博士論文（要約）

論文題目 On the large-scale production of Na^+,K^+-ATPase in cultured mammalian cells
（哺乳類培養細胞による Na^+,K^+-ATPase の大規模生産）

氏　名　ニールセン　ティア　キルケゴール
Tea Kirkegaard Nielsen

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Na^+,K^+-transporting adenosine triphosphatase (Na^+,K^+-ATPase), also known as the sodium pump, is an ATP driven ion pump present in all animal cells. It transports 3 Na^+ from the cytoplasm to the extracellular medium and 2 K^+ in the opposite direction per ATP hydrolysed. Na^+,K^+-ATPase is thus responsible for generating gradients for Na^+ and K^+ across the plasma membrane. These gradients are used for many fundamental biological processes, notably, in nerve cell excitation. Na^+,K^+-ATPase belongs to P-type ATPase superfamily, which includes Ca^{2+}-ATPase (SERCA) as another representative member. Structural and even biochemical studies on Na^+,K^+-ATPase run far behind those on SERCA. This is partly because Na^+,K^+-ATPase is a far more complex protein than SERCA. It consists of α and β subunits and an auxiliary FXYD protein. The α-subunit is the catalytic subunit, with a very similar architecture to SERCA1a. The β-subunit is heavily glucosylated, and is considered to be the molecular chaperone of the α-subunit. The FXYD protein is an auxiliary regulatory protein for fine tuning of catalytic activity and stability. The other reason, why Na^+,K^+-ATPase's structural information is limited, is that no good system is established for large-scale production of pumps. Such a system is absolutely needed for studying ion pumping mechanism in detail. It is needless to say that atomic structure for human proteins will be very advantageous for drug development.

For heterologous expression of mammalian membrane proteins, many different systems have been explored. For Na^+,K^+-ATPase, a yeast system using *Pichia pastoris* has seen some success. Yet, there are serious problems arising from a low expression level and different lipid environment. An efficient expression system using mammalian cells is highly desirable. An adenovirus-COS cell system developed in applicant's laboratory has proven to be very useful for SERCA proteins. Yet, it is not straightforward at all to apply the system to Na^+,K^+-ATPase, as it is a multi-subunit protein residing in the plasma membrane, whereas SERCA is a single polypeptide protein staying in the sarcoplasmic reticulum membrane(or cytoplasmic membranes). Furthermore, as Na^+,K^+-ATPase is present all animal cells, an efficient tag is absolutely needed to separate the recombinant from the endogenous protein. Here I describe my effort towards the large scale

production of human Na^+,K^+-ATPase in mammalian cells using recombinant adenovirus system.

Results

a. Quantitation of endogenous Na^+,K^+-ATPase in mammalian cells

The cell line for heterologous expression should be chosen based on its ability for producing target protein and the amounts of endogenous protein of the same kind. For this purpose, four cell lines (COS1, COS7, MDCKI and MDCKII) were examined for the amounts of endogenous Na^+,K^+-ATPase using two types of antibodies against the α-subunit. The expression level of endogenous Na^+,K^+-ATPase is similar in the four cell types, about 1% of total proteins in the plasma membrane, and mostly the α1 isoform. This means that the contribution of endogenous Na^+,K^+-ATPase is significant and complementation with recombinant protein should be considered.

b. Selection of tag and its position

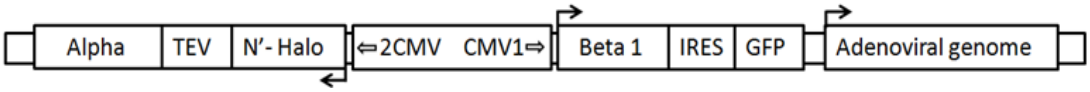
We chose Halo-tag, as it has proven to be very efficient for large scale production of SERCA1a. For the α-subunit it should be attached to the N-terminus, as the C-terminus has an important structural role. For the β-subunit 3 possibilities (i.e. none, N-terminus and C-terminus) should be examined. These possibilities were examined by visualising the localisation using confocal microscopy and fluorescent ligands for Halo-tag. When Halo-tag was attached to the N- or C-terminus of the β-subunit, major proportion of recombinant Na^+,K^+-ATPase remained in ER, indicating that Halo-tag should be attached to the α-subunit.

c. Ratio of α- and β-subunits

It is possible to use two separate viruses for the α- and β-subunits or a single virus harbouring both subunits. To see if the amounts of plasmid affect the localisation of recombinant Na^+,K^+-ATPase, COS7 cells were transfected by various ratio of plasmids for the two subunits. Corroborating our expectation, a significant fraction of the α-subunit is correctly transported to the plasma membrane, presumably complemented with the endogenous β, even when no plasmid for β was included. Yet, also as expected, proper localisation was observed only when sufficient amounts of β plasmid were included. Localisation was correct when bidirectional expression vector was used.

d. Construction of recombinant adenovirus

These observations lead us to construct the following plasmid that was incorporated into recombinant adenovirus.



In this construct, a bidirectional CMV promoter is used. Halo-tag is attached to the N-terminus of α with a TEV protease site to remove the Halo-tag during purification. GFP is also included, following the internal ribosome entry site (IRES), for efficient monitoring of expression level.

e. Overexpression of human Na^+,K^+-ATPase in different cell lines

To find the best cell line for expressing human Na^+,K^+-ATPase, 6 cell lines (COS1, COS7, MDCKI, MDCKII, MC1 and MC2) were infected with the recombinant adenovirus. Expression levels in MDCK cell lines were low, consistent with paucity of CAR-receptors for adenovirus. Contrary to our experience with SERCA proteins, COS1 was superior to COS7 in expressing human Na^+,K^+-ATPase, about 5 times more than endogenous one.

f. Large scale production of human Na^+,K^+-ATPase

After optimising the amount of virus to infect the cells and the ratio of the two viruses, one for FXYD protein and the other for the α- and β-subunits, large scale production of human Na^+,K^+-ATPase were performed with 40 dishes of 15 cm diameter. Three isoforms of the α-subunit (α1-α3) were expressed with the β1 subunit and FXYD1.

g. Solubilisation and purification of human Na^+,K^+-ATPase

Purification of recombinant Na^+,K^+-ATPase with Halo-resin after solubilisation with $C_{12}E_8$ went out smoothly and yielded very pure preparation, as judged by SDS-PAGE. Blue native gel electrophoresis confirmed that the α- and β-subunits made a complex. Deglycosylation of the β-subunit yielded a very similar pattern to that of the pig kidney ATPase, showing that glycosylation took place properly. Nevertheless, the ATPase activity of the purified enzyme was rather low (0.3 μmol Pi/min/mg). Preliminary experiments to find optimum detergent and lipid environment were only partially successful.

Discussion

As far as we are aware, this is the first attempt to produce large quantity of a mammalian membrane protein that resides in the plasma membrane using

mammalian cultured cells.

The expression level of recombinant Na^+,K^+-ATPase was not high enough to be able to neglect contribution of the endogenous ATPase. As complementation of the recombinant α by endogenous β occurs, one tag attached to the α-subunit is insufficient. One possible way for overcoming this problem is to attach another tag (e.g. 6 His) to the N-terminus of β or express the same β-subunit as the endogenous one.

The ouabain-sensitive ATPase activity was substantially lost by solubilisation and purification. One possible cause is insufficient amount of phospholipids and cholesterol. I plan to mimic exactly the lipid composition in the crystals of the pig kidney ATPase.

Conclusion

A recombinant adenovirus was designed for efficient expression of human Na^+,K^+-ATPase in mammalian cells. In this construct, a bidirectional expression vector is used for producing the α- and β-subunits at 1:1 ratio, and a Halo-tag is attached to the N-terminus of α. Of the six cell lines tested, COS-1 appeared to be the best for expressing Na^+,K^+-ATPase. Using this system, large scale production of human Na^+,K^+-ATPase (α1-α3/β1/FXYD1) was successful. Expressed proteins were properly transferred to the plasma membrane and purified to homogeneity using the Halo-tag. In the most successful case, 0.63 mg purified protein was obtained from 40 plates of 15 cm dishes (equivalent to 0.92 L of culture medium). As scaling up to 120 plates is practical, it can be concluded that this system is applicable to crystallisation of human Na^+,K^+-ATPase.