Amino acid sequence and the cellular location of the Na^+-dependent D-glucose symporters (SGLT1) in the ovine enterocyte and the parotid acinar cell

Patrick S. TARPEY, I. Stuart WOOD, Soraya. P. SHIRAZI-BEECHEY* and R. Brian BEECHEY

Epithelial Function and Development Group, Institute of Biological Sciences, The University of Wales, Aberystwyth, Dyfed SY23 3DD, U.K.

The Na^+-dependent D-glucose symporter has been shown to be located on the basolateral domain of the plasma membrane of ovine parotid acinar cells. This is in contrast to the apical location of this transporter in the ovine enterocyte. The amino acid sequences of these two proteins have been determined. They are identical. The results indicated that the signals responsible for the differential targeting of these two proteins to the apical and the basal domains of the plasma membrane are not contained within the primary amino acid sequence.

INTRODUCTION

We have previously described the preparation of a fraction of osmotically active membrane vesicles from ovine parotid glands [1,2]. The ouabain-sensitive K^+-activated phosphatase activity was concentrated 30–40-fold in this fraction and there were low levels of enzymes associated normally with other intra-cellular membranes. It was proposed that the vesicles were derived from the basolateral region of the ovine parotid acinar cells. These vesicles could utilize a transmembrane gradient of Na^+ to concentrate D-glucose and orthophosphate within the intra-vesicular space. The Na^+-dependent D-glucose symporter is electrogenic and is inhibited by phlorizin. This symporter has properties similar to the Na^+-dependent D-glucose symporters, SGLT1 type proteins, which are located on the apical membranes of absorptive kidney proximal tubule cells and intestinal epithelia in a number of species, including pre-ruminant lambs, humans, pigs, rats and rabbits [3–6]. In rabbits, the kidney and intestinal apically located SGLT1 proteins appear to arise from a single gene [7]. It has been concluded that the information for targeting the rabbit intestinal SGLT1 protein to the apical membrane is contained in the primary amino acid sequence of the SGLT1 protein [8]. The location of the Na^+-dependent D-glucose symporter on the basal membrane of the secretory acinar cells of the parotid gland presents an opportunity to investigate the mechanism(s) whereby the apically and the basally located proteins are targeted to these two different domains of the plasma membrane.

In this paper we present further evidence to support the conclusion that the ovine parotid plasma membrane vesicles are derived from the basolateral region of the acinar cells. We have determined the sequence of two SGLT1-type proteins, one derived from an ovine intestinal cDNA library and the other from a parotid gland cDNA library. They have identical amino acid sequences. Furthermore, the portions of the 3′-untranslated regions of cDNA that have been sequenced are identical. We conclude that the signal(s) responsible for the differential targeting of these two proteins is not contained within the primary amino acid sequence. Some of these results have been presented at various scientific meetings [2,9–12].

MATERIALS AND METHODS

Preparation of plasma membrane vesicles from the ovine parotid gland

Basolateral membrane vesicles from sheep parotid acini were prepared using the procedure described previously [1]. The final protein concentration was 4–8 mg/ml.

Estimation of protein

Protein was assayed by its ability to bind Coomassie Blue according to the BioRad assay technique for soluble proteins. Bovine γ-globulin (1-100 μg of protein) was used as the standard. The protein values achieved by this procedure were the same as those observed after solubilization of the sample with 0.02% (v/v) Triton X-100 before assay. This indicates there is complete solubilization of the membrane-bound proteins.

Western blotting

Membrane proteins were separated by SDS/PAGE using 8.0% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS, with 4.3% (w/v) polyacrylamide stacking gel. After electro-transference of the proteins to a nitrocellulose membrane, the protein-binding sites on the membranes were blocked by immersion in PBS containing 0.5% (w/v) non-fat dried milk (Oxoid) and 0.05% (v/v) Tween 20 for 1 h. Primary antibodies were detected using secondary antibodies conjugated to horseradish peroxidase raised in either rabbit (for SGLT1 and Na^+/K^+-ATPase) or mouse (for RLA Class I antigen). The immunoblots were developed by Enhanced Chemiluminescence (ECL), using a commercially available kit (Amersham International), following the manufacturer's instructions.

Primary antibodies

The SGLT1-antibody was raised in rabbits [13] against a synthetic hydrophilic nonadecapeptide which corresponds to amino acid residues 402–420; a proposed extra-membranous loop of the

Abbreviations used: MeGlc, methyl α-D-glucopyranoside; ECL, enhanced chemiluminescence.

The amino acid sequence provided in this paper was derived from nucleotide sequences which have been deposited in the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under the accession numbers X82410 (parotid gland), X82411 (small intestine).

* To whom correpondence should be addressed.

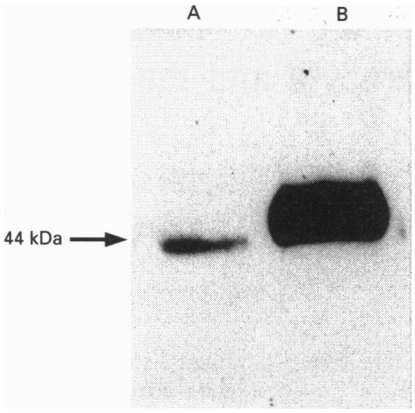

Figure 1 Immunodetection of the RLA class I major histocompatibility antigen on the ovine parotid gland basolateral plasma membrane vesicles

Samples were dissolved in SDS and the component proteins were separated by SDS/PAGE. After transferring the proteins on to nitrocellulose membrane, the immunodetection of the RLA class I major histocompatibility antigen was carried out as described in the Materials and methods section. Blots were incubated with monoclonal antibodies raised against the 44 kDa light-chain subunit. Primary antibodies were detected using a horseradish peroxidase-conjugated secondary antibody and detected by ECL. Lane A, parotid homogenate (20 μg of protein); lane B, parotid basolateral membrane vesicles (20 μg of protein).

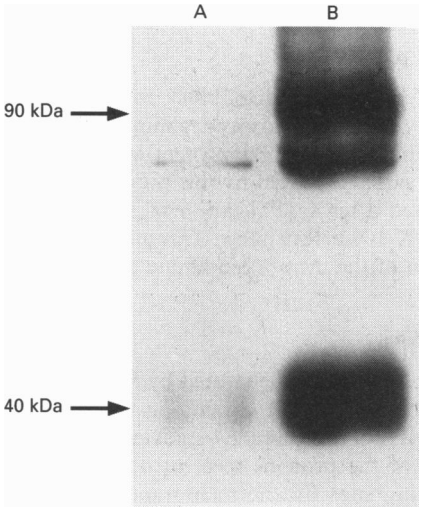

Figure 2 Immunodetection of the Na^+/K^+-ATPase on the ovine parotid gland basolateral plasma membrane vesicles

Samples were dissolved in SDS and the component proteins were separated by SDS/PAGE. After transferring the proteins on to nitrocellulose membrane, the immunodetection of the Na^+/K^+-ATPase was performed as described in the Materials and methods section. Blots were incubated with antibodies raised against purified lamb kidney Na^+/K^+-ATPase. A horseradish peroxidase-conjugated secondary antibody was used and detected by ECL. Lane A, parotid homogenate (20 μg of protein); lane B, parotid basolateral membrane vesicles (20 μg of protein).

rabbit and ovine intestinal Na^+-dependent D-glucose symporters [4,9,14]. Antibodies to the Na^+/K^+-ATPase were a gift from Dr. W. J. Ball (University of Cincinnati, Cincinnati, OH, U.S.A.). The polyclonal antibodies to the purified lamb kidney Na^+/K^+-ATPase [15] were raised in rabbits. Antibodies (VPM19), raised in mice to the major histocompatibility, RLA Class I antigen, were a gift from Dr. J. Hopkins (University of Edinburgh, Edinburgh, Scotland, U.K.).

Immunohistochemical investigation of the ovine parotid gland

Tissue preparation

To prepare sections for light microscopy, parotid tissue segments were fixed in 4% (w/v) paraformaldehyde/PBS at room temperature for 2 h. The tissue was then dehydrated step-wise by placing it in 50% (v/v) aqueous ethanol for 15 min, followed by three steps in 75% (v/v) ethanol for 15 min. The samples were embedded in Unicryl resin (British Biocell, Cardiff, Wales, U.K.) and 4 μm sections were cut on an LKB 11800 Pyramitome using a glass knife.

Immunohistochemical localization

Tissue sections were labelled by immunogold staining which was enhanced with silver. The tissue was counter-stained with a polychromatic staining kit (British Biocell) following the instructions of the manufacturer.

Na^+/K^+-ATPase

Non-specific binding sites were blocked by incubating the sections for 1 h with PBS containing 5% (w/v) non-fat milk powder (Oxoid) plus 0.5% (v/v) Tween 20. The sections were then incubated for 1 h with the Na^+/K^+-ATPase antibody. After a brief wash, secondary antibody, goat anti-(rabbit IgG) colloidal gold (5 nm), was incubated with the sections for 1 h. The sections were washed and the gold label enhanced using a silver enhancement kit (British Biocell) following the manufacturer's instructions. Control sections were processed in parallel using normal rabbit serum to replace the primary antibody in the procedure.

SGLT1

Sections of parotid gland were blocked for 1 h in buffer containing 500 mM NaCl, 10 mM Tris/HCl (pH 7.5), 0.15% (v/v) Tween 20 and 5% (w/v) non-fat dried milk (Oxoid). The sections were then incubated with the primary antibody for 1 h and were washed briefly and the secondary antibody, goat anti-(rabbit IgG) conjugated to gold (5 nm), was added for 1 h. The sections were washed again and the gold particles enhanced with silver. To determine the specificity of the staining, control sections were incubated with pre-immune rabbit serum rather than primary antibody.

Preparation of RNA

Total cellular RNA from ovine parotid gland and intestinal mucosal scrapings was prepared by a single-step guanidinium isothiocyanate/phenol/chloroform extraction procedure [16]. Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography [17].

Northern blot analysis

This was performed as described previously [18].

Cloning of the parotid gland Na^+-dependent D-glucose symporter

Ovine parotid mRNA was fractionated by centrifugation through a 10–40% sucrose density gradient. mRNA of 1–6 kb in size was used to synthesize a cDNA library in λgt10 bacteriophage vector. A 940 bp DNA fragment was isolated from the cDNA library by PCR amplification with Biotaq DNA polymerase (Bioline,

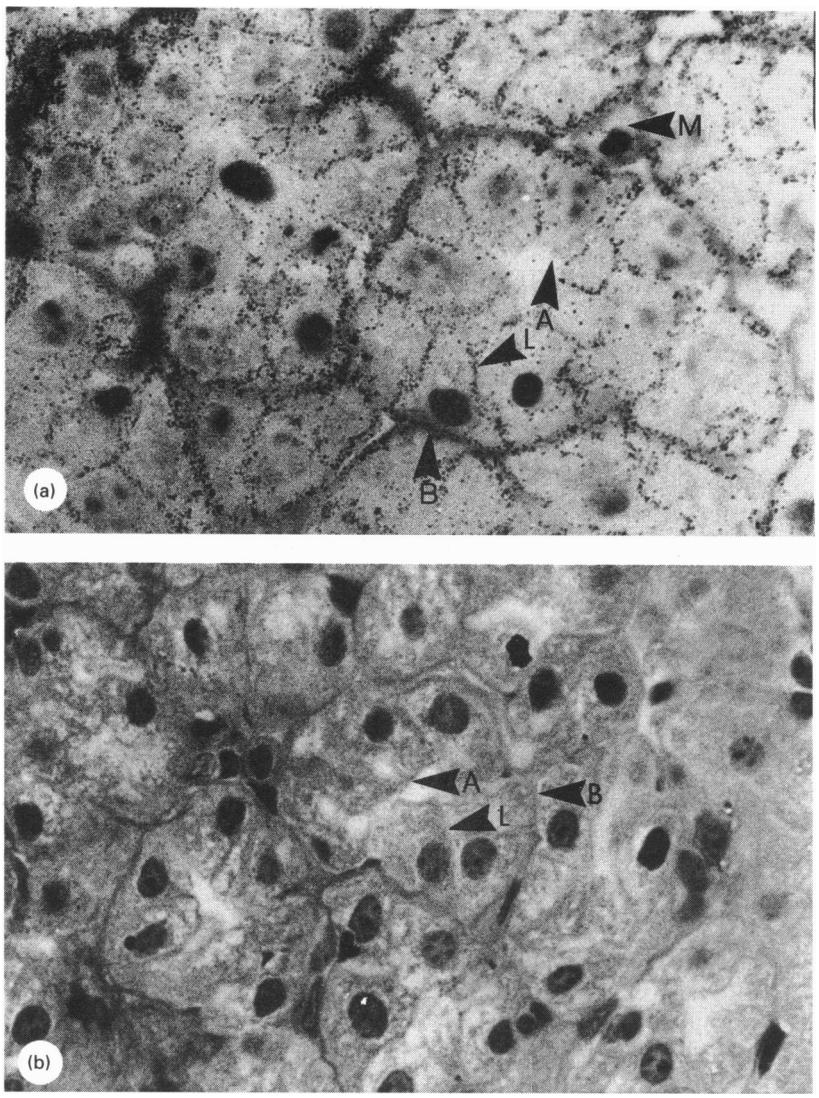

Figure 3 Localization of the Na^+/K^+-ATPase in the ovine parotid gland

Parotid gland tissue segments were fixed, dehydrated, embedded in Unicryl resin and sections, 4 μm thick, were cut (see Materials and methods section). The sections were incubated with an antibody raised against lamb kidney Na^+/K^+-ATPase (**a**). Control sections were incubated with normal rabbit serum (**b**). Gold-labelled goat anti-(rabbit IgG) was used as a secondary antibody. The gold label was enhanced with silver to enable visualization by light microscopy. Specific immune reactions were observed on the basal (B) and lateral (L) aspects of the plasma membrane of the acinar cells (see part **a**). Cross-reactivity was observed neither on the apical plasma membranes (A) of these cells nor on the myoepithelial cells (M). Gold labelling was not observed on the control section (see part **b**). Magnification $\times 700$.

London, U.K.) using primers A and B (see the Oligonucleotides section). This fragment was labelled and used to screen the library for full-length clones encoding the parotid Na^+-dependent D-glucose symporter. Following high-stringency screening, a 3.7 kb clone was identified and isolated. Both strands of the clone were sequenced on a Model 373A automated DNA sequencer using a 'Taq DyeDeoxy Terminator' cycle sequencing kit (Applied Biosystems). Comparison with the rabbit intestinal SGLT1 nucleotide sequence revealed this clone to be 400 bp short of the initiation codon. Sequence data for this 400 bp region were obtained from the 940 bp PCR fragment.

Cloning of the intestinal Na^+-dependent D-glucose symporter

A 2.0 kb *Eco*RI–*Mlu*I fragment which encoded for the rabbit intestinal SGLT1 protein coding region was isolated from the plasmid pMJC424 [14]. It was labelled by random priming with [α-^{32}P]dCTP using an oligonucleotide labelling kit (Pharmacia). cDNA was synthesized from lamb jejunal mRNA using a cDNA synthesis kit (Pharmacia) and was used to construct a library in the bacteriophage λgt10 (Stratagene). The library was screened with the cDNA probe at high stringency [42 °C in 50% (v/v) formamide hybridization buffer] [18]. Positive clones were isolated and subcloned into pBluescript II KS+ (Stratagene).

The sequence of the 5′-end region was obtained by a PCR-based strategy using 'Biotaq' DNA polymerase (Bioline). An internal primer, B, and the λgt10 forward primer were used to amplify products from the intestinal cDNA library. Products were subcloned into the plasmid vector pGEM-T (Promega) and screened by Southern blot analysis using the rabbit cDNA probe. Clones containing the remaining 5′ protein coding sequence were isolated and the nucleotide sequence determined. DNA

sequencing, on both strands, was carried out by the chain-termination method [19] using either Sequenase v2.0 (USB/Amersham) or T7 DNA polymerase (Pharmacia).

Oligonucleotides

The following oligonucleotides were used: λgt10 forward primer, 5′-AGCAAGTTCAGCCTGGTTAAG-3′; primer A, 5′-TGGACAGCAGCACTTTGAGC-3′, derived from rabbit kidney SGLT1 [20]; primer B, 5′-GCAGCCACCCTTCACGTGAGA-3′, derived from human intestinal SGLT1 [21].

RESULTS AND DISCUSSION

We have previously isolated a vesicular membrane fraction from ovine parotid glands which is enriched in Na^+/K^+-ATPase. These vesicles were capable of transporting D-glucose in an Na^+-dependent manner. In these studies we have confirmed all of the experimental data presented previously [1,2]. However, the validity and significance of arguments to be presented later in this paper are totally dependent upon the cellular origin of these membrane vesicles. Hence, further experiments were devised to confirm the cellular origin of the membranes.

Association of the major histocompatibility antigen RLA Class 1 with the parotid gland plasma membrane vesicles

The RLA Class 1 major histocompatibility antigen is located on the basolateral membrane of polarized epithelial cells [22]. We examined the parotid gland membrane vesicles for the presence of this protein. Samples of the parotid homogenate and basolateral membranes were dissolved in SDS and the constituent proteins separated by SDS/PAGE. The proteins were electrotransferred to a nitrocellulose membrane and blotted with antibodies against the RLA Class 1 antigen. Figure 1 shows cross-reaction with a protein, migrating at 44 kDa, in sheep parotid membrane vesicles (lane B) and cellular homogenate (lane A). The abundance of the band in each track was estimated by densitometry using Phloretix 1D densitometry software. The results indicate that the RLA Class 1 antigen is enriched 18–20-fold in the membrane vesicles with respect to the homogenate.

Characterization of the components of the Na^+/K^+-ATPase associated with the parotid gland plasma membrane vesicles

The ouabain-sensitive K^+-activated phosphatase, a partial reaction of the Na^+/K^+-ATPase [23], has been used as a measure of the presence of the Na^+/K^+-ATPase complexes in these membranes [1]. We have used Western blot analysis to demonstrate the presence of the subunits of the Na^+/K^+-ATPase in the parotid gland membrane vesicles. Samples of the ovine parotid gland homogenates and the derived basolateral membrane vesicles were subjected to SDS/PAGE. The proteins were then transferred to nitrocellulose and probed with a polyclonal antibody raised against a purified preparation of lamb kidney Na^+/K^+-ATPase. The results are presented in Figure 2. Bands were observed at apparent molecular masses of 90 kDa, the α-subunit (often two separated bands), and 55 kDa (broad band), the β-subunit [24]. These cross-reactions demonstrate the presence of the subunits of the conventional Na^+/K^+-ATPase complex. Densitometric scans of the blots showed that there was a 19-fold enrichment of the Na^+/K^+-ATPase α- and β-subunits in the parotid basolateral membrane vesicles (lane B) compared with the homogenate (lane A). This correlates with the previous observation that there was a significant concentration of the activity of this enzyme in the final membrane preparation [1].

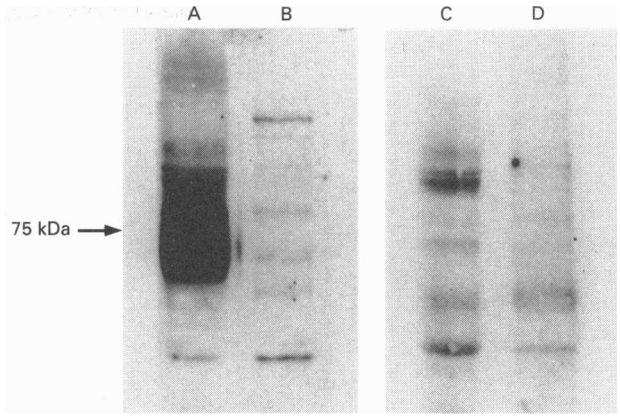

Figure 4 The immunodetection of an SGLT1-type protein in the ovine parotid gland basolateral plasma membrane vesicles

Samples were dissolved in SDS and the component proteins were separated by SDS/PAGE. After transferring the proteins on to nitrocellulose membrane, the immunodetection of SGLT1-type proteins was carried out as described in the Materials and methods section. Blots were incubated with an antibody raised against a synthetic peptide corresponding to residues 402–420 of the rabbit or ovine intestinal SGLT1 sequence. Incubations were performed in either the presence (lanes C and D) or absence (lanes A and B) of the immunizing peptide. A horseradish peroxidase-conjugated secondary antibody was used and detected by ECL. Lanes A and C, parotid basolateral membrane vesicles; lanes B and D, parotid homogenate.

Immunohistochemical localization of the Na^+/K^+-ATPase

The use of the Na^+/K^+-ATPase activity as an indicator of the presence of the basolateral membranes in the vesicle preparations used in these studies has been validated directly. The localization of the Na^+/K^+-ATPase within the sheep parotid gland acinar cells was investigated by immunohistochemical analysis. Fresh tissue was fixed and embedded in Unicryl resin as described in the Materials and methods section. Sections were incubated with the antibody to the purified lamb kidney Na^+/K^+-ATPase. The localization of the antibody within the tissue was established using goat anti-(rabbit IgG) labelled with 5 nm gold particles as a secondary antibody. Control sections were processed in parallel and normal rabbit serum was used to replace the primary antibody in the procedure.

The results of the study described above are shown in Figure 3. In Figure 3(a) it can be seen that there is a well-defined dense localization of the gold particles along the length of the basal and lateral regions of the plasma membrane of the acinar cells. There is no ordered association of the gold particles with the apical region of the plasma membrane. No specific localization of the Na^+/K^+-ATPase was detected on the plasma membrane of the myoepithelial cells. In Figure 3(b) (control) no gold labelling was observed. These observations validate the use of the Na^+/K^+-ATPase activity as an indicator of the presence of the basolateral membranes in the vesicle preparations used in these studies.

In summary, further evidence for the basolateral origin of the membranes is based upon the localization of the Na^+/K^+-ATPase on the basolateral region of the plasma membrane, as demonstrated by immunohistochemistry, and the use of Western blotting to demonstrate the co-localization of the subunits of the Na^+/K^+-ATPase and the RLA Class 1 antigen in the membrane vesicles.

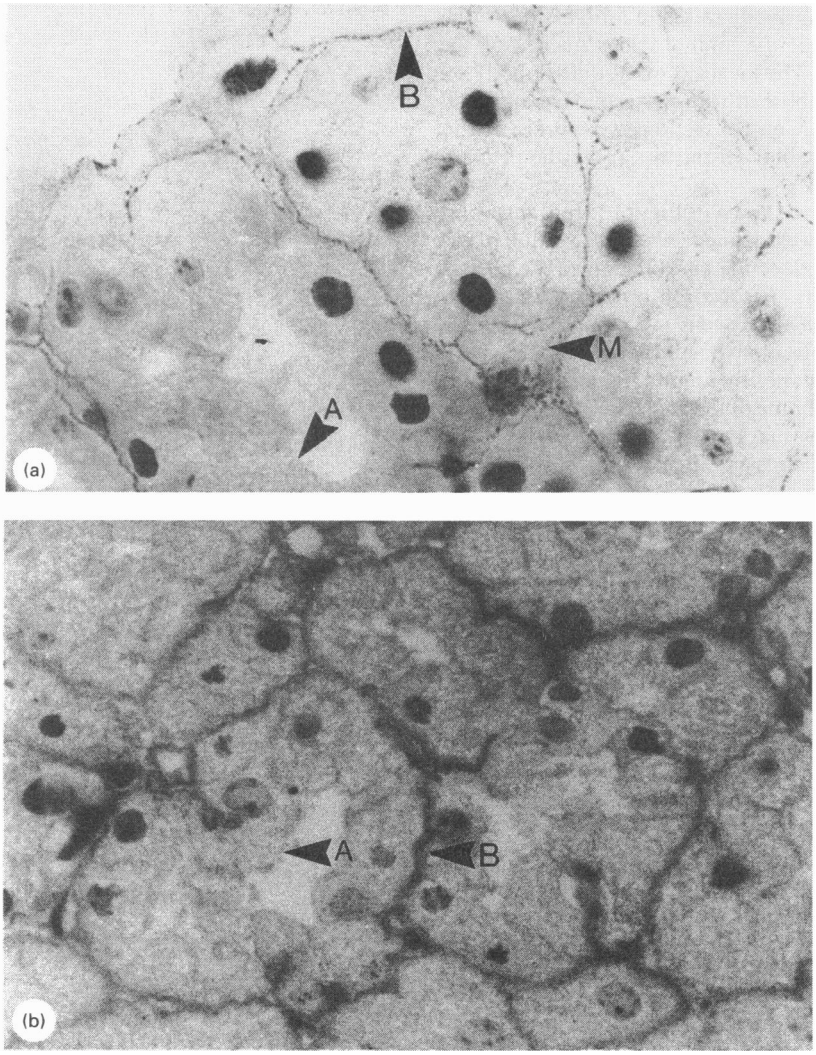

Figure 5 Localization of SGLT1-type proteins in the ovine parotid gland

Parotid gland tissue segments were fixed, dehydrated, embedded in Unicryl resin and sections, 4 μm thick, were cut (see Materials and methods section). The sections were incubated with an antibody raised against a peptide fragment (residues 402–420) of the amino acid sequence of the rabbit or ovine SGLT1 (**a**). Control sections were incubated with normal rabbit serum (**b**). Gold-labelled goat anti-(rabbit IgG) was used as a secondary antibody. The gold label was enhanced with silver to enable visualization by light microscopy. Specific immuno-reactivity is observed on the basal (B) plasma membranes of the acinar cells. No SGLT1 protein was seen on the apical plasma membrane (A) of the acinar cells. There was no labelling on the myo-epithelial plasma membranes (M) (see part **a**). Gold labelling was not observed in the control section (see part **b**). Magnification $\times$ 875.

The presence of SGLT1-type proteins in the parotid gland basolateral membrane vesicles

The basolateral location of the Na^+-dependent D-glucose symporter in the parotid plasma membrane is unusual. Hence, experiments were devised to detect and confirm the presence of SGLT1-type proteins in these basolateral membrane vesicle preparations.

Immunodetection of SGLT1-type proteins

Parotid gland basolateral membrane vesicles were dissolved in SDS and the proteins separated by SDS/PAGE. They were electrotransferred to a nitrocellulose membrane and probed with either the anti-SGLT1 antibody or the antibody which had been pre-absorbed with the immunizing peptide antigen. The reaction of this pre-absorbed antibody with any protein on the blot is regarded as a non-specific interaction. The results are presented in Figure 4. It can be seen that the antibody specifically recognized a single diffuse band, typical of a glycoprotein, migrating at an apparent molecular mass of 75 kDa. A similar cross-reaction is observed in brush-border plasma membranes isolated from the intestine of pre-ruminant sheep [25]. The rabbit intestinal SGLT1 is also a glycoprotein which has an apparent molecular mass of approx. 75 kDa [14]. The abundance of the 75 kDa band was negligible in the parotid homogenate. The co-enrichments of the Na^+/K^+-ATPase, the RLA Class-1 antigen and SGLT1 in the isolated parotid vesicles indicate that these proteins are all localized on the basolateral membrane of the ovine parotid acinar cells.

Immunohistochemical localization of SGLT1-type proteins in the basolateral membranes

Fresh ovine parotid tissue was fixed and embedded in Unicryl, as described in the Materials and methods section. Sections were

treated with the polyclonal antibody raised in rabbits against the nonadecapeptide fragment of the amino acid sequence of the rabbit intestinal SGLT1 [3]. The localization of the antibody on the parotid section was established using a secondary antibody labelled with gold particles. Control sections were processed in parallel using pre-immune rabbit serum to replace the primary antibody in the procedure.

The results of this study are shown in Figure 5. In Figure 5(a) it can be seen that there is a well-defined dense localization of the gold particles along the length of the basal region of the plasma membrane of the acinar cells. Association of the gold particles with the lateral aspect of the acinar cells is seen rarely. There is no apparent specific association of the antibody with the apical region of the plasma membrane. In Figure 5(a) there is a clear separation of the basal membrane of the acinar cell from adjacent cells (indicated by the arrow marked B). There is defined gold labelling of this region of the plasma membrane. This confirms that the labelling seen in the bulk of the tissue is not associated with the myoepithelium. In Figure 5(b) (control) no labelling was observed.

We conclude that a protein immunologically related to SGLT1 is located on the basal region of the ovine parotid acinar cell plasma membrane. In marked contrast, in ovine enterocytes the SGLT1 proteins are located exclusively on the apical pole of these cells [4].

This raises the question of what the mechanism(s) are whereby these proteins are targeted to opposite poles of the plasma membrane in a secretory and an absorptive epithelial cell. To approach this we set out to identify, isolate and clone species of cDNA, from both ovine jejunum and parotid glands, that were similar to the cDNA of the SGLT1 protein isolated from rabbit intestine. The amino acid sequences were deduced and inspected for the presence of potential targeting signals.

Determination of the primary amino acid sequences of the SGLT1-type proteins which are present in the ovine parotid basal plasma membrane and the ovine enterocyte apical plasma membrane

Northern blot analysis of the ovine parotid gland RNA

Northern blot analysis of parotid gland RNA was performed to determine if there was any sequence identity between the cDNA encoding the rabbit intestinal SGLT1 protein and components of the parotid RNA preparations. Ovine parotid RNA was fractionated by electrophoresis on an agarose gel. The RNA was transferred to a nylon membrane and probed with labelled cDNA encoding the rabbit intestinal SGLT1. The results are shown in Figure 6. It can be seen that hybridization occurred with a major transcript at approximately 4.0 kb in both parotid total RNA and mRNA. A transcript of approximately 1 kb was observed routinely in the mRNA sample. The significance of this mRNA species is not clear since it cannot encode the complete amino acid sequence of the glucose transporter. This band, which is always present, is unlikely to originate from the degradation of mRNA. There is no other indication of the degradation of the mRNA, as judged by reversible staining of the mRNA on the nylon membrane. These results confirm that there are components in the ovine parotid gland mRNA which hybridize with the rabbit intestinal SGLT1 cDNA.

Northern blotting has been performed on lamb intestinal mRNA and five transcripts were noted over the range 2.4–5.5 kb [9,18,26]. The significance of this large number of transcripts is obscure. It is clear that the ovine small intestine contains mRNA species that have significant similarity to the rabbit SGLT1 cDNA.

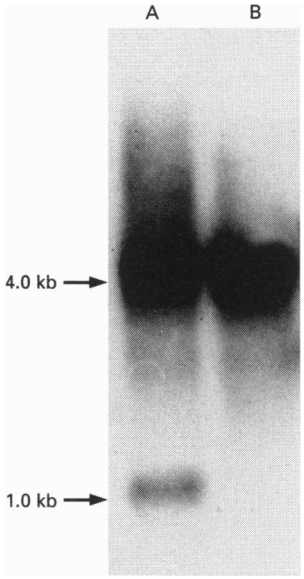

Figure 6 Northern blot analysis of ovine parotid gland RNA with rabbit intestinal SGLT1 cDNA

Parotid RNA was fractionated by electrophoresis on a denaturing 1% (w/v) agarose gel, transferred to nylon membrane and probed with the radiolabelled SGLT1 cDNA. Lane A, ovine parotid mRNA (3 μg); lane B, ovine parotid RNA (10 μg).

Cloning and sequencing of the ovine parotid and intestinal Na^+-dependent D-glucose symporters

Parotid gland Na^+-dependent D-glucose symporter

A sheep parotid cDNA library was constructed in λgt10. The mRNA used to construct the library was size-selected (2–6 kb) in order to include the 4.0 kb transcript identified by Northern blot analysis (see Figure 6). A 940 bp fragment was obtained from the parotid cDNA library by PCR amplification using oligonucleotide primers A and B. This fragment was used to screen the sheep parotid cDNA library at high stringency. A single 3.7 kb clone was identified. Double-stranded sequencing was carried out on both the 940 bp PCR fragment and on the 3.7 kb λgt10 clone. The 3.7 kb clone was truncated 400 bp short of the start codon, relative to the rabbit SGLT1 sequence. Sequence data for this region were obtained from the 940 bp PCR product. The composite sequence derived from overlapping clones shows that the cDNA encodes for a protein containing 664 amino acid residues, Figure 7. This exhibits 85% identity and 93% similarity to the amino acid sequence of the rabbit intestinal SGLT1 protein (Table 1).

Intestinal Na^+-dependent D-glucose symporter

A cDNA library was prepared in the bacteriophage λgt10. On probing the library with the labelled rabbit SGLT1 cDNA, a clone containing a 2.1 kb cDNA insert was isolated. This was subsequently used to re-screen the library and a clone containing a 3 kb insert was identified. Both the 2.1 and 3 kb inserts were sequenced, however, neither spanned the entire protein-coding region. When compared with the rabbit intestinal sequence they were missing approximately the first 800 and 650 nucleotides respectively. The 5′-end of the cDNA was cloned by a PCR procedure using the λgt10 cDNA library as a template and a

```
        10          20          30          40          50          60
MDSSTWSPPA TATAEPLQAY ERIRNAADIS VIVIYFVVVM AVGLWAMFST NRGTVGGFFL

        70          80          90         100         110         120
AGRSMVWWPI GASLFASNIG SGHFVGLAGT GAAAGIATGG FEWNALILVV LLGWVFVPIY

       130         140         150         160         170         180
IKAGVVTMPE YLRKRFGGQR IQVYLSVLSL VLYIFTKISA DIFSGAIFIN LALGLDLYLA

       190         200         210         220         230         240
IFILLAITAL YTITGGLAAV IYTDTLQTVI MLLGSFILTG FAFHEVGGYS AFVTKYMNAI

       250         260         270         280         290         300
PTVTSYGNTT VKKECYTPRA DSFHIFRDPL KGDLPWPGLI FGLTIISLWY WCTDQVIVQR

       310         320         330         340         350         360
CLSAKNMSHV KAGCIMCGYM KLLPMFLMVM PGMISRILFT EKVACTVPSE CEKYCGTKVG

       370         380         390         400         410         420
CTNIAYPTLV VELMPNGLRG LMLSVMLASL MSSLTSIFNS ASTLFTMDIY TKIRKKASEK

       430         440         450         460         470         480
ELMIAGRLFM LVLIGVSIAW VPIVQSAQSG QLFDYIQSIT SYLGPPIAAV FLLAIFCKRV

       490         500         510         520         530         540
NEPGAFWGLI IGFLIGVSRM ITEFAYGTGS CMEPSNCPTI ICGVHYLYFA IILFVITIIV

       550         560         570         580         590         600
ILAISLFTKP IADVHLYRLC WSLRNSKEER IDLDAEDEDI QDAREDALEI DTEASEEKKG

       610         620         630         640         650         660
CLRQAYDMFC GLDQQKGPKM TKEEEAAMKL KMTDTSEKRL WRMVVNINGI ILLAVAVFCH

 664
AYFA
```

Figure 7 Amino acid sequence of the ovine intestinal/parotid gland Na^+-dependent D-glucose symporter

Table 1 Comparison of the amino acid sequences of the ovine parotid and intestinal SGLT1 proteins with other SGLT1-type proteins

Source of tissue	Identity (%)	Similarity (%)	No. of amino acids	Reference
Ovine intestine, parotid	100	100	664	[4] and this paper
Rabbit kidney, intestine	85	93	662	[14,20,31]
Rat kidney	88	94	665	[30]
Human intestine	86	94	664	[21]
Pig kidney	89	95	662	[32,33]

combination of the λgt10 forward primer and the internal sequence primer B (see the Materials and methods section).

The amino acid sequences of the intestinal and parotid plasma membrane proteins are presented in Figure 7. They are identical, indicating that they derive from the same gene. A similar situation exists in the rabbit, where the amino acid sequence of the apically located kidney and intestinal sequences are identical. It can be seen from the information presented in Table 1 that the ovine parotid and intestinal proteins are members of the SGLT1 family. All the proteins in this group have 662–665 amino acid residues, they exhibit an extraordinary degree of similarity (> 93 %), they all have 12 putative membrane spanning regions and the potential glycosylation site, Asn^{248}, is conserved in all these sequences. This common site for glycosylation is predicted to lie extracellularly. The Asp^{28}, which is changed to asparagine in the condition known as glucose–galactose malabsorption [27,28], is also conserved in the ovine proteins. The nonadecapeptide segment corresponding to amino acid residues 402–420 in the rabbit intestinal protein sequence is identical at the amino acid level in the ovine sequences. The amino acid sequences of the SGLT1 family do not include any obvious cleavable signal sequences [29].

cRNA prepared from human, rabbit and rat SGLT1 clones has previously been injected into *Xenopus* oocytes with the consequent expression of SGLT1 activity [14,21,30]. We have shown that the injection of either ovine parotid or intestinal

mRNA into oocytes of *Xenopus laevis* resulted in the expression of an Na^+-dependent D-glucose symporter activity on the oocyte plasma membrane. Injection of 50 ng of parotid mRNA resulted in a 7-fold increase in methyl α-D-glucopyranoside (MeGlc) uptake over water-injected controls. This glucose analogue was used because it is specifically transported by the Na^+-dependent transporter and is incapable of being metabolized by the oocytes. In the presence of 1 mM phlorizin, a competitive inhibitor of Na^+-dependent glucose transport, uptake was reduced by 86% [2]. Similarly, injection of 50 ng of ovine jejunal mRNA resulted in a 6-fold increase in MeGlc uptake over water-injected controls. This transport was reduced by 59% in the presence of 1 mM phlorizin (I. S. Wood, unpublished work). The high degree of similarity between the amino acid sequences of all these Na^+-dependent glucose symporters and the ovine proteins described here, is strong support for the assumption that the ovine proteins are Na^+-dependent glucose symporters (see Table 1).

The identity of the amino acid sequences of the ovine parotid and intestinal proteins directed to the opposite poles of the epithelial cells must indicate that there is no inherent targeting signal present within the protein structure. This conclusion conflicts with that drawn by Kong et al. [8]. These authors inserted the entire rabbit SGLT1 cDNA into a mammalian expression vector and demonstrated that when this vector was used to transfect MDCK cells, SGLT1 activity was expressed. Over 93% of the expressed recombinant SGLT1 protein was directed to the apical membrane. They concluded that the primary amino acid sequence of the SGLT1 protein contains the information necessary to target the mature protein to the apical membrane. Our results show that this cannot be the case for the ovine proteins that we have investigated. In the experiments described by Kong et al. [8] the expressed insert contained 153 nucleotides in the 3′-untranslated region, excluding the poly(A^+) tail. It is possible that this untranslated sequence contains an apical targeting signal. However, the 3′-untranslated region of the ovine cDNA is virtually identical over the first 270 nucleotides. We conclude that this region of the 3′-untranslated region cannot contain a target sequence. If the 3′-untranslated section of the ovine mRNA does contain targeting signals then they must lie beyond the region that we have sequenced. We are currently investigating this possibility.

We are grateful to Drs. W. J. Ball and J. Hopkins for the provision of antibodies, Dr. Ap Gwyn for help and advice on immunohistochemistry, P. J. Barker for raising antibodies to SGLT1, Dr. W. Rees for guidance with the automated sequencer, Professor E. M. Wright for the provision of rabbit SGLT1 cDNA and Dr. A. Morrison for help and encouragement. E. Howells provided sheep parotid glands. P.S.T. and I.S.W. are BBSRC funded research students. S.P.S.-B. is a Wellcome Trust Senior Lecturer. The financial support of the Wellcome Trust is gratefully ackowledged.

REFERENCES

1 Vayro, S., Kemp, R., Beechey, R. B. and Shirazi-Beechey, S. P. (1991) Biochem. J. **279**, 843–848
2 Tarpey, P. S., Vayro, S., Shirazi-Beechey, S. P. and Beechey, R. B. (1992) Biochem. Soc. Trans. **20**, 328S
3 Hirayama, B. A., Wong, H. C., Smith, C. D., Hagenbuch, B. A., Hediger, M. A. and Wright, E. M. (1991) Am. J. Physiol. **261**, C296–C304
4 Shirazi-Beechey, S. P., Wood, I. S., Dyer, J., Scott, D. and King, T. P. (1995) in Ruminant Physiology: Digestion, Metabolism, Growth and Production (Englehardt, W. V., ed.), pp. 115–132, Enke-Verlag, Stuttgart
5 Shirazi-Beechey, S. P., Davies, A. G., Tebbutt, K. et al. (1990) Gastroenterology **98**, 676–685
6 Mullins, J., Beechey, R. B., Gould, G. W., Campbell, F. C. and Shirazi-Beechey, S. P. (1992) Biochem. J. **285**, 785–790
7 Hediger, M. A., Budarf, M., Emmanuel, B., Mohandas, T. and Wright, E. M. (1989) Genomics **4**, 296–300
8 Kong, C.-T., Varde, A. and Lever, J. E. (1993) FEBS Lett. **333**, 1–4
9 Wood, I. S., Scott, D., Beechey, R. B. and Shirazi-Beechey, S. P. (1994) Biochem. Soc. Trans. **22**, 266S
10 Tarpey, P. S., Shirazi-Beechey, S. P. and Beechey, R. B. (1994) Biochem. Soc. Trans. **22S**, 264S
11 Tarpey, P. S., Wood, I. S., Shirazi-Beechey, S. P. and Beechey, R. B. (1994) Proc. Soc. Nutr. Physiol. **3**, 79
12 Wood, I. S., Beechey, R. B., Scott, D. and Shirazi-Beechey, S. P. (1994) Proc. Soc. Nutr. Physiol. **3**, 96
13 Lachmann, P. J., Strangeways, L., Vyakarnam, A. and Evan, G. I. (1986) Ciba Found. Symp. **119**, 25–40
14 Hediger, M., Coady, M. J., Tyson, S., Ikeda, T. S. and Wright, E. M. (1987) Nature (London) **330**, 379–381
15 Sun, Y. and Ball, W. J. (1992) Am. J. Physiol. **262**, R77–R84
16 Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
17 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, New York
18 Freeman, T. C., Wood, I. S., Sirinathsinghji, D. J. S., Beechey, R. B., Dyer, J. and Shirazi-Beechey, S. P. (1993) Biochim. Biophys. Acta **1146**, 203–212
19 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
20 Morrison, A. L., Panayotova-Heiermann, M., Feigl, G., Scholermann, B. and Kinne, R. K. H. (1991) Biochim. Biophys. Acta **1089**, 121–123
21 Hediger, M. A., Turk, E. and Wright, E. M. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 5748–5752
22 Moktari, S., Ferraci, H., Gorvel, J.-P., Mishal, Z. and Maroux, S. (1986) J. Membr. Biol. **89**, 53–64
23 Skou, J. C. (1975) Q. Rev. Biophys. **7**, 401–434
24 Koob, R., Kraemer, D., Trippe, G., Aebi, U. and Drenckhahn, D. (1990) Eur. J. Cell Biol. **53**, 93–100
25 Shirazi-Beechey, S. P., Hirayama, B. A., Wang, Y., Scott, D., Smith, M. W. and Wright, E. M. (1991) J. Physiol. **437**, 699–708
26 Lescale-Matys, L., Dyer, J., Scott, D., Freeman, T. C., Wright, E. M. and Shirazi-Beechey, S. P. (1993) Biochem. J. **291**, 435–440
27 Turk, E., Zabel, B., Mundlos, S., Dyer, J. and Wright, E. M. (1991) Nature (London) **350**, 354–356
28 Wright, E. M., Turk, E., Zabel, B., Mundlos, S. and Dyer, J. (1991) J. Clin. Invest. **88**, 1435–1440
29 Hediger, M. A., Mendlein, J., Lee, H. S. and Wright, E. M. (1991) Biochim. Biophys. Acta **1064**, 360–364
30 Lee, W.-S., Kanai, Y., Wells, R. G. and Hediger, M. A. (1994) J. Biol. Chem. **269**, 12032–12039
31 Coady, M. J., Pajor, A. M. and Wright, E. M. (1990) Am. J. Physiol. **259**, C605–C610
32 Ohta, T., Isselbacher, K. J. and Rhoads, D. B. (1990) Mol. Cell. Biol. **10**, 6491–6499
33 Wright, E. M., Loo, D. D. F., Panayotova-Heiermann, M. et al. (1994) J. Exp. Biol. **196**, 197–212

Received 16 March 1995/4 July 1995; accepted 25 July 1995