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Investigating the Surface Expression of the Renal Type IIa Na^+/P_i-Cotransporter in *Xenopus laevis* Oocytes

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Abstract. We have combined a functional assay, surface labeling and immunocytochemical methods to compare total and surface-exposed renal type IIa Na^+/P_i cotransporter protein. The wild-type type cotransporter (NaPi-IIa) and its functionally comparable cysteine mutant S460C were expressed in *Xenopus* oocytes. S460C contains a novel cysteine residue that, when modified by preincubation with methanethiosulfonate reagents, leads to complete suppression of cotransport function. This allowed surface labeling of the S460C using MTSEA-Biotin and confirmation by electrophysiology on the same cell. Protein was analyzed by Western blotting before and after streptavidin precipitation and by immunocytochemistry and immunogold electronmicroscopy. MTSEA-Biotin treatment resulted in a complete inhibition of S460C-mediated Na^+/P_i-cotransport activity, which indicated that all transporters at the surface were biotinylated. After biotinylation, only a small fraction of total S460C protein was precipitated by streptavidin compared with the total amount of S460C protein detected in the lysate. Light- and electron-microscopy analysis of oocytes showed a large amount of WT and S460C transporter protein beneath the oocyte membrane. These data indicate that the apparent weak labeling efficiencies of surface-biotinylation-based assays of membrane proteins heterologously expressed in oocytes can be related to diminished incorporation of the protein in the oolemma.

Key words: *Xenopus laevis* oocytes — Surface labeling — NaPi-IIa — Electronmicroscopy — Immunocytochemistry — Biotinylation

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Introduction

Xenopus laevis oocytes are commonly used for heterologous expression of membrane transport proteins to provide information on their function, regulation and routing to the cell surface (for review, *see* Romero et al., 1998; Wagner et al., 2000). For the analysis of functional properties such as substrate specificity or affinity of native and mutant membrane proteins, the detection of transport activity that exceeds endogenous levels is an indication of adequate surface expression. However, at least 'semi-quantitative' information on surface location is required for making conclusions that relate, for example, to transport capacity or routing of native or mutant membrane proteins to and from the oocyte surface.

In a number of heterologous expression studies using *Xenopus* oocytes information on surface expression of transport protein was obtained by either immunocytochemical (e.g., Lostao et al., 1995; Bai et al., 1996; Turk et al., 1996; Chillaron et al., 1997; Mulders et al., 1998,1997; Forster et al., 1999; Lambert et al., 1999*a,b*), surface labeling (e.g., Lostao et al., 1995; Pajor, Sun & Valmonte, 1998, 1999; Lambert et al., 1999*a;* Singer-Lahat et al., 2000), and/or by membrane isolation procedures (e.g., Wall and Patel, 1989; Chillaron et al., 1997; Kamsteeg et al., 1997). Also the analysis of glycosylation of the proteins of interest was used for a similar purpose (e.g., Magagnin et al., 1992; Hayes et al.,1994; Bai et al., 1996; Kamsteeg et al., 1997; Turk et al., 1996; Chillaron et al., 1997; Mulders et al., 1997, 1998).

To illustrate some of the limitations of biochemical (surface biotinylation) and/or morphological techniques (immunofluorescence, electron microscopy) for the detection of protein surface expression in oocytes, we have investigated the type IIa inorganic phosphate (P_i) Na^+-coupled cotransporter (NaPi-IIa). In the mammalian kidney, this protein provides the principal pathway at the

lumen of the proximal tubule by which filtered P_i in the glomerular filtrate is reabsorbed (for review, *see* Murer et al., 2000). The functional properties of NaPi-IIa have been extensively characterized and previous studies have shown that NaPi-IIa is an electrogenic, secondary active cotransporter (e.g., Busch et al., 1994, 1995; Forster et al., 1997, 1998; for review, *see* Murer et al., 2000). Regulation of renal P_i reabsorption is mediated through the targeting of a number of hormonal and nonhormonal factors to the NaPi-IIa protein directly or indirectly (Murer et al., 2000).

In this study, we employed a functionally equivalent mutant construct of NaPi-IIa (S460C), the transport activity of which can be fully inhibited through modification of the cysteine residue substituted at site 460, by preincubation in methane thiosulfonate (MTS) reagents (Lambert et al., 1999*b*; Köhler et al., 2000). This allowed us to surface-label S460C using MTSEA-Biotin and confirm the labeling by electrophysiology. We then found that after biotinylation only a small fraction of S460C protein could be precipitated by streptavidin, which suggested that a concomitantly small fraction of transport protein was actually at the surface. Light- and immunogold-electron microscopy of oocytes that expressed either WT NaPi-IIa or the S460C mutant confirmed this finding.

Materials and Methods

In Vitro cRNA Synthesis

In vitro synthesis and capping of cRNAs were performed by incubating the rat NaPi-IIa constructs, previously linearized by Not I digestion, in the presence of 40 U of T7 RNA polymerase (Promega) and Cap Analog (NEB) (Werner et al., 1990).

Oocyte Preparation and Injection

Stage V-VI oocytes (Dumont, 1972) were prepared as previously described (Magagnin et al., 1992). Oocytes were incubated in modified Barth's solution (*see below*). Typically 10 ng of cRNA in 50 nl of water were injected per oocyte and experiments performed 4–6 days postinjection.

Solutions and Reagents

All standard chemicals and reagents were obtained from either Sigma or Fluka (Buchs, Switzerland). The biotin labeled methanethiosulfonate (MTS) reagent, N-Biotinylaminoethyl methanethiosulfonate hydrobromide (MTSEA-Biotin) was obtained from Toronto Research Chemicals (Downsview, ON, Canada) and freshly prepared in dimethyl sulfoxide (DMSO). The concentration of DMSO did not exceed 1% and control experiments indicated no effect on transport function by DMSO at this concentration.

The solution compositions (in mM) were as follows: (i) oocyte incubation (modified Barth's solution): NaCl (88); KCl (1); $CaCl_2$ (0.41); $MgSO_4$ (0.82); $NaHCO_3$ (2.5); $Ca(NO_3)_2$ (2); HEPES (7.5); adjusted to pH 7.6 with HCl and supplemented with antibiotics (10 mg/l penicillin, streptomycin). (ii) control superfusate (ND100): NaCl (100); KCl (2); $CaCl_2$ (1.8); $MgCl_2$ (1); HEPES (5) and adjusted to pH 7.4 with KOH. (iii) substrate test solutions: inorganic phosphate at 1 mM P_i was added to ND100 from a 1 M K_2HPO_4/KH_2PO_4 stock preadjusted to pH 7.4. PFA was added to ND100 solution from a frozen 100 mM stock to give a final concentration of 3 mM and the pH was readjusted to pH 7.4.

Electrophysiology

A two-electrode voltage clamp was used as previously described (Forster et al., 1998) to quantify function of surface-expressed transport protein. Oocytes were mounted in a small recording chamber (volume 100 μl) and continuously superfused (5 ml/min) with test solutions precooled to 20°C. The freshly prepared MTSEA-Biotin was applied to the oocyte chamber using a 0.5 mm diameter cannula positioned near the cell and fed by gravity. The steady-state response of an oocyte to P_i was always measured at a holding potential (V_h) = −50 mV in the presence of 100 mM Na^+. Data were acquired online using pClamp v 8.1 software and Digidata 1200A compatible hardware (Axon Instruments, Foster City, CA) and sampled at more than twice the recording bandwidth. Recorded currents were prefiltered using an 8-pole low pass Bessel filter (Frequency Devices, Havenhill, NJ) which was set to 20 Hz for the steady-state measurements reported here.

Immunoblotting of Oocyte Homogenates

Yolk-free homogenates were prepared 3 days after injection (H_2O or cRNA). Pools of 5 oocytes were lysed together with 100 μl of homogenization buffer [1% Elugent (Calbiochem) in 100 mM NaCl, 20 mM Tris/HCl, adjusted to pH 7.6], by pipetting the oocytes up and down (Turk et al., 1996). To pellet the yolk proteins, samples were centrifuged at 16,000 × *g* for 3 min at 22°C; 10 μl of the supernatant in 2 × loading buffer (4% SDS, 2 mM EDTA, 20% glycerol, 0.19 M Tris/HCl pH 6.8, 2 mg/ml bromphenol blue) were separated on an SDS-PAGE gel and separated proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Switzerland). The membrane was then processed according to standard procedures (Sambrook, Fritsch & Maniatis et al., 1989) using a rabbit polyclonal antibody raised against an NH_2-terminal synthetic peptide of the rat NaPi-IIa cotransporter. The specificity of the antibody has been demonstrated previously (Custer et al., 1994). Immunoreactive proteins were detected with a chemiluminescence system (Pierce, Rockford, IL).

Streptavidin Precipitation of Biotinylated Protein

Groups of 7 oocytes that express S460C or the WT protein were incubated for 5 min in 100 μM MTSEA-Biotin. Biotin-streptavidin precipitation was performed as previously described (Hayes et al., 1994). Briefly, after taking a sample for Western blotting, the oocyte homogenate was incubated for 2 hr with streptavidin beads and precipitated proteins were eluted with 2 × loading buffer at 95°C for 5 min. Samples were loaded on an SDS-gel and immunoblotted after protein separation.

Immunocytochemistry

The fixation of *Xenopus laevis* oocytes has been previously described (Hayes et al., 1994). Briefly, the oocytes were immersed for 30 min in PBS containing 3% paraformaldehyde. After rinsing in cold PBS, the

eggs were frozen onto thin cork slices using liquid-nitrogen-cooled liquid propane. Sections of 5 μm were cut at −23°C using a cryomicrotome, and mounted on chromalaum-gelatin-coated glass slides. For immunostaining, after preincubation in PBS containing 3% milk powder and 0.02% Triton X-100, the sections were first incubated overnight with a rabbit anti-NaPi-IIa antibody (Custer et al., 1994) directed against the NH_2-terminus (dilution: 1:500), followed by incubation with a swine anti-rabbit IgG-conjugated fluorescein-isothiocyanate (FITC) secondary antibody (Dakopatts). The same antiserum was used to detect the S460C mutant. Finally, sections were coverslipped using DAKO Glycergel (Dakopatts) plus 2.5% 1,4-diazabicyclo(2.2.2)octane (Sigma) as a fading retardant. Unspecific binding of the secondary antibodies to the oocytes was tested by omitting the primary antibody. All control incubations were negative.

Immunogold Electron Microscopy

For electron microscopy *Xenopus laevis* oocytes were immersed for 2 hr in the fixative described above plus 0.1% glutaraldehyde. Afterwards fixed oocytes were infiltrated with 2 M sucrose containing 10% (wt/vol) polyvinylpyrrolidone. The oocytes were rapidly frozen in liquid nitrogen; ultrathin sections of 80 nm were cut at −120°C according to Griffiths et al., 1983, using a Leica Ultracut UCT (Leica, Switzerland). The sections were transferred to polyvinylchloride-polyvinylacetate covered nickel grids and stored on 1% gelatine at 4°C overnight. To quench free aldehyde groups the grids were incubated for 5 min in 50 mM NH_4Cl and afterwards incubated for 10 min in PBS containing 3% bovine serum albumin, 0.02% Triton X-100 and 0.01% Tween-20 (Solution A). Thereafter, the grids were transferred for 1 hr at 37°C with the primary antibodies (dilution 1:500 in solution A), washed in PBS and incubated for 1 hr at room temperature with goat anti-rabbit IgG coupled to 8 nm gold particles at a dilution (in solution A) that corresponded to an OD_{525} of 0.06. Conjugation to gold particles was performed according to Slot and Geuze (1985). Finally the grids were rinsed with PBS and post-fixed for 10 min in 1% glutaraldehyde in PBS, again rinsed first with PBS and secondly with cacodylate buffer and thirdly with distilled water. The sections were contrasted in 2% (wt/vol) methylcellulose containing 0.2% uranyl acetate for 10 min and examined on a Phillips CM 100 electron microscope.

Results

Effect of MTSEA-Biotin on the Electrogenic Response of NaPi-IIa and S460C

The Na^+/P_i cotransport function of the S460C mutant construct is completely blocked by externally applied MTSEA (Lambert et al., 1999). This characteristic provided us with a functional test to indicate the extent of surface biotinylation by MTSEA-Biotin (Fig. 1). For both WT and S460C expressing oocytes, prior to MTSEA-Biotin incubation, application of 1 mM P_i induced an inward current at −50 mV holding potential. Furthermore, 3 mM phosphonoformic acid (PFA), a competitive inhibitor of Na^+-coupled P_i-cotransport (Busch et al., 1995), induced an apparent outward current in ND100 solution at $V_h = -50$ mV, relative to the oocyte holding current. The PFA-induced change in holding current has been shown to arise from block of an uncoupled, inward,

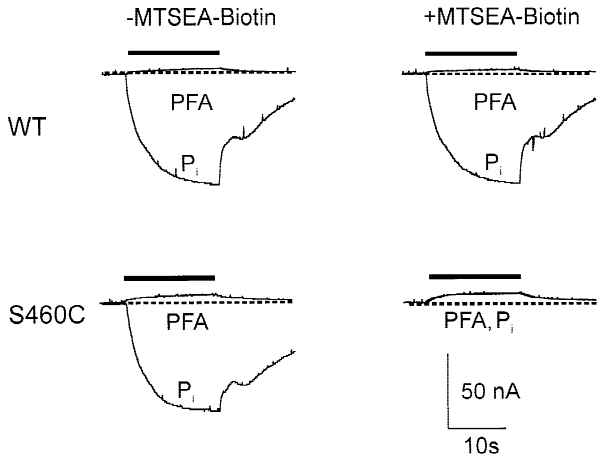

Fig. 1. The effect of incubation in MTSEA-Biotin on the electrogenic response of *Xenopus* oocytes injected with WT NaPi-IIa or S460C cRNA. Representative traces showing control response to 1 mM P_i and 3 mM PFA superimposed, before (left) and after (right) incubation in 100 μM MTSEA-Biotin for 5 min added to ND96 solution. Oocytes were continuously voltage-clamped at a holding potential $V_h = -50$ mV. Test substrates were applied for 20 sec as indicated by solid bars. *Upper traces:* oocyte injected with WT NaPi-IIa cRNA; *bottom traces:* oocyte injected with S460C cRNA. Cells tested in this experiment were subsequently used for streptavidin precipitation (*see* Fig. 2).

Na^+-dependent slippage or leak current that is an intrinsic property of type IIa cotransporters (Forster et al., 1998). As previously established, the P_i-induced current of oocytes that express WT NaPi-IIa was not affected after MTSEA incubation (Lambert et al., 1999*b*). This indicated that the WT protein lacks functionally critical cysteines that are accessible by externally applied MTSEA. In contrast, for S460C, after incubation in MTSEA-Biotin, P_i induced the same change in holding current recorded in the presence of PFA, before and after incubation. We have previously interpreted this behavior as an indication that the modification of Cys-460 has fully inhibited the cotransport mode (Lambert et al., 1999*b*). Furthermore, the equivalence of the postincubation P_i and PFA-induced currents indicated that the Cys-460 in each surface-exposed S460C protein has been appropriately modified and, by implication, would now be biotinylated.

Precipitation of Biotinylated S460C Protein

Under the assumption that only functionally active proteins are present at the oocyte surface, the above labeling procedure (i.e., the addition of MTSEA-Biotin) allowed us to make a 'semi-quantitative' assay of S460C protein located at the surface after its biotinylation and precipitation by streptavidin. Protein was detected by means of standard Western blotting using antibodies raised against the N-terminal of NaPi-IIa (Custer et al., 1994).

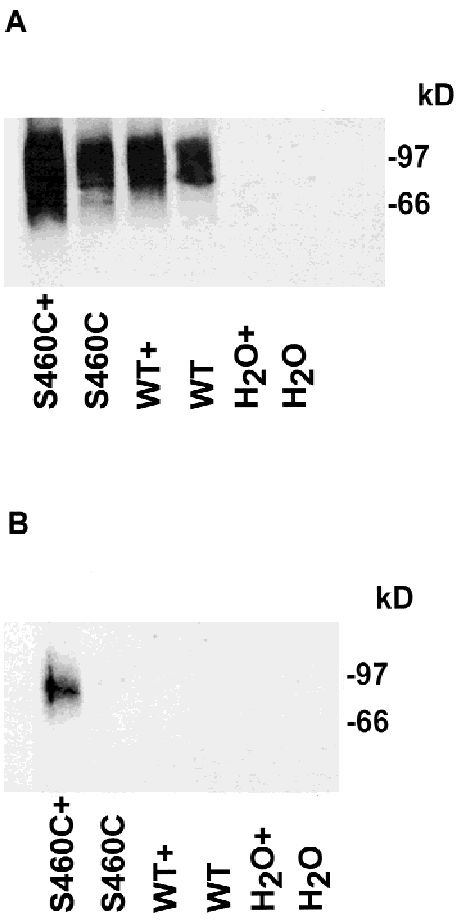

Fig. 2. Immunodetection of WT NaPi-IIa and S460C protein. (*A*) Western blot under reducing conditions of 10 μl of oocytes lysate from a pool of 7 ooyctes injected with either water, WT NaPi-IIa, or S460C cRNA, incubated with (+) or without MTSEA-Biotin (100 μM). NaPi-IIa and S460 C protein gave bands at about 90 kD. (*B*) strepavidin-precipitation of the same pool of oocytes revealed a single band at 90 kD indicating that the biotin-labeled protein was S460C. 130 μl of each lysate was incubated with streptavidin beads. The immunoreactive proteins were blotted with an antibody raised against the NH_2-terminus of the rat NaPi-IIa.

Figure 2*A* shows Western blots of total oocyte lysate before streptavidin precipitation. Comparable levels of the glycosylated forms of WT and S460C were observed at 80-100 kDa; this immunostaining corresponds to the molecular weight of the functional unit of NaPi-IIa (Köhler et al., 2000). Incubation of MTSEA-Biotin did not affect the expression levels of either protein (Fig. 2*A*). As indicated in Fig. 2*B*, after incubation in MTSEA-Biotin, only the S460C protein could be precipitated by streptavidin.

The intensity of the Western blot signal obtained after streptavidin precipitation (Fig. 2*B*) was small compared to the total amount of transporter protein present in the oocyte lysate (Fig. 2*A*); for the analysis of the total amount of protein we used only 10 μl of oocyte lysate, whereas a 130 μl aliquot was used for streptavidin precipitation of lysate and subsequent immunoblotting. Since the functional test described above established that transporters at the oocyte surface were fully accessed by the biotin reagent, we concluded that only a very minor fraction of total lysate S460C protein at the surface was biotinylated and thus precipitated. Similar observations were made by using different batches of oocytes (*data not shown*), although there was a batch to batch variability in the amount of total functional protein expressed, as evidenced from the concomitant variations in electrogenic response to P_i (typically this varied from −50 to −150 nA between batches with up to 30% variation within any batch). Attempts to increase the precipitation efficiency (e.g., by increasing the amount of streptavidin beads) were not successful.

Morphological Detection of WT and S460C in *Xenopus laevis* Oocytes

To gain further insight into the distribution of NaPi-IIa protein in the oocyte, we employed two morphological techniques: immunohistochemistry and immunogold labeling.

As illustrated in Fig. 3, labeling of WT (*A*) and S460C (*B*), using antiserum directed against the N-terminal of the respective proteins, resulted in a bright immunofluorescence staining at the oocyte surface. In H_20-injected oocytes, no significant NaPi-IIa specific staining was observed (*C*). From the low resolution views (*see* respective insets, Fig. 3), one might conclude that most of NaPi-IIa protein is indeed located almost exclusively at the membrane, however, at a higher magnification, grainy immunolabeling was detectable directly beneath the oocyte membrane (arrows, Fig. 3). The intensity and pattern of immunostaining was comparable for both proteins. These observations indicated that only part of WT or S460C protein was membrane bound. This is consistent with the streptavidin precipitation results.

Finally, to obtain higher resolution views of NaPi-IIa distribution, we applied the immunogold labeling technique (*see* Materials and Methods). As shown in Fig. 4 for oocytes that express the WT (*A* and *C*) and S460C (*B* and *D*), gold particles were present along the microvilli of the oocyte membrane as well as at the base of microvilli. Additionally, they were detected in the submembranous area, consistent with the immunohistochemical findings. We also found that both proteins could also be detected in deeper parts of the ooyctes located between yolk platelets and dark pigment particles (Fig. 4*C* and *D*). The subcellular distribution of the S460C cotransporters was similar to that of NaPi-IIa.

Discussion

In this study we combined functional measurements (electrogenic transport activity), surface labeling and im-

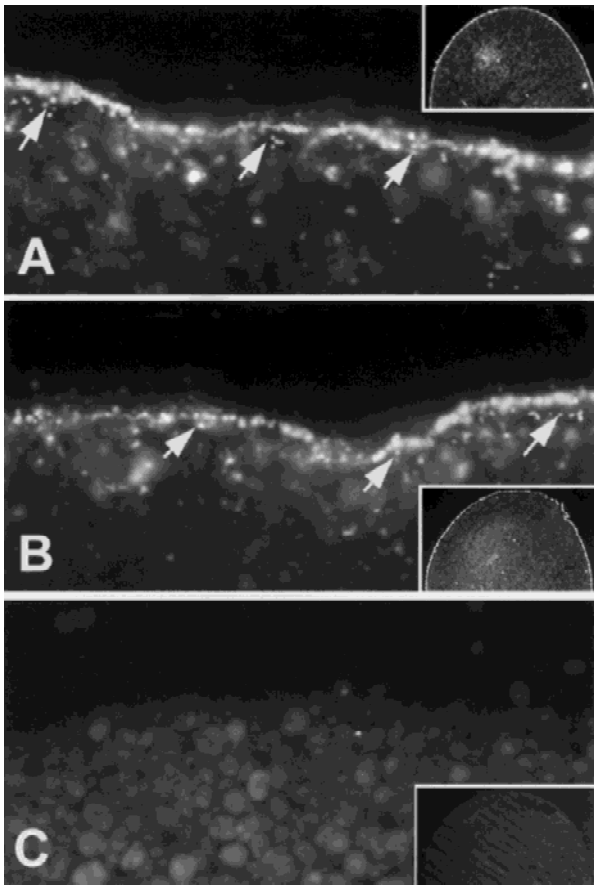

Fig. 3. Immunocytochemical detection of WT NaPi-IIa (*A*) or S460C (*B*) in *Xenopus* oocytes. Specific immunostaining appears in the oolemma and directly below the oocyte surface in fluorescent "granules" (arrows). No specific staining was seen for H_2O injected oocytes (*C*). Insets show overviews of the respective immunostainings. Magnification ≈ 900×.

munocytochemical methods to provide information on the distribution of NaPi-IIa protein when expressed in *Xenopus* oocytes. To detect and label functionally active protein, we used a mutant form of NaPi-IIa (S460C) that shows essentially the same functional characteristics as the WT (e.g., apparent substrate affinity, voltage dependency etc.) (Lambert et al., 1999*b*). Because chemical modification of Cys-460 by MTSEA-Biotin leads to a complete suppression of the cotransport function of this mutant and simultaneously labels it with biotin, we used this construct for assaying functional surface expression by subsequent streptavidin precipitation.[1] The labeling procedure was performed under incubation conditions previously established to give complete suppression of $^{32}P_i$ uptake and the P_i-induced inward current (Lambert et al., 1999*b*).

To allow conclusions to be made concerning surface expression of NaPi-IIa protein, based on protein analysis after streptavidin precipitation and in total cell lysate assays, the following assumptions were made. First, MTSEA-Biotin was assumed to react with Cys-460 only—i.e., for each S460C protein, only Cys-460 was labeled. This is important because the biotin-labeling procedure *per se* cannot distinguish between cysteine residues in functionally important sites and any other native cysteine residue that happens to be accessible, but which, after modification, does not result in a detectable change in function. Our finding that neither externally applied MTSEA nor MTSEA-Biotin have any effect on WT protein function and, moreover, that the WT protein remained undetectable after streptavidin precipitation would support this assumption. Second, despite the reported membrane permeability of MTSEA-Biotin (Seal, Leighton & Amarg, 1998), we assumed that it did not modify cysteine residues accessible from the intracellular face (e.g., Holmgren et al., 1996; Seal et al. 1998; Kaplan et al., 2000). In a previous study on the S460C mutant we established that cysteine modification by MTS reagents from the intracellular side was unlikely since the suppression of transport function was the same when incubated with the impermeant reagent MTSET as for MTSEA (Lambert et al., 1999*b*). We therefore conclude that biotin labeling using MTSEA-Biotin of the NaPi-IIa protein occurred exclusively at site Cys-460 and that only surface labeling from external application of MTSEA-Biotin took place.

In our experiments, the low level of protein in the streptavidin precipitate reflects a concomitantly low amount of surface expression of functional protein. Ideally, to make quantitative statements about the fraction of surface-expressed protein compared with that in the whole cell lysate, a documentation of the efficiency of the streptavidin precipitation protocol would be indicated. We are unable to provide such information as our attempts to determine the total amount of surface biotinylated protein before and after streptavidin precipitation

[1] A biophysical alternative to determine the amount of functional transport protein in the oocyte surface should also be considered. If the moveable charges associated with the transport cycle remain independent of the experimental conditions, presteady-state charge measurements can provide information about the number of functional transporters at the cell surface (Loo et al., 1993; Zampighi et al., 1994; Forster et al., 1999). This approach provides a straightforward assay for surface expression but the interpretation of such data must also be treated with caution. For example, Zampighi et al. (1994) reported a significant difference between the number of transporters estimated from presteady-state kinetics alone and that found by counting SGLT-1-related particles in freeze fracture membrane preparations from the same oocytes. Nevertheless, for assessing relative changes in surface expression after the induction of endocytosis (e.g., Hirsch et al., 1996; Forster et al., 1999), this biophysical approach may prove useful.

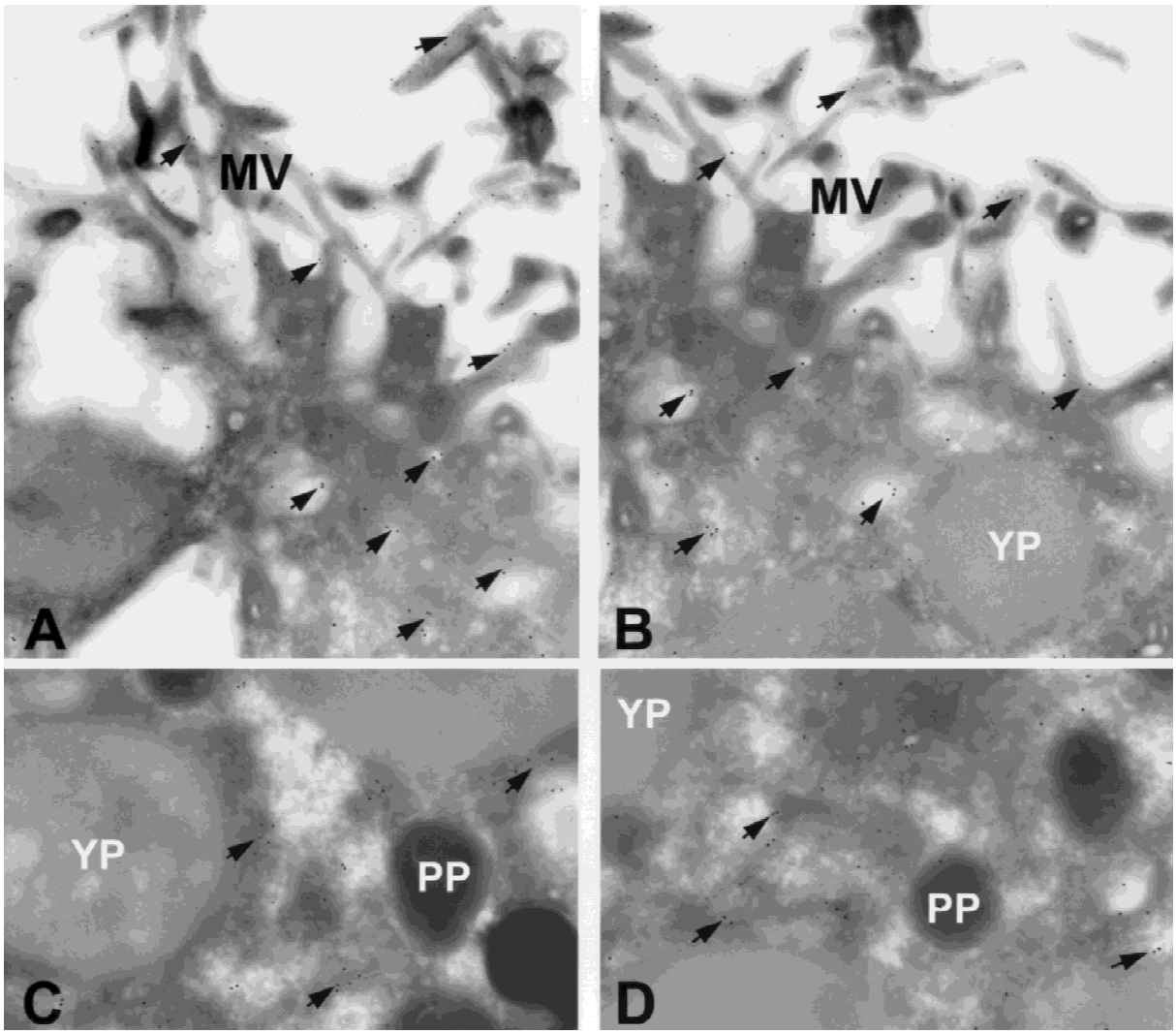

Fig. 4. Labeling of WT NaPi-IIa (*A* and *C*) and S460C (*B* and *D*) by the immunogold technique on ultrathin cryosections of *Xenopus* oocytes. Many NaPi-IIa or S460C bound gold particles (arrows) are detected along the oocyte microvilli (MV) and in the region below each microvillus. In the deeper parts of the submembranous region, specific immunogold-labeling is occasionally found between yolk platelets (YP) and pigmented granules (PP). Magnification ≈ 12000×.

were unsuccessful due to the high background interactions found from the assay made prior to streptavidin precipitation. For example, by using an antibody raised against streptavidin itself, under the assumption that only the surface biotin-labelled S460C would then be detected, we found that both WT expressing and water-injected oocytes, gave comparable signals to that from S460C expressing oocytes. Therefore we would expect any assay that detects biotin in the whole cell lysate would also be contaminated with endogenous biotin (*data not shown*). Apparent low efficiencies of surface biotinylation (and thus low surface expression) have been reported in several other oocyte expression studies, for example for the Na^+/dicarboxylate cotransporter (NaDC-1; Pajor et al., 1998) and for the glucose transporter (GLUT4, Marshall et al., 1993).

As a confirmation of an apparent low surface expression of the S460C protein compared with total cell lysate, immunocytochemistry of S460C protein revealed a significant intracellular pool of protein. This was also true for the WT. Apart from immunofluorescence staining in the oolemma, both proteins were located in a patchy pattern directly below the oocyte membrane and this was confirmed by immunogold-labeling.

Conclusions

This study has revealed an important property of the NaPi-IIa protein when expressed in *Xenopus* oocytes. Our findings may also have consequences for studying other transport proteins in the same expression system.

For studies involving characterization of transport capacities, for example, those involving altered protein routing to and from the membrane, information on both surface expression and function is required. In view of the small fraction of type IIa Na^+/P_i cotransporter protein present at the surface, a change in apparent transport capacity could quite easily be explained by a small redistribution of the protein from the submembranous regions and vice versa. For example, by induction of membrane retrieval of NaPi-IIa through PKC activation (Forster et al., 1999) and membrane insertion and retrieval induced by PKC and PKA activation respectively, for SGLT-1 isoforms (Hirsch, Loo & Wright, 1997). It also makes studies on the isolated oocyte surface membranes rather difficult because quantitation of specific protein content in such preparations can be influenced by contamination from endomembranes.

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