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An SH3 binding region in the epithelial Na^+ channel (αrENaC) mediates its localization at the apical membrane

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The amiloride-sensitive Na^+ channel constitutes the rate-limiting step for Na^+ transport in epithelia. Immunolocalization and electrophysiological studies have demonstrated that this channel is localized at the apical membrane of polarized epithelial cells. This localization is essential for proper channel function in Na^+ transporting epithelia. In addition, the channel has been shown to associate with the cytoskeletal proteins ankyrin and α-spectrin in renal epithelia. However, the molecular mechanisms underlying the cytoskeletal interactions and apical membrane localization of this channel are largely unknown. In this study we show that the putative pore forming subunit of the rat epithelial (amiloride-sensitive) Na^+ channel (αrENaC) binds to α-spectrin *in vivo*, as determined by co-immunoprecipitation. This binding is mediated by the SH3 domain of α-spectrin which binds to a unique proline-rich sequence within the C-terminal region of αrENaC. Accordingly, the C-terminal region is sufficient to mediate binding to intact α-spectrin from alveolar epithelial cell lysate. When microinjected into the cytoplasm of polarized primary rat alveolar epithelial cells, a recombinant fusion protein containing the C-terminal proline-rich region of αrENaC localized exclusively to the apical area of the plasma membrane, as determined by confocal microscopy. This localization paralleled that of α-spectrin. In contrast, microinjected fusion protein containing the N-terminal (control) protein of αrENaC remained diffuse within the cytoplasm. These results suggest that an SH3 binding region in αrENaC mediates the apical localization of the Na^+ channel. Thus, cytoskeletal interactions via SH3 domains may provide a novel mechanism for retaining proteins in specific membranes of polarized epithelial cells.

Key words: epithelial Na^+ channel/proline-rich region/SH3 domains/α-spectrin

Introduction

The activity of amiloride-sensitive Na^+ channels constitutes the rate limiting step for Na^+ transport in epithelia. Na^+ transport plays a major role in fluid reabsorption in the colon and kidney, and in fluid clearance from the alveolar space at birth or during pulmonary edema (O'Brodovich, 1991; Saumon and Basset, 1993 and references therein). The epithelial Na^+ channel is not related to the Na^+ channel of excitable tissues. Its activity is regulated by hormones which stimulate PKA/cAMP as well as by changes in intracellular Ca^{2+}, H^+ and Na^+ concentrations, and by G proteins (reviewed in Smith and Benos, 1991). This channel mediates cellular entry of Na^+ from the apical (lumen) surface, and therefore must be localized at the apical membrane for vectorial transport of Na^+ in polarized epithelial cells. Indeed, immunolocalization studies have identified Na^+ channels in the apical (but not basal-lateral) membranes of renal collecting ducts, sweat gland ducts, A6 cells (Tousson *et al.*, 1989), olfactory epithelial cells (Menco and Benos, 1989) and alveolar epithelial cells (Matalon *et al.*, 1993). Moreover, electrophysiological studies using Ussing chambers and patch clamp techniques have demonstrated that Na^+ channels are localized at the apical membrane of alveolar epithelial cells (O'Brodovich *et al.*, 1991; Orser *et al.*, 1991).

Several lines of evidence have suggested an association between the epithelial Na^+ channel and the cytoskeleton. Disruption of actin microfilaments, addition of actin fragments or actin–gelsolin complexes alter Na^+ channel activity in A6 cells (Cantiello *et al.*, 1991) and impair Na^+ channel regulation in frog skin (Els and Chou, 1993). In addition, a decrease in Na^+ transport and number of apical membrane Na^+ channels in alveolar epithelial cells has been shown to be associated with disruption of the actin cytoskeletal network (Compeau *et al.*, 1994). Importantly, the channel has been shown to co-segregate with the cytoskeletal proteins actin, ankyrin and α-spectrin (α-fodrin) to the apical microvilli in renal epithelia, and to remain associated with ankyrin and α-spectrin after biochemical purification (Smith *et al.*, 1991).

Recently, the primary sequence of the rat amiloride-sensitive epithelial Na^+ channel (rENaC) was determined by functional expression cloning (Canessa *et al.*, 1993a, 1994; Lingueglia *et al.*, 1993), and shown to be composed of three homologous subunits: α, β and γ. The α-subunit (αrENaC) was proposed as the pore forming subunit of the channel, as its expression in *Xenopus* oocytes by itself was sufficient to induce amiloride-sensitive Na^+ currents (Canessa *et al.*, 1993a). Co-expression of all three subunits markedly increased the magnitude of these currents, suggesting that the β and γ subunits represent integral proteins of the Na^+ channel complex (Canessa *et al.*, 1994).

The C-terminal region of αrENaC contains two proline-rich sequences that resemble the SH3 binding motifs found in several proteins involved in signal transduction, including 3BP1 (Ren *et al.*, 1993), SOS, mSOS-1 and

hSOS-1 (Simon *et al.*, 1991; Bowtell *et al.*, 1992; Chardin *et al.*, 1993), dynamin (Gout *et al.*, 1993), p62 (Wong *et al.*, 1992) and p85 of PI3-kinase (Chen *et al.*, 1993). SH3 domains are sequences conserved in several signal transducing and/or cytoskeletal proteins, and are often found in proteins which also contain SH2 domains (Mayer and Baltimore, 1993; Pawson and Schlessinger, 1993 and references therein). SH3 domains mediate protein−protein interactions by binding to specific proline-rich sequences (Yu *et al.*, 1994). An example of such an interaction is the association between the guanine nucleotide exchange factor hSOS with the SH3 domains of GRB2 and the formation of a complex which participates in the activation of Ras by tyrosine kinase receptors (reviewed in Pawson and Schlessinger, 1993). Other examples include the association of 3BP1 with the SH3 domain of c-abl (Ren *et al.*, 1993), v-src with the SH3 domain of p85 (Liu *et al.*, 1993) and dynamin with the SH3 domains of GRB2 and PLCγ (Gout *et al.*, 1993). SH3 domains are also found in several cytoskeletal proteins, including the non-erythroid α-spectrin (α-fodrin) (Lehto *et al.*, 1988; Dubreuil *et al.*, 1989; Wasenius *et al.*, 1989). Since α-spectrin was previously shown to co-purify with the kidney epithelial Na^+ channel (Smith *et al.*, 1991), we have investigated whether the proline-rich C-terminal region of αrENaC could bind to the SH3 domain of α-spectrin. Accordingly, we report here that αrENaC binds α-spectrin in living cells, and that this binding is mediated by the SH3 domain of α-spectrin which binds to a proline-rich, unique sequence within the C-terminal region of αrENaC. Moreover, we show that the C-terminus of αrENaC, when microinjected into polarized alveolar epithelial cells, is sufficient to mediate apical localization. This could represent a novel mechanism to localize (or retain) the channel at the apical membrane, which is essential for proper Na^+ channel activity.

Results

Binding of αrENaC to α-spectrin in intact cells

Previous studies (Smith *et al.*, 1991) have shown that the kidney epithelial Na^+ channel co-segregates and co-purifies with the cytoskeletal protein α-spectrin. To investigate whether αrENaC can bind α-spectrin *in vivo*, we tested if αrENaC can co-immunoprecipitate α-spectrin from MDCK cells overexpressing αrENaC (C.M.Canessa and B.C.Rossier, unpublished). For this purpose, we used polyclonal antibodies directed against a glutathione S-transferase (GST) fusion protein prepared from the N-terminal region of the channel (see Figure 2A and B). These antibodies, called αN, recognize a broad band of ~75–90 kDa in immunoblots of αrENaC-transfected MDCK cells (Figure 1A, lane 2), which is not recognized by pre-immune serum (Figure 1A, lane 3), and is not observed in lysate of untransfected MDCK cells (Figure 1A, lane 1). As the expected size of αrENaC is 78 kDa (Canessa *et al.*, 1993a), the appearance of a broad band probably represents different glycosylation forms of the channel (Canessa *et al.*, 1993b). αrENaC was immunoprecipitated from lysate of αrENaC-overexpressing transfected MDCK cells with affinity purified αN antibodies. Following separation of proteins by SDS−PAGE and transfer to nitrocellulose membrane, blots were incubated with either αN (Figure 2B, lane 1) or anti α-spectrin monoclonal antibodies (mAb101-AA6, Ylikoski *et al.* 1990) (Figure 2B, lanes 2 and 3). As seen in Figure 2B (lane 3), αrENaC co-immunoprecipitated a 240 kDa (and a proteolytic ~150 kDa) protein which was recognized by the α-spectrin antibodies, and showed the same migration pattern as α-spectrin from MDCK cell lysate (Figure 2B, lane 2). These results therefore show that αrENaC binds α-spectrin *in vivo*.

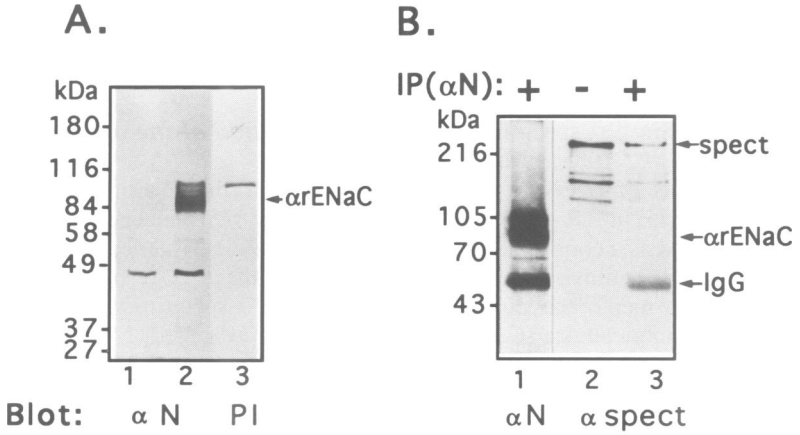

Fig. 1. Binding of αrENaC to α-spectrin *in vivo*. (**A**) Characterization of anti-αrENaC antibodies. Twenty micrograms of cell lysate from untransfected (lane 1) or αrENaC-transfected MDCK cells (lanes 2 and 3) were separated on a 7.5% SDS−polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were then incubated with either affinity purified rabbit polyclonal antibodies (called αN) raised against a GST fusion protein containing amino acids 1–76 of the N-terminus of αrENaC (Canessa *et al.*, 1993) (lanes 1 and 2), or with a pre-immune serum (lane 3). Following incubation with alkaline phosphatase-conjugated goat anti-rabbit secondary antibodies, proteins were detected with NBT/BCIP solutions, as described in Materials and methods. (**B**) Co-immunoprecipitation of α-spectrin with αrENaC. Affinity purified αN antibodies, bound to protein A−Sepharose, were incubated with lysate from transfected MDCK cells. Following thorough washes, proteins were separated by 8% SDS−PAGE, transferred to nitrocellulose membranes and blotted with either αN antiserum (lane 1) or anti-α-spectrin monoclonal (101-AA6) antibodies (Ylikoski *et al.*, 1990) (lane 3). In lane 2, ~30 μg of cell lysate from transfected MDCK cells were blotted with the α-spectrin antibodies (101-AA6). Blots were then incubated with peroxidase-conjugated goat anti-rabbit (lane 1) or goat anti-mouse (lanes 2 and 3) secondary antibodies, followed by ECL detection.

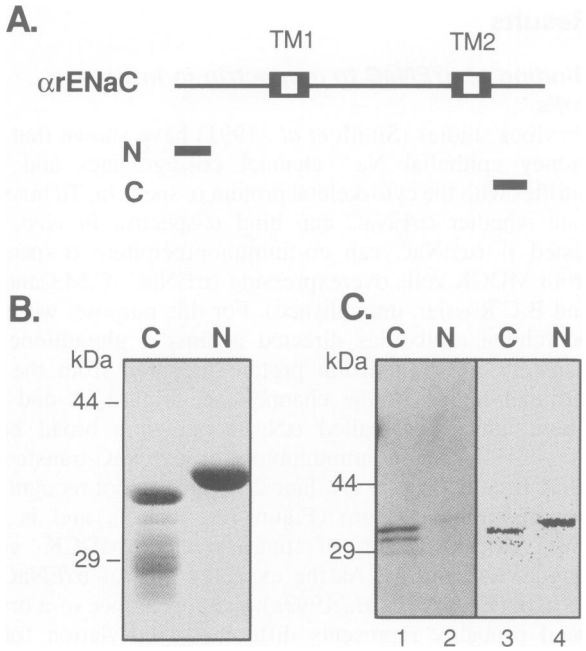

Fig. 2. Binding of the C-terminus of αrENaC to α-spectrin SH3 *in vitro*. (**A**) Schematic representation of the N-terminal (N) and C-terminal (C) constructs of αrENaC (I). Construct borders: N-terminus = nucleotides 1–228; C-terminus = nucleotides 1842–2070 of αrENaC cDNA (Canessa *et al.*, 1993a). TM1 and TM2: putative transmembrane domains. (**B**) GST fusion proteins of the N-terminus (N) and C-terminus (C) of αrENaC. The C-terminal fusion protein was partially degraded due to its proline-rich content. (**C**) Binding of biotinylated α-spectrin GST−SH3 to the C-terminus of the αrENaC in a filter binding assay. Lane 1: C-terminal fusion protein (C). Lane 2: N-terminal fusion protein (N); lanes 3 and 4 show the Ponceau S staining of the same blot shown in lanes 1 and 2 (demonstrating equal loading of the C- and N-terminal fusion proteins on the gel). For the filter binding assays, 0.5–1 μg of N- or C-terminal fusion proteins of αrENaC were separated by 10% SDS−PAGE, transferred to nitrocellulose and incubated with biotinylated GST−α-spectrin SH3 (2 μg/ml) followed by streptavidin–biotinylated peroxidase complex and ECL detection.

Binding of the SH3 domain of α-spectrin to the C-terminus of αrENaC in vitro

The C-terminal region of αrENaC contains proline-rich sequences (Canessa *et al.*, 1993a). Since α-spectrin contains an SH3 domain (Lehto *et al.*, 1988), we first investigated whether the C-terminus of αrENaC could bind the SH3 domain of α-spectrin *in vitro*. The N-terminal portion of αrENaC was used as a control in these studies because both the C- and N-terminal regions of the protein, bordering the two transmembrane domains (Figure 2A), were suggested to be intracellular (Canessa *et al.*, 1993b). GST fusion proteins containing either the N-terminal (control) or the proline-rich C-terminal region of αrENaC were prepared (Figure 2A and B). These proteins (~0.5–1 μg) were separated by SDS−PAGE, transferred to nitrocellulose membranes and probed with biotinylated GST fusion protein of the SH3 domain of α-spectrin (~2 μg/ml), previously prepared from chicken brain α-spectrin (Merilainen *et al.*, 1993). Figure 2C shows that the SH3 domain of α-spectrin was able to bind to the C-terminal fusion protein of αrENaC, but not to the control N-terminal protein. There was also no detectable binding to the GST fusion protein alone (not shown). These results demonstrate that the SH3 domain of α-spectrin binds to the proline-rich C-terminus of αrENaC *in vitro*.

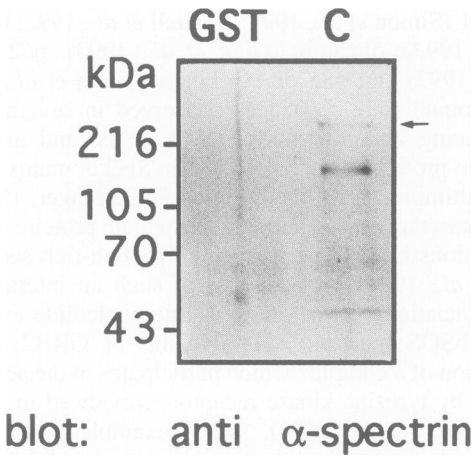

Fig. 3. Binding of αrENaC−C-terminus to intact α-spectrin. GST or GST−αrENaC−C-terminus (C), immobilized on glutathione−agarose beads, were incubated with alveolar epithelial cell lysate. Following washes and separation by 10% SDS−PAGE, proteins were blotted with anti-α-spectrin monoclonal antibodies (101-AA6) followed by peroxidase-conjugated goat anti-mouse secondary antibodies and ECL detection. The arrow marks the 240 kDa α-spectrin band. A proteolytic product (~150 kDa band) of α-spectrin is also shown.

To test whether the C-terminal region of αrENaC can bind intact α-spectrin in cells, alveolar epithelial cells grown for 1–2 days in primary culture (see below) were lysed and the lysate incubated either with αrENaC−C-terminal fusion protein or with GST alone (control) immobilized on glutathione−agarose beads. Following washes, separation by SDS−PAGE and transfer to nitrocellulose, the blot was incubated with an α-spectrin monoclonal antibody (mAb 101-AA6). The results (Figure 3) show a ~240 kDa band (and a ~150 kDa proteolytic product) corresponding to the expected size of α-spectrin, which bound to the C-terminal fusion protein but not to the GST control protein. Thus, the C-terminal region of αrENaC is sufficient to mediate binding to intact α-spectrin.

Identification of the α-spectrin-SH3 binding motif in αrENaC

Analysis of the C-terminal region of αrENaC reveals two proline-rich sequences: PTSPPPSLP (named P1) and PPLALTAPPPA (named P2) (Figure 4A). To determine which of these is responsible for binding to the SH3 domain of α-spectrin, a series of constructs (GST fusion proteins) was generated which encompass either one or both of the P1 and P2 regions, as well as flanking sequences (Figure 4A and B, lanes 1–4). These GST fusion proteins (0.5 μg each) were separated on a 10% SDS−polyacrylamide gel, transferred to nitrocellulose membrane and probed with biotinylated α-spectrin SH3 (~2 μg/ml), as described above. The results show (Figure 4C) that whereas the SH3 domain of α-spectrin avidly bound to constructs III, IV and V (all containing the P2 region), it was not able to bind to construct II (which lacks the P2 region). This suggests that the P2 region, but not the P1 region, is responsible for binding to α-spectrin SH3. These results are also consistent with the observation that a synthetic peptide (PTSPPPSLP)

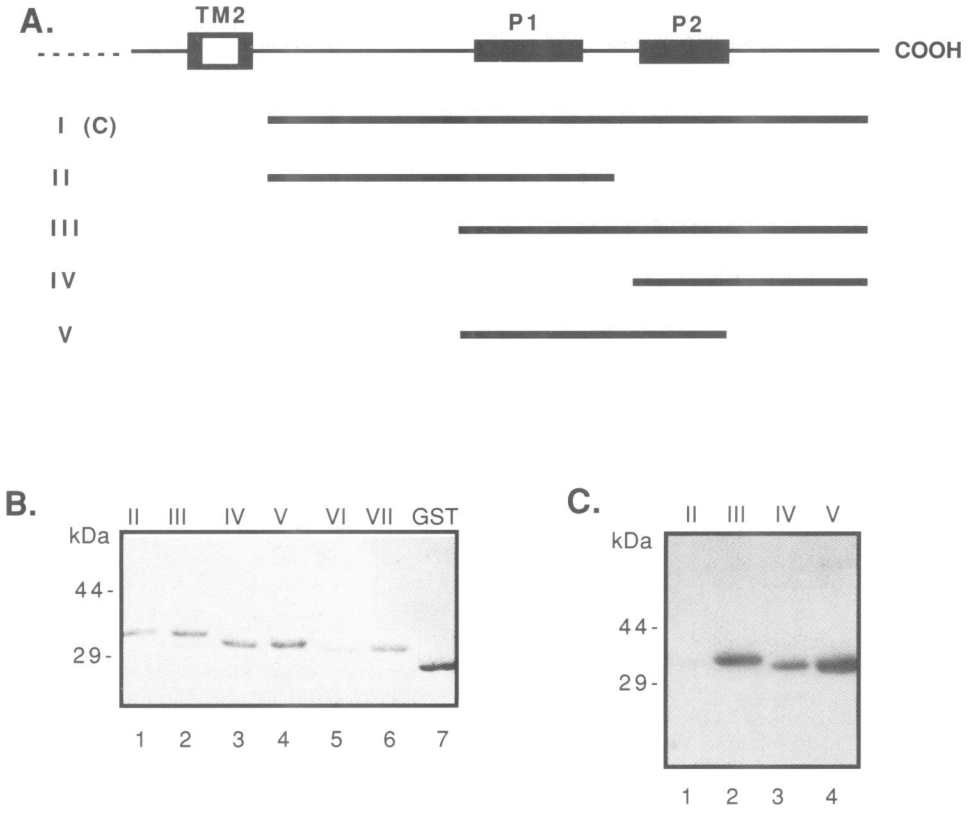

Fig. 4. Identification of the specific sequence in αrENaC which binds to the SH3 domain of α-spectrin. (**A**) Schematic representation of the various constructs prepared from the different regions of the C-terminus of αrENaC (Canessa *et al.*, 1993a): construct I is the same as construct C in Figure 2; construct II encompasses nt 1842–1974, construct III nt 1935–2070, construct IV nt 1977–2070, and construct V nt 1935–2025. (**B**) GST fusion proteins of the various constructs depicted in (A), and of constructs VI and VII which are described in Figure 5. (**C**) Binding of biotinylated α-spectrin GST-SH3 (2 μg/ml) to 0.5 μg of the different constructs depicted in (A) and (B). Binding was determined by the filter binding assay as described in Figure 2.

corresponding to the P1 region failed to inhibit the binding of α-spectrin to the C-terminus of αrENaC (data not shown). They also demonstrate that the sequences flanking the proline-rich regions are not necessary for binding; construct II (which also contains sequences upstream of P1) did not bind at all, and both constructs IV (which contains sequences downstream of P2) and V (which does not) were able to bind to the SH3 domain of α-spectrin equally well. In the C-terminus of αrENaC, an additional sequence containing proline residues (GPSAPPL) is located downstream of the P2 region. This sequence is not necessary for SH3 binding, however, since a construct lacking it (construct V, see Figure 4) was able to bind to the SH3 domain of α-spectrin.

To verify further that the prolines in the P2 region mediate the binding to α-spectrin SH3, new GST fusion protein constructs were prepared in which either four (construct VII) or all five (construct VI) prolines of the P2 region were deleted or mutated (Figure 5A and 4B, lanes 5 and 6). As shown in Figure 5A, the first pair of prolines of the P2 region was deleted and the subsequent triple proline sequence (PPP) was mutated to either SGS (construct VI) or SGP (construct VII). These two new constructs were otherwise identical to construct IV (see Figure 5A). As shown in Figure 5B, elimination of the five prolines in the P2 region indeed inhibited binding to the SH3 domain of α-spectrin. Since similar inhibition (Figure 5C) was observed when the last proline of the P2 region was left intact (construct VII), it is unlikely that this proline is required for SH3 binding.

Apical localization of the proline-rich C-terminus of αrENaC

The epithelial Na^+ channel must be localized at the apical membrane of polarized epithelial cells for proper Na^+ transport to occur. Previous studies (Sormunen *et al.*, 1994) have demonstrated by immunofluorescence and immunoelectron microscopy that α-spectrin is localized at the apical (but not basal) membrane of type I and type II alveolar epithelial cells. An example of such apical localization of α-spectrin in type II cells using immunogold labelling is depicted in Figure 6b. We therefore investigated whether the ability of the C-terminus of αrENaC to bind to the SH3 domain of α-spectrin may help mediate the proper localization of the Na^+ channel at the apical membrane of polarized Na^+-transporting epithelial cells, such as alveolar epithelium. For these studies, we used freshly isolated rat fetal alveolar epithelial cells grown briefly (1–2 days) in primary cultures. When these cells are seeded on a permeant filter support they become polarized and actively transport Na^+ (O'Brodovich *et al.*, 1991); accordingly, they have been shown to possess amiloride-sensitive Na^+ channels in their apical membrane (Orser *et al.*, 1991), to have whole cell amiloride-sensitive sodium currents (Wang *et al.*, 1993a) and to express transcripts for αrENaC (O'Brodovich *et al.*, 1993).

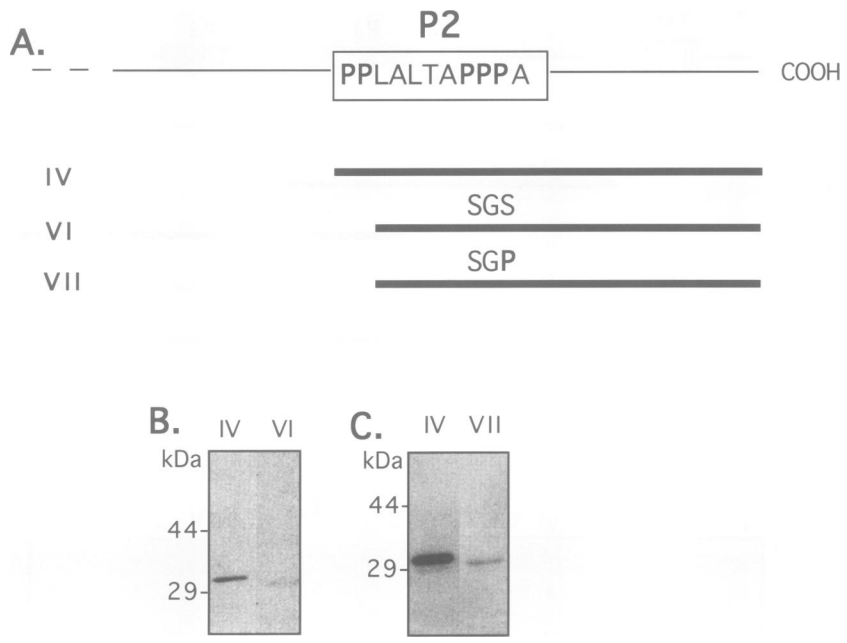

Fig. 5. Requirement of prolines in the P2 region for SH3 binding. (**A**) Design of constructs VI and VII modeled after construct IV, only the first two prolines (P661 and P662) deleted (Canessa *et al.*, 1993a) and proline triplet (P668, P669, P670) mutated to SGS (construct VI) or SGP (construct VII). The GST fusion proteins of constructs VI and VII are shown in Figure 4B, lanes 5 and 6. (**B** and **C**) Comparison of binding biotinylated α-spectrin SH3 (2 μg/ml) to 0.5 μg of constructs VI and IV (B) or to 0.5 μg of constructs VII and IV (C). Experimental protocol is detailed in Figure 2 and in Materials and methods.

Morphologically, these cells have their apical membrane on the top and sides, with tight junctions being very close to the bottom of the cell (Figure 6a). Confluent polarized cells on permeant filters were then microinjected with either the N-terminal (control) or C-terminal GST fusion proteins of αrENaC. The morphology of the microinjected cells, as determined by phase microscopy, was indistinguishable from that of the surrounding uninjected cells (not shown). The distribution of the injected proteins was then localized by indirect immunofluorescence using rabbit anti-GST primary antibodies and FITC-labelled donkey anti-rabbit secondary antibodies. Figure 7 shows fluorescence images, determined by conventional fluorescence microscopy, of cells injected with either the N- or C-terminal fusion protein. As seen in Figure 7a, the N-terminal (control) fusion protein was evenly distributed throughout the cytoplasm and was excluded from the nucleus or lamellar bodies. In contrast, the C-terminal protein showed a markedly different localization pattern, with most intense staining at the periphery of the cell (Figure 7b). The total amount of fluorescence in cells injected with either the N- or the C-terminal protein was similar, suggesting that an equivalent amount of proteins was delivered into each cell.

To follow the subcellular distribution of the injected fusion proteins, confocal microscopy was employed with optical sectioning performed in both the $x-y$ and $x-z$ orientations. Figure 8 depicts $x-y$ confocal images taken from the middle and from the apical (top) regions of the cells injected with either the N-terminal control fusion protein (panels a and c) or the C-terminal fusion protein (panels b and d). These sections are presented side by side for comparison. As seen in Figure 8c which depicts an $x-y$ section from the middle of the cell, the N-terminal fusion protein was evenly distributed in the cytoplasmic space with clear exclusion from the nucleus and lamellar bodies; there was hardly any protein detected in the basal or apical (Figure 8c) membranes of these polarized cell. Accordingly, an $x-z$ reconstruction of a series of cross-section images taken (at 0.5 μm intervals) from the cells depicted in Figure 8 reveals that the N-terminal control protein was indeed concentrated in the cytoplasmic space with no preference towards the apical or basal regions of the cell (Figure 8e). In contrast, the distribution of the proline-rich C-terminal protein was markedly different: the injected protein was confined almost exclusively to the plasma membrane, as evident from the $x-y$ confocal image taken through the middle of the cell (Figure 8b). Moreover, sequential optical sections taken at the $x-y$ orientation revealed that the C-terminal protein was absent from the basal membrane, but present in the apical membrane (Figure 8d), including in the microvilli which are typically located at the apical membrane of these alveolar epithelial cells (see Figure 6a). In accordance, a confocal $x-z$ image reconstruction showed that the C-terminal fusion protein was localized exclusively at the apical side of the plasma membrane, with no protein detected in the basal membrane (Figure 8f). It will be important in the future to follow the localization of a mutant αrENaC−C-terminal protein with deleted or mutated prolines in the P2 region, to ascertain that this specific region within the C-terminus is indeed the one responsible for the apical localization *in vivo*. Taken together, our data show that the proline-rich C-terminus of αrENaC mediates localization of the protein at the apical membrane of polarized alveolar epithelial cells; this polarized distribution parallels the distribution of α-spectrin in alveolar epithelial cells from lung tissue sections (Sormunen *et al.*, 1994; see also Figure 6b). Due to the high background fluorescence emitted from the permeable filters on which

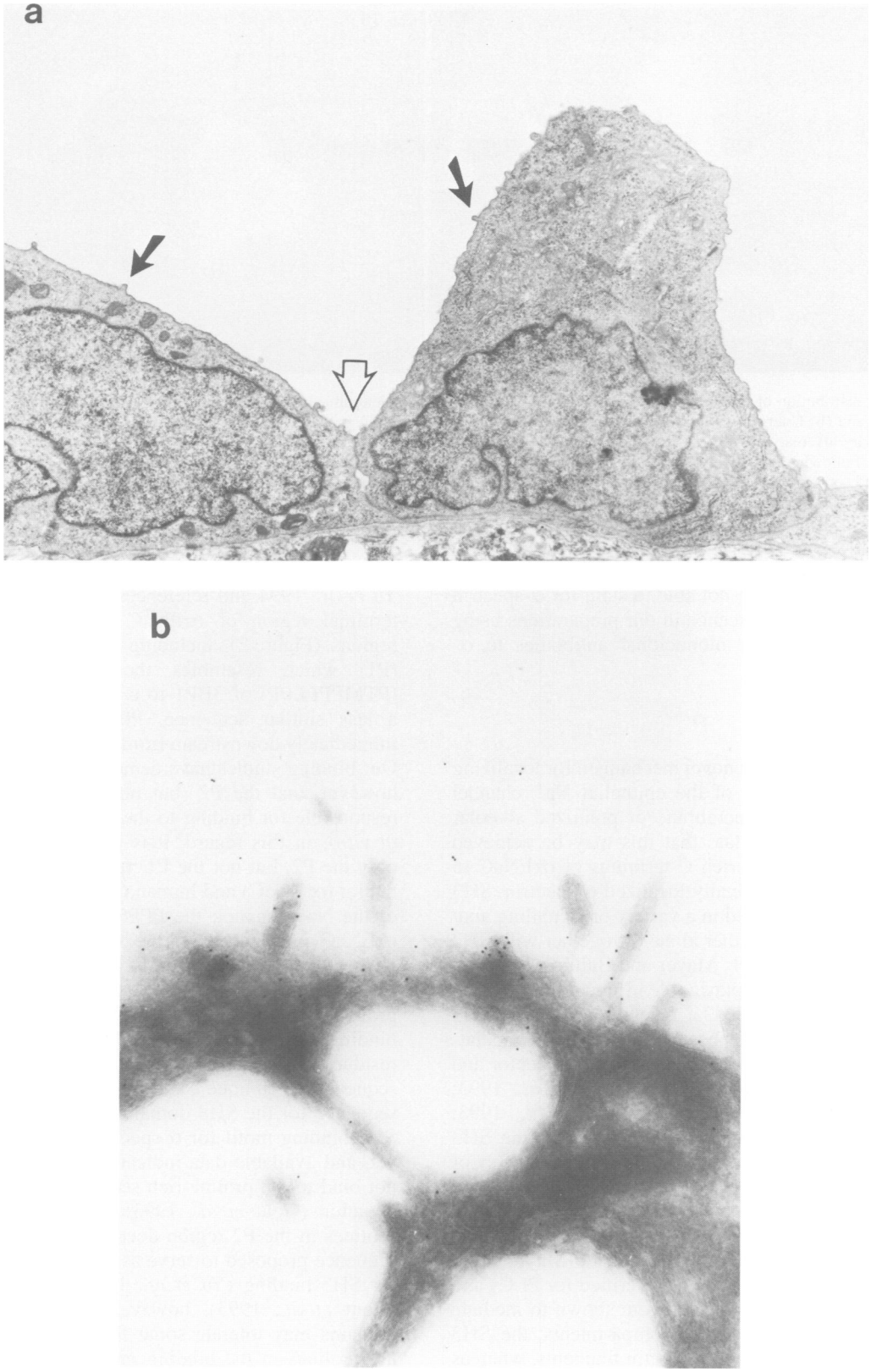

Fig. 6. (**a**) Morphology of primary alveolar epithelial cells seeded on a permeable support. Transmission electron micrograph (~8000 × magnification) of a monolayer of rat alveolar epithelium (type II cells) grown on a permeant filter for 2 days after cell harvest. Characteristic apical microvilli (filled arrows) are present. The apical membrane extends to the tight junctions (empty arrow) that are present on the lateral side of the cultured cells. (**b**) Distribution of α-spectrin in alveolar epithelial cells. Immunoelectron micrograph showing apical distribution of α-spectrin in human alveolar epithelial (type II) cells, as determined by immunogold labelling. (Magnification = × 64 000.)

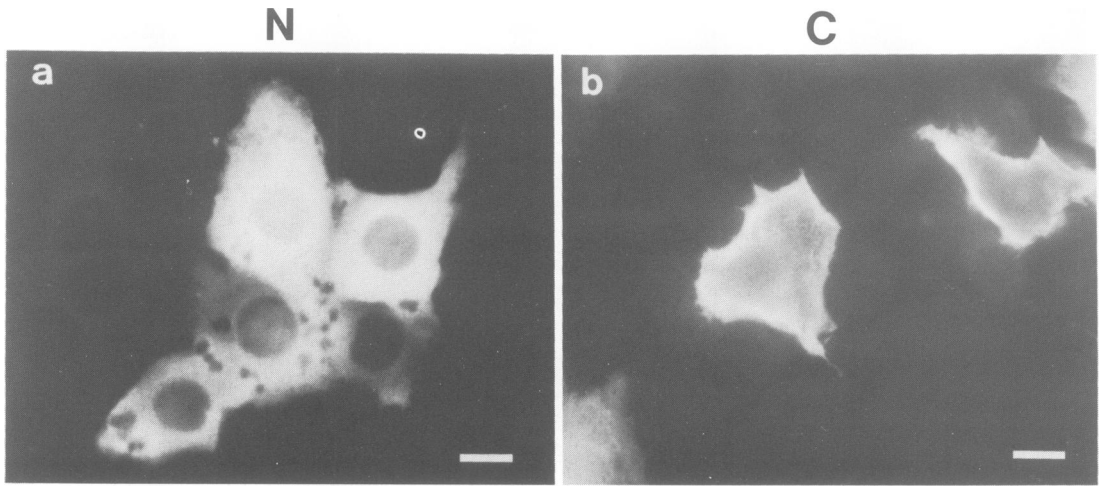

Fig. 7. Cellular distribution of microinjected N- and C-terminal fusion proteins determined by fluorescence microscopy. Distribution of (**a**) N-terminal (N) and (**b**) C-terminal (C) fusion proteins following microinjection into polarized alveolar epithelial cells similar to those shown in Figure 6a. Primary alveolar epithelial cells were seeded on permeable filters, allowed to become polarized and then microinjected with the indicated fusion proteins. Following recovery, the injected cells were fixed and stained with rabbit anti-GST primary antibodies followed by donkey anti-rabbit FITC-conjugated secondary antibodies as detailed in Materials and methods. Concentrations of fusion proteins used for microinjections were 2 mg/ml and 16 mg/ml for the N- and C-terminal proteins respectively, but the amount of proteins actually delivered into each cell was similar, as indicated by the total amount of fluorescence emitted from cells in panels a and b. Bar = 10 μm.

the primary alveolar epithelial cells were grown (to ensure cell polarization), we were not able to stain for α-spectrin by indirect immunofluorescence in our preparations using either affinity purified or monoclonal antibodies to α-spectrin.

Discussion

In this report we describe a novel mechanism for localizing the pore forming subunit of the epithelial Na^+ channel (αrENaC) at the apical membrane of polarized alveolar epithelial cells. We postulate that this may be achieved by binding of the proline-rich C-terminus of αrENaC to the SH3 domain of the apically localized α-spectrin. SH3 domains have been detected in a variety of signalling and/or cytoskeletal proteins, either alone or together with SH2 domains (Koch *et al.*, 1991; Mayer and Baltimore, 1993), suggesting that some may play a role in signal transduction via tyrosine kinases. Indeed, a recent role for the SH3 domains of GRB2/sem5 has been demonstrated in mediating the signalling pathway between the EGF receptor and Ras (Buday and Downward, 1993; Chardin *et al.*, 1993; Egan *et al.*, 1993; Gale *et al.*, 1993; Li *et al.*, 1993; Rozakis-Adcock *et al.*, 1993); in that pathway, the SH3 domains of GRB2 binds to the proline-rich motif(s) of hSOS-1. Since a number of SH3-containing proteins are part of the cytoskeleton or are involved in cytoskeletal organization, it has been suggested that SH3 domains mediate some cytoskeletal functions (Mayer and Baltimore, 1993). This was recently verified for PLCγ and GRB2, where their SH3 domains were shown to mediate binding to specific cytoskeleton compartments; the SH3 domain of PLCγ was localized to actin filaments, whereas GRB2 was localized to membrane ruffles, a localization that was abrogated in GRB2 proteins with SH3 loss of function point mutations (Bar-Sagi *et al.*, 1993).

SH3 domains bind to target proteins by associating with proline-rich motifs, such as those found in 3BP1 (Ren *et al.*, 1993), the above-mentioned SOS proteins, dynamin (Gout *et al.*, 1993) and others (Gout *et al.*, 1993; Yu *et al.*, 1994 and references therein). Similarly, the C-terminal region of αrENaC contains two proline-rich regions (Figure 2), including the sequence PTSPPPSLP (P1) which resembles the SH3 binding sequence (PTMPPPLPP) of 3BP1 to c-abl (Ren *et al.*, 1993), and a less similar sequence, PPLALTAPPPA (P2), found immediately downstream from the first proline-rich region. Our binding studies have demonstrated (Figures 4 and 5), however, that the P2 (but not P1) sequence is the one responsible for binding to the SH3 domain of α-spectrin *in vitro*. In this regard, it is particularly interesting that only the P2, but not the P1, region is conserved between the rat (αrENaC) and human (αhENaC) cDNA sequences of the Na^+ channel; the PPPSLP sequence within P1 of αrENaC is replaced by the sequence LSL in αhENaC (Canessa *et al.*, 1993a; Voilley *et al.*, 1994).

The P2 region in αrENaC is hydrophobic and rich in prolines, alanines and leucines. Although several SH3 binding regions from other proteins contain hydrophobic residues (L/I/V) around the conserved prolines, the P2 sequence is unique, and may represent the binding sequence for the SH3 domain of α-spectrin. The specific SH3 binding motif for α-spectrin has not been described yet, and available data indicate that α-spectrin SH3 does not bind to the proline-rich sequences of 3BP1, SOS and dynamin (Gout *et al.*, 1993). The spacing between the prolines in the P2 region does not conform to the PXXP sequence proposed to serve as a minimal consensus motif for SH3 binding (Yu *et al.*, 1994). As suggested earlier (Gout *et al.*, 1993), however, it is possible that SH3 domains may tolerate some flexibility in the spacing of the prolines in the binding motif. Analysis of the three-dimensional structure of α-spectrin together with the relevant ligand may help resolve this issue.

Localization of specific proteins at specialized membranes in polarized epithelial cells is a complex and incompletely understood process. It has been suggested that proteins are either directly targeted to the appropriate

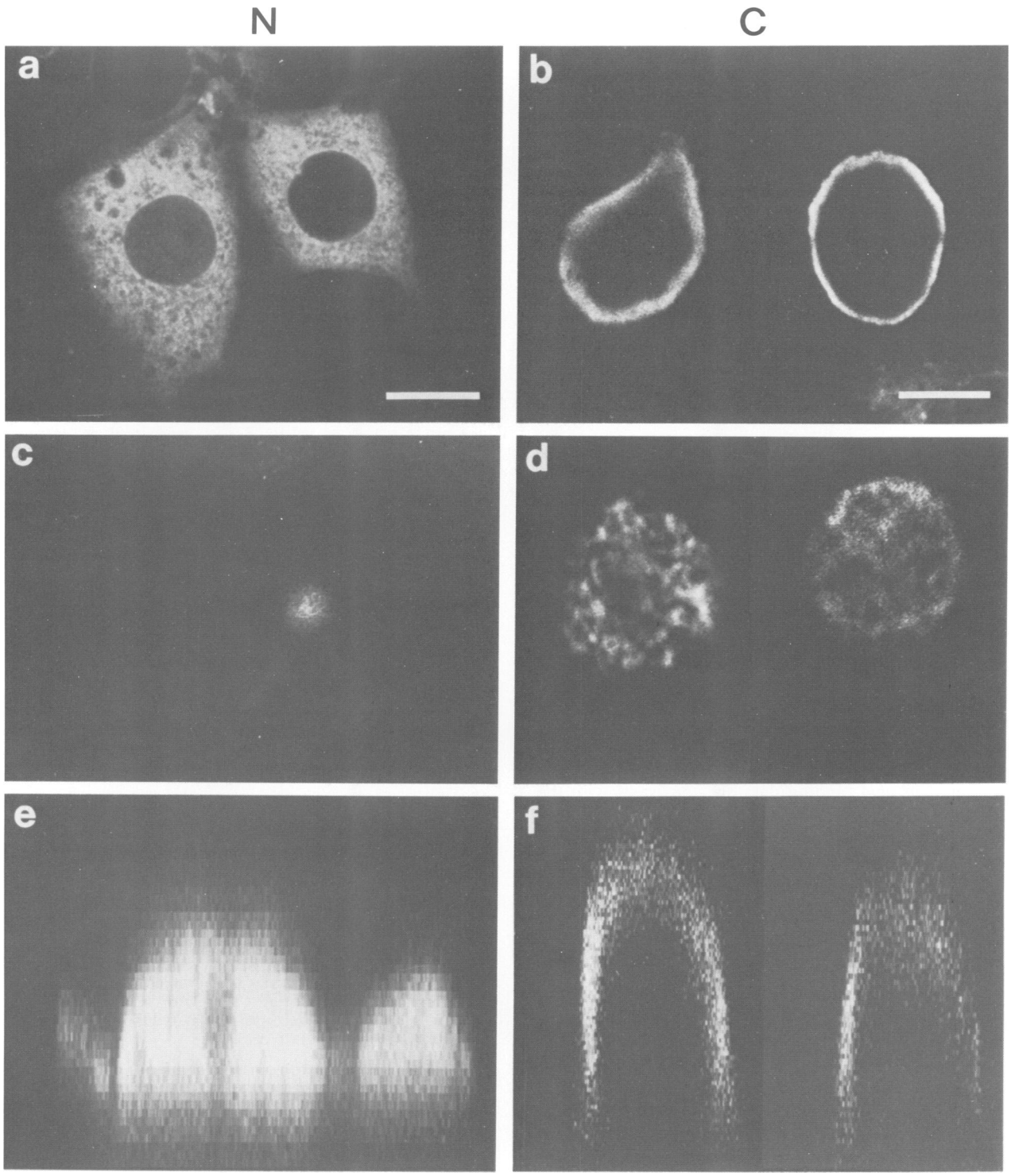

Fig. 8. Subcellular distribution of N- and C-terminal fusion proteins determined by confocal microscopy. (**a**–**d**) Cross-section (x–y) fluorescent images were taken through the middle (panels a and b) and through the apical (panels c and d) regions of cells injected with the N-terminal protein (panels a and c) or C-terminal protein (panels b and d). Microinjections and staining of the injected polarized alveolar epithelial cells were described in Figure 7 and in Materials and methods. (**e** and **f**) Confocal x–z image reconstruction showing vertical distribution of the N- and C-terminal injected proteins. Micrographs depict reconstruction of a series of x–z images taken at 0.5 μm intervals from the same cells shown in panels a–d, which were injected with either the N-terminal (e) or C-terminal (f) fusion proteins. The x–z images are presented as stretches (twice normal height) in the vertical direction to illustrate differences in the subcellular distribution of the N- and C-terminal proteins. The photomicrographs are representative of multiple (10–20) cells observed on each filter from at least three separate cell preparations done on different days. Injected and non-injected neighbouring cells appeared similar, as determined by phase microscopy (not shown), but could not be photographed due to optical interference from the permeable filters on which the cells were seeded. Bar = 10 μm.

Table I. Proline-rich sequences resembling SH3 binding motifs found in ion transporting or ion conducting proteins

Protein	Proline-rich sequences	Reference
Epithelial cells		
NHE2	PPSTVPAPR PPKPPPRLVRR	Wang *et al.* (1993b)
$Na^+/K^+/2Cl^-$	PPPPATPLRP	Xu *et al.* (1994)
Skeletal muscle cells		
ClC-1	PVPPPSPEVP	Steinmeyer *et al.* (1991)
Excitable cells		
NMDAR-2D	PPPAKPPPPPQPLP RPPPGPAPFVPR PLSPPTTQPPQKPPP PGFPSPPAPP PTAPPPRRAAPPP PPPPPWAAGPPPRR PPPAPTSR	Ishii *et al.* (1993)

membrane via the Golgi apparatus, or that they are randomly distributed to membranes and then retained at the relevant membrane by selective retention signals (Nelson, 1993, and references therein). In our study, the normal trafficking process of αrENaC (or segments of it) was bypassed by microinjecting the proteins directly into the cytoplasmic space, yet the C-terminal protein was localized at the apical membrane of polarized epithelial cells. This suggests that it was probably retained there by some retention signal(s), possibly by the proline-rich SH3 binding region of the channel. Although we currently cannot exclude the possibility that SH3 domains from other proteins may be involved in the process, both our *in vitro* and *in vivo* binding data are consistent with the notion that it is the SH3 domain of α-spectrin that may mediate binding to αrENaC and hence retention at the apical membrane.

Cytoskeletal association via SH3 domains could represent a novel mechanism for retaining proteins in specific membranes of polarized or other specialized cells. We have recently identified proline-rich, potential SH3 binding motifs in several transmembrane proteins involved in ion transport in epithelia and excitable (or other) tissues (Table I). These include the NHE2 isoform (but not the NHE1, NHE3 or NHE4 isoforms) of the Na^+/H^+ antiport (Wang *et al.*, 1993b), the rat muscle Cl^- channel ClC-1 (Steinmeyer *et al.*, 1991), the recently cloned $Na^+/K^+/2Cl^-$ cotransporter (Xu *et al.*, 1994) and the D subunit (but not other subunits) of the NMDA receptor 2 (Ishii *et al.*, 1993). Although the ability of any of these proline-rich sequences to bind SH3 domains has not been shown experimentally, their presence in proteins with a requirement for specific membrane localization may suggest their involvement in the determination of that localization. Thus, SH3 binding could represent an as yet undescribed mechanism to localize or retain proteins at specific membranes in polarized or specialized cells.

Materials and methods

Preparation of GST fusion protein constructs

All GST fusion proteins were prepared by PCR amplification of the appropriate regions of αrENaC cDNA (Canessa *et al.*, 1993a), and then subcloning them into the *Bam*HI and *Eco*RI sites of pGEX-2TK (Pharmacia), essentially as described (Smith and Johnson, 1988). The insert-containing plasmid was used to transform the HB101 strain of *Escherichia coli*. Expression of fusion proteins was induced with 0.2 mM IPTG, and bacteria were collected and lysed. As the proline-rich C-terminal fusion proteins were easily degraded (even when a lon^- strain of *E.coli* was used for transformation), high doses of protease inhibitors were used during lysis of bacteria (1 mM PMSF, 100 μg/ml of leupeptin and pepstatin, 0.05% aprotinin, 10 mM benzamidine, 10 μg/ml trypsin inhibitor, 10 μM antipain and 50–100 mM EDTA). Fusion proteins were purified from bacterial lysate with glutathione–agarose beads, as described (Smith and Johnson, 1988). For constructs with mutated prolines, all mutations were carried out by PCR and verified by sequencing.

αrENaC antibodies

Rabbit polyclonal antibodies (αN) were raised against a GST fusion protein encompassing the first 76 amino acids of the N-terminus of αrENaC (see Figure 2). This region of αrENaC shows poor homology to the β and γ subunits of the channel (Canessa *et al.*, 1994). To test the antibodies, 20 μg of cell lysate, prepared either from control untransfected MDCK cells, or from MDCK cells stably transfected with αrENaC in pLK-neo (C.M.Canessa and B.C.Rossier, unpublished) and induced to express the transfected protein with 1 μM dexamethasone for 18 h, were separated on a 7.5% SDS–polyacrylamide gel. After transfer to nitrocellulose membrane, blots were incubated with either pre-immune serum or αN affinity purified antibodies, followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit secondary antibodies. Proteins were visualized with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP), according to the manufacturer's instructions (Bio-Rad).

Precipitation of α-spectrin from epithelial cell lysate

Alveolar epithelial cells, isolated as described below, were lysed in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin). Lysate from ~2–3 × 10^6 cells was then added to 5–10 μg of GST or GST–αrENaC–C-terminal fusion proteins, immobilized on glutathione–agarose beads. Following incubation (90 min, 4°C) the beads were washed three times, and proteins were separated on a 10% SDS–polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were then blotted with anti-α-spectrin mAbs 101-AA6 (Ylikoski *et al.*, 1990), followed by peroxidase-conjugated goat anti-mouse secondary antibodies and ECL detection, according to kit instructions (Amersham).

For co-immunoprecipitations, affinity purified αN antibodies, conjugated to protein A–Sepharose, were incubated (90 min, 4°C) with 100 μl (~3 mg proteins) lysate from MDCK cells overexpressing αrENaC. Beads were then washed four times with HNTG (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol), proteins separated on 8% SDS–polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were then incubated with either αN antiserum (1:500) or with mAbs (101-AA6) to α-spectrin (1:4000), followed by incubation with goat anti-rabbit or anti-mouse (respectively) secondary antibodies and ECL detection.

Filter binding assays

N- or C-terminal fusion proteins of αrENaC (0.5–1.0 μg) or 0.5 μg of construct II–VII proteins, were separated on a 10% SDS–polyacrylamide gel, transferred to nitrocellulose membrane, blocked overnight with TBS-BSA (10 mM Tris, pH 7.4, 150 mM NaCl, 5% BSA) (Sigma) and incubated with biotinylated GST–α-spectrin SH3 (1–2 μg/ml) for 2 h (24°C). Biotinylations were carried out with the NHS-LC biotinylation kit (Pierce). Blots were then washed and incubated with streptavidin-biotinylated peroxidase complex followed by ECL detection.

Preparation of primary cultures of lung epithelial cells

Primary alveolar epithelial cells from 20 day-old rat fetuses were prepared as described (O'Brodovich *et al.*, 1991). Briefly, lungs were removed and cells liberated using a 0.125% trypsin and 0.002% DNase digestion, and were passed through a Nitex 100 mesh filter. After incubation with 0.1% collagenase epithelial cells were separated from fibroblasts using a differential adsorption and centrifugation technique. Epithelial cells were then seeded at confluence onto permeable filter supports (Falcon) and cultured overnight in αMEM + 10% fetal bovine serum (37°C, 5% CO_2 in air) to allow them to attach to the filters and

to become polarized prior to microinjection. These polarized alveolar epithelial (type II) cells actively transport sodium (O'Brodovich *et al.*, 1991), possess amiloride-sensitive Na^+ channels in their apical membrane (Orser *et al.*, 1991), have whole cell amiloride-sensitive Na^+ currents (Wang *et al.*, 1993a) and express transcripts for αrENaC (O'Brodovich *et al.*, 1993).

Microinjections

Polarized alveolar epithelial cell (prepared as described above), seeded on a permeable filter support, were microinjected with the appropriate fusion proteins diluted in PBS, using glass capillaries drawn to a tip diameter of <1 μm, as described (Wang *et al.*, 1982). Each cell received an injected volume of ~10^{-15} μl. The concentrations of injected proteins were 2 and 16 mg/ml for the N-terminal and C-terminal protein, respectively. The latter high concentration was required because the C-terminal protein tended to adhere to the glass pipette used for microinjections. However, the amount of protein actually injected into the cells was comparable with that of the N-terminus, as is also suggested by the similar total amount of fluorescence seen in cells in Figure 7a and b.

Immunofluorescence microscopy

Injected cells (on permeant filters) were transferred to αMEM +10% FBS and kept at 37°C for 15–40 min. Filters were then washed with PBS, fixed with 3% paraformaldehyde, incubated with 100 mM glycine to quench free aldehyde and permeabilized with 0.1% Triton X-100 in PBS containing 0.1% BSA. Permeabilized cells were blocked with 5% goat serum and incubated with rabbit anti-GST antiserum (1:100 dilution), followed by fluorescently labelled (FITC) goat or donkey anti-rabbit secondary antibodies. Injected cells were then observed using conventional fluorescence and confocal microscopy. Injected and non-injected vicinal cells appeared similar, as determined by phase microscopy (not shown), but could not be photographed due to optical interference from the filters on which the cells were grown.

Confocal fluorescence microscopy

Fixed and stained cells, prepared as described above, were viewed using a Bio-Rad 600 laser scanning confocal imaging system mounted on a Leitz Metallux microscope using × 63 (n.a. = 1.3) and × 100 (n.a. = 1.32) PlanApo oil objectives (Leitz). Light for fluorescence excitation was provided by a krypton–argon ion laser (Bio-Rad) and the fluorescent light intensity detected by a photomultiplier after an emission filter (530–580 nm). Serial 0.5 μm optical images using a Kelman averaging protocol were acquired of each cell by scanning at ascending z levels. Beginning at the basal cell surface (i.e. that attached to the substratum), a minimum of 10 optical sections were obtained per cell. The image size was 768 × 512 pixels with an 8-bit gray level resolution (256 gray levels). Images were photographed using Kodak TriX pan film with a Nikon camera attached to a video monitor. The photomicrographs are representative of multiple (10–20) cells observed on each coverslip from at least three separate cell preparations done on different days. The x–z confocal images are stretched (to twice their normal length) in the vertical direction.

Immunoelectron microscopy

Tissues from peripheral lungs were fixed, frozen in liquid nitrogen and sectioned. Ultrathin sections were then incubated with 10% FBS and 20 mM glycine, labelled with anti-α-spectrin polyclonal antibodies (K62) (Merilainen *et al.*, 1993) followed by protein A–gold. The sections were embedded in methylcellulose and examined in a Phillips 410 LS transmission electron microscope.

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References

Bar-Sagi,D., Rotin,D., Batzer,A., Mandiyan,B. and Schlessinger,J. (1993) *Cell*, **74**, 83–91.
Bowtell,D., Fu,P., Simon,M. and Senior,P. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 6511–6515.
Buday,L. and Downward,J. (1993) *Cell*, **73**, 611–620.
Canessa,C.M., Horisberger,J.D. and Rossier,B.C. (1993a) *Nature*, **361**, 467–470.
Canessa,C.M., Merillat,A.-M., Horisberger,J.-D. and Rossier,B.C. (1993b) *J. Am. Soc. Nephrol.*, **4**, 846.
Canessa,C.M., Schild,L., Buell,G., Thorens,B., Gautschi,I., Horisberger,J.-D. and Rossier,B.C. (1994) *Nature*, **367**, 463–467.
Cantiello,H.F., Stow,J.L., Prat,A.G. and Ausiello,D.A. (1991) *Am. J. Physiol.*, **261**, C882–888.
Chardin,P., Camonis,J.H., Gale,N.W., Aelst,L.V., Schlessinger,J., Wigler,M.H. and Bar-Sagi,D. (1993) *Science*, **260**, 1338–1343.
Chen,J., Lane,W., Brauer,A., Tanaka,A. and Schreiber,S. (1993) *J. Am. Chem. Soc.*, **115**, 12591–12592.
Compeau,C.G., Rotstein,O.D., Tohda,H., Marunaka,Y., Rafii,B., Slutsky,A.S. and O'Brodovich,H.M. (1994) *Am. J. Physiol.*, **266**, C1330–C1341.
Dubreuil,R.R., Byers,T.J., Sillman,A.L., Bar-Zvi,D., Goldstein,L.S.B. and Branton,D. (1989) *J. Cell Biol.*, **109**, 2197–2205.
Egan,S.E., Giddings,B.W., Brooks,M.W., Buday,L., Sizeland,A.M. and Weinberg,R.A. (1993) *Nature*, **363**, 45–51.
Els,W.J. and Chou,K.-Y. (1993) *J. Physiol.*, **462**, 447–464.
Gale,N.W., Kaplan,S., Lowenstein,E.J., Schlessinger,J. and Bar-Sagi,D. (1993) *Nature*, **363**, 88–92.
Gout,I. *et al.* (1993) *Cell*, **75**, 25–36.
Ishii,T. *et al.* (1993) *J. Biol. Chem.*, **268**, 2836–2843.
Koch,C.A., Anderson,D., Moran,M., Ellis,C. and Pawson,T. (1991) *Science*, **252**, 668–673.
Lehto,V.P., Wasenius,V.M. and Saraste,M. (1988) *Nature*, **334**, 388.
Li,N., Batzer,A., Daly,R., Yajnik,V., Skolnik,E., Chardin,P., Bar-Sagi,D., Margolis,B. and Schlessinger,J. (1993) *Nature*, **363**, 85–88.
Lingueglia,E., Voilley,N., Waldmann,R., Lazdunski,M. and Barbry,P. (1993) *FEBS Lett.*, **318**, 95–99.
Liu,X., Marengere,L.E.M., Koch,C.A. and Pawson,T. (1993) *Mol. Cell. Biol.*, **13**, 5225–5232.
Matalon,S., Bauer,M., Benos,D., Kleyman,T., Lin,C., Cragoe,E.J.J. and O'Brodovich,H.M. (1993) *Am. J. Physiol.*, **264**, L357–364.
Mayer,B.J. and Baltimore,D. (1993) *Trends Cell Biol.*, **3**, 8–13.
Menco,B. and Benos,D.J. (1989) *J. Cell Biol.*, **109**, 254(a).
Merilainen,J., Palovuori,R., Sormunen,R., Wasenius,V. and Lehto,V.P. (1993) *J. Cell Sci.*, **105**, 647–654.
Nelson,W.J. (1993) *Science*, **258**, 948–955.
O'Brodovich,H.M. (1991) *Am. J. Physiol.*, **261**, C555–564.
O'Brodovich,H.M., Hannam,V. and Rafii,B. (1991) *Am. J. Respir. Cell Mol. Biol.*, **5**, 377–384.
O'Brodovich,H.M., Canessa,C., Ueda,J., Rafii,B., Rossier,B.C. and Edelson,J. (1993) *Am. J. Physiol.*, **265**, C491–496.
Orser,B.A., Bertlik,M., Fedorko,L. and O'Brodovich,H.M. (1991) *Biochim. Biophys. Acta*, **1094**, 19–26.
Pawson,A. and Schlessinger,J. (1993) *Curr. Biol.*, **3**, 434–442.
Ren,R., Mayer,B., Cicchetti,P. and Baltimore,D. (1993) *Science*, **259**, 1157–1161.
Rozakia-Adcock,M., Fernley,R., Wade,J., Pawson,T. and Bowtell,D. (1993) *Nature*, **363**, 83–85.
Saumon,G. and Basset,G. (1993) *J. Appl. Physiol.*, **74**, 1–15.
Simon,M.A., Bowtell,D., Dodson,G.S., Laverty,T.R. and Rubin,G.M. (1991) *Cell*, **67**, 701–716.
Smith,D.B. and Johnson,K.S. (1988) *Gene*, **67**, 31–40.
Smith,P.R. and Benos,D.J. (1991) *Annu. Rev. Physiol.*, **53**, 509–530.
Smith,P.R., Saccomani,G., Joe,E.-H., Angelides,K.J. and Benos,D.J. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 6971–6975.
Sormunen,R., Paakko,P., Palovuori,R., Soini,Y. and Lehto,V. (1994) *Am. J. Respir. Cell Mol. Biol.*, **11**, 75–84.
Steinmeyer,K., Ortland,C. and Jentsch,T. (1991) *Nature*, **354**, 301–304.
Tousson,A., Alley,C.D., Sorscher,E.J., Brinkley,B.R. and Benos,D.J. (1989) *J. Cell Sci.*, **93**, 349–362.
Voilley,N., Lingueglia,E., Champigny,G., Mattéi,M.G., Waldmann,R., Lazdunski,M. and Barbry,P. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 247–251.
Wang,K., Faramisco,J.R. and Ash,J.F. (1982) *Methods Enzymol.*, **85**, 514–562.

Wang,X., Kleyman,T., Tohda,H., Marunaka,Y. and O'Brodovich,H.M. (1993a) *Can. J. Physiol. Pharmacol.*, **71**, 58–62.
Wang,Z., Orlowski,J. and Shull,G. (1993b) *J. Biol. Chem.*, **268**, 11925–11928.
Wasenius,V.M., Saraste,M., Salven,P., Eramaa,M., Holm,L. and Lehto,V.P. (1989) *J. Cell Biol.*, **108**, 79–93.
Wong,G., Muller,O., Clark,R., Conroy,L., Moran,M.F., Polakis,P. and McCormick,F. (1992) *Cell*, **69**, 551–558.
Xu,J., Lytle,C., Zhu,T., Payne,J., Benz,E. and Forbush,B. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 2201–2205.
Ylikoski,J., Pirvola,U., Narvanen,O. and Virtanen,I. (1990) *Hearing Res.*, **43**, 199–203.
Yu,H., Chen,J., Feng,S., Dalgarno,D., Brauer,A. and Schreiber,S. (1994) *Cell*, **76**, 933–945.

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Note added in proof

We have recently demonstrated inhibition of binding of αrENaC C-terminal fusion proteins lacking the P2 region to intact α-spectrin.