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Na^++K^+-ATPase in gills of the blue crab *Callinectes sapidus*: cDNA sequencing and salinity-related expression of α-subunit mRNA and protein

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Summary

Many studies have shown that hyperosmoregulation in euryhaline crabs is accompanied by enhanced Na^++K^+-ATPase activity in the posterior gills, but it remains unclear whether the response is due to regulation of pre-existing enzyme or to increased gene transcription and mRNA translation. To address this question, the complete open reading frame and 3′ and 5′ untranslated regions of the mRNA coding for the α-subunit of Na^++K^+-ATPase from the blue crab *Callinectes sapidus* were amplified by reverse transcriptase/polymerase chain reaction (RT-PCR) and sequenced. The resulting 3828-nucleotide cDNA encodes a putative 1039-amino-acid protein with a predicted molecular mass of 115.6 kDa. Hydrophobicity analysis of the amino acid sequence indicated eight membrane-spanning regions, in agreement with previously suggested topologies. The α-subunit amino acid sequence is highly conserved among species, with the blue crab sequence showing 81–83 % identity to those of other arthropods and 74–77 % identity to those of vertebrate species. Quantitative RT-PCR analysis showed high levels of α-subunit mRNA in posterior gills 6–8 compared with anterior gills 3–5. Western blots of gill plasma membranes revealed a single Na^++K^+-ATPase α-subunit protein band of the expected size. The posterior gills contained a much higher level of α-subunit protein than the anterior gills, in agreement with previous measurements of enzyme activity. Immunocytochemical analysis showed that the Na^++K^+-ATPase α-subunit protein detected by α5 antibody is localized to the basolateral membrane region of gill epithelial cells. Transfer of blue crabs from 35 to 5 ‰ salinity was not accompanied by notable differences in the relative proportions of α-subunit mRNA and protein in the posterior gills, suggesting that the enhanced Na^++K^+-ATPase enzyme activity that accompanies the hyperosmoregulatory response may result from post-translational regulatory processes.

Key words: Na^++K^+-ATPase, gills, blue crab, *Callinectes sapidus*, cDNA sequencing, salinity, osmoregulation, quantitative polymerase chain reaction.

Introduction

The Na^+-plus-K^+-dependent adenosine triphosphatase (Na^++K^+-ATPase) is a transmembrane protein composed of three subunits (α, β, γ) responsible for pumping three Na^+ out of animal cells in exchange for two K^+ or NH_4^+ per ATP hydrolyzed. Situated in the basolateral membrane of most epithelial cells, the Na^++K^+-ATPase not only mediates electrogenic transfer of Na^+ from the cytosol to the extracellular fluid and K^+ or NH_4^+ from the extracellular fluid to the cytosol but also establishes electrochemical gradients used by apical and basolateral transporters such as the Na^+/H^+ exchanger, $Na^+/K^+/2Cl^-$ cotransporter and various ion channels. Originally described enzymatically in homogenates of nerves from the shore crab *Carcinus maenas* (Skou, 1957), the Na^++K^+-ATPase α- and β-subunits were first cloned and sequenced from mammalian kidney (Shull et al., 1985, 1986). The α-subunit provides the catalytic function of the Na^++K^+-ATPase, binding and hydrolyzing ATP and itself becoming phosphorylated during the transport cycle. The α-subunit also binds ouabain, a highly specific inhibitor. The β-subunit is thought to participate in anchoring the complex in the basolateral membrane and the γ-subunit, not always present, appears to serve a regulatory role (for a review, see Therien and Blostein, 2000).

Many studies have shown that the enzymatic activity of the Na^++K^+-ATPase in homogenates of ion-transporting gill tissue is enhanced when euryhaline crustaceans are subjected to osmoregulatory stress following transfer from sea water to dilute salinities (for reviews, see Towle, 1990; Péqueux, 1995). For example, the specific Na^++K^+-ATPase activity in homogenates or microsomal fractions of posterior gills of

Fig. 1. cDNA and predicted amino acid sequence of the Na^++K^+-ATPase α-subunit from gills of the blue crab *Callinectes sapidus*. Stop codons in the 5′ untranslated region (including one in-frame, underlined), the putative stop site and stop codons downstream from the stop site are indicated in red. A Kozak consensus sequence around the likely start codon (underlined) is shown in green. Transmembrane domains predicted by hydrophobicity analysis are shown in blue. The location of the degenerate primers NAK10F and NAK16R, employed in the initial amplification, are indicated by right- and left-pointing arrowheads respectively. The polyadenylation signal in the 3′ untranslated region is indicated in violet. GenBank Accession Number AF327439

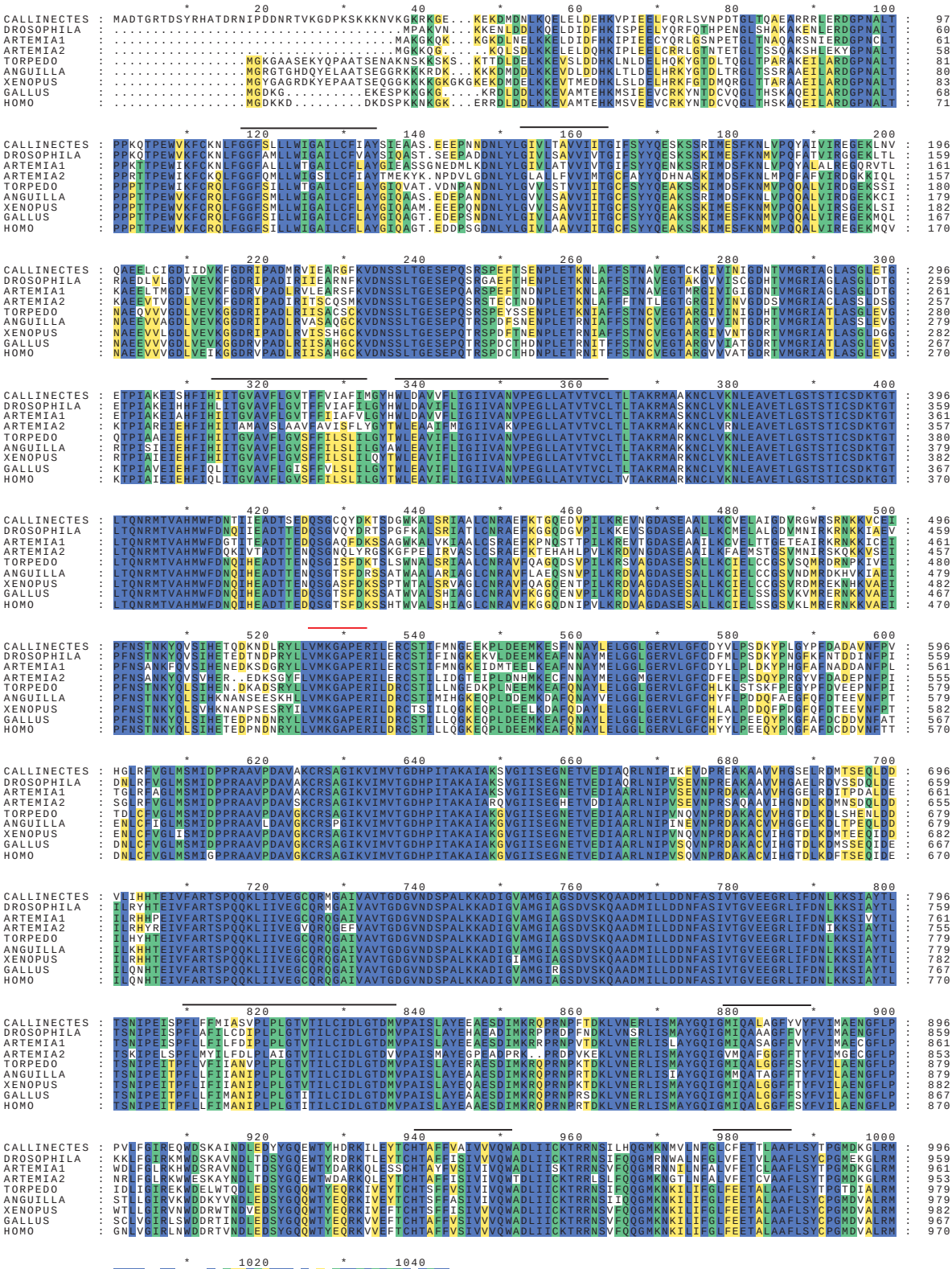

Fig. 2. Multiple alignment of the *Callinectes sapidus* α-subunit amino acid sequence with examples from other arthropods (*Drosophila melanogaster* and two isoforms from *Artemia franciscana*) and from vertebrates (*Torpedo californica*, *Anguilla anguilla*, *Xenopus laevis*, *Gallus gallus* and *Homo sapiens*). Alignment was produced using ClustalW and GeneDoc software. Blue, 100 % agreement; green, 80 %; yellow, 60 %. Putative transmembrane domains are indicated by solid black lines; the likely ATP binding site is indicated by a solid red line (Horisberger et al., 1991).

Callinectes sapidus approximately doubles following transfer of the animals from 35 to 5‰ salinity (Neufeld et al., 1980; Piller et al., 1995; Towle et al., 1976). The increased Na^++K^+-ATPase activity is thought to drive uptake of Na^+ and Cl^- across the gill epithelium, leading to hyperosmoregulation of the hemolymph in low-salinity environments. However, it remains unclear whether the activity of pre-existing Na^++K^+-ATPase molecules is regulated in response to a change in salinity or whether the observed increase in enzymatic activity (and presumably in pumping activity) may be the result of enhanced gene transcription and/or translation. In the present study, we used reverse transcription and the polymerase chain reaction (RT-PCR) to identify and characterize Na^++K^+-ATPase α-subunit cDNA prepared from gills of the euryhaline blue crab *Callinectes sapidus*. Quantitative RT-PCR, western blotting and immunocytochemistry were employed to investigate the correlation between α-subunit mRNA and protein abundance in relation to the osmoregulatory response. Some of our data have appeared in abstract form (Paulsen et al., 2000).

Materials and methods

Blue crabs (*Callinectes sapidus* Rathbun) were obtained from Gulf Specimens, Panacea, Florida, USA, and were maintained in recirculating biologically filtered aquaria at 20 °C containing natural sea water at salinities of either 35 or 5‰. Crabs were fed cleaned mussels twice weekly. Prior to the removal of the gills, the crabs were anesthetized on ice for 30 min.

Total RNA was extracted from the gills under RNAse-free conditions using materials provided by Promega Corporation (Chomczynski and Sacchi, 1987). Poly(A) mRNA in the total RNA preparation was reverse-transcribed to cDNA using oligo-dT primer and Superscript II reverse transcriptase (Life Technologies). Degenerate primers for the polymerase chain reaction (PCR) were based on conserved regions of published Na^++K^+-ATPase α-subunit sequences from other arthropods, including *Drosophila melanogaster* (Lebovitz et al., 1989) and *Artemia franciscana* (Baxter-Lowe et al., 1989; Macías et al., 1991). Primers were designed with the assistance of Primer Premier software and synthesized by Operon Technologies. Polymerase chain reactions were carried out in an MJ Research thermocycler using Sigma RedTaq DNA polymerase and nucleotides. The usual incubation protocol included an initial denaturation at 92 °C for 5 min followed by cooling to 60 °C and addition of the polymerase. Using degenerate primers, the reaction tubes were cycled 30 times through 1 min at 92 °C, 1 min at 45 °C and 2 min at 72 °C, followed by an extension at 72 °C for 5 min and storage at 4 °C. With non-degenerate primers, the annealing temperature was raised from 45 to 55 °C. PCR products were separated electrophoretically on agarose gels, visualized by ethidium bromide staining and extracted from the gels using the QiaQuick protocol (Qiagen).

An initial 700-base-pair PCR product obtained with degenerate primers NAK10F (5′-ATGACIGTIGCICAYATG-TGG-3′) and NAK16R (5′-GGRTGRTCICCIGTIACCAT-3′) was sequenced at the Marine DNA Sequencing Center of the Mount Desert Island Biological Laboratory using ABI 377 and 3100 automated sequencers. *Callinectes*-specific primers were then designed to complete the sequencing with the aid of 3′ rapid amplification of cDNA ends (3′-RACE) (Life Technologies) and 5′-RACE (Ambion) techniques. Sequence assembly and analysis were performed with DNASTAR and DNASIS software packages. Comparison with published sequences in GenBank was made with the BLAST algorithm (Altschul et al., 1997) and multiple alignments were produced with ClustalW (http://antheprot-pbil.ibcp.fr/ie_sommaire.html) and GeneDoc software (http://www.psc.edu/biomed/genedoc/). Putative transmembrane regions were identified by hydrophobicity analysis using AnTheProt (http://antheprot-pbil.ibcp.fr/ie_sommaire.html).

The abundance of α-subunit mRNA in total RNA extracts was estimated by quantitative RT-PCR using identical amounts of total RNA in each reverse transcription reaction. The polymerase chain reaction was carried out with biotinylated dUTP replacing a portion of the dTTP, under conditions in which product formation was directly dependent on cDNA template availability (Towle et al., 1997). Following transfer to nylon membranes, biotinylated products were visualized with the Phototope protocol (New England Biolabs). A lower limit of considerably less than a twofold difference in mRNA abundance can be detected using this method (Weihrauch et al., 2001).

Identification of α-subunit protein was achieved by western blotting of partially purified plasma membranes (Lucu and Flik, 1999) and detection with the α5 monoclonal antibody against a highly conserved cytosolic epitope of the α-subunit of avian Na^++K^+-ATPase (Lebovitz et al., 1989). The α5 antibody was obtained from Developmental Studies Hybridoma Bank or was kindly provided by Dr D. M. Fambrough. Identical quantities of membrane protein were loaded into each well of the polyacrylamide gel, as assayed using Coomassie Blue binding and bovine serum albumin as standard (Bradford, 1976).

Immunocytochemical localization of α-subunit protein was carried out with the α5 antibody using previously published methods (Lignot et al., 1999; Ziegler, 1997) following fixation in Bouin's fixative. Transverse sections (3 μm) were incubated in primary antibody, followed by washing and incubation in secondary antibody (fluorescein-isothiocyanate-conjugated goat anti-mouse IgG; Jackson Immunoresearch), and examined in a Leitz Diaplan fluorescence microscope. Controls included omitting primary antibody, revealing only a low background of autofluorescence.

Results

Amplification of the α-subunit cDNA from total RNA of *C. sapidus* gill was accomplished initially with degenerate primers NAK10F and NAK16R, yielding a 700-base-pair product. After sequencing and a BLAST search of GenBank, the initial PCR product was clearly identified as encoding a fragment of the Na^++K^+-ATPase α-subunit. Synthesis of

Callinectes-specific primers enabled the amplification of the complete α-subunit cDNA using 3′- and 5′-RACE techniques. The resulting 3828-nucleotide cDNA encodes a putative 1039-amino-acid polypeptide with a predicted molecular mass of 115.6 kDa (Fig. 1). A Kozak consensus sequence (CAACCATGG) brackets the likely start codon, an A replacing the second position C in the most conserved arrangement (Kozak, 1991). Several stop codons, one in-frame, occur prior to the putative start site. The predicted translational stop site is followed by six nucleotides and then two additional in-frame stop codons. A polyadenylation signal (ATTAAA, the second-most common polyadenylation signal) (Wickens, 1990) precedes the start of the poly-A tail by 13 nucleotides. No evidence of additional isoforms or alternative splicing products was observed.

Hydrophobicity analysis of the α-subunit amino acid sequence led to a prediction of eight transmembrane domains (Fig. 1), in agreement with other topological models of the Na^++K^+-ATPase α-subunit (for a review, see Horisberger et al., 1991). The *C. sapidus* α-subunit amino acid sequence was aligned with α-subunit sequences from other invertebrates including *Artemia franciscana* (Baxter-Lowe et al., 1989; Macías et al., 1991) and *Drosophila melanogaster* (GenBank Accession No. AF044974) and vertebrate species including *Torpedo californica* (Kawakami et al., 1985), *Anguilla anguilla* (Cutler et al., 1995), *Xenopus laevis* (Verrey et al., 1989), *Gallus gallus* (Takeyasu et al., 1990) and *Homo sapiens* (Ovchinnikov et al., 1988). The multiple alignment reveals extensive regions of highly conserved amino acid sequence, particularly in the fourth predicted transmembrane region and in the putative cytosolic domain, which includes the ATP binding site (Fig. 2). Statistical analysis of the alignment using GeneDoc software showed 81–83 % amino acid identity of the *C. sapidus* α-subunit sequence with other arthropod α-subunit sequences and 74–77 % identity with vertebrate sequences.

The posterior ion-transporting gills of euryhaline crabs are known to have substantially higher Na^++K^+-ATPase enzymatic activity than the anterior respiratory gills. Furthermore, the enzymatic activity in the posterior gills is enhanced upon transfer of crabs from high to low salinity (for reviews, see Towle, 1990; Péqueux, 1995). To determine whether gene activation might be responsible for the observed differences in enzymatic activity, we measured the abundance of α-subunit mRNA using quantitative RT-PCR. Using arginine kinase mRNA as an invariant internal standard for comparison (Kotlyar et al., 2000), we found that the relative proportion of Na^++K^+-ATPase α-subunit mRNA is much higher in the posterior gills (gills 6–8) than in the anterior gills (gills 3–5) (Fig. 3), supporting the conclusion that transcriptional regulation or differential mRNA stability may be responsible for the gill differences in Na^++K^+-ATPase enzymatic activity. However, little difference in α-subunit mRNA abundance was noted following transfer from high (35 ‰) to low (5 ‰) salinity (Fig. 3). Although it is likely that our quantitative RT-PCR technique was insufficiently sensitive to recognize small differences in mRNA abundance, it is clear that the doubling of enzymatic activity following a reduction in salinity (Neufeld et al., 1980; Piller et al., 1995; Towle et al., 1976) is not the result of a doubling of α-subunit mRNA levels.

The α-subunit protein was identified in *C. sapidus* gills by western blotting of partially purified plasma membrane proteins separated by SDS–polyacrylamide gel electrophoresis. The α5 antibody detected a single polypeptide of approximately 114 kDa (on the basis of a comparison with Bio-Rad Kaleidoscope prestained standards) (Fig. 4), consistent with the size of the α-subunit protein predicted from the cDNA sequence. Membrane preparations from the posterior gills contained a much greater proportion of α-subunit protein than those from the anterior gills, paralleling our estimations of α-subunit mRNA abundance. However, acclimation salinity appeared to have little effect on the relative amount of α-subunit protein in the posterior gills (Fig. 4). Although visual inspection suggested a modest increase in levels of the α-subunit protein following transfer from 35 to 5 ‰ salinity, digitizing the band

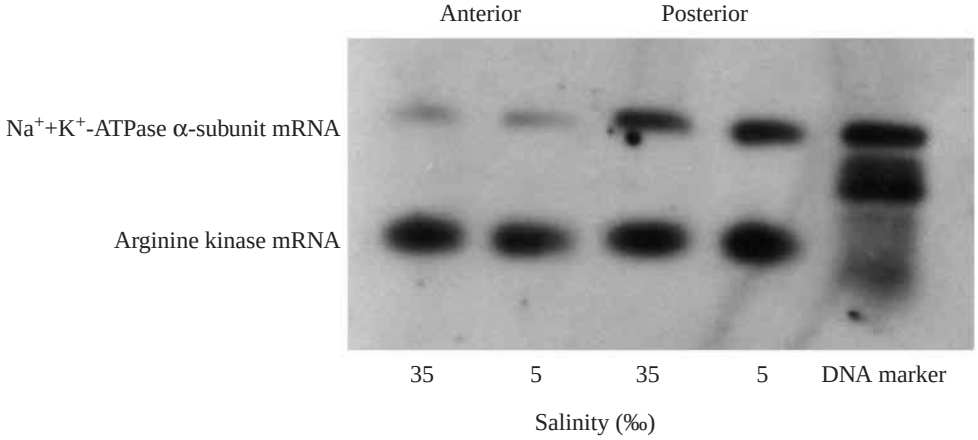

Fig. 3. Estimate of Na^++K^+-ATPase α-subunit mRNA abundance in total RNA extracts of anterior and posterior gills of *Callinectes sapidus* acclimated for at least 2 weeks to 35 or 5 ‰ salinity. Gills 3–5 (anterior) and 6–8 (posterior) were pooled from three individuals for each RNA preparation. mRNA levels were evaluated by duplex quantitative RT-PCR under conditions of limiting template, with arginine kinase mRNA serving as an invariant standard (Kotlyar et al., 2000). The primers employed to amplify Na^++K^+-ATPase α-subunit cDNA were NAK10F and NAK16R and for arginine kinase AKF5 (5′-CGCTGAGTCTAAGAAGGGATT-3′) and AKCALLR1 (5′-CCCAGGCTTGTCTTCTTGTCC-3′). Biotinylated PCR products were visualized after 22 cycles using a streptavidin/alkaline phosphatase procedure (Phototope, New England Biolabs). The data are representative of three separate experiments.

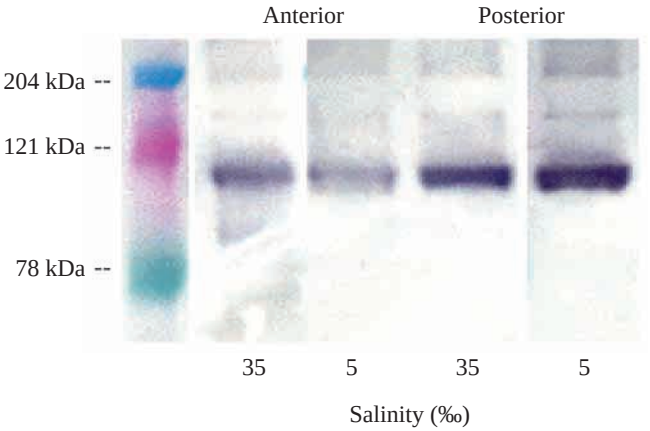

Fig. 4. Western blot analysis of Na^++K^+-ATPase α-subunit protein in partially purified plasma membrane preparations from anterior and posterior gills of *Callinectes sapidus* acclimated for 2 weeks to 35 or 5‰ salinity. Gills 3–5 (anterior) and 6–8 (posterior) from four crabs were pooled for each treatment. Following SDS–polyacrylamide gel electrophoresis and transfer to nylon membranes, α-subunit protein was detected by incubation with α5 monoclonal antibody. The left-hand lane contains Kaleidoscope prestained standards with the indicated molecular masses. The data are representative of three separate experiments.

densities with NIH Image software (Scion Corporation) failed to support such a conclusion.

Immunocytochemical analysis of α-subunit localization in cross sections of posterior gill lamellae indicated its predominance in basolateral membrane regions of the epithelium and its absence from the apical region (Fig. 5), in agreement with earlier ultracytochemical studies (Towle and Kays, 1986). Comparison of immunostaining between crabs acclimated to 35 or 5‰ salinity revealed little difference in intensity, consistent with western blot analysis of α-subunit protein abundance. An examination of anterior gills by immunocytochemistry indicated very low levels of α-subunit protein (data not shown).

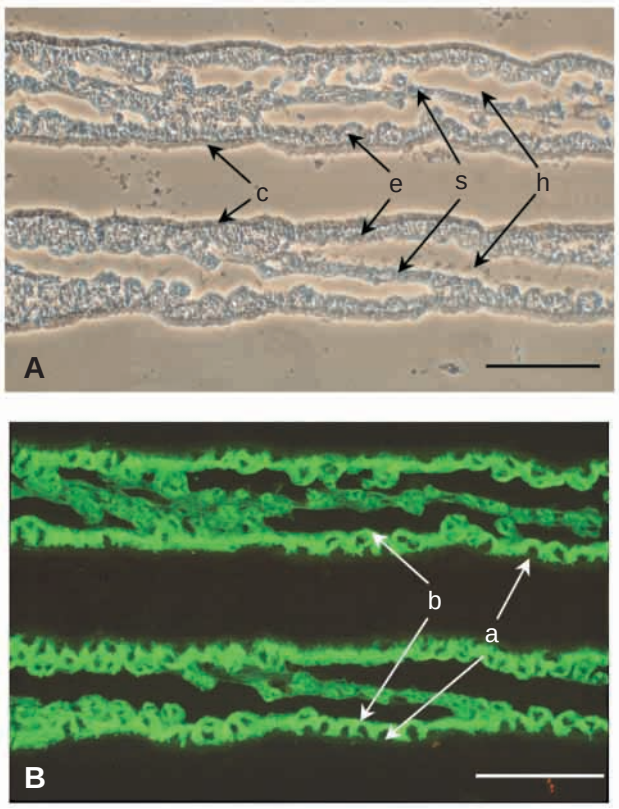

Fig. 5. Immunocytochemical localization of Na^++K^+-ATPase α-subunit protein in the basolateral membrane of gill epithelial cells in the blue crab *Callinectes sapidus*. (A) Phase-contrast photomicrograph of portions of two cross-sectioned gill lamellae. c, cuticle; e, epithelial layer; s, intralamellar septum; h, hemolymph space. (B) Immunocytochemical identification of α-subunit protein using the α5 monoclonal antibody against a highly conserved cytosolic epitope. Intense fluorescence is evident in the basolateral membrane region (b) but not the apical membrane region (a) of the epithelial cells. Scale bars, 100 μm.

Discussion

Ion-transporting cells in the branchial epithelium of brachyuran crabs are identified by an abundance of mitochondria and high specific Na^++K^+-ATPase activity localized in the basolateral membrane (Péqueux, 1995; Towle and Kays, 1986). These cells are arranged in a recognizably dark-colored ‘patch’ adjacent to the afferent blood vessel in each lamella of the posterior gills (Barra et al., 1983; Compère et al., 1989; Copeland and Fitzjarrell, 1968). The anterior gills are essentially devoid of the ion-transporting cell type and are thought to play a major role in respiratory processes (Taylor and Taylor, 1992). The area of the dark-colored patch in the posterior gills is known to increase following transfer of blue crabs from high to low salinity, reaching a maximum at approximately 7 days post-transfer (Aldridge and Cameron, 1982; Towle and Burnett, 2001). Thus, it might be expected that Na^++K^+-ATPase activity would demonstrate a parallel increase following a reduction in environmental salinity; indeed, such a change has been documented in a broad variety of euryhaline crab species (for reviews, see Harris and Bayliss, 1988; Lucu, 1993; Péqueux, 1995; Towle, 1990).

Despite numerous studies of salinity-related changes in Na^++K^+-ATPase activity in crustacean gills, it remains unclear whether the measured increases in activity result from *de novo* synthesis of Na^++K^+-ATPase mRNA and/or protein or from post-translational processes such as subunit assembly, membrane trafficking or cell signaling. Amplification and sequencing of the α-subunit cDNA has allowed us to assess whether differences in mRNA availability might explain some of the variation in enzymatic activity. It is clear that the high Na^++K^+-ATPase activity in the posterior gills compared with the anterior gills can be explained largely by the much greater abundance of α-subunit mRNA in total RNA extracts of posterior gills. Whether the high level of α-subunit mRNA in the posterior gills is the result of higher gene transcription rates

or of greater mRNA stability cannot be assessed by our experiments. However, the 3′ untranslated region of the α-subunit cDNA contains no adenosine- and uridine-rich instability elements such as are found in short-lived mRNAs (Chen and Shyu, 1995), suggesting that this mRNA may be quite long-lived.

Identification of the *Callinectes sapidus* Na^++K^+-ATPase α-subunit protein with a heterologous antibody against the avian α-subunit revealed a single protein band of approximately the expected size on western blots. Because the monoclonal α5 antibody is directed against the highly conserved cytosolic domain of the α-subunit, it is not surprising that it would react strongly with the crustacean protein. Indeed, the antibody has been employed to characterize the α-subunit of another arthropod, *Drosophila melanogaster* (Lebovitz et al., 1989). We can therefore be confident that the α5 antibody is detecting the appropriate protein in the western blots of *C. sapidus* gill cell membranes. The detected band is located at approximately 114 kDa, in close agreement with the size of the α-subunit protein (115.6 kDa) predicted from the cDNA sequence. The α5 antibody also identifies a protein that is restricted to the basolateral membrane of the gill epithelial cells, in agreement with the previously demonstrated ultracytochemical localization of the Na^++K^+-ATPase (Towle and Kays, 1986).

A comparson of the relative abundance of α-subunit protein in membranes from posterior and anterior gills showed clearly that the posterior gills contain a much higher level of α-subunit protein, consistent with the higher expression of α-subunit mRNA in posterior gills. We tentatively conclude on the basis of these observations that the process of cellular differentiation leading to the production and maintenance of ion-transporting cells in the posterior gill epithelium depends at least in part on transcriptional activation of the α-subunit gene in these cells, yielding high levels of both mRNA and protein. Assembly of the α-subunit protein with the β and possibly γ subunits (if the latter exists in the Na^++K^+-ATPase of *C. sapidus*) and targeting to the basolateral membrane would yield a functional pump protein poised to energize Na^+ uptake (and NH_4^+ excretion) across the posterior gill epithelium. Reduced transcription of the α-subunit gene in the anterior gills would lead to specializations other than ion transport.

Acclimation salinity appears to have little effect on the expression of α-subunit mRNA and protein in the gills of the blue crab. Although it is difficult to make strictly quantitative determinations using either the RT-PCR technique or western blotting, we have shown that a twofold difference in mRNA abundance is easily detected by our RT-PCR method (Weihrauch et al., 2001). If variation in α-subunit mRNA availability were the underlying cause of the reported differences in Na^++K^+-ATPase activity, we should have detected at least some change in mRNA levels. Under our experimental conditions, neither α-subunit mRNA nor protein levels appeared to vary very much with salinity.

Because we standardized both the RT-PCR method and the western blot with equivalent amounts of total RNA (reverse-transcribed to cDNA) and membrane protein, respectively, it is conceivable that our approach masked the differentiation of additional ion-transporting cells in the gill epithelium. Such an ontogenetic change following a reduction in salinity would probably recruit a large number of transport and regulatory proteins involved in transepithelial ion movements, with the α-subunit as one component of the recruitment process. Thus, the relative proportion of the α-subunit to total RNA and protein could remain constant even in the event of substantial upregulation of transcription and translation.

However, most published measurements of Na^++K^+-ATPase activity are specific activity measurements, factoring in the concentration of total protein in the homogenate or membrane fraction. Thus, the apparent lack of change in α-subunit protein content with salinity suggests that post-translational processes may play an important role in the response of Na^++K^+-ATPase activity to salinity change. In mammalian tissues, a wide variety of mechanisms regulating the Na^++K^+-ATPase have been studied. These include interaction with the γ-subunit and cytoskeletal elements, endogenous ouabain-like inhibitors and hormones that may act through protein kinases and phosphatases (for a review, see Therien and Blostein, 2000).

Studies of crustacean gills have revealed several candidate regulatory molecules that may participate in controlling the function of the Na^++K^+-ATPase. Dopamine and cyclic AMP have been implicated by several authors (Bianchini and Gilles, 1990; Lucu and Flik, 1999; Mo et al., 1998; Sommer and Mantel, 1988, 1991). A sinus gland component, later identified as crustacean hyperglycemic hormone, has been shown to enhance Na^+ transport and Na^++K^+-ATPase activity in perfused gills of the intertidal crab *Pachygrapsus marmoratus* (Eckhardt et al., 1995; Spanings-Pierrot et al., 2000). Methyl farnesoate levels increased in the hemolymph of *Carcinus maenas* following transfer from sea water to dilute salinity (Lovett et al., 2001); whether levels of Na^++K^+-ATPase activity are affected by this molecule is unknown. Future studies are therefore necessary to identify the transport-regulating factors and their mode of action on the Na^++K^+-ATPase as well as other ion transporters.

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