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In vitro biosynthesis of the β-subunit of the Na^+/K^+-ATPase in developing brine shrimp: glycosylation and membrane insertion

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We demonstrate here translation, glycosylation, and membrane insertion of the β-subunit of the Na^+/K^+-ATPase of the developing brine shrimp, *Artemia*, in a reticulocyte lysate translation system. The apparent molecular weight of the primary translation product as determined by SDS-PAGE is 33000 ± 1000 ($n = 7$). When microsomal membranes are present during the entire translation period, a new band with an apparent molecular weight of 37000 ± 1000 ($n = 7$) appears. This change in apparent molecular weight is due to the addition of about two N-linked oligosaccharides. The temporal relationship between protein synthesis and glycosylation have also been examined. Glycosylation and membrane insertion could be achieved if membranes were added after completion of about 70% of the peptide chain. However, glycosylation did not occur if membranes were added after the completion of translation of the β-subunit. The β-subunit was synthesized on membrane-bound polysomes, where about two N-linked oligosaccharides were added to the growing polypeptide chain. These studies demonstrate that in vitro translation systems will be useful for studying the biosynthesis of the β-subunit of the brine shrimp, which is a good model system to examine the developmental regulation of the Na^+/K^+-ATPase.

Introduction

The Na^+- and K^+-stimulated adenosine triphosphatase (Na^+/K^+-ATPase (EC 3.6.1.3)) is an integral membrane protein which effects the coupled transports of Na^+ and K^+ against electrochemical gradients. This action results in the maintenance of a low [Na^+] and a high [K^+] inside the cell, which subserve many important physiological processes. The Na^+/K^+-ATPase contains a large subunit, α, and a smaller subunit, β (for reviews, see Refs. 1–4). The α-subunit contains the binding sites for K^+, Na^+, nucleotides, cardiac glycosides, and vanadate. The function of the β-subunit is unknown, but it appears to be essential for enzymatic activity.

Because of the significant role of the Na^+/K^+-ATPase in cellular function, the elucidation of the molecular mechanisms of biosynthesis and regulation of this enzyme is very important. Early studies suggested that the α-subunit of some species may by synthesized on free ribosomes [5,6], but this has not been substantiated by more recent investigations [7–9]. The β-subunit of

Abbreviations: Na^+/K^+-ATPase, sodium- and potassium-activated adenosine triphosphatase; EDTA, ethylenediaminetetraacetic acid; VRC, vanadyl ribonucleoside complex; RNA, ribonucleic acid; cDNA, complementary deoxyribonucleic acid; SDS, sodium dodecyl sulfate.

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several species also appears to be synthesized on bound polysomes [5,6,8].

One model system which has been useful in studying the developmental regulation of the Na^+/K^+-ATPase is brine shrimp embryos, in which dramatic increases in the activity and synthesis of the Na^+/K^+-ATPase are observed during the first 24 h of development [10,11]. As part of our long-range study of developmental regulation of the brine shrimp Na^+/K^+-ATPase, utilizing a molecular biological approach, we present data here on the synthesis, glycosylation, and membrane insertion of the β-subunit in a cell-free system.

Materials and Methods

Isolation of RNA

Brine shrimp (San Francisco Bay Brand) were grown for 16 h as described previously [12] except that the sea water was supplemented with 25 mg/liter streptomycin sulfate (Sigma) and 500 units/ml penicillin G (Sigma). Brine shrimp were collected, rinsed with distilled water, and homogenized with a Waring blender. Total polysomes were isolated by a modification of the method of Ramsey and Steele [13] as described by Fisher et al. [7], except that centrifugation steps for separation of free and membrane-bound polysomes were omitted. RNA was incubated with proteinase K (Boehringer Mannheim) and extracted with phenol as described previously [7].

Cell-free translation system

The reticulocyte lysate was purchased from Green Hectares (Oregon, WI) and treated with micrococcal nuclease [14]. The cell-free translations were carried out as described previously [7] except that RNA was not incubated with CH_3HgOH, the lysate concentration was 61–64% (v/v), and the incubation temperature was 30°C. Rough microsomes were isolated from dog pancreas, treated with micrococcal nuclease, and extracted in the presence of EDTA as described previously [15]. Microsomal membranes were added to indicated cell-free translation mixtures to yield 6.25 A_{280}/ml.

Synchronized cell-free translation system

A large-scale, cell-free translation mixture was prepared, and 2 min after the addition of the RNA, 7-methylguanosine 5-phosphate (Sigma) was added in order to inhibit additional initiation of translation [16]. Microsomal membranes were added to aliquots removed at indicated times, and incubation at 30°C was continued as indicated. Termination of translation was achieved by placing samples on ice until immunoprecipitation was initiated.

Immunoprecipitation, electrophoresis, and fluorography

After completion of cell-free translation aliquots (30 to 60 μl) were adjusted to a total volume of 160 μl containing 2% (w/v) SDS and 0.78 mg/ml trasylol (aprotinin, Boehringer Mannheim). After incubation at room temperature for 10 min, immunoprecipitation was accomplished using antibody and conditions which were described previously [7]. Electrophoresis of immunoprecipitated samples was performed on a discontinuous slab gel system [17] as described by Hokin et al. [18] except that the separating gels contained 12% acrylamide. The gel shown in Fig. 2 was prepared as described above except that the concentration of bisacrylamide was doubled. Gels were prepared for fluorography in incubation with EnHance (New England Nuclear), dried, overlaid with preflashed Kodak X-Omat AR-5 film, and stored at −70°C until autoradiograms were developed. Molecular weight standards (BRL, lysozyme, β-lactoglobulin, chymotrypsinogen, ovalbumin, bovine serum albumin, phosphorylase *b*, and myosin were included in two lanes on each gel. The estimates of molecular weight of the β-subunits are presented as the mean ± S.D. of *n* gels.

Endoglycosidase F treatment

Mature β-subunit was isolated as described previously [12] except that the final step of the purification was electrophoresis and electroelution of the β-subunit from the gel. The purified subunit was labelled with [^{14}C]formaldehyde [19], and endoglycosidase F digestions were carried out with 6000 dpm β-subunit in 0.1 M sodium phosphate (pH 6.1), 0.05 M EDTA, 1% (v/v) Nonidet P-40, 0.1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol,

and 0.75 units endoglycosidase F (New England Nuclear). This preparation of endoglycosidase F is actually a mixture of endo-β-*N*-acetylglucosaminidase F and *N*-glycosidase F [20]. Samples were incubated at 37°C for 26 h.

Endoglycosidase H treatment

Translations were carried out as described above, and 54 μl of the mixture were adjusted to a total volume of 160 μl containing 2% (w/v) SDS, 0.78 mg/ml aprotinin, 2 μM PMSF, 0.28 M Tris (pH 6.0), and 0.5 units endoglycosidase H (endo-β-*N*-acetylglucosaminidase H, New England Nuclear). Samples were incubated at 37°C for 23 h; β-subunits were immunoprecipitated and examined by gel electrophoresis.

Results

Cell-free translation products

Numerous studies have demonstrated that the addition of microsomal membranes to cell-free translation systems can result in membrane insertion and glycosylation of newly synthesized proteins. We have utilized this approach to examine the biosynthesis and membrane insertion of the β-subunit of the Na^+/K^+-ATPase of developing brine shrimp. Total polysomal RNA was translated in a reticulocyte lysate system, and the products were immunoprecipitated with antibodies raised against highly purified mature β-subunit. The immunoprecipitated products were separated by electrophoresis on SDS-polyacrylamide gels (Fig. 1). In most experiments, immunoprecipitates produced a single, broad band which migrated with an average apparent molecular weight of 33000 ± 1000 ($n = 7$). The broad band could be resolved into two bands by increasing the concentration of bisacrylamide in the gels (Fig. 2).

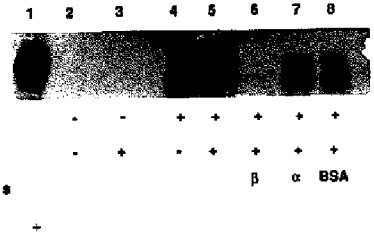

Fig. 1. Immunoprecipitable cell-free translation products synthesized in the presence and absence of microsomal membranes. No immunoprecipitable proteins were detected in samples which did not contain RNA (lanes 2 and 3). Addition of RNA resulted in a broad band of 33000 ± 1000 (mean ± S.D., $n = 7$). A second band with an apparent molecular weight of 37000 ± 1000 (mean ± S.D., $n = 7$) was observed in samples containing microsomal membranes. Addition of 10 μg of a highly purified mature form of the β-subunit to cell-free translation mixtures prior to addition of antibody resulted in displacement of the bands (lane 6). Addition of 30 μg of highly purified α-subunit or 100 μg of bovine serum albumin had no effect (lanes 7 and 8, respectively). Lane 1 contains a highly purified β-subunit which was isolated from brine shrimp and externally labelled with ^{14}C. BSA, bovine serum albumin.

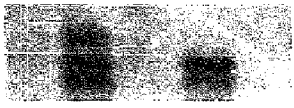

Fig. 2. Two primary cell-free translation products. Examination of the cell-free translation products on gels containing additional cross-linker (see Methods) allowed resolution of the primary cell-free translation products into two bands. Samples shown were translated in the presence (+) or absence (−) of microsomal membranes. All procedures were carried out as described in Methods.

Glycosylation

When microsomal membranes were included in the cell-free translation mixture, a band of average apparent molecular weight of 37000 ± 1000 ($n = 7$) was also present in the immunoprecipitates (Fig. 1). Since the mature β-subunit is in the glycosylated form [12], the larger molecular weight cell-free translation product could be a glycosylated form of the primary translation product. This possibility was investigated by incubation of the cell-free translation products with endoglycosidase H (Fig. 3), which catalyzes the hydrolysis of the chitobiose core of high mannose oligosaccharides [21]. After incubation with endoglycosidase H, the larger apparent molecular weight product was reduced in size and its migration was indistinguishable from that of the primary cell-free translation product (Fig. 3, lane 4). This indicates that the larger cell-free translation product is a β-subunit which has been glycosylated.

Fig. 3. Endoglycosidase digestion of β-subunits. ^{14}C-labeled mature β-subunit was treated with endoglycosidase F as described in Experimental Procedures. Samples shown are no incubation (lane 1), incubation with endoglycosidase F buffer only (lane 2), or incubation with endoglycosidase F (lane 3). Cell-free translation products were incubated with endoglycosidase H and immunoprecipitated as described in Experimental Procedures. Samples were immunoprecipitated directly (lane 6), incubated with endoglycosidase H buffer only (lane 5), or incubated with endoglycosidase H (lane 4).

Glycosylation was not quantitative, and one possible explanation for this observation is that the amount of membranes present in the system was inadequate to achieve complete glycosylation. However, increasing the amount of microsomal membranes resulted in inhibition of protein synthesis (data not shown). A total of five different preparations of microsomal membranes (three isolated as described in Experimental Procedures and two purchased from commercial sources (Amersham)) were examined (data not shown). In all cases, only a portion of the immunoprecipitable β-subunit was detected as the glycosylated form.

The mature form of the β-subunit had an apparent molecular weight which was slightly greater than the glycosylated cell-free translation product (Fig. 1, lane 1). In order to investigate the possibility that these differences in apparent molecular weight were related to processing of the oligosaccharides, mature β-subunit was incubated with endoglycosidase F. This enzyme catalyzes the hydrolysis of glycosidic bonds of core structures of a variety of asparagine-linked complex oligosaccharides [20]. After incubation with endoglycosidase F, the apparent molecular weight of mature β-subunit was reduced (Fig. 3, lane 3), but it was not identical to that of the primary cell-free translation product (Fig. 3).

Immunocompetition

Immunocompetition was utilized to investigate the identity of all bands. Addition of highly purified β-subunit to samples prior to the addition of antibodies resulted in displacement of all radioactive bands (Fig. 1, lane 6). In contrast, addition of much larger quantities of purified α-subunit or bovine serum albumin had no effect (Fig. 1, lanes 7 and 8). A slight difference in band intensity (lanes 5, 7, and 8) may be caused by expected small variations in sample recovery.

Membrane insertion

Since glycosylation occurs only after the protein has crossed the microsomal membrane, the presence of oligosaccharides indicates that the β-subunit is inserted into the membrane. This was confirmed by experiments in which all of the glycosylated form of the β-subunit remained associated with microsomal membranes after alkaline extraction, which results in exclusive retention of integral membrane proteins (data not shown).

Previous attempts to determine the site of synthesis of the β-subunit of brine shrimp by isolation of free and membrane-bound polysomes suggested that the β-subunit was synthesized on free polysomes [7]. Isolation of intact polysomes required the use of vanadyl ribonucleoside complex (VRC) to inhibit the high ribonuclease levels present in brine shrimp embryos. However, high levels of VRC also led to aggregation of polysomes. The VRC concentration curve suggested that in the absence of VRC, $mRNA_\beta$ was associated with free ribosomes. To circumvent the ribonuclease problem, we use here an alternative method of examining the synthesis, i.e., cell-free systems.

The relationship between the extent of biosynthesis and the ability of the β-subunit to be glycosylated and inserted into the membrane was examined in a synchronized cell-free translation system. In the synchronized system, most of the β-subunit appeared as full-length product after about 12 to 15 min of translation (Fig. 4). This corresponds to a rate of synthesis of about 20 to 25 amino acids incorporated into protein for each minute of synthesis.

Microsomal membranes were added to the synchronized cell-free translation system after completion of synthesis or at various intervals during the synthesis of the β-subunit. If the membranes were added after synthesis was completed (30

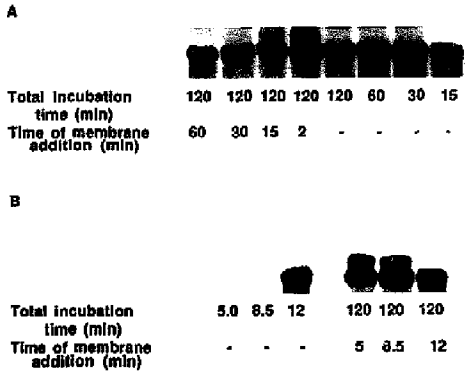

Fig. 4. Membrane insertion and glycosylation of the β-subunit in a synchronized translation system. Cell-free translation was started in a single tube and 7-methylguanosine 5-phosphate was added after 2 min in order to inhibit additional initiation of translation. At indicated times, aliquots were removed for termination of translation or continued incubation in the presence of microsomal membranes. Separate experiments were performed for time points greater than 15 min (Part A) or less than 15 min (Part B).

min), the newly synthesized β-subunit was not glycosylated (Fig. 4). If the membranes were added after 8.5 min of synthesis, when the β-subunit was about 55 to 70% completed, maximal levels of glycosylation and membrane insertion were achieved. If membranes were added after 12 to 15 min of translation, a period when some β-subunits are complete while others are nearing completion, a portion of the β-subunit was glycosylated and inserted into the membrane (Fig. 4).

Discussion

This study has utilized an in vitro system to study the mechanism of biosynthesis of the β-subunit of the Na^+/K^+-ATPase of brine shrimp. We have shown in an in vitro system that the β-subunit of brine shrimp is cotranslationally inserted into microsomal membranes, where it is glycosylated. The data clarify earlier studies which suggested that the site of synthesis of the β-subunit of brine shrimp was on free polysomes [7]. The early studies attempted to identify the site of synthesis of the β-subunit of brine shrimp by determining the distribution of $mRNA_\beta$ in preparations of free and membrane-bound polysomes. However, it was observed that this distribution could be altered by changing the concentration of a ribonuclease inhibitor, VRC, present in the homogenization buffer. We suggested that high concentrations of the VRC may have produced aggregation of polysomes resulting in an artifactual appearance of $mRNA_\beta$ in the bound polysome fraction. Also, ribonuclease may have remained active when low levels of inhibitor were present, and partial degradation of RNA resulted in an artifactual appearance of the $mRNA_\beta$ in the free polysome fraction. Since the in vitro studies reported here show that the β-subunit can still be inserted into membranes and glycosylated when up to 70% of the peptide chain is completed, another interpretation of the earlier data is that the presence of $mRNA_\beta$ in the free polysome fraction was due to synthesis of a large portion of the β-subunit prior to association with the membranes. The in vitro studies reported here clearly indicate that the β-subunit can be synthesized on membrane-bound polysomes.

The temporal relationship between biosynthesis of the peptide chain of the β-subunit and its association with the membranes has also been studied in another model system, amphibian bladder [8]. In contrast to the present study, that study showed that insertion of the β-subunit into the membrane could only be achieved if microsomal membranes were present at very early stages of synthesis of the β-subunit. We have no explanation for the discrepancy between the two studies at this time, partly because too little is known about the molecular mechanisms involved in the insertion of proteins into membranes.

Two immunoprecipitable primary translation products were detected. This phenomenon was also described in one other model system, A6 cells [8]. Possible explanations for two primary translation products include partial degradation of the primary translation product, premature termination of translation, the presence of isoforms of the subunits, or impurities in the immunoprecipitates. Additional studies will be required to distinguish between these possibilities.

N-linked glycosylation of the primary cell-free translation product was achieved in the in vitro system, and the difference between the apparent

molecular weights of the primary and glycosylated forms of the β-subunit would indicate the addition of two oligosaccharides. Analysis of the amino acid sequences of β-subunits from several other species has revealed the presence of three to four N-glycosylation sites [25–30]. It is possible that one or two of these sites has not been conserved or utilized in the brine shrimp. This would be consistent with the observation that the β-subunit of brine shrimp contains less carbohydrate than that detected in other species [12]. Sequencing of a cDNA encoding the β-subunit of the brine shrimp is currently in progress, and the amino acid sequence deduced from the nucleotide sequence may help to resolve this question.

The glycosylated cell-free translation product had a slightly smaller apparent molecular weight than the mature form of the β-subunit. This is not unexpected since the oligosaccharides present on the mature form of the β-subunit probably resulted from additional processing. However, removal of oligosaccharide chains from the mature form of the β-subunit by incubation with endoglycosidase F did not produce a form of the β-subunit which comigrated with the primary translation product. There are several possible explanations for this observation, including incomplete digestion by endoglycosidase F or the presence of an oligosaccharide which is resistant to endoglycosidase F-catalyzed hydrolysis. Additional studies will be required to resolve this question.

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