Mutual dependence of Na,K-ATPase α- and β-subunits for correct posttranslational processing and intracellular transport

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Received 20 June 1990

In this study, we have followed the fate of newly synthesized α- and β-subunits of Na,K-ATPase in *Xenopus* oocytes injected with α and/or β cRNA to examine whether assembly of the two subunits is needed for a correct folding and/or for intracellular transport of Na,K-ATPase. Our data indicate that (1) assembly of α- and β-subunits occurs at the level of the ER, (2) β-subunits are needed for the newly synthesized α-subunit to adopt a stable configuration and (3) α- and β-subunits mutually depend on each other to be transported out of the ER.

Na,K-ATPase; Protein biosynthesis; Protein oligomerization; Glycosylation processing; Protein degradation; *Xenopus laevis*; Oocyte

1. INTRODUCTION

Na,K-ATPase is an ubiquitous plasma membrane enzyme found in animal cells which is responsible for cellular Na^+ and K^+ homeostasis [1].

The minimal functional enzyme unit is a heterodimer of tissue-specific isoform composition [2]. The catalytic α-subunit comprises all functional properties so far characterized, namely, it hydrolyzes ATP and it binds Na and K as well as the specific cardiac glycoside inhibitors. The functional role of the glycosylated β-subunit is much less well defined. There is no doubt that the β-subunit is needed for the expression of functional enzymes [3,4]. However, so far there exists no convincing evidence that the β-subunit is directly involved in the catalytic cycle. Rather, it might have an indirect regulatory role at the plasma membrane as recently suggested by Gloor et al. [5] who have identified β2 isoforms as adhesion molecules in glial cells.

Besides such modulatory effects on the active enzyme it is possible that the β-subunit has a distinct role in the structural and functional maturation of the enzyme (for review, see [6]). This hypothesis is supported by data obtained with other oligomeric proteins which suggest that assembly of individual subunits is a prerequisite for an oligomeric protein to attain functional maturity and/or to exit from the endoplasmic reticulum [7].

Since very little is known whether this concept holds true also for Na,K-ATPase, we have exploited in this study the *Xenopus* oocyte to overexpress exogenous Na,K-ATPase subunits and to characterize the effect of subunit assembly on the structural maturation of the α-subunit by measuring the half-life of the catalytic subunit newly synthesized in the presence or absence of β-subunit.

In addition, by following the posttranslation processing of the β-subunit as a glycoprotein, we tested whether the two subunits mutually depend on each other to be efficiently transported out of the ER.

2. MATERIALS AND METHODS

Stage VI oocytes were obtained from *Xenopus* females and prepared for injection as previously described [3]. α and β cRNA were transcribed [8] from linearized *Xenopus* α1- and β1-cDNA templates subcloned into plasmid $pGEM_2$ [3,9]. Fifty nl containing 3 ng β-cRNA and/or 9 ng α-cRNA were injected into the vegetal pole of oocytes. After injection, oocytes were labeled for 3 or 4 h with 3 mCi/ml ^{35}S-methionine in MBS medium [10] and subjected to different chase periods in the presence of 10 mM methionine. After the incubation period, Triton X-100 oocyte extracts were prepared [10]. In some instances, oocytes were frozen at −70°C and bisected along the animal-vegetal equator before extraction of individual oocyte halves as described by Ceriotti and Coleman [11]. Aliquots of Triton extracts were denatured with SDS and then immunoprecipitation, SDS-PAGE, and autoradiography were performed as previously described [12]. Autoradiographs were quantitated by laser densitometry. Antibodies against *Xenopus* α1-subunits were prepared in rabbits against fusion proteins expressed from pEx vectors containing *Eco*RI/*Pvu*II fragments of *Xenopus* α cDNA encoding 98 amino acids of the N-terminus of α1-subunits. A β-galactosidase/β1-subunit fusion protein was constructed by cloning a *Bam*H_1/*Pst*1 fragment of the *Xenopus* β1-subunit encoding extracellular domains of the β1-subunit into the pEx vector. DNA was transformed into *E. coli* (strain POP) and the fusion proteins were isolated as described by Seth [13].

3. RESULTS AND DISCUSSION

3.1. *β-subunits confer a stable configuration to newly synthesized α-subunits*

To test whether assembly of the β-subunit in the endoplasmic reticulum imposes a conformational change

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

on the α-subunit, we compared the degradation rate of α-subunits newly synthesized in oocytes which had been injected with α cRNA alone or with α and β cRNA.

Fig. 1A shows a typical autoradiography of α-subunit immunoprecipitates obtained from oocytes labeled for 4 h and subjected to various chase periods. For the determination of the half-life of the α-subunit, results from several similar experiments were compiled and represented in a semilogarithmic plot. Despite some important variations between different batches of oocytes, our data clearly indicate that the half-life of the α-subunit synthesized in oocytes injected with $\alpha+\beta$ cRNA is considerably longer ($t_{\frac{1}{2}}$ at least 20 h) than in oocytes injected with α cRNA alone (Fig. 1B). The half-life of the α-subunit in oocytes injected with α cRNA alone is in fact biphasic. About 90% of the α-subunit is degraded within 5 h ($t_{\frac{1}{2}} \sim 2$ h), while the remaining population is highly stable, resembling the α-subunit synthesized in α- and β-cRNA injected oocytes (Fig. 1B). We believe that this small stable population represents the newly synthesized α-subunit which is associated to oocyte β-subunits while the large rapidly degraded α-subunit population represents the α-subunit synthesized from injected α cRNA in excess over endogenous β-subunit.

The increase of the half-life of the α-subunit by the concomitant synthesis of the β-subunit suggests that association of the β-subunit provokes a structural change in the α-subunit. This result is in good agreement with our previous observations that the highly trypsin-sensitive endogenous α-subunit of oocytes synthesized in excess over β-subunits can be rendered trypsin-resistant by injection of β cRNA [3]. In addition, it supports the hypothesis that the acquisition of trypsin-resistance of the α-subunit soon after its synthesis, which we observe in differentiated cells synthesizing stoichiometric amounts of α- and β- subunits [14] might be due to subunit assembly. The present data indicate that Na,K-ATPase is likely to be subjected to the same constraints as other oligomeric proteins which have been shown to be incompletely folded when synthesized as individual subunits, and thus to be inherently more sensitive to proteolytic action (for review, see [7]).

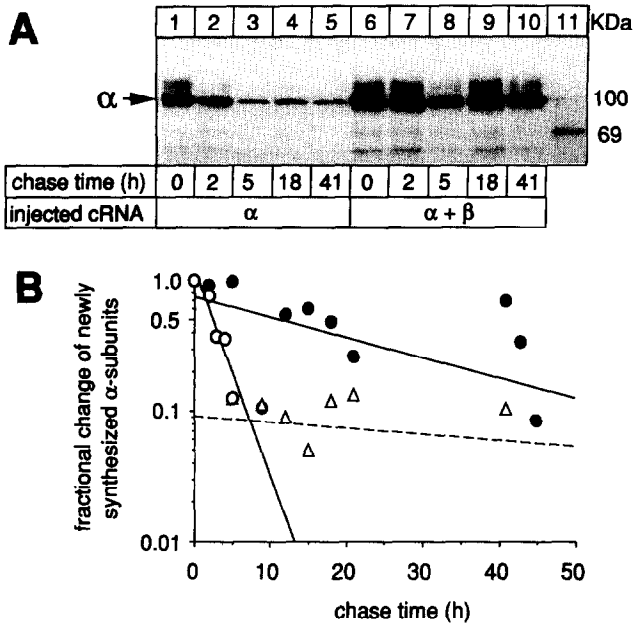

Fig. 1. Degradation of newly synthesized α-subunits in *Xenopus* oocytes. (A) Autoradiography of immunoprecipitated α-subunits after different chase times. Oocytes were injected either with 9 ng α cRNA (lanes 1-5) or with 9 ng α cRNA plus 3 ng β cRNA (lanes 6-10), labeled for 4 h with ^{35}S-methionine and subjected to indicated chase periods. Extraction of oocytes, immunoprecipitation and SDS-PAGE, as described in Materials and Methods. The same number of counts were subjected to immunoprecipitation at each time point. Specific radioactivity of total cellular proteins did not change significantly during the chase period. Lane 11: protein markers. (B) Half-life of α-subunits. Results from several experiments shown in A were quantitated and represented as fractional change of α-subunits during various chase periods in oocytes injected with α cRNA (○, $y=1.26\cdot10^{-16}\times$; △, $y=0.09\cdot10^{-0.005}\times$) or with α plus β cRNA (●, $y=0.73\cdot10^{-0.015}\times$).

3.2. *Subunit assembly as a prerequisite for exit out of the ER of Na,K-ATPase*

In an attempt to assess the competence for intracellular transport of α- and β-subunits synthesized individually or together, we injected α, β or $\alpha+\beta$ cRNA into the vegetal pole of oocytes and followed the glycosylation processing of the β-subunit and the appearance of newly synthesized subunits in the opposite animal pole after a 3 h pulse and various chase periods.

Fig. 2 (lanes 2, 4, 6) shows that the β-subunit synthesized in the vegetal pole, injected with β cRNA alone, remains in its core glycosylated form, up to a 21 h chase, indicating that it is confined to the endoplasmic reticulum. On the other hand, β-subunits synthesized in oocytes injected with β and α cRNA are progressively processed to their fully glycosylated form (Fig. 2, lanes 8,10,12), suggesting that they have passed to a distal Golgi compartment, competent for complex type glycosylation (for review, see [15]). Interestingly, we could never detect β-subunits either synthesized alone (Fig. 2, lanes 1, 3, 5) or in parallel with α-subunits

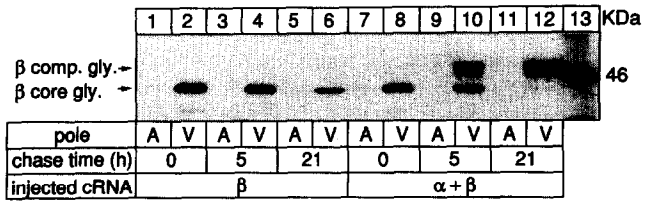

Fig. 2. Intracellular transport of newly synthesized β-subunits in *Xenopus* oocytes. Oocytes were injected into the vegetal pole with β-cRNA and after a 3 h labeling and various chase periods bisected into vegetal and animal halves as described in Materials and Methods. The same number of counts were subjected to immunoprecipitation from animal (A) and vegetal (V) halves. Lane 13: protein markers.

(Fig. 2, lanes 7, 9, 11) in the animal pole, even after a 21 h chase or up to a 45 h chase (data not shown). Similar negative results were obtained for the α-subunit synthesized alone or together with β-subunits (data not shown). In agreement with observations made previously by Ceriotti and Coleman [11], we must conclude from these data that in the oocyte there exists no physical continuity of the secretory apparatus.

In order to strengthen the hypothesis that the β-subunit becomes transport competent only during concomitant α-subunit synthesis, we performed more experiments on extracts of whole oocytes injected with β cRNA (Fig. 3A, lanes 1-4) or with $\alpha+\beta$ cRNA (Fig. 3A, lanes 5-8) and have analyzed the kinetics of the glycosylation processing of the β-subunit. Fig. 3B is a compilation of data of several experiments which shows the relative distribution of core- and fully glycosylated forms within the total β-subunit population immunoprecipitated after different chase periods. It becomes apparent that β-subunits synthesized concomitantly with α-subunits are entirely processed into fully glycosylated forms within about 17 h of chase. In contrast, β-subunits synthesized in the absence of α-subunits are nearly exclusively in their core glycosylated form up to a 21 h chase. Apparently, at later time points, the proportion of fully glycosylated β-subunits increased. It is likely that the small proportion of fully glycosylated forms of β-subunits seen in oocytes injected with β-cRNA alone represents the β-subunit population which associates to oocyte α-subunits and thus becomes transport competent. In addition, the core glycosylated β-subunit is more rapidly degraded ($t_{\frac{1}{2}} \sim 9$ h, as determined from experiments similar to the one shown in Fig. 3, lanes 1-3) than the fully glycosylated form ($t_{\frac{1}{2}} > 20$ h, as estimated from chase experiments up to 96 h). Thus, we overestimate the proportion of fully glycosylated forms. This might explain their apparent increase at later chase periods (Fig. 3B).

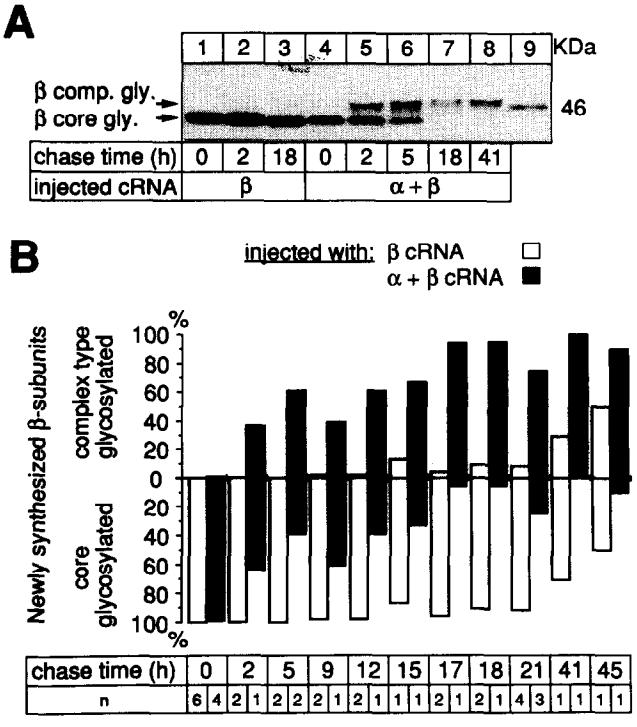

Fig. 3. Glycosylation processing of β-subunits in *Xenopus* oocytes. (A) Autoradiography of immunoprecipitated β-subunits. Oocytes were injected with 3 ng β-cRNA (lanes 1-3) or 3 ng β cRNA plus 9 ng α-cRNA (lanes 4-8). Other details as in Fig. 1. (B) Quantification of the glycosylation pattern of β-subunits. Results from several experiments shown in A were quantitated. The distribution of core and fully glycosylated β-forms in the total β-subunit pool synthesized in oocytes injected with β cRNA (▭) or α-plus β-cRNA (■) are shown.

Considering the half-life of the unassembled α-subunit on the one hand ($t_{\frac{1}{2}} \sim 2$ h), and the time needed for α-β complexes to be transported to a distal Golgi compartment ($t_{\frac{1}{2}} \sim 10$ h) on the other hand, it is indeed likely that degradation of the unassembled α-subunit occurs at the level of the endoplasmic reticulum or at least in a nearby pre-Golgi compartment.

In conclusion, the data of the present study are consistent with the hypothesis that α- and β-subunits of Na,K-ATPase mutually depend on each other for the adoption of a correct configuration likely to be necessary to leave the endoplasmic reticulum. Indeed, the β-subunit does not become fully glycosylated in the absence of the α-subunit and the α-subunit in turn is rapidly degraded in the absence of the β-subunit. Considering the half-life of the unassembled α-subunit on the one hand ($t_{\frac{1}{2}} \sim 2$ h), and the time needed for α-β complexes to be transported to a distal Golgi compartment ($t_{\frac{1}{2}} \sim 10$ h) on the other hand, it is indeed likely that degradation of the unassembled α-subunit occurs at the level of the endoplasmic reticulum or at least in a nearby pre-Golgi compartment.

Interestingly, core glycosylated unassembled β-subunits, though more rapidly degraded than fully glycosylated β-subunits, have a longer half-life than unassembled α-subunits. Further experiments are needed to decide whether this fact reflects a difference in their site of degradation and/or in their retention mechanism. In any case, the longer half-life of the newly synthesized β-subunit might be a potentially important feature defining the efficiency of the subunit assembly reaction.

Acknowledgements: We would like to thank Mrs Nicole Skarda-Coderey for her excellent secretarial work. This study was supported by the Swiss National Fund for Scientific Research Grant 31-26241.89.

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