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Rapid Report

N-linked glycosylation is not required for Na^+/glucose symport activity in LLC-PK_1 cells

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Abstract

The role of N-linked glycosylation in Na^+/glucose symporter function was investigated using LLC-PK_1 cell cultures. Tunicamycin treatment did not inhibit Na^+-dependent glucose transport or phlorizin binding activity assayed in intact LLC-PK_1 cells. However apical membrane vesicles derived from tunicamycin-treated cells had no detectable Na^+-dependent glucose transport activity but retained unchanged phlorizin binding to the symporter. These observations suggest that N-linked glycosylation is not required for transport function or insertion in the membrane in intact cells but may play a role in maintaining symporter transport activity in isolated membranes

Key words: Sodium ion/glucose cotransport; Glucose transport; Phlorizin; Tunicamycin; Apical membrane; (LLC-PK_1 kidney epithelial cell)

Na^+/glucose symporters located in the apical membrane of intestinal and renal proximal tubule cells catalyze active transport of glucose coupled to a Na^+ gradient across the membrane [1]. Complementary DNA's encoding Na^+/glucose symporters (SGLT1) from rabbit [2] and human [3] intestine contain two potential N-linked glycosylation sites, but it appears that only Asn^{248} is glycosylated [4]. SGLT1 sequences are also expressed in kidney [5,6]. Densely confluent cultures of LLC-PK_1, a cell line with properties of renal proximal tubule, express Na^+/glucose symport activity [7]. LLC-PK_1 SGLT1 exhibits 92% amino acid sequence similarity with that of rabbit intestine [8], and retains the glycosylation site at Asn^{248}.

An important question concerns the functional role of this carbohydrate moiety in either the transport activity or the normal targeting of this protein to the apical membrane. While results from in vitro chemical and enzymatic deglycosylation studies using intestinal brush-border membrane vesicles [9] and assay of mutant SGLT1 mRNA constructs defective in glycosylation of Asn^{248} in an oocyte expression system [4] indicated that glycosylation was not required for Na^+/glucose symporter activity, no information is currently available concerning the role of glycosylation in the membrane insertion and transport function of the symporter in its normal cellular environment. An opposite conclusion was reached by Birnir et al. [10] based on loss of transport activity after tunicamycin treatment of COS-7 cells transiently transfected with recombinant SGLT1 cDNA. However, this study did not control for nonspecific inhibitor effects on protein synthesis. In the case of the facilitative glucose transporter, GLUT1, a number of studies have indicated that glycosylation either influences [11] or plays an essential role [12,13] in glucose transport activity.

The antibiotic tunicamycin, which blocks the first step in the dolichol pathway required for synthesis of the core sequence of N-glycosidically linked oligosaccharides [14], has been widely used to investigate the role of carbohydrate residues in glycoprotein function. The present study examines the effects of tunicamycin treatment on the functional expression of the Na^+/glucose symporter in LLC-PK_1 cultures.

The LLC-PK_1 pig kidney epithelial cell line (ATCC CL101) was grown as described previously [15]. Addi-

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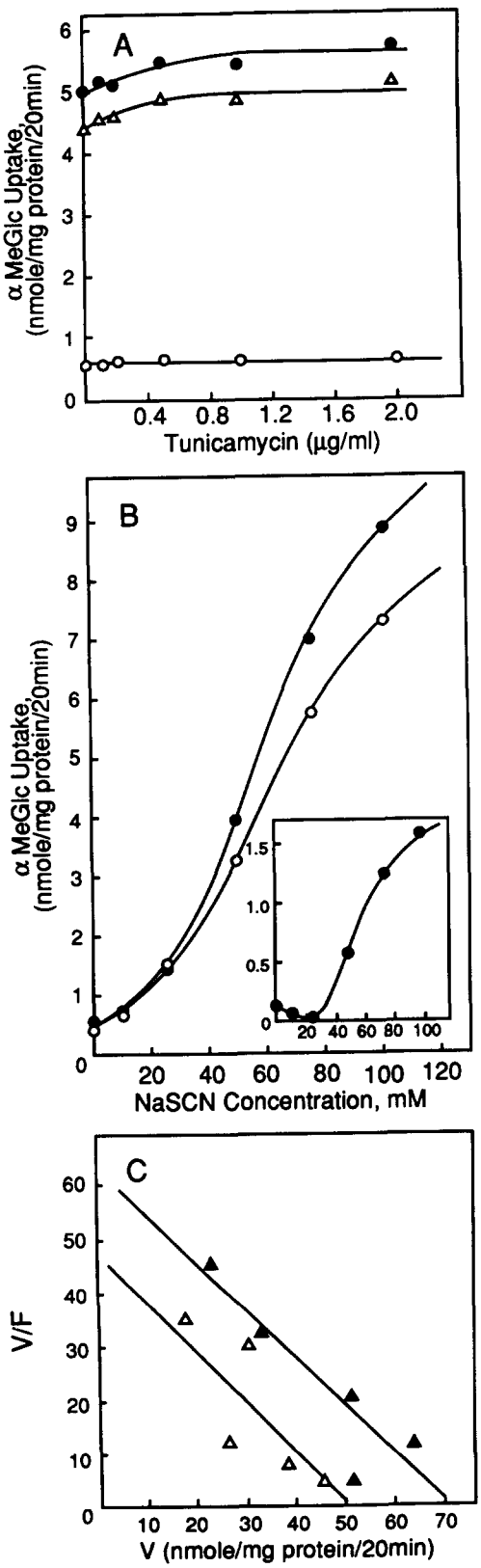
tion of tunicamycin for 24 h to confluent LLC-PK$_1$ monolayers resulted in a dose-dependent inhibition of [^{3}H]glucosamine incorporation into macromolecules with minimal effects on protein biosynthesis assessed by [^{3}H]leucine incorporation (not shown). Tunicamycin treatment did not affect the protein content per dish, assayed as described previously [16]. Maximal inhibition of glycosylation (80%), assessed by [^{3}H]glucosamine incorporation, was observed after 24 h exposure to tunicamycin concentrations above 0.7 μg/ml. A 24 h exposure to 1 μg/ml tunicamycin immediately before assay or membrane isolation was utilized in subsequent experiments.



The nonmetabolizable glucose analog methyl α-D-glucopyranoside (αMeGlc) was used as a specific substrate to assay Na^+-dependent glucose transport in intact cells. Tunicamycin treatment did not inhibit Na^+-dependent glucose uptake, assayed in intact cells as a function of tunicamycin concentration (Fig. 1A) or as a function of external Na^+ (Fig. 1B). In fact, a modest but reproducible stimulation of symporter function was observed in underglycosylated cells. No effect on Na^+-independent uptake was observed. Scatchard analysis of Na^+-dependent uptake as a function of αMeGlc concentration indicated that tunicamycin-treated cells exhibited a V_{max} of 70.8 nmol/mg per 20 min compared with a value of 51.4 nmol/mg per 20 min for control monolayers, with no appreciable effect on apparent K_m (1 mM) (Fig. 1C). No significant difference in intracellular volume, assayed by equilibration with 1 mM 3-*O*-methyl-D-[1-^{3}H]glucose, was observed between untreated cells (1.06 ± 0.06 μl/mg protein) and tunicamycin-treated cells (1.16 ± 0.06 ml/mg protein). Furthermore, no difference in permeation of [^{14}C]sucrose was noted (not shown). The number of phlorizin binding sites has been used to estimate the number of Na^+/glucose symporter units in intact cells [17]. No significant difference in the number of phlorizin binding sites per cell was observed between control and tunicamycin-treated cultures (not shown). Taken together, these results suggested that N-linked glycosylation was not required for transport function or insertion into a polarized membrane. We have previously demonstrated that tunicamycin treatment of

Fig. 1. Tunicamycin treatment does not inhibit Na^+/glucose symport activity in LLC-PK$_1$ cultures. (A) Confluent monolayers grown on 35 mm dishes were exposed for 24 h to the indicated concentration of tunicamycin. Uptake of ^{14}C-αMeGlc was measured in Na^+-free (○) or Na^+-containing (●) uptake buffer as described previously [15]. Uptake buffer contained 0.5 mM ^{14}C-αMeGlc, 0.125 M sucrose, 5 mM $MgCl_2$, 10 mM K^+-Hepes (pH 7.2), 1.36 mM $CaCl_2$, and either 100 mM NaSCN or 100 mM KSCN. Na^+-dependent uptake is shown (△) after subtracting Na^+-free uptake. (B) Na^+-dependent αMeGlc uptake was measured in control monolayers (○) and in cells treated with 1 μg/ml tunicamycin (●) as a function of Na^+ concentration. The inset represents the difference between the two curves. (C) Scatchard analysis of αMeGlc uptake in control (△) and tunicamycin-treated (▲) monolayers after subtracting Na^+-independent uptake.

MDCK cell monolayers did not impair functions related to vectorial fluid transport [18].

We next tested whether normal Na^+/glucose symporter activity was observed in apical membrane vesicles isolated from tunicamycin-treated cells.

Fig. 2 shows that apical membrane vesicles from tunicamycin-treated cultures were completely devoid of Na^+-stimulated glucose transport activity whereas vesicles from control cultures exhibited this transport activity. Na^+-independent uptake was slightly decreased in vesicles from tunicamycin-treated cells. These findings differ from those of Yusufi et al. [20] who observed that inhibition of glycoslyation in rats treated with swainosine did not influence renal brush-border membrane vesicle Na^+/glucose symport activity or the activities of the brush-border hydrolases γ-glutamyltranspeptidase and leucine aminopeptidase.

The number of Na^+-dependent phlorizin binding sites was nearly identical in vesicles from control and tunicamycin-treated vesicles (B_{max} values of 12 and 13 pmol/mg protein; K_d values for phlorizin of 1.1 and 1.2 μM, respectively). These values are in good agreement with those reported previously for control LLC-PK_1 apical membranes [19]. The observation of unchanged numbers of phlorizin binding sites per mg protein indicates that a normal recovery of apical membranes was obtained after isolation from tunicamycin-treated cells. Also this result indicated that normal levels of the Na^+/glucose symporter protein, detectable by binding of its specific high-affinity ligand phlorizin, were retained in the vesicles from tunicamycin-treated cells. Thus it is unlikely that tunicamycin reduced the steady-state levels or altered the turnover of the symporter protein.

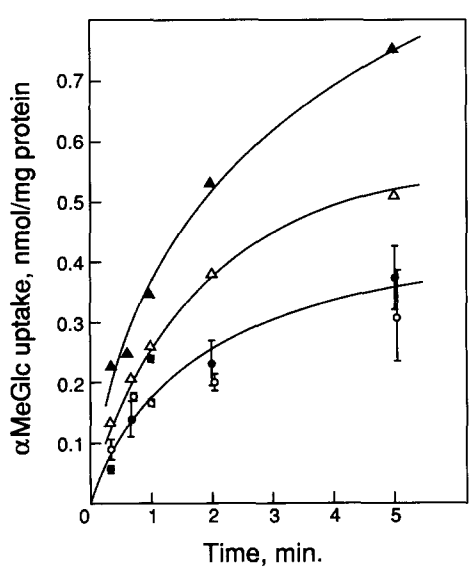


Fig. 2. Absence of Na^+-dependent αMeGlc transport activity in apical membrane vesicles from tunicamycin-treated LLC-PK_1 cells. ^{14}C-αMeGlc transport was assayed in apical membrane vesicles (150 μg) prepared as described previously [15] from control (triangles) and tunicamycin-treated cells (circles). Uptake activity in the presence of either 100 mM NaSCN (filled symbols) or 100 mM KSCN (open symbols) is shown as a function of time. Uptake was assayed as described previously [14,15] in incubation mixtures containing 0.125 M sucrose, 5 mM $MgCl_2$, 10 mM K-Hepes (pH 7.2), 0.5 mM ^{14}C-αMeGlc, 1.5 μCi/ml, and 100 mM of either NaSCN or KSCN.

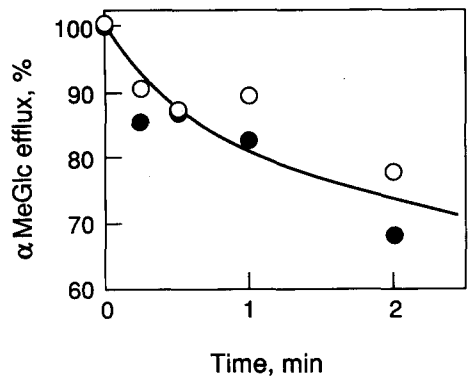


Fig. 3. Apical membranes from tunicamycin-treated cells do not exhibit increased leakiness. Apical membrane vesicles (150 μg) from control (●) and tunicamycin-treated (○) cells were assayed for efflux of ^{14}C-αMeGlc as a function of time, in the presence of external 10 μM phlorizin to block specific efflux via the symporter. Membranes were incubated with methyl α-D-[^{14}C]glucopyranoside (αMeGlc), 0.5 mM, 1.5 μCi/ml, for 20 min at 21°C in a mixture containing 100 mM NaCl, 0.125 M sucrose, 5 mM $MgCl_2$ and 10 mM Hepes (pH 7.2) in a volume of 100 μl. Then, mixtures were diluted 5-fold with 10 μM phlorizin in 0.125 M sucrose, 5 mM $MgCl_2$, 10 mM K^+-Hepes (pH 7.2). At the indicated times, efflux was terminated by addition of 3 ml of ice-cold 0.8 M NaCl, 10 mM Tris-HCl (pH 7.5) followed by rapid filtration through a 0.45 μm pore size nitrocellulose filter and a second wash with 3 ml of the same buffer.

Inability to measure Na^+-dependent transport was not due to increased vesicle leakiness after tunicamycin treatment. Control and underglycosylated membranes were loaded with ^{14}C-αMeGlc and then efflux via leakage pathways was measured in the presence of external phlorizin to block specific efflux via the symporter (Fig. 3). Similar rates of efflux were observed in both preparations. These results indicate that membranes from tunicamycin-treated cells were not appreciably leakier than control membranes, although the possibility that a Na^+ leak pathway has been activated cannot be excluded. Also, both preparations excluded [^{14}C]sucrose to the same extent (not shown).

Western blot analysis of extracts from either tunicamycin-treated LLC-PK_1 cells (not shown) or from MDCK cells stably transfected with recombinant SGLT1 from rabbit intestine [21] did not detect any change in apparent molecular weight of the SGLT1 subunit compared with untreated controls. These observations indicate that lack of the SGLT1 carbohydrate moeity does not lead to increased susceptibility to proteolysis under these conditions.

Our results indicate that N-linked glycosylation is not required for Na^+/glucose symporter function or

insertion into the apical membrane of living cells. The failure to observe transport activity in apical membrane vesicles derived from tunicamycin-treated cells despite the presence of unchanged levels of the symporter protein indicates that the carbohydrate moeity does contribute to the functional integrity of the symporter complex. Perhaps the loss of SGLT1 transport activity after membrane isolation from tunicamycin-treated cells results from altered charge or conformation of the underglycosylated symporter.

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References

[1] Crane, R.K. (1977) Rev. Physiol. Pharmacol. 78, 99–159.
[2] Hediger, M.A., Coady, M.J., Ikeda, T.S. and Wright, E.M. (1987) Nature 330, 379–381.
[3] Hediger, M.A., Turk, E. and Wright, E.M. (1989) Proc. Natl. Acad. Sci. USA 86, 5748–5752.
[4] Hediger, M.A., Mendlein, J., Lee, H.S. and Wright, E.M. (1991) Biochim. Biophys. Acta 1064, 360–364.
[5] Coady, M.J., Pajor, A.M. and Wright, E.M. (1990) Am. J. Physiol. 259, C605–C610.
[6] Morrison, A.I., Panayotova-Heiermann, M., Feigl, G., Scholermann, B. and Kinne, R.K.H. (1991) Biochim. Biophys. Acta 1089, 121–123.
[7] Mullin, J.M., Weibel, J., Diamond, L. and Kleinzeller, A. (1980) J. Cell. Physiol. 104, 375–389.
[8] Ohta, T., Isselbacher, K.J. and Rhoads, D.B. (1990) Mol. Cell. Biol. 10, 6491–6499.
[9] Hirayama, B.A. and Wright, E.M. (1992) Biochim. Biophys. Acta 1103, 37–44.
[10] Birnir, B., Lee, H.S., Hediger, M.A. and Wright, E.M. (1990) Biochim. Biophys. Acta 1048, 100–104.
[11] Asano, T., Katagiri, H., Takata, K., Lin, J.L., Ishihara, H., Inukai, K., Tsukuda, K., Kikuchi, M., Hirano, H., Yazaki, Y. and Oka, Y. (1992) J. Biol. Chem. 266, 24632–24636.
[12] Feugeas, J.P., Neel, D., Goussault, Y. and Derappe, C. (1991) Biochim. Biophys. Acta 1066, 59–62.
[13] Feugeas, J.P., Neel, D., Pavia, A.A., Laham, A., Goussault, Y. and Derappe, C. (1990) Biochim. Biophys. Acta 1030, 60–64.
[14] Struck, D.K. and Lennarz, W.J. (1977) J. Biol. Chem. 252, 1007–1013.
[15] Lever, J.E. (1982) J. Biol. Chem. 257, 8680–8686.
[16] Lever, J.E. (1987) Methods Enzymol. 146A, 376–383.
[17] Amsler, K. and Cook, J.S. (1985) J. Cell. Physiol. 122, 254–258.
[18] Lever, J.E. and Sari, C.E. (1983) Biochim. Biophys. Acta 762, 265–271.
[19] Moran, A., Davis, L. and Turner, R.J. (1988) J. Biol. Chem. 263, 187–192.
[20] Yusufi, A.N., Szczepanska-Konkel, M. and Dousa, T.P. (1988) J. Biol. Chem. 263, 13683–13691.
[21] Kong, C.-T., Varde, A. and Lever, J.E. (1993) FEBS Lett. 333, 1–4.