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Cell-surface biotinylation of GLUT4 using bis-mannose photolabels

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New cell-impermeant bis-mannose photolabels have been developed with biotinyl groups attached to 4-(1-azi-2,2,2-trifluoroethyl)-benzoyl-1,3-bis(D-mannos-4-yloxy)-2-propylamine (ATB-BMPA) by either a polyethoxy spacer (Bio-ATB-BMPA) or an additional hexanoic acid spacer (Bio-LC-ATB-BMPA). The half-maximal inhibition constants, K_i values, for inhibition of glucose transport activity in insulin-stimulated rat adipocytes were determined to be 359 ± 10 and 273 ± 28 μM for Bio-ATB-BMPA and Bio-LC-ATB-BMPA, respectively. These values are similar to those previously reported for the non-biotinylated compound ATB-BMPA. Following UV-irradiation-induced cross-linking of the biotinylated photolabels to rat adipocytes, the biotinylated glucose transporter isoform 4 (GLUT4) could be detected by non-radioactive and radioactive methods that utilized the interaction with streptavidin. Biotinylated GLUT4 from 1–2 μg of adipose cell membranes, precipitated onto magnetic streptavidin beads, could be sensitively and quantitatively detected using an electrochemiluminescent assay method. This utilized a ruthenium-tagged anti-GLUT4 antibody that on excitation at an electrode generated an electrochemiluminescent signal in an ORIGEN analyser. Alternatively, surface-biotinylated GLUT4 could be easily, but less sensitively, detected in streptavidin agarose precipitates which were analysed by conventional GLUT4 Western blotting. Data obtained using the non-radioactive methods compared favourably with those using tritiated versions of the biotinylated probes. Insulin treatment of adipocytes increased the levels of signals from surface biotinylated GLUT4 by ~10-fold or ~20-fold, respectively, when the electrochemiluminescent or the Western blot detection methods were used and these signals were blocked by cytochalasin B.

INTRODUCTION

Radioactive bis-mannose photolabels, including 4-(1-azi-2,2,2-trifluoroethyl)-benzoyl-1,3-bis(D-mannos-4-yloxy)-2-propylamine (ATB-BMPA), have been previously used to detect and quantify levels of glucose transporters at the plasma membrane surface of mammalian cells [1–3]. Because the compounds are impermeable, they do not have access to those glucose transporters that are sequestered in intracellular membrane compartments. This is a particularly useful property when analysing the distribution of the insulin-stimulated glucose transporter isoform 4 (GLUT4). This is present in intracellular membrane compartments in non-stimulated cells. Following stimulation with insulin it is translocated to the cell surface [4–6]. UV-induced cross-linking of the probe in intact cells only labels the GLUT4 that is accessible at the cell surface. The labelling technique can therefore quantify the magnitude of the insulin response on translocation without the need to subcellularly fractionate the cells to obtain separate purified plasma membranes and low-density microsomes. The latter procedure is often technically difficult and can lead to cross-contamination of fractions which consequently under-estimate the level of insulin stimulated GLUT4 translocation [7].

Sensitive methods for quantification of cell-surface levels of those glucose transporters that contribute to transport activity are required as these transporters may be up- or down-regulated in response to hormonal regulation and in disease states [8–11]. Under conditions in which GLUT4 is down-regulated, there is a compensating change in GLUT1, which is up-regulated [12,13], and both transporters may influence the transport activity. In addition, in human adipocytes [14] and muscle [15], even in the absence of disease, levels of glucose transporters are much lower than in the corresponding rat tissues. Therefore, more sensitive methods for detecting and distinguishing the contributions to transport activity that these isoforms make are required. To progress towards the development of improved methods, we now report the synthesis and characterization of two novel biotinylated bis-mannose photolabels. These can be used to detect GLUT4 by sensitive non-radioactive methods that utilize the strong interaction with the biotin-binding protein, streptavidin. Hatanaka et al. [16–18] have developed a biotinylated azitrifluoroethylbenzoic acid (Bio-ATB) compound in which the benzoic acid group is linked by a polyethoxy spacer to biotin. This bifunctional reagent combines the advantages of biotin as a tag for binding to streptavidin conjugates and the diazirine photoreactive group, which is now recognized as being superior to the more conventionally used phenylazides [18,19]. The diazirine on UV irradiation gives a carbene that is more reactive than the nitrene derived from irradiation of the phenylazides [20].

To prepare the bis-mannose derivatives, the carboxylic acid group of Bio-ATB has been coupled to the 2-propylamine linker between the two mannose moieties of BMPA to give Bio-ATB-BMPA (Figure 1, compound C). Since the binding pocket of streptavidin is in a deep cleft [21], we have also prepared a derivative with an additional hexanoic acid spacer arm, Bio-LC-ATB-BMPA (Figure 1, compound D). We have compared the interaction of this compound and the shorter Bio-ATB-BMPA with the GLUT4 present in adipose cells.

The non-radioactive detection methods that we have used to

Abbreviations used: GLUT, glucose transporter isoform; ATB, 4-(1-azi-2,2,2-trifluoroethyl)benzoyl; BMPA, 1,3-bis(D-mannos-4-yloxy)-2-propylamine; Bio-ATB-BMPA, 4,4′-O-[2-[2-[2-[2-[2-(biotinylamino)ethoxy]ethoxy]ethoxy]-4-(1-azi-2,2,2,-trifluoroethyl)benzoyl]amino-1,3-propanediyl]bis-D-mannose; Bio-LC-ATB-BMPA, 4,4′-O-[2-[2-[2-[2-[2-[6-(biotinylamino)hexanoyl]amino]ethoxy]ethoxy]ethoxy]-4-(1-azi-2,2,2,-trifluoroethyl)benzoyl]amino-1,3-propanediyl bis-D-mannose; DMF, dimethylformamide.

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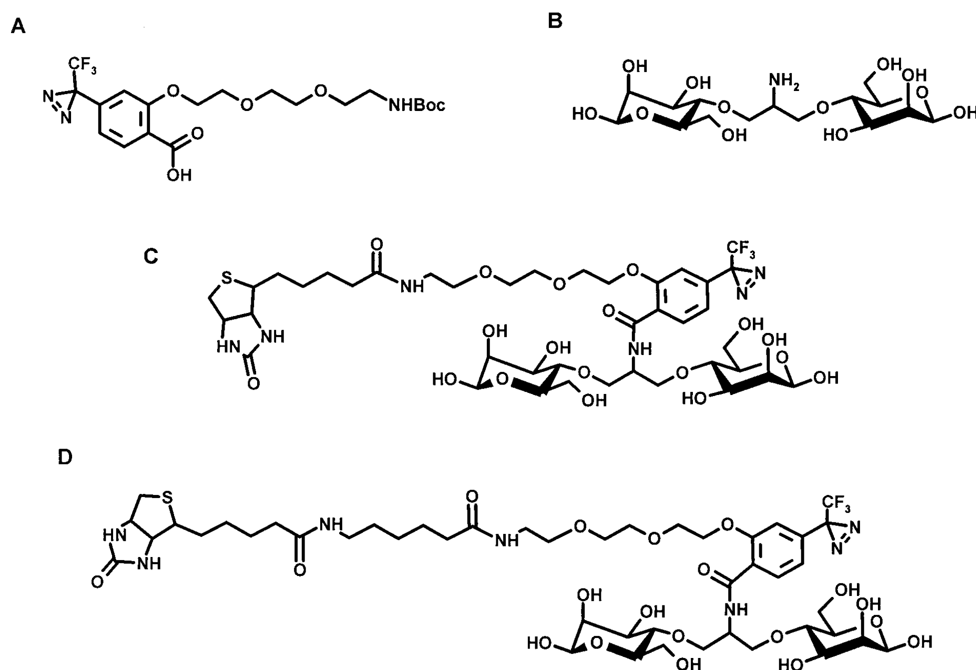

Figure 1 Structures of biotinylated bis-mannose photolabels and their precursors

Compound A is the Boc-amino protected 2-(polyethoxy)-4-(1-azi-2,2,2-trifluoroethyl)benzoic acid described by Hatanaka et al. [16]. Compound B is the bis(D-mannosyloxy)propylamine compound described by Holman and Midgley [23]. Compound C is the Bio-ATB-BMPA while compound D is the Bio-LC-ATB-BMPA. Boc, butoxycarbonyl.

quantify the surface exposed GLUT4 include a development of the novel electrochemiluminescent technique introduced by IGEN Technology, Gaithersburg, MD, U.S.A. [22]. This method is very sensitive but depends on the availability of the appropriate instrumentation. As an alternative we have also developed a procedure that measures the biotinylated GLUT4 that is precipitated by streptavidin agarose and detected by conventional Western blotting. These non-radioactive detection techniques are compared with the previously established methods employing tritiated ligands.

MATERIALS AND METHODS

Synthesis of biotinylated photolabel precursors

Hatanaka et al. [16] have described the preparation of the *N*-hydroxysuccinimide ester of 2-[2-[2-(2-biotinylaminoethoxy)-ethoxy]ethoxy]-4-(azitrifluoroethyl)benzoic acid. A similar method was used here, although the compound was prepared from compound A (Figure 1) rather than the route via the methyl ester [16]. Following deprotection to remove the amino-protecting t-butoxycarbonyl group, direct coupling with biotin *N*-hydroxysuccinimide ester was carried out. This method is described here only for the synthesis of the preparation of the equivalent precursor of the Bio-LC-ATB-BMPA with the additional aminohexanoyl spacer (Compound D, Figure 1): 50 mg (0.1 mmoles) of compound A was mixed with 80% trifluoroacetic acid in water for 2 h at room temperature. The trifluoroacetic acid was removed by repeated rotary evaporation from methanol/water (2:1, v/v). The resulting amine was dissolved in 300 μl of dimethylformamide (DMF) with 15 μl of *N*-methylmorpholine and 60 mg (0.13 mmoles) of biotinylaminohexanoic acid *N*-hydroxysuccinimide ester in 200 μl of DMF were added. The reaction was left at room temperature for 20 h. The resulting product was rotary evaporated to dryness in a stream of nitrogen and then purified by flash chromatography using ethyl acetate, methanol and water (15:2:1 v/v as eluant). This gave 73 mg (89% yield) of the required 2-[2-[2-[2-[6-(biotinylaminohexanoyl)]aminoethoxy]ethoxy]ethoxy]-4-(1-azi-2,2,2-trifluoroethyl)benzoic acid. 0.3 R_F in ethylacetate/methanol/water (15:2:1, v/v); *m/z* (fast atom bombardment MS) 717 (M+1).

Synthesis of Bio-ATB-BMPA

15 mg (0.025 mmol) of 2-[2-[2-(2-biotinylaminoethoxy)ethoxy]-ethoxy]-4-(azitrifluroethyl)benzoic acid in 300 μl DMF were mixed with 3.7 mg (0.032 mmol) of *N*-hydroxysuccinimide and 6.6 mg (0.032 mmol) of dicyclohexylcarbodiimide and left at room temperature for 20 h. Dicyclohexylurea was removed by centrifugation and the remaining *N*-hydroxysuccinimide ester in DMF was mixed with 20 mg (0.044 mmol) of 1,3-bis-(D-mannos-4-yloxy)-2-propylamine [23] and 5 μl of *N*-methylmorpholine. The solution was left at room temperature for 20 h, rotary evaporated to dryness in a stream of nitrogen and then the product was purified by paper chromatography (butan-1-ol/ethanol/water; 5:1:2, v/v), R_F 0.6, 12.3 *mg* (43% *yield*); *m/z* (fast atom bombardment MS) 1001 (M+1).

Synthesis of Bio-LC-ATB-BMPA

30 mg (0.036 mmol) of 2-[2-[2-[2-[6-(biotinylaminohexanoyl)]-aminoethoxy]ethoxy]ethoxy]-4-(1-azi-2,2,2-trifluoroethyl)-benzoic acid in 300 μl of DMF were mixed with 5.4 mg (0.047 mmol) of *N*-hydroxysuccinimide and 10.3 mg (0.05 mmol) of dicyclohexylcarbodiimide, and the solution was left at room temperature for 20 h. Dicyclohexylurea was removed by centrifugation and the remaining *N*-hydroxysuccinimide ester in DMF was mixed with 30 mg (0.066 mmol) of BMPA and 7 μl of *N*-methylmorpholine. The solution was left at room temperature

for 20 h, rotary evaporated to dryness in a stream of nitrogen and then the product was purified by paper chromatography (butan-1-ol/ethanol/water; 5:1:2, v/v), R_F 0.7, 27.6 mg (56 % yield); m/z (fast atom bombardment MS) 1114 (M+1).

Adipose cell preparations

Rat adipocytes were prepared from 180 to 200 g male Wistar rats by collagenase digestion of epididymal fat pads as previously described [24,25]. Cells were suspended at 40 % cytocrit in an albumin–Hepes buffer (140 mM NaCl, 4.7 mM KCl, 1.25 mM $MgSO_4$, 2.5 mM $CaCl_2$, 2.5 mM NaH_2PO_4, 10 mM Hepes, and 1 % (w/v) BSA, pH 7.4) at 37 °C. They were maintained in the presence or absence of 20 nM of insulin for 20 min at 37 °C and then used in glucose transport assays or for cell-surface photolabelling.

Glucose transport assays

The uptake of 50 μM 2-deoxy-D-glucose for 1 min into 50 μl aliquots of 40 % cell suspensions of rat adipose cells was determined at 37 °C and at the indicated range of inhibitor concentrations. Since the substrate concentration was low compared with its affinity constant, the ratio of the uptake rate determined in the absence (v_o) or presence (v) of inhibitor could be plotted against the inhibitor concentration (I) to give the K_i value, assuming that the inhibition conforms to the simple Michaelis–Menten competitive type. The K_i was determined from the equation $v_o/v = 1 + I/K_i$.

Photolabelling with tritiated biotinylated bis-mannose ligands

1 ml suspensions of rat adipose cells at 40 % cytocrit in albumin/Hepes buffer were mixed with 270 μCi or 108 μCi (as specified in the figure legends) of Bio-ATB-[2-^{3}H]BMPA (specific activity ~ 10 Ci/mmol) for 1 min at 18 °C in small polystyrene culture dishes (Nunclon) and then irradiated for 1 min in a Rayonet photochemical reactor using 300 nm lamps. The suspensions were then washed three times in albumin/Hepes buffer and then solubilized in 1.5 ml of Thesit detergent buffer (containing 2 % (w/v) $C_{12}E_9$, 5 mM sodium phosphate and 5 mM EDTA, pH 7.2, and with the proteinase inhibitors antipain, aprotinin, pepstatin and leupeptin each at 1 mg/ml) at room temperature. Following centrifugation at 20000 ***g*** for 20 min at 4 °C, insoluble material and the fat cake were removed and the clear supernatant was subjected to immunoprecipitation with 30 ml of Protein A–Sepharose coupled with 50 μl of anti-GLUT4 antiserum [2]. The antisera were raised against a peptide corresponding in sequence to the GLUT4 C-terminal segment. After incubation for 2 h at 0–4 °C, the immunoprecipitates were washed three times with 1.0 % (w/v) and then once in 0.1 % (w/v) Thesit detergent buffer. Labelled GLUT4 was then released from the antibody complexes with electrophoresis sample buffer (2 % (w/v) SDS, 8 M urea, 10 % (w/v) glycerol and 10 % (v/v) 2-mercaptoethanol in 115 mM Tris/HCl, 1 mM EDTA, pH 6.8) and subjected to electrophoresis on 9 % (w/v) acrylamide gels. The radioactivity of the gel was extracted from the gel slices and estimated by liquid-scintillation counting [2].

Photolabelling for non-radioactive detection methods

200 μl suspensions of rat adipose cells at 40 % cytocrit in albumin/Hepes buffer were mixed with 1 mM Bio-ATB-BMPA or Bio-LC-ATB-BMPA for 1 min at 18 °C in 35 mm polystyrene culture dishes and then irradiated for 1 min in a Rayonet photochemical reactor, using 300 nm lamps. The suspensions were then washed three times in 15 ml of albumin/Hepes buffer, once in Tes buffer (10 mM Tris/HCl, 5 mM EDTA, 250 mM sucrose, pH 7.2) and were then homogenized with 15 strokes in a Potter–Elvehjem homogenizer (Thomas Instruments) in Tes buffer in a final volume of 3 ml. The membranes were then sedimented by centrifugation at 554000 ***g*** for 30 min at 4 °C and resuspended at 1 mg/ml in Tes buffer.

Labelling of anti-GLUT4 antibody with ruthenium(II) tris(bipyridine)-NHS ester.

0.7 mg of affinity purified anti-GLUT4-C-terminal peptide antibody in 1 ml PBS (154 mM NaCl, 12.5 mM sodium phosphate, pH 7.2) was mixed with a 10:1 molar excess of ruthenium(II) (tris)bipyridine-NHS ester (32 μl of a 3 mg/ml DMSO solution; IGEN Inc.). The mixture was maintained at room temperature for 45 min in the dark. The reaction was then quenched by the addition of 20 μl of 2 M glycine and maintained for an additional 15 min at room temperature. The unbound ruthenium label was removed by dialysis overnight against PBS. The amount of ruthenium label bound to the antibody was calculated after measuring the absorbance of the ruthenium(II) (tris)bipyridinyl substituent at 455 nm. The resulting ruthenium-label:antibody ratio was usually ~ 6:1.

Electrochemiluminescent detection

1–5 μg of cell-surface biotin labelled or unlabelled total membrane preparations isolated from rat adipose cells in Tes buffer (pH 7.2) were solubilized in 100 μl of Thesit solution (1 % (w/v) Thesit, 130 mM NaCl). After adding 25 μg of streptavidin-coated magnetic beads (Dynabeads M-280-Streptavidin), the suspension was incubated for 2 h at room temperature with continuous mixing. The ruthenium-labelled anti-GLUT4 antibody was then added, to a final concentration of ~ 9 μg/ml. To decrease non-specific interactions between the components of the assay, 5 μg/ml of non-immune rabbit IgG were added with the anti-GLUT4 antibody. This solution was mixed by shaking for a further 1 h. 100 μl of a proprietary solution containing tripropylamine and Triton X-100 in PBS (ORIGEN Assay Buffer; IGEN Inc.) was then added. The resulting electrochemiluminescent signal was detected in an ORIGEN Analyser (IGEN Inc.).

Western blotting

40 μg of membranes were solubilized in 500 μl of 2 % (w/v) Thesit detergent buffer and 40 μl of a 50 % slurry of immobilized streptavidin on 6 % beaded agarose (Pierce). The samples were mixed by slow rotation for 20 h at 4 °C and then the precipitates were washed twice in 1 % (w/v) Thesit detergent buffer, twice in 0.1 % (w/v) Thesit detergent buffer and finally in PBS. The biotinylated protein was then solubilized at 95–100 °C for 20 min in electrophoresis sample buffer and subjected to electrophoresis on 9 % (w/v) acrylamide gels. The proteins were transferred to nitrocellulose (Nova Blot) and stained with Ponceau S (0.1 % (w/v) Ponceau S in 3 % (w/v) trichloroacetic acid). The nitrocellulose was blocked for 30 min in Tris-buffered saline, 0.1 % (v/v) Tween 20 and 5 % (w/v) milk protein, pH 7.4, washed briefly with Tris-buffered saline, 0.1 % (v/v) Tween 20, pH 7.4. Immunoreactive GLUT4 was blotted using affinity-purified anti-C-terminal peptide antibody at 1.5 mg/ml in Tris-buffered saline, 0.1 % (v/v) Tween 20 and 1 % (w/v) BSA, which was followed by washing (six times for 3 min in Tris-buffered saline, 0.1 % (v/v) Tween 20, pH 7.4). The bound antibody was de-

tected using enhanced chemiluminescence detection reagents (Amersham International) after incubation for 30 min with anti-(rabbit-peroxidase) coupled secondary antibody (Sigma) in Tris-buffered saline, 0.1 % (v/v) Tween 20, pH 7.4.

RESULTS

To estimate the affinity of the biotinylated photolabels for the glucose transporters present in adipocytes, the probes were used as inhibitors in 2-deoxy-D-glucose transport assays. The experiments were carried out in dim light and under conditions where light activation of the probes was insignificant. Using this approach, the half-maximal inhibition values for inhibition of 2-deoxy-D-glucose uptake were determined (Figure 2). In insulin-treated adipocytes, the affinity for the Bio-LC-ATB-BMPA ($K_i = 273 \pm 28\ \mu$M; three experiments) was slightly higher ($P < 0.05$) than for the Bio-ATB-BMPA ($K_i = 359 \pm 10\ \mu$M; three

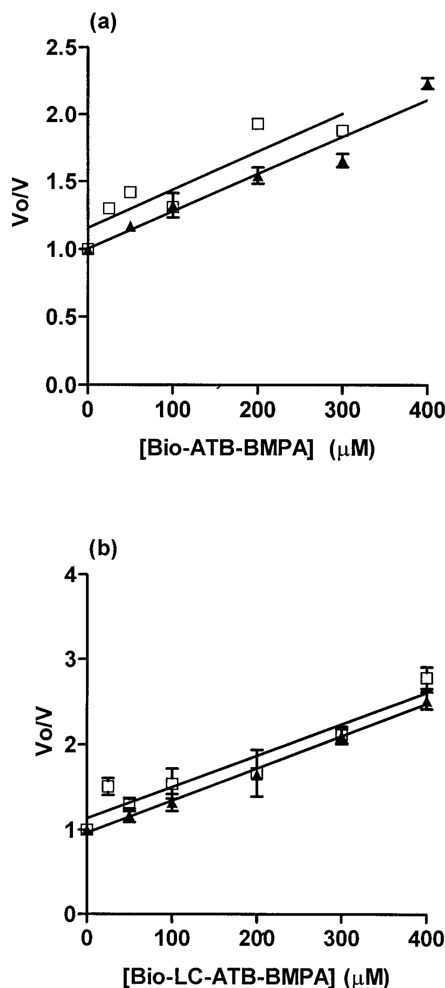

Figure 2 Determination of half-maximal inhibition values for biotinylated bis-mannose photolabels

The uptake rates of 50 μM 2-deoxy-D-glucose in basal (□) or insulin-treated rat adipocytes (▲) were determined at the indicated range of inhibitor concentrations of (**a**) Bio-ATB-BMPA and (**b**) Bio-LC-ATB-BMPA. Data are plotted as the ratio of uptake in the presence (v) and absence (v_0) of inhibitor. The v_0/v values were the mean $\pm$S.E.M. for three experiments except for the basal values for Bio-ATB-BMPA, which were from a single experiment. K_i values were determined by least-squares curve fitting to separate experiments and the mean K_i values (see Results) were from three separate experiments except for the basal Bio-ATB-BMPA (one experiment).

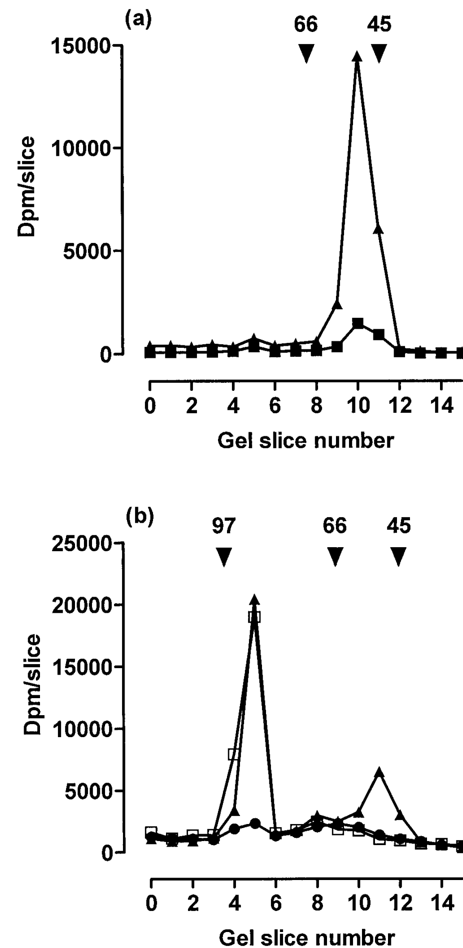

Figure 3 Radiolabelling of rat adipocytes GLUT4 by biotinylated bis-mannose photolabels

Rat adipocytes in the basal state or after treatment with 10 nM insulin were cell-surface photolabelled with 270 μCi (**a**) or 108 μCi (**b**) of Bio-ATB-[2-^{3}H]-BMPA. The labelled cells were washed in Tes buffer and then solubilized in Thesit detergent buffer. In (**a**) GLUT4 from basal (■) or insulin-treated cells (▲) was precipitated using an anti-C-terminal antibody. In (**b**) biotinylated proteins from insulin-treated cells treated either without or with 20 μM cytochalasin B (□) were precipitated with streptavidin agarose either in the absence (▲) or presence (●) of 1 mM biotin. The labelled proteins were resolved on 9% (w/v) acrylamide gels.

experiments). The half-maximal inhibition constants were unaltered by insulin. In the basal state the K_i values for Bio-ATB-BMPA and Bio-LC-ATB-BMPA were 353 μM (single experiment) and $260 \pm 5\ \mu$M (three experiments), respectively. Both compounds inhibit with similar affinities to that of the non-biotinylated compound, ATB-BMPA, which has a K_i of $\sim 250\ \mu$M for both GLUT4 [2] and GLUT1 [1,26].

To evaluate further the utility of the new reagents before developing non-radioactive assays, we used the radiolabelled version of Bio-ATB-BMPA for initial characterization and comparison with tritiated ATB-BMPA. Following labelling of either basal or insulin-stimulated rat adipocytes, the labelled GLUT4 was precipitated by an anti-C-terminal peptide antibody. The Bio-ATB-[2-^{3}H]BMPA, at 38.6 μM, labelled GLUT4 and produced a signal that was very similar to that obtained with ATB-BMPA at the same concentration (Figure 3a) [2]. To test the extent to which streptavidin could precipitate this material, we compared these precipitations (Figure 3b) with those obtained using the GLUT4 antibody. The streptavidin precipitated two

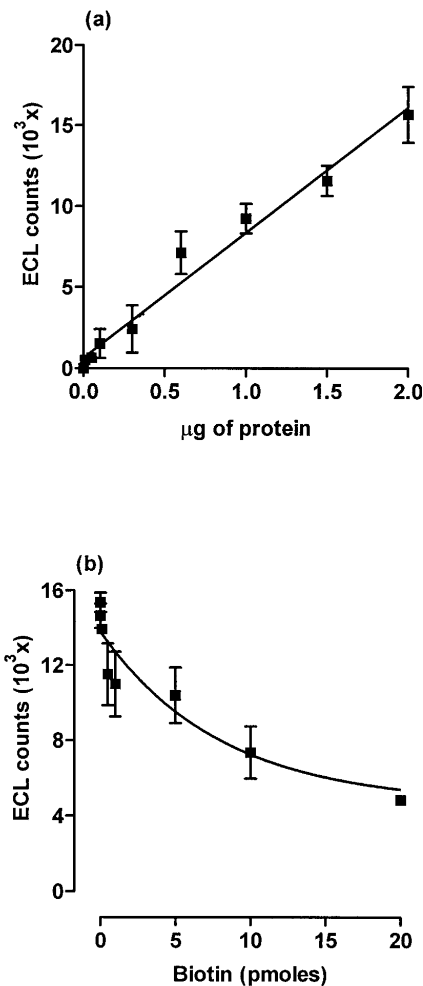

Figure 4 Electrochemiluminescent detection of cell-surface biotinylated GLUT4 in rat adipocyte membranes

Rat adipocytes in the basal state or after treatment with insulin were cell-surface biotinylated with Bio-LC-ATB-BMPA and then washed to remove excess ligand. The cell suspensions were then homogenized and total cell membranes were isolated. In (**a**) detergent solubilized membrane protein samples from Bio-LC-ATB-BMPA-labelled cells were mixed with streptavidin magnetic beads and assayed for interaction with ruthenium-tagged anti-GLUT4 antibody in an ORIGEN electrochemiluminesence analyser. Results are the mean ±S.E.M. from three experiments. In (**b**) the Bio-LC-ATB-BMPA-labelled GLUT4 was displaced from streptavidin magnetic beads by the indicated range of biotin concentrations and was detected using the ruthenium-tagged antibody. Results are the mean ±S.E.M. from three experiments.

Table 1 Analysis of cell-surface biotinylated GLUT4 in rat adipose cell membranes

Intact adipocytes in the basal state, following insulin stimulation in presence or absence of 10 μM cytochalasin B, were labelled with Bio-ATB-BMPA or Bio-LC-ATB-BMPA. After isolation of total cell membranes the electrochemiluminescent (ECL) signal was detected in an ORIGEN electrochemiluminesence analyser using streptavidin magnetic beads and ruthenium-tagged anti-GLUT4 antibody. The results are means ±S.E.M. The number in parentheses indicate the number of separate experiments per condition. The fold insulin stimulation is the average of three or five paired experiments for Bio-ATB-BMPA or Bio-LC-ATB-BMPA, respectively.

	Bio-ATB-BMPA		Bio-LC-ATB-BMPA	
	10^{-3} ECL counts	Fold insulin stimulation	10^{-3} ECL counts	Fold insulin stimulation
Basal	2.33±1.67 (3)	–	3.12±1.07 (5)	–
Insulin	15.24±8.14 (3)	13.73	37.95±6.02 (5)	18.9
Insulin+cytochalasin B	5.57±1.30 (2)	–	3.72±1.50 (2)	–

biotinylated proteins, GLUT4 at ~50 kDa and an ~80 kDa non-specifically labelled protein. A similar pattern of labelling was obtained with ATB-BMPA as determined by SDS/PAGE of membrane samples [2]. The identity of the 80 kDa band has not been determined. The labelling of the GLUT4 transporter peak was reduced by inclusion of 20 μM cytochalasin B in the cell incubation medium. Both the 80 kDa and the transporter labelling were reduced by inclusion of 1 mM biotin in the incubation medium with the streptavidin agarose beads. The transporter peak precipitated by streptavidin was similar in size to that immunoprecipitated by the GLUT4 antibody, suggesting that the spacer arm on the ligand was long enough to allow the labelled transporter to be efficiently recognized by streptavidin.

In developing the non-radioactive assays we assumed that it was necessary to label a larger proportion of the available surface sites than could be achieved with the radioactive method (where a tracer concentration of ligand was used that was below the apparent binding K_d). To saturate the majority of the binding sites, but at the same time conserve material, we used a concentration of 1 mM of these compounds in assays where the biotinylated transporter was detected by electrochemiluminescence, or Western blotting. The data in Figure 4(a) indicate the levels of sensitivity that was achieved in the electrochemiluminescent assay. The detection of Bio-LC-ATB-BMPA was approximately linear up to a concentration of 2 μg of membrane protein. At higher levels of protein the response was non-linear with increasing protein amounts, possibly because of saturation of the streptavidin sites on the magnetic beads by a combination of biotinylated GLUT4, the non-specifically labelled protein at 80 kDa, shown in radiolabelling experiments (Figure 3b), and by any residual carry-over of non-covalently bound reagent. To estimate the saturation point of the streptavidin beads, the binding of 2 μg of biotinylated membranes was displaced by a range of biotin concentrations. Although the displacement had an irregular dependence on the biotin concentration (possibly because of the multisite binding in streptavidin tetramers), the half-maximal displacement occurred at around 10 pmoles/assay of biotin (Figure 4b).

The Bio-LC-ATB-BMPA gave a higher signal than the Bio-ATB-BMPA, but when either reagent was used, the level of increase in the signal following insulin treatment of the cells was ~10-fold in paired experiments (Table 1). The signals in both cases were reduced by ~90% by inclusion of 20 μM cytochalasin B in the cell incubation.

It was also possible to absorb the biotinylated GLUT4 onto streptavidin agarose beads so that the cell-surface biotinylated GLUT4 could be separated from the bulk of the GLUT4. This procedure therefore removed any non-biotinylated GLUT4 in the intracellular membrane compartment, which was also present in the total membrane sample. A wash step was carried out to remove any non-specifically adsorbed GLUT4 and then the biotinylated protein was removed by heat denaturation at 95–100 °C for 20 min, and subjected to SDS/PAGE. Western blotting with an anti-GLUT4 C-terminal peptide antibody and detection with a chemiluminescent second antibody then revealed the GLUT4 that had been biotinylated at the cell surface (Figure 5). Using this approach there appeared to be essentially no signal in the unlabelled sample and the level of insulin stimulation was

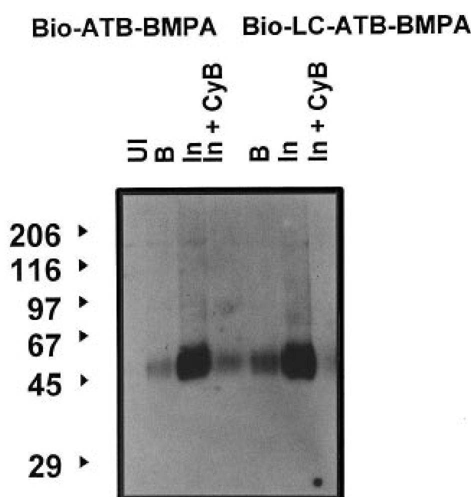

Figure 5 Western blot analysis of cell-surface biotinylated GLUT4 that associates with streptavidin agarose

Rat adipocytes in the basal state (B), after treatment with insulin (In), or after treatment with insulin and 10 μM cytochalasin B (CyB) were cell-surface biotinylated with either Bio-ATB-BMPA or Bio-LC-ATB-BMPA. A sample of 40 μg of isolated membranes was then solubilized in 2% Thesit detergent buffer and the biotinylated proteins were isolated on streptavidin agarose, subjected to electrophoresis on 9% (w/v) acrylamide gels and Western blotted with anti-GLUT4 antibody. The immune complexes were detected using a peroxidase-coupled secondary antibody and Enhanced Chemiluminescence detection reagents (Amersham International).

$\sim$ 20-fold (two separate experiments). The signal was reduced by $\sim$ 90%, by inclusion of 20 μM cytochalasin B in the cell incubation medium.

DISCUSSION

Methods utilizing the biotin interaction with avidin and streptavidin have emerged as very powerful detection techniques and have been applied to many biological systems. Part of the attraction of this approach is the very strong interaction between the biotinylated ligands and either avidin or streptavidin molecules, which allows complexes to be formed in the detergent systems used to extract the biological samples. However, in the case of application of this technology to analyse glucose transporters there is a major problem. The low affinity of the glucose transporters for their substrates, typically around 3–10 mM for D-glucose [27], means that a sugar derivative bearing only a biotin group is unlikely to bind strongly enough to survive the procedures used to isolate the transporters.

As a solution to this type of problem, Hatanaka et al. [16] developed a heterobifunctional reagent (Bio-ATB) that is a diazirine-based photolabel with biotin attached to a benzoic acid moiety via a polyethoxy spacer arm. The photolabel, when irradiated with UV light, efficiently cross-links to the protein of interest so that detection is then only dependent on the favourable biotin interaction with avidin and streptavidin. The Bio-ATB is hydrophilic because the spacer leading to the biotin is a polyethoxy ether. The abundant hydroxy groups in bis(D-mannosyloxy)propylamine provides additional hydrophilicity. Using the radioactive form of the Bio-ATB-hexoses we have confirmed that these photolabels are cell impermeant at 18 °C (results not shown).

One of the disadvantages of radioactive methods for detecting bis-mannose photolabelled transporters is that only a small fraction of the added label becomes covalently associated with the transporter. We determined that the efficiency of cross-linking between the occupied transporter and the ligand was high (approaching 100% efficiency), but because the affinity of the transporter was only 250 μM, most of the compound remained in the cell-suspension medium and one needed extensive washing to dispose of the tritium that was not incorporated into the transporter. In addition to the improved safety aspects associated with the uses of non-radioactive detection techniques, the electrochemiluminescence method described here was very rapid and did not require extensive sample preparation procedures. The method was very sensitive and could detect a difference in cell-surface labelling of basal and insulin-treated samples in as little as 100 ng of total adipose cell membrane. Since the levels of GLUT4 were approx. 50 pmol/mg [28], the assay can, in effect, detect 5 pmoles of GLUT4 in 1 μg (the optimum level for the assay) and $\sim$ 500 fmoles at the lowest usable level. Non-linearity in the electrochemiluminescent signal above 2 μg of protein probably occurs because of saturation of the streptavidin beads. Competitive biotin displacements have indicated that the half-maximal saturation of the beads occurred at $\sim$ 10 pmol of biotin (a value that was in the same order as the calculated amount of GLUT4 present). The non-radioactive method, employing Western blotting, was less sensitive and 40 μg of total cellular membrane were required to give a quantitatively detectable signal. However, this method would be convenient to use in the absence of the electrochemiluminescence analysis instrument.

The tritiated Bio-ATB-BMPA has been used to label the GLUT1 isoform in human erythrocytes (data not shown). The efficiency of the labelling was observed to be similar to that obtained previously with ATB-BMPA [1] and to the results presented here for the detection of GLUT4 in rat adipocytes.

The compound with the longer spacer arm, Bio-LC-ATB-BMPA, was much more easily detected than the compound without the additional aminohexanoic acid spacer, 2-fold differences were observed in paired experiments. This improvement was probably not because of the difference in affinity. At the level of 1 mM, calculations based on the half-maximal inhibition constants of 359 and 273 μM suggest that Bio-ATB-BMPA and Bio-LC-ATB-BMPA would occupy 74 and 78% of the available sites, respectively. The binding site within streptavidin is within a deep cleft [21] and a long spacer has been shown to be important for binding to biotin in solution. In the case of recognition of the biotinylated GLUT4, the spacer has to be long enough to bridge two deep clefts: those of the transporter and the streptavidin. In addition, the two proteins will be adjacent to one another and any extraneous glycoprotein may well cause additional steric problems. The distance between the site of attachment to the transporter, the trifluoroethylbenzoyl carbene, and the biotin moiety are approx. 18 and 26 Å for the Bio-ATB-BMPA and the Bio-LC-ATB-BMPA, respectively. Studies have been made in which two avidin molecules are linked together with bis-biotin compounds with spacers of different lengths. It has been estimated that to form a connecting bridge long enough to form avidin polymers, a spacer to the biotin corresponding to 12 methylenes ($\sim$ 18 Å) is required [29]. Because the surface topology of the heavily glycosylated glucose transporters is likely to be complex, the case can probably be made for an even longer spacer arm in the biotinylated bis-hexose photolabels, but this has not been investigated so far.

We have been able to use the electrochemiluminescent technique in the analysis of levels of GLUT4 in isolated muscle and in fat cell tissue in which we do not have the advantage of being able to work with isolated cells, as is the case with the isolated adipose cells studied here (J. Yang and G. D. Holman, unpublished work). Because of the high sensitivity of the elect-

rochemiluminescence technique for analysis of cell surface GLUT4, the technique is likely to be applicable to the study of biopsy material where levels of GLUT4 (or other transporters) are altered in disease states [30].

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