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Transport function and subcellular distribution of purified human erythrocyte glucose transporter reconstituted into rat adipocytes

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In order to delineate the insulin-independent (constitutive) and insulin-dependent regulations of the plasma membrane glucose transporter concentrations in rat adipocytes, we introduced purified human erythrocyte GLUT-1 (HEGT) into rat adipocytes by poly(ethylene glycol)-induced vesicle-cell fusion and its transport function and subcellular distribution in the host cell were measured. HEGT in adipocytes catalysed 3-O-methylglucose equilibrium exchange with a turnover number that is indistinguishable from that of the basal adipocyte transporters. However, insulin did not stimulate significantly the HEGT function in adipocytes where it stimulated the native transporter function by 7–8-fold. The steady state distribution and the transmembrane orientation assays revealed that more than 85% of the HEGT that were inserted in the physiological, cytoplasmic side-in orientation at the adipocytes plasma membrane were moved into low-density microsomes (LDM), while 90% of the HEGT that were inserted in the wrong, cytoplasmic side-out orientation were retained in the plasma membrane. Furthermore, more than 70% of the LDM-associated HEGT were found in a small subset of LDM that also contained 80% of the LDM-associated GLUT-4, the insulin-regulatable, native adipocyte glucose transporter. However, insulin did not cause redistribution of HEGT from LDM to the plasma membrane under the condition where it recruited GLUT-4 from LDM to increase the plasma membrane GLUT-4 content 4–5-fold. These results demonstrate that the erythrocyte GLUT-1 introduced in adipocytes transports glucose with an intrinsic activity similar to that of the adipocyte GLUT-1 and/or GLUT-4, and enters the constitutive GLUT-4 translocation pathway of the host cell provided it is in physiological transmembrane orientation, but fails to enter the insulin-dependent GLUT-4 recruitment pathway. We suggested that the adipocyte plasma membrane glucose transporter concentration is constitutively kept low by a mechanism where a cell-specific constitutent interacts with a cytoplasmic domain common to GLUT-1 and GLUT-4, while the insulin-dependent recruitment requires a cytoplasmic domain specific to GLUT-4.

Introduction

Transport of glucose across the plasma membrane of animal cells is a highly regulated process [1]. It is mediated by a family of tissue-specific transmembrane proteins (transporters) [2–5], and further regulated by insulin in muscle and adipose cells [1,6,7]. In erythrocytes and liver cells, where glucose transport is not regulated by insulin, the transport process is very fast, and does not limit the rate of cellular glucose utilization [8,9]. In muscle and adipose cells, on the other hand, the transport process is much slower in the absence of insulin (basal or constitutive regulation), and insulin regulates the cellular glucose utilization by stimulating the transport process [7,10]. Since insulin can only stimulate the transport process in these cells,

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Abbreviations: CB, cytochalasin B; HDM, high-density microsomes; HEGT, human erythrocyte glucose transporter; KRH, Krebs-Ringer-Hepes buffer; LDM, low-density microsomes; NM, nuclear and mitochondrial membrane; PC, phosphatidylcholine; PEG, poly(ethylene glycol); PM, plasma membrane; TBS, Tris-buffered saline; WGA, wheat germ agglutinin.

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the constitutively maintained low level of the glucose transport activity in these cells is an essential prerequisite for the insulin-mediated regulation.

Glucose transporters in these insulin-sensitive cells are mostly (more than 90%) stored in intracellular vesicles in the absence of insulin, and insulin stimulates glucose transport function largely by recruiting transporters from this storage pool to the plasma membrane (Refs. 11 and 12; Hah, J.S., Jo, I.H. and Jung, C.Y., unpublished data). This is in contrast to the insulin-insensitive cells where glucose transporters are located mostly, if not entirely, in the plasma membrane even in the absence of insulin [13,14]. It thus appears that both the constitutive and the insulin-mediated regulation of glucose transport function in animal cells are largely achieved by the regulation of the steady-state distribution of glucose transporters between the intracellular storage pool and the plasma membrane. How adipocytes constitutively maintain most of their glucose transporters in the storage pool and how insulin redistributes the transporters to the cell surface are currently not known.

Adipocytes express GLUT-4 as the major isoform with a small amount (5–10%) of GLUT-1 [15]. Like GLUT-4, GLUT-1 is largely stored intracellularly in the absence of insulin and recruited to the plasma membrane in response to insulin [15]. GLUT-1 is the major isoform of transformed cells as well as of human erythrocytes where glucose transport is not regulated by insulin. GLUT-1 in these cells are mostly in the plasma membrane [1], indicating that an isoform can be distributed differently in different cells.

Whether it is a particular transporter isoform, or a cell-specific environment that determines the constitutive and insulin-mediated distribution of glucose transporters is an important unanswered question. This question has been approached by transfecting GLUT-1 transporter gene into oocytes [16] or cultured cell lines (3T3-L1 cells or CHO cells) [17,18]. Results of these studies show that the GLUT-1 gene products are either insulin-insensitive [16] or insulin-sensitive [17,18] in host cells. In these studies, transporter gene, rather than transporter protein, was introduced into host cells. Consequently, cell-specific, posttranslational modification may have modified the gene product, and thus its behavior. More importantly, the effects of insulin on the glucose transport function of these host cells could be quite different from those of freshly isolated rat adipocytes, the best studied cell model for the glucose transport regulations by insulin.

We describe here a novel approach which would complement these gene transfection experiments: We introduced HEGT (human erythrocyte glucose transporter), a GLUT-1 protein purified from human erythrocytes, directly into intact rat adipocytes by a PEG-induced, vesicle-cell fusion protocol, and studied its transport function and its subcellular distribution in host cells. Our results demonstrate that the erythrocyte GLUT-1 in adipocytes transports glucose with an intrinsic activity similar to that of the basal adipocyte GLUT-1 and GLUT-4, and enters into the basal GLUT-4 translocation pathway of adipocytes, provided it is in physiological transmembrane orientation, but fails to enter into the insulin-mediated GLUT-4 translocation pathway.

Experimental procedures

Materials. Monoclonal antibody, 64C7 specific to human erythrocyte GLUT-1 or HEGT, was prepared as described [19]. This antibody does not react with adipocyte GLUT-1. Monoclonal antibody, 1F8, specific to GLUT-4 [20], was kindly supplied by Dr. D.E. James, Washington University, St. Louis, MO and also obtained from Eastacres (Boston, MA). Protein A, *Staphylococcus aureus*, was purchased from Calbiochem (San Diego, CA). Anti-mouse IgG-coated Magnisort-M was obtained from DuPont (Wilmington, DE). Insulin (porcine, crystalline) was a gift from Eli Lilly & Co. (Indianapolis, IN). PEG, trypsin, phenylmethylsulfonyl fluoride, cytochalasins B and E were supplied by Sigma (St. Louis, MO). Wheat germ agglutinin (WGA) was purchased from E-Y Laboratories. Sepharose 4B and Percoll were from Pharmacia. [^{3}H]Cytochalasin B (22 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). ^{125}I-labeled goat anti-mouse IgG, ^{125}I-labeled protein A and [^{14}C]PC were from New England Nuclear (Boston, MA). [*Carboxyl*-^{14}C]Inulin and ^{14}C-labeled 3-O-MeGlc were from ICN Pharmaceuticals, Inc. (Irvine, CA). All other chemicals were reagent grade and from standard sources.

Preparation and radiolabeling of HEGT in vesicles. HEGT vesicles were prepared as described [21]. The preparation contained protein and phospholipid at a mass ratio of 0.31 ± 0.16 (mean $\pm$ S.E., $n = 6$). For protein labeling of HEGT vesicles, photoincorporation of [^{3}H]CB was performed as described [21]. For lipid labeling, aliquots of [^{14}C]PC in a mixture of toluene and ethanol (1 : 1, v/v) were dried under a stream of nitrogen gas, suspended directly with octylglucoside-solubilized HEGT and sonicated for 1 min. HEGT-lipid micellar solution was diluted 20-fold with 100 mM NaCl, 50 mM Tris-HCl (pH 7.4), containing 1 mM dithiothreitol and 0.1 mM EDTA, and centrifuged at $170000 \times g$ for 60 min to remove detergent. Protein- and lipid-labeled HEGT vesicles were washed twice with KRH (130 mM NaCl, 4.7 mM KCl, 1.25 mM $MgSO_4$, 2.5 mM NaH_2PO_4, 2.5 mM $CaCl_2$ and 10 mM Hepes, adjusted to pH 7.4 with 10 M NaOH).

Isolation and subcellular fractionation of rat adipocytes. Male Sprague-Dawley rats (160–220 body weight

g) were killed. Adipocytes were isolated from epididymal fat pads as described [22]. For subcellular fractionation, cells were washed once with STEP buffer (0.25 M sucrose, 2 mM EGTA and 10 mM Tris-HCl, pH 7.4 at 20°C, with 10 μM phenylmethylsulfonyl fluoride) at room temperature, resuspended in 15–20 ml of this same buffer, and homogenized at 2000 rpm using 10 up-and-down strokes of a teflon pestle (Thomas Scientific). Unless otherwise noted, all further steps in the fractionation procedure were carried out using STEP buffer and at 4°C. The homogenate was centrifuged at $2000 \times g$ for 30 s and all fat cake was removed. Infranatant and pellet were again centrifuged at $16000 \times g$ for 15 min. The resulting pellet (NM-PM) was further fractionated into PM and NM, according to Belsham et al. [23]. HDM and LDM were prepared from the $16000 \times g$ supernatant according to Simpson et al. [14], with a slight modification as detailed previously [24].

Isolation and subcellular fractionation of hepatocytes. Rat hepatocytes were prepared from in situ collagenase perfused liver as described by Seglen [25]. Isolated rat hepatocytes were subfractionated according to Belsham et al. [23], and Fleisher and Kervina [26].

Measurement of 3-O-MeGlc flux. Equilibrium exchange influx of 3-O-MeGlc in adipocytes either before or after fusion was measured using 3-O-[^{14}C]MeGlc as a tracer, and a modification of oil flotation method [6] as detailed elsewhere [22].

Fusion of HEGT vesicles with adipocytes and hepatocytes. HEGT vesicles were sonicated in ice-cold water using a bath-type sonicator for 2 min. For fusion with adipocytes, the cell suspension was centrifuged at $230 \times g$ for 30 s using an International Clinical Centrifuge (Needham Heights, NY) to pack the cells at approx. 50% cytocrit. 8–10 ml of this cell suspension (containing adipocytes from 8–10 rats) was mixed with a varying amount (up to 200 μl) of HEGT vesicle suspension (1 mg protein/ml), and transferred into 5 ml pipet tips whose bottoms were closed with parafilm (American Can Company, Greenwish, CT). Each 5 ml pipet tip received 2 ml of the mixture at most. The mixtures were overlaid with 10% PEG 8000 (mol. wt. 1000–8000) in KRH buffer, shaken head-to-head twice and held for 2–3 min. The mixtures were transferred into a syringe containing at least 10 times the mixture volume of KRH buffer, held for 3–5 min at room temperature to allow the cells to float, and the infranatant was removed. This washing procedure was repeated 3–5-times to remove all unfused vesicles. The entire fusion protocol including washing took 30–40 min. For the fusion with hepatocytes, 1 ml of cell hepatocyte suspension (cytocrit of approx. 80% in KRH) was mixed with 50–100 μg protein of sonicated HEGT vesicles. The mixture was overlaid with 20% (final) PEG 8000 and incubated for 5 min at 20°C. The mixture was then diluted at least 10-fold with KRH buffer and held for 10 min. Fused hepatocytes were recovered as pellets by centrifugation at $200 \times g$ for 4 min. This washing procedure was repeated 4–5-times to free unfused HEGT vesicles and PEG.

Incorporation of HEGT to adipocytes after fusion was assessed using HEGT vesicles photolabeled with [^{3}H]CB. Fused cells were centrifuged at $9000 \times g$ for 1 min with a Beckman Microfuge B (Beckman, Fullerton, CA). Cell layers were collected, dissolved with 0.1% Triton X-100 and incubated in ice for 30 min. Triton X-100 extracts were centrifuged and the fat cakes were removed carefully. Both infranatant and pellet were used to measure protein amounts and associated radioactivities.

Immunoadsorption assay. This was used to assess the transmembrane orientation of HEGT in the plasma membrane of intact fused adipocytes. Cells were incubated with an excess of 6HC7 (10 μg/mg cell protein) at 4°C for 30 min with occasional shaking, washed three times with 10 vol. of KRH buffer, then incubated with [^{125}I]protein A (5 μg/mg cell protein, 7.3 μCi/μg) at 4°C for 30 min with occasional shaking. After removing the infranatant, a portion of the cells was applied directly to a microfuge tube containing 100 μl silicone oil, and centrifuged for 1 min. The remaining portion of the cells was used to measure cytocrit and protein. Cell-associated radioactivities were counted with a Packard gamma counter (Chicago, IL). Bound protein A was calculated from % bound radioactivities and the specific radioactivity of the ^{125}I-protein A. The immunoadsorption assay detected $51 \pm 6\%$ (mean $\pm$ S.E., $n = 3$) of total HEGT, or $93 \pm 6\%$ (mean $\pm$ S.E., $n = 5$) of accessible (cytoplasmic side out) HEGT in intact HEGT vesicles (assessed by susceptibility to tryptic digestion).

Tryptic susceptibility assay. A mild digestion with trypsin which cleaves HEGT only from the cytoplasmic side, was used to assess transmembrane orientation of HEGT in HEGT vesicles or subcellular fractions of fused adipocytes, using [^{3}H]CB-labeled HEGT. Without sonication or freezing-thawing, vesicles were resuspended into 150 mM NaCl, 5 mM sodium phosphate buffer (pH 8.0), at protein concentrations of 1 mg/ml, and incubated with TPCK-treated trypsin (10 μg/ml) at 37°C for 60 min with occasional shaking. Proteolysis was stopped by the addition of 50 μg/ml of phenylmethylsulfonyl fluoride. The mixture was diluted 5-fold with 5 mM sodium phosphate buffer, and centrifuged at $170000 \times g$ for 60 min. The pellets were solubilized and the proteins were separated by gel electrophoresis according to Laemmli [27] using 12% polyacrylamide. A more rigorous digestion condition including sonication was used for digestion of the entire population of HEGT.

WGA-Sepharose affinity column chromatography.

WGA-Sepharose was prepared by coupling WGA to cyanogen bromide-activated Sepharose 4B (5 mg WGA/ml gel) according to Porath et al. [28], poured to a column (1 × 10 cm), and equilibrated with the buffer. Without sonication or freezing-and-thawing, fractionated vesicles were resuspended into 150 mM NaCl, 5 mM sodium phosphate buffer (pH 8.0) at a protein concentration of 1 mg/ml, applied to the column, and unbound vesicles were removed from the column by wash with the same buffer. The bound vesicle population was eluted with *N*-acetyl-D-glucosamine.

Immunoprecipitation of HEGT and GLUT-4 vesicles in LDM. Anti-mouse IgG-coated Magnisort-M was washed four times with TBS (pH 7.4) using a magnet (Bio-Rad). Washed Magnisort-M was then incubated with 1% BSA at room temperature for 1 h with shaking, and again washed four times with TBS. Microsomal fractions (LDM or the 48000 × *g* supernatant) isolated from fused adipocytes were first incubated with prewashed Magnisort-M to remove any materials which bind nonspecifically to Magnisort-M. The washed microsomes (200 μg protein/ml) were then incubated with 64C7 or 1F8 (25–30 μg protein/ml) overnight at 4°C. BSA-treated Magnisort-M was then added to this mixture and incubated at room temperature for 2 h with shaking. The supernatant was removed from Magnisort beads and the beads were washed four more times with TBS. The bound microsomal vesicles were released by lowering pH to 3.1, solubilized by Laemmli solubilizing solution and applied to 10% SDS/PAGE for immunoblotting.

TABLE I

Effects of fusion on adipocyte transport function

Equilibrium exchange influx of 5 mM 3-O-MeGlc was measured at 37°C by following the tracer equilibrium exchange time course measured at six different time points (time zero, then every 3–4 s for insulin-treated cells and every 10–15 s for basal cells) and at 30 min. Data were then analyzed in a semilog plot to obtain the half-equilibration time ($t_{1/2}$) as described elsewhere [22]. Rates of exchange (k) were calculated from the relationship, $k = 0.693/t_{1/2}$, and expressed relative to that of the basal transport activity of unfused control.

Experimental conditions	$t_{1/2}$ (s)		k (relative)
Control (non-treated)	95.0 ± 5.1 [a]	(22)	1.00 ± 0.08
HEGT vesicles only	90.5 ± 15	(23)	1.05 ± 0.40
+ PEG (10%, 8000) only	88.3 ± 8.4	(7)	1.12 ± 0.11
+ Insulin [b]	12.5 ± 1.3	(5)	7.68 ± 0.78
+ PEG + insulin	11.0 ± 1.12	(3)	7.91 ± 0.81
Fused [c]	23.6 ± 2.1	(23)	3.52 ± 0.28
Fused [c] + insulin	8.07 ± 1.2	(3)	11.90 ± 1.71

[a] Values are means ± S.E., with the number of measurements shown in parentheses.
[b] Incubated with 7 nM insulin for 30 min at 37°C.
[c] 20 μg HEGT per ml cells and 10% PEG 8000 were used.

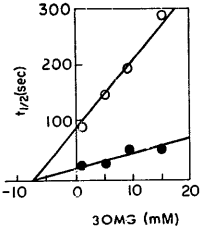

Fig. 1. Determination of kinetic parameters V_{max} and K_m by a linear transformation of the Michaelis-Menten equation [22]. Equilibrium exchange time courses were measured at 1, 5, 10 and 15 mM 3-O-MeGlc, and $t_{1/2}$ were calculated. Without (○) and with (●) fusion. Each data point represents the tracer exchange time course measured at six different time points as detailed in the legend to Table I.

SDS gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli [27]. After staining and destaining, gel lanes were serially sliced and radioactivities were counted in LKB liquid scintillation counter (Pharmacia LKB 1209, Rackbeta, MD) as described [21].

Immunoblot analysis. Proteins on SDS-polyacylamide gels were transferred to nitrocellulose paper in buffer containing 20% methanol, 192 mM glycine, 25 mM Tris (pH 8.3). The paper was blocked with 3% BSA in TBS containing 0.2% Tween 20 for 1 h, incubated with antibody in TBS with 2% BSA overnight at room temperature, and washed four times with TBS in the presence of 0.2% Tween 20 [29]. The antigen-antibody complexes were quantitated using ^{125}I-protein A for 64C7 and with goat anti-mouse ^{125}I-IgG for 1F8. Nitrocellulose paper was washed four times with TBS, dried and autoradiographed at −70°C. Radioactivities associated with blots were then counted in Packard Auto-gamma counter. Protein was assayed by the method of Bradford [30] using γ-globulin as standard.

Results

Glucose transport function of HEGT-fused adipocytes

HEGT was reconstituted into intact adipocytes by a PEG-induced fusion protocol as described in Experimental procedures. Equilibrium exchange influx of 3-O-MeGlc by adipocytes was measured prior to and after fusion with HEGT vesicles (Table I). The transport rate was increased significantly (3–4-fold) after fusion. The rate of 3-O-MeGlc exchange was not affected when adipocytes were incubated with HEGT vesicles without PEG, or with PEG in the absence of HEGT vesicles. Kinetic analysis (Fig. 1) indicated that

the sugar exchange in control and fused adipocytes show an essentially identical affinity with an estimated K_m value of 7 mM.

The effect of insulin on adipocytes fused with HEGT was also studied (Table I). In control (nonfused) adipocytes, insulin treatment caused a large (7–8-fold) increase in 3-O-MeGlc exchange rate. Adipocytes treated with PEG alone (without HEGT vesicles) increased the transport rate by the same extent after the insulin treatment. This demonstrates that the insulin-sensitivity of adipocytes was not affected by PEG. Furthermore, the 3-O-MeGlc exchange in fused cells was also stimulated by insulin treatment. Fusion followed by insulin treatment increased the flux by 11.9-fold, whereas fusion alone increased the flux by 3.5-fold (Table I).

The transport activity of fused cells increased with an increasing amount of HEGT used for fusion (Table II). With HEGT up to 100 μg per ml cell suspension, the increase was linearly proportional to the amount of HEGT used (Table II). Further increase in the amounts of HEGT beyond 100 μg per ml of the cell suspension, however, increased transport rate only slightly.

Subcellular distribution of HEGT in fused cells

The steady-state subcellular distributions of HEGT incorporated in adipocytes were measured with the cells fused with [^{3}H]CB-labeled HEGT vesicles and incubated for 45–60 min at 37°C (Fig. 2). The relative specific distributions (per mg protein and normalized against that of PM) of HEGT in NM, PM, HDM and LDM were 0.06 ± 0.01, 1.00, 2.34 ± 0.17 and 1.36 ± 0.14 (mean ± S.E., n = 3), respectively. More than 32% of the total cellular HEGT incorporation was recovered in LDM. Very little HEGT was found in NM, and a large portion (up to 49% of the total cellular incorporation) of HEGT was found in HDM. Significantly, HEGT was more concentrated in LDM than in PM.

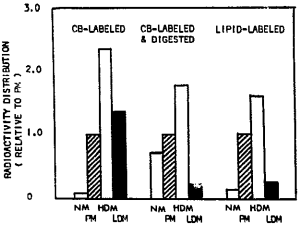

Fig. 2. Subcellular distribution of HEGT, trypsin-digested-HEGT and lipid-components of HEGT vesicles after incorporation into adipocytes by fusion. HEGT vesicles were labeled with [^{3}H]CB and [^{14}C]PC to tag the protein and lipid, respectively. The trypsin-digested HEGT was prepared by sonicating the digestion mixture for 10–20 s at 4°C to introduce trypsin into the inside of HEGT vesicles, then digesting in a low ionic-strength buffer containing 5 mM sodium phosphate (pH 8.0). Under these conditions the entire population of HEGT of the HEGT-vesicles was digested. Labeled HEGT vesicles were fused with adipocytes and incubated for 30 or 60 min at 37 C (stabilization), followed by subcellular fractionation as described in Experimental procedures. Data were analyzed and calculated as relative distribution per mg protein in each fraction relative to that in PM. Essentially identical results were obtained for the 30 and 60 min post-fusion incubations.

TABLE II

Calculation of the turnover number of fused HEGT relative to that of the native adipocyte glucose transporter

HEGT [a] added	Transporter concentrations in PM [b]		Relative transport activities [c]		Relative specific transport activities [d]		Relative turnover numbers [e] HEGT/AGT
	HEGT	AGT	overall	HEGT contribution	HEGT	AGT	
0	0	7.96	1.0	0	0	0.125	
10	9.6	7.96	2.1	1.1	0.115	0.125	0.92
20	18.7	7.96	3.4	2.4	0.128	0.125	1.036
40	31.4	7.96	4.8	3.8	0.121	0.125	0.968
100	49.6	7.96	7.1	6.1	0.123	0.125	0.984
300	202	7.96	7.7	6.7	0.034	0.125	0.272

[a] HEGT (μg) used for fusion per ml of packed cells.

[b] Amounts of HEGT (pmol/mg of protein) were assessed from incorporation of the radioactivity of [^{3}H]CB-labeled HEGT vesicles in fusion, and from the specific CB binding activity (16 nmol per mg protein) of HEGT vesicles. The amount of native adipocyte glucose transporters abbreviated as AGT here, was quantitated by measuring glucose-sensitive cytochalasin B binding activity, which was 7.96±0.9 pmol/mg of unfused adipocyte plasma membrane protein (mean ± S.E., n = 5). This value is used for all fused adipocytes.

[c] Equilibrium exchange influx of 5 mM 3-O-MeGlc was measured at 37°C as illustrated in Fig. 1. Relative transport activities are calculated as the ratio of the half-equilibration time ($t_{1/2}$) of control to that of fused cells for overall, and ($t_{1/2}$ control/$t_{1/2}$ fused)−1 for HEGT contribution. In this expression, contribution of native adipocyte transporter is 1.0.

[d] Ratio of relative contribution to transport activity to transporter concentration.

[e] Relative turnover numbers were calculated from the ratio of relative specific transport activity of HEGT to that of AGT.

When [^{14}C]PC-labeled HEGT vesicles were used in fusion experiments, the subcellular distribution of the radioactivity in host cells was quite different from that of [^{3}H]CB-labeled HEGT vesicles (Fig. 2), showing 0.13 ± 0.01, 1.00, 1.74 ± 0.09 and 0.41 ± 0.14 (mean ± S.E., n = 3) in NM, PM, HDM and LDM, respectively. Less than 12% of the incorporated lipid label was recovered in LDM, this is significantly less than the [^{3}H]CB label found in LDM (> 32%). This clearly indicates that the bulk lipid and the protein in fused HEGT vesicles went to LDM largely by separate and independent routes and not as an intact HEGT vesicle.

Finally, the relative subcellular distribution of fused HEGT in adipocytes was found to be greatly affected by a pretreatment of HEGT vesicles with trypsin at a low ionic strength, the condition known to cleave the cytoplasmic domain of the entire population of HEGT regardless of its transmembrane orientation (see Fig. 2 legends). When such a trypsin-digested, [^{3}H]CB-labeled HEGT was used for fusion, the relative distribution of the truncated HEGT was shown to be 0.71 ± 0.12, 1.00, 1.81 ± 0.21 and 0.32 ± 0.04 (mean ± S.E., n = 3) in NM, PM, HDM and LDM, respectively (Fig. 2). Only 7–8% of the HEGT went to LDM. Two points should be noted here. First, there was a great reduction in HEGT distribution in LDM compared with that of the non-digested HEGT-fusion. Second, except for an increase in HEGT-association with the NM fraction, the distribution pattern was very similar to that of the [^{14}C]PC-labeled-lipid component of HEGT vesicles.

When HEGT vesicles were fused to rat hepatocytes, very little HEGT went to the hepatocyte LDM (Fig. 3). A steady-state distribution of 1.00, 0.21 ± 0.04, 0.05 ± 0.01 (mean ± S.E., n = 3) in PM, HDM and LDM, respectively, was calculated. Less than 2% of the total cellular incorporation of HEGT was recovered in LDM fraction.

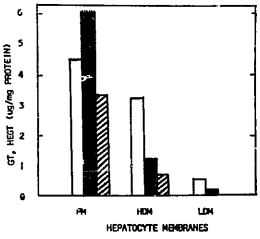

Fig. 3. Subcellular distribution of HEGT after incorporation into rat hepatocytes by fusion. HEGT-vesicles were labeled with [^{3}H]CB. Fusion was performed using 60 μg HEGT for 3 ml of 80% hepatocyte suspension with 20% PEG 8000. Results of three independent experiments were shown separately (open, solid and hatched).

TABLE III

Susceptibilities of HEGT in the PM, HDM and LDM of fused adipocytes and in HEGT vesicles to trypsin digestion and WGA-affinity binding

Adipocytes were fused with [^{3}H]CB-labeled HEGT and subjected to subcellular fractionation. Each fraction, and HEGT vesicles as well, was digested with trypsin and separated on SDS-gel electrophoresis, or subjected to WGA-Sepharose chromatography as detailed in Experimental procedures. The degree of the trypsin digestion was estimated from radioactivities of the 45–66 kDa band (undigested HEGT) and those of the 19 kDa band (digested fragment). The degree of WGA binding was calculated from the total radioactivity applied and the radioactivity retained in the column. Values are mean ± S.E., with the number of experiments shown in parentheses.

Samples	% Trypsin Digestion%	Binding to WGA
Fused adipocyte PM [a]	71.9 ± 9.5 (3)	32.2 ± 6.3 (3)
Fused adipocyte HDM	60.7 ± 9.4 (3)	41.6 [b]
Fused adipocyte LDM	86.7 ± 8.8 (3)	10.9 ± 2.1 (3)
Intact HEGT vesicles	55.3 ± 8.1 (4)	98.6 ± 6.5 (3)

[a] Relative adbundances of inside-out and right-side-out vesicles of the adipocyte plasma membrane are not known.

[b] Single determination.

Transmembrane orientation of fused HEGT in adipocytes

Because of the well-known molecular asymmetry of HEGT across the cell membrane [7], an interesting and relevant question in reconstitution of HEGT into cells via fusion concerns its transmembrane orientation in host cell membranes. The HEGT in the vesicle preparation used here is known to be in a mixed transmembrane orientation [32,33]. Results of controlled trypsin-digestion indicated that approx. 55% of HEGT in this preparation are in cytoplasmic side-out orientation and the remaining 45% are in cytoplasmic side-in orientation (Table III).

The transmembrane orientation of fused HEGT in the host cell plasma membrane was determined by measuring the binding of a HEGT-specific monoclonal antibody 64C7 to intact, fused adipocytes (Table IV). This antibody is known to bind to HEGT only at the cytoplasmic domain [19]. Analysis of the antibody binding and other relevant data shown in Table IV indicated that the HEGT in the plasma membrane of fused adipocytes are mostly (88.3%) in non-physiological transmembrane orientation exposing its cytoplasmic domain to the extracellular milieu.

The transmembrane orientation of HEGT in the LDM of fused adipocytes was determined from the data of controlled trypsin digestion and WGA-Sepharose affinity column chromatography (Table III). LDM isolated from the adipocytes fused with [^{3}H]CB-labeled HEGT vesicles was digested with trypsin under the controlled condition where only those HEGT whose cytoplasmic domain is exposed are digested as described in Experimental procedures. The results summarized in Table III clearly indicate that the suscepti-

bility of HEGT to the controlled trypsin digestion is much greater in LDM than in the intact HEGT-vesicles used for fusion. This demonstrates that HEGT in LDM of fused adipocytes are mostly (87%) in cytoplasmic side-out orientation in LDM. This is not expected if HEGT in LDM were largely contaminating intact HEGT vesicles entered through non-fusion pathways. The tryptic susceptibility of HEGT in HDM, on the other hand, was only slightly greater than that of HEGT in intact HEGT vesicles (Table III). This suggests that HEGT in HDM are largely contaminating intact HEGT vesicles. WGA binds to the cell surface-specific carbohydrate moiety (*N*-acetyl-D-glucosamine) of glycoproteins [27]. When LDM was isolated from adipocytes fused with [^{3}H]CB-labeled HEGT, then applied to WGA-Sepharose column, very little radioactivity was retained in the column (Table III), again indicating that HEGT in LDM is mostly (89%) in the cytoplasmic-side-out orientation.

Isolation of HEGT-containing intracellular storage vesicles in fused adipocytes

In an effort to identify the putative vesicular transport pathway for fused HEGT to enter into LDM in relation to the native, GLUT-4 transport pathway to the storage vesicles in adipocytes, we examined if fused HEGT and GLUT-4 share the storage vesicles in fused adipocytes. LDM was isolated from the adipocytes fused with [^{3}H]CB-labeled HEGT vesicles, then subjected to immunoadsorption with 64C7 in KRH buffer with no detergent. When immunoadsorbed vesicles were precipitated using anti-mouse IgG-coated Magnisort-M beads, as much as 76.2 ± 4.1% (mean ± S.D. for four determinations) of [^{3}H]CB radioactivity of the LDM was recovered. This precipitate contained only 4.2 ± 1.2% (mean ± S.D. for four measurements) of the total LDM protein. When this immunoprecipitate and the resulting supernatant were subjected to semi-quantitative Western blot analysis using monoclonal antibodies specific to HEGT (64C7) and the native adipocyte glucose transporter (1F8), most of the immunoreactivities in LDM were recovered in the precipitate for both antibodies (72 ± 11 and 81 ± 9%, with 64C7 and IF8, respectively, $n = 4$) and little remained in the supernatants (Fig. 4). When these experiments were repeated with LDM of unfused adipocytes, 64C7 showed no immunoprecipitation and no reactivity in Western blots (not illustrated). Essentially similar findings were obtained when 1F8 instead of 64C7 was used for the immunoprecipitation step (not illustrated). These results suggest that the erythrocyte GLUT-1 in fused adipocyte share a common intracellular storage compartment with the native adipocyte GLUT-4.

TABLE IV

Calculation of % abundance of fused HEGT that is in cytoplasmic side-out orientation at the plasma membrane of intact adipocytes based on 64C7 immunoadsorption data

64C7 binding to intact fused adipocytes (pmol/ml cells)	17.4 ± 0.62 [a] ($n = 3$)
HEGT incorporation (μg/mg PM protein)	1.17 ± 0.21 ($n = 18$)
Total protein recovered in PM (mg/ml cells)	0.236 [b]
PM protein recovery (%)	23.2 [c]
Calculated PM protein (mg/ml cells)	1.052 (0.236/0.232)
HEGT recovered (μg/ml cells)	1.23 (1.17 × 1.052)
HEGT (pmol/ml cells)	19.7 [d] (16.0 × 1.23)
Calculated detectability (%)	88.3 (17.4/19.7) × 100

[a] HEGT-specific antibody, 64C7 was incubated with intact fused cells in suspension at 4°C for 30 min. Bound antibody was detected using ^{125}I-protein A as detailed in Experimental procedures.

[b] Calculated from PM protein recovery (mg/rat) of 0.118 ± 0.013 (mean ± S.E., $n = 32$) and adipocyte recovery (ml/rat) of 0.484 ± 0.033 (mean ± S.E., $n = 31$).

[c] Data from Belsham et al. [23].

[d] The total cytochalasin B binding activity of purified HEGT measured by Scatchard analysis was 16.0 ± 0.12 pmol/μg protein (mean ± S.E., $n = 12$).

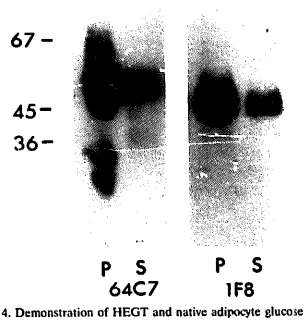

Fig. 4. Demonstration of HEGT and native adipocyte glucose transporter in a common subpopulation of microsomes of fused adipocytes by immunoblotting. 200 μg (protein) of LDM of fused adipocytes was immunoprecipitated with 25 μg of 64C7 as described in the text. Both the immunoprecipitate and the supernate were divided into two equal parts, run on 10% acrylamide gel, transferred to nitrocellulose paper and immuno-blotted with 64C7 and IF8 (5 μg/ml each). Lanes P and S are immunoprecipitate and the corresponding supernatant, respectively. Positions of three molecular weight markers are indicated on the left margin of the blots with molecular masses in kDa. Nonimmune sera did not give any detectable blot (not illustrated).

Effects of insulin on subcellular distribution of fused HEGT in adipocytes

Adipocytes fused with HEGT were incubated with or without 7 nM insulin at 37°C for 20–40 min, and the

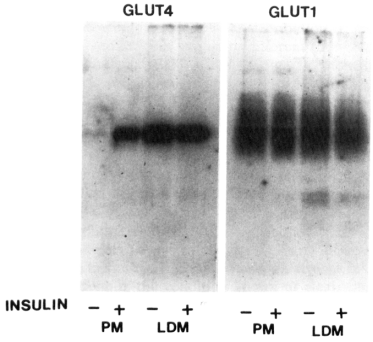

Fig. 5. Effects of insulin treatment on the steady-state distribution of HEGT (GLUT-1) and native adipocyte glucose transporter (GLUT-4) between NM-PM and LDM in fused adipocytes. NM-PM and LDM (45 μg protein each) were immunoblotted as in Fig. 4 with 64C7 and IF8. Fused adipocytes were incubated for 30 min at 37°C with (+) or without (−) 7 nM insulin prior to subcellular fractionation.

subcellular distribution of native GLUT-4 and fused HEGT in NM-PM and LDM were measured by semi-quantitative Western blot analysis using 1F8 and 64C7, respectively (Fig. 5). The native adipocyte transporter in fused adipocytes was translocated from LDM to PM in response to insulin; ^{125}I-goat anti-mouse IgG associated with the blots were 642 ± 61 and 7388 ± 980 cpm in NM-PM and LDM, respectively, without insulin, and 3137 ± 361 and 4088 ± 781 cpm in NM-PM and LDM, respectively, with insulin (n = 3). HEGT distribution, on the other hand, was not affected significantly by the insulin treatment under the same conditions; ^{125}I-protein A associated with the blots were 4863 ± 663 and 6433 ± 896 cpm in NM-PM and 5DM, respectively, without insulin, and 4804 ± 731 and 5896 ± 831 cpm in NM-PM and LDM, respectively, with insulin (n = 3).

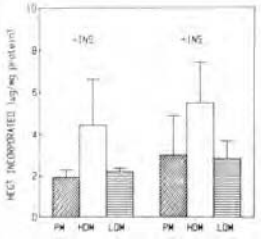

Fig. 6. The effects of insulin treatment on the subcellular redistribution of [^{3}H]CB-labeled HEGT in fused adipocytes. Fused cells were prepared using 20 μg HEGT protein for each ml of adipocyte suspension of 50% cytocrit as described in Experimental procedures, and incubated with (+INS) or without (−INS) 7 nM of insulin at 37°C for 1 h prior to subcelluur fractionation. Results of four independent sets of experiments were pooled. Values shown are mean with S.E. (vertical bars).

Effects of insulin on HEGT distribution in fused adipocytes were also studied using [^{3}H]CB-labeled HEGT vesicles for fusion (Fig. 6). There was no significant redistribution of HEGT among different subcellular fractions of fused adipocytes after the insulin treatment. In hepatocytes, the subcellular distributions of both HEGT and the native hepatocyte transporter (GLUT-2) were also not affected by insulin (not illustrated).

Discussion

Reconstitution of erythrocyte GLUT-1 into adipocytes by fusion

Adipocytes incorporated with HEGT by PEG-induced fusion showed a large, dose-dependent increase in basal glucose transport function, and this was further stimulated by insulin (Table I). Treatment with PEG alone or HEGT vesicles alone did not produce any significant change in glucose transport activity for both basal and insulin-treated adipocytes (Table I), clearly demonstrating that the fusion protocol does not derange either host cell glucose transport function or its response to insulin. Furthermore, the increase in transport activity is exclusively due to an increase in V_{max} with no change in K_m (Fig. 1). This increase is directly proportional to the amount of HEGT inserted in the host cell plasma membrane provided fusion does not exceed a specified maximum (Table II). These findings demonstrate that this PEG-induced, vesicle-cell fusion protocol can be used to reconstitute and study the function of a purified membrane protein in any given animal cell environment.

Transport activity of erythrocyte GLUT-1 in adipocytes

HEGT incorporated into adipocytes by fusion is highly functional catalyzing 3-O-MeGlc exchange in host cells. Since no change in transport affinity was observed by fusion with HEGT, the turnover numbers of the native adipocyte glucose transporters and the fused HEGT in adipocytes can be compared by knowing the amounts of the native transporter and fused HEGT in host cell plasma membrane, and the transport activities of nonfused adipocytes and HEGT-fused adipocytes. The calculation illustrated in Table II suggests that the turnover numbers of HEGT and of native adipocyte transporters are not greatly different if not equal for the basal adipocytes. This calculation, however, assumes that fused HEGT are equally functional regardless of their transmembrane (inside-out or

right-side-out) orientation (Table IV). The basal adipocyte plasma membrane contains approximately 70% GLUT-4 and 30% GLUT-1 [15]. The practicaliy identical turnover numbers of HEGT and the native adipocyte transporters in fused adipocytes observed here demonstrate that the intrinsic activities of erythrocyte GLUT-1 and GLUT-4 are not too different. There have been conflicting findings on the turnover rates of GLUT-1 and GLUT-4. It has been suggested that the adipocyte GLUT-1 has a greater intrinsic activity than GLUT-4 in adipocyte [15], although a mRNA injection experiment suggested that the turnover number of GLUT-1 is 10-times larger than that of GLUT-4 in oocytes [39].

Cell-specific constitutive redistribution of fused erythrocyte GLUT-1

The subcellular distribution of HEGT was studied after 45–60 min incubation at 37°C in KRH buffer. This incubation (stabilization incubation) was necessary for fused cells to regain a low basal transport activity typical to unfused adipocytes. During this incubation, like other fused integral proteins [40], HEGT fused to the host cell surface membrane have redistributed themselves from the plasma membrane to intracellular organelles (Fig. 2). Adipocyte glucose transporters photolabeled with cytochalasin B were shown to redistribute themselves in intact adipocytes in response to insulin [41] (Fig. 6). Immunoblotting data (not illustrated) show that the relative distribution of adipocyte GLUT-4 between LDM and PM in fused adipocytes is the same as that in unfused control adipocytes, demonstrating that the fusion protocol did not damage the adipocyte transporter distribution pathways.

In adipocytes, a large portion (29–32%) of fused HEGT protein goes to LDM, while very little HEGT vesicle-lipid (10–12% of the cellular incorporation) goes to LDM (Fig. 2). This indicates that at least 80% of the HEGT association in LDM is via a protein-specific route. This pathway requires intact cytoplasmic domain of HEGT, as very little trypsin digested HEGT (7–8% of the total cellular incorporation) goes to LDM (Fig. 2). Very little HEGT goes to LDM in hepatocytes (Fig. 3). This suggests that HEGT enters into LDM in adipocytes via a cell-specific pathway of adipocytes.

Moreover, the fact that both the fused HEGT and the native adipocyte GLUT-4 are concentrated in the same small subset of LDM in adipocytes (Fig. 4) demonstrates that HEGT enters into this compartment via the host cell's GLUT-4 translocation pathway. It is important to note here that 64C7 does not immunoreact with the adipocyte GLUT-1. In rat adipocytes, the native GLUT-1 and GLUT-4 are known to occur in two distinct subsets of intracellular vesicles, the latter being the predominant isoform recruited by insulin [42]. In 3T3-L1 adipocytes, however, the two isoforms apparently share a common intracellular pool [43].

Importance of the cytoplasmic domain for cell-specific redistribution of HEGT in adipocytes was further indicated by the fact that only those HEGT in physiological orientation is translocatable (Tables III and IV). The internalization of receptors for low-density lipoprotein [44], epithelial growth factor [45] and transferrin [46] are all known to require intact, specific cytoplasmic domains, although the internalization of transporters and receptors are probably dictated by distinct structural determinants in cytoplasmic domain. The HEGT fused to adipocyte PM in cytoplasmic side-out, non-physiological orientation are mostly retained in PM (Table IV), whereas most of the HEGT fused to the host cells in cytoplasmic side-in, physiological orientation move to LDM (Table III). If only those HEGTs that are fused in the physiological transmembrane orientation are considered, they are 9.8-times more concentrated in LDM than in PM. These relative, steady-state pool sizes are very similar to that of GLUT-4 in adipocytes in the absence of insulin, 7–10 times higher in LDM than in PM [42,47,48].

The following sequence of events is consistent with the observations discussed above. Upon fusion, HEGT in the host cell (adipocytes or hepatocytes) plasma membrane are expected to be oriented nearly randomly as in purified HEGT vesicles (where approx. 45% are in the physiological orientation, Table III). For adipocytes, most the GLUT-1 fused in the physiological orientation at the plasma membrane move to the GLUT-4 specific intracellular storage compartment via the GLUT-4 specific vesicular transport pathway. For hepatocytes, very little of the fused GLUT-1 are found intracellularly (Fig. 5), simulating the constitutive distribution of the native hepatocyte glucose transporter, GLUT-2 [11,12]. The findings clearly indicate that a cell-specific factor(s) rather than a specific transporter isoform is a primary determinant for the constitutive (non insulin-stimulated) distribution of glucose transporter in adipocytes.

The large accumulation of HEGT found in HDM, on the other hand, most likely represents the contamination of intact (unfused) HEGT vesicles entered during subcellular fractionation through cell surface adhesion. The following observations support this contention: The accumulation is not affected by trypsin-digestion of HEGT and is practically identical to the accumulation of HEGT vesicle-lipid (Fig. 2). The tryptic susceptibility of HEGT in HDM and that in free HEGT vesicles are very similar (Table III), indicating that they are in the same randome transmembrane orientation. The accumulation of HEGT in HDM was not affected by insulin either in immunoblot assay (not illustrated) or in [^{3}H]CB label distribution assay (Fig. 6).

Insulin does not affect the transport activity and the subcellular distribution of erythrocyte GLUT-1 in adipocytes

Insulin stimulated the 3-O-MeGlc flux of fused adipocytes by an inerement of 8.38 ± 2.01 in arbitrary units, while the same insulin treatment increase the flux in control adipocytes with an increment of 6.68 ± 0.92 units (Table I). The difference between the two is not statistically significant. Since the fusion protocol of itself (in the absence of HEGT) did not affect the native adipocyte transporter function and its response to insulin stimulation (Table I), one may attribute this difference in increments to the response of HEGT to insulin. The increase in the flux rate due to the fused HEGT observed for the basal adipocytes was 2.52 ± 0.30 units (Table I). Approx. 12% of the HEGT in the plasma membrane are in physiological orientation (Table IV). If only these HEGT were responding to insulin as the native adipocyte transporter did (7-fold), this difference in increments is expected to be approx. 2.1 units. These considerations indicate that our flux data are not exact enough to determine if HEGT in adipocytes is responding to insulin.

Our immunoblot experiments, however, clearly demonstrate that erythrocyte GLUT-1 reconstituted in adipocytes does not move from LDM to PM in response to insulin (Figs. 5 and 6), where the native GLUT-4 are highly responsive to the insulin treatment (Fig. 6). This suggests that a specific isoform of transporter is required for the insulin-mediated recruitment of glucose transporter in adipocytes. One cannot rule out, however, the possibility that HEGT may have lost its insulin responsiveness during fusion procedures. The transport activity of HEGT in human erythrocytes is not stimulated by insulin [1]. However, erythrocytes do not have any intracellular organelles, and the insulin-insensitivity in this cell does not necessarily mean that erythrocyte GLUT-1 is inherently non-responding to insulin-mediated recruitment. Glucose transport in HepG2 cells is also shown to be insensitive to insulin [17]. This cell has intracellular organelles and the major glucose transporter isoform is GLUT-1 [17]. HepG2 cells, however, may lack the putative cellular factor(s) needed for the insulin-induced recruitment mechanism.

Specific features of glucose transporter recycling pathway

Surface membrane receptors such as transferrin and mannose-6-phosphate receptors are known to constantly recycle via the coated pit/coated vesicle pathway [49,50]. It is quite likely that the glucose transporters also recycle between the plasma membrane and the intracellular pool via a vesicle transport pathway similar (but may not be identical) to those known for many membrane proteins [51,52], and insulin may shift this steady state distribution toward the plasma membrane either by slowing down the endocytotic rate, or by enhancing the exocytotic rate, or both. It is also known that glucose transporters are much more concentrated in the intracellular storage vesicles (where it amounts to 16% of the total protein) than in the plasma membrane (where it is only 0.1% of the total protein) [42]. Any useful model for the glucose transporter recycling pathway in adipocytes should account for this concentration mechanism. In addition, the model should account for the following two important features disclosed in the present study: First, insulin recruits GLUT-4 (Fig. 6), but not HEGT (Figs. 5 and 6) in fused adipocytes even though both occur in the same intracellular vesicles (Fig. 4). This suggests that the recruitment involves not only vesicle transport but also an isoform-specific modulation of individual transporters in vesicles. How this isoform-specific regulation of individual transporters is achieved is not known, and it may be only speculated at this time: it may involve an insulin-induced, selective retention of GLUT-4 but not HEGT at the plasma membrane. At the molecular level, this retention may be achieved by an isoform-specific modulation of the GLUT-4 movement (lateral diffusion) in the lipid bilayer either upon fusion or prior to endocytic scission. At the biochemical level, this modulation may involve an isoform-specific phosphorylation-dephosphorylation by a protein kinase and a protein phosphatase. It is relevant to note here a recent proposal [53] that insulin may induce a change in GLUT-4 protein conformation (unmasking) as an essential step of the recruitment mechanism. This study [53] indicates that glucose transporters in isolated rat adipocytes are located mostly (95%) in plasma membrane invaginations and surface-connected vesicles even in the absence of insulin, and that insulin treatment causes the flow of GLUT-4 in the plane of the membrane out of these specialized loci to the cell surface with little evidence for vesicle transport. Second, the physiological transmembrane orientation is required for fused HEGT to enter the host cell intracellular storage vesicles. This strongly suggests that the interaction of glucose transporter at its cytoplasmic domain with certain cytosolic constituents is required for the constitutive retainment of glucose transporter in the storage compartment.

Usefulness of vesicle-cell fusion as an experimental system

The results discussed here strongly suggest that by the fusion protocol described here one can introduce in principle any purified membrane protein into any cell type. This is in contrast to protein expression by gene transfection which can be achieved only with propagating cell lines and oocytes; it is not possible, for example, to introduce different glucose transporter isoforms into rat adipocytes or hepatocytes by gene trans-

fection. It is quite likely, on the other hand, that different transporter isoforms such as GLUT-4 and GLUT-2 will soon be purified and functionally reconstituted in vesicles. Our fusion protocol will then permit us to introduce a given isoform into a given cell type such as adipocytes and hepatocytes, and study isoform-specific and tissue-specific regulations of this important protein function in details.

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