0014-4754/94/070664-05$1.50 + 0.20

Identification of organ-specific glycosylation of a membrane protein in two tissues using lectins

M. Benallal and B. M. Anner*

Laboratory of Experimental Cell Therapeutics, Geneva University Medical School, CH-1211 Geneva 4 (Switzerland)
Received 22 December 1993; accepted 18 February 1994

Abstract. Since glycosylation of proteins is performed by the host cell, and variable sugar groupings can confer heterogeneity on the same polypeptide, we wished to see whether membrane proteins, in particular the ubiquitous transmembrane Na,K-ATPase, could be glycosylated differently in different organs. Using a highly sensitive enzyme-linked antibody detection system of bound digoxigenin-labelled lectins on nitrocellulose sheets containing electroblotted α and β subunits of kidney and brain Na,K-ATPase, isolated from various rat strains, in combination with isoform-specific immunoblots, we discovered that brain Na,K-ATPase was highly mannosylated in contrast to renal Na,K-ATPase. Thus, we describe the existence of organ-related glycoforms of an integral ubiquitous membrane protein, i.e. diversification of the same polypeptide by organ-typical sugars. At the same time, the presence of the same glycosylation pattern can make distinct protein isoforms occurring in a same organ more homogeneous. Such organ-related glycoforms may serve for tissue identification and as tissue-specific receptors.
Key words. Lectin recognition; renal and brain Na,K-ATPase; distinct glycosylation; complex sugars in kidney; mannosylation in brain.

Cell surface carbohydrates form points of attachment for cells, infectious agents, toxins, hormones, lectins and other molecules[1,2]. Glycomoieties of membrane proteins mediate cell-identification, cell-recognition, cell-cell interaction and adhesion[3]. On the other hand, lectins are ubiquitous proteins which recognize and bind to specific sugar groupings of glycoproteins[4,5] just as antibodies recognize proteinaceous epitopes. Hence, lectins have revealed heterogeneous glycosylation of soluble proteins such as renin[6] and acetylcholinesterase[7]. No reports on organ-specific glycosylation of membrane proteins have appeared.

The Na,K-ATPase (EC 3.6.1.37) or sodium pump is a vital ubiquitous integral membrane transport system composed of a 110 kD α subunit, a 50–60 kD β glycoprotein, phospholipids and cholesterol[8]. It is responsible for the establishment and maintenance of the transmembrane Na/K gradient which participates in the regulation of the membrane potential and has an important signalling function. The high intracellular K/Na ratio built up by the sodium pump is required for the modulation of gene expression[9] for the synthesis of macromolecules and for cell development, growth and differentiation[10]. Furthermore, the system serves as a receptor for cardioactive steroids of exogenous and endogenous origin[11–13]. The Na,K-ATPase exists in various isoforms whose functional differences are unknown, encoded by distinct genes[14]. The kidney contains essentially α-1 and β-1 forms whereas the brain contains α-1, α-2, α-3, β-1 and β-2 forms[15]. The β subunit is a known glycoprotein; the glycosylation pattern of the renal β-1 form is currently being examined by chemical analysis[16]. The neuronal β-2 isoform has been found to be homologous to the adhesion molecule on glia[17]. Glycosylation of the α-1 form has been reported recently and shown to be essentially on the cytoplasmic side of the membrane[18,19].

To look for hypothetical organ-typical sugar groupings, Na,K-ATPase was isolated from brain and kidney, and the sugar groupings on the enzyme samples from the two organs differentiated by specific lectins covalently bound to digoxigenin and detectable by antidigoxigenin antibody conjugated to alkaline phosphatase[20]. The use of highly selective lectins, combined with Western-blotting, revealed organ-specific glycoforms of Na,K-ATPase. The use of lectin-blots for Na,K-ATPase analysis has been communicated in preliminary form[21,22].

Materials and methods

Source of animals and Na,K-ATPase. Genetically hypertensive rats from Milan (H) and their normotensive controls (N) were received from Prof. P. Bianchi; genetically hypertensive SHR rats (S) and their normotensive controls Wistar Kyoto (W) controls were from Iffa-Credo, Lyon, France, and common outbred Wistar (O) rats were from the local animal house. Na,K-ATPase was isolated from brain and kidney by the dodecyl sulphate (SDS) extraction microprocedure[23].

Quantitative gel electrophoresis and α subunit normalization. Alpha and β subunits of the isolated Na,K-ATPase were separated in the presence of SDS on minigels (6 cm x 5.3 cm, 0.45 mm thick), 8–25% gradient (Pharmacia automated Phastsystem), and stained by Coomassie Blue R350. The use of high density minigels,

convenient for miniaturised electroblotting in an automated electrophoresis and blotting system, caused an unusually close migration of α and β subunits as well as the upwards bending of the α subunit. The α subunit was quantified by laser densitometry and a second gel was run with α-normalized amounts; 7 to 10 µg of protein was added per lane.

Quantitative electroblotting. The proteins were then quantitatively transferred to BA 850 nitrocellulose sheets by the Western technique of Towbin et al.[24] in the electroblotting unit of a Pharmacia Phastsystem. The process was checked by measuring the protein remaining on the original gel as well as the proteins arriving on nitrocellulose by Ponceau S (Boehringer, Mannheim) staining and Immunopure Lane Marker (Pierce, Oud-Beijerland); the protein transfer efficiency was at least 80%.

Immunoblotting. The nitrocellulose sheet was then sequentially incubated with rabbit anti-rat antisera (UBI) to the α-1 (No.11522), α-2 (gift from Dr. A. Klip), α-3 (No. 11554), β-1 (No. 11143) and β-2 (No. 11171) isoform subunits of rat Na,K-ATPase (1:500) for 16 h at 4 °C and with alkaline phosphatase conjugated anti-rabbit immunoglobulin G (1:3000) for 2 h at 4 °C. The proteins on the sheet were stained with 4-nitroblue tetrazolium as substrate. The isoforms detected on immunoblots (not shown) are indicated in figures 1 and 3.

Lectin blotting. Specific lectin-blotting was performed according to Haselbeck et al.[20]. Briefly, the nitrocellulose sheets were submerged in a solution of Tris-HCl 0.05M, NaCl 0.15 M, pH 7.5 (solution A) with 0.5% casein for at least 30 min to block the remaining sites. The blots were washed for 10 min twice in solution A and once with solution A containing (in mM) 1 $MgCl_2$, 1 $MnCl_2$, 1 $CaCl_2$, pH 7.4, and then incubated with the digoxigenin labelled lectins GNA, SNA, MAA, PNA or DSA (Boehringer) for 60 min at 22 °C. After three washing cycles with solution A, the blots were incubated with polyclonal sheep antidigoxigenin Fab fragments conjugated with alkaline phosphatase, for 60 min at 22 °C. After a further washing process, the conjugated alkaline phosphatase was detected by the reduction of the 4-nitroblue tetrazolium salt in the presence of 5-bromo-4-chloro-3-indoyl-phosphate to a blue precipitate. Non-specific binding of alkaline phosphatase conjugated antidigoxigenin antibody with the various Na,K-ATPase preparations tested was ruled out by incubating the Na,K-ATPase preparations blotted on nitrocellulose directly with the antidigoxigenin antibody, without prior exposition to lectins. No reaction could be obtained under such conditions. This test seemed necessary since Na,K-ATPase is the pharmacological receptor for cardioactive steroids (reviewed in ref. 11).

Glycosidase treatment. Treatment of Na,K-ATPase preparations with endoglycosidase H (EC.3.2.1.96) from *Streptomyces griseus* (Seigakagu Kogyo Co., Ltd, Tokyo) was carried out directly on nitrocellulose sheets[25]. The proteins were blotted onto the sheets, which were immersed for 120 min at 37 °C in citric acid-Na_2HPO_4 buffer, pH 5.0, with endoglycosidase H, 50 mU/ml. Treatment with N-glycosidase F (PNGase, EC.3.2.2.18), recombinant, from *Flavobacterium meningosepticum* (Boehringer, Mannheim) was done according to Pedemonte et al.[18]: 40 µg Na,K-ATPase protein from an MNS rat was dissolved in 7.5 µl 1% dodecyl sulphate then diluted with 12.5 µl 200 mM EDTA and 4% (vol./vol.) 2-mercaptoethanol, pH 7.5, (Tris) and 22.5 µl (0–10 units) of N-glycosidase F at 22 °C for 32–36 h. As a control, 40 µg of carboxypeptidase Y was treated in parallel; this enzyme also lost its GNA-sensitivity, confirming that N-glycan chains had been cleaved.

Results and discussion

Figure 1A shows the protein profiles of Na,K-ATPases isolated from the brains of Milan hypertensive (H) and Milan normotensive (N) rats, separated by gel electrophoresis and stained with Coomassie Blue. The isoforms were identified by immunoblotting (not shown). Figure 1B shows the replicas of α and β subunits, revealed by alkaline phosphatase reaction after *Galanthus nivalis* agglutinin (GNA) lectin-blotting of the

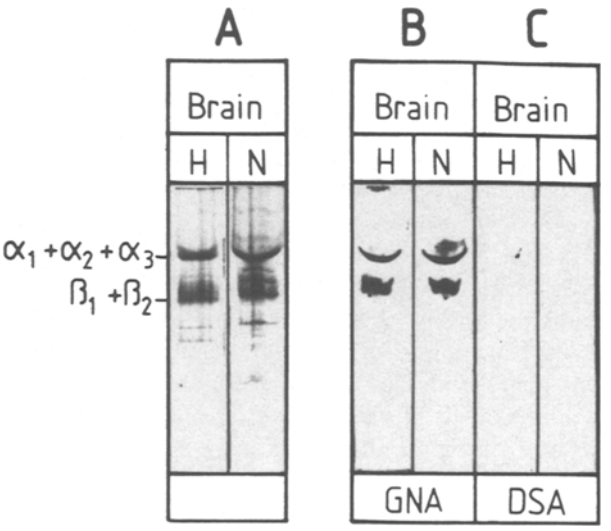

Figure 1. GNA binds to brain Na,K-ATPase only.

A Coomassie Blue stained Na,K-ATPase separated by gel electrophoresis. The isoforms identified by immunoblotting are indicated. Alpha and β subunits of Na,K-ATPase from brains of Milan hypertensive (H) and normotensive (N) rats are seen.

B GNA-stained Na,K-ATPase by lectin-blotting.

C DSA does not recognize any brain Na,K-ATPase isoform. Enzyme isolation, gel electrophoresis, electrotransfer, immunoblotting and lectin-blotting were performed as described in 'Materials and methods'. Typical experiments are shown which were repeated at least 3 times using Na,K-ATPase preparations which had been stored at −70 °C or were freshly prepared.

proteins after they had been transferred quantitatively to nitrocellulose sheets. Clearly, the contour of the lectin-revealed protein can be superimposed on the Coomassie Blue stained surface, indicating that the same protein had interacted with the lectin. If the positive GNA-reaction was due to minor contaminants migrating by coincidence below the Na,K-ATPase protein, such a strong superimposable positive reaction would not be expected since calibration tests of GNA with carboxypeptidase Y showed only a faint reaction below 0.1 μg protein (not shown).

That the strongly positive reaction of the mannose-specific GNA was due to specific sugar and not to some non-specific alkaline phosphatase reaction was further documented by the totally negative reaction of the galactose-specific lectin *Datura stramonium* agglutinin (DSA) (fig. 1C) applied under the conditions used for GNA and previously tested for a positive reaction with asialofetuin and fetuin (not shown). The specificity of the mannose-specific GNA reaction became even more apparent when *Maackia amurensis* agglutinin (MAA), *Sambucus nigra* agglutinin (SNA) and *Arachis hypogeae* (peanut) agglutinin (PNA) were tested and all reacted negatively with Na,K-ATPase under conditions shown to give positive reactions with control glycoproteins (not shown). The hypertensive (H) and normotensive (N) rat strains reacted identically.

To check the specificity of the surprising and striking response of both α and β subunits to GNA, the lectin was pretreated with α-methyl-mannopyrannoside, a sugar known to compete with the glycoprotein's mannose residues. Figure 2A shows that the GNA-Na,K-ATPase interaction was abolished by the added sugar.

To further ascertain the high-mannose glycosylation status of brain Na,K-ATPase α- and β-subunits, and to find out whether N or O linkages were involved, the sugars were removed by selective glycosidases prior to lectin binding. Endoglycosidase H cleaves the diacetylchitobiose linkage of N-linked carbohydrates with narrow specificity in that it hydrolyses only high-mannose type oligosaccharides[25]. This glycosidase abolished the GNA sensitivity of the enzyme from normo- and hypertensive rats (fig. 2B) thus confirming the high-mannose status indicated by GNA.

On the other hand, N-glycosidase F from *Flavobacterium meningosepticum* has a broad specificity and cleaves virtually all N-linked glycans, provided they are accessible to the enzyme[26]. The GNA sensitive component was removed totally by N-glycosidase F, indicating the predominance of N-linkages. Na,K-ATPase, incubated under the same conditions but without glycosidase, retained its reactivity towards GNA, indicating that the observed loss of label was indeed due to sugar cleavage and not to non-specific degradation (fig. 2C).

Brain Na,K-ATPase was also screened for terminal sialic acid with SNA and MAA lectins; neither the α- nor the β subunit gave a positive reaction (fig. 5) under conditions where the control glycoproteins transferrin and fetuin were positive (not shown).

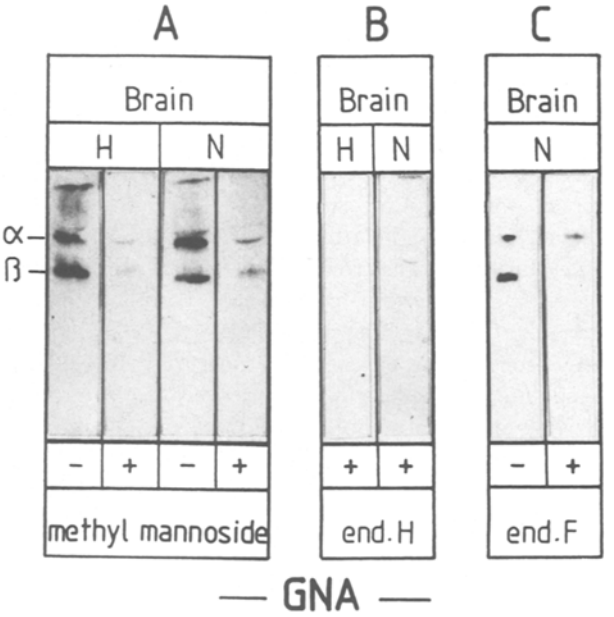

Figure 2. Control experiments for brain Na,K-ATPase.
A Prevention of interaction of GNA with Na,K-ATPase of hypertensive (H) or normotensive (N) Milan rats by pretreatment of the lectin with 0.5 α-methyl-D-mannopyrannoside.
B Endoglycosidase H removes all GNA-sensitive sugars.
C N-Glycosidase F removes all GNA-sensitive sugars.

PNA recognizes the core disaccharide galactose-β(1-3)-N-acetyl-galactosamine of O-glycans, provided putative masking terminal sialic acids are removed; after removal of sialic acid by mild acid treatment of blots no reaction occurred, i.e., O-glycans seemed to be absent. This agreed with the potent action of N-glycosidase F, which hydrolyzes only N-glycans (fig. 2C). Taken together, the control experiments shown in figure 2 substantiate the view that brain Na,K-ATPase is highly mannosylated. Interestingly, this corresponds to a status of precursor glycoproteins prior to demannosylation, and hence prior to further processing to complex and hybrid glycoproteins[27].

Figure 3A shows Coomassie blue stained α and β subunits of renal outer medulla Na,K-ATPase isolated from four inbred (Milan hypertensive, H; Milan normotensive, N; Spontaneous hypertensive, S; Wistar Kyoto controls, W) and a outbred rat strain (Wistar outbred, O). Interestingly, a band migrating above the α subunit is only faintly seen in outbred animals but becomes more pronounced with inbreeding, as repeatedly observed in our laboratory; since this protein with slower migration also reacted positively with anti-α 1 antibody (not shown), it probably corresponds to the anomalously migrating Na,K-ATPase protein also reported by other laboratories[28]. After transfer to nitrocellulose and blotting with digoxigenin-labelled *Datura*

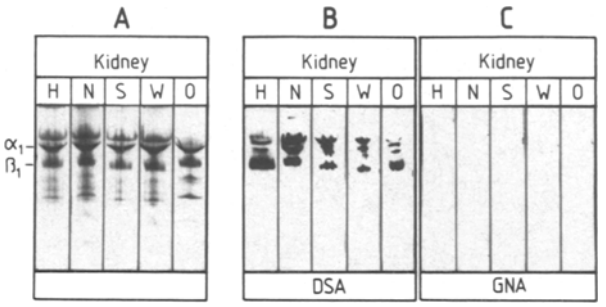

Figure 3. DSA lectin binds to kidney Na,K-ATPase only.
A Coomassie Blue stained Na,K-ATPase isolated from renal outer medulla. The isoforms identified by immunoblotting are indicated. Genetically hypertensive rats from Milan (H); normotensive controls (N), genetically hypertensive SHR rats (S) and their normotensive controls Wistar Kyoto (W) controls; common outbred Wistar rats (O). Na,K-ATPase isolation, immunoblotting and lectin-blotting were performed as described in legend to figure 1.
B Positive reaction with DSA which recognizes specifically galactose-β(1-4)-N-acetylglucosamine of complex and hybrid glycans or individual N-acetyl-glucosamine residues.
C Negative reaction to GNA.
Typical experiments are shown which were repeated at least 3 times using Na,K-ATPase preparations which had been stored at -70 °C or were freshly prepared.

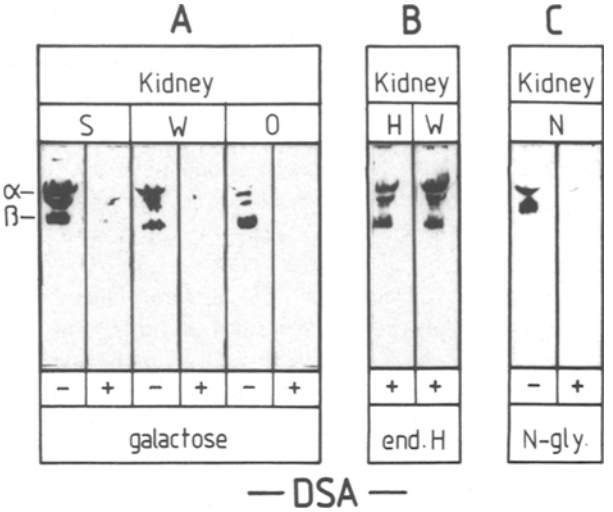

Figure 4. Control experiments for kidney Na,K-ATPase.
A D-galactose (0.5 M) prevents DSA-Na,K-ATPase interaction.
B Endoglycosidase H does not remove DSA-sensitive sugars. Endoglycosidase H treatment was performed on nitrocellulose as described in 'Materials and methods'.
C N-glycosidase F removes the DSA sensitive sugars indicating the presence of N-glycans; the treatment was performed as described in 'Materials and methods'.

stramonium agglutinin (DSA), both the α and β subunits of all rat strains reacted positively, indicating the presence of galactose-β(1-4)-N-acetylglucosamine residues of complex N-glycans[20]. No difference was seen between Na,K-ATPase from genetically hypertensive and normal animals. In contrast to brain Na,K-ATPase, no reaction took place with the mannose-sensitive GNA-lectin (fig. 3B), which ruled out the presence of hybrid-glycans.

Galactose prevented the DSA-Na,K-ATPase interaction (fig. 4A), thus proving its sugar specificity. The mannose-cleaving endoglycosidase H did not alter the lectin-sensitivity of the renal enzyme (fig. 4B), which is an additional argument for the predominance of galactose-β(1-4)-N-acetyl-glucosamine glycosylation. Like the brain enzyme, the kidney Na,K-ATPase was sensitive to N-glycosidase F treatment (fig. 4C), an indication for N-glycans.

Several consensus sequences for putative N-glycosylation sites are found in the derived amino acid sequences of both renal and brain α subunit[29]. Along the same lines, and in harmony with brain Na,K-ATPase, no reaction occurred with PNA (data not shown), ruling out the presence of O-glycans, in agreement with recent results of Pedemonte et al.[19]. The α subunits were resistant to MAA and SNA, lectins which recognize sialic acid (fig. 5). Na,K-ATPase isolated from renal cortex showed the same lectin-sensitivity as the renal outer medulla enzyme (not shown).

However, in contrast to that of brain, the β-1 subunit of almost all kidney samples reacted positively with MAA as well as with SNA, indicating the presence of terminal

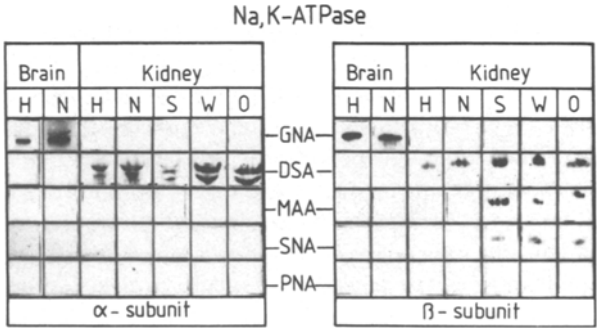

Figure 5. Comparative lectin-sensitivity of brain and kidney α and β subunits. The rats strains are defined in legends to figure 3. PNA, which recognizes the core disaccharide galactose-β(1-3)-N-acetyl-galactosamine of O-glycans, reacts negatively with both subunits, even after removal of putative masking terminal sialic acid by mild acid treatment on blots (0.05 N H_2SO_4, -70 °C for 60 min) according to Kijimoto-Ochiai et al. (30). SNA and MAA bind to kidney β subunits but do not react with brain β subunits, thus excluding terminal sialic on brain Na,K-ATPase. No lectin besides the mannose-recognising GNA interacts with brain Na,K-ATPase, thus confirming the presence of terminal mannose only. SNA and MAA react positively with β subunits from S, W, and O strains but not with H and N strains.

sialic acid linked to galactose by α(2-6) and α(2-3) bonds respectively. The Na,K-ATPase beta subunits of Milan rats, which reacted negatively with MAA and SNA (fig. 5), were an exception.

Taken together, the results show that the kidney Na,K-ATPase contains complex N-glycans and that its β subunit also contains glycans terminated by sialic acids. In contrast, the brain enzyme contains only unsialylated precursor-type mannose glycoforms of Na,K-ATPase.

The mutually exclusive reaction of GNA and DSA (fig. 5), together with the complementary control experiments shown in figures 2 and 4 and the testing of the lectins by control glycoproteins (not shown), indicates that only mannose-glycans occur in brain Na,K-ATPase, and only galactose-beta(1-4)-N-acetylglucosamine-glycans or individual N-acetylglucosamines in kidney Na,K-ATPase, despite the presence of α-1 and β-1 isoforms in both preparations.

Organ-related glycoforms of integral membrane proteins have not yet been described, to our knowledge. The rat brain Na,K-ATPase, which is mannosylated, seems to have a more primitive glycosylation status than the kidney enzyme, and to correspond to a precursor stage of glycoprotein synthesis[27]. The rat kidney Na,K-ATPase, in contrast, carries elaborated complex glycans with terminal sialic acid on the β subunit. Such distinct glycomoieties might participate in modulating the life-time of proteins; terminal sialic acid, for instance, appears to prevent protein clearance in the organism[2]. The brain, which is located in an area highly protected by the blood-brain barrier, might not require excessive glycan protection of its vital Na,K-ATPase, in contrast to kidney, which is a clearance organ par excellence.

Furthermore, organ-typical glycoforms might have specific tissue identification and receptor functions, since heterogeneous protein isoforms can be homogeneous with regard to their glycosylation status. On the other hand, the same polypeptide can be glycosylated distinctly in different organs. Thus, organ-typical glycosylation could confer homogeneity on heterogeneous protein isoforms co-expressed in the same organ, or it could confer heterogeneity on the same protein isoform expressed in several organs. Information gained from combined immuno- and lectin-blotting of proteins from other species, organs and tissues, normal or diseased, may tell us whether our theory can be extended and refined, and whether glycosylation patterns of Na,K-ATPase could be used to develop new diagnostic tools.

Acknowledgments. We thank Prof. G. Bianchi for the gift of Milan hypertensive and normotensive rat strains, Mrs M. Moosmayer for help with Na,K-ATPase isolation, Mrs D. M. Lacotte and Mr F. Pillonel for skilful artwork and Dr. R. Anner for reading the manuscript. Supported by Swiss National Science Foundation grants No. 31-25666.88, 31-37552.93 and by the Ernest and Lucie Schmidheiny Foundation

* To whom correspondence should be addressed

1 Monsigny, M., Kieda, C., and Roche, A.-C., Biol. Cell *47* (1983) 95.
2 Paulson, J. C., TIBS *14* (1989) 272.
3 Feizi, T., and Childs, R. A., Biochem. J. *245* (1987) 1
4 Lis, H., and Sharon, N., A. Rev. Biochem. *55* (1986) 35.
5 Sharon, N., and Lis, H., Science *246* (1989) 227.
6 Hosoi, M., Kim, S., and Yamamoto, K. K., Clin. Sci. *81* (1991) 393.
7 Liao, J., Heider, H., Sun, M., and Brodbeck, U., J. Neurochem. *58* (1992) 1230.
8 Jørgensen, P. L., Meth. Enzym. *31* (1974) 277.
9 Nakagawa, Y., Rivera, V., and Larner, A. C., J. biol. Chem. *267* (1992) 8785.
10 Takagi, K., Cell Struct. Funct. *11* (1986) 235.
11 Anner, B. M., Biochem. J. *227* (1985) 1.
12 Anner, B. M., Rey H.G., Moosmayer, M., Meszoely, I., and Haupert, G. T., Am. J.Physiol. *258* (1990) 144.
13 Goto, A., Yamada, K., Yagi, N., Yoshioka, M., and Sugimoto, T., Pharmac. Rev. *44* (1992) 377.
14 Lingrel, J. B., Orlowski, J., Shull, M. M., and Price, E. M., Progr. Nucleic Acid Res. *38* (1990) 37.
15 Sweadner, K. J., Biochim. biophys. Acta *988* (1989) 185.
16 Treuheit, M. J., Costello, C. E., and Kirley, T. L., J. biol. Chem. *268* (1993) 13914.
17 Gloor, S., Antonicek, H., Sweadner, K. J., Pagliusi, S., Frank, R., Moos, M., and Schachner, M., J. Cell Biol. *110* (1990) 165.
18 Pedemonte, C. H., Sachs, G., and Kaplan, J. H., Proc. natl, Acad. Sci. USA *87* (1990) 9783.
19 Pedemonte, C. H., and Kaplan, J. H., Biochemistry *31* (1992) 10465.
20 Haselbeck, A., Schikaneder, E., Von der Eltz, H., and Hösel, W., Anal. Biochem. *191* (1990) 25.
21 Benallal, M., and Anner, B. M., Experientia *49* (1993) A50.
22 Benallal, M., and Anner, B. M., Biol. Chem. Hoppe-Seyler *374* (1993) 620.
23 Dzahdzhugazyan, K. N., and Jørgensen, P. L., Biochim. biophys. Acta *817* (1985) 165.
24 Towbin, H., Staehelin, T., and Gordon, J., Proc. natl Acad. Sci. USA *76* (1979) 4350.
25 Trimble, R. B., and Maley, F., Anal. Biochem. *141* (1984) 515.
26 Tarentino, A. L., Gomez, C. M., and Plummer, T.H.Jr., Biochemistry *24* (1985) 4665.
27 Roth, J., Biochim. biophys. Acta *906* (1987) 405.
28 Cortas, N., Elstein, D., Markowitz, D., and Edelman, I. S., Biochim. biophys. Acta *1070* (1991) 223.
29 Shull, G. E., Greeb, J., and Lingrel, J. B., Biochemistry *25* (1986) 8125.
30 Kijimoto-Ochiai, S., Kagiri, Y. V., and Ochiai, H., Anal. Biochem. *147* (1985) 222.