Interaction between the N-terminal Domain of Gastric H,K-ATPase and the Spectrin Binding Domain of Ankyrin III*

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We screened a cDNA bank of rabbit gastric fundic mucosa by two-hybrid assays looking for binding partners of the N-terminal domain of the rabbit gastric H,K-ATPase. We extracted five clones sharing more than 90% sequence identity. The longest clone codes for a protein sharing a high identity (96 and 96.8%, respectively) with a fragment of the membrane domain, from Arg-835 to Ser-873, plus the major part of the “spectrin binding domain” going from Glu-874 to Leu-1455 of human and mouse ankyrin III. We conclude that the membrane and spectrin binding domains of the rabbit ankyrin III are candidates for the binding partner of the N-terminal domain of the rabbit gastric H,K-ATPase. To validate the ankyrin-ATPase interaction and to test its specificity, we produced both domains in yeast and bacteria, coimmunoprecipitated them with an anti-ATPase antibody, and copurified them by affinity chromatography. The sequence of rabbit ankyrin III was not known, and this is the first report demonstrating that the ankyrin III and the H,K-ATPase interact with no intermediate. The interaction involves the N-terminal domain of the ATPase on one hand and the spectrin binding domain of the ankyrin on the other.

The sequestration of molecules in intracellular compartments is a fundamental capacity of all living cells, and in polarized cells, the sequestration is directional. For instance, the plasma membrane that envelops all epithelial cells has two domains, basal and apical. Both correspond to different components. Membrane proteins must be targeted to one or the other domain after synthesis. The mechanisms of targeting are not yet fully understood, but the cell skeleton has a major role, at least by leading proteins to, and holding them in, their membrane domains (1–3).

Linkers are required for the interaction of some membrane proteins such as AE1 (anion exchange 1 or band 3 protein) and Na,K-ATPase with skeleton components. The AE1 and Na,K-ATPase linker is ankyrin (4–6). Smith et al. (7) previously demonstrated the colocalization of ankyrin and H,K-ATPase in parietal cells and the co-immunoprecipitation of the gastric pump with exogenous erythrocyte ankyrin. This supports the idea that the gastric ATPase can bind ankyrin as its parent protein, the Na,K-ATPase, does. However, it is not known how ATPase and ankyrin interact and whether minor cellular components act as linkers.

In this study, we demonstrate that the rabbit gastric H,K-ATPase binds to ankyrin III. We map the binding site in the N-terminal (Nt)1 domain of ATPase on one hand and in the spectrin binding domain of ankyrin III on the other hand. The spectrin binding domain of the rabbit ankyrin III is cloned and has more than 90% identity with the analogous domains of the mouse and human ankyrin.

EXPERIMENTAL PROCEDURES

Library Construction—Stomachs were collected from Nembutal-injected rabbits. Gastric fundic mucosa was scraped and immediately frozen in liquid nitrogen. Samples were sent to CLONTECH Laboratories, Inc. for preparation of the cDNA bank with (dT) and random oligomers to limit the overexpression of 3’ extremities of mRNA.

cDNAs were ligated in the prey plasmid pGAD 10 using EcoRI adapters and incorporated in DH5α Escherichia coli strain by efficiency transformation. The library had about 1.7 million independent clones, of which 30% were empty.

Construction of the Bait Plasmid—The DNA fragment for the first 98 residues of the rabbit gastric H,K-ATPase was obtained by PCR from the complete cDNA. The PCR conditions were as follows: (i) denaturation at 95 °C for 10 min; (ii) 35 cycles each consisting of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and elongation at 72 °C for 30 s using Vent polymerase (New England Biolabs); and (iii) a final elongation at 72 °C for 10 min.

The PCR product with the expected size of 320 base pairs was digested with BamHI and SalI and purified on a 4% agarose gel with a QIAquick gel extraction kit (Qiagen) according to the manufacturer’s instructions. It was inserted in the BamHI/SalI site of the pFBL23 plasmid (a gift of J. Camonis, Institut Curie, Paris, France), and the product was sequenced (Genome Express, Grenoble, France).

Library Screening—Yeast strain HF7c (a donation of J. Camonis, Institut Curie, Paris, France) was transformed to Tryp prototrophy with pFBL23-Nt using the lithium acetate method (8). The resulting strain was further transformed to Trp, Leu, His prototrophy with the pGAD10 library plasmid. The transformation mix (100 μl) was plated on a 10-cm Petri dish and incubated at 30 °C for 5 days. Aliquots of each mix were taken before plating and used to determine the transformation efficiency on Trp- and Leu-free medium.

His+ colonies were screened for β-galactosidase activity using the filter assay method. Positively transformed colonies eliciting 50-unit absorbance at 600 nm were blotted onto Whatman 3MM paper. The filter was frozen in liquid nitrogen and thawed at room temperature.

***The abbreviations used are: Nt, N-terminal or N terminus; PCR, polymerase chain reaction; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; SBD, spectrin binding domain; aa, amino acids.
The freeze-thaw cycle was repeated three times. The filter was soaked in 60 mM NaHPO4, 40 mM Na2HPO4, 10 mM KCl, and 1 mM MgCl2 containing 2 mg/mL 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) and then incubated overnight at 37 °C. The clones expressing the β-galactosidase were selected only when the Lex A DNA binding domain was present.

Plasmids were extracted and recovered by electroporation in the leu E. coli strain 1066 (a gift of C. Cullin, Centre Génétique Moléculaire, CNRS, Gif sur Yvette, France). The plasmids were then used to transform the H7 strains alone or with pFBL23 in the presence of Nt or a control protein (laminin, hydrophobic protein). The clones expressing the Nt of H,K-ATPase were selected when they were positive for Trp, Leu, His prototrophy and β-galactosidase activity. The plasmids were sequenced (Genome Express).

Coimmunoprecipitation of the Two-hybrid Proteins Expressed from Yeast—Yeast extracts were prepared from liquid cultures in SD-Trp-Leu-His medium (CLONTECH Laboratories, Inc.). Cultures were grown to an absorbance at 600 nm of 0.8. The yeast extracts were centrifuged (5,000 g for 20 min), and the supernatants were incubated for 4 h at 4 °C in the presence of 10 μg of the anti-Nt H,K-ATPase antibody (95A3). Then, 10 μg of IgG2 against mouse IgG were added and incubated for 4 h at 4 °C. Immune complexes were collected on protein A-Sepharose (Amersham Pharmacia Biotech), separated by SDS-PAGE, and blotted onto nitrocellulose.

Construction of Vectors Expressing the Nt of H,K-ATPase—The DNA sequence encoding for the Nt (amino acids 1–98) was obtained from the complete cDNA of rabbit gastric H,K-ATPase cloned in Bluescript vector by PCR amplification. The PCR conditions were the same as for the bait plasmid. The final product of 320 base pairs was digested with NcoI and BglII and purified on a 4% agarose gel with a QIAquick gel extraction kit (Qiagen) using the manufacturer’s instructions. This cDNA was inserted between the restriction sites NcoI and BglII of the prokaryotic expression vector pQE 60 (Qiagen) to generate a plasmid encoding for an in-frame fusion protein composed of Nt and 6 histidines in the C-terminal extremity. The plasmid with the insert was checked by sequencing (Genome Express).

Expression of the Nt of H,K-ATPase—JS 238 E. coli strain (a gift of O. Fayet, Laboratoire de Microbiologie et Génétique Moléculaire, Toulouse, France) overexpressing the lactose operon repressor (LacI q mut) was transformed by the CaCl2 method with pQE 60 containing mouse ankyrin III sequences (Fig. 1). The sequence shows little variation between the human and rabbit ankyrin III domain of the rabbit gastric H,K-ATPase, we selected the membrane and spectrin binding domains of the rabbit ankyrin III. A 320-bp fragment of ankyrin was obtained by the two-hybrid assays; (i) 100 μg of bacterial extract containing the Nt plus 2 μl of yeast extract containing the SBD; (ii) 100 μg of bacterial extract containing the Nt plus 2 μl of yeast extract containing the SBD; (iii) 100 μg of bacterial extract without the Nt plus 2 μl of yeast extract without the SBD.

Ten μg of 95A3 were added and incubated for 4 h at 4 °C. Then, 10 μg of IgG2 anti-mouse IgG were added for 4 h at 4 °C. Immune complexes were collected on protein A-Sepharose (Amersham Pharmacia Biotech), separated by SDS-PAGE, and blotted onto nitrocellulose by electro-elution, and analyzed.

Affinity Chromatography—Four columns of 500 μL of nickel resin (superflow, Qiagen) were prepared and equilibrated with 50 mM NaHPO4, 300 mM NaCl, pH 8. In columns A and B, 10 μl of the bacterial extract containing the Nt domain of the ATPase were poured. In columns C and D, 10 μl of the bacterial extract free of the Nt domain of the ATPase were poured. All columns were washed according to the manufacturer’s protocol. Three liters of a yeast culture containing the SBD fragment of ankyrin were poured in columns A and C, and 3 liters of the same culture medium free of SBD were poured in columns B and D. All columns were rinsed with 50 mM NaHPO4, 300 mM NaCl, pH 8 and eluted with 500 μL imidazole. The four eluates were analyzed by SDS-PAGE and Western blots.

Western Blots—Nitrocellulose sheets were incubated in 1× phosphate-buffered saline, 0.1% bovine serum albumin, 0.1% Tween 20 in the presence of antibodies, either the 95A3 mouse IgG monoclonal antibody against the Nt of the H,K-ATPase or the rabbit IgG polyclonal antibody against the SBD of ankyrin III (a gift of K. Kordeli, Laboratoire de Biologie Cellulaire, Institut J. Monod, Faculté de Jussieu, Paris, France). For staining, secondary species antibodies conjugated with horseradish peroxidase were used, and the stain was revealed with the Pierce chemiluminescence kit (reference number 34080, Pierce) using the manufacturer’s protocol.

RESULTS

Screening of the Gastric cDNA Bank

Seven million gastric cDNA clones were screened against the Nt fragment of the H,K-ATPase by the two-hybrid assays; 7 million is four times the amount of independent clones of the cDNA bank and should thus be statistically sufficient to screen all the different cDNA fragments. Selected were 144 positive clones, which amount to 20 positive clones per million. Five clones had more than 90% identity. Three were identical (1,791 base pairs) and corresponded to 597 aa. The two others were shorter and overlapped the previous sequence on 498 aa (1,494 base pairs). Those fragments have a high identity (≥96%) with the “membrane domain” of the human and mouse ankyrin III from Arg-835 to Ser-873 and with the major part of the spectrin binding domain going from Glu-874 to Leu-1455 (9).

By looking for a protein interacting with the N-terminal domain of the rabbit gastric H,K-ATPase, we selected the membrane and spectrin binding domains of the rabbit ankyrin III. The sequence shows little variation between the human and mouse ankyrin III sequences (Fig. 1).

Characterization of the ATPase-Ankyrin Interaction

Coimmunoprecipitation of Fusion Proteins—To check whether the activation of the reporter genes of the two-hybrid assay is the consequence of a specific interaction between the N-terminal domain of the ATPase and the ankyrin fragment, we first tested whether the Nt domain (expressed as Lex A DNA binding domain-Nt) and the SBD domain (expressed as SBD-gal4-activating domain) can coprecipitate when they are
incubated in the presence of a specific anti-ATPase antibody. Both proteins are recovered in the same precipitate (Fig. 2, lanes 1 and 4). This precipitate is absent when the extracts of the bait Lex A DNA binding domain-Nt cotransfected with gal4-activating domain (SBD-free) and of the prey SBD-gal4-activating domain cotransfected with Lex A DNA binding domain (Nt of ATPase-free) are used. This absence indicates that the immunoreaction is specific. Thus the activation of the reporter gene of the two-hybrid assays is due to the direct interaction of the Nt domain of the gastric ATPase with the ankyrin fragment.

**Coimmunoprecipitation of the Isolated Domains**—To corroborate the previous conclusions, both proteins were expressed in the absence of their fusion partners: the activating and binding domains. The SBD (597 aa) was expressed in yeast, and the Nt of the ATPase (98 aa) was expressed in *E. coli*. After mixing the yeast and *E. coli* extracts, the Nt anti-ATPase antibody (95A3) was added. Precipitation was induced as described under “Experimental Procedures.” The SBD of ankyrin was recovered in the ATPase precipitate (Fig. 3, lanes 1 and 2). If the ATPase is absent, the ankyrin fragment is not precipitated, which implicates that the coprecipitation requires a specific interaction (Fig. 3, lanes 3–6).

**Affinity Chromatography**—To further check the specificity of the interaction, we tested whether the Nt-SBD complex was recovered by affinity chromatography on nickel resin (Fig. 4). In two columns (A and B), we poured the bacterial extract containing the Nt fragment of the ATPase tagged with 6 histi-

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**Table:**

| Protein          | Accession   | Identity | Sequence |
|------------------|-------------|----------|----------|
| ANK III H.sapiens| Q12965      | 486      | EVLDMDSDDGVKANAPENLSDQGEYISDVYESEDAMTGDQDK  |
| ANK III mouse    | O61399      | 526      | EVLDMDSDDGVKANAPENLSDQGEYISDVYESEDAMTGDQDK  |
| rabbit sequence  | 1           |          |          |
| ANK III H.sapiens| Q12965      | 486      | YLGFDQDLKELGDSLPSLEAGYMGFSLGARSALSLSFSNDFDSY |
| ANK III mouse    | O61399      | 486      | YLGFDQDLKELGDSLPSLEAGYMGFSLGARSALSLSFSNDFDSY |
| rabbit sequence  | 1           |          |          |
| ANK III H.sapiens| Q12965      | 254      | TLRNRRSSYDSMMIEELLVPSEKQHLTFREDSSDNSLHYIS   |
| ANK III mouse    | O61399      | 254      | TLRNRRSSYDSMMIEELLVPSEKQHLTFREDSSDNSLHYIS   |
| rabbit sequence  | 254         |          |          |
| ANK III H.sapiens| Q12965      | 130      | WAAADTVLNVNLVSSPISHFGFLVSMVDSARGSSMRGSRHSGM |
| ANK III mouse    | O61399      | 130      | WAAADTVLNVNLVSSPISHFGFLVSMVDSARGSSMRGSRHSGM |
| rabbit sequence  | 130         |          |          |
| ANK III H.sapiens| Q12965      | 1022     | VEVMPGAPAGOFGLPVIEIPHFOSMRRKIRILRLSENGET    |
| ANK III mouse    | O61399      | 1022     | VEVMPGAPAGOFGLPVIEIPHFOSMRRKIRILRLSENGET    |
| rabbit sequence  | 1022        |          |          |
| ANK III H.sapiens| Q12965      | 1022     | KEKEVQFNSKNDLELLTNGMEDELSEPLGGKRKIRIKIKK  |
| ANK III mouse    | O61399      | 1022     | KEKEVQFNSKNDLELLTNGMEDELSEPLGGKRKIRIKIKK  |
| rabbit sequence  | 1022        |          |          |
| ANK III H.sapiens| Q12965      | 1207     | ALRDKRVLQGDAPPICPDIVKVLGKATLLSPQVTEPRPRR   |
| ANK III mouse    | O61399      | 1207     | ALRDKRVLQGDAPPICPDIVKVLGKATLLSPQVTEPRPRR   |
| rabbit sequence  | 1207        |          |          |
| ANK III H.sapiens| Q12965      | 1207     | KEKEVQFNSKNDLELLTNGMEDELSEPLGGKRKIRIKIKK  |
| ANK III mouse    | O61399      | 1207     | KEKEVQFNSKNDLELLTNGMEDELSEPLGGKRKIRIKIKK  |
| rabbit sequence  | 1207        |          |          |
| ANK III H.sapiens| Q12965      | 1207     | TSPAQKGDTTTPLTFKICOVSFTTNTXSARFLWADCHQVL   |
| ANK III mouse    | O61399      | 1207     | TSPAQKGDTTTPLTFKICOVSFTTNTXSARFLWADCHQVL   |
| rabbit sequence  | 1207        |          |          |
| ANK III H.sapiens| Q12965      | 1207     | ETYGLATQLYRESILCVPYMAKAFVFAKMKNDPVESLRLCFCM |
| ANK III mouse    | O61399      | 1207     | ETYGLATQLYRESILCVPYMAKAFVFAKMKNDPVESLRLCFCM |
| rabbit sequence  | 1207        |          |          |
| ANK III H.sapiens| Q12965      | 1207     | TDDKVDKTEOSNFLKILEVEARSKIDIVLGSKPYYDCYGNLA |
| ANK III mouse    | O61399      | 1207     | TDDKVDKTEOSNFLKILEVEARSKIDIVLGSKPYYDCYGNLA |
| rabbit sequence  | 1207        |          |          |
| ANK III H.sapiens| Q12965      | 1207     | PLTKGQQLQVFNSFLFSENRLPSKIKIRDTSSEQCRPSLSFL |
| ANK III mouse    | O61399      | 1207     | PLTKGQQLQVFNSFLFSENRLPSKIKIRDTSSEQCRPSLSFL |
| rabbit sequence  | 1207        |          |          |

**Fig. 1.** Alignment of the rabbit sequence with the sequences of the SBD of human and mouse ankyrin III. The rabbit sequence is the reference, and the nonidentical residues are in gray boxes. *H. sapiens*, *Homo sapiens*. 

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*Individual sections of the text have been removed to ensure the natural reading of the document.*
The stained band is the Nt fragment of the ATPase. The stained band is as in lane 4.

All columns were then rinsed and eluted. The presence of the Nt fragment of the ATPase and of the SBD fragment of ankyrin in the elution was demonstrated by SDS-PAGE and by Western blots. The fractions eluted from column A contain both proteins (Fig. 4a, lanes 1 and 2). Similar bands are absent from the elution of column C (Fig. 4c, lanes 1 and 2) and of column D (result not shown). Therefore, the Nt domain of the ATPase binds to the nickel resin and is able to hold back the SBD fragment. Moreover, the Western blot of the fractions loaded on, and eluted from, columns B, C, and D (Fig. 4, b and c; results from column D are not shown) argue for the specificity of the interaction because, in the absence of the Nt, of the SBD, or of both fragments, no immunoreactive protein is held back on the columns, as revealed by the antibodies.

Therefore, we conclude that the interaction between ATPase and ankyrin fragments is specific. The Nt domain of the gastric ATPase and the SBD of rabbit ankyrin III are responsible for this interaction. Because the Nt and SBD fragments are the only proteins recovered by affinity chromatography, we further conclude that the interaction is direct and does not require a third partner (Fig. 5).

DISCUSSION

Looking for cytoplasm partners of the N-terminal domain of the rabbit gastric H,K-ATPase, we screened a cDNA bank of rabbit fundic mucosa by two-hybrid assays. We identified the SBD of rabbit ankyrin III. Previous studies strongly suggested an interaction between the H,K-ATPase and an ankyrin. Smith et al. (7) demonstrated by indirect immunofluorescence and by confocal microscopy on isolated gastric glands that the H,K-ATPase colocalized with ankyrin in resting and stimulated parietal cells. In the resting cells, the distribution of both proteins is diffuse in the cytoplasm, whereas after stimulation, they are both located at the apical membrane. In the same work, Smith et al. (7) demonstrated that exogenous erythrocyte ankyrin coimmunoprecipitates with the parietal cell microsomes. Because the main protein of gastric microsomes is the proton pump, this H,K-ATPase was a good candidate for an interaction with ankyrin.

In this work we make the first direct demonstration that the ankyrin III and the H,K-ATPase interact and that their interaction does not require a third partner. Moreover, we demonstrate that the 80 residues of the N terminus of the α subunit of the gastric H,K-ATPase bind to a fragment of ankyrin that contains the spectrin binding domain of ankyrin.

It has been demonstrated that the Na,K-ATPase and ankyrin interact by way of several sites (10). The ATPase sites were mapped on the second (aa 140–290) and catalytic (aa 345–784) loops of the α subunit. It was further demonstrated that the ATPase interacts with the repeat domain (89 kDa) and the SBD of ankyrin (10). The existence of at least two sites explains the high affinity ($K_d$, 25 nm) of the complex, and the destruction of
one site decreases the affinity constant by 20-fold. The gastric H,K-ATPase and the Na,K-ATPase are two homologous ionic pumps. They have a similar structure, with two subunits sharing a high degree of sequence identity (60–65% on the whole sequence). Homology is high, especially in the domains carrying the ankyrin Na,K-ATPase interaction sites. Therefore, the gastric H,K-ATPase could have ankyrin binding sites on the second loop (aa 155–300) and/or the catalytic domain (aa 357–777). Indeed, the catalytic domain of the H,K-ATPase has a 19-residue fragment conserved in the Na,K-ATPase and in the band 3 protein also. This fragment has been identified as an ankyrin binding site.

By two-hybrid assays, we tested whether loop II and the catalytic domain of the gastric ATPase interact with the SBD of ankyrin III. The ATPase fragments (residues Gln-161 to Thr-293 for loop II and Thr-357 to Arg-777 for the catalytic loop) failed to interact with the SBD. Two explanations can be offered. Either none of those H,K-ATPase domains binds to the SBD of ankyrin III, or the two-hybrid assays are inefficient. The latter hypothesis cannot be ruled out because, if the N-terminal of the ATPase is short and polar and thus has a good probability to be expressed as a native fold, loop II and the catalytic domain are longer and more hydrophobic. The expression of hydrophobic fragments often leads to inclusion bodies because the native fold is not reached.2 Lastly, it could be argued that this part of the H,K-ATPase binds to other domains of ankyrin (repeat domain, regulatory domain) that we did not use in our assays.

The N-terminal domain of the H,K-ATPase and the spectrin binding domain of ankyrin interact. This interaction is specific to the gastric pump, because the N terminus of the Na,K-ATPase does not interact. Actually, no interaction of the N terminus of the Na,K-ATPase was detected when the whole ankyrin III was used. The N-terminal fragments of both ionic pumps are the most different parts of their sequences. This could indicate different functions. The N terminus of the H,K-ATPase could be a regulatory element in the activation of parietal cells by acting in the translocation of the pump to the apical membrane. Indeed, this phenomenon is specific to the H,K-ATPase. It relies upon the recruitment of the gastric parietal cell skeleton by as yet unclear mechanisms. Ankyrin III could be a piece of the puzzle.

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Fig. 4. Western blots of the 11% SDS-PAGE of the fractions eluted from columns A, B, and C. a, column A was loaded with the bacterial extract containing the Nt of H,K-ATPase and rinsed, and the yeast extract containing the SBD of rabbit ankyrin III was poured. Lanes are as in a. b, column B was loaded with the bacterial extract containing the Nt of H,K-ATPase and rinsed, and then the yeast extract containing the SBD fragment was poured. Lanes are as in a. c, column C was loaded with the bacterial extract containing the Nt of H,K-ATPase and rinsed, and then the yeast extract containing the SBD fragment was poured. Lanes are as in a.

Fig. 5. Coomassie Blue staining of the SDS-PAGE of the fractions eluted from columns A–D coupled with the corresponding Western blots. Lane T, initial bacterial extract; lane F, dead volume of column A; lane EA, column A elution; lane EB, column B elution; lane EC, column C elution; lane ED, column D elution. Lane 1, lane EA probed with the anti-H,K-ATPase antibody and a secondary anti-rabbit antibody conjugated with horseradish peroxidase. Lane 2, lane EA probed with the anti-H,K-ATPase antibody and a secondary anti-mouse antibody conjugated with horseradish peroxidase. Lane 3, lane EB probed with the anti-SBD polyclonal antibody and a secondary anti-rabbit antibody conjugated with horseradish peroxidase. Lane 4, lane EB probed with the anti-H,K-ATPase antibody and a secondary anti-mouse antibody conjugated with horseradish peroxidase. Western blots of lanes EC and ED were negative. Each lane was also tested against the secondary antibody alone, and all results were negative.
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