Fractionation of endocytic vesicles and glucose-transporter-containing vesicles in rat adipocytes

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We subfractionated intracellular vesicles from rat adipocytes in order to examine the subcellular distribution of endocytic vesicles or endosomes with respect to insulin-regulatable glucose-transporter (GT)-containing vesicles [James, Lederman & Pilch (1987) J. Biol. Chem. 262, 11817–11824]. Vesicles mediating fluid-phase endocytosis sedimented as a single major peak of greater density than the single distinct peak of GT-containing vesicles. This difference was also apparent during cellular insulin exposure and after insulin removal. Endocytosis of insulin and IGF (insulin-like growth factor) II was also examined. In sucrose gradients, IGF II-containing vesicles were less dense than those containing internalized insulin. Receptor-mediated endocytic vesicles were distinct from fluid-phase endocytic vesicles, but overlapped with the GT-containing vesicles. Vesicles containing internalized ligand were further fractionated by agarose-gel electrophoresis after various times of internalization. At least three different vesicle subpopulations containing the iodinated ligands were resolved after 5 min of internalization. Endocytic vesicles containing rapidly internalized insulin (1.5 min at 37 °C) consistently co-migrated with GT-containing vesicles. These data indicate that fluid-phase and receptor-mediated endocytosis occur via different pathways in adipocytes. Furthermore, whereas the intracellular GT-containing vesicles are distinct from fluid-phase vesicles, a rapidly labelled pool of insulin-containing vesicles consistently co-fractionated with GT-containing vesicles when separation techniques based on size, density and charge were used. This suggests that the insulin receptor may directly interact with the intracellular GT-containing vesicles after insulin-induced endocytosis.

INTRODUCTION

Insulin stimulates the translocation of glucose transporters and IGF II receptors from an intracellular pool to the plasma membrane in rat adipocytes (Cushman & Wardzala, 1980; Suzuki & Kono, 1980; Karnieli et al., 1981; Kono et al., 1981; Wheeler et al., 1982; Lienhard et al., 1982; Simpson et al., 1983; Wardzala et al., 1984; Oka et al., 1984). More recently, it has been shown, by using immuno-gold labelling of ultrathin cryosections, that an insulin-responsive glucose transporter is located within the trans-Golgi reticulum (TGR) of 3T3-L1 adipocytes (Blok et al., 1988). This organelle has previously been shown to contain the mannose 6-phosphate receptor (Geuze et al., 1986), which is now believed to be identical with the IGF II receptor (Morgan et al., 1987), and the TGR also contains the asialoglycoprotein receptor (Geuze et al., 1983). Thus it has been suggested that the TGR may be a sorting pool for receptors after dissociation from their ligands, which is thought to occur in an adjacent or contiguous organelle known as the 'compartment of uncoupling receptors and ligands' (CURL). The presence of the insulin-responsive glucose transporter in the TGR is a potentially important observation, because it provides a mechanism by which this protein may be juxtaposed directly with its stimulatory counterpart, namely the insulin receptor. However, confirmation of the existence of the glucose transporter in the TGR will require alternative approaches employing subcellular fractionation to co-localize markers for this organelle and the glucose transporter in an insulin-responsive cell type.

In the present study, we have attempted to fractionate subpopulations of endocytic vesicles in rat adipocytes and to compare their characteristics with those of the intracellular glucose-trasporter-containing vesicles. Both receptor-mediated and fluid-phase endocytosis were examined in adipocytes under short- and long-term labelling conditions. The glucose-transporter-containing vesicles did not co-purify with the major population of vesicles in the fluid-phase pathway. However, vesicles containing rapidly internalized insulin and IGF II co-purified with the glucose-transporter-containing vesicles.

EXPERIMENTAL

Materials

Pig monocomponent insulin was given by Dr. R. Chance, Eli Lilly, Indianapolis, IN, U.S.A., ^{125}I-labelled goat anti-rabbit IgG was from Du Pont–New England Nuclear, Boston, MA, U.S.A. ^{125}I-IGF II was kindly supplied by Dr. B. Posner, McGill University, Montreal, Canada. ^{125}I-insulin labelled in the B16 position was kindly supplied by Dr. S. Waugh, Boston University, Boston, MA, U.S.A. Na^{125}I was purchased from Amersham. Fluorescein isothiocyanate (FITC) conjugated to dextran (M_r 70000) was from Sigma. Seakem

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high-gelling-temperature agarose was purchased from FMC BioProducts, Rockland, ME, U.S.A.; nitrocellulose was from Schleicher and Schuell; M_r standards were from Bethesda Research Laboratories; collagenase was from Cooper Biomedical, Freehold, NJ, U.S.A. All electrophoresis reagents were obtained from Bio-Rad.

Preparation of adipocytes and subsequent incubations

Adipocytes were isolated from epididymal fat-pads of 170–200 g male Sprague–Dawley rats (Taconic Farms, Germantown, NY, U.S.A.) by the method of Rodbell (1964) as modified by Cushman (1970). All incubations were carried out in a modified Krebs–Ringer buffer containing 12.5 mM-Hepes, 120 mM-NaCl, 6 mM-KCl, 1.2 mM-MgSO$_4$, 1 mM-CaCl$_2$, 1 mM-sodium phosphate, 2 mM-sodium pyruvate and 2% (w/v) bovine serum albumin (radioimmunoassay grade), pH 7.4. After digestion of pads in crude collagenase, cells were washed three times, resuspended in 5 vol. of buffer, and incubated at 37 °C for 30 min with gentle shaking to establish basal conditions. When added, insulin was present at a concentration of 10 nM.

Labelling of endosomes

Fluid-phase endocytosis was studied by using the marker FITC–dextran, which was extensively dialysed before use. Adipocytes were resuspended in Krebs–Ringer buffer (1:5, v/v), incubated with FITC–dextran, at a final concentration of 10–15 mg/ml, for various times as described in the text. Cells were washed three times with HES buffer (20 mM-Hepes, 1 mM-EDTA and 255 mM-sucrose, pH 7.4) at 12 °C and homogenized in HES at 4 °C as previously described (James et al., 1987). In some experiments, insulin was included in the incubation media for 15 min before addition of FITC–dextran. In pulse–chase studies, cells were incubated with FITC–dextran for 45 min, washed three times in Krebs–Ringer buffer at 12 °C, and incubated for a further 15 min in the absence or the presence of insulin at 37 °C. Where cells were studied during the reversal of insulin action, adipocytes were washed three times with Krebs–Ringer buffer at 12 °C after incubation for 15 min with insulin at 37 °C. A 200-fold molar excess (over insulin) of anti-insulin antibody was immediately added together with the FITC–dextran for 10 or 45 min. Controls were run in parallel under identical experimental conditions except for addition of insulin.

To study receptor-mediated endocytosis, adipocytes were incubated with either [125I]-insulin or [125I]-IGF II for 30–40 min at 12 °C. Previous studies have shown that incubation of adipocytes at 15 °C results in inhibition of insulin internalization (Suzuki & Kono, 1979). For these studies, adipocytes were diluted in 3 vol. of modified Krebs–Ringer buffer. Iodinated ligands (5 × 10^6 c.p.m.) were added to 2 ml of cell suspensions at a final concentration of 0.1 M. After this incubation, cells were washed once in a large volume of buffer (15 ml) at 12 °C, immediately resuspended in 3 ml of buffer at 37 °C and incubated for different times (1.5–15 min) at 37 °C. At the end of the incubation, the cells were immediately combined with 15 ml of untreated adipocytes, preincubated at 12 °C, and diluted in 3 vol. of Krebs–Ringer buffer pre-equilibrated to 12 °C. The addition of these cells served to decrease the temperature immediately to 12 °C and also aided as a source of carrier membrane in the subsequent fractionation. The combined cells were washed three times in HES at 12 °C and homogenized. The specificity of [125I]-insulin and [125I]-IGF II binding was measured by binding ligands for 40 min at 12 °C to adipocytes in the presence and the absence of excess unlabelled ligand (> 1 μM). Insulin bound to adipocytes was measured by using the oil-flotation method (Gliemann et al., 1972). Non-specific binding constituted 25% and 27% for [125I]-insulin and [125I]-IGF II respectively.

Subcellular fractionation

Adipocytes were fractionated into plasma and microsomal membranes by the method of McKeel & Jarett (1970) as modified by Simpson et al. (1983). All subcellular fractions were washed and resuspended in HES buffer. Plasma membranes, low-density and high-density microsomes (microsomal fractions) and the mitochondria/nuclei fraction were resuspended at a final protein concentration of 3–6 mg/ml. The low-density microsomes were further fractionated by sucrose-density-gradient centrifugation and agarose-gel electrophoresis as previously described (James et al., 1987).

Immunoblotting

Vesicle fractions, prepared in the absence of reductant, were subjected to SDS/polyacrylamide-gel electrophoresis in 5–13%-acrylamide resolving gels and transferred to nitrocellulose as previously described (James et al., 1987). Prestained M_r markers were always included for determination of M_r. Nitrocellulose was blocked for 1 h in 5% (w/v) powdered milk, incubated with antisera for 1 h at 37 °C, washed three times in phosphate-buffered saline containing 1% Triton X-100, incubated with [125I]-labelled goat anti-rabbit IgG (25000 c.p.m./ml) for 2 h at 37 °C, and then washed four times as above. The nitrocellulose was air-dried and subjected to autoradiography.

Assays

Glucose-6-phosphatase was assayed by the method of Nordlie & Sukalski (1983). β-Hexosaminidase was assayed by the method of Koldovsky & Palmieri (1971). Protein was measured by the bicinchoninic acid assay (Pierce Chemical Co.), with bovine serum albumin as standard. Fluorescence was measured at an emission wavelength of 515 nm and an excitation wavelength of 485 nm. Triton X-100 was added at a final concentration of 1% to all samples before measurement of fluorescence in order to quench light-scattering. In addition, control membranes or sucrose gradients with no FITC–dextran were always run in parallel, and these values were subtracted as background. The integrity of iodinated ligands was measured by examining precipitability in 10% trichloroacetic acid.

RESULTS

Fluid-phase endocytosis

Fluid-phase endocytosis was examined in isolated adipocytes with FITC–dextran as the marker (reviewed by Mellman et al., 1986). As a control, we first verified that the inclusion of FITC–dextran in the incubation media had no effects on basal glucose transport, on insulin-stimulated glucose transport, and on the reversal of insulin-stimulated glucose transport (results not shown). By using a differential-centrifugation technique,
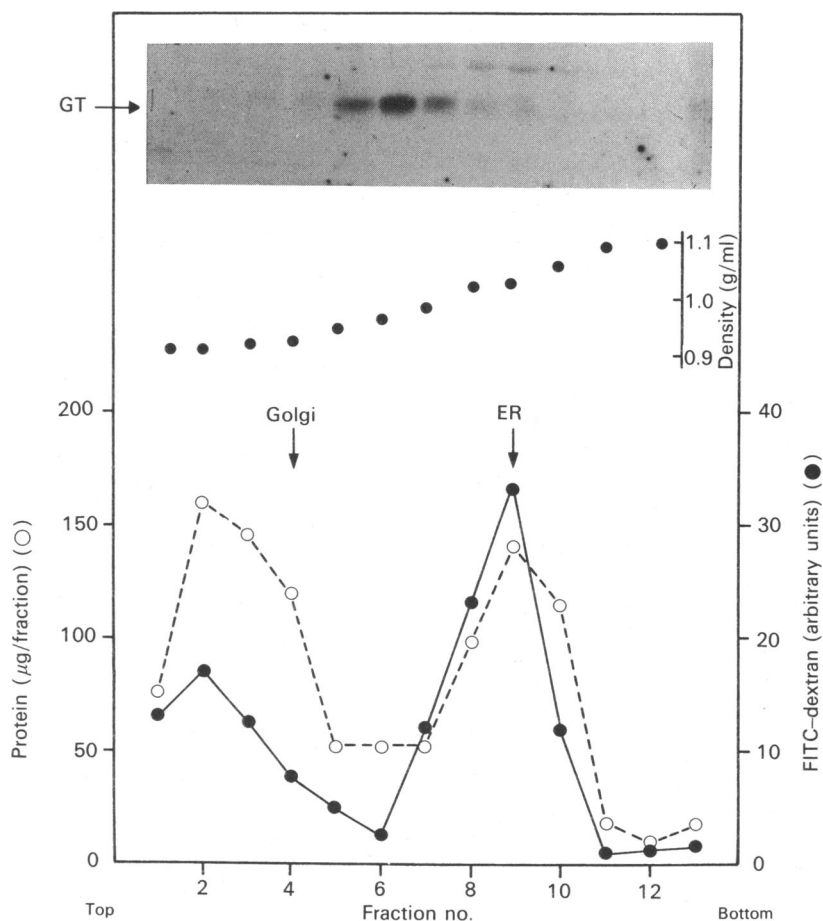
Fig. 1. Sucrose-gradient fractionation of low-density microsomes

Rat adipocytes were incubated with FITC–dextran for 5 min at 37 °C. Low-density microsomes were obtained and sedimented through a discontinuous sucrose gradient as described in the Experimental section. Fractions were collected after centrifugation (1 h at 91000 g) and analysed. The relative migration of glucose-transporter-containing vesicles (GT) in the sucrose gradient, as determined by immunoblotting using a glucose-transporter-specific antiserum, is shown at the top of the Figure. The positions of the Golgi and endoplasmic reticulum (ER), as determined by measurement of the marker enzymes galactosyltransferase and glucose-6-phosphatase respectively, are denoted by arrows.

two different microsomal fractions were isolated from rat adipocytes (low- and high-density microsomes). On the basis of the size of vesicles in these fractions (Simpson et al., 1983) and the isolation conditions compared with those used by others for isolation of endocytic vesicles (Wall & Hubbard, 1985; Marsh et al., 1987), the low-density microsomes are most likely to contain the endosomal component of rat adipocytes. After brief incubation of adipocytes with FITC–dextran (1.5–5 min), this marker was located in both the high- and low-density microsomes (results not shown), whereas markers of the receptor-mediated endocytic pathway were identified primarily in the low-density microsomes under similar incubation conditions (see below). It is noteworthy that the high-density microsomes are contaminated with plasma membrane upon isolation as determined by the presence of marker enzymes (Simpson et al. 1983). Thus, although we cannot exclude the possibility that some endocytic vesicles co-fractionate with the high-density microsomes, it is more likely that the presence of endoycytic markers in this fraction is due to contamination with plasma-membrane vesicles. In addition, our primary object in the present study was to examine the relationship between endocytic vesicles and

the intracellular vesicles enriched in the glucose transporter, and the latter are isolated exclusively in the low-density microsomes (Simpson et al., 1983; James et al., 1987).

The endocytic vesicles labelled by a 5 min incubation with FITC–dextran were resolved into one major peak after sucrose-density-gradient centrifugation of the low-density microsomes (Fig. 1). This peak, which was consistently labelled to the same magnitude in four different experiments, was well separated from the glucose-transporter-containing vesicles (Fig. 1). The endocytic-vesicle peak overlapped with the smaller of the two protein peaks and with the marker for endoplasmic reticulum, glucose-6-phosphatase (Fig. 1). Glucose-6-phosphatase activity in the low-density microsomes constitutes a small (20 %) proportion of the total cellular activity, the majority being present in the high-density microsomes (Simpson et al., 1983). A smaller peak of FITC–dextran was also present at the top of the gradient (Fig. 1). This is presumably free FITC–dextran, since this peak varied considerably in amplitude between different experiments. In addition, no vesicles were present in these sucrose-gradient fractions, as determined by electron microscopy (results not shown).

Table 1. Modulation of fluid-phase endocytosis in rat adipocytes

Rat adipocytes were isolated and incubated under a variety of experimental conditions with FITC–dextran at 37 °C to examine fluid-phase endocytosis. After these incubations, adipocytes were subjected to subfractionation by differential centrifugation. The low-density microsomes obtained from this procedure, which were enriched in glucose-transporter and endocytic vesicles, were subjected to sucrose-gradient centrifugation. FITC–dextran migrated as one major peak (Fig. 1). Shown below is the area under this peak of internalized FITC–dextran. The experimental conditions noted below are as follows: Basal, cells incubated in the absence of insulin; Insulin, cells incubated in the presence of 10 nM-insulin for 15 min before addition of FITC–dextran; Post-insulin, cells incubated in the presence of FITC–dextran for 3, 10 or 45 min after addition of an excess of insulin antibody; Chase-basal, cells incubated without insulin but with FITC–dextran for 45 min, washed with buffer at 12 °C, and incubated for a further 15 min at 37 °C; Chase–insulin, the same as Chase-basal, except that insulin was included in the incubation media after the wash at 12 °C. The numbers in parentheses refer to the number of times each experiment was performed. Results are means ± S.D.

Experimental conditions	Incubation time at 37 °C (min)	Area under peak (arbitrary units)
Basal	1.5 (2)	0.6, 0.8
	3 (3)	1.2 ± 0.2
	10 (3)	1.8 ± 0.4
	45 (4)	4.5 ± 0.5
Insulin	3 (2)	2.1 ± 0.2
	45 (3)	5.9 ± 0.3
Post-insulin	3 (3)	2.1 ± 0.3
	10 (3)	3.3 ± 0.3
	45 (2)	5.5, 5.9
Chase-basal	45 + 15 (2)	2.2, 2.4
Chase-insulin	45 ± 15 (2)	1.5, 1.8

Several different experimental protocols were employed in an effort to label the intracellular vesicles containing the glucose transporter with the fluid-phase marker. The area under the sucrose-gradient peak of endocytosed FITC–dextran for each of these experiments is presented in Table 1. FITC–dextran continued to accumulate in endocytic vesicles with incubation times up to 45 min. In addition, preincubation of cells with insulin for 15 min before addition of FITC–dextran resulted in a 30–70 % increase in the accumulation of the marker in the endosomal fraction (Table 1). This effect was demonstrable with short-term (3 min) and long-term (45 min) labelling. Similar data have been reported by others (Gibbs & Lienhard, 1984; Gibbs et al., 1986), where insulin was found to stimulate the internalization rate of radioactive sucrose by 30 % in adipocytes. When insulin was included in the incubation media during a chase of FITC–dextran from the cell, there was an increase in the rate of FITC–dextran release (Table 1). This may be due to accelerated endocytosis, exocytosis or both. Finally, the accumulation of FITC–dextran in the endosomal fraction was also increased by 70–80 % during the reversal phase after cells were stimulated with insulin, and then washed thoroughly to remove insulin

from the media (Table 1). However, irrespective of the incubation conditions used, as described above, the peak corresponding to endocytosed FITC–dextran (Fig. 1) did not coincide with the intracellular vesicles enriched in glucose transporter when resolved by sedimentation in sucrose. Furthermore, there was no significant difference in FITC–dextran concentration, in sucrose-gradient fractions enriched in glucose transporter, between any of the experiments described above.
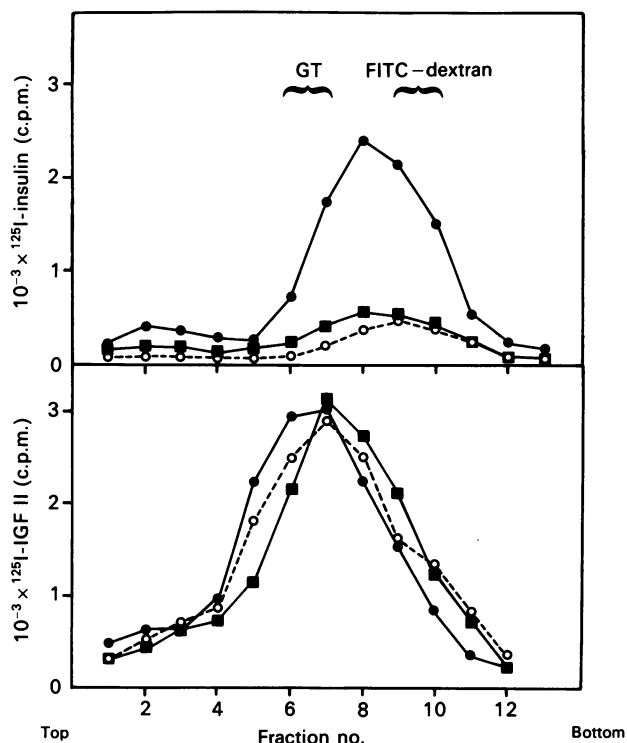
Receptor-mediated endocytosis

The low-density microsomes appear to contain most of the receptor-mediated endocytic vesicles in adipocytes (Table 2). The ^{125}I-insulin and ^{125}I-IGF II in the low-density microsomes was > 92 % precipitable with trichloroacetic acid at all incubation times examined, suggesting little degradation of ligand in this pool. This is consistent with the distribution of the lysosomal enzyme marker, β-hexosaminidase, which is highest in the high-density microsomal and mitochondria/nuclei fractions (results not shown).

Differences in the kinetics and the intracellular loci for internalization of ^{125}I-IGF-II compared with that of ^{125}I-insulin were revealed upon sedimentation of the low-density microsomes in sucrose (Fig. 2). The endocytic vesicles labelled by short exposure (1.5–5 min) to either ^{125}I-insulin or -IGF II were resolved into a single peak in sucrose gradients. However, despite considerable overlap between these peaks, the ^{125}I-IGF II peak sedimented at a slightly higher density than that of ^{125}I-insulin (Fig. 2). In addition to a difference in sedimentation characteristics between these two ligands, ^{125}-IGF II was internalized more rapidly than insulin, apparently reaching a plateau in the endocytic pool after incubation for 1.5 min at 37 °C. Insulin internalization, on the other hand, proceeded for at least 3–5 min before reaching a plateau (Fig. 2). Others have shown that saturation of internalization for ^{125}I-insulin in rat adipocytes occurs with a half time of 3.5 min (Marshall, 1985b). Accumulation of ^{125}I-insulin in endocytic vesicles reached a peak between 3 and 5 min after incubation at 37 °C, but by

Table 2. Subcellular distribution of iodinated ligands

Adipocytes were isolated and incubated with iodinated ligands at 12 °C for 30–40 min. Cells were then washed at 12 °C, incubated at 37 °C for 5 min and immediately washed with buffer at 12 °C and homogenized. Subcellular fractionation was performed by using differential centrifugation to obtain plasma membranes (PM), low-density microsomes (LDM), high-density microsomes (HDM) and a mitochondria/nuclei fraction (M/N). Values shown are means ± S.D. for the percentage of total radioactivity recovered in each membrane fraction. The numbers of individual experiments are shown in parentheses.

Ligand	Radioactivity in fraction (% of total)				$10^{-4} \times$ Total radio-activity (c.p.m.)
	PM	LDM	HDM	M/N	
Insulin (3)	37.9 ± 9.7	38.5 ± 8.2	15.9 ± 2.3	6.7 ± 3.8	3.9
IGF II (2)	23.0, 34	60.1, 50.8	12.7, 12.7	3.4, 2.4	3.9

Fig. 2. Sucrose-gradient fractionation of receptor-mediated endocytic pools

Rat adipocytes were incubated with either ^{125}I-insulin or ^{125}I-IGF II for 30–40 min at 12 °C. Adipocytes were washed with Krebs–Ringer buffer containing no iodinated ligands, at 12 °C, and transferred to 37 °C for 1.5 min (○), 5 min (●) or 15 min (■). Adipocytes were further washed at 12 °C and homogenized. Low-density microsomes were obtained and sedimented in sucrose gradients as described in the Experimental section. The resolution of ^{125}I-insulin-containing endocytic vesicles is shown in the upper panel, and that of ^{125}I-IGF II-containing endocytic vesicles in the lower panel. The positions of the immunoblotted glucose transporter (GT) and of FITC–dextran in the sucrose gradient are also shown for comparison.

15 min was substantially diminished. In contrast, there was essentially no decrease in the accumulation of ^{125}I-IGF II in endocytic vesicles between 5 and 15 min (Fig. 2).

After sucrose-gradient centrifugation, the sucrose fractions containing ^{125}I-insulin or ^{125}I-IGF II were concentrated and subjected to agarose-gel electrophoresis. The peak of ^{125}I-insulin from the gradient (fractions 6–11, Fig. 2), obtained after 5 min of internalization at 37 °C, was further resolved into three different subpopulations of endocytic vesicles in agarose (Fig. 3): a sharp peak (fraction 2) which did not migrate into the gel, a broader fast-migrating peak (fractions 13–17) and a smaller slower-migrating component (fractions 11–13), which reproducibly migrated as a shoulder of the faster-migrating peak of ^{125}I-insulin (fractions 13–17, Fig. 3). In the agarose-gel profile of internalized ^{125}I-insulin, after incubation of cells for 5 min at 37 °C, a small peak of ^{125}I-insulin was observed in fraction 9 (Fig. 3). However, this was not a reproducible finding. These individual subpopulations were kinetically distinct. After 1.5 min of

internalization at 37 °C, only the more slowly migrating peak, which corresponded to the shoulder of the 5 min peak, was clearly present, as determined by electrophoresis in agarose (Fig. 3). After internalization at 37 °C for up to 15 min, a greatly diminished amount of ^{125}I-insulin was observed in all three subpopulations (Fig. 3). Endocytic vesicles containing ^{125}I-IGF II were resolved into three similar subpopulations by agarose-gel electrophoresis (results not shown). However, these subpopulations were not kinetically distinct, as was the case for ^{125}I-insulin, once again indicating that internalization of ^{125}I-IGF II proceeds more rapidly than does that of ^{125}I-insulin, and the amount internalized does not diminish over the course of this experiment.

Relationship between glucose transport and receptor-mediated and fluid-phase endocytic vesicles

Fluid-phase endocytic vesicles reached a peak in fractions quite distinct from the peak of glucose-transporter-containing vesicles and from the peak of receptor-mediated endocytic vesicles on sedimentation in sucrose (Figs. 1 and 2). The peak of internalized ^{125}I-insulin was shifted, albeit overlapping, with respect to the peak of FITC–dextran (Fig. 2). On the other hand, both peaks of internalized iodinated ligands overlapped somewhat with the peak of glucose-transporter-containing vesicles when resolved by sucrose gradient centrifugation. The IGF II peak overlapped with both the peak of glucose transporter and that of ^{125}I-insulin (Fig. 2). The ^{125}I-insulin peak did not directly overlap with the glucose-transporter peak, but was consistently shifted by one fraction on the dense side of this peak (Fig. 2). The sucrose-gradient fractions containing the peak of either ^{125}I-insulin or ^{125}I-IGF II were concentrated and further subjected to agarose-gel electrophoresis. The insulin peak, corresponding to endocytic vesicles labelled after incubation for 5 min at 37 °C, obtained by sucrose-density-gradient sedimentation (Fig. 2) was further resolved into three different subpopulations by agarose-gel electrophoresis (Fig. 3). The glucose-transporter-containing vesicles, detected by immunoblotting, were subdivided into two components, one at the origin of the agarose gel and another overlapping with the shoulder peak of internalized ^{125}I-insulin (Fig. 3). However, when the sucrose-gradient fractions containing glucose transporter alone (fractions 5–7, Fig. 1) were subjected to agarose-gel electrophoresis (Fig. 4), no glucose transporter was detected at the origin of the gel. Additional low-M_r bands were detected in immunoblots (Fig. 4). However, this was due to non-specific binding of the labelled secondary antibody (results not shown). Furthermore, when the remaining sucrose-gradient fractions (fractions 8–11) containing internalized ^{125}I-insulin were subject to agarose-gel electrophoresis, no significant glucose transporter was detected at the origin (results not shown). Thus these data suggest that the glucose transporter at the origin of the agarose gel shown in Fig. 3 may be due to vesicle trapping. This phenomenon has been noted previously by others using this technique to resolve clathrin-coated vesicles (Rubenstein et al., 1981).

DISCUSSION

We have examined the relationship between the intracellular vesicles containing the insulin-regulatable glucose
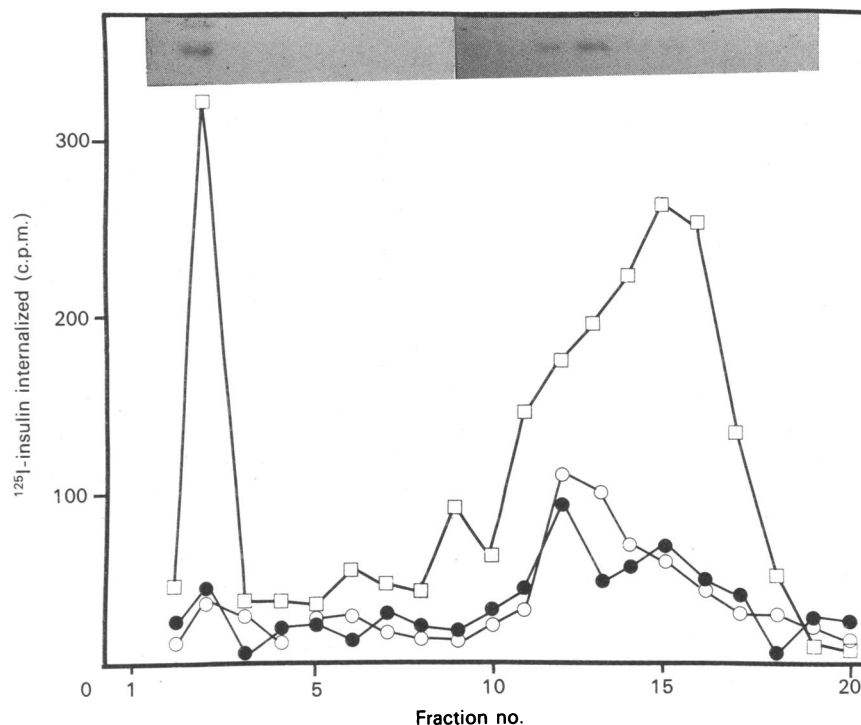
Fig. 3. Agarose-gel electrophoresis of receptor-mediated endocytic vesicles fractionated by differential centrifugation and sucrose gradients
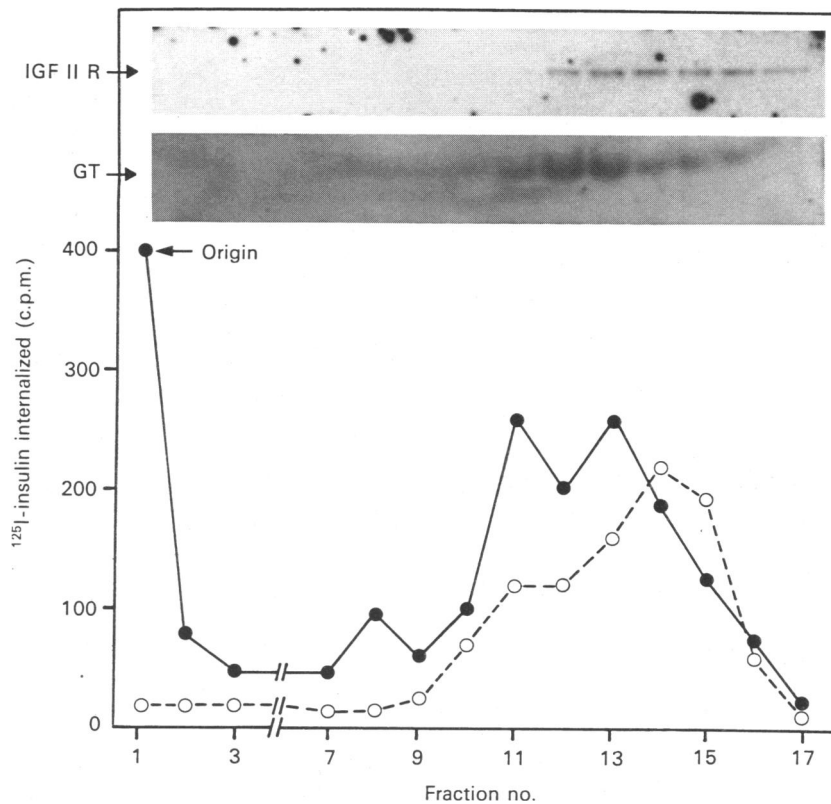
Adipocytes were incubated with ^{125}I-insulin for 30–40 min at 12 °C, washed and incubated at 37 °C for 1.5 min (○), 5 min (□) or 15 min (●). Low-density microsomes were obtained from each and further fractionated by sedimentation in sucrose (see Fig. 2). The peak sucrose-gradient fractions containing ^{125}I-insulin (fractions 6–11, Fig. 2) were concentrated by centrifugation and subjected to agarose-gel electrophoresis as described in the Experimental section. Fraction 2 denotes the origin of the gel. Vesicles migrated toward the anode during electrophoresis. The agarose gel was sliced into 0.5 cm fractions and counted for ^{125}I radioactivity. Shown in the upper portion of the Figure is the migration of the glucose-transporter-containing vesicles in agarose as determined by immunoblotting.

transporter and those vesicles participating in receptor-mediated and fluid-phase endocytosis in isolated rat adipocytes. The glucose-transporter-containing vesicles were distinct from the major intracellular pool of fluid-phase endocytic vesicles. In addition, receptor-mediated endocytic vesicles were distinct from fluid-phase endocytic vesicles. However, an intracellular pool containing rapidly internalized insulin consistently co-fractionated with intracellular glucose-transporter-containing vesicles.

The present data implicate divergence between the fluid-phase and receptor-mediated endocytic pathways in rat adipocytes (Fig. 2). This divergence was demonstrable at an early time point in the endocytic pathway (5 min). Hence it may reflect specificity in vesicle budding at the cell surface or exclusive sorting of these endocytic markers within a very early endosomal compartment. In support of the former, Sandvig et al. (1987) have shown that acidification of the cytosol inhibits endocytosis of transferrin while having only a minor effect on the uptake of fluid-phase markers.

Insulin stimulated FITC–dextran uptake into adipocytes by 30–70%, in agreement with previous studies (Gibbs & Lienhard, 1984; Gibbs et al., 1986). A similar phenomenon was also evident when cells were incubated with insulin and then washed to examine reversal after insulin stimulation (Table 1). However, irrespective of the experimental conditions, most of the vesicles con-

taining internalized FITC–dextran were of higher average density than vesicles containing the glucose transporter. Hence it is likely that endocytosis of the glucose transporter for the most part undertakes a different route to the fluid-phase marker, FITC–dextran. It is unlikely that the rate of re-entry of glucose-transporter-containing vesicles is insignificant compared with the overall fluid-phase-endocytic rate of the adipocyte. Fluid-phase endocytosis in rat adipocytes corresponds to the formation of approx. 2000 endocytic vesicles of 100 nm diameter per cell per min (Gibbs & Lienhard, 1984). In the presence of insulin there are about 2×10^6 molecules of glucose transporter inserted into the plasma membrane per cell (Simpson et al., 1983), and the half-time for reversal after insulin stimulation is 10 min (Karnieli et al., 1981). Thus the net rate of internalization of glucose transporters can be calculated to be around 10^5 molecules of glucose transporter/cell per min during the reversal phase. If there were 100–200 molecules of glucose transporter per vesicle, this particular endocytic pool should contribute substantially to the net endocytic rate. In addition, this calculation does not take into account the possible cycling of the glucose transporter between the intracellular compartment and the plasma membrane during this phase. The possibility that a small amount of FITC–dextran is delivered to the glucose-transporter-containing vesicles cannot be excluded from the present analyses. In fact, assuming that most of the intracellular

Fig. 4. Agarose-gel electrophoresis of glucose-transporter-containing vesicles and receptor-mediated endocytic vesicles fractionated by sucrose gradients

Adipocytes were incubated with ^{125}I-insulin for 30–40 min at 12 °C, washed and incubated at 37 °C for 5 min. Low-density microsomes were obtained by differential centrifugation and further fractionated by sedimentation in sucrose (see Fig. 2). Sucrose-gradient fractions containing the glucose transporter (fractions 5–7, Fig. 2) were concentrated and subjected to agarose-gel electrophoresis (O). The remaining sucrose-gradient fractions containing ^{125}I-insulin (fractions 8–11, Fig. 2) were simultaneously subjected to agarose-gel electrophoresis (●). Fraction 2 denotes the origin of the gel. Vesicles migrated toward the anode during electrophoresis. The agarose gel was sliced into 0.5 cm fractions and counted for ^{125}I radioactivity. These fractions were then subjected to SDS/polyacrylamide-gel electrophoresis and immunoblotting to detect the relative mobilities of the glucose transporter (GT) and the IGF II receptor (IGF II R). Immunoblots of agarose-gel fractions representing the glucose-transporter-containing vesicles obtained from sucrose gradients are shown at the top.

glucose transporters are located in the TGR (Blok et al., 1988), one would expect this to be the case, because it has recently been shown that only 4 % of internalized ricin is delivered to the TGR in BHK-21 cells (van Deurs et al., 1988). Hence the present data provide further confirmation for the existence of glucose transporters within a unique organelle, such as the TGR.

Receptor-mediated endocytic vesicles were resolved into three different subpopulations by agarose-gel electrophoresis (Fig. 3), consistent with there being heterogeneity in charge among these individual pools. This is consistent with other reports of heterogeneity within the endosomal pool (Marsh et al. 1987). The subpopulations of endocytic vesicles isolated in the present study were kinetically defined. These pools presumably correspond to peripheral or early endosomes and internal or late endosomes (Wall & Hubbard, 1985). The early-endo-somal fraction containing both ^{125}I-insulin (Fig. 3) and ^{125}I-IGF II (results not shown) co-migrated with the glucose-transporter vesicles when subjected to both sucrose-gradient centrifugation and agarose-gel electrophoresis. This fraction is unlikely to be derived from the TGR or the Golgi/lysosome region of the cell, because

ligands do not appear in these organelles for at least 5 min after endocytosis from the cell surface (Wall & Hubbard, 1985). The IGF receptor was present in this fraction (Fig. 4). In addition, it is likely that the internalized insulin receptor and insulin are still in the same vesicle after incubation for 1.5 min at 37 °C. Further investigation will be required to verify the co-localization of rapidly internalized insulin receptors and the glucose transporter, because such an association may serve as part of the insulin signalling mechanism. The time course for the appearance of ^{125}I-insulin in the intracellular vesicles isolated in the present study (Fig. 3) is adequate to account for insulin-stimulated glucose transport (Karnieli et al., 1981). Furthermore, the kinase activity of the internalized insulin receptor in adipocytes remains demonstrable in the low-density microsomal fraction for at least 3–4 min (Klein et al., 1987). However, we cannot rule out the possibility that the glucose transporter and rapidly internalized insulin or IGF II are in different vesicles of similar size, density and charge.

It has previously been concluded that the subcellular distribution of internalized ^{125}I-insulin and the intracellular glucose transporter are different (Ezaki & Kono,

1984). This conclusion was drawn from data similar to those described here, except that only sedimentation in sucrose was used to resolve subcellular fractions. However, in the present study we show that the single peak of ^{125}I-insulin, obtained by sedimentation in sucrose, can be further separated into three different pools, one of which consistently co-migrates with the glucose transporter.

Sedimentation of the low-density microsomes in sucrose revealed differences in the migration of vesicles containing internalized ^{125}I-insulin and -IGF II (Fig. 2). In addition, marked differences in the kinetics of internalization for each of these ligands was demonstrable (Fig. 2). This is of particular interest, in view of data suggesting that the IGF II receptor and the mannose 6-phosphate receptor may be the same protein (Morgan et al., 1987). Therefore it is possible that in adipocytes, as shown for other cells (Sahagian, 1984; von Figura et al., 1984; Pfeffer, 1987), the IGF II receptor cycles not only between an intracellular pool and the cell surface (Oka et al., 1984), but also between the Golgi and endosomes. Furthermore, it has been shown that the mannose 6-phosphate receptors in the Golgi, endosomes and plasma membrane are in rapid equilibrium (Sahagian, 1984; von Figura et al., 1984; Pfeffer, 1987), consistent with the kinetics of internalization of ^{125}I-IGF II as shown here (Fig. 2). In contrast, internalization of the insulin receptor is largely ligand-dependent (Marshall, 1985a). Hence one would predict, as shown in Fig. 2, that the kinetics of internalization of the insulin–receptor complex would be slower than that of the IGF II–receptor complex.

In conclusion, the results reported here clearly dissociate most of the endocytic vesicles participating in the fluid-phase pathway from both the glucose-transporter-containing vesicles and the bulk of the vesicles mediating receptor–ligand endocytosis. It is possible that the glucose transporter resides in an early endosome-like vesicle, which may be one of the earliest recipients of the incoming insulin–insulin-receptor complex or a signal derived from this complex. Alternatively, these vesicles may simply share morphological and biochemical similarities, such that separation based on size, density and charge of membranes is not possible.

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