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Preparation and Use of a Photoactivatable Glucose-6-Phosphate Analogue

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Abstract—A benzophenone-containing derivative of glucose-6-phosphate, 6-[(3-([2,3-$^3H_2$]-*p*-benzoyl dihydrocinnamidoylpropyl-1-oxy)phosphoryl]-D-glucopyranose ([^{3}H]BZDC-Glc-6-P) was synthesized and employed to photoaffinity label proteins on intact rat liver microsomes. The use of a non-photoactivatable, UV-transparent desoxy analogue of BZDC, named *p*-benzyldihydrocinnamoyl (BnDC), is introduced as a general method to achieve competition when hydrophilic ligands are modified with hydrophobic photophores.

The homeostatic regulation of blood glucose levels is in part dependent upon the release of glucose from the liver.[1] Surprisingly, hepatic glucose production, derived from either gluconeogenesis or glycogenolysis, requires that glucose-6-phosphate (Glc-6-P) be imported into the lumen of the endoplasmic reticulum (ER) of hepatocytes and cleaved there by a glucose-6-phosphatase.[2] The glucose units are then re-released to the cytoplasm and finally exported across the hepatocyte plasma membrane to the bloodstream. This transformation of Glc-6-P to glucose is thus dependent upon a glucose-6-phosphatase with an intralumenal active site and a Glc-6-P transporter, which is responsible for the translocation of Glc-6-P from the cytoplasm to the lumen of the ER where cleavage takes place.

Until recently, this multi-component substrate-transport model was controversial. However, recent data and analyses of a family of human genetic disorders have substantiated its validity. Mutations in the glucose-6-phosphatase gene[3] were found in glycogen storage disease (GSD)-type 1a patients, but not in a closely related form of the disease (GSD 1b), in which transport appears to be defective but normal phosphatase activity is seen when the ER membrane is permeabilized. Complementing this finding, a human cDNA that encodes a 46 kDa transmembrane protein homologous to bacterial Glc-6-P transporters was identified.[4] In two patients with GSD 1b, mutations in this protein were detected. Furthermore, linkage analysis mapped the GSD 1b locus[5] and the putative transporter[6] to human chromosome 11q23. Subsequent work has substantiated the localization of the glucose-6-phosphatase active site to the lumen of the ER[7] and established the functional significance of the Glc-6-P transporter.[8]

However, the tissue distribution of the Glc-6-P transporter is broader than the distribution of glucose-6-phosphatase.[6] Interestingly, subjects with GSD 1b show deficiencies in neutrophil behavior[9], perhaps related to defects in Ca^{2+} signaling.[10,11] In addition, the import of Glc-6-P into microsomes from both liver and other tissue has been demonstrated and shown to be accompanied by an increase in levels of sequestered Ca^{2+}.[12] Therefore, it appears that the Glc-6-P transporter could have significance beyond its role in glucose homeostasis.

One approach to the further study of the Glc-6-P transporter is to utilize radioactive photoactivatable probes to selectively label it. Photoactivatable derivatives of chlorogenic acid, a highly-specific competitive inhibitor of Glc-6-phosphatase activity in intact, but not disrupted, rat and human microsomes, were used to label a high-affinity binding site in intact rat liver microsomes.[13] We describe herein the synthesis of a tritium-labeled *p*-benzoyldihydrocinnamoyl (BZDC) derivative[14] of Glc-6-P. The benzophenone photophore probes offer significant advantages for photoaffinity labeling in biological systems,[15] particularly in terms of synthesis, handling, and efficiency of covalent modification

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of the target proteins.[16] In addition, we introduce the utilization of the novel desoxy *p*-benzyldihydrocinnamoyl (BnDC) group as a non-activatable, UV-transparent mimic of the BZDC photophore. The use of these probes with rat liver microsomes, however, produced an unexpected result. Rather than preferentially labeling an integral membrane protein with the characteristics of the Glc-6-P transporter, two proteins of molecular sizes 34 and 40 kDa were labeled with the [^{3}H]BZDC Glc-6-P probe. These proteins were dislodged from ER microsomes by treatment with salt and localized to the aqueous portion of a phase separation with Triton X-114. Thus, [^{3}H]BZDC Glc-6-P appears to selectively modify two peripheral proteins of unknown function associated with the ER membranes, but fails to covalently modify the Glc-6-P transporter.

Synthesis of BZDC-Glc-6-P (1) and its Non-Photoactivatable Analogue BnDC-Glc-6-P (2) (Fig. 1)

The unsaturated succinimido-*p*-benzoylcinnamate (**7**), previously prepared by DCC coupling of *p*-benzoylcinnamic acid with *N*-hydroxysuccinimide,[14] was more conveniently synthesized using diphenyl succinimidyl phosphate.[17] Catalytic hydrogenation (10% Pd/C in EtOAc) of **7** reduced the double bond and also converted the benzophenone carbonyl group into a methylene group, giving succinimido-*p*-benzyl-dihydrocinnamate (BnDC) (**8**) in 69% yield. Benzyl 2,3,4-*tri*-*O*-benzyl-α-D-*arabino*-hexopyranoside (**3**) was prepared by sequential treatment of D-glucose with benzyl alcohol and benzaldehyde followed by benzylation and cleavage of the benzylidene acetal with lithium aluminum hydride-aluminum chloride. Intermediate **3** could be obtained as white crystals from D-glucose with an overall yield of ca. 5%. Reaction of **3** with benzyloxy-[*N*-Cbz-3-amino-1-propyloxy](*N*,*N*-di*iso*propylamino)phosphine and 1H-tetrazole in CH_2Cl_2, followed by oxidation with *m*CPBA, afforded fully protected intermediate **4**. Hydrogenolytic deprotection of **4** gave precursor **5**, which was employed to make the photoactivatable and photoinert Glc-6-P probes. Coupling **5** with [^{3}H]BZDC-NHS (**6**) gave [^{3}H]BZDC-Glc-6-P (**1**) and reaction of **5** with BnDC-NHS reagent **8** gave the non-photoactivatable BnDC-Glc-6-P (**2**).

Photoaffinity Labeling of Intact Microsomal Proteins with [^{3}H]BZDC-Glc-6-P

Microsomes were prepared from rat liver, and then fractionated by density gradient centrifugation to enrich for microsomes from the smooth endoplasmic reticulum (SER), rough endoplasmic reticulum (RER), and the Golgi apparatus.[18] A fraction enriched in mitochondria was also prepared. Each fraction was incubated with [^{3}H]BZDC Glc-6-P, photoaffinity labeled, separated by SDS-PAGE, and processed for fluorography. Coomasie-blue-stained gels demonstrated that the pattern of major proteins found in each of the three organellar-specific microsomal preparations were distinct and different from that of the mitochondrial fraction (Fig. 2). The fluorograms (Fig. 2B) showed labeling primarily of proteins at 34 and 40 kDa. The labeling of each of the two bands was enhanced in preparations enriched for SER (lane 2) and Golgi (lane 4). The bands were not seen in fractions enriched for the RER or mitochondrial fractions (lanes 3 and 5).

One approach to determine whether this labeling was dependent upon the Glc-6-P portion of the probe, labeling with [^{3}H]BZDC Glc-6-P was compared to that seen with another BZDC coupled probe, [^{3}H]BZDC-inositol (1,4,5) trisphosphate (IP_3).[19–22] The [^{3}H]BZDC-IP_3 probe selectively labeled a 60-kDa band, while the Glc-6-P analogue again labeled predominantly bands at 34 and 40 kDa (Fig. 2C). Each of these experiments used 800 μg of protein and 0.5 μCi of radioligand with a 60 min irradiation time. The specificity of photolabeling by the [^{3}H]BZDC Glc-6-P probe was determined by competitive displacement experiments using 100 mM Glc-6-P, unlabeled BZDC Glc-6-P, or the photoinert BnDC Glc-6-P (**2**). The presence of 1 mM of either unlabeled **1** or **2** completely displaced the photocovalent

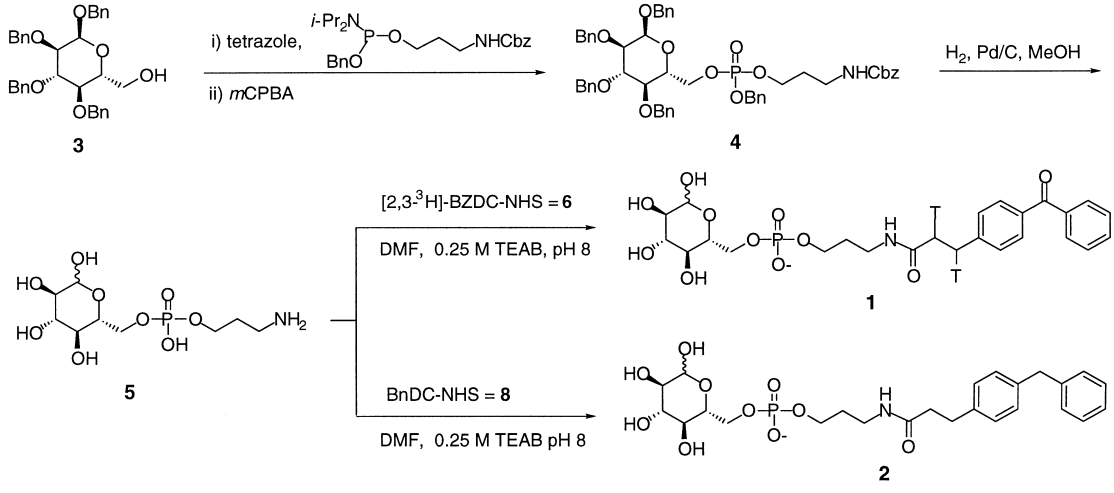

Figure 1. Synthesis of [^{3}H]BZDC-Glc-6-P (**1**) and BnDC-Glc-6-P (**2**).

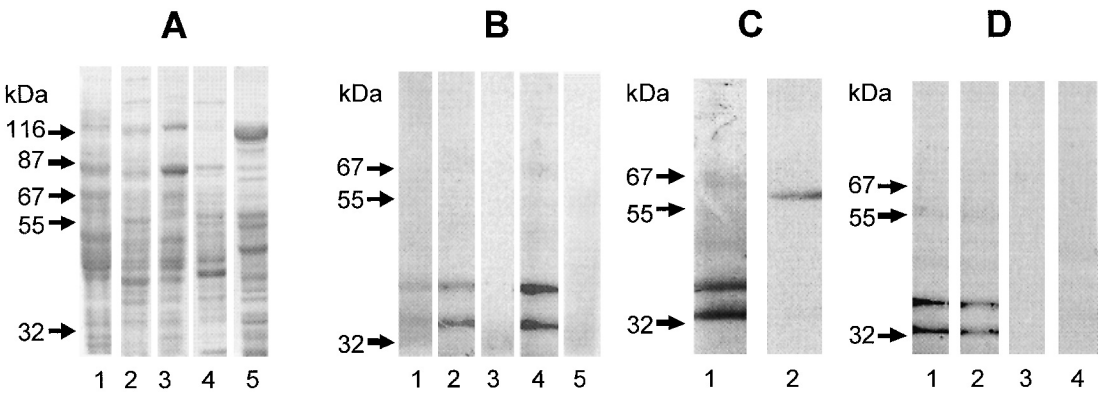

Figure 2. [^{3}H]BZDC Glc-6-P labeling of rat liver subcellular fractions. Panel A: Coomassie-blue staining of 10% SDS-PAGE of 15 μg protein per lane: lane 1, total microsomes; lane 2, SER; lane 3, RER; lane 4, Golgi; lane 5, mitochondria. Panel B: Fluorography of photoaffinity labeling reaction (800 μg protein per 0.5 μCi 1). Panel C: Comparison of fluorograms for [^{3}H]BZDC Glc-6-P labeling (lane 1) and [^{3}H]BZDC-IP$_3$ (lane 2) labeling of SER microsomes. Panel D: Fluorogram showing specificity of [^{3}H]BZDC Glc-6-P labeling of SER: no competitor (lane 1), 100 mM Glc-6-P (lane 2), 1 mM BZDC Glc-6-P (lane 3), 1 mM BnDC-Glc-6-P (lane 4).

modification of the 32 and 40 kDa proteins (Fig. 2D). In contrast, 100 mM Glc-6-P was only partially effective in suppressing the photolabeling. This suggests that the photophore is modifying a hydrophobic region of the target proteins[20,21] proximal to the Glc-6-P recognition site.

Recent studies have demonstrated that the Glc-6-P transporter is an integral ER membrane protein with a M_r of approximately 46 kDa.[4,6] In order to determine if the two proteins labeled preferentially by the photoaffinity probe were themselves integral membrane proteins, we conducted phase separation experiments with Triton X-114, a method that permits discrimination between hydrophobic and hydrophilic proteins.[23] Integral membrane proteins are found exclusively in the detergent phase, while hydrophilic proteins are recovered in the aqueous phase. Integral membrane proteins can be separated from peripheral proteins associated with the ER using sodium carbonate.[24] After Na_2CO_3 treatment, integral membrane proteins are retained in the pellet and peripheral proteins move to the supernatant. When the ER vesicles were treated with Triton X-114, the two photolabeled proteins were found exclusively in the aqueous phase. These two proteins were also recovered in the supernatant following Na_2CO_3 pretreatment. These data (not shown) suggest that the two proteins are peripherally associated with the cytoplasmic surfaces of the ER.

It appeared that the primary proteins labeled by the [^{3}H]BZDC Glc-6-P probe did not include the Glc-6-P transporter. Often, failure to photolabel a target with an externally-appended label suggests that the photophore or attachment chemistry interferes with the interaction between the normal ligand and the target protein.[16] We thus considered four other known Glc-6-P binding proteins: phosphoglucomutase (62 kDa), glucose-6-phosphatase (39 kDa), Glc-6-P-dehydrogenase (72 kDa), and phosphoglucosisomerase (132 kDa). Among these, Glc-6-phosphatase has a molecular mass similar to one of the proteins labeled with [^{3}H]BZDC Glc-6-P, although it had been shown to be an integral membrane protein.[7] The larger of the photolabeled proteins was however, not detected by an antibody specific for Glc-6-phosphatase. This antibody did nonetheless detect a protein found exclusively in the hydrophobic portion of the Triton X-114 phase separation.

Conclusion

We sought to selectively label the Glc-6-P transporter with the [^{3}H]BZDC Glc-6-P analogue using SER-enriched microsomes. Indeed, the probe selectively labeled two proteins that are preferentially associated with the SER and Golgi, but which had molecular masses distinct from that of the Glc-6-P transporter. The identity of these peripheral, not integral, membrane proteins has not yet been determined. The selectivity of the labeling was established using the non-photoactivatable and UV transparent BnDC group, since the free Glc-6-P ligand lacking the hydrophobic moiety failed to competitively displace the photolabeling of the 34 and 40 kDa proteins. Even though soluble Glc-6-P was ineffective as a competitive inhibitor, the absence of labeling of these two proteins by [^{3}H]BZDC IP$_3$ suggests that the Glc-6-P head group must indeed be in part responsible for the recognition of [^{3}H]BZDC Glc-6-P by the labeled proteins. In other words, the basis for the affinity between these proteins and [^{3}H]BZDC Glc-6-P involves both the hydrophobic photophore and the soluble Glc-6-P head group. This property has previously been exploited with analogues of Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$, in which affinity for the lipid-containing phosphoinositide ligands was subsequently demonstrated.[16,19,20] Although Glc-6-P is not a constituent of any known hydrophobic or lipid-containing biochemical structure, the linker modified Glc-6-P is congruent (epimeric at one OH group) with Man-6-P-phosphoethanolamine, the site of protein attachment to glycosylphosphatidylinositol (GPI) anchored proteins. While the identities of the 34 and 40 kDa proteins remain to be determined, it is an intriguing possibility that they could be proteins that interact with the attachment site for GPI-anchored, non-integral membrane proteins.

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17. Experimental details for synthesis: **Protected Glc-6-P analogue (4)**. A solution of benzyl 2,3,4-tri-*O*-benzyl-α-D-*arabino*-hexopyranoside (**3**) (1.00 g, 1.85 mmol), benzyloxy-[(*N*-Cbz-3-amino-1-propyl)oxy](*N*, *N*-di*iso*propylamino)phosphine (0.826 g, 1.85 mmol) and 1H-tetrazole (0.259 g, 3.70 mmol) in CH_2Cl_2 (50 mL) was stirred at rt under Ar for 2 h. The reaction mixture was cooled to −40 °C and a solution of *m*CPBA (57%, 1.12 g, 3.7 mmol) in CH_2Cl_2 (20 mL) was added via cannula. After stirring at −40 °C for 30 min, the mixture was warmed to rt, washed (satd aq Na_2SO_3, satd aq $NaHCO_3$, dried ($MgSO_4$), concentrated, and purified on SiO_2 (EtOAc: hexane, 3:2, v:v) to give **4** (1.47g, 88%) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 7.20–7.40 (m, 30H), 5.15–5.25 (m, 1H), 4.75–5.10 (m, 7H), 3.95–4.30 (m, 4H), 3.75–3.85(m, 1H), 3.40–3.55 (m, 2H), 3.21 (ddd, J = 6, 6, 6 Hz, 2H), 1.79 (dddd, J = 6, 6, 6, 6, Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 156.3, 139.5, 137.9, 137.8, 136.9, 136.4, 135.7, 135.5, 128.5, 128.4, 128.3, 128.1, 127.9, 127.8, 127.7, 127.6, 127.5, 95.4, 95.3, 81.6, 79.7, 79.6, 76.9, 75.6, 75.0, 72.8, 69.5, 69.3, 69.2, 69.1, 66.4, 66.1 (m), 65.0, 64.9, 36.9, 29.8. **Aminopropyl Glc-6-P (5)**. Precursor **4** (1.37 g, 1.52 mmol) in MeOH (40 mL) and H_2O (10 mL) was hydrogenated at 50 psi over 10% Pd/C for 24 h. The mixture was filtered and concentrated; the brown residue was dissolved in H_2O, filtered through a 0.45 μm filter, passed through a Chelex (Na^+ form) column, and concentrated to give the product **5** as a white solid (0.543 g, 99%) as a mixture of α,β isomers (α:β 1:1.3). ^{1}H NMR (D_2O) δ 5.06 (d, J = 4 Hz, H_1 of α isomer), 4.49 (d, J = 8 Hz, H_1 of β isomer), 3.70-4.15 (m, 4H), 3.05–3.60 (m, 4H), 2.99 (dd, J = 7, 7 Hz, 2H), 1.85 (dddd, J = 6, 6, 6, 6 Hz, 2H). **Succinimido-*p*-benzoylcinnamate (7)**. To a suspension of *p*-benzoylcinnamic acid (0.100 g, 0.396 mmol) and diphenyl succinimidyl phosphate (0.206 g, 0.595 mmol) in CH_3CN (15 mL) was added Et_3N (0.060 g, 0.595 mmol) and stirred (rt, 16 h). The reaction mixture was then concentrated and the crude product was purified by radial chromatography (EtOAc:hexane, 1:2, v:v) to yield unsaturated product **7** (0.137 mg, 99%) as white crystals. ^{1}H NMR ($CDCl_3$) δ 7.45–7.90 (m, 9H), 7.98 (d, J = 16 Hz, 1H), 6.70 (d, J = 16 Hz, 1H), 2.90 (s, 4H). **BnDC-NHS (8)**. Ester **7** (0.100 g, 0.285 mmol) was dissolved in EtOAc (10 mL) and hydrogenated over 10% Pd/C at 50 psi for 24 h. The mixture was filtered through Celite, concentrated, and crude product purified by radial chromatography (EtOAc:hexane, 1:2, v:v) and afforded product **8** (0.67 g, 69%) as a white solid. ^{1}H NMR ($CDCl_3$) δ 7.10–7.35 (m, 9H), 3.95 (s, 2H), 2.95–3.05 (m, 2H), 2.80–2.95 (m, 2H), 2.82 (s, 4H). ^{13}C NMR ($CDCl_3$) δ 169.1, 167.8, 139.4, 136.7, 129.0, 128.7, 128.3, 128.2, 125.9, 41.5, 32.4, 29.8, 25.4. **BnDC-Glc-6-P (2)**. Aminopropyl Glc-6-P (**5**) (0.029 g, 0.077 mmol) was dissolved in TEAB buffer (5 mL, 0.2 M, pH 8.0), and a solution of BnDC-NHS (**8**) (0.026 g, 0.077 mmol) in DMF (5 mL) was added. After stirring at rt for 24 h, the reaction was concentrated in vacuo, the residue was dissolved in water, loaded onto a DEAE column, and eluted with linear gradient (0–100 mM) of TEAB buffer (pH 8.0). Fractions containing organic phosphate were combined, concentrated, redissolved in water, and passed through a Chelex (Na^+ form) column. Concentration of the filtrate under vacuum yielded product **2** (0.025 g, 65%) as a glass-like solid. NMR showed it was a mixture of α,β isomers (α:β 1:1.4). ^{1}H NMR (D_2O) δ 6.80–7.15 (m, 9H), 5.05 (d, J = 4 Hz, H_1 of α isomer), 4.48 (d, J = 8 Hz, H_1 of β isomer), 3.50–4.00 (m, 5H), 3.56 (s, 2H), 3.20–3.40 (m, 3H), 3.05–3.20 (m, 1H), 2.97 (dd, J = 7, 7 Hz, 2H), 2.63 (dd, J = 7, 7 Hz, 2H), 2.48 (s, 1H), 2.25 (dd, J = 7, 7 Hz, 2H), 1.43 (m, 2H). ^{31}P NMR (D_2O) δ 4.1. **[^{3}H]BZDC-Glc-6-P (1)**. A solution of [^{3}H]BZDC-NHS (**6**) (1.0 mCi, 0.025 μmol) in EtOAc (0.30 mL) was transferred to a 0.5 mL plastic centrifuge tube and dried with N_2 flow. To the centrifuge tube was then added a solution of amine **5** (0.042 mg, 0.125 μmol) in a mixture (0.020 mL) of 1:1 DMF:TEAB buffer (0.25 M, pH 8.0). After stirring at rt for 24 h, the reaction was concentrated under vacuum to dryness. The resulting residue was dissolved in water (0.5 mL), loaded onto a small DEAE column, and eluted with linear gradient (0-0.10 M) of TEAB buffer (pH 8.0). Radioactivity was eluted at ca. 0.05 M TEAB to give radioligand **1** (0.14 mCi, 14% radiochemical yield).
18. Experimental details for biochemistry. **Preparation of subcellular fractions**. Total liver microsomes were isolated from livers of female Sprague–Dawley rats (250–300 g) by homogenization at 4 °C with a Polytron, speed set at 5, using a 20-s burst per 1 g tissue in 8 volumes of buffer A containing 1 mM HEPES, pH 7.5, 0.3 M sucrose, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was then centrifuged at 10000×*g* for 30 min. The resulting supernatant was centrifuged at 100000×*g* for 60 min, the pellet resuspended in buffer A, and recentrifuged at 100,000×*g* for 60 min. The final pellet was dissolved in buffer B (25 mM Tris/HCl pH 7.4, 1 mM EDTA, 1 mM K_2HPO_4). To isolate SER and RER, 10 mL of supernatant resulting from the 10,000×*g* centrifugation was floated over layers of 1.4 mL of 0.6 M sucrose–15 mM CsCl and 6 mL of 1.3 M sucrose–15 mM CsCl and centrifuged at 80,000×*g* for 1.5 h at 4 °C. SER was

collected at the lower interface and RER from the pellet. These fractions were resuspended and centrifuged at 100000×*g* for 60 min. To isolate mitochondria, liver homogenate was centrifuged at 600×*g* for 10 min. The resulting supernatant was centrifuged at 10300×*g* for 10 min. The pellet was suspended in buffer C containing 5 mM HEPES, pH 7.4, 250 mM mannitol, 0.5 mM EGTA, 0.1% BSA and then loaded onto a cushion of 30% Percoll in buffer D, containing 25 mM HEPES, pH 7.4, 225 mM mannitol, 1 mM EGTA, 0.1% BSA. This was centrifuged at 95000×*g* for 30 min. The lower part of the dense, brownish yellow mitochondrial band was collected and washed with buffer C. The final centrifugation at 6300×*g* for 10 min. **Photoaffinity labeling and electrophoresis**. Irradiations were conducted in a 96-well plate under dark conditions. To each well was added 43 μL (800 μg of protein) of a subcellular fraction, 50 μL of 2×buffer B, and 7 μL of [^{3}H]BZDC-Glc-6-P. They were incubated on ice for 10 min, and then photoactivated for 60 min using a 100-W long wavelength (365 nm) UV lamp. Next, 6×SDS sample buffer was added to the samples and they were separated on 10% SDS-PAGE. Following fixation, the [^{3}H]BZDC-Glc-6-P labeled gels were prepared for fluorography using Entensify (NEN Life Science Products, Boston, MA), dried, and placed on X-ray film at −80 °C for 7–10 days. **Treatment of membranes with Triton X-114 or Na_2CO_3**. Samples (1 mg/mL) in buffer containing 10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 2% Triton X-114 were kept on ice for 5 min and subsequently incubated at 30 °C for 3 min. Mixtures were then centrifuged at 3000×*g* for 10 min. The resulting sample was resolved into aqueous and detergent-rich phases, which were then separated by SDS-PAGE for further analysis. Aliquots of smooth ER (1 mg/mL) were treated with Na_2CO_3. The resulting pellet and supernatant were collected and analyzed by SDS-PAGE. **Immunoblot analysis**. Equal aliquots of Triton X-114 and Na_2CO_3 treated samples were fractionated by SDS-PAGE and electrotransferred to PVDF membrane for immunodetection by an antiserum specific for glucose-6-phosphatase. Electrotransferred proteins on PVDF membrane were incubated with sheep antiserum against rat Glc-6-Pase, and visualized with goat anti-sheep antibodies conjugated with horseradish peroxidase in conjunction with detection by luminescence using a commercial ECL kit.

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