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Localization of the binding domain of the inhibitory ligand forskolin in the glucose transporter GLUT-4 by photolabeling, proteolytic cleavage and a site-specific antiserum

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The binding domain of forskolin in the adipocyte/muscle-type glucose transporter (GLUT-4) was localized with the aid of the photoreactive derivative, [^{125}I]IAPS-forskolin (3-[^{125}I]iodo-4-azidophenethylamido-7-*O*-succinyldeacetyl-forskolin). Plasma membranes from insulin-treated rat adipocytes containing predominantly the GLUT-4 isoform were irradiated with UV light in the presence of [^{125}I]IAPS-forskolin. The covalently labeled glucose transporters were isolated by immunoprecipitation with specific antiserum and partially digested with trypsin and elastase. The fragments were separated by gel electrophoresis, transferred on to nitrocellulose membranes, and identified by direct autoradiography and by immunoassay with antiserum against a peptide sequence corresponding to the C-terminus of GLUT-4. Digestion with a high-purity grade trypsin generated two photolabeled fragments with apparent molecular weights of 21 and 16 kDa. Since the antiserum detected two fragments with identical electrophoretic mobility, both labeled fragments appeared to contain the intact C-terminus of GLUT-4. In contrast, digestion with elastase generated only one photolabeled fragment with intact C-terminus at 21 kDa, and a smaller unlabeled fragment with intact C-terminus at 15 kDa. A less pure trypsin preparation generated two labeled (21 and 17 kDa) and one unlabeled (15 kDa) fragment with intact C-terminus. These data suggest that the site of covalent binding of IAPS-forskolin in the GLUT-4 is located within a region of 1–6 kDa defined by the difference between the unlabeled C-terminal fragment (15 kDa) and the labeled fragments (21, 17 and 16 kDa). Based on a tentative allocation of the fragments to the sequence of the GLUT-4, it is suggested that the covalent binding site of IAPS-forskolin is located between the membrane spanning helices 7–9, possibly in the proximity of helix 9.

Introduction

The diterpene forskolin inhibits the facilitated diffusion of glucose in adipocytes, erythrocytes, and platelets [1–3]. This effect has been shown to occur independently from changes of adenylate cyclase activity, and to parallel the binding of forskolin to the cytochalasin B binding site of the glucose transporter [2–5]. Furthermore, glucose transporters were covalently labeled by photolysis of membranes in the presence of tritiated forskolin [6] or the photoreactive, iodinated forskolin derivative IAPS-forskolin [7,8]. Thus, it was concluded that a forskolin binding site at the transporter mediates the inhibitory effect of the diterpene on the stereospecific glucose transport.

Binding of forskolin to glucose transporters appears to occur at a site with crucial importance for the function of the transporters, since it is inhibited by glucose and cytochalasin B [4]. Interestingly, the different transporter isoforms bind the diterpene with different affinities in the order GLUT-4 > GLUT-1 > GLUT-3 ≫ GLUT-2 [9]. For GLUT-1, GLUT-2 and GLUT-4, this ranking order of affinities corresponds with that of the different K_m values for glucose [10]. Thus, forskolin appears to label a domain at the core of the transporter involved in the binding of glucose. It has previously been suggested that the core of the GLUT-1 is located between the membrane spanning helices 7 and 10 [11]. Furthermore, evidence based on microsequencing of a small fragment of the GLUT-1 labeled with IAPS-forskolin has indicated that the photoreactive forskolin derivative binds at a site near or within the membrane spanning helix 10, possibly at tryptophan-388 [12]. The present study was designed to identify the binding region of IAPS-forskolin in the GLUT-4. Since we could not prepare GLUT-4 in

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amounts sufficient for sequencing of a tryptic fragment, we employed an experimental strategy involving the partial proteolysis of GLUT-4 and a site-specific antibody against its C-terminus. With this approach, we detected labeled and unlabeled fragments of the GLUT-4 which still contained the C-terminus. Since the largest unlabeled fragment (15 kDa) and the labeled fragments (16, 17 and 21 kDa) differed by only 1–6 kDa, the covalent binding site of IAPS-forskolin can be narrowed down to a small region of the GLUT-4.

Materials and Methods

Synthesis of IAPS-forskolin. 3-[^{125}I]Iodo-4-azidophenetylamido-7-*O*-succinyldeacetyl-forskolin (IAPS-forskolin) was prepared as described [7] by coupling of 2-(3-[^{125}I]iodo-4-azidophenyl)ethylamine to *N*-hydroxysuccinimidyl-7-*O*-succinyl-7-deacetyl-forskolin. Carrier-free label was used (theoretical specific activity 2200 Ci/mmol) for radiosynthesis. The product was stored in ethanol solution at −20°C.

Preparation of plasma membranes from isolated adipocytes. Male rats (170–200 g, Wistar strain, fed ad libitum) were used throughout. Isolated adipocytes were prepared from epididymal fat pads with collagenase (Worthington Biochemicals) according to Rodbell [13] with modifications as described previously [14,15]. Adipocytes obtained from 20 rats were incubated at 37°C in a KRBH buffer (pH 7.4), containing 10 mM sodium bicarbonate, 30 mM Hepes, 2.5 mM glucose, 200 nM adenosine, and 1% bovine serum albumin (Cohn Fraction V) [15]. Insulin (crystalline porcine zinc insulin, courtesy of Hoechst, Frankfurt) was added to a final concentration of 10 nM. After 30 min, cells were washed and homogenized, and plasma membranes were prepared by differential centrifugation as previously described in detail [16,15]. In some of the membrane preparations, glucose transport activity was assessed in the resealed membrane vesicles [14] or after reconstitution into lecithin vesicles as described previously [17].

Photolabeling with IAPS-forskolin. Samples of 300 μl containing approx. 300 μg of membrane protein were incubated with [^{125}I]IAPS-forskolin (2 μl, final concentration 20–50 nM) for 30 min on ice. The samples were photolyzed for 3 min with a high-pressure mercury lamp (HBO 50, Zeiss, Oberkochen, Germany). The reaction was immediately quenched by addition of 2 μl 10% β-mercaptoethanol, and the samples were diluted with 1 ml of ice-cold Tris buffer (10 mM). Membranes were separated by centrifugation in a refrigerated microfuge (15 000 rpm, 30 min). For immunoprecipitation of GLUT-4, the pellets were solubilized by incubation for 30 min on ice in buffer containing (mM): Hepes 50, NaCl 150, and Triton X-100 (1%, w/v). The samples were centrifuged (15 000 × *g*, 30 min), and anti-GLUT-4 antiserum pre-adsorbed to a suspension of protein-A sepharose (1.5 μl serum per 100 μg of membrane protein) was added to the supernatants. After 2 h at 4°C, the protein-A sepharose was separated by centrifugation and washed once with buffer containing 50 mM Hepes and 0.1% (w/v) Triton X-100, then with the same buffer supplemented with 300 mM NaCl, and finally with buffer containing 50 mM Hepes, 300 mM NaCl and 0.1% SDS. Immunocomplexes were eluted with electrophoresis sample buffer containing (w/v) 4% SDS, 20% glycerol, 0.05% Bromophenol blue, and (mM) Tris 125, EDTA 5 and dithioerythrol 200.

Proteolytic enzymes. Trypsin (tissue culture grade) was purchased from Biochrom, Berlin, Germany (cat. No. 2113, specific activity 6 U/mg. Other activities according to manufacturer: elastase 0.3 U/mg, chymotrypsin 0.003 U/mg). Trypsin (analytical grade, cat. No. 109819, specific activity 110 U/mg) and elastase (cat. No. 1027891, specific activity 105 U/mg) were from Boehringer Mannheim, Mannheim, Germany. Elastase was stored desiccated at 4°C and was used no longer than 2 months after purchase.

Proteolytic cleavage of immunoprecipitated glucose transporter GLUT-4. Immunoprecipitated glucose transporters GLUT-4 were eluted from the protein A-Sepharose with Laemmli's sample buffer. Complete proteolysis was performed by an overnight incubation of the eluates with trypsin (0.5 μg/μl). Partial proteolysis in the presence of SDS was performed with a modification of the previously described procedure [18]. The proteolytic enzymes were added as desired (5–100 ng/μl), and the samples were immediately loaded into the wells of a stacking gel (3.9% polyacrylamide). The gel (mini-gel apparatus, Biometra, Göttingen) was run under thoroughly controlled conditions (21°C, 7.5 mA/gel) for exactly 50 min. Thereafter the samples had entered the separating gel (12–14% polyacrylamide), and the current was raised to 20 mA/gel. In some experiments, gradient gels were used as indicated (Sigma technical manual). The separation was completed, and the samples were transferred on to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) in a semi-dry blotting apparatus (Sartorius, Göttingen, Germany). Labeled GLUT-4 and its labeled fragments were detected by autoradiography for 6–21 days. Nitrocellulose membranes were stored for 1–2 half-lives of ^{125}I before the immunoassay of the GLUT-4 was performed.

Antisera and immunoblotting of the glucose transporter GLUT-4. Antisera against the GLUT-4 were raised with peptides corresponding to the C-terminus (sequence in one letter code: STELEYLGPDEND) or the N-terminus (MPSGFQQIGSED) of GLUT-4 coupled to keyhole limpet hemocyanin. Nitrocellulose membranes were blocked for 2 h with buffer containing 10 mM Tris (pH 7.4), 0.05% (w/v) Tween 20, and 150 mM NaCl, and were incubated with the respective

antiserum at a dilution of 1:200 for 2 h at room temperature. After three washes with a buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 0.2% SDS, 0.5% Triton X-100, and 0.5% albumin, the nitrocellulose was incubated with ^{125}I-protein A (0.25 μCi/ml, Amersham) for 2 h, thoroughly washed and autoradiographed for 1–2 days.

Results

Generation of fragments (21 and 17 kDa) of the photolabeled GLUT-4 containing the C-terminus by partial proteolytic digestion

Photolabeling of adipocyte membranes with [^{125}I]IAPS-forskolin generates a number of labeled proteins unrelated to the glucose transporter. Since these proteins would interfere with the identification of proteolytic fragments, the GLUT-4 was immunoprecipitated after photolabeling in all experiments presented here. As is illustrated in Fig. 1 (upper panel), the immunoprecipitates of the GLUT-4 contained a single labeled band at 48 kDa (Fig. 1, lane 1 of upper panel). As anticipated, the labeling of this band was markedly inhibited by glucose (Fig. 1, lane 3 of upper panel).

Conditions for partial proteolysis with trypsin in the presence of SDS during electrophoresis in the stacking gel [18] were established in preliminary experiments. These experiments indicated that the tryptic digestion of the GLUT-4 generated fragments which were reproducibly detected with antiserum against the C-terminus of the protein (data of the preliminary experiments not shown, see Fig. 1, upper panel) provided that the temperature, the concentration of enzyme and the running time in the stacking gel were carefully controlled. After proteolysis and separation by SDS-PAGE, the proteins were transferred on to nitrocellulose membranes; this procedure allowed the detection of the fragments by direct autoradiography and by immunoassay in the same samples. As is illustrated in Fig. 1 (upper panel, lane 2), digestion of the immunoprecipitated GLUT-4 generated two labeled fragments at 21 and 17 kDa; labeling of these fragments was markedly inhibited by glucose (Fig. 1, lane 3 of upper panel). After decay of the radioactivity, an immunoassay with serum against the C-terminus of GLUT-4 was performed (lower panel). The antiserum detected fragments of the GLUT-4 at 21, 17 and 15 kDa; the 21 and 17 kDa fragment were exactly superimposable to the two labeled fragments of identical electrophoretic mobility (upper panel). An additional band detected with protein A at 28 kDa corresponds with a degradation product of the IgG from the immunoprecipitation (control samples in lane 5 and 6, lower panel of Fig. 1).

Attempts to digest the GLUT-4 before electrophoresis produced mainly fragments devoid of the C-terminus (data not shown, see also Fig. 4). Furthermore, we failed to detect a major fragment with an intact N-terminus by immunoblotting with specific antiserum (data not shown). Thus, trypsin appears to preferentially remove the N-terminus of the GLUT-4 as a small fragment, probably by cleavage after Arg-19.

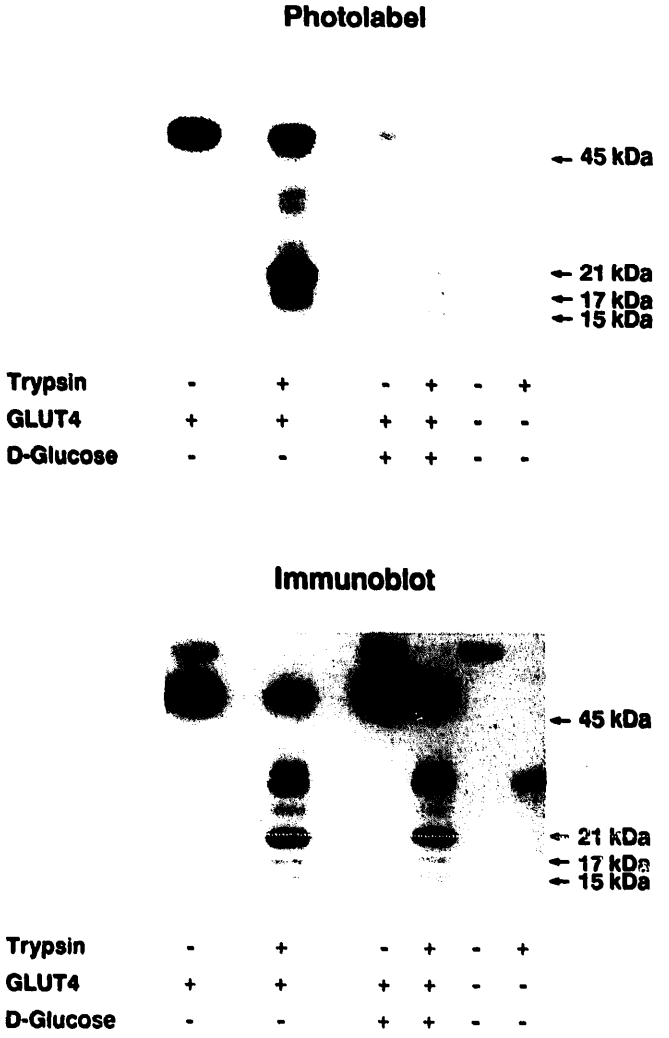

Fig. 1. Partial tryptic digestion of the adipocyte glucose transporter (GLUT-4) after photolabeling with IAPS-forskolin. Plasma membranes obtained from insulin-treated adipocytes were incubated with or without 500 mM D-glucose as indicated, and photolysis was performed as described in Materials and Methods in the presence of 20 nM [^{125}I]IAPS-forskolin. The glucose transporter was isolated by immunoprecipitation, and digested with trypsin (tissue culture grade, 0.05 μg/μl) during the migration through a stacking gel of the SDS-PAGE (12% separating gel). As a control for protein-A-binding fragments of the immunoglobulins, undigested and digested immunoglobulin from the antiserum was run in lane 5 and 6, respectively. The proteins were transferred on to nitrocellulose membranes, and incorporated photolabel was detected by autoradiography (upper panel; exposure time 17 days at −80°C with enhancing screen). After decay of the radiography, a protein A assay was performed with antiserum against the C-terminus of GLUT-4 (lower panel) and subjected to autoradiography for 3 days at room temperature.

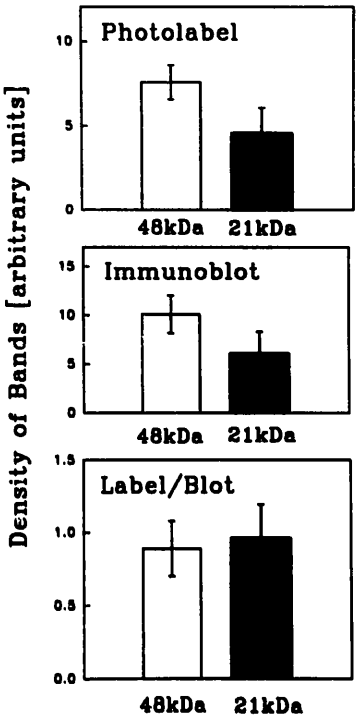

Fig. 2. Comparison of the specific activities of the photolabeled GLUT-4 and its 21 kDa tryptic fragment. GLUT-4 from adipocyte membranes was photolabeled and digested as described in the legend of Fig. 1. The digested transporter was separated by SDS-PAGE, and the fragments were detected by autoradiography or after immunoassay with antiserum against the C-terminus of GLUT-4. The films were quantitated by densitometry. Upper panel: Photolabel incorporated into starting material (48 kDa) and 21 kDa fragment. Middle panel: Immunoreactivity of starting material (48 kDa) and 21 kDa fragment. Lower panel: 'specific activity' of starting material and 21 kDa fragment. The data represent means ± S.E. of four different digestions.

Evidence that IAPS-forskolin exclusively labels the C-terminal half of the GLUT-4: comparison of the specific activity of the labeled GLUT-4 and its 21 kDa fragment

In order to assess the specific activity of the labeled 21 kDa fragment and to compare it with that of the intact GLUT-4, autoradiographs were quantitated by densitometry (Fig. 2). For both intact GLUT-4 (48 kDa) and 21 kDa fragment, the data from the incorporated photolabel were normalized per immunoreactivity (Fig. 2, lower panel). According to this calculation, the specific activity of the 21 kDa fragment was not lower than that of the undigested GLUT-4. Thus, the 21 kDa fragment retains most, if not all, of the photolabel. It appears reasonable to conclude, therefore, that IAPS-forskolin was mainly linked to the C-terminal half of the glucose transporter. Consequently, all smaller fragments containing the photolabel are very likely derived from the C-terminal half of the GLUT-4; this limitation considerably facilitates the allocation of the fragments.

Generation of a 15 kDa unlabeled fragment and a 16 kDa labeled fragment of the photolabeled GLUT-4 by partial proteolysis with elastase and trypsin

When the proteolytic cleavage of the GLUT-4 was repeated with an analytical-grade trypsin, the pattern of fragmentation was strikingly different than that shown in Fig. 1. As is illustrated in Fig. 3 (lane 3 of upper panel), the high-purity trypsin generated two photolabeled fragments with apparent molecular weights of 21 and 16 kDa. Both fragments appeared to contain the intact C-terminus of the GLUT-4, since two proteins with identical electrophoretic mobility (16 and 21 kDa) cross-reacted with the specific antiserum (lower panel of Fig. 3, lane 3). However, tryptic diges-

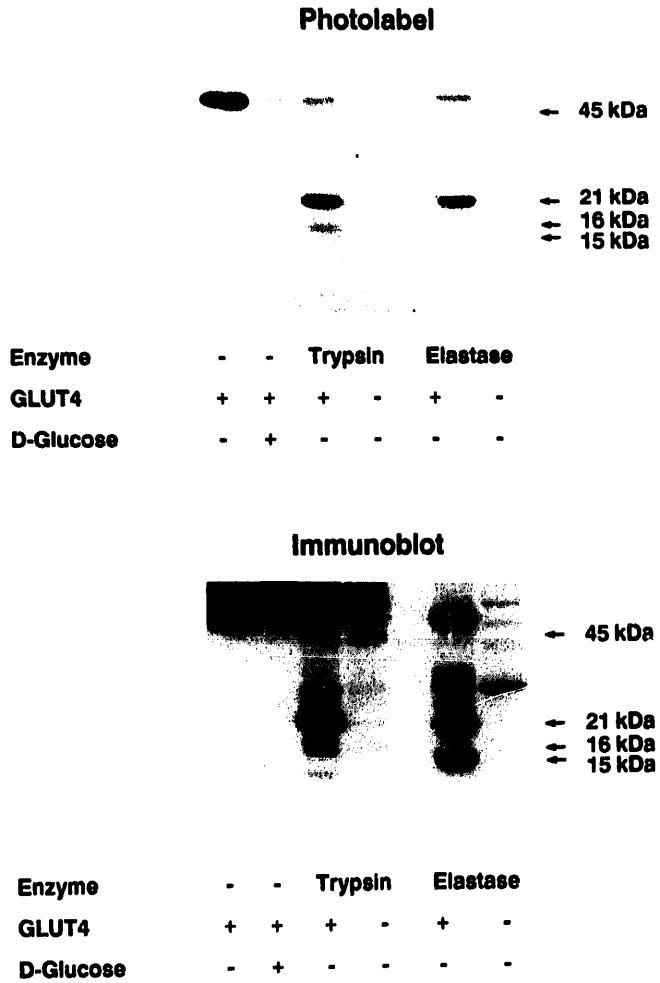

Fig. 3. Partial digestion of the adipocyte glucose transporter (GLUT-4) with trypsin (analytical grade) and elastase after photolabeling with IAPS-forskolin. GLUT-4 from adipocyte membranes was photolabeled and digested with analytical-grade trypsin (0.1 $\mu g/\mu l$) or elastase (0.1 $\mu g/\mu l$) as described in the legend of Fig. 1 during migration through a stacking gel of the SDS-PAGE (separating gel 14%). Undigested and digested immunoglobulin from the antiserum was run in lane 4 and 6, respectively. The fragments were detected by autoradiography (upper panel; exposure time 6 days at −80°C with enhancing screen) or, after decay of the radioactivity, by immunoassay with antiserum against the C-terminus of GLUT-4 (lower panel; exposure time 2 days at room temperature).

tion under more rigorous conditions (see description of Fig. 4) suggested that a 16 kDa fragment devoid of the C-terminus can be generated. Thus, the possibility cannot be discounted that the labeled 16 kDa fragment depicted in Fig. 3 is devoid of the C-terminus. In that case, the immunoreactive band at 16 kDa (Fig. 3, lower panel) would correspond to a different C-terminal fragment co-migrating exactly with the labeled fragment.

Experiments in which the two trypsin preparations were run in parallel (data not shown) indicated that the low-purity trypsin had indeed generated different fragments than the high purity trypsin (21/17/15 kDa and 21/16 kDa, respectively). According to a specification of the supplier, the trypsin employed in the initial experiments (Fig. 1) was contaminated with elastase (5%) and chymotrypsin (0.05%). Therefore, we investigated the effects of pure preparations of these enzymes. Chymotrypsin produced the same pattern of fragmentation as trypsin (data not shown). Elastase generated two fragments (21 and 15 kDa) reacting with antiserum against the C-terminus of GLUT-4 (lower panel of Fig. 3, lane 5). Like the 15 kDa fragment generated by the low-purity trypsin (Fig. 1), the 15 kDa fragment produced by elastase did not contain the photolabel (upper panel of Fig. 3, lane 5). This finding is crucial for the identification of the binding region, since it excludes a large portion (15 kDa) of the C-terminus. Furthermore, it appears reasonable to assume that the 15 kDa fragment produced by the low-purity trypsin (Fig. 1) was generated by a contamination with elastase.

It has to be noted that we have no information as to what enzyme produced the 17 kDa fragment which was observed after digestion with low-purity trypsin (Fig. 1). Analytical-grade trypsin as well as chymotrypsin and elastase, the enzymes contaminating the low-purity trypsin, failed to generate a corresponding fragment.

Full tryptic digestion of the immunoprecipitated GLUT-4

In order to produce a maximal proteolytic digestion of the GLUT-4 with trypsin, immunoprecipitates were incubated in buffer overnight at room temperature with the enzyme. Thereafter the samples were separated by electrophoresis, transferred on to nitrocellulose, and autoradiographed. As is illustrated in Fig. 4 (upper panel), the smallest photolabeled fragment generated under these conditions migrated at an apparent molecular weight of 10 kDa. This fragment failed to react with antiserum against the C-terminus of GLUT-4. In addition, a photolabeled fragment of 16 kDa was detected. Only a weak cross-reactivity, if any, with the antiserum was detected in the corresponding region of the immunoblot (Fig. 4, lower panel). Thus, under rigorous conditions a photolabeled fragment of 16 kDa devoid of the C-terminus of the GLUT-4 appears to be formed. It cannot be excluded, therefore, that the 16 kDa fragment generated by partial proteolysis (Fig. 3) consists of two different proteins migrating at the same apparent molecular weight: one fragment devoid of the C-terminus and another one retaining the C-terminus of GLUT-4.

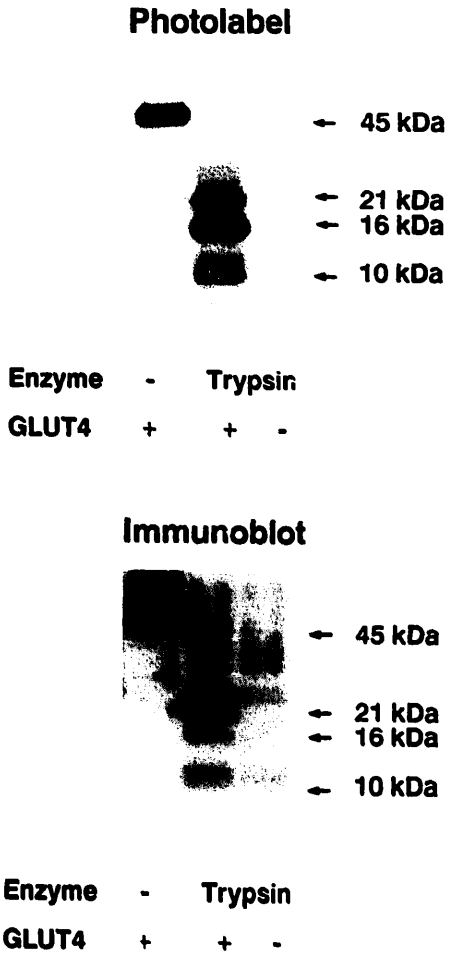

Fig. 4. Full tryptic digestion of the adipocyte glucose transporter (GLUT-4) after photolabeling with IAPS-forskolin. GLUT-4 from adipocyte membranes was photolabeled, isolated by immunoprecipitation, and digested with analytical-grade trypsin by an 18 h incubation at room temperature in the presence of 0.5 $\mu g/\mu l$ trypsin. The samples were separated by SDS-PAGE (gradient gel 10–16%), and the fragments were detected by autoradiography (upper panel). After decay of the radioactivity, an immunoassay with antiserum against the C-terminus of GLUT-4 was performed (lower panel).

Discussion

The data presented above indicate that the apparent molecular weight of the largest unlabeled fragment with intact C-terminus is 15 kDa. Consequently, the photolabel must be linked to a site 'upstream' a proteolytic (elastase) cleavage site generating a C-terminal fragment of 15 kDa. Furthermore, the photolabel was completely recovered in a C-terminal fragment of 21 kDa (Fig. 2). Thus, our experimental approach has narrowed the binding site of IAPS-forskolin within the GLUT-4 down to a range of 6 kDa, probably corre-

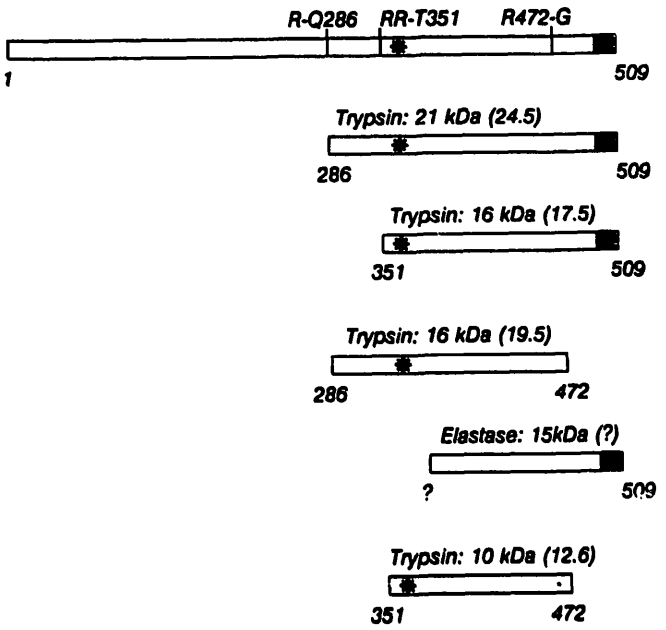

Fig. 5. Schematic presentation of the labeled and unlabeled fragments of the GLUT-4 generated by digestion with trypsin or elastase. Putative tryptic cleavage sites are located at glutamine-286 (R-Q286), threonine-351 (RR-T351), and glycine-473 (R472-G). Asterisks symbolize the presence of radioactivity from labeling with IAPS-forskolin. The shaded area represents the domain detected by the antiserum against a peptide corresponding with the C-terminus. The molecular weights given in kDa were determined as apparent molecular weights according to their electrophoretic mobility in SDS-polyacrylamide gels; numbers in parenthesis represent the molecular weights deduced from the sequence of GLUT-4.

sponding with the membrane spanning helices 7–9 (see below). Considering the labeled fragments of 16 and 17 kDa generated by trypsin, one might further narrow down the binding region to a range of 1–2 kDa, probably located in the proximity of the membrane spanning helix 9. However, we cannot fully exclude the possibility that the 16 kDa is devoid of the C-terminus and co-migrates with a different unlabeled fragment which retains the C-terminus (see Results, descriptions of Figs. 3 and 4).

A tentative identification of the fragments we have obtained in the different experiments is given in Fig. 5. We assume that the 21 kDa fragment is generated by cleavage at Arg-Gln-286 at the end of the large intracellular loop of the glucose transporter. Furthermore, we assume that no cleavage occurred at two consensus sites close to helix 9 (Arg-Val-377) and helix 12 (Arg-Val-468). Based on these assumptions, it can be concluded that the range of 6 kDa defined by the unlabeled 15 kDa fragment and the labeled 21 kDa fragment corresponds with the membrane spanning helices 7–9. Based on the assumption that the labeled 16 kDa fragment retains the intact C-terminus, the covalent binding site of IAPS-forskolin would be further defined to a range of 1–2 kDa near the membrane spanning helix 9. It has to be emphasized, however, that the allocation of the fragments must be considered tentative, since it is merely based on their apparent molecular weights and on the assumption of several preferential tryptic cleavage sites; all attempts to obtain an amino acid sequence of one of the fragments have failed so far.

In order to test the validity of the above described conclusions, alternative correlations of the fragments with the sequence of the GLUT-4 have to be discussed. Firstly, it has to be considered whether the largest fragment might have been generated by cleavage at Arg-Thr-351. In that case, the apparent molecular weight of the 21 kDa fragment would be much higher than the calculated weight of a Thr-351–Asp-509 fragment (17.5 kDa). Such a difference appears to be unlikely, however, since the glucose transporter (48 kDa) and also its deglycosylated form (43 kDa) run at lower apparent molecular weight than calculated from its sequence (55 kDa), probably because of the high abundance of hydrophobic amino acids. Therefore, it appears reasonable to assume that the apparent molecular weights of the fragments of the GLUT-4, like that of the intact protein, are lower than those calculated from their sequence.

Secondly, it has to be considered whether the 16 kDa fragment containing the C-terminus was generated by cleavage at Arg-Val-377. In that case the calculated molecular weight of the fragment (15 kDa) would be only slightly lower than its observed apparent molecular weight (16 kDa). However, there would be a marked discrepancy in the difference between the 21 kDa fragment and the 16 kDa fragment (5 kDa as obtained from the apparent molecular weights vs. 9 kDa as calculated from the sequence of a 286–509 fragment and a 377–509 fragment). On the basis of these considerations, we feel that there is no better alternative to the allocation of the observed fragments with the sequence of the GLUT-4 than that outlined in Fig. 5, since this correlation provides the most consistent relation between the calculated and the observed molecular weights.

Several investigators have used covalent labeling techniques in order to locate the sugar recognition site and the cytochalasin B binding site in the GLUT-1 [11,19–22]. These sites appear to be related to the binding site of forskolin, since binding of the diterpene is inhibited by D-glucose as well as by cytochalasin B [2,4]. Based on the fragmentation pattern of the GLUT-1 photolabeled with cytochalasin B and an impermeable photoreactive mannose derivative, it was suggested that the cytochalasin B binding site is located in the internal region of the transmembrane helix 10, and that the exofacial sugar recognition site is located in the outer domain of helix 9 [11]. Indeed, it has been demonstrated very recently by site directed mutagenesis that a tryptophan in helix 10 (Trp-388) is

involved in the binding of cytochalasin B [23]. Furthermore, the binding site of IAPS-forskolin in the erythrocyte-type glucose transporter (GLUT-1) has previously been identified by full tryptic digestion and microsequencing of a small labeled fragment [12]. In contrast to our conclusions concerning the GLUT-4, these data suggested that the label was covalently bound to a site within the helix 10.

Our conclusion as to the covalent binding site of IAPS-forskolin in the GLUT-4 does not necessarily contradict those locating the ligand binding sites of GLUT-4 in helix 10. Firstly, it appears possible that the binding site of IAPS-forskolin is structurally different in GLUT-1 and GLUT-4, since there is an almost 10-fold difference in the binding affinity of tritiated forskolin to GLUT-1 and GLUT-4 [9]. Secondly, the diterpene may bind to more than one site in the glucose transporters, as was suggested for cytochalasin B on the basis of structure-activity relationships [24]. This assumption is supported by the finding that mutation of Trp-388 in the GLUT-1 reduced the inhibitory potency of cytochalasin B on glucose transport, but failed to fully abolish the effect [23]. Finally, the contact sites of the reversible binding must not necessarily be identical with those responsible for covalent binding of the photoreactive ligand. Thus, even an exact localization of the site of covalent binding will define the ligand binding region with some uncertainty. On the basis of these considerations, we tentatively suggest that forskolin binds to more than one contact site in a region formed by the membrane spanning helices 9 and 10. This binding domain is located within the region previously defined as the core of the transporter (helix 7–10 [11]).

Acknowledgements

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