankyrin binding regions in the two proteins. eAE1 (16, 17, 21), which probably accounts for the very limited interaction of kAE1 cytoplasmic N terminus compared with that of other biophysical analyses suggested a different conformation (for review see Ref. 34). The HEK293 cell model was mainly used in our study because, despite many attempts, we could not establish an inducible kAE1 expression in stably transfected MDCK cells. Moreover, HEK293 cells have been proved to be a more accurate model than MDCK cells in functional studies using stopped flow spectrofluorometry (2, 10). The kAE1-delABS mutant was not detectable at the cell surface, but in immunofluorescence experiments using anti-cytosolic N terminus antibody, this protein was revealed mainly on cytoplasmic structures and partly at the plasma membrane where it colocalized with endogenous ankyrin-G. These data suggest that kAE1-delABS can traffic, although inefficiently, to the plasma membrane. The negative PLA results obtained with this mutant may thus be considered as relevant to a disruption of interaction between its cytoplasmic N terminus and ankyrin-G. Importantly, the reliability of PLA data on kAE1 was strengthened by showing that the previously demonstrated interaction of RhBG with ankyrin-G (1) could be also evidenced here by PLA. RhBG and kAE1 are associated not only through their common interaction with ankyrin-G but also directly, as shown by PLA. Similarly, proteins of the erythroid Rh complex (Rh and RhAG) coimmunoprecipitate with eAE1 in the RBC membrane (4). We also observed that ankyrin-G binding to RhBG and/or kAE1 is not a prerequisite to their own association.

In our HEK293 cotransfectant cells, RhBG plasma membrane expression was not modulated by kAE1 coexpression. Thus, in a nonpolarized cell system, RhBG and kAE1 are most probably independently expressed and targeted to the plasma membrane. Previously, we proposed a model in which phosphorylation of Tyr429 in the C terminus of RhBG is essential for its targeting to the basolateral membrane of kidney epithelial cells, and then Tyr429 dephosphorylation allows anchoring of the protein to the underlying spectrin-based skeleton via ankyrin-G (2). Likewise, kAE1 basolateral membrane location in polarized MDCK cells has been shown to be controlled by reversible phosphorylation of two tyrosine residues of the protein, and the authors suggested that this regulation operates in vivo according to the systemic acid-base homeostasis (35). Moreover, our present data in this kidney epithelial cell model clearly showed that the kAE1-delABS mutant was exclusively retained on cytoplasmic structures, similarly to the RhBG-F419A/L420A/D421A mutant previously reported (1). We could therefore anticipate that RhBG and kAE1 are also inde-
Hindley, C., Morgan, A., and Bell, A. J. (2007) Ankyrin–G relies on ankyrin–B for cardiac Nav1.5 and neurofascin. Proc. Natl. Acad. Sci. U.S.A. 104, 8661–8666.

10. Baines, A. J., and Bennett, V. (2001) Spectrin and ankyrin-based pathways: the biology of ankyrin–G. Annu. Rev. Physiol. 63, 515–548.

11. Liu, Z., Peng, J., Mo, R., Hui, C., and Huang, C. H. (2001) Rh type B glycoprotein is a new member of the Rh superfamily and a putative ammonium transport protein. Proc. Natl. Acad. Sci. U.S.A. 98, 1353–1357.

12. Bennett, V., Cartron, J. P., Le Van Kim, C., and Colin, Y. (2005) The renal RhBG anion exchanger: functional studies using a flocculent-stained cell sorter Influx 500 (BD Biosciences), and Julien Picot and Sylvain Bigot (Institut National de la Transfusion Sanguine, Paris, France) for flow cytometry analysis. We also thank Dr. Wassin El Nemer (INSERM U1134) and Dr. Jean-Philippe Semblat (Institut National de la Transfusion Sanguine, Paris, France) for helpful advice on PLA experiments and immunofluorescence confocal microscopy, respectively.

Acknowledgments—We thank Nicole Boggetto (Flow cytometry platform, Institut Jacques-Monod, Paris, France) for cell sorting experiments using a fluorescence-activated cell sorter Influx 500 (BD Biosciences), and Julien Picot and Sylvain Bigot (Institut National de la Transfusion Sanguine, Paris, France) for flow cytometry analysis. We also thank Dr. Wassin El Nemer (INSERM U1134) and Dr. Jean-Philippe Semblat (Institut National de la Transfusion Sanguine, Paris, France) for helpful advice on PLA experiments and immunofluorescence confocal microscopy, respectively.

REFERENCES

1. Lopez, C., Métral, S., Eladari, D., Drevensek, S., Gane, P., Chambrey, R., Bennett, V., Cartron, J. P., Le Van Kim, C., and Colin, Y. (2005) The ammonium transporter RhBG: requirement of a tyrosine-based signal and ankyrin–G for basolateral targeting and membrane anchoring in polarized kidney epithelial cells. J. Biol. Chem. 280, 8221–8228.

2. Sohet, F., Colin, Y., Genetet, S., Ripoche, P., Métal, S., Le Van Kim, C., and Lopez, C. (2005) Phosphorylation and ankyrin–G binding of the C-terminal domain regulate targeting and function of the ammonium transporter RhBG. J. Biol. Chem. 283, 26557–26567.

3. Alper, S. L. (2009) Molecular physiology and genetics of Na+/H+-exchange anion transporters. Exp. Biol. 212, 1672–1683.

4. Bruce, L. J., Beckmann, R., Ribeiro, M. L., Peters, L. L., Chasis, J. A., Delaunay, J., Mohandas, N., Anstee, D. J., and Tanner, M. J. (2003) A band 3-based macromolecular complex of integral and peripheral proteins in the RBC membrane. Blood 101, 4180–4188.

5. Ripoche, P., Bertrand, O., Gane, P., Birkenmeier, C., Colin, Y., and Carton, J. P. (2004) Human Rhesus-associated glycoprotein mediates facilitated transport of NH₃ into red blood cells. Proc. Natl. Acad. Sci. U.S.A. 101, 17222–17227.

6. Nicolas, V., Le Van Kim, C., Gane, P., Birkenmeier, C., Carton, J. P., Colin, Y., and Mouro-Chateloup, I. (2003) Rh-RHAG/ankyrin–R, a new interaction site between the membrane bilayer and the red cell skeleton, is impaired by Rh(null)-associated mutation. J. Biol. Chem. 278, 25526–25533.

7. Brosius, F. C., 3rd, Alper, S. L., Garcia, A. M., and Lodish, H. F. (1989) The major kidney band 3 gene transcript predicts an amino-terminal truncated band 3 polypeptide. J. Biol. Chem. 264, 7784–7787.

8. Alper, S. L., Natale, J., Gluck, S., Lodish, H. F., and Brown, D. (1989) Subtypes of intercalated cells in rat kidney collecting duct defined by antibodies against erythroid band 3 and renal vacuolar H+-ATPase. Proc. Natl. Acad. Sci. U.S.A. 86, 5429–5433.

9. Verlander, J. W., Miller, R. T., Frank, A. E., Royaux, I. E., Kim, Y. H., and Weiner, I. D. (2003) Localization of the ammonium transporter proteins RhBG and RhCG in mouse kidney. Am. J. Physiol. Renal Physiol. 284, F323–F337.

10. Zidi-Yahiaoui, N., Mouro-Chanteloup, I., D’Ambrosio, A. M., Lopez, C., Gane, P., Le van Kim, C., Carton, J. P., Colin, Y., and Ripoche, P. (2005) Human Rhesus B and Rhesus C glycoproteins: properties of facilitated ammonium transport in recombinant kidney cells. Biochem. J. 391, 33–40.

11. Liu, Z., Peng, J., Mo, R., Hui, C., and Huang, C.-H. (2001) Rh type B glycoprotein is a new member of the Rh superfamily and a putative ammonium transporter in mammals. J. Biol. Chem. 276, 1424–1433.

12. Bennett, V., and Baines, A. J. (2001) Spectrin and ankyrin-based pathways: metazoan inventions for integrating cells into tissues. Physiol. Rev. 81, 1353–1392.

13. Mohler, P. J., Gramolini, A. O., and Bennett, V. (2002) Ankyrins. J. Cell Sci. 115, 3961–3972.

The Renal RhBG-kAE1-Ankyrin-G Complex

The association of the three proteins is functional because RhBG and possibly kAE1 carry out their transport activity only when interacting with the spectrin-based skeleton through ankyrin-G. Future investigations will indicate whether the RhBG-kAE1-ankyrin-G complex could fulfill the functions of a metabolon that would participate in the regulation of acid/base homeostasis by excreting ammonium and protons in urine.
The Renal RhBG-kAE1-Ankyrin-G Complex

115, 1565–1566
14. Ding, Y., Casey, J. R., and Kopito, R. R. (1994) The major kidney AE1 isoform does not bind ankyrin (Ank1) in vitro: an essential role for the 79 NH2-terminal amino acid residues of band 3. J. Biol. Chem. 269, 32201–32208
15. Wang, C. C., Moriyama, R., Lombardo, C. R., and Low, P. S. (1995) Partial characterization of the cytoplasmic domain of human kidney band 3. J. Biol. Chem. 270, 17892–17897
16. Zhang, D., Kiyatkin, A., Bolin, J. T., and Low, P. S. (2000) Crystallographic structure and functional interpretation of the cytoplasmic domain of erythrocyte membrane band 3. Blood 96, 2925–2933
17. Pang, A. J., Bustos, S. P., and Reithmeier, R. A. (2008) Structural characterization of the cytosolic domain of kidney chloride/bicarbonate anion exchanger 1 (kAE1). Biochemistry 47, 4510–4517
18. Frumence, E., Genetet, S., Ripoche, P., Iolascon, A., Andolfo, I., Le Van Bridu, J., Parry, C. S., Smith, J. A., Lybrand, T. P., Hustedt, E. J., and Beth, A. H. (2006) Clarkin, J. Q., and Bennett, V. (2000) Crystal structure of a 12 ANK repeat stack from human ankyrinR. J. Biol. Chem. 275, 23952–23958
19. Davis, J. Q., and Bennett, V. (2005) Ankyrin-B coordinates kAE1 membrane expression and function in heart. J. Biol. Chem. 285, 28723–28730
20. Su, Y., Al-Lamki, R. S., Blake-Palmer, K. G., Best, A., Golder, Z. J., Zhou, A., and Karet Frankl, F. E. (2015) Physical and functional links between anion exchanger-1 and sodium pump. J. Am. Soc. Nephrol. 26, 400–409
21. Zhou, D., Lambert, S., Malen, P. L., Carpenter, S., Boland, L. M., and Bennett, V. (1998) Ankyrin-G is required for clustering of voltage-gated Na channels at axon initial segments and for normal action potential firing. J. Cell Biol. 143, 1295–1304
22. Jenkins, S. M., and Bennett, V. (2001) Ankyrin-G coordinates assembly of the spectrin-based membrane skeleton, voltage-gated sodium channels, and L1 CAMs at Purkinje neuron initial segments. J. Cell Biol. 155, 739–746
23. Rasmussen, H. B., Freksa-Jensen, C., Jensen, C. S., Jensen, H. S., Jorgensen, N. K., Misonou, H., Trimmer, J. S., Olesen, S. P., and Schmitt, N. (2007) Requirement of subunit co-assembly and ankyrin-G for M-channel localization at the axon initial segment. J. Cell Sci. 120, 953–963
24. Kizhatil, K., Davis, J. Q., Davis, L., Hoffman, J., Hogan, B. L., and Bennett, V. (2007) Ankyrin-G is a molecular partner of E-cadherin in epithelial cells and early embryos. J. Biol. Chem. 282, 26552–26561
25. Mohler, P. J., Davis, J. Q., and Bennett, V. (2005) Ankyrin-B mutation causes type 4 long-QT cardiac arrhythmia and sudden cardiac death. Nature 421, 634–639
26. Mohler, P. J., Splawski, I., Napolitano, C., Bottelli, G., Sharpe, L. M., and Söderberg, O. (2014) Analysis of protein interactions in situ by proximity ligation assays. Curr. Top. Microbiol. Immunol. 377, 111–126
27. Williamson, R. C., Brown, A. C., Mawby, W. J., and Toye, A. M. (2008) Human kidney anion exchanger 1 localisation in MDCK cells is controlled by the phosphorylation status of two critical tyrosines. J. Cell Sci. 121, 3422–3432
28. Doshi, J., Chien, C. T., Sternglanz, R., and Fields, S. (1993) Identification of false positives that arise in using the two-hybrid system. BioTechniques 14, 920–924