Two Novel Peripheral Membrane Proteins, Pasin 1 and Pasin 2, Associated with Na\textsuperscript{+},K\textsuperscript{+}-ATPase in Various Cells and Tissues

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Abstract. Purification of pig kidney Na\textsuperscript{+},K\textsuperscript{+}-ATPase at low concentrations of SDS (0.5%) allowed copurification of several peripheral membrane proteins. Some of these associated proteins were identified as components of the membrane cytoskeleton. Here we describe two novel globular proteins of Mr 77,000 (pasin 1) and Mr 73,000 (pasin 2) which copurify and coimmunoprecipitate with Na\textsuperscript{+},K\textsuperscript{+}-ATPase and can be stripped off Na\textsuperscript{+},K\textsuperscript{+}-ATPase microsomes by 1 M KCl. Pasin 1 and pasin 2 were detected by immunoblot analysis in various cells and tissues including erythrocytes and platelets. Immunostaining revealed colocalization of pasin 1 and Na\textsuperscript{+},K\textsuperscript{+}-ATPase along the basolateral cell surface of epithelial cells of kidney tubules and parotid striated ducts (titers of pasin 2 antibodies were too weak for immunocytochemistry). In erythrocytes, pasin 1 and pasin 2 are minor components bound to the cytoplasmic surface of the plasma membrane. Pasin 1 showed the same electrophoretic mobility as protein 4.1b. However, both proteins have different isoelectric points (pasin 1, pI 6; protein 4.1, pI 7), different chymotryptic fragments, and are immunologically unrelated. Short pieces of sequence obtained from pasin 1 and pasin 2 were not found in any other known protein sequence. The occurrence of pasin 1 and pasin 2 in diverse cells and tissues and their association with Na\textsuperscript{+},K\textsuperscript{+}-ATPase suggests a general role of these proteins in Na\textsuperscript{+},K\textsuperscript{+}-ATPase function.

\textbf{N}a\textsuperscript{+},K\textsuperscript{+}-ATPase is a transmembrane heterodimeric protein complex that consists of two subunits, the catalytic \(\alpha\)-subunit (90–100 kD) and the noncatalytic \(\beta\)-subunit (50–60 kD). Na\textsuperscript{+},K\textsuperscript{+}-ATPase is essential for the establishment and maintenance of the gradients for Na\textsuperscript{+} and K\textsuperscript{+} across the plasma membrane and thus for generation of the membrane potential, regulation of cell volume, and transport (coupled to influx of Na\textsuperscript{+}) of various ions and organic solutes (Cantley, 1981; Pedersen and Carafoli, 1983a, b).

In most epithelial cells, such as columnar epithelial cells of the kidney, intestine, or exocrine glands, Na\textsuperscript{+},K\textsuperscript{+}-ATPase represents the major driving force for the net resorption of Na\textsuperscript{+} and several Na\textsuperscript{+}-cotransported molecules across the epithelial layer. This function of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase depends on the restriction of the pump to the basolateral cell surface of polarized epithelial cells. The involvement of Na\textsuperscript{+},K\textsuperscript{+}-ATPase in diverse physiological processes requires mechanisms that regulate its activity and place it to specialized domains of the cell surface. Recent observations indicate that analogs of ankyrin and spectrin are colocalized along the lateral cell surface of kidney epithelial cells indicating that Na\textsuperscript{+},K\textsuperscript{+}-ATPase may be placed at the basolateral plasma membrane by linkage via ankyrin to the spectrin-based membrane cytoskeleton (Koob et al., 1987). This view has been further supported by binding of erythrocyte ankyrin to purified kidney Na\textsuperscript{+},K\textsuperscript{+}-ATPase (Koob et al., 1988; Nelson and Veshnock, 1987; Morrow et al., 1989).

However, direct evidence for the specific association of Na\textsuperscript{+},K\textsuperscript{+}-ATPase with components of the cytoskeleton or any other peripheral membrane protein in situ has so far not been provided. This is also true for a 35-kD protein, calnaktin, that may be involved in Ca\textsuperscript{2+}-dependent inhibition of Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity (Yingst, 1988).

To address this problem we have purified Na\textsuperscript{+},K\textsuperscript{+}-ATPase at low concentrations of detergents (0.05 % SDS) in order to allow peripheral membrane proteins to remain attached to the pump during the procedure of purification. Here we describe two novel peripheral membrane proteins, pasin 1 and pasin 2 (from ATPase associated), that are associated with Na\textsuperscript{+},K\textsuperscript{+}-ATPase in various tissues such as kidney, parotid gland, brain, platelets, and erythrocytes.

\textbf{Materials and Methods}

\textbf{Materials}

The listed materials were obtained from the indicated suppliers: PMSF (Fluka AG, Buchs, Switzerland); Aprotinin (Trasylol) (Boehringer Mannheim GmbH, FRG); complete or incomplete Freund's adjuvans (Behring, Marburg, FRG); reagents for gel electrophoresis (Biorad, München, FRG); Nitrocellulose (Schleicher & Schuell, Dassel, FRG); 125I protein A (NEN-Dupont, Dreieich, FRG); peroxidase-labeled goat anti-mouse IgG, tetramethylrhodamine isothiocyanate (TRITC)-conjungated goat anti-mouse IgG, and TRITC-conjugated goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO); anti-rabbit IgG and rabbit peroxidase antipsrroxidase (PAP) complex (Dakopatts, Hamburg, FRG); isoelectric focusing calibra-

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**Methods**

**Purification of Na⁺,K⁺-ATPase.** All tissues were obtained within a period of 30 min after stunning. All tissues were homogenized in 10 vol of buffer A (0.25 M sucrose, 25 mM imidazole, pH 7.5) and centrifuged for 30 min at 10,000 g. Typically, eight kidneys were used for one preparation. The supernatant was collected and centrifuged for 30 min at 48,000 g. The resulting membrane pellet was suspended in buffer A and adjusted to a final protein concentration of 5 mg/ml (Bradford, 1976). Membranes were then solubilized by addition of Na-dodecylsulfate (0.4 mg SDS/mg protein) and diluted in buffer B (30 mM imidazole, 1 mM EDTA) to give a final concentration of 1.25 mg/ml. After incubation for 45 min at room temperature 6 ml of extracted membranes were layered on top of a discontinuous sucrose gradient of 40% (8 ml), 30% (5 ml), and 25% (3 ml) sucrose in buffer B. In some experiments the sucrose gradient was replaced by 40% (15 ml) and 25% (15 ml) sucrose in buffer B. After centrifugation for 4 h at 220,000 g two main protein bands were visible in both continuous and discontinuous gradients. The lower band (30–40%) contained microsomes enriched with Na⁺,K⁺-ATPase activity assay as described above for tissue pieces (Burnet, 1983). The upper band (20–30%) contained oat-solubilized Na⁺,K⁺-ATPase activity assay as described elsewhere (Drenckhahn and Wagner, 1986). The higher band (20–30%) contained oat-solubilized Na⁺,K⁺-ATPase activity assay as described above for tissue pieces (Burnet, 1983). The upper band (20–30%) contained oat-solubilized Na⁺,K⁺-ATPase activity assay as described elsewhere (Drenckhahn and Wagner, 1986).

**Preparation of Membranes.** Membranes and supernatants contained microsomes enriched with Na⁺,K⁺-ATPase activity assay as described above for tissue pieces excised from pig kidney. Membranes of pig erythrocytes and platelets were exactly similar as described in detail elsewhere (Friedrichs et al., 1989). The membranes were extracted with SDS and layered on top of the discontinuous sucrose gradient exactly as described above for pig kidney membranes. After centrifugation, there was only one major band accumulating at the 30–40% border. Glandular tissue of 10 pig parotid glands was cut into small cubes (3 × 3 mm) which were treated the same way as described above for tissue pieces excised from pig kidney. Membranes of pig erythrocytes and platelets were collected for cryosectioning and staining by indirect immunofluorescence as described elsewhere (Drenckhahn and Wagner, 1986).

**Isoelectric Focusing, Two-dimensional Gel Electrophoresis.** For isoelectric focusing, proteins were loaded on a lane of a minigel slab gel and separated in the first dimension by isoelectric focusing as described above. Afterwards, the lane was excised, equilibrated with SDS-sample buffer (15–30 min), and then subjected to SDS-PAGE (7.5%) in the second dimension (O'Farrell, 1975). The separated proteins were visualized by silver staining (Rabilloud et al., 1988).

**Antibodies.** Specificity of antibodies raised against the α-subunit of pig kidney Na⁺,K⁺-ATPase, pig erythrocyte band 3, and bands 4.1 has been described previously (Friedrichs et al., 1989; Koob et al., 1987). Antibodies against pig kidney pasin 1 (77 kD) and pasin 2 (73 kD) were obtained by immunizing rabbits and mice with 100 μg (rabbit) and 10 μg (mice) of the pasin 1 and pasin 2 bands. The bands were excised from 7.5% SDS-PAGE loaded with peripheral membrane proteins extracted from Na⁺,K⁺-ATPase microsomes by 1 M KCl (4°C, 1 h). The excised bands were homogenized and emulsified with complete (first injection) and incomplete (further injections) Freund's adjuvants and injected subcutaneously at various sites of the back. All antibodies were blot affinity purified (Drenckhahn and Franz, 1986).

**Immunoprecipitation.** 75 μg SDS-solubilized Na⁺,K⁺-ATPase were suspended for 10 h at 4°C in 50 μl PBS (pH 7.4) containing 0.5% (wt/vol) BSA (Grade V; Sigma Chemical Co., St. Louis, MO). Then 25 μl of rabbit anti-Na⁺,K⁺-ATPase serum or 25 μl rabbit nonimmune serum were added to the suspension and left at room temperature for 1 h. Afterwards, 100 μl of a suspension of protein A-Sepharose was added (representing ~50 μl of packed beads). After a 60-min incubation period at room temperature samples were centrifuged for 2 min at 100 g, and the resulting pellet of sepharose beads was washed five times with 1 ml PBS (pH 7.4) containing 0.05% Tween 20. The final pellet was dissolved in an equal volume of electrophoresis sample buffer and subjected to SDS-PAGE (7.5% acrylamide) and immunoblotting using antibodies to Na⁺,K⁺-ATPase (α-subunit), pasin 1, and pasin 2.

**Immunoprecipitation.** 75 μg SDS-solubilized Na⁺,K⁺-ATPase were suspended for 10 h at 4°C in 50 μl PBS (pH 7.4) containing 0.5% (wt/vol) BSA (Grade V; Sigma Chemical Co., St. Louis, MO). Then 25 μl of rabbit anti-Na⁺,K⁺-ATPase serum or 25 μl rabbit nonimmune serum were added to the suspension and left at room temperature for 1 h. Afterwards, 100 μl of a suspension of protein A-Sepharose was added (representing ~50 μl of packed beads). After a 60-min incubation period at room temperature samples were centrifuged for 2 min at 100 g, and the resulting pellet of sepharose beads was washed five times with 1 ml PBS (pH 7.4) containing 0.05% Tween 20. The final pellet was dissolved in an equal volume of electrophoresis sample buffer and subjected to SDS-PAGE (7.5% acrylamide) and immunoblotting using antibodies to Na⁺,K⁺-ATPase (α-subunit), pasin 1, and pasin 2.

**Immunopeptide Maps.** Spectrin-stripped inside-out vesicles of pig erythrocytes (Fiedrichs et al., 1989) were extracted with 1.5 M KCl, 1 mM EDTA, 5 mM Na-phosphate (pH 7.5) for 30 min at 37°C. The 48,000 g supernatant was dialyzed against 5 mM Na-phosphate, 1 mM EDTA (pH 7.5) for 24 h at 4°C. The protein concentration was adjusted to 200 μg/ml. 400 μg of the KCl-stripped protein was digested for 30 min at 4°C with 1 μg a-chymotrypsin. Digest was terminated by addition of 1 mM PMSF. Proteins were precipitated with 60% ethanol, the pellet dissolved in 200 μl of electrophoresis sample buffer and then subjected to SDS-PAGE (10% acrylamide) and subsequent immunoblot analysis using affinity-purified antibodies to pig erythroid protein 4.1 and pig kidney pasin 1.

**Immunocytocytochemistry.** Small pieces of the outer medulla of pig kidney were shock frozen in liquid nitrogen. Tissue from the pig parotid gland was cut into 1-mm³ pieces and fixed for 20 h at 4°C with a solution containing 2% formaldehyde and 0.1% glutaraldehyde in PBS (pH 7.4). After immersion in 20% sucrose (10 h), tissue pieces were frozen in liquid nitrogen and processed for cryosectioning and staining by indirect immunofluorescence as described elsewhere (Drenckhahn and Wagner, 1986).

**Results**

**Peripheral Membrane Proteins Associated with Kidney Na⁺,K⁺-ATPase.** In the present study we have purified pig kidney Na⁺,K⁺-ATPase at low concentrations of SDS (0.05%). Under these rather mild conditions Na⁺,K⁺-ATPase microsomes were found to be associated with several protein bands not belonging to the β-subunit (40–50 kD), α-subunit (90–100 kD), or the α,β-heterodimer (140–150 kD) of Na⁺,K⁺-ATPase. Three of the associated protein bands have been identified as proteins related or identical to brain spectrin (235–240 kD), erythrocyte ankyrin (210 kD), and actin (42 kD) (Koob et al., 1990). Two major associated polypeptide bands showed an apparent molecular weight in SDS-PAGE of 77,000 and 73,000, respectively, which is slightly below the position of protein 4.1a (80,000) and 4.2 (75,000) of pig erythrocyte membranes (Figs. 1 and 2). Both pasin 1 and pasin 2 remained associated with Na⁺,K⁺-ATPase microsomes under conditions of low-stringency electrophoresis, proteins were loaded on a lane of a mini slab gel and separated in the first dimension by isoelectric focusing as described above. Afterwards, the lane was excised, equilibrated with SDS-sample buffer (15–30 min), and then subjected to SDS-PAGE (7.5%) in the second dimension (O'Farrell, 1975). The separated proteins were visualized by silver staining (Rabilloud et al., 1988).
Steps of the purification of pig kidney Na⁺,K⁺-ATPase and its associated peripheral membrane proteins (7.5% SDS-PAGE, Coomassie blue stain). (lane 1) Crude membrane fraction of homogenized kidney outer medulla (10,000 g supernatant); (lane 2) fraction of light microsomes taken from the 25%/30% sucrose interface (low Na⁺,K⁺-ATPase activity); (lane 3) Na⁺,K⁺-ATPase microsomes taken from the 30%/40% interface; (lane 4) KCl (1 M)-stripped Na⁺,K⁺-ATPase microsomes; (lane 5) corresponding peripheral membrane proteins extracted from Na⁺,K⁺-ATPase with 1 M KCl; (lane 6) EDTA-insensitive peripheral membrane proteins of Na⁺,K⁺-ATPase microsomes obtained by first removing EDTA (1 mM)-sensitive proteins and then stripping the pelleted Na⁺,K⁺-ATPase microsomes with 1 M KCl. Bands at 240 and 210 kD (lanes 1, 3, 5) represent kidney spectrin and ankyrin, respectively. The 95 and 55-kD bands (lanes 1, 3, 4) represent the α-subunit (95 kD) and β-subunit (55 kD) of Na⁺,K⁺-ATPase. The prominent 42-kD band is actin. Erythrocyte membranes served as molecular weight standard (see Fig. 2).

that removed spectrin and actin (1 mM EDTA, pH 7.5, 37°C, 30 min). Complete removal of pasin 1 and pasin 2 was obtained at 1 M KCl (4°C, 1 h). In this fraction of EDTA-insensitive peripheral membrane proteins pasin 1, pasin 2, and a still unknown 50-kD protein were the major Coomassie blue-stained polypeptide bands (Fig. 1).

Two dimensional SDS-PAGE of KCl-stripped proteins showed single protein spots at the Mr level of pasin 1 and pasin 2, respectively (Fig. 3). As shown by combined isoelectric focusing and immunoblotting (see below) the isoelectric point of pasin 1 is ~pH 6 which corresponds well to the position of the 77-kD spot seen in two-dimensional SDS-PAGE. The isoelectric point of pasin 2 has not yet been determined (because of antibody problems) but as judged from the 73-kD spot in two-dimensional SDS-PAGE it is around pH 8.

Characterization of Antibodies to Pasin 1 and Pasin 2
Antibodies raised against pasin 1 and pasin 2 excised from SDS-PAGE of KCl-extracts were used to further characterize both protein bands by immunoblotting. As shown in Fig. 4 the pasin 1 antibodies (raised in mice and rabbits) reacted selectively with the 77-kD band (pasin 1), but only weakly or not at all with the 73-kD band (pasin 2). In addition, the pasin 1 antibody labeled varying numbers of bands of lower molecular weight (mainly running 40,000-70,000) which are most likely proteolytic breakdown products of pasin 1.

Mouse and rabbit antibodies raised against pasin 2 showed strong immunoreactivity with pasin 2, weak cross-reactivity with pasin 1 and only negligible immunoreactivity with...
bands of lower molecular weight. After blot-affinity purification, the pasin 2 antibodies still showed often a weak cross-reaction with pasin 1. This weak immunological cross reactivity is probably due to contamination of the pasin 2 antiserum with antibodies against proteolytic fragments of pasin 1 that are present in the pasin 2 band (pasin 1 is a much better immunogen than pasin 2).

**Cosedimentation and Coimmunoprecipitation of Pasin 1 and Pasin 2 with Na⁺,K⁺-ATPase**

To see whether pasin 1 and pasin 2 are confined to Na⁺,K⁺-ATPase microsomes or whether both proteins are also associated with other protein components of kidney membranes, SDS (0.05%)-extracted kidney membranes were separated by discontinuous and continuous sucrose gradient

(25-40%) centrifugation. The gradients were pumped out, collected in 24 aliquots, and assayed for Na⁺,K⁺-ATPase activity and protein contents. Every second fraction was subjected to immunoblot analysis with antibodies to Na⁺,K⁺-ATPase (α-subunit), pasin 1, and pasin 2. Immunoblots were quantified by densitometry. In both continuous and discontinuous sucrose gradients Na⁺,K⁺-ATPase, pasin 1, and pasin 2 displayed nearly identical curves of distribution throughout the gradient. Peaks for these proteins as well as for Ouabain-sensitive Na⁺,K⁺-ATPase activity occurred in the same fractions of the gradients (fractions 6-9, 30-40% sucrose) (Figs. 5-7).

To exclude the possibility that pasin 1 and pasin 2 are bound to a contaminating fraction of membrane proteins (microsomes) we immunoprecipitated SDS-solubilized Na⁺,K⁺-ATPase and subjected the immunoprecipitated proteins to
immunoblot analysis. As documented in Fig. 8, pasin 1 as well as pasin 2 coimmunoprecipitated with Na⁺,K⁺-ATPase, whereas all three proteins were absent from immunoprecipitates in which the Na⁺,K⁺-ATPase antibodies were replaced by nonimmune IgG.

**Demonstration of Pasin 1 and Pasin 2 in Other Cells and Tissues**

Immunoblot analysis of membrane fractions of pig platelets, erythrocytes, parotid gland, and brain demonstrated the presence of pasin 1 and pasin 2 as well as the α-subunit of Na⁺,K⁺-ATPase in these cells and tissues (Fig. 9). The labeled bands displayed the same apparent molecular masses as described above for pasin 1 (77 kD), pasin 2 (73 kD) and the α-subunit of Na⁺,K⁺-ATPase (90-100 kD) of the kidney. In all cells and tissues assayed, antipasin 2 showed weak to moderate cross-reactivity with the pasin 1 band. In erythrocyte membranes pasin 1, pasin 2, and Na⁺,K⁺-ATPase appeared to be minor components as judged from the rather high amounts of protein (80 μg) that had to be loaded per lane to obtain well-labeled immunoreactive bands. Since pasin 1 cross reacted in erythrocyte membranes with a polypeptide band in the position of band 4.1 we dissected bands 4.1a and bands 4.1b from SDS-PAGE gels (stained with copper) and reelectrophoresed the dissected proteins on separate lanes of another gel. Immunoblot analysis showed that antipasin 1 reacted selectively with band 4.1b but not with band 4.1a (Fig. 10). The presence of pasin 1 and 2 in erythrocytes allowed determination of the sideness of both proteins. Incubation of intact erythrocytes with trypsin (10 μg/ml, 12 h) did not cause disappearance of pasin 1 and pasin 2 in immunoblots. However, incubation of leaky erythrocyte ghosts (membranes) with trypsin resulted in complete loss of pasin 1 and pasin 2 immunoreactivity (not shown).

**IEF and Immunopeptide Maps**

As shown in the preceding paragraph antibodies to pasin 1 cross reacted with a component of erythrocyte band 4.1b. Likewise, in kidney membranes antibodies against protein 4.1 labeled polypeptides in the position of pasin 1 (Friedrich et al., 1989), suggesting a certain degree of relationship between pasin 1 and protein 4.1. However, separation of erythrocyte and kidney peripheral membrane proteins (striped off by 1 M KCl) by IEF and identification of pasin 1 and protein 4.1 by subsequent immunoblotting clearly showed that pasin 1 in kidney and erythrocytes are rather acidic proteins (pI ~6) with an isoelectric point slightly below that of protein 4.1 (pI ~7). As seen in Fig. 11, kidney pasin 1 appeared as multiple bands in IEF. These bands are probably caused by proteolytic breakdown products of pasin 1 with similar acidity.

Further proof for our conclusion that pasin 1 and protein 4.1 are unrelated proteins was obtained by one-dimensional peptide maps of KI-extracted peripheral membrane proteins of pig erythrocytes. The extracted proteins were cleaved with α-chymotrypsin and analyzed by immunoblotting using polyclonal antibodies to pig protein 4.1 and pasin 1. At a protein to protease ratio of 1:400 (wt/wt) protein 4.1 was cleaved in three major fragments of 55, 43, and 29 kD, whereas pasin 1 displayed only two major fragments at 68 and 45 kD (Fig. 12). The pasin 1 antibody did not cross react with any of the chymotryptic fragments of protein 4.1 and vice versa.

**Immunocytochemistry**

Subcellular localization of pasin 1 and pasin 2 was studied by immunofluorescence in tissue sections of pig kidney and parotid gland (Fig. 13). The antibodies did not cross react with rat tissues. Only the pasin 1 antibodies (raised in rabbits and mice, respectively) caused significant immunostaining of tissue sections. The titer of the pasin 2 antibodies was...
probably too low for detection by immunofluorescence. The antibodies against pasin 1 reacted selectively with the basolateral cell surface of virtually all epithelial cells of the kidney. The brightest stain was associated with the basolateral membranes of the straight and convoluted distal tubule and the collecting duct. Antibodies against Na⁺,K⁺-ATPase showed virtually the same staining pattern (Fig. 13, a and b). In sections of the parotid gland, antibodies against pasin 1 and Na⁺,K⁺-ATPase (α-subunit) displayed strong affinity for the folded basolateral surface of the striated duct epithelium (Fig. 13, c and d). No significant immunofluorescence was observed in association with the glandular epithelium of the parotid gland.

**Rotary Shadowing**

EDTA-resistant peripheral membrane proteins were stripped off Na⁺,K⁺-ATPase microsomes by 1 M KCl (4°C, 1 h). The resulting supernatant consisted mainly of pasin 1, pasin 2, and several minor polypeptide bands of lower molecular weight. The majority of these minor bands have been identified by immunoblotting as breakdown products of pasin 1. After dialysis against 30% (vol/vol) glycerol containing 0.1 M KCl, 10 mM imidazole, and 1 mM NaN₃ (pH 7.4), KCl-extracted proteins were sprayed on freshly cleaved micas, dried under vacuum at room temperature, and rotary shadowed with platinum at an angle of 10° followed by carbon (Aebi et al., 1983).

In contrast to the EDTA-stripped protein fraction which contained numerous highly elongated spectrin-like molecules (Koob et al., 1990), the pasin-enriched protein fraction consisted exclusively of globular molecules. Two fractions of particles were observed, a fraction of small particles 5-7 nm in size and a fraction of large particles 9-11 nm in size. The larger particles often formed pairs, two of which are shown in Fig. 14.

**Sequence Data**

Pasin 1 and pasin 2 bands, electrotransferred to PVDF-
membranes were kindly sequenced by Prof. Dr. Jany (Technical University, Darmstadt, FRG). Short pieces of sequences could be obtained, indicating that the transferred bands represent relatively pure proteins and do not contain significant amounts of other contaminating proteins of similar electrophoretic mobility: pasin 1, H$_2$N-PIVE$\text{\textbeta}$VTX-LVAEGE-COOH; and pasin 2, H$_2$N-XKTINV$\text{\textbeta}$VTLMDA-ELXFAE-COOH.

**Discussion**

In the present study we have purified Na$^+$,K$^+$-ATPase by lowering concentration of SDS down to 0.05%. Under these conditions, Na$^+$,K$^+$-ATPase copurified with several peripheral membrane proteins. Three of the copurifying proteins were identified as the main components of the spectrin-based membrane cytoskeleton, i.e., actin, nonerythroid spectrin, and ankyrin (Koob et al., 1990). The two major remaining proteins that cosedimented with Na$^+$,K$^+$-ATPase in sucrose gradients and coimmunoprecipitated with Na$^+$,K$^+$-ATPase had an apparent molecular weight of 77,000 (pasin 1) and 73,000 (pasin 2), respectively. The lack of immunological cross-reactivity of pasin 1 antibodies with pasin 2 indicates that both proteins represent distinct molecules.

Pasin 1 and pasin 2 are peripheral membrane proteins which dissociated from Na$^+$,K$^+$-ATPase vesicles at 1 M KCl but remained attached under conditions that removed...
Figure 14. Rotary shadowing of a protein fraction highly enriched in pasin 1 and pasin 2 (corresponding to lane 6 of Fig. 1). Only molecules with globular morphology are seen. Bar, 0.1 μm.

actin and kidney spectrin (1 mM EDTA, 37°C). Since pasin 1 and pasin 2 were degraded by trypsin only in disrupted cells or isolated membranes, but not by incubation of intact cells with trypsin (studies with erythrocytes), both proteins have to be considered as components bound to the cytoplasmic face of the plasma membrane. Pasin 1 and pasin 2 share several features of proteins 4.1 and 4.2 of pig erythrocyte membranes, such as similar molecular mass, globular morphology, association with erythrocyte membranes, and dissociation from the membrane at 1 M KCl but not at 1 mM EDTA. However, for the following reasons we do not believe that pasin 1 and pasin 2 are related to either proteins 4.1 and 4.2, nor to any other known protein.

In immunoblots of erythrocyte membranes antipasin 2 labeled a 73-kD band clearly located below band 4.2 (75-kD). Likewise, antibodies against protein 4.2 labeled an Mr 75 band in kidney membranes (Friedrichs et al., 1989) but did not react with the pasin 2 band (73 kD). Antibodies to pasin 1 reacted with a single polypeptide band in erythrocyte membranes that was located in the position of protein 4.1b (77 kD) but not 4.1a (80 kD). Since proteins 4.1a and 4.1b are probably identical in sequence (Conboy et al., 1988; Goodman et al., 1982), but differ by still unknown posttranslational modifications (Inaba and Maede, 1988; Mueller et al., 1987), one should expect that polyclonal antibodies cross reacting with protein 4.1b will also cross react with protein 4.1a and vice versa. By IEF and subsequent immunoblotting, pasin 1 could be unequivocally separated from proteins 4.1a and 4.1b. The isoelectric point of kidney and erythrocyte pasin 1 was pH 6, whereas that of protein 4.1a and 4.1b was pH 7, which is consistent with previously published data on the isoelectric point of human erythrocyte protein 4.1 (Harell and Morrison, 1979). Further support for the conclusion that pasin 1 and protein 4.1 are unrelated proteins was provided by differences of the chymotryptic cleavage pattern of both proteins.

Another cytosolic protein that is partly found in association with the plasma membrane of various tissues, including erythrocytes, and has a molecular weight of ~80,000 is tissue transglutaminase (EC 2.3.2.13, protein-glutamine:amine y-glutamyltransferase). Preliminary data indicate a certain degree of sequence homology between guinea pig transglutaminase and protein 4.2 (Korsgren et al., 1990). However, peak fractions of isolated human erythrocyte transglutaminase (prepared according to Brenner and Wold, 1978 and assayed according to Korner et al., 1989) did not cross react in immunoblots with either antibodies against proteins 4.1 and 4.2 or with antibodies against pasin 1 and pasin 2 (not documented).

Recently, a complex of three peripheral membrane proteins (named catenins) has been observed in immunoprecipitates of the basolateral membrane protein uvomorulin (Ozawa, 1989). Whether pasin 1 is related to α-catenin, an 80-kD protein, is under current investigation. However, the absence of uvomorulin from kidney Na+,K+-ATPase vesicles and erythrocytes (not shown) does not speak in favour of a relation between pasin 1 and the uvomorulin-binding catenins. Moreover, pasin 1 is probably also not related to a recently discovered 80-kD protein that is associated with the gastric H+,K+-ATPase (Urushidani et al., 1989). Unlike pasin 1 and pasin 2, the gastric 80-kD protein shares several features of an integral membrane protein and is absent from brain, kidney, and erythrocytes (Hanzel et al., 1989).

The small pieces of amino acid sequence obtained from pasin 1 and pasin 2 were not found in any other sequence of proteins submitted to the NBRF-protein databank until December 1989. Thus, it is reasonable to assume that pasin 1 and pasin 2 are novel proteins distinct from any other proteins so far sequenced.

Our suggestion that pasin 1 and pasin 2 are associated with Na+,K+-ATPase is mainly based on three observations. (a) Kidney pasin 1 and pasin 2 cosedimented with Na+,K+-ATPase in continuous and discontinuous sucrose gradients. Similar observations were obtained with brain and parotid membranes in which pasin 1 and pasin 2 became considerably enriched by SDS extraction (0.05%) and subsequent sucrose gradient centrifugation. Both proteins were detected in the peak fraction of Na+,K+-ATPase. (b) Pasin 1 and pasin 2 coimmunoprecipitated with Na+,K+-ATPase. (c) In tissue sections of the kidney and parotid gland, pasin 1 and Na+, K+-ATPase were precisely colocalized along the basolateral membrane surface of columnar epithelial cells (parotid striated duct, kidney tubules). Presently we do not know whether pasin 2 has the same subcellular location because the titer of the pasin 2 antibodies was too low for immunostaining.

At present we cannot exclude the possibility that pasin 1 and pasin 2 are associated with a minor contaminating component associated with Na+,K+-ATPase rather than with Na+,K+-ATPase itself. However, if such a contaminating membrane protein does exist it should have properties very similar to Na+,K+-ATPase, i.e., copurification, commounoprecipitation, same subcellular location (basolateral membrane of transporting epithelia), and occurrence in the same tissues and cells (including erythrocytes) in which pasin 1, pasin 2, and Na+,K+-ATPase were demonstrated. Finally, we wish to mention that to our knowledge Na+,K+-ATPase has not been previously demonstrated in erythrocytes and platelets by immunoblotting or any other biochemical approach except measurement of activity and Ouabain binding.

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