Embryonic Expression of the Putative γ Subunit of the Sodium Pump Is Required for Acquisition of Fluid Transport Capacity during Mouse Blastocyst Development

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Abstract. The sodium/potassium pump, Na^+,K^+-ATPase, is generally understood to function as a heterodimer of two subunits, a catalytic α subunit and a noncatalytic, glycosylated β subunit. Recently, a putative third subunit, the γ subunit, was cloned. This small protein (6.5 kD) coimmunoprecipitates with the α and β subunits and is closely associated with the ouabain binding site on the holoenzyme, but its function is unknown. We have investigated the expression of the γ subunit in preimplantation mouse development, where Na^+,K^+-ATPase plays a critical role as the driving force for blastocoel formation (cavitation). Using reverse transcriptase-polymerase chain reaction, we demonstrated that the γ subunit mRNA accumulates continuously from the eight-cell stage onward and that it cosediments with polyribosomes from its time of first appearance. Confocal immunofluorescence microscopy revealed that the γ subunit itself accumulates and is localized at the blastomere surfaces up to the blastocyst stage. In contrast with the α and β subunits, the γ subunit is not concentrated in the basolateral surface of the polarized trophectoderm layer, but is strongly expressed at the apical surface as well. When embryos were treated with antisense oligodeoxynucleotide complementary to the γ subunit mRNA, ouabain-sensitive K^+ transport (as indicated by $^{86}Rb^+$ uptake) was reduced and cavitation delayed. However, Na^+,K^+-ATPase enzymatic activity was unaffected as determined by a direct phosphorylation assay ("back door" phosphorylation) applied to plasma membrane preparations. These results indicate that the γ subunit, although not an integral component of Na^+,K^+-ATPase, is an important determinant of active cation transport and that, as such, its embryonic expression is essential for blastocoel formation in the mouse.

Na^+,K^+-ATPase is a plasma membrane enzyme that uses the energy from hydrolysis of ATP to transport Na^+ and K^+ in opposite directions across the membrane and against their electrochemical gradients. This enzyme, also known as the sodium pump, is required for normal functioning of all animal cells where the ion gradients maintained by the enzyme are used for diverse functions such as regulation of cell volume and pH, secondary active transport, and excitability (Jørgensen, 1986; Fambrough et al., 1987). Na^+,K^+-ATPase is generally considered to consist of two obligatory subunits: a catalytic α subunit and a noncatalytic, glycosylated β subunit (for review see Mercer, 1993). In addition to ATP binding and phosphorylation sites, the α subunit also bears a site that binds the cardiac glycoside ouabain, a specific inhibitor of sodium pump activity. Although the β subunit lacks catalytic activity, it is nonetheless required for production of functional holoenzyme. Mammals have at least four isoforms of the α subunit and three of the β subunit, all encoded by separate genes (Mercer et al., 1986; Kent et al., 1987; Martin-Vasallo et al., 1989; Malo et al., 1990; Shamraj and Lingrel, 1994; Malik et al., 1996).

A third putative subunit of Na^+,K^+-ATPase, the γ subunit, was cloned more recently (Mercer et al., 1993; Béguin et al., 1997). This small polypeptide (predicted mass = 6.5 kD) copurifies and coimmunoprecipitates with the α and β subunits and is closely associated with the ouabain binding site of the holoenzyme. It is a type I transmembrane protein (its NH_2 terminus is extracellular) whose association with α/β heterodimers influences the K^+ activation of the enzyme (Béguin et al., 1997). Its physiological function, however, is unknown. Experiments involving heterologous expression of combinations of α, β, and γ subunits in yeast failed to reveal any role of the γ subunit in ouabain binding, enzymatic activity, or ion transport (Scheiner-Bobis and Farley, 1994). Yeasts do

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not contain an endogenous Na^+,K^+-ATPase, however, so it remains possible that the function of the γ subunit will only be revealed through experimentation with animal cells. For example, the γ subunit might be required for physiological regulation of sodium pump activity or for polarized deployment of sodium pumps in epithelia.

The preimplantation mouse embryo offers a unique opportunity to explore the function of the γ subunit. After approximately five cleavage divisions, the outer cells of the embryo become specialized as a transporting epithelium, the trophectoderm (for review see Wiley et al., 1990). Na^+,K^+-ATPase becomes concentrated in the basolateral membranes of the trophectoderm where it takes on a morphogenetic role: it drives fluid transport across the cell layer to form the blastocoel (Watson and Kidder, 1988; MacPhee et al., 1997). This process, called cavitation, leads to the development of a blastocyst capable of initiating implantation. Cavitation is sensitive to a variety of perturbations affecting Na^+,K^+-ATPase, such as those that interfere with the localization of the enzyme in the basolateral membranes (Watson et al., 1990*a*) or those that interfere with sodium pump activity, as in the case of ouabain treatment (Dizio and Tasca, 1977; Manejwala et al., 1989; MacPhee et al., 1997). Furthermore, cavitation requires expression of embryonic genes, and is accompanied by de novo synthesis of the α and β subunits of the enzyme (Kidder and McLachlin, 1985; Watson et al., 1990*b*; MacPhee et al., 1994, 1997; Khidhir et al., 1995). We reasoned that if the putative γ subunit is a determinant of Na^+,K^+-ATPase function, then perturbing the embryonic synthesis of γ subunits should have a noticeable effect on blastocyst development. Here we show that this is indeed the case. Our results provide strong support that γ subunit has an important influence on active cation transport in mammalian cells.

Materials and Methods

Embryo Collection and Subcellular Fractionation

Random-bred CF-1 females (Charles River Canada Ltd., St. Constant, Québec or Harlan Sprague-Dawley, Indianapolis, IN) were superovulated with pregnant mare's serum gonadotropin and human chorionic gonadotropin (hCG)[1] (both purchased from Sigma Chemical Co., St. Louis, MO; 5–10 IU each, separated by 46 to 48 h) and mated with $CB6F_1/J$ males (The Jackson Laboratory, Bar Harbor, ME). Embryos were flushed from the reproductive tract on days 1–4 as described previously (MacPhee et al., 1994). Embryos were washed five times through Ca^{2+}- and Mg^{2+}-free PBS containing 3 mg/ml polyvinylpyrrolidone (PBS-PVP) before further processing. The timing of embryo collection was as follows: oocytes, 18 h post-hCG; two-cell, 48 h; four-cell, 60 h; eight-cell, 65 h; morula, 80 h; early blastocyst, 90 h.

For fractionation into subribosomal and polyribosomal RNP fractions, 300–500 embryos were immediately lysed with detergent solution and subjected to differential centrifugation as described previously (De Sousa et al., 1993; MacPhee et al., 1994). The resulting RNP fractions were used for RNA isolation.

RNA Isolation and Reverse Trancriptase (RT)–PCR

RNA was isolated from embryo lysates and from embryo RNP fractions by pelleting through CsCl, and the RNA reverse-transcribed, as described previously (De Sousa et al., 1993; MacPhee et al., 1994; Davies et al., 1996). The absence of genomic DNA from each cDNA preparation was confirmed by PCR using primers that amplify an intron of the β-actin gene (De Sousa et al., 1993). The γ subunit upstream primer was 5′-CCCTTC-GAGTACGACTATGA-3′ and the downstream primer was 5′-TTGAC-CTGCCTATGTTTCTT-3′. These primers were designed using Oligo™ Primer Analysis Software (National Biosciences, Plymouth, MN) to amplify a 134-bp fragment of the mouse cDNA (Mercer et al., 1993). A standard amplification cycle consisted of denaturation at 94°C for 45 s, annealing at 54°C for 40 s, and extension at 72°C for 45 s. For a profile of γ subunit presence throughout preimplantation development, 10 embryo equivalents were used for each stage at 40 cycles of amplification. The amplicons were analyzed on 3% agarose gels (3:1 low melting point agarose/agarose) containing 0.75 μg/ml ethidium bromide. The identity of the amplicons was confirmed by restriction enzyme digestion (StuI) and by direct sequencing (GenAlyTic sequencing service, the University of Guelph, Guelph, Ontario) following purification using a QIAquick Spin™ PCR purification kit (QIAGEN Inc., Chatsworth, CA).

To confirm that γ subunit mRNA increases quantitatively during preimplantation development and is thus a product of embryonic transcription, a semi-quantitative RT-PCR method was used to compare mRNA levels in different stages (Davies et al., 1996). A fixed amount of rabbit α-globin mRNA (25 pg per 100 embryos; GIBCO BRL, Burlington, Ontario) was added to each embryo batch as an internal standard before RNA extraction. After RNA isolation and reverse transcription, the cDNA preparations were amplified using both γ subunit and α-globin primers in the same reaction tube. This necessitated using a "primer-dropping" method (Wong et al., 1994) where α-globin primers were dropped into reaction mixtures for 20 cycles of amplification after 20 cycles had already been completed with γ subunit primers alone. The γ subunit and α-globin amplicons were separated by electrophoresis, imaged using a fluorescent gel documentation system (Bio-Rad Gel Doc 1000), and the ratio between their peak areas determined using Molecular Analyst software (Bio-Rad Laboratories Canada Ltd., Mississauga, Ontario). Final ratios were determined by averaging the data from five twofold serial dilutions of each cDNA preparation.

Wholemount Immunofluorescence Microscopy

Embryos were fixed in a 2:1 dilution of methanol in PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 1 mM $MgCl_2$, pH 6.9) for 2–3 min followed by fixation in 100% methanol for an additional 3 min. They were then washed three times in PHEM buffer followed by a final treatment for 45 min with blocking solution (0.01% Triton X-100, 0.1 M lysine, 1% goat serum in PHEM). After washing in PHEM for 5 min, embryos were treated overnight at 4°C with a 1:100 dilution of a rabbit antibody (Ab-G17; Mercer et al., 1993) that was raised against a synthetic peptide corresponding to amino acids 6–22 of the sheep γ subunit. This antibody was supplied by R. Mercer (Washington University, St. Louis, MO). Other embryos were immunostained in the same way with a 1:50 dilution of a mouse monoclonal antibody raised against the α1 subunit of Na^+,K^+-ATPase (supplied by M. Caplan, Yale University, New Haven, CT). After primary antibody treatment, embryos were washed three times for 10 min each through a solution of 0.002% Triton X-100, 1% goat serum in PHEM, and left in the final wash at 4°C for 2 h. This was followed by incubation for 1 h at 4°C in a 1:50 dilution of fluorescein isothiocyanate-conjugated goat anti–rabbit IgG or rat anti–mouse IgG, as appropriate (ICN Pharmaceuticals Canada Ltd., Montréal, Québec). The embryos were washed three times again through 0.002% Triton X-100, 1% goat serum in PHEM, and kept in the final wash overnight at 4°C. Finally, they were mounted in SlowFade (Molecular Probes, Inc., Eugene, OR) and viewed with a Bio-Rad MRC 600 confocal microscope.

Antisense Treatments

The antisense phosphorothioate oligodeoxynucleotide (ODN) used in this study was designed using Oligo™ software to be complementary to 15 bases including and downstream of the initiation codon of the mouse γ subunit mRNA (Mercer et al., 1993). The sequence of this ODN was 5′-CTICACAGCCACCAT-3′; inosine replaced guanine at the third residue in order to reduce the stability of self-complementarity within the ODN. A randomized sequence ("nonsense") ODN (5′-CACCCT-ACIGACATC-3′) having the same base composition as the antisense ODN was also used. To facilitate uptake of ODNs we used the strategy developed by Khidhir et al. (1995) that involves permeabilizing the em-

1. *Abbreviations used in this paper*: hCG, human chorionic gonadotropin; KSOM, potassium-augmented simplex optimization medium; ODN, oligodeoxynucleotide; PVP, polyvinylpyrrolidone; RT, reverse transcription.

bryos for a brief period with lysolecithin. Late 4-cell embryos, flushed from the oviducts at 60 h post-hCG, were exposed to 0.001% lysolecithin (Sigma Chemical Co.) for 2 min. They were then washed in potassium-augmented simplex optimization medium (KSOM; Erbach et al., 1994) and incubated continuously with 0.5 μM ODN in KSOM in a humidified chamber with an atmosphere of 5% CO_2 in air. Development of the embryos in microdrops under oil was monitored for 40 h; embryos were scored at 2 h intervals for the presence or absence of a blastocoel (cavitation). At 100 h post-hCG the embryos were washed five times through PBS-PVP and fixed immediately for wholemount immunofluorescence.

Ouabain-sensitive ^{86}Rb Uptake Assay

Embryos treated with antisense or nonsense ODN were removed from culture at 100 h post-hCG and transferred to a medium consisting of KSOM with K^+ salts replaced with equimolar NaCl, and BSA replaced with 1.5 mg/ml polyvinylpyrrolidone. They were cultured in this medium with or without 1.0 mM ouabain (Sigma Chemical Co.), for 15 min before the addition of ^{86}RbCl (0.5–1.5 mCi/mg; Amersham Canada, Oakville, Ontario). Embryos were assayed for $^{86}Rb^+$ uptake as described previously (Van Winkle and Campione, 1991). Briefly, five embryos were incubated for 30 min in drops of KSOM containing 0.35 mM ^{86}RbCl and 0.35 mM KCl, with or without ouabain, and with polyvinypyrrolidone replacing BSA. The embryos were then washed four times in PBS-PVP, lysed in 2% sodium dodecyl sulfate, and counted for $^{86}Rb^+$ uptake. A sample of the final wash solution equal to the volume in which the embryos were transferred was also counted, with the counts per minute associated with the final wash being subtracted from the cpm for each group of embryos. Because the epithelial trophectoderm of blastocysts acts as a barrier to ouabain (DiZio and Tasca, 1977), blastocysts were collapsed in 0.5 μg/ml cytochalasin D (Sigma Chemical Co.) for 30 min before being assayed for $^{86}Rb^+$ uptake as described above. The data from antisense- and nonsense-treated embryos were compared statistically using Student's *t* test. Some blastocysts were cultured for an additional 8 h after treatment with cytochalasin and $^{86}Rb^+$ to look for deleterious effects on development; none were noted.

Membrane Isolation and "Back Door" Phosphorylation Assay

Antisense- and nonsense–treated embryos were removed from culture at 100 h post-hCG, washed five times through PBS-PVP, and stored frozen at −80°C. Plasma membrane fractions were prepared using a modification of the method of Resh (1982); all steps were carried out at 4°C. The embryos were homogenized by repeated pipetting in 500 μl buffer (255 mM sucrose, 20 mM Tris-HCl, 1 mM EDTA, pH 7) and then the homogenate was centrifuged at 16,000 *g* for 15 min using the JS 13.1 rotor of a Beckman J2-HS centrifuge. Pellets were resuspended in homogenization buffer and centrifuged at 16,000 *g* for an additional 15 min. The resulting pellets were again resuspended in homogenization buffer (100 μl), layered over 100 μl of a 33.5% sucrose cushion in Tris–EDTA buffer, and spun at 70,000 *g* for 1 h using the TLA-100 rotor of a Beckman tabletop ultracentrifuge. The top 150 μl of each supernatant was collected, diluted twofold with Tris–EDTA buffer, and centrifuged for 30 min at 50,000 *g*. Plasma membrane pellets were suspended in a small amount of Hepes–Mg^{2+} buffer (100 mM Hepes-Tris, 5 mM $MgCl_2$, pH 7.4) and stored at −80°C. Kidney membranes were isolated by the same procedure. Protein concentration was determined with a protein assay kit (Bio-Rad Laboratories).

Membranes were phosphorylated by the method of Resh (1982) where 1 μg of membrane protein preparation was incubated in a volume of 80 μl with 10 μM H_3PO_4, with or without 1 mM ouabain, for 30 min before phosphorylation with 20 μCi of [^{32}P]orthophosphate (NEN Life Science Products, Guelph, Ontario), which had been prefiltered through a 0.22-μm filter. The reaction was quenched after 10 min by the addition of 50 μg BSA (as carrier) and 500 μl ice-cold 5% trichloroacetic acid, 0.1 M H_3PO_4 and placed on ice for 5 min before centrifugation for 2 min at 10,000 *g*. Pellets were rinsed three times with 5% trichloroacetic acid, 0.1 M H_3PO_4 and then rinsed quickly with 0.3 ml of 0.15 M KH_2PO_4, pH 2. The final pellets were suspended in sample buffer (250 mM sucrose, 35 mM 1-hexadecylpyridinium chloride, 100 mM KH_2PO_4, 128 mM β-mercaptoethanol, pH 4) for liquid scintillation counting or for analysis by gel electrophoresis.

Electrophoretic analysis of phosphorylated plasma membrane proteins was carried out using acidic polyacrylamide gels as described by Blackshear (1984). The gels were run at 40 mA for 6 h, treated for 5 min in 1% glycerol, dried for 45 min, and exposed to Kodak XAR-5 film with an intensifier screen at −80°C. Gel lanes run with marker proteins were separated from the gel, stained with 0.25% Coomassie blue in 10% acetic acid and 10% methanol, and destained before being dried.

Results

The γ Subunit in Preimplantation Embryos Is a Product of Embryonic Gene Expression

Our first step was to confirm that the γ subunit, like the α and β subunits, is a product of embryonic gene expression. PCR primers designed using the mouse cDNA sequence (Mercer et al., 1993) were used to amplify the expected 134-bp amplicon from reverse-transcribed RNA of selected stages of preimplantation development. Although γ subunit mRNA was detected in unfertilized oocytes, it was not detected in cleaving embryos until the eight-cell stage (Fig. 1 *A*). It is then present continuously through the blastocyst stage. To confirm that γ subunit mRNA present after the four-cell stage is a product of continuous embryonic transcription and to estimate its rate of accumulation, we used a semi-quantitative RT-PCR approach (Davies et al., 1996) to compare mRNA levels between different

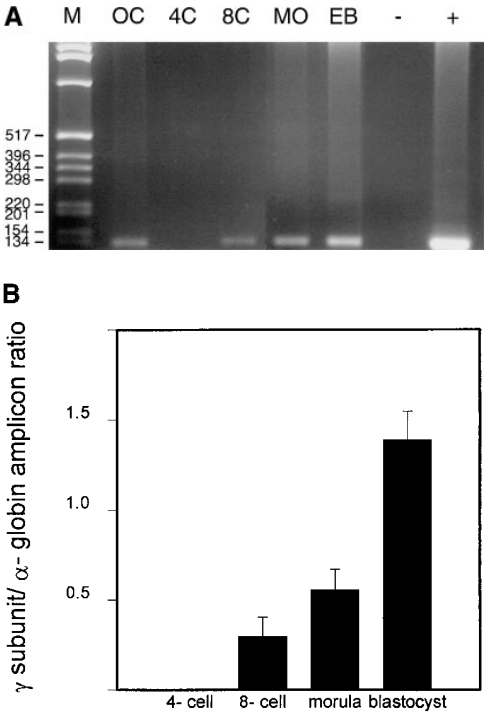

Figure 1. γ Subunit mRNA accumulates from the eight-cell stage onward and is thus a product of embryonic transcription. (*A*) An amplicon of the expected size (134 bp) was amplified from reverse-transcribed RNA of oocytes (*OC*), eight-cell embryos (*8C*), morulae (*MO*), and early blastocysts (*EB*), but not of four-cell embryos (*4C*). *M*, molecular size markers; −, negative control (water blank); +, positive control (mouse kidney cDNA as template). 10 oocyte or embryo equivalents of cDNA were used for each amplification (40 cycles). (*B*) Semi-quantitative RT-PCR demonstrates that the amount of γ subunit mRNA approximately doubles on a per embryo basis in each successive stage. The mean γ subunit/α-globin amplicon ratio was taken as an indication of the relative mRNA level for each stage. The error bars indicate standard deviation derived from five determinations.

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atggtggctgtgcaggggactgagaatcccttcgagtacgactatgaaacagtccgcaaa 60
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ggaggcatgatattagcgggcctggccttcgtcgtgggcctcctcatcattctcagcaaa 93

aggttccgctgtgggggcggtaagaaacataggcaggtcaatgaagatgaactgtgacag 180
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aggttccgctgtgggggcggtaagaaacataggcaggtcaa..................... 134

aagagccagcagtgcc                                             196
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Figure 2. The sequence of the γ subunit amplicon from blastocysts is 97% identical to the sequence of the corresponding portion of the published mouse kidney cDNA (Mercer et al., 1993). The primer sequences are underlined. These sequence data are available from Genbank/EMBL/DDBJ under accession number X70060.

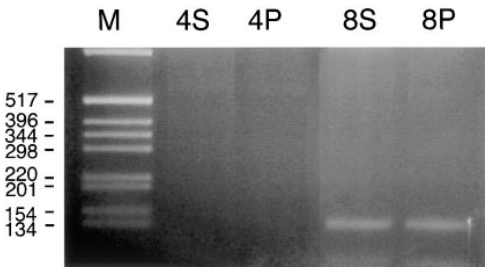

Figure 3. γ Subunit mRNA cosediments with polyribosomes from its time of first appearance in the eight-cell stage, indicating de novo synthesis. Four- and eight-cell uncompacted embryos were fractionated into subribosomal supernatant (*S*) and polyribosomal pellet (*P*) fractions which were assayed for γ subunit mRNA by RT-PCR. Each amplification reaction used 32.5 embryo equivalents of cDNA, with amplification for 40 cycles. *M*, molecular size markers.

stages. A fixed amount of α-globin mRNA was added to each embryo batch before lysis to control for variation in RNA recovery and the efficiency of reverse transcription and PCR amplification. Because the amount of α-globin mRNA added was the same for each stage on a per embryo basis, the ratio between the γ subunit and α-globin amplicons is a relative measure of the amount of γ subunit mRNA. As shown in Fig. 1 *B*, the amount of γ subunit mRNA approximately doubles (on a per embryo basis) in each succeeding stage from the eight-cell stage onward. Thus the γ subunit gene is actively transcribed throughout this developmental period.

The identity of the 134-bp amplicon was confirmed by direct sequencing. As shown in Fig. 2, the amplicon from blastocyst cDNA showed 97% sequence identity with the corresponding segment of the published sequence cloned from mouse kidney cDNA (Mercer et al., 1993). We presume that the few mismatches result from errors in PCR amplification or sequencing, but we cannot rule out the possibility that the preimplantation embryo expresses an isoform of the γ subunit different from that found in kidney.

The presence of γ subunit mRNA does not necessarily indicate that de novo synthesis of the polypeptide is occurring. To eliminate this uncertainty, we used a previously established protocol (De Sousa et al., 1993) to prepare subribosomal supernatant and polyribosomal pellet fractions from four- and eight-cell embryos and assayed them for γ subunit mRNA by RT-PCR (Fig. 3). Both fractions from eight-cell embryos were found to contain the mRNA, although we could not detect it in either fraction from four-cell embryos. This result strongly suggests that γ subunit mRNA is translated from the time of its first appearance in the eight-cell stage. The fact that a portion of the mRNA does not cosediment with polyribosomes suggests that the mRNA is not being translated with maximal efficiency, at least in the eight-cell stage. A similar finding was reported for the mRNA encoding the α subunit (MacPhee et al., 1994).

The developmental profile of the γ subunit itself was explored by confocal immunofluorescence microscopy (Fig. 4). We first confirmed that the antibody produces a specific pattern of immunostaining of mouse kidney (Fig. 4 *E*; Mercer et al., 1993). As expected, strongest staining was seen in individual nephron sections with much lighter staining of glomeruli. The same antibody was then applied to preimplantation embryos. As shown in Fig. 4 *A*, a low level of immunostaining, presumably resulting from a lingering oogenetic contribution, can be detected in four-cell embryos. The intensity of immunostaining increases thereafter. Although there is some cytoplasmic immunoreactivity, the fluorescent signal is concentrated in the cell peripheries, including both apical (facing outward) and basolateral (adjacent to the blastocoel) membranes of the trophectoderm (Fig. 4 *D*). There does not appear to be a significant difference in the amount of γ subunit between inside and outside cells of morulae (Fig. 4 *C*) nor between inner cell mass and trophectoderm of blastocysts. Embryos stained with preimmune serum (Fig. 4 *F*) showed no specific immunoreactivity.

Antisense Disruption of γ Subunit Accumulation Delays Cavitation

As a test of the hypothesis that embryonic synthesis of the γ subunit is required for cavitation, we treated embryos with antisense ODN designed to disrupt translation of γ subunit mRNA. Treatment began in the four-cell stage, before the mRNA begins to accumulate, to maximize the effect. A nonsense (scrambled sequence) ODN was used as a control for nonspecific toxicity. Embryos were permeabilized with lysolecithin to facilitate uptake of the ODN. In preliminary experiments, we determined that a lysolecithin concentration of 0.001% is optimal; greater concentrations proved toxic. This concentration differs from that used by Khidhir et al. (1995), probably reflecting differences in the strain of mice used and between particular lots of lysolecithin.

Results of one experiment, performed in triplicate, are plotted in Fig. 5. Antisense treatment caused a delay in cavitation, an effect that was apparent by 98 h post-hCG. The effect was still evident at 100 h, when the number of cavitating embryos in the antisense group of this experiment was only ~50% of that in the groups treated with nonsense ODN or lysolecithin alone ("control"). Those embryos that had not started to cavitate at 100 h in the antisense treated group appeared otherwise normal when viewed by phase contrast microscopy. In the course of nine experiments, a delay in cavitation was consistently seen in the antisense groups although the quantitative effect varied, with the frequency of cavitation in antisense groups

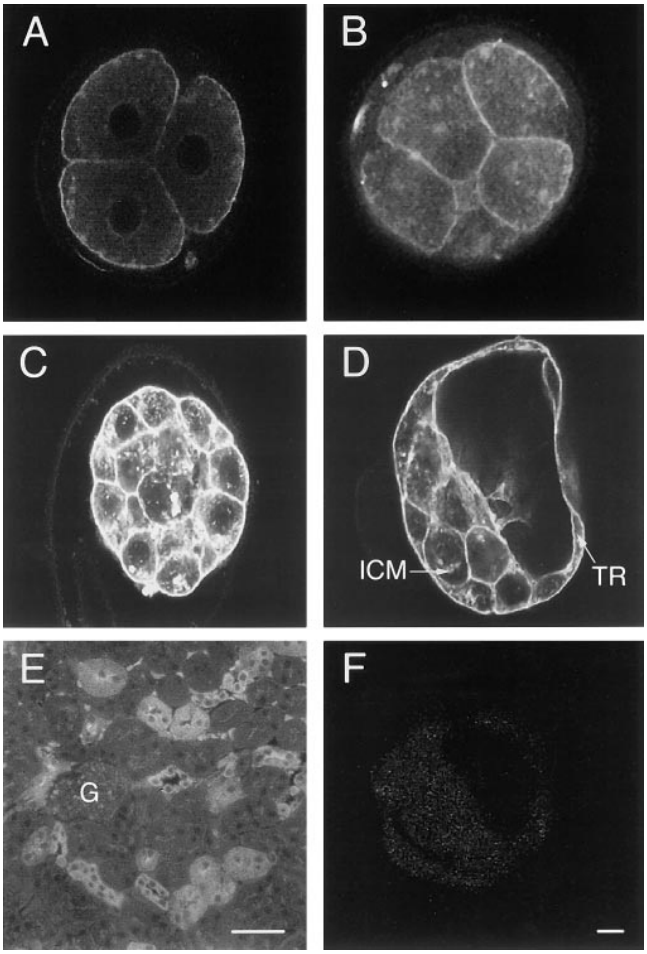

Figure 4. The γ subunit accumulates from the eight-cell stage onward and is localized in both apical and basolateral membrane domains of trophectoderm. Confocal images were made at the same magnification and using comparable microscope settings for all stages to allow comparison of signal intensities between stages. Shown are representative four- (*A*) and eight-cell (*B*) embryos, a compacted morula (*C*), and a blastocyst (*D*). *ICM*, inner cell mass; *TR*, trophectoderm. A section of mouse kidney showing a glomerulus (*G*), stained with the same antibody is shown in *E* (Bar, 25 μm). The image in *F*, in which the signal intensity was enhanced for better visibility, shows a blastocyst treated with preimmune serum. Bar, 10 μm.

ranging between 45 and 73% of that in nonsense groups at the 100 h time point (mean = 62%). The effect was not sustained, however, because by 108 h post-hCG, virtually all of the embryos in all three treatment groups of each experiment were cavitating and there was no longer any difference between groups. In two experiments, some embryos were removed from culture at 80 h post-hCG and RNA was isolated for semi-quantitative RT-PCR. The amount of γ subunit mRNA in the antisense-treated groups was found to have been reduced by 34% in one experiment and 46% in the other, as compared with the respective nonsense-treated groups.

Confocal immunofluorescence microscopy of embryos taken from the treatment groups at 100 h post-hCG demonstrated that the antisense treatment had caused an obvious reduction in the amount of γ subunit (Fig. 6, *A* and *B*) as compared with embryos treated with nonsense ODN

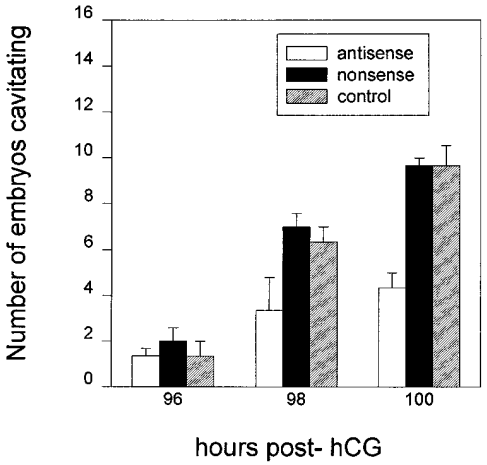

Figure 5. Antisense disruption of γ subunit accumulation interferes with blastocyst development. Embryos treated with antisense ODN, but not nonsense ODN or lysolecithin alone (*control*), suffer a delay in the onset of cavitation. The experiment was done in triplicate with each embryo drop containing 20 embryos.

(Fig. 6, *D* and *E*). In contrast, we could not detect any effect on the α subunit after disrupting accumulation of the γ subunit (Fig. 6, *C* and *F*). This finding makes it unlikely that, in this system, the γ subunit is required for deployment of the enzyme in plasma membranes.

The importance of trophectodermal Na^+,K^+-ATPase for cavitation is assumed to reflect its ability to pump Na^+ into the blastocoel in exchange for K^+, which is pumped

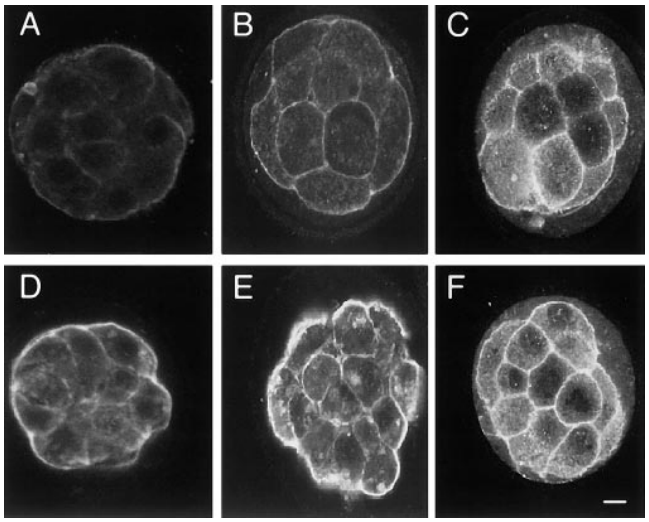

Figure 6. Treatment with antisense oligodeoxynucleotide caused an obvious reduction in the amount of γ subunit (but not α subunit) in the blastomere membranes. Confocal images were made at the same magnification and using comparable microscope settings for all stages in order to allow comparison of signal intensities between treatments. Embryos in *A–C* were treated with antisense ODN whereas those in *D–F* were treated with nonsense ODN; all were taken out of culture at 100 h post-hCG. The embryos were immunostained with γ subunit antibody except those in *C* and *F*, which were immunostained with α subunit antibody. Embryos in *A* and *D* were from one experiment whereas embryos in *B*, *C*, *E*, and *F* were from a separate experiment. Bar, 10 μm.

Table I. Effect of Antisense Treatment on $^{86}Rb^+$ Uptake

Experiment number	Treatment	Morulae not cavitating, cpm/embryo (± SD)			Morulae cavitating, cpm/embryo (± SD)		
		Total	+ Ouabain	Ouabain sensitive	Total	+ Ouabain	Ouabain sensitive
1	Antisense	12.1 ± 5.5*	8.3 ± 4.3‡	3.8	—	—	—
	Nonsense	39.5 ± 4.6	4.3 ± 1.6	35.2	—	—	—
2	Antisense	14.6 ± 3.0*	3.3 ± 1.7‡	11.4	18.3 ± 5.7*	3.4 ± 1.1‡	14.9
	Nonsense	37.9 ± 2.8	3.6 ± 3.3	34.4	45.0 ± 2.0	3.3 ± 0.1	41.7
3	Antisense	22.0 ± 2.4*	2.9 ± 0.8‡	20.1	48.2 ± 0.7*	7.6 ± 3.2‡	40.6
	Nonsense	42.9 ± 6.6	6.8 ± 3.1	35.1	64.1 ± 3.1	4.5 ± 0.8	59.6

*Significantly different from the nonsense-treated value in the same experiment ($P < 0.01$).
‡Not significantly different from the nonsense-treated value in the same experiment ($P > 0.10$).

into the trophectoderm cells. Previously, sodium pump activity in preimplantation embryos has been measured by monitoring ouabain-sensitive $^{86}Rb^+$ uptake, Rb^+ acting as a surrogate for K^+ (Van Winkle and Campione, 1991). We used this same method to determine the extent to which the antisense treatment had affected the function of the sodium pump. As summarized in Table I, the rate of ouabain-sensitive $^{86}Rb^+$ uptake in the first two experiments was severalfold lower in late morulae in which the onset of cavitation had been delayed by the antisense treatment; in the third experiment, the reduction was 43%. In the second and third experiments we also analyzed embryos that had succeeded in initiating cavitation by 100 h despite the antisense treatment, and found similar reductions. These data indicate that the γ subunit is an important determinant of the function of the sodium pump and that embryonic expression of the γ subunit is a requirement for cavitation.

The Rb^+ uptake assay is a measure of Na^+,K^+-ATPase activity in intact cells and, as such, can reflect changes brought about by effectors of pump activity independent of the number of active enzyme molecules. Hence, a reduction in ouabain-sensitive Rb^+ uptake could indicate a direct effect on the enzyme itself or some change in a physiological effector. In order to ascertain whether the enzymatic activity of the enzyme itself had been affected by the reduction of γ subunit accumulation, we used the "back door" phosphorylation assay (Resh, 1982) to measure Na^+,K^+-ATPase activity in isolated plasma membrane preparations. This assay is based on the fact that, in the presence of ouabain and Mg^{2+}, the α subunit of Na^+,K^+-ATPase is uniquely phosphorylated to form an alkali–labile intermediate that is identical to that which is generated during the enzyme's forward reaction cycle. Since the enzyme must pass through at least a partial reaction cycle to be phosphorylated, only active enzyme is detected. As shown in Fig. 7, a single polypeptide of ~95,000 M_r was phosphorylated when plasma membrane preparations from embryos (100 h post-hCG) or from adult kidney were incubated with [^{32}P]orthophosphate and Mg^{2+} in the presence of ouabain. Phosphorylation of the 95-kD polypeptide was minimal in the absence of ouabain. As expected, the bound phosphate was released when the membranes were incubated in 2 mM ATP (Resh, 1982).

Measurements of Na^+,K^+-ATPase activity in antisense-treated embryos using this phosphorylation assay are summarized in Table II. Four experiments were carried out; in the first, duplicate assays were performed for each treatment whereas in the other three experiments the assays were done in triplicate. In each experiment, the assay failed to demonstrate any difference in enzymatic activity between antisense- and nonsense-treated groups. These data make it clear that the reduced availability of γ subunits in antisense-treated embryos had no effect on Na^+,K^+-ATPase activity per se, making it doubtful that the γ subunit is an integral component of the enzyme.

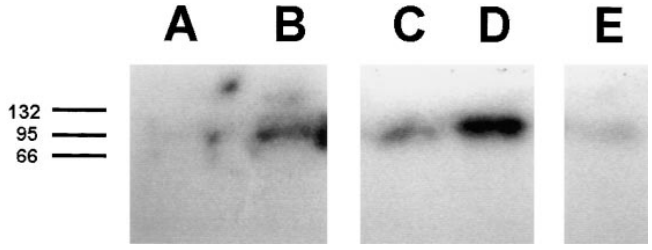

Figure 7. The α subunit of Na^+,K^+-ATPase is uniquely labeled by [^{32}P]orthophosphate in the presence of ouabain. Lanes *A* and *B* contain equal amounts of embryo (morulae and blastocysts, 100 h post-hCG) plasma membrane protein and the autoradiogram was exposed for 6 h; lanes *C–E* contain equal amounts of adult mouse kidney plasma membrane protein with exposure of the autoradiogram for 4 h. In lanes *B*, *D*, and *E*, the membranes were incubated in the presence of 1 mM ouabain before labeling. In lane *E* the kidney membranes were incubated with 2 mM ATP for 5 min after labeling.

Discussion

In preimplantation embryos, Na^+,K^+-ATPase is both a

Table II. Effect of Antisense Treatment on Na^+,K^+-ATPase Activity

Experiment number	Treatment	cpm bound per μg of embryo membrane
1	Antisense	4,570:4,278
	Nonsense	4,965:5,319
2	Antisense	14,635 ± 2,332*
	Nonsense	10,883 ± 667
3	Antisense	12,479 ± 340‡
	Nonsense	12,641 ± 1,150
4	Antisense	18,231 ± 790§
	Nonsense	19,652 ± 728

*Not significantly different from the nonsense-treated value in the same experiment ($P = 0.32$).
‡Not significantly different from the nonsense-treated value in the same experiment ($P = 0.89$).
§Not significantly different from the nonsense-treated value in the same experiment ($P = 0.83$).

"housekeeping" enzyme, responsible for maintaining the electrochemical gradients of Na^+ and K^+ across the plasma membrane, and an agent of morphogenesis. Sodium pumps deployed in the basolateral membranes of the epithelial trophectoderm work in concert with a variety of Na^+ entry routes arrayed in the apical domain to generate a transepithelial flow of Na^+ and water to form the blastocoel (Watson and Kidder, 1988; Manejwala et al., 1989). Hence, treatments that interfere with the establishment of a polarized distribution of trophectodermal sodium pumps or with the activity of those pumps can prevent cavitation (Manejwala et al., 1989; Watson et al., 1990*a*; MacPhee et al., 1997). We have taken advantage of the dependence of blastocyst development on embryonic expression of sodium pump subunits to test the hypothesis that the putative γ subunit of Na^+,K^+-ATPase is an important determinant of the enzyme's function and, as such, is required for cavitation. Embryonic transcription of the γ subunit gene, cosedimentation of the mRNA with polyribosomes, and increasing γ subunit immunoreactivity with development after the eight-cell stage all served as evidence that the γ subunit is embryonically expressed in the mouse, a prerequisite finding before an antisense approach could be applied. The temporal pattern of expression of the γ subunit gene, with mRNA present in oocytes being degraded after fertilization to reappear during cleavage of the zygote, is typical of genes expressed in preimplantation embryos and reflects the transition from oogenetic to embryonic control of development (for review see Kidder, 1993).

Antisense disruption of γ subunit accumulation delayed cavitation, indicating that synthesis of the γ subunit is essential for blastocyst development. Antisense-treated morulae collected at 100 h post-hCG, when the cavitation delay was most apparent, were found to have reduced immunoreactivity for the γ subunit as well as reduced sodium pump function as indicated by ouabain-sensitive $^{86}Rb^+$ uptake. Failure of the antisense ODN to completely block cavitation probably reflects the fact that synthesis of the γ subunit was not completely abolished; this could be due to incomplete destruction of the mRNA, although we do not know to what extent translation of the remaining mRNA might have been impaired. In any case, antisense-treated morulae retained some capacity for active cation transport. Those embryos that were able to initiate extracellular fluid accumulation on schedule with nonsense-treated or control embryos are likely to have been the most advanced in their cohort and as such would have had more time to accumulate fluid using their reduced Na^+ transport capacity. It is also possible that the more advanced embryos, which would have been the first to cleave to the eight-cell stage, might have initiated γ subunit expression before the antisense inhibition had become fully established. This interpretation is supported by the observation that when the antisense treatment was delayed until the eight-cell stage, there was no discernable effect on the timing of cavitation (results not shown).

Using the *Xenopus* oocyte expression system, Béguin et al. (1997) demonstrated that the γ subunit is not required for the assembly of α/β heterodimers or their insertion into the plasma membrane as functional enzyme molecules. These results are in agreement with our observation that antisense attenuation of γ subunit accumulation did not affect the level of α subunit immunoreactivity at the cell surface. However, Béguin et al. (1997) also showed that stable expression and plasma membrane insertion of the γ subunit in oocytes depends on its association with nascent α/β heterodimers. If the same is true in preimplantation mouse embryos, then it remains to be discovered how the γ subunit could accumulate in both the apical and basolateral surfaces of the trophectoderm when α1 and β1 subunits are concentrated in the basolateral surface (Watson and Kidder, 1988; MacPhee et al., 1997). One explanation might be that nascent γ subunits are delivered to both surfaces in association with α/β heterodimers, but that this association is lost in the apical domain when the latter are not retained there. In polarized MDCK cells, for example, localization of Na^+,K^+-ATPase is achieved by preferential retention of nascent α/β heterodimers in the basolateral domain whereas they are selectively removed from the apical domain (Hammerton et al., 1991).

The fact that the γ subunit is present in both apical and basolateral trophectoderm surfaces suggests that it may also function separately from Na^+,K^+-ATPase. Although there are few studies examining the cellular distribution of the γ subunit, it has consistently been found in association with the α and β subunits in those cells and tissues where it has been detected (Mercer et al., 1993). Hence we expected to find the γ subunit concentrated in the basolateral (juxtacoelic) surface of the trophectodermal epithelium. The fact that it is not suggests that the γ subunit may associate with other membrane proteins or have functional properties not requiring the α and β subunits. Expression studies using *Xenopus* oocytes (Noguchi et al., 1987; Béguin et al., 1997), yeast (Scheiner-Bobis and Farley, 1994; Pedersen and Jørgensen, 1992), and insect cells (DeTomaso et al., 1993) have all indicated that functional Na^+,K^+-ATPase activity can be obtained by expressing only the α and β subunits. Interestingly, the amino acid sequence of the γ subunit places it in a recently recognized family of membrane proteins, characterized by single transmembrane domains that mediate transmembrane ion movements. Other members of the family include phospholemman (Palmer et al., 1991), channel-inducing factor (Attali et al., 1995), and Mat-8 (Morrison et al., 1995). When expressed in *Xenopus* oocytes, phospholemman and Mat-8 induce Cl^- selective currents while channel-inducing factor induces a K^+ current. Early evidence suggested that these proteins may activate endogenous channels; however, it is now known that phospholemman itself forms a taurine-selective ion channel when incorporated into a synthetic lipid bilayer (Moorman et al., 1995). More recently, it has been demonstrated that in the absence of taurine, phospholemman can function as a cation or anion selective channel (Kowdley et al., 1997). The channel undergoes voltage-dependent transitions among conformations with distinct ion selectivities, possibly explaining the very different ion selectivity properties of the members of this family of proteins. Consistent with these observations, it has been determined that the γ subunit induces monovalent cation (Na^+, or K^+) selective channels when expressed in *Xenopus* oocytes (Mercer, R., personal communication). The possible functioning of the γ subunit as a cation channel is compatible with our observations and could explain the discrepancy between our assay data obtained

from intact cells and those from isolated plasma membranes. In the intact embryo, the γ subunit may increase Na^+,K^+-ATPase activity by supplying intracellular Na^+ to the enzyme such that, when its expression is inhibited, transepithelial Na^+ transport is adversely affected; such an effect could not be detected by assaying plasma membrane preparations in vitro. In this respect, trans-trophectodermal Na^+ movement in blastocysts may be similar to transepithelial transport in the kidney in which Na^+ entry at the apical membrane is often rate limiting (for review see Stanton and Kaissling, 1989). Thus the presence of the γ subunit in the apical membranes of the blastocyst may be relevant to its function during cavitation. Alternatively, the γ subunit associated with the sodium pumps in the basolateral membranes may serve to recycle intracellular K^+ into the blastocoel to sustain pump function.

Whatever the role of the γ subunit turns out to be, it has an important influence on Na^+,K^+-ATPase activity and transepithelial Na^+ transport, though probably not as an integral component of the enzyme. Furthermore, our results demonstrate that the γ subunit is an essential component of the cellular machinery that drives fluid transport during blastocoel formation. Future experiments will be aimed at trying to clarify what role the γ subunit plays in influencing sodium pump activity during preimplantation development.

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