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Characterization of vesicles containing insulin-responsive intracellular glucose transporters isolated from 3T3-L1 adipocytes by an improved procedure

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Our previously described immunoadsorption method for the isolation of vesicles containing the insulin-responsive intracellular glucose transporters from 3T3-L1 adipocytes has been improved in two ways. First, the minimal number of *g* minutes required to sediment the plasma membranes from the cell homogenate has been determined and, as a result, the supernatant used for immunoadsorption in the new procedure contained twice as much of the intracellular transporters. Second, the immunoadsorption has been performed with affinity-purified antibodies directed against the carboxy terminal peptide of the transporter, rather than against the entire protein. 10^7 cells (10 mg protein) yielded about 12 μg of vesicular protein and 11 μg of vesicular phospholipid. The transporter constituted 3% of the protein in the vesicles; this amount equates to approx. eight copies of the transporter per 50 nm vesicle. The polypeptide composition of the vesicles was determined by gel electrophoresis and protein staining. Major components, other than the glucose transporter, are polypeptides of M_r 270000, 245000, 165000 and 115000. The vesicles contained several phosphoproteins; the major ones have a M_r of 245000, 190000, 115000 and 25000. Insulin treatment of adipocytes did not significantly change the phosphoprotein composition of the vesicles. The vesicles were not enriched in the Golgi marker enzyme, galactosyltransferase. The cellular content of the marker for the *trans*-Golgi reticulum, sialyltransferase, was too low to detect.

Introduction

The rapid increase in glucose transport elicited by insulin in fat and muscle cells is due, at least in part, to the translocation of intracellular glucose transporters to the plasma membrane (reviewed in Ref. 1). We have described a procedure for the isolation of vesicles containing the insulin-responsive, intracellular glucose transporters (IIGT) from a cellular supernatant of 3T3-L1 adipocytes depleted of plasma membranes [2]. The procedure consists of immunoadsorption of the vesicles onto

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Abbreviations: Buffer A, 150 mM KCl/20 mM Hepes/2 mM $MgCl_2$ (pH 7.2); Buffer B, 50 mM Hepes/10 mM $Na_4P_2O_7$/100 mM NaF/4 mM Na_2EDTA/2 mM Na_3VO_4 (pH 7.4); $C_{12}E_8$, dodecyl octaethylene glycol ether; DMEM, Dulbecco's modified Eagle medium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IgG, immunoglobulin; IIGT, insulin-responsive, intracellular glucose transporter; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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formaldehyde-fixed *Staphylococcus aureus* cells coated with anti-transporter antibodies. The present report describes a procedure for isolation of the IIGT vesicles that has been improved in two ways. First, the yield is twice as large. Second, the vesicles are isolated with antibodies against the carboxy terminal peptide of the rat/human glucose transporter [3], rather than with antibodies against the glucose transporter itself. A recent article suggested that antibodies against the glucose transporter may be contaminated with antibodies against membrane lipids, which were present in the glucose transporter used for immunization and that, therefore, the vesicles isolated through the use of these antibodies may be impure [4]. Although the characteristics of the vesicle preparation described in Ref. 2 are not consistent with this hypothesis, the use of affinity-purified antibodies against the carboxy terminal peptide eliminates this possibility.

The larger yield has allowed further characterization of the vesicles in several ways. We have determined the amounts of protein and phospholipid in the vesicles, their glucose transporter content and their polypeptide and phosphoprotein compositions. Because insulin may exert many of its effects through changes in the phosphorylation state of specific proteins (reviewed in Ref. 5), we compared the phosphoprotein composition of vesicles isolated from basal and insulin-treated cells in an effort to identify a protein involved in control of the translocation process. Finally, because the results from immunocytochemistry of 3T3-L1 adipocytes with antibodies to the glucose transporter indicate that the vesicles are derived from the *trans*-Golgi reticulum [6], potential marker enzymes for this organelle and the Golgi have been assayed.

Materials and Methods

Materials. Formaldehyde-fixed Staph A cells (Pansorbin, Calbiochem) were extracted twice at 95°C for 30 min with 150 mM NaCl/1.4 M 2-mercaptoethanol/104 mM SDS/50 mM Tris-Cl (pH 7.2) [7], washed with 150 mM KCl/20 mM Hepes/2 mM $MgCl_2$ (pH 7.2) (Buffer A), frozen in liquid nitrogen and stored at −70°C. The capacity of these *S. aureus* cells for binding of purified rabbit IgG (Sigma) was determined by incubating the cells with IgG for 2 h, pelleting the *S. aureus* cells, and measuring unadsorbed IgG by the Lowry method. It was found to be 9 μg IgG/μl *S. aureus* cells. The affinity-purified rabbit antibodies against the purified human erythrocyte glucose transporter (anti-GT antibodies) and against the carboxy terminal peptide (residues 477–492) of the human/rat glucose transporter (anti-GT C peptide antibodies) were preparations that have previously been described [8,3]. 2,6-Sialyltransferase was purchased from Genzyme. Uridine diphosphate [U-^{14}C]galactose and cytidine 5′-monophosphate [9-^{3}H]sialic acid were from New England Nuclear. Ortho[^{32}P]phosphate was obtained from ICN Pharmaceuticals.

Cell culture and fractionation. 3T3-L1 cells were carried as fibroblasts and differentiated into adipocytes as described in Ref. 9. The adipocytes were used between days 7 and 11 after initiation of differentiation. Insulin stimulation of 2-deoxyglucose uptake by the adipocytes during this period was routinely about 10-fold. Plates were incubated at 37°C in serum-free Dulbecco's modified Eagle medium (DMEM) for 2 h just before use.

The adipocytes on a 10-cm plate were washed twice with 4 ml of cold buffer A and then scraped off the plate with a rubber spatula in 4 ml cold buffer A containing the proteinase inhibitors phenylmethanesulfonyl fluoride (200 μM)/pepstatin A (2 μM)/L-*trans*-epoxysuccinylleucylamido(3-methyl)butane (0.1 mM). This mixture was transferred to a 30-ml homogenizer tube with a teflon pestle (A.H. Thomas 3431-D88, clearance 0.13–0.18 mm) at room temperature. The dish was washed once with 4 ml cold buffer A, which was combined with the cells, and the suspension was homogenized at room temperature by 25 hand strokes. Insulin-treated cells were exposed to 160 nM insulin for 15 min in DMEM and homogenized in buffer A containing both the proteinase inhibitors and 160 nM insulin. For routine preparation of the IIGT vesicles, the homogenate in a 12-ml tube was centrifuged for 20 min at 11 500 rpm in a Sorvall SS34 rotor ($16\,000 \times g_{max}$). The fat on the surface was aspirated as well as possible with a blunt end needle, and the supernatant was then transferred with a glass pasteur pipet to

another centrifuge tube, from which aliquots were taken. By these successive transfers most of the remaining fat was left behind in the tubes and on the pipets. This 11 500 rpm supernatant was used as the source of the IIGT vesicles.

Labeling of 3T3-L1 adipocytes with ortho[^{32}P] phosphate. Cell monolayers on a 60-mm dish were washed three times with 4 ml of serum- and P_i-free DMEM, and were then incubated with 2 ml of the same medium. After 30 min in an incubator (10% CO_2 at 37° C), 1.0 mCi of carrier-free [^{32}P]P_i was added. Total incubation time in [^{32}P]P_i was 2 h. Insulin was added directly to this medium for the times indicated in the legend to Fig. 5.

Immunoadsorptions. S. aureus cells were washed twice with 10 mg/ml bovine serum albumin in buffer A and then loaded by incubation for 2 h at room temperature with the IgG's (either the anti-GT C peptide antibodies or purified nonimmune rabbit IgG). The loaded *S. aureus* cells were washed once with buffer A and then incubated for 2 h at room temperature with the 11 500 rpm supernatant, with continuous mixing on a rotating wheel. The *S. aureus* cells were then separated from the medium and washed once with buffer A. *S. aureus* cells were pelleted at each stage by centrifuging in an Eppendorf 5412 microfuge for 1.5 min ($13\,000 \times g_{max}$) or at 6000 rpm for 10 min in a SS34 rotor of a Sorvall centrifuge ($4000 \times g_{max}$).

For the isolation of IIGT vesicles used in the determination of the amount of protein and phospholipid and the polypeptide composition, this procedure was modified slightly to reduce nonspecific binding. First, the 11 500 rpm supernatant (7 ml from a single 10 cm plate) was 'precleared' by slow rotation with *S. aureus* cells carrying nonimmune IgG (1 μl cells with 2 μg nonimmune IgG per ml supernatant) for 30 min. Second, after incubation of the 'precleared' supernatant with *S. aureus* cells bearing immune or, in the control, nonimmune IgG (1 μl cells with 2 μg IgG per ml supernatant) for 2 h, the *S. aureus* cells were pelleted and washed twice with 1 ml buffer A. Finally, the vesicles were solubilized by treatment of the 7 μl of washed *S. aureus* cells with 37 μl 19 mM $C_{12}E_8$/150 mM NaCl/20 mM Hepes/2 mM $MgCl_2$ (pH 7.4) with 0.3 mM phenylmethanesulfonyl fluoride/1 μM pepstatin A/0.1 mM L-*trans*-epoxysuccinylleucylamido-(3)methyl)butane for 15 min. The *S. aureus* cells were pelleted and the supernatant was used for the analyses.

Fractionation and immunoadsorption from [^{32}P]P_i-labeled cells. An identical procedure, including the preclearing step, was used to isolate IIGT vesicles from cells labeled with [^{32}P]P_i except that the volume and composition of the homogenization buffer was changed. Buffer A was replaced with a buffer designed to inhibit phosphatase action [10]: 50 mM Hepes/10 mM $Na_4P_2O_7$/100 mM NaF/4 mM Na_2EDTA/2 mM Na_3VO_4 (pH 7.4) (buffer B). Since smaller dishes were used for [^{32}P]P_i-labeled cells, the homogenization was performed in only 3.4 ml buffer B to maintain similar protein concentrations. We refer to this alternate protocol as the modified procedure.

Gel electrophoresis and immunoblotting. Polypeptides were separated by SDS-gel electrophoresis according to Laemmli [11]. [^{32}P]P_i-labeled phosphoproteins were visualized by autoradiography as described previously [10]. For immunoblotting, the polypeptides were transferred to nitrocellulose, and the nitrocellulose was probed with the affinity purified antibodies to the glucose transporter or glucose transporter carboxyl terminal peptide and then with ^{125}I-labeled goat antibodies against rabbit IgG. The details of the procedures for the preparation of SDS samples, electrophoresis, immunoblotting and quantitation of the relative amounts of glucose transporter in the lanes on the immunoblot by cutting and counting the glucose transporter band have been described previously [2]. The only modification was that the SDS sample buffer included a mixture of proteinase inhibitors (1 mM diisopropyl fluorophosphate/1 μM pepstatin A/0.1 mM L-*trans*-epoxysuccinylleucylamido(3-methyl)butane).

Generally, the affinity-purified antibodies against the glucose transporter, rather than those against the carboxy terminal glucose transporter peptide, were used for immunoblotting. In one experiment the same samples of the 11 500 rpm supernatant and pellet from basal and insulin-treated cells were analyzed for relative glucose transporter content by immunoblotting with each preparation of antibodies (see Fig. 1 for this type of experiment). The values for the distribution of

the glucose transporter determined with the two preparations were the same, and thus both preparations of antibodies recognize the same antigen, which must be the glucose transporter. In addition, the actual values for the radioactivity in the glucose transporter band given by the two preparations of antibodies were the same within 20%. Subsequently, it was found that most of the antibodies elicited by the purified glucose transporter were against its carboxy terminus, since immunoblotting in the presence of a 350-fold molar excess of glucose transporter peptide 477–492 over anti-GT antibodies decreased the labeling of the glucose transporter by 85%.

In some experiments the absolute amount of glucose transporter on the immunoblot was determined by running 3T3-L1 membranes that contained a known amount of glucose transporter, as measured by cytochalasin B binding, on adjacent lanes. The details of this method are described in Ref. 8.

Other assays. 5′-Nucleotidase activity was assayed by a slight modification of the method of Simpson et al. [12]. Samples in buffer A were incubated with 0.3% Triton X-100/5 mM 2′,3′-AMP/0.2 mM [^{3}H]5′-AMP (1 μCi/ml) at 37°C. At 15, 30 and 60 min, aliquots (200 μl) were removed and added to 50 μl 0.25 M $ZnSO_4$, followed by the addition of 100 μl 0.125 M $BaSO_4$. The radioactivity in a sample of the supernatant was measured by liquid scintillation spectrometry. The concentration of protein was adjusted so that the rate of adenosine release was linear with time. The specific activity of the homogenate was about 10 nmol/h per mg protein under these conditions. The activity of this enzyme in 3T3-L1 adiocytes is much lower than that in rat adipocytes (250 nmol/h per mg homogenate protein [12]). Galactosyltransferase was assayed as described by Fleischer [13], with 60 mM 4-morpholineethanesulfonic acid as the buffer [12]. Sialyltransferase was assayed by the method of Weinstein et al. [14], with the modification that the acceptor substrate was 4 mg/ml asialofetuin [15].

Total phosphate was determined by acid digestion and colorimetric assay according to the method described in Ref. 16. The amount of phospholipid was calculated on the assumption that 1 μg phosphorus derived from 26 μg phospholipid. Protein was measured by a modification of the Lowry method [17].

Results

Subcellular distribution of 5′ nucleotidase and glucose transporter in basal and insulin-treated adipocytes

In order to determine the optimal condition for separating the plasma membranes from the HGT vesicles, cell homogenates were centrifuged for various *g* minutes. The pellets and supernatants were assayed for glucose transporter content and for 5′-nucleotidase activity, which is largely located in the plasma membrane of rat adipocytes [12] and was assumed to be similarly located in the 3T3-L1 adipocytes. In order to illustrate the methodology, representative results for the centrifugation procedure found to be most appropriate (11 500 rpm for 20 min) are presented in Fig. 1. The glucose transporter, which migrates upon gel electrophoresis with an average M_r 55 000 [2], is the only band found in the immunoblot. By quantitation of the radioactivity in the glucose trans-

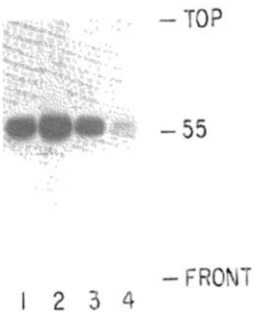

Fig. 1. Distribution of the glucose transporter between cell fractions. 3T3-L1 adipocytes were homogenized as described in the Materials and Methods, and the homogenate was centrifuged at 11 500 rpm for 20 min. The pellet was resuspended in a volume that was the same as that of the original homogenate, and samples for SDS-PAGE were prepared by trichloroacetic acid precipitation. An amount of sample equivalent to 0.6% of the original homogenate from a 10-cm plate was run on each lane; in this case, the blot was probed with 1 μg/ml of anti-GT C peptide antibodies. Lanes 1 and 2, pellets from basal and insulin-treated cells, respectively; lanes 3 and 4, supernatants from basal and insulin-treated cells, respectively. The glucose transporter band in lanes 1–4 contained 817, 1257, 613 and 230 cpm, respectively, after correction for the blank value of about 280 cpm.

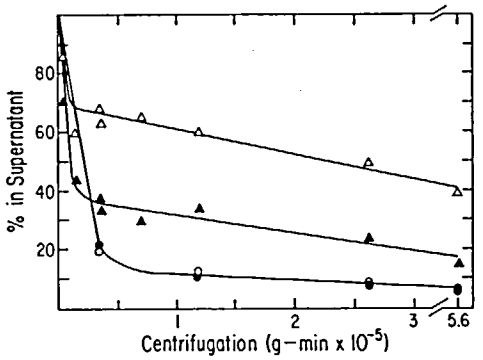

Fig. 2. Effect of centrifugation g minutes on the distribution of 5′-nucleotidase activity (○, ●) and glucose transporter (△, ▲) between pellet and supernatant. Open symbols represent basal cells; filled symbols, insulin-treated ones. Cells were homogenized as described in the Materials and Methods. For determination of the 5′-nucleotidase activity, 7.2 ml of the homogenate was centrifuged at 4000 rpm for 20 min in a SS 34 rotor of a Sorvall centrifuge. The pellet was resuspended in 0.7 ml buffer A for the nucleotidase assay. This procedure was continued with centrifugation of the successive supernatants at 6000, 8000 and 11500 rpm for 20 min. Finally, the membranes remaining in the 11500 rpm supernatant were sedimented at 45000 rpm for 75 min in a Beckman 50 Ti rotor (180000× g_{max}). Each pellet was assayed for 5′-nucleotidase activity, its activity was expressed as a percent of the total activity in all the pellets, and from these values the percent of activity remaining in the supernatant after each centrifugation was calculated. It was necessary to assay the pellets rather than the supernatants for 5′-nucleotidase, because the activity was so low that a concentrative step was required. The glucose transporter content in a sample of the supernatant after each centrifugation was determined by quantitative immunoblotting, as described in the Materials and Methods and legend of Fig. 1 and is expressed as a percentage of the amount in the homogenate.

porter band, the distribution of glucose transporter in basal cells was 55% in the pellet (lane 1) and 45% in the supernatant (lane 3). Insulin treatment shifted the distribution to 85% in the pellet (lane 2) and 15% in the supernatant (lane 4). For both the basal and insulin-treated cells, the pellet contained 94% of the 5′-nucleotidase activity and the supernatant only 6%.

The data in Fig. 2 summarize several experiments of this type. The results indicate that centrifugation of the homogenate at 11500 rpm for 20 min (about $3 \cdot 10^5$ g_{max} minutes) was optimal for separation of the IIGT vesicles from the plasma membranes, since a substantial portion of the glucose transporter (about 50% in the basal case) remained in the supernatant, whereas only 6% of the plasma membranes, as measured by 5′-nucleotidase activity, was present in the supernatant. The supposition that the unsedimented glucose transporter is in IIGT vesicles is supported by the observation that insulin treatment of cells decreased the amount of glucose transporter in the supernatant to about 40% of that for basal cells, with a corresponding increase in the pellet (Figs. 1 and 2). Over the course of this study, 19 separate experiments in which the relative glucose transporter content in the 11500 rpm supernatant from basal and insulin-treated cells was measured have been performed; the glucose transporter content of the supernatant from insulin-treated cells was 46 ± 9% (S.D.) of that from basal cells.

It is worth pointing out here how this procedure is an improvement on our previously described method [2]. Previously, the cell homogenate was centrifuged at 11500 rpm for 20 min and then the supernatant was centrifuged at 19000 rpm for 30 min. This 19000 rpm supernatant served as the source of the IIGT vesicles. The 19000 rpm supernatant from basal cells contained only 25% of the total glucose transporter. Thus, the use of the 11500 rpm supernatant, which contains 50% of the total glucose transporter, for immunoadsorption (see below) increased the yield of IIGT vesicles by a factor of two. Moreover, as assessed by the effect of insulin on the glucose transporter content of the two supernatants, the purity of the IIGT vesicles in the two supernatants did not differ. The glucose transporter content of the 19000 rpm supernatant from insulin-treated cell is 58% of that from basal cells [2], whereas, as described above, the corresponding value in the case of the 11500 rpm supernatant was 46%.

Immunoadsorption of the IIGT vesicles

IIGT vesicles in the 11500 rpm supernatant were adsorbed onto the surface of formaldehyde-fixed *S. aureus* cells that contained anti-GT C peptide antibodies bound to the protein A. Preliminary experiments in which the extent of immunoadsorption was determined as a function of the amount of antibodies showed that 2 μg anti-GT C peptide antibodies on 1 μl *S. aureus* cells was required to absorb most of the IIGT vesicles

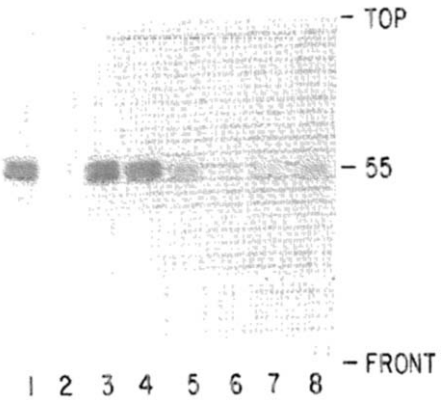

Fig. 3. Immunoadsorption of the IIGT Vesicles. *S. aureus* cells (1 μl) were loaded with 2 μg anti-GT C peptide antibodies or irrelevant rabbit IgG. 1 ml amounts of the 11500 rpm supernatant from basal or insulin-treated cells were incubated with the *S. aureus* cells as described in the Materials and Methods. Samples of the immunoadsorbed supernatant, as well as samples that had not been treated with *S. aureus* cells, were converted to samples for SDS-PAGE by trichloroacetic acid precipitation and subjected to immunoblotting for the glucose transporter. Lane 1, untreated supernatant from basal cells; lane 2, supernatant from basal cells after immunoadsorption with anti-GT C peptide antibodies; lane 3, untreated supernatant from basal cells; lane 4, supernatant from basal cells after immunoadsorption with irrelevant IgG; lanes 5–8, the corresponding samples from insulin-treated cells. After correction for the blank (about 240 cpm), the glucose transporter band in lanes 1–8 contained 1214, 273, 1448, 1292, 781, 365, 657 and 687 cpm, respectively.

in 1 ml supernatant. Fig. 3 presents the results of an immunoadsorption experiment performed under these conditions. In this particular experiment, 78% of the glucose transporter in the supernatant from basal cells and 53% of that in the supernatant from insulin-treated cells were adsorbed onto the *S. aureus* cells bearing anti-GT C peptide antibodies (compare lane 1 to 2 and lane 5 to 6). Much less glucose transporter was removed by nonspecific adsorption on the *S. aureus* cells bearing irrelevant IgG (11% for the supernatant from basal cells and 0% for that from insulin-treated cells; compare lane 3 to 4 and 7 to 8). This experiment was repeated several times; the values for the percent glucose transporter adsorbed ± S.D. (*n* experiments) were: basal supernatant with specific antibodies, 80 ± 7(6); basal supernatant with irrelevant antibodies, 23 ± 8(6); insulin supernatant with specific antibodies, 62 ± 9(4); insulin supernatant with irrelevant antibodies, 18 ± 12(4). Since 1 ml of the 11500 rpm supernatant from basal cells contains about 60 ng glucose transporter (see below), there was about a 10-fold molar excess of anti-GT C peptide antibodies over glucose transporter under the conditions used for the typical immunoadsorption.

In immunoadsorption experiments of the type just described, we usually did not determine the amount of glucose transporter associated with the *S. aureus* cells after immunoadsorption, since it is reasonable to conclude that the glucose transporter disappearing from the supernatant upon adsorption with the anti-GT C peptide antibodies was present on the *S. aureus* cells. However, in one experiment the IIGT vesicles adsorbed on *S. aureus* cells from the supernatant of basal adipocytes were solubilized with SDS sample buffer containing 6 M urea and then immunoblotted for glucose transporter. All of the glucose transporter adsorbed from the supernatant was found in the pellet sample (data not shown). This result is the same as that described with the original procedure for isolation of the IIGT vesicles (Fig. 2 of Ref. 2).

It was hoped that the adsorbed IIGT vesicles could be released from the *S. aureus* cells with the carboxy terminal peptide against which the antibodies used for immunoadsorption were raised. Unfortunately, this approach proved unsuccessful. In three separate experiments *S. aureus* cells to which IIGT vesicles had been bound were incubated at 25°C for 1 h and/or for 24 h with 1500-fold molar excess of glucose transporter peptide 477–492 over the bound antibodies, and then the supernatant and *S. aureus* cells from this incubation were analyzed for glucose transporter content by immunoblotting. No detectable release of the glucose transporter occurred (data not shown). The failure to release the IIGT vesicles may be due to the existence of multiple antigen-antibody interactions between the *S. aureus* cells and the vesicles, which are estimated to contain at least eight copies of the glucose transporter (see below).

In our previous study, we found that some proteins in the cellular supernatant adsorbed to the *S. aureus* cells nonspecifically [2]. Consequently, in order to determine the amount of vesicular protein and phospholipid, the IIGT

TABLE I

PROTEIN AND PHOSPHOLIPID COMPOSITION OF IIGT VESICLES

The 11500 rpm supernatants from 10-cm plates of basal and insulin-treated cells were precleared with *S. aureus* cells bearing nonimmune IgG and then adsorbed with *S. aureus* cells coated with nonimmune or immune IgG (2 μg on 1 μl *S. aureus* cells per ml supernatant). The adsorbed material was released with $C_{12}E_8$ and assayed for protein and phospholipid, as described in the Materials and Methods. Results are expressed as μg per 10-cm plate. bas, basal cells; ins, insulin-treated cells; I, adsorption with immune IgG; NI, adsorption with nonimmune IgG.

Expt.	Samples	Protein		Phospholipid	
		total	specific	total	specific
1	bas, I	16.2	10.5	16.6	8.7
	NI	5.7		7.9	
	ins, I	9.9	2.7	11.4	2.4
	NI	7.2		9.0	
2	bas, I	20.1	15.0	25.8	17.6
	NI	5.1		8.2	
	ins, I	7.5	3.9	12.8	7.6
	NI	3.6		5.2	
3	bas, I	13.6	10.0	12.1	6.6
	NI	3.6		5.5	
	ins, I	7.3	2.1	8.4	2.4
	NI	5.2		6.0	
Average expts. of 1–3	bas		11.7		11.0
	ins		2.9		4.1

vesicles on the *S. aureus* cells were solubilized with the nonionic detergent $C_{12}E_8$ and the *S. aureus* cells were separated from the extract. The data in Table I show that about 11 μg of IIGT vesicular protein and phospholipid were obtained from a 10-cm plate of basal adipocytes (about 10^7 cells and 10 mg protein). Insulin-treated adipocytes yielded about 3 μg of each. This reduction in yield was expected since, as noted above, the 11500 rpm supernatant from insulin-treated cells contained 46% as much transporter as that from basal cells.

Glucose transporter content of the IIGT vesicles. The glucose transporter content of the 11500 rpm supernatant from basal cells was determined by quantitative immunoblotting with sampels containing amounts of transporter known from cytochalasin B binding as standards (see Materials and Methods). Three separate experiments gave values of 460, 490 and 490 ng glucose transporter in the supernatant from the cells on a 10-cm plate. Since 80% of this was immunoadsorbed in the standard procedure for isolation of the IIGT vesicles, the preparation of vesicles from a single 10-cm plate contained 380 ng glucose transporter per 12 μg protein (Table I). Thus, the transporter constituted about 3% of the protein in the vesicles.

The extent to which the transporter is enriched in the IIGT vesicles, relative to total membranes in the 11500 rpm supernatant, was determined as follows. The total amount of membrane protein in this supernatant from a 10-cm plate of basal cells, measured by sedimenting the membranes at $180000 \times g_{max}$ for 1.25 h and then assaying for protein, was found to be 330 μg. Thus, the transporter constituted 0.15% of the protein in the total membranes and so was enriched 20-fold in the IIGT vesicles.

An estimate of the number of transporter molecules per IIGT vesicle can also be made. If it is assumed that the size of the vesicles isolated here is the same as that found for the IIGT vesicles isolated by our previous procedure (50 nm, as determined by thin-section electron microscopy

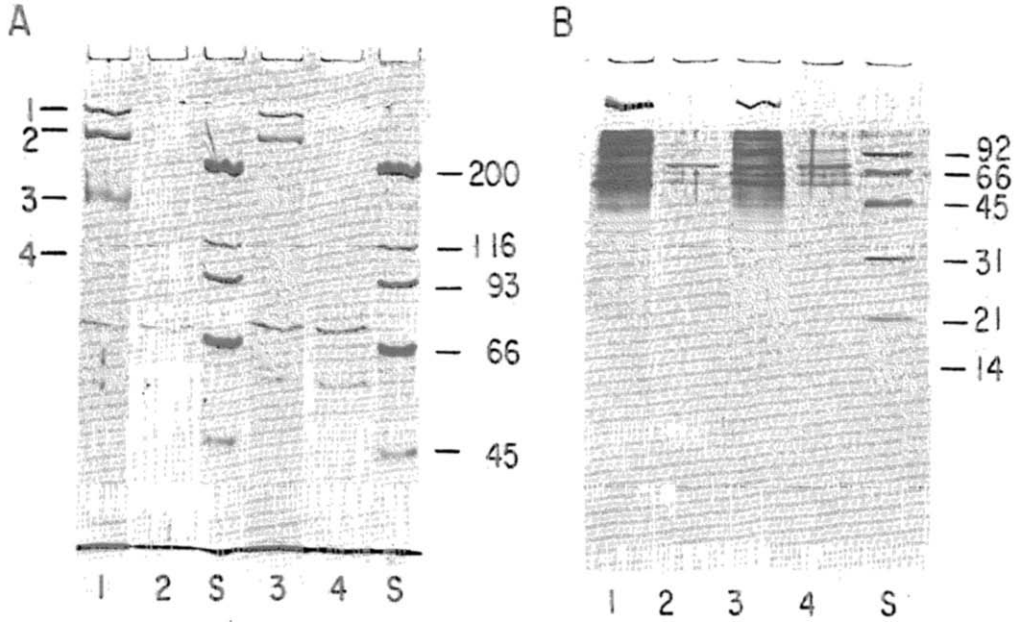

Fig. 4. Polypeptide composition of the IIGT vesicles. IIGT vesicles were adsorbed onto *S. aureus* cells and then the polypeptides were released with 1% $C_{12}E_8$, as described in the Materials and Methods. These were run on 7.5% (part A) and 15% (part B) acrylamide gels and stained with Coomassie blue. Lanes 1 and 2 show the polypeptides released from *S. aureus* cells bearing immune and nonimmune IgG, respectively, that had been incubated with the 11500 rpm supernatant from basal cells. Lanes 3 and 4 are the corresponding samples derived from the supernatant of insulin-treated cells. The amount on each lane is that from 2.6 ml of supernatant (33% of the cells on a 10-cm plate). The lanes marked S contain molecular mass standards (about 100 ng of each polypeptide), and the right-hand scale gives the values for these in kDa. The left-hand numbers designate the major polypeptides specifically associated with the IIGT vesicles. This experiment is representative of three entirely separate ones giving the same results.

[2]) * and that the typical phospholipid has a volume of 1.25 nm^3 [18], then a vesicle contains $1.5 \cdot 10^7$ daltons of phospholipid [18]. With this value and those for the glucose transporter and lipid content of the IIGT vesicles (Table I), we can calculate that one vesicle contains eight transporters.

Polypeptide composition of the IIGT vesicles

In our previous study, the low amounts of vesicular polypeptides in the $C_{12}E_8$ extract of the *S. aureus* immunoadsorbate necessitated their detection by radiolabeling with ^{125}I-Bolton-Hunter reagent [2]. In this study, the method of $C_{12}E_8$ solubilization has been applied to a larger amount of IIGT vesicles and, consequently, it has been possible to identify some of the vesicular proteins by protein staining. Fig. 4A and B shows 7.5 and 15% polyacrylamide gels of the polypeptides released by $C_{12}E_8$ treatment. Comparison of the samples isolated with immune and nonimmune IgG (lane 1 vs. lane 2 and lane 3 vs. lane 4 of each gel) shows that there are a number of polypeptides specific to the vesicles. Polypeptides that stand out as major components of the vesicles have apparent M_r values of greater than 270000, 245000, 165000 and 115000 (bands 1–4 of Fig. 4A, respectively). These presumably correspond to the polypeptides of greater than 250000, 177000 and 110000 that were radiolabeled in our previous study [2]. Although it is not as evident in the photograph, the staining of many bands in the sample from in-

* Some of the vesicles are probably larger, since, as described in the text, the improved preparation includes IIGT vesicles that were lost by sedimentation at 19000 rpm in the original method.

sulin-treated cells (lane 3 of each gel) was less intense than that of the corresponding bands in the sample from basal cells (lane 1). This finding is expected on the basis of the observation that the supernatant from insulin-treated cells yields only about a third as much IIGT vesicle protein as that from basal cells (Table I).

The gels of the $C_{12}E_8$-extracted proteins do not show either the glucose transporter or the antibodies. Experiments in which the proteins in the $C_{12}E_8$ extract and on the *S. aureus* cells after extraction (released with SDS-urea) have been immunoblotted showed that in this nondenaturing detergent the antibodies remain bound to the protein A and the glucose transporter remains bound (or binds, if not originally bound while in the vesicles) to the antibodies. In fact the conditions here are similar to those that we previously employed to immunoprecipitate glucose transporter from membranes solubilized in $C_{12}E_8$ [10].

Phosphoprotein composition of the IIGT vesicles

The phosphoprotein composition of the IIGT vesicles was determined by labeling cells with [^{32}P]P_i and then isolating the vesicles, as described in the Materials and Methods. Since the composition of the homogenization buffer was changed by the inclusion of phosphatase inhibitors, it was first necessary to ascertain that the fractionation of the IIGT vesicles was not changed. By the modified procedure, the glucose transporter content of the 11 500 rpm supernatant from cells treated with 100 nM insulin for 20 min was $40 \pm 15\%$ (S.D., $n = 7$) of that from basal cells. This value is similar to that of 46% obtained by the standard protocol (see above). Moreover, the polypeptide composition of the IIGT vesicles isolated by the modified procedure (data not shown) was similar to that of the vesicles isolated by the standard protocol (Fig. 4). Consequently, the IIGT vesicles fractionated in the same way in either buffer.

As illustrated in Fig. 5 the IIGT vesicles contained several phosphoproteins, designated bands 1 to 5. Two of these (bands 1 and 4) have mobilities identical to the major Coomassie blue-staining polypeptides of M_r 245 000 and 115 000 (Fig. 4A) and, presumably, are the same proteins. In addition, the major phosphoproteins of M_r 190 000 and 25 000 (bands 2 and 5) may correspond to bands that stain more weakly with Coomassie blue (Fig. 4). A faint band (band 3) at M_r 165 000 may correspond to the protein of this molecular weight found by Coomassie blue staining (Fig. 4A).

Treatment of the cells with insulin for various periods did not result in a marked change in the pattern of phosphoproteins in the IIGT vesicles (Fig. 5, compare lanes 4, 6 and 8 with lane 2). The weak band at M_r 165 000 (band 3) in Fig. 5A is more intense in cells treated with insulin for 1 min (compare lanes 2 and 4); however, this minor difference was observed in only two out of four experiments. There was an overall increase in intensity of the phosphoprotein bands in cells after 30 min of insulin exposure (Fig. 5, compare lanes 2 and 8). Presumably, this was due to the 1.6-fold increase in the specific activity of the ATP that occurs under exactly these conditions [10].

Thus, we were unable to identify a protein in the IIGT vesicles that consistently changed its phosphorylation state in response to insulin and so might be involved in regulation of the translocation process. A potential complication of this approach is the possibility that if the change in the phosphorylation state of a protein in an IIGT vesicle triggers the fusion of the vesicle with the plasma membrane, then the IIGT vesicles isolated after insulin exposure may not contain this protein. In the hope that the putative change in the phosphorylation state of a vesicle protein might occur more rapidly after insulin exposure than the fusion of the vesicle, we examined the phosphoprotein composition of vesicles isolated from insulin-treated cells under two conditions where translocation had not yet occurred. One of these was brief exposure to insulin. The glucose transporter content of the 11 500 rpm supernatant from cells treated for only 1 min was $92 \pm 10\%$ (S.D., $n = 3$) of that in basal cells. As shown in Fig. 5 (lane 4), the IIGT vesicles isolated from cells exposed to insulin for 1 min had the same phosphoprotein composition as those from basal cells. The second condition was exposure of cells to insulin for a longer period at a temperature lower than 37°C. In two separate experiments in which the glucose transporter content of the 11 500 rpm supernatant from cells treated with 1 μM insulin at 18°C for 20 min was examined, the glucose transporter content was the same as that of the

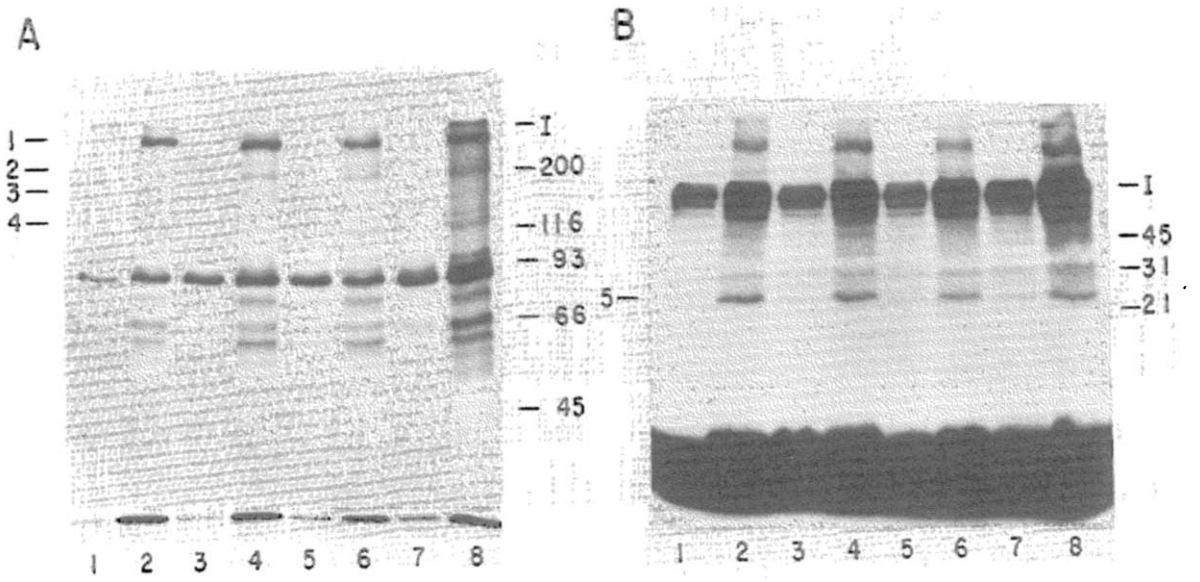

Fig. 5. Phosphoprotein composition of the IIGT vesicles. IIGT vesicles from [^{32}P]P_i-labeled cells were adsorbed onto *S. aureus* cells and then the polypeptides were released with 1% $C_{12}E_8$, as described in the Materials and Methods. These were run on 7.5% (part A) and 15% (part B) acrylamide gels and autoradiographed for 72 h. Phosphoproteins released from *S. aureus* cells bearing nonimmune (odd-numbered lanes) and immune (even-numbered lanes) IgG, respectively, that had been incubated with the 11500 rpm supernatant from basal cells (lanes 1 and 2), or cells treated with 100 nM insulin for 1 min (lanes 3 and 4), 4 min (lanes 5 and 6) or 30 min (lanes 7 and 8) are shown. The amount on each lane is that from 0.37 ml of supernatant (11% of the cells on a 6-cm plate). The right-hand scale gives the values for the molecular mass standards in kDa; I designates the interface between the stacking and separating gels. The numbers of the left-hand side designate the major phosphoproteins specifically associated with the IIGT vesicles. This experiment is representative of four separate ones giving the same results.

basal cells. The phosphorylation patterns of the proteins in IIGT vesicles isolated from basal cells and those exposed to 1 μM insulin for 5 and 20 min at 18° C were identical and similar to those for cells treated at 37° C (data not shown).

Although these results indicate that a change in the state of phosphorylation of a vesicle protein is not involved in the regulation of translocation, they do not rigorously eliminate the possibility. For example, it is possible that such a phosphoprotein was not detected because it underwent dephosphorylation during the isolation of the vesicles, even though the homogenization medium contained inhibitors of phosphatases for both phosphotyrosine and phosphoserine [10]. Also, the amount of such a protein may be below the level of detection. We have previously shown that the glucose transporter itself is not phosphorylated in the absence or presence of insulin [10].

Marker enzymes and the IIGT vesicles

In rat adipocytes the intracellular glucose transporters fractionate similarly, although not identically, with the Golgi membranes, as measured by the marker enzyme galactosyltransferase [12,19]. This finding prompted us to determine whether galactosyltransferase activity was immunoadsorbed from the 11500 rpm supernatant along with the IIGT vesicles. The supernatant (8 ml) from a 10 cm plate of basal cells was divided in half, and one-half was treated with 3 μg anti-GT C peptide antibodies on 1.5 μl *S. aureus* cells per ml, as described in the Materials and Methods. After the immunoadsorption, the membranes in the untreated and glucose transporter-depleted supernatants were sedimented at 180000 $\times g_{max}$ for 1.25 h, and then resuspended and assayed for galactosyltransferase. As a control, the supernatant from a second plate was treated in the

same way, except that the immunoadsorption was with nonimmune rabbit IgG. In both cases, 75% of the galacosyltransferase activity remained in the supernatant after the immunoadsorption step; and thus most of the galactosyltransferase is not associated with the IIGT vesicles. The loss of 25% of the activity in the immunoadsorptions with both immune and nonimmune IgG may have been due to some nonspecific adsorption of Golgi membranes on the *S. aureus* cells. The specific activity of the enzyme in the membranes from the 11 500 rpm supernatant was 37 nmol/mg protein per h, a value which is about half that reported for the low-density microsomes from adipocytes (62 nmol/mg per h [12]).

A recent study employing immunoelectron microscopy indicates that the IIGT vesicles may be derived from the *trans*-Golgi reticulum [6]. An enzyme that is localized in this organelle in some cell types is α-2,6-sialyltransferase [20,21]. Unfortunately, no sialyltransferase activity was detectable in either the homogenate or in the membranes sedimented from the 11 500 rpm supernatant at $180000 \times g_{max}$ for 1.25 h. Control assays with purified 2,6-sialyltransferase showed that activities as low as 0.1 and 0.5 nmol/mg per h in the homogenate and in the membranes sedimented from the 11 500 rpm supernatant, respectively, would have been detected.

Discussion

The method for isolation of the IIGT vesicles has been improved so that the yield is about twice that obtained by the original method [2]. For the first time, the protein, lipid and glucose transporter contents of the vesicles have been determined. Moreover, the polypeptide composition has been more firmly established by electrophoresis of sufficient protein to allow staining with Coomassie blue. The phosphoprotein composition of the vesicles has been determined; insulin treatment of cells has no detectable effect on this composition.

The recent analysis of the distribution of the glucose transporter in 3T3-L1 adipocytes by quantitative immunocytochemistry indicates that insulin increases the glucose transporter content in the plasma membrane from 15 to 43% of the total in the cell [6]. The source of this glucose transporter was tentatively identified as the *trans*-Golgi reticulum; the glucose transporter content of this organelle correspondingly decreases from about 45 to 16% of the total [6]. The value for the difference between the glucose transporter content of the *trans*-Golgi reticulum in the basal and insulin-treated state (26% of the total) is very similar to that for the difference between the glucose transporter content of 11 500 rpm supernatant from basal and insulin-treated cells (27% of the total). Thus, the improved method described here is one that probably leads to the isolation of most of the insulin-responsive intracellular glucose transporter in 3T3-L1 adipocytes. Moreover, a large portion of the IIGT vesicles isolated from basal cells (at least that portion not obtained from insulin-treated cells) must be derived from the *trans*-Golgi reticulum. Immunocytochemical analysis of the distribution of the glucose transporter also showed that about 25% of the glucose transporter in both basal and insulin-treated adipocytes is located in cytoplasmic vesicles of an undefined nature [6]. Presumably, the IIGT vesicles isolated from both basal and insulin-treated cells also include some portion of these.

The higher yield of vesicular protein may allow the development of antibodies against other polypeptides besides the glucose transporter in the vesicles, and these will be useful to determine whether these polypeptides are also translocated to the plasma membrane in response to insulin. In this regard, we have reported that insulin elicits the translocation of transferrin receptors in 3T3-L1 adipocytes [22] and recently found that this is also the case for the insulin-like growth factor II/mannose 6-phosphate receptor (Tanner, L.I. and Lienhard, G.E., unpublished results). In a study to be reported elsewhere, we found that the IIGT vesicles isolated by the method described here contain intracellular populations of both these receptors and that the polypeptide of M_r 245 000 is the insulin-like growth factor II/mannose 6-phosphate receptor (Tanner, L.I. and Lienhard, G.E., unpublished results).

Finally, we note that the combined results of this study and that of the distribution of the glucose transporter by immunocytochemistry [6] suggest a possible interesting effect of insulin on

the *trans*-Golgi reticulum. Insulin may cause the membrane area of the reticulum to decrease by about 60%; presumably the lost membrane largely appears in the plasma membrane. The rationale for this suggestion is the finding that the amount of IIGT vesicles isolated from insulin-treated adipocytes was 60% less, as assayed by the yield of membrane lipid (Table I). If the glucose transporter had simply redistributed by itself to the plasma membrane in response to insulin, then its density in the *trans*-Golgi reticulum and in each vesicle derived from it would have been less but the actual number of vesicles containing glucose transporter may have been unchanged. A general effect of insulin causing the redistribution of membrane between the reticulum and the plasma membrane would also account for the redistribution of the receptors described above. In the future, it may be possible to test for the occurrence of this effect through selective staining of the *trans*-Golgi reticulum and measurement of its area.

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