Intramolecular Fusion of Na Pump Subunits Assures Exclusive Assembly of the Fused $\alpha$ and $\beta$ Subunit Domains into a Functional Enzyme in Cells also Expressing Endogenous Na Pump Subunits*

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Experiments designed to identify Na pump structural features which tag the molecule for asymmetric cell-surface localization are inherently complex because either subunit, or both, may contain targeting information and because the cells which recognize those targeting signals and maintain asymmetric plasma membrane domains also express their own Na pumps, the subunits of which can assemble into hybrid pump molecules with pump subunits expressed from transfected cDNA clones. Cotransfecting cDNA for both subunits only complicates matters further by resulting in expression of four distinct dimeric molecular species. To eliminate the potential for cross-assembly in these and other experiments we have constructed cDNA encoding a "single-subunit" Na pump (called $\alpha$-$\beta$) in which the $\alpha$ and $\beta$ subunits are joined by a linker of 17 amino acids. By all criteria tested $\alpha$-$\beta$ functioned as a normal heterodimeric Na pump. It was expressed in a variety of mammalian cell lines as a single, high molecular weight polypeptide located primarily on the surface membrane, with the $\beta$ subunit exposed to the extracellular medium. Binding of the conformation-sensitive monoclonal antibody 24 to the $\beta$ subunit indicated that the fusion protein was folded as a properly "assembled" sodium pump. Expression of $\alpha$-$\beta$ in ouabain-resistant mouse L cells resulted in high affinity ouabain binding and ouabain-sensitive, sodium-dependent rubidium transport. The enzyme was properly targeted to the basolateral plasma membrane in polarized epithelial cells. The functional integrity of the fusion protein renders it suitable for site-directed mutagenesis studies of targeting and enzymology where control of subunit assembly is desired. These results also support topological models in which the carboxyl terminus of the $\alpha$ subunit is cytoplasmic.

The Na,K-ATPase (Na pump) is an enzyme located in the plasma membrane of all animal cells. It couples the energy of ATP hydrolysis to the transport of sodium and potassium ions against potential gradients. In this way it maintains normal transmembrane gradients of these ions and provides energy for electrical signal transduction in excitable cells or for the coupled transport of other solutes across the plasma membrane (for reviews, see Horisberger et al. (1991c) and Jorgenson and Anderson (1988)). To effect the vectorial transport of sodium across epithelia, the Na pumps of epithelial cells are located asymmetrically with respect to the plane of the epithelial wall, usually in the basolateral domain of the plasma membrane (Matlin and Caplan, 1992). The Na pump holoenzyme is a dimer comprised of two dissimilar subunits. The $\alpha$, or catalytic, subunit is a $\sim$110-kDa polypeptide which crosses the membrane 8 or 10 times. Much of its mass is contained within a large central cytoplasmic domain between the fourth and fifth transmembrane segments. The $\alpha$ subunit contains the domains directly involved in ion translocation and ATP hydrolysis as well as binding sites for substrates and inhibitors of enzyme function. The $\beta$ subunit is a $\sim$55-kDa polypeptide comprised of a short cytoplasmic amino-terminal domain, a single membrane span, and a glycosylated extracellular domain.

Complimentary DNAs encoding both subunits have been cloned from a wide variety of sources (Horisberger et al., 1991c; Lingrel et al., 1990). Comparison of the amino acid sequences for the proteins encoded by these cDNAs revealed three isoforms for the $\alpha$ subunit and two or three (Good et al., 1990) $\beta$ subunit isoforms. Although a reason for the existence of multiple isoforms has not been identified, each isoform has been found to have a characteristic tissue distribution, and differences have been observed between $\alpha$ subunit isoforms in substrate affinities and ouabain sensitivity. These characteristics suggest that the Na pumps in a particular tissue may be precisely tuned kinetically to the conditions of the cellular environment in that tissue by means of the selective expression of particular isoforms. In some tissues these isoforms may be differentially regulated while the other is not, consistent with a role for distinct isoforms in establishing separate, regulated, and constitutively expressed pools of Na pumps in those tissues (Ghosh et al., 1991).

Experiments designed to discern the contributions of different $\alpha$ or $\beta$ subunit isoforms to Na pump function typically involve measurements of Na pump activity or metabolism in cultured cells expressing specific subunit isoforms or mutant isoforms constructed from cloned cDNA (e.g. Price and Lingrel (1988), Horisberger et al. (1991b), and Gottardi and Caplan (1993)). A significant limitation in this type of experiment is that the exogenous subunits can assemble with the recipient cells' own Na pump subunits, even when both $\alpha$ and $\beta$ subunits are expressed at high levels.

To eliminate cross-assembly as a confounding factor in studies of the properties of specific sodium pump subunits expressed from cDNA, we have designed a "single-subunit"
**Single-subunit Na Pump**

**RESULTS**

Fig. 2 shows an autoradiograph of Na pump polypeptides immunoprecipitated with mAb 24 from Cos-1 cells transiently expressing α-β. Only a single polypeptide of M, 135,000 is evident (A). The corresponding lane from a mock transfection is blank (B). In other experiments the apparent molecular mass of the immunoprecipitated peptide was in the range of 135–149 kDa. Expression of the α-β protein in this experiment was controlled by the vaccinia T7 promoter. The exogenous protein is expressed at high levels, and a large amount appears on the surface of the cell when visualized by immunofluorescence microscopy with mAb 24 (not shown, but see Figs. 3 and 4). α-β expressed in mouse L cells and immunoprecipitated with mAb 24 was also uncleaved (not shown). The presence of only a single large peptide in Fig. 2A indicates
Strong intracellular mAb 29 labeling was present in a few cells, however, which appeared to express the construct at extraordinarily high levels (not shown).

Fig. 4 shows two confocal optical sections of mAb 24 immunofluorescence through a single saponin-permeabilized LLC-PK1 cell transiently expressing α-β. The cell is one of a confluent monolayer of cells grown on a glass coverslip. A section through a plane parallel to the coverslip midway through the cell (lower panel) shows bright labeling of the surface membrane only. A vertical section through the middle of the cell (upper panel) shows strong labeling of the basal and lateral membranes and complete absence of label on the apical surface. These results show that the targeting signals which direct Na pumps to the basolateral membrane in epithelial cells are intact in the fusion protein.

Because of the vast difference in affinity for ouabain between the chicken α3 and the endogenous mouse α subunits in 0 mM extracellular potassium (K\textsubscript{o+}), the ouabain binding sites associated with α-β on the surface of mouse L cells can be titrated at low concentrations of ouabain, where negligible binding of ouabain to the L cell Na pumps occurs. Fig. 5 shows that \( L(\alpha_3\cdot\beta_1) \) cells expressed high affinity ouabain-binding sites (\( K_D \approx 33 \text{ nM} \) in 0 K\textsubscript{o+} at ~170,000 sites per cell after 3 days of butyrate induction. By contrast, specific \(^{3} \text{H} \) ouabain binding to untransfected L cells subject to identical butyrate treatment was not detectable over the range of \(^{3} \text{H} \) ouabain concentrations studied.

Fig. 6 shows that butyrate-induced \( L(\alpha_3\cdot\beta_1) \) cells expressed a Rb-uptake component with ~1000-fold higher ouabain sensitivity than the butyrate-treated untransfected L cells. Both components were enhanced ~2-fold by 15 μg/ml monensin, demonstrating that they are Na-sensitive (Pressley et al., 1986) The highly ouabain-sensitive component was inhibited half-maximally by 100 nM ouabain, comparable to the \( K_D \) for ouabain binding obtained in Fig. 5. The difference may be attributable to the presence of 80 μM KCl in the Rb-uptake...
We have shown that the chicken α3-β1 fusion construct is expressed in mammalian cells as a single molecular unit. The expressed molecule is an active Na-dependent, ouabain-sensitive ion pump with high affinity for ouabain, a characteristic of Na pumps containing the chicken α3 subunit. The enzyme is efficiently targeted to the plasma membrane of L cells, and to the basolateral membrane of LLC-PK1 cells when expressed at moderate levels. These results suggest that the fused α and β subunit domains are folded and assembled as they are in the native enzyme.

Folding and assembly into a functional enzyme implies that the proper transmembrane topology of the two subunits is preserved in the fusion protein. Because the β subunit is known to have its amino terminus on the cytoplasmic side, the carboxyl terminus of the α subunit must be cytoplasmic as well, consistent with most current topological models (Lingrel et al., 1990).

That the α-β fusion protein preserves the enzymatic and cell biological characteristics of the wild-type ATPase renders it well suited to studies designed to tease apart the roles of the α and β subunits in the enzyme mechanism or intracellular processing of the heterodimer. One approach to such studies has involved coexpression of defined pairs of α and β isoforms in cells which lack sodium pumps of their own, for example yeast (Horowitz et al., 1990). Such a system removes the problem of cross-assembly of exogenous and host Na pump subunits. A potential limitation, however, is the fact that post-translational processing of the ATPase in these cells may be much different than in animal cells and may have effects on enzyme properties which will also have to be assessed. The use of this approach for studies of cell biology may also be limited by failure of the host cells to process properly targeting signals on the foreign molecule. A second approach is to use α subunits with precisely defined mutations to convert them into ouabain-resistant forms. These can be expressed in animal cells, even those of the same tissue as that from which the clone was derived, and studied in the presence of ouabain at a concentration high enough to inhibit only the endogenous pumps. This type of experiment has been useful in discriminating subtle differences in substrate dependencies among the three α isoforms (Jewel and Lingrel, 1991). There is no way to eliminate cross-assembly with endogenous β subunits, however, and no simple way to assess its effects on the observed enzymatic properties. This problem may be circumvented by using a ouabain-resistant α subunit linked directly to a defined β subunit.

An important question at present concerns the identification of the signals which direct the enzyme to the apical or basolateral domain in polarized epithelial cells. Gottardi and Caplan (1993) have addressed this question as it pertains to the gastric H,K-ATPase, a heterodimeric P-type ion-motive ATPase which is mechanistically and structurally homologous to the Na pump. Both the Na and the H,K pumps are expressed in gastric epithelial cells but, whereas the Na pump is located in the basolateral plasma membrane, the H,K pump is expressed apically (Forte and Soll, 1989). Gottardi and Caplan showed that the H,K pump β subunit contains information which results in apical localization in LLC-PK1 cells (a porcine proximal tubule epithelial line) when expressed in the absence of the H,K pump α subunit. They have also shown that a chimera ("H519N") of the Na pump α subunit, in which the amino-terminal half is replaced with the corresponding region of the H,K pump, is targeted apically in these cells in association with the endogenous Na pump β subunit. Thus both the α and the β subunits of the H,K pump contain
apical localization signals, and if the Na pump β subunit contains an analogous basolateral localization signal, it is subordinate to the positive apically directing signal in the α subunit chimera.

Identifying the mechanism of basolateral placement of the Na pump may be comparatively difficult, and the α-β fusion protein may provide a simplified approach. Despite overall structural homology, the targeting mechanisms may be quite different for the Na and the H,K pumps. It may be that a positive basolateral targeting signal on the Na pump was replaced with an apically directing signal in the H519N chimera. If this is true it should be a simple matter to replace the apical targeting signal on the H,K α subunit with the Na pump basolaterally directing segment and observe delivery of this chimera entirely to the basolateral surface. Alternatively the Na pump may be delivered to the basolateral membrane as a part of the "default" pathway for membrane proteins without positive targeting signals, or it may be delivered nonpreferentially to both domains but accumulate basolaterally by virtue of a stable association with other basolateral components such as the spectrin-based cortical cytoskeleton. In either of these cases addition of a positive apical targeting signal would redirect all newly synthesized Na pumps to the apical membrane, consistent Gottardi and Caplan's results.

The presence of an actual basolateral targeting signal on the Na pump is likely to be demonstrated by the effects of its removal. If association with the cytoskeleton is what keeps the Na pumps at the basolateral surface, as Hammerton et al. (1991) have proposed, then disruption of the ankyrin binding component(s) on the α subunit, once they are identified, should result in a uniform steady-state distribution of the modified pumps over the entire cell surface in polarized cells. The appearance of pumps on the apical membrane could most readily be observed in permeabilized cells by immunofluorescence microscopy with antibodies to an external epitope of the transfected protein. Of these, antibodies to the α subunit are rare. Fusion of the β to the α subunit has the effect of attaching an extracellular epitope to the α subunit at the same time as it eliminates cross-assembly with the endogenous β subunits. This provides a simple means to quantify the amount of transfected Na pump on each domain by direct radioimmunolabeling of either surface of a confluent monolayer of polarized cells grown on filters. The standard method of quantitation, surface biotinylation, is inapplicable because the efficiency of biotinylation differs between the two membrane surfaces (Gottardi and Caplan, 1992). Quantitation of the distribution of α subunit antibodies in permeabilized cells from three-dimensional reconstructions of confocal images is only approximate. Finally, the ability to coexpress α/β double mutants in the form of a single molecular entity will greatly simplify experimental design even if both the α and β subunits contain targeting information. Thus the α-β fusion construct provides a simple, quantitative means to assess the role of the cytoskeleton in the distribution of Na,K-ATPase molecules in polarized epithelial cells by means of conventional techniques without cytoskeletal disruption and without the need to permeabilize the cells to expose internal epitopes.

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