Immunohistochemical Studies of Na^+/D-glucose Cotransporters in the Intestine and Kidney of *Squalus acanthias* and *Leucoraja erinacea*

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In preceding studies[3] different characteristics for the Na^+/D-glucose cotransport in the kidneys of the little skate (*Leucoraja erinacea*) and the spiny dogfish (*Squalus acanthias*) were detected. The transporter in the skate showed a high affinity for D-glucose (K_m=0.12 mM) and an apparent coupling ratio of 2 Na^+ to 1 D-glucose, whereas the affinity of the shark transporter was much lower (K_m=1.90 mM) and the coupling ratio was determined as 1:1. It was therefore suggested that Na^+-dependent uptake of glucose in the skate is carried out by SGLT1, whereas SGLT2 is in charge of the Na^+/D-glucose cotransport in the dogfish, because these transporters display the aforementioned transport characteristics in mammals. We have earlier cloned the corresponding genes from the shark and skate kidney. Comparison of the DNA sequences revealed that the shark transporter has a high homology to SGLT2, while the skate sequence is strongly homologous to SGLT1. Furthermore we detected expression of SGLT1 in the skate intestine by RT-PCR, but no expression of any SGLT in the shark intestine.[1] To investigate these species-specific characteristics of Na^+-dependent glucose uptake in more detail, we generated polyclonal antibodies in rabbits against different extracellular loops of the cloned transporters (see Althoff *et* al., this bulletin).

Immunohistochemistry was performed on frozen sections of shark and skate tissues from animals of either sex. The tissues were fixed with 4% paraformaldehyde (in PBS, pH6.8) for 3-4 hours at room temperature. Cryosections (7 µm thick) on slides were blocked for one hour in 3% Carnation© (in PBS) and subsequently incubated for another hour with specific primary antibodies (diluted 1:100 in PBS + 1% Triton-X-100). The primary antibodies were detected with fluorescent-labelled secondary antibodies (Alexa-488 anti rabbit IgG, Molecular Probes), diluted as described above. The DNA stain 4',6-Diamidino-2-phenylindole (DAPI) was used to counterstain the nuclei. To label the endogenous alkaline phosphatase, a marker for the brush border membrane (BBM), the Vectastain ABC-AP kit (Vector Laboratories) was applied according to the manufacturer's manual.

We used 6 different antibodies to immunostain frozen sections of the skate and shark, respectively. One antibody for each species, showing the least background, was selected and used in the following studies. The selected antibodies were those against loop 8 of the shark protein (rabbit anti SH L8) and loop 13 of the skate SGLT (rabbit anti SK L 13c).

The SGLT2 protein could not be detected in frozen sections of the spiral valve from the spiny dogfish (*Squalus acanthias*; figure 1). Figure 1 only shows an unspecific background signal that did not differ from controls using only the secondary antibody (data not shown). Whereas staining of the alkaline phosphatase resulted in a bright signal at the apical membrane of the villi (figure 1b), immunostainings on the same sections with these antibodies against shark SGLT2 showed no signal at all. This is in accordance with earlier findings, where SGLT2 could be cloned from shark kidney, but not from the shark intestine.

Consequently two different isoforms seem to be involved in glucose uptake in the shark kidney and intestine, respectively. In fact, it is known that Na^+-dependent transport of glucose in mammals is achieved by SGLT1 in the intestine and by SGLT1 and SGLT2 in the kidney[6].

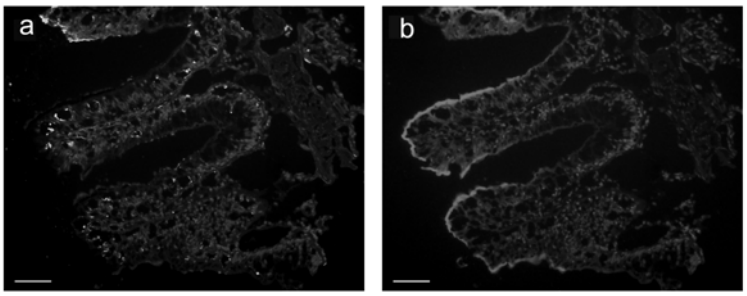

Figure 1: Immunostaining of the shark intestine. A Specific antibody against shark SGLT2 (SH L8 5288) was used to detect the protein in frozen sections (7µm) of the shark intestine, but we only detected unspecific background (a). To stain the apical membrane of the intestinal villi, the Vectastain ABC-AP kit (Vector Laboratories) was applied to label the endogenous alkaline phosphatase, a marker for the brush border membrane (b). The nuclei were counterstained using DAPI (bars = 100 µm)

In sections of the shark kidney, we obtained a signal in particular renal segments after staining with specific antibodies against SGLT2 (figure 2a). When compared to staining of the BBM on the same sections (figure 2b), it is obvious that the transporter is located in tubules, which are negative for alkaline phosphatase, meaning they do not have a BBM. In the mesial part of the kidney (figures 2a and b) the alkaline phosphatase was detected in the segments PIIa and PIIb of the proximal tubule, whereas SGLT2 is localized to the late distal tubules (LDT). However, in the bundle sheath (figure 2c), we detected SGLT2 in the early parts of the proximal tubule (PIa), as well as in the collecting tubule. Interestingly, alkaline phosphatase activity was detected in the basal part of the tubules in the bundle sheath. This has been described earlier for the kidney of the Prussian carp.[2] In mammals, early parts of the proximal tubule (PIa and PIb) have been shown to be the major site of glucose reabsorption from the primary urine.[5]

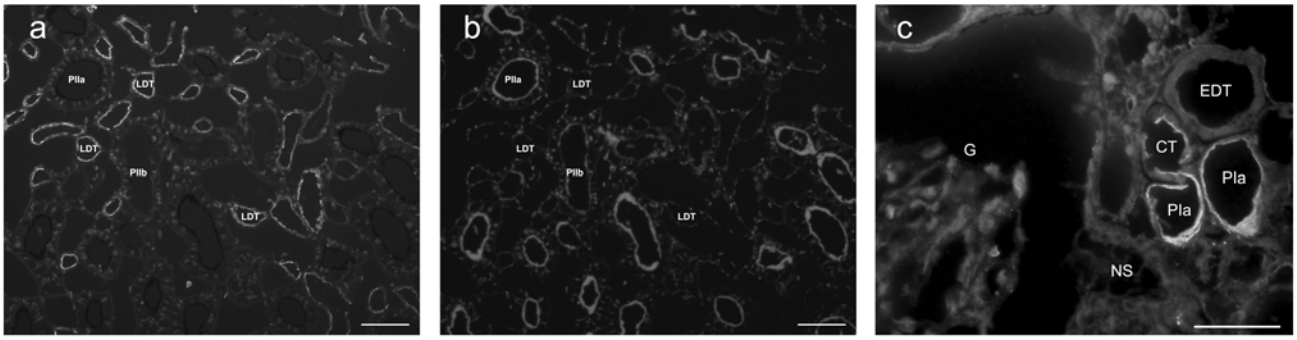

Figure 2: Distribution of SGLT2 in the shark kidney. SGLT2 in the shark kidney was detected by immunostaining (a) of frozen sections (7µm). Alkaline phosphatase was labelled with the ABC-AP stainining solution to display the bush border membrane (b). Panel c shows the distribution of SGLT2 within the bundle sheath. Specific segments of the kidney are labelled (bars a+b = 100µm; c = 50 mm). CT = collecting tubule; EDT = early distal tubule; G = glomerulus; LDT = late distal tubule; NS = neck segment; PIa, PIIa, PIIb = different elements of the proximal tubule

Our immunochemical investigations on frozen sections of the skate (*Leucoraja erinacea*) spiral valve revealed a localization of SGLT1 in the apical membrane of the villi (figure 3a) that was stained by labelling the alkaline phosphatase (figure 3b). A closer look at the sections at a higher magnification (630x), lead to the detection of SGLT1 in intracellular vesicles (figure 3c).

It has been recently described, that an intracellular pool of SGLT1 located within endosomes exists in a human intestinal cell line.[4] It was therefore suggested, that the activity of SGLT1, like that of other membrane transporters (e.g. CFTR) is regulated by intracellular trafficking. Our findings suggest, that Na^+-dependent D-glucose uptake in the shark intestine might be regulated by recycling of the responsible transporter, as well.

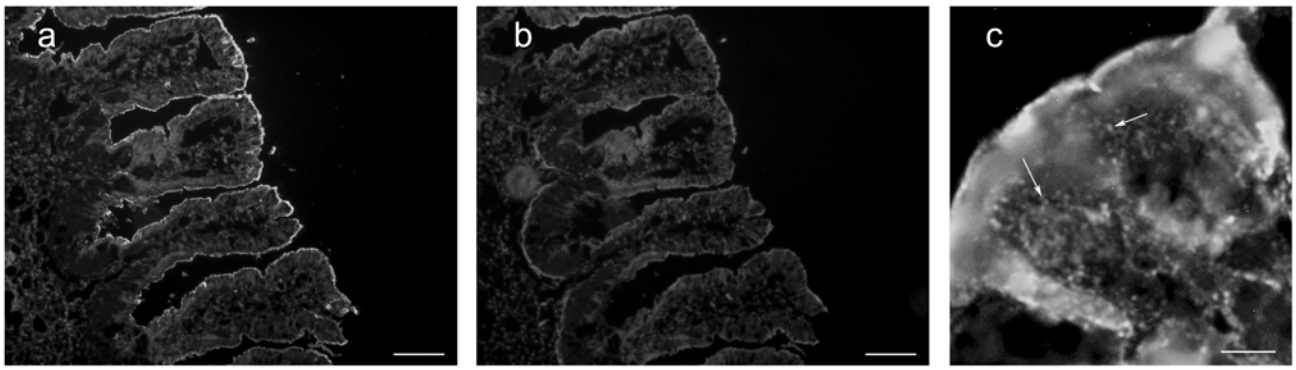

Figure 3: Detection of SGLT1 in the skate intestine. SGLT1 in the skate intestine was identified using the specific antibody against loop 13 (SK L13c 5296) and a fluorescent secondary antibody (Alexa-488 anti-rabbit) (a), (c). ABC-AP stainining solution was used to display the alkaline phosphatase in the apical membrane (b). SGLT1 is localized in the apical membrane (a), as well as in intracellular vesicles (c, highlighted by arrows; bars a+b = 100 µm; c = 10 µm).

The immunohistochemical analysis of SGLT1 in frozen sections of the skate kidney revealed a distribution within the renal tubules, comparable to that of SGLT2 in the kidney of the dogfish. In the mesial zone SGLT1 was identified in the late distal tubule (figure 4a) and to some part in late segments of the proximal tubule (PIIb, not shown). The alkaline phosphatase (figure 4b) activity was again detected exclusively in the apical membrane of the PIIa tubules. The distribution in the bundle sheath (figure 4c) is the same as in the shark. SGLT1, here was localized in the apical membrane of PIa and the collecting tubule. Therefore, although driven by a different isoform of the transporter, Na^+-dependent glucose transport seems to take place in the same parts of the skate and shark nephron, respectively.

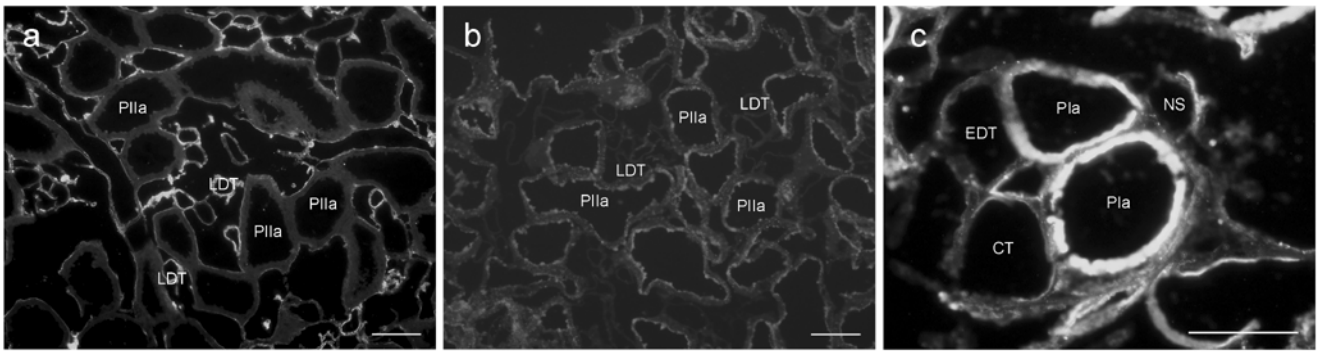

Figure 4: Immunostaining of SGLT1 in the skate kidney. Staining of SGLT1 (a,c) and alkaline phosphatase (b) in frozen sections of the skate kidney. SGLT1 in the mesial zone (a) and in the bundle sheath (c) was labelled using the rabbit anti L13c antibody and Alexa-488 anti rabbit secondary antibody. The bush border membrane is displayed by staining the marker enzyme alkaline phosphatase (b). (bars a + b= 100µm; c= 50 µm)

The presented studies give further support to our earlier findings that Na^+-/D-glucose cotransport in the skate and shark is mediated by two different isoforms of the SGLT family. Using specific antibodies we detected the SGLT1 protein in the skate kidney and intestine, and the SGLT2 protein in the shark kidney. However, these experiments still leave the question unanswered which transporter is in charge of glucose transport in the shark intestine, as we were not able to detect SGLT1 or SGLT2, respectively, in this tissue.

In addition, we obtained more information about the localization of the transporters within the tissues. In the skate intestine, SGLT1 was shown to be localized in the apical membrane, as well as in intracellular vesicles. The regulation of SGLT1 by intracellular recycling, in the skate, seems to be specific for the intestine, as the protein is restricted to the apical membrane in renal tubules. SGLT1 and SGLT2, respectively, reside in the same parts of the nephron, namely the early parts of the proximal tubule, PIa, the collecting tubule and the late distal tubule. Considering the close relation of the two species, this equal distribution is not very surprising. The physiological role of glucose transport in the proximal tubule has been described before (e.g. for the mammalian kidney), whereas the existence of SGLT proteins in the late distal tubule leads to speculation. Further insight into the physiology of Na^+-dependent glucose transport in the kidney and intestine of the shark/skate can be obtained by using the presented antibodies in additional studies on tissue cultures, Western blots or sequential sections.

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