The Insulin-sensitive Glucose Transporter, GLUT4, Interacts Physically with Daxx

TWO PROTEINS WITH CAPACITY TO BIND Ub9 AND CONJUGATED TO SUMO1*

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In this study we have used the yeast two-hybrid system to identify proteins that interact with the carboxyl-cytoplasmic domain (residues 464–509) of the insulin-sensitive glucose transporter GLUT4 (C-GLUT4). Using as bait C-GLUT4, we have isolated the carboxyl domain of Daxx (C-Daxx), the adaptor protein associated with the Fas and the type II TGF-β (TβRII) receptors (1, 2). The two-hybrid interaction between C-GLUT4 and C-Daxx is validated by the ability of in vitro translated C-GLUT4 to interact with in vitro translated full-length Daxx and C-Daxx. C-Daxx does not interact with the C-cytoplasmic domain of GLUT1, the ubiquitous glucose transporter homologous to GLUT4. Replacement of alanine and serine for the dileucine pair (Leu489-Leu490) in the C-cytoplasmic domain of GLUT1, the ubiquitous glucose transporter homologous to GLUT4. Replacement of alanine and serine for the dileucine pair (Leu489-Leu490) critical for targeting GLUT4 from the trans-Golgi network to the perinuclear intracellular store as well as for its surface internalization by endocytosis inhibits 2-fold the interaction of C-GLUT4 with Daxx. Daxx is pulled down with GLUT4 immunoprecipitated from lysates of 3T3-L1 fibroblasts stably transfected with GLUT4 and 3T3-L1 adipocytes expressing physiological levels of the two proteins. Similarly, GLUT4 is recovered with anti-Daxx immunoprecipitates. Using an established cell fractionation procedure we present evidence for the existence of two distinct intracellular Daxx pools in the nucleus and low density microsomes. Confocal immunofluorescence microscopy studies localize Daxx to promyelocytic leukemia nuclear bodies and punctate cytoplasmic structures, often organized in strings and underneath the plasma membrane. Daxx and GLUT4 are SUMOlated as shown by their reaction with an anti-SUMO1 antibody and by the ability of this antibody to pull down Daxx and GLUT4.

The cytoplasmic domain of membrane proteins plays important roles in their transport, signal transduction, organization of protein scaffolds, and regulation of their turnover. Trafficking of GLUT4 in adipose and skeletal muscle cells is regulated by insulin and muscle contraction and is critical for the control of glucose levels in blood. Upon increase in insulin levels and muscle contraction the GLUT4 retained in intracellular stores is translocated to the plasma membrane, where it facilitates glucose transport (3). Trafficking of GLUT4 is mediated by motifs localized to the amino and carboxyl-cytoplasmic domains of the protein, though their characterization and the identification of the factors involved in their reading is incomplete. SUMO (also called sentrin, PIC1, and GMP1), a 101-amino acid ubiquitin-like modifier protein that is highly conserved from yeast to human, appears to control protein turnover and compartmentalization (4). Three members of the SUMO family have been described in vertebrates. They show major structural differences in the sequences of their N-extensions, which are absent in ubiquitin. It has been shown recently that Ubc9, the only E2-type SUMO1-conjugating enzyme described in vertebrates, interacts with the carboxyl-cytoplasmic domain of GLUT4 as part of a mechanism that slows its turnover (5). Overexpression of Ubc9 increases GLUT4 abundance 8-fold, probably as result of the conjugation of SUMO1 to GLUT4 and the resistance of the conjugate to degradation (5). Interestingly, overexpression of Ubc9 decreases the levels of GLUT1, the ubiquitous glucose transporter homologous to GLUT4, by 2-fold. Ubc9 binds to a highly conserved sequence of 11 amino acids contained in the C-cytoplasmic domains of GLUT4 (RV-PETRGRFTFD) and GLUT1 (KVPETKGRFTFD) (5).

Here we report that Daxx,1 an adaptor protein associated with the Fas receptor and TβRII that mediates activation of JNK and cell apoptosis (1, 2, 6) and is distributed between the PD10 nuclear bodies enriched in SUMOlated proteins (7–13) and the antiapoptotic cytoplasm (2, 14–16), interacts physically with C-GLUT4 but not with C-GLUT1. As GLUT4, a small population of Daxx is conjugated to SUMO1. Microscopy studies localize Daxx to the nucleus and to punctate cytoplasmic structures identified as low density microsomes by cell fractionation studies. The binding of Daxx to GLUT4 is discussed in the framework of their demonstrated SUMOlation.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—EcoRI and BamHI restriction sites were introduced by PCR at positions 1495 and 1748 in the cDNA of GLUT4. The same technique was used to introduce EcoRI and XhoI sites at positions 1552 and 1736 in the cDNA of GLUT1. The cDNA encoding C-GLUT4 (residues 464–509) and C-GLUT1 (residues 443–491) were cloned into the EcoRI/BamHI and EcoRI/XhoI sites, respectively, of the vector pGEX-2T (Amersham Biosciences). The fusion proteins glutathione S-transferase–Daxx and glutathione S-transferase–GLUT4 were purified as described previously (22).

1 The abbreviations used are: Daxx, death-associated protein; PML, promyelocytic leukemia; JNK, c-Jun NH2-terminal kinase; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; c, carboxyl domain; LDM, low density microsomes; HDN, high density nuclei; HA, hemagglutinin; PM, plasma membrane; TβRII, type II TGF-β receptor; GLUT, glucose transporter.

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pLexA plasmid and used to study their two-hybrid interactions with Daxx. Subcloning of C-GLUT4 into the HindIII and XhoI sites of the pcDNA3 vector was also performed by PCR. The open reading frame of full-length human Daxx, the kind gift of Dr. A. F. Fluta (University of Maryland), was subcloned into the two-hybrid pB242AD plasmid and into the pcDNA3.H6C vector as described (7). The vector was transformed into E. coli DH5α (residues 1–572) cloned into the BamHI and HindIII sites of the pRSETA vector and subcloned into the EcoRI and XhoI sites of the pB242AD plasmid. All the DNA subcloned or amplified by PCR were sequenced before use.

**Yeast Two-hybrid Cloning**—The two-hybrid screen of a human heart MATCHMAKER LexA library was performed according to the instructions of the manufacturer (CLONTECH, Palo Alto, CA) with minor modifications. Appropriately 0.5 mg of the library DNA was transformed into the yeast strain EGY48 carrying the pLexA-C-GLUT4 plasmid. The first 5 × 10⁷ co-transformants were spread on SD/−His−Trp−Ura plates, and then the Leu− yeast colonies were spread on SD/Gal/Rafβ-gal/−His−Leu−Trp−Ura plates. Grown blue colonies were isolated for the identification of the library plasmids and further study. Positive library plasmids were transformed for second round into EGY48 to assay the two-hybrid interactions with the pLexA: C-GLUT4 plasmid.

**Measurement of the Strength of the Two-hybrid Interactions by the Quantitative β-Galactosidase Assay**—The relative strength of the two-hybrid interaction between Daxx and C-GLUT4 was measured as described (17). The C-GLUT4 mutants were quantified using the liquid β-galactosidase assay as described (17). The C-GLUT4 mutants developed included C-GLUT4(Arg⁴⁸³-Ala⁴⁸⁴), C-GLUT4(Ala⁴⁸⁹-Ser⁴⁹⁰), C-GLUT4(Ala⁵⁰⁵) and C-GLUT4Δs. They were developed by substituting Arg-Ala for the Phe⁴⁶⁵-Arg⁴⁶⁶ pair, by replacing the pair Ala-Ser for the Leu⁵⁰⁵-Leu⁵⁰⁶ pair, by substituting Ala for Tyr⁴⁴⁶, and by removing the last five C-residues of GLUT4, respectively. EcoRI and BamHI sites were introduced by PCR in all the mutants to clone them into the corresponding C-residues of GLUT4, respectively.

**In Vitro Translation and in Vitro Binding Assays**—Full-length DaxxH6C, C-DaxxH6C (residues 661–740), N-DaxxH6C (residues 1–572), and C-GLUT4 were in vitro synthesized and ³⁵S-labeled by the transcription/translation of the pcDNA2.H6C.Daxx, pcDNA3.H6C.C- Daxx, and pDNA2/C-GLUT4 in the TNT rabbit reticulocyte lysate system (Promega). The ³⁵S-labeled Daxx proteins were incubated with 10 μl of the Co⁺⁺-based Talon affinity resin (CLONTECH) and then for 60 min at 4 °C in 50 mM Tris, pH 8, 10% glycerol, 250 mM NaCl with or without ³⁵S-labeled C-GLUT4. After washing the resin with 15 mM imidazole, the retained proteins were released by incubation with 200 mM imidazole and then resolved by SDS-PAGE and analyzed by autoradiography.

**Cell Culture**—Cells were grown on plastic dishes or glass coverslips. 3T3-L1 fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 4 mM glutamine, 50 μg/ml streptomycin, 100 IU/liter penicillin and nonessential amino acids (complete medium) at 37 °C in a humidified CO₂ incubator. The 3T3-L1 adipocytes were induced for 3 days in vitro to separate the nonadipocytes from the postnuclear supernatants, developed by a 5-min centrifugation at 600 g. The postnuclear supernatant was subjected to 10% sucrose cushion and centrifuged 3 min at 300 × g to separate the detergent and the aqueous phases (20). The two phases as well as the sucrose interphase were harvested for their content in Daxx by Western analysis using the rabbit polyclonal anti-Daxx M-112 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

**In Vitro Translated C-Daxx and Full-length Daxx Interact with in Vitro Translated C-GLUT4**—The ability of Daxx to interact with C-GLUT4 was further assayed through a biochemical analysis. C-Daxx (residues 1–572) and full-length Daxx were incubated with His₉ g-agarose in vitro translated and labeled with [³⁵S]-methionine/cysteine. Samples of each Daxx product were first incubated with 10 μl of a Co⁺⁺-based Talon resin and then with ³⁵S-labeled C-GLUT4 to pull down the His-tagged protein complexes. The products retained by the resin were eluted and studied by autoradiography. The results showed that full-length Daxx and C-Daxx (Fig. 1B) but not N-Daxx (data not shown) interacted with C-GLUT4.
more, the coincidence between the sizes of full-length Daxx, C-Daxx, and GLUT4 calculated from their electrophoretic mobility and from their amino acid sequences excluded their modification during their translation in vitro and showed that the interaction between Daxx and GLUT4 requires no prior modification of the proteins.

Interaction of Daxx with GLUT4 Involves the Dileucine Motif in the Transporter Carboxyl-Cytoplasmic Domain—C-GLUT4 contains a few transport motifs required for the intracellular sorting and endocytosis of GLUT4 (22). Among these motifs are: the dileucine-based motif (23), which mediates the targeting of GLUT4 from the trans-Golgi network to the pericentriolar storage compartment (PC-GSC) (18) as well as its surface expression (24); and clusters of acidic residues (25), including the last five C-residues that together with the adjacent Tyr462 play a role in the retention of GLUT4 in the PC-GSC (18). To study if any of these motifs were involved in the interaction of C-GLUT4 with C-Daxx, they were separately inactivated during their translation in vitro and showed that the interaction between Daxx and C-GLUT4 requires no prior modification of the proteins.

Interaction of Daxx with GLUT4 Involves the Dileucine Motif in the Transporter Carboxyl-Cytoplasmic Domain—C-GLUT4 contains a few transport motifs required for the intracellular sorting and endocytosis of GLUT4 (22). Among these motifs are: the dileucine-based motif (23), which mediates the targeting of GLUT4 from the trans-Golgi network to the pericentriolar storage compartment (PC-GSC) (18) as well as its surface expression (24); and clusters of acidic residues (25), including the last five C-residues that together with the adjacent Tyr462 play a role in the retention of GLUT4 in the PC-GSC (18).

To study if any of these motifs were involved in the interaction of C-GLUT4 with C-Daxx, they were separately inactivated (see Fig. 2). The dileucine-based motif, \( \text{RRXXXL} \) (26), was inhibited by introducing an Ala between the two Arg (GLUT4Arg483-Ala484 mutant), and by replacing the pair Leu489-Leu490 by Ala-Ser (GLUT4Leu489-Leu490 mutant). The motifs involved in the retention of GLUT4 in the PR-GSC were inhibited by replacing an Ala for Tyr462 (GLUT4Ala462 mutant) and by deleting the last five PDEND residues (GLUT4Δ5 mutant). The wild-type C-GLUT4 and the C-GLUT4 mutants were then cloned into the pLexA plasmid and introduced into yeast EGY48 cells previously transformed with the Daxx:pB42AD plasmid. The relative strength of two-hybrid interactions was quantified with the liquid \(-\text{galactosidase} \) assay (Fig. 2). The interaction of C-Daxx with C-GLUT4 was significantly inhibited, 2-fold, by the substitution of the Leu489-Leu490 pair by Ala-Ser, but none of the other three mutants developed significantly affected the interaction.

In Vivo Interaction between Daxx and GLUT4—We also measured the ability of Daxx to interact physically with GLUT4 in clonal 3T3-L1 fibroblasts stably transfected with HA-GLUT4 and expressing levels of the transporter comparable with those measured in 3T3-L1 adipocytes and adipose tissue (Fig. 3) (18). For this purpose, postnuclear lysates were separately incubated with a monoclonal antibody against the HA tag introduced in GLUT4 and with a polyclonal antibody against Daxx, and the content of Daxx and GLUT4 in the immunoprecipitates was measured by Western using specific antibodies (Fig. 3). We observed that the immunoprecipitation of GLUT4 with the anti-HA antibody brought down a small amount of Daxx (Fig. 3A). Furthermore, Daxx was not immunoprecipitated when the incubation was repeated using a lysate from 3T3-L1 fibroblasts that were not expressing GLUT4 (Fig. 3A). Moreover, the immunoprecipitation of Daxx with an anti-Daxx antibody also brought down a small amount of GLUT4 (Fig. 3). The relative strengths of the two-hybrid interaction of C-Daxx with C-GLUT4, with the C-GLUT4/HA-GLUT4, and with the empty vector pLexA were measured using the liquid \(-\text{galactosidase} \) assay. Bars represent the mean deviation of the \( \beta \)-galactosidase levels measured in five clones.
with a monoclonal antibody against the nuclear antigen NA or with rabbit preimmune serum brought down neither GLUT4 nor Daxx (Fig. 3). It was interesting that lysates and immunoprecipitates contained two GLUT4 species of 64 kDa and 61 kDa. The 64-kDa species was dominant and was preferentially immunoprecipitated by the anti-HA antibody (Fig. 3A).

When the above experiment was repeated using 3T3-L1 adipocytes and monoclonal antibodies to stain the proteins (Fig. 3B), the results obtained were comparable with those in fibroblasts, and small amounts of GLUT4 and Daxx were reproducibly found in pull downs of Daxx and GLUT4, respectively. Again, a mock immunoprecipitation performed with rabbit preimmune serum brought down neither Daxx nor GLUT4 (Fig. 3B). Stimulation of adipocytes with 100 nM insulin for 20 and 40 min did not change the amount of GLUT4 pull down by the anti-Daxx antibody or the amount of Daxx immunoprecipitated by the anti-GLUT4 antibody (Fig. 3C).

**Daxx Does Not Interact with GLUT1**—The liquid β-galactosidase assay was also used to study the two-hybrid interaction between C-Daxx and C-GLUT1, the ubiquitous glucose transporter homologous to GLUT1. This was of interest since C-GLUT1 as C-GLUT4 interacts with Ubc9 and appears to be SUMOlated. The two-hybrid assay showed that C-GLUT1 did not interact physically with C-Daxx (Fig. 2). Taken together this result and the results of the C-GLUT4 experiments showed that the interaction between C-Daxx and C-GLUT4 was specific. Furthermore, this observation also excludes the fact that the 11-residue-long Ubc9 domain shared by C-GLUT4 and C-GLUT1 is involved in the interaction of Daxx with C-GLUT4.

**Daxx Is Localized to the Nucleus and Low Density Microsomes**—The cellular distribution of Daxx is controversial. Most published studies indicate that Daxx is entirely nuclear (7–13). However, recent studies have traced Daxx to the cytoplasm and showed that its distribution between cytoplasm and nucleus can be regulated (2, 14–16). The interaction of Daxx with C-GLUT4 led us to compare the cellular distributions of Daxx and GLUT4 in cellular fractions (Figs. 4 and 5) and by microscopy (see Fig. 7). Fractions of 3T3-L1 fibroblasts and 3T3-L1 adipocytes enriched in nuclei, cytoplasm, HDM, and LDM were scrutinized for Daxx using the anti-Daxx polyclonal antibody.
from the cytosol and HDM fractionated from fibroblasts discarded the fact that the Daxx detected in LDM was produced by contamination with the protein released from broken nuclei. It remains unclear if the traces of Daxx in HDM isolated from 3T3-L1 adipocytes were produced by the contamination of HDM with LDM. In contrast with Daxx, GLUT4 was detected in HDM, LDM, and plasma membrane (Fig. 4B).

The association of Daxx with LDM was studied by incubating the LDM with 1% Triton X–114 at 30 °C, treatment which provokes the partition of integral membrane proteins and peripheral membrane proteins with the detergent and aqueous phase, respectively (20). As shown in Fig. 4C, Daxx was quantitatively recovered with the aqueous phase indicating, therefore, that it is peripherally associated with LDM membranes.

The cellular distribution studies described above were further extended to examine whether or not the stimulation of adipocytes with insulin changed the distribution of Daxx among the HDM, LDM, and PM fractions. The result was negative; Daxx remained confined in the LDM fraction before and after the stimulation with insulin. In neither situation Daxx was detected in the PM fraction (Fig. 5). We found it interesting in this experiment that, whereas the HDM fraction and to a lesser extent the PM contained a GLUT4 species of 90 kDa (see also below), this species was virtually absent from LDM, which instead contained two species of 76 kDa and 100 kDa (Fig. 5).

The cellular distribution of Daxx was further studied by immunofluorescence microscopy in 3T3-L1 fibroblasts using the anti-Daxx M-112 antibody, which recognizes Daxx as the major protein species both in 3T3-L1 fibroblasts stably transfected with GLUT4 (Fig. 6A). The cells were fixed-permeabilized with cold methanol (4°C) and stained with the anti-Daxx antibody. The pattern of stained fibroblasts and adipocytes was similar but not identical. In agreement with previous studies Daxx was localized to the PML nuclear bodies, which were displayed as small, round, bright fluorescent spots (7, 9, 11, 13), and to the nucleoplasm (Fig. 6B and C). Furthermore, consistent with the results of the cell fractionation studies the anti-Daxx antibody stained numerous punctate structures in the cytoplasm (Fig. 6, B–D). These structures were easily visualized near the plasma membrane where the cytoplasm is thin and were often arranged in rows of different length and stained the contour of the plasma membrane (Fig. 6D). Clonal fibroblasts stably transfected with the GLUT4(Ala489-Ser490) and GLUT4(Ala483-Ala484) displayed similar staining (data not shown). The pattern of Daxx staining in 3T3-L1 adipocytes was analogous to the pattern
observed in 3T3-L1 fibroblasts. Daxx was localized to the nucleus and to many punctate structures distributed throughout the cytoplasm (Fig. 7, A, D, and G). Double staining of adipocytes for Daxx and GLUT4 showed that the only significant co-distribution of the two proteins was limited to a few punctate structures in the area of the GLUT4 perinuclear storage compartment (18) (Fig. 7, C, F, and I). In addition, we observed significant differences between the Daxx-positive PML nuclear bodies in adipocytes and fibroblasts, their size being much larger in the former (compare Fig. 7, K and L). The sonication of adipocytes to produce plasma membrane lawns blew out most of the Daxx-positive structures, but the few remaining attached to the plasma membrane were clearly distinguished from the much more abundant clusters of GLUT4 molecules (Fig. 7, J).

**Covalent Modification of GLUT4 and Daxx by Conjugation to the Ubc9 Substrate, SUMO1**—It has been reported that anti-GLUT4 immunoprecipitates prepared from membranes of 3T3-L1 adipocytes solubilized with detergent contain a 90-kDa protein that reacts with anti-SUMO1 antibodies (5). Due to the lack of reactivity of this 90-kDa protein with anti-GLUT4 antibodies it was not possible to distinguish in that study if this species was conjugated to SUMO1. We note that HDM-Purified from clonal 3T3-L1 fibroblasts are enriched in a 90-kDa GLUT4 species as shown by Western analysis. (Fig. 5). To study if this species was conjugated to SUMO, SUMO1lated proteins and GLUT4 were immunoprecipitated from HDM us-

ing specific antibodies and studied by Western using antibodies against GLUT4 and SUMO1, respectively. The study showed that the 90-kDa GLUT4 species was precipitated by SUMO1 and GLUT4 antibodies and reacted with the two antibodies as shown by Western (Fig. 8).

It has been recently shown that Daxx is immunoprecipitated from BOSC23 cells transfected with FLAG epitope-tagged SUMO1 using an anti-FLAG antibody (21). We therefore explored if the Daxx contained in the postnuclear supernatant and accessible to GLUT4 was SUMO1lated. We found that the anti-SUMO1 antibody was able to immunoprecipitate two of the three closely spaced Daxx polypeptide species contained in the postnuclear supernatant (Fig. 9). Moreover, the same two polypeptides contained in the anti-Daxx immunoprecipitate were found to react with the anti-SUMO1 antibody by Western (Fig. 9). These results were reproduced in postnuclear supernatants from both clonal 3T3-L1 fibroblasts stably transfected with GLUT4 and Chinese hamster ovary cells (data not shown).

**DISCUSSION**

In this study we have shown that the insulin-sensitive glucose transporter GLUT4 interacts physically with Daxx, the adaptor protein whose function has been associated with apoptosis (1, 2, 10, 27–29). The co-immunoprecipitation of Daxx and GLUT4 from extracts of 3T3-L1 fibroblasts stably transfected with GLUT4 and from 3T3-L1 adipocytes expressing constitutive levels of the proteins assesses the physiological meaning of the two-hybrid interaction between Daxx and GLUT4. The small amount of Daxx/GLUT4 complexes detected in Daxx and GLUT4 immunoprecipitates suggests that the interaction implicates a small population of the proteins. This agrees with the limited colocalization of the two proteins observed in the microscopy studies.

The results of the study of Daxx in cellular fractions and by microscopy, indicate the existence of large amounts of Daxx outside the nucleus. This observation extends, therefore, recent results showing the presence of Daxx in the cytoplasm (2, 14–16). The punctate staining of the cytoplasm of 3T3-L1 fibroblasts and 3T3-L1 adipocytes incubated with antibodies against Daxx agrees with the localization of Daxx in LDM, the cellular fraction that enriched in endosomes contains a sizable part of GLUT4 (30). The localization of Daxx to endosomes and the identification of endosomes carrying ligand-receptor complexes as the sites where signal transduction is often initiated (31) suggests that the interaction of Daxx with proteins such as Fas receptor TβRII could also occur at endosomes. The recovery of Daxx with the aqueous phase upon treatment of LDM with Triton X-114 indicates that its association with LDM membranes is peripheral, a result not unexpected since Daxx is soluble and interacts with the cytoplasmic domains of Fas receptor TβRII (1, 2) and GLUT4.

Whereas Daxx and partly GLUT4 are localized to punctate structures distributed throughout the cytoplasm, it is interesting that in adipocytes double-immunostained for GLUT4 and Daxx the colocalization of the two proteins is confined to a few punctate structures localized in the vicinity of the PC-GSC that stores the bulk of GLUT4. The meaning of this is not clear. While the lack of extensive overlapping between the distributions of Daxx and GLUT4 could be an artifact and reflect the masking of their C-domains (involved in their interaction and containing the epitopes recognized by the antibodies), the small amount of Daxx/GLUT4 complexes found in the GLUT4 and Daxx-immunoprecipitates is in agreement with the results of the microscopy studies. Daxx could interact quickly and reversibly with GLUT4 to regulate its trafficking as it moves through one or more intra-
cellular compartments. The contrast between the translocation of GLUT4 from intracellular stores to the plasma membrane and the staying of Daxx in LDM after stimulation of adipocytes with insulin discards that Daxx moves shoulder to shoulder with GLUT4 and reaffirms that their interaction is transient and occurs intracellularly.

The ability of the anti-SUMO1 antibody to immunoprecipitate the 90-kDa GLUT4 species detected in crude cell lysates confirms the previous detection of SUMO1 in anti-GLUT4 immunoprecipitates (5) and assesses the SUMOlation of GLUT4. GLUT4, therefore, may belong to the group of SUMOlated proteins whose ability to bind to Daxx has been separately documented by methods that include the yeast two-hybrid-based trap, immunoprecipitations, analysis of cellular fractions, and microscopy. The interaction of Daxx with in vitro translated C-GLUT4 and with SUMO1 (21) suggest that Daxx may bind simultaneously to both. On the other hand, the lack of reactivity of GLUT1 with Daxx and its probable conjugation to SUMO1 (5) suggests that Daxx does not interact with all the SUMOlated proteins and that additional binding determinants are involved in the binding of Daxx. Nevertheless the demonstration of the SUMOlation of Fas (32) and the interaction of

Fig. 7. Double immunostaining of 3T3-L1 adipocytes with anti-Daxx and anti-GLUT4 antibodies. In vitro differentiated adipocytes cultured in complete medium (A–I, K) and plasma membrane lawns (J) were fixed-permeabilized with cold (−20 °C) methanol. Daxx and GLUT4 were stained with the rabbit polyclonal M-112 antibody (fluorescein channel) and the mouse monoclonal antibody 1F8 (Texas Red channel), respectively. Nuclei from 3T3-L1 adipocytes (K) and 3T3-L1 fibroblasts (L) grown in complete medium were stained for Daxx with antibody M-112 (fluorescein channel). The stained cells were studied by confocal microscopy as described under “Experimental Procedures.” The cell shown in A–I produced 15 optical sections of 0.4 μm each. Sections are numbered starting from the plane of cell attachment. White arrows mark yellow punctate structures containing Daxx and GLUT4. Bars A, D, G: 13 μm; Bars B, E, H: 3.6 μm; Bars C, F, I: 1.16 μm; Bar J: 2.1 μm; Bars K, L: 2.65 μm.
and of the SUMOlated 90-kDa GLUT4 in HDM. The localization of the 79- and 100-kDa GLUT4 species in LDM and its dissociation from Daxx and its rapid transfer to HDM. The conjugation of GLUT4 to SUMO occurs in LDM and provokes a demonstrable interaction with the centromeric protein CENP-C during interphase (7). In fact, the interaction between CENP-C and Daxx and the recently demonstrated binding of Daxx to SUMO makes it more comprehensible that the first reported member of the SUMO family, the Saccharomyces cerevisiae SMT3, was cloned in a screen for suppressors of a temperature-sensitive allele of MIF2, a gene encoding an homologue of the mammalian CENP-C (35).

With regard to this it is interesting to know if the interaction of the Daxx contained in LDM blocks the targeting of GLUT4 to lysosomes in a manner regulated by SUMO.

The reported interaction of GLUT4, GLUT1, and Daxx with Ubc9, the SUMO-conjugating enzyme, is likely to reflect the involvement of Ubc9 in their conjugation to SUMO1. Overexpression of Ubc9, the SUMO-conjugating enzyme, has been shown to increase dramatically the levels of GLUT4 and to decrease the levels of GLUT1 (5). It is not known if this difference reflects their different abilities to interact with Daxx. With regard to this it would be interesting to know if the interaction of the Daxx contained in LDM blocks the targeting of GLUT4 to lysosomes in a manner regulated by SUMO.

The recent demonstration that SUMO inhibits the interaction between Daxx and GLUT4 makes it more comprehensible that the first reported member of the SUMO family, the Saccharomyces cerevisiae SMT3, was cloned in a screen for suppressors of a temperature-sensitive allele of MIF2, a gene encoding an homologue of the mammalian CENP-C (35).

In addition to GLUT4, Daxx has been reported to interact with a broad array of proteins (28). A 139-amino acid region from the N-end of ETS1 has been shown to contain a PLL-TPSSK motif (Daxx interacting domain) conserved as PSVLL-DAK in the CENP-C sequence (27) and contained as PSLEEQVK in C-GLUT4. It is interesting that substitution of AS for LL in C-GLUT4 inhibited the two-hybrid interaction between Daxx and GLUT4. Because the inhibition was not complete it is likely that other determinants in C-GLUT4 may mediate its interaction with Daxx.

Computer-aided alignment of the human C-GLUT4 sequence and the sequences of another six Daxx-binding proteins, including H-CENP, H-ASK1, H-PML, H-ETS1, H-Pax3, and H-Fas receptor, reveals a three-block consensus within a domain of less than 73 residues (see Fig. 10). While the domain of C-GLUT4 that binds to Ubc9 has been identified (5) and C-GLUT4 contains sequences that could be involved in the regulation of SUMO conjugation (4), the only putative SUMO1 acceptor sequences in the GLUT4 molecule (KXX or E, and P or G residues two to five sites upstream or downstream of the acceptor lysine) are localized to its large cytoplasmic loop (KDEKRRKLEREP).

While the almost exclusive association of Daxx with cytoplasmic and nuclear mechanisms that control apoptosis may reflect the current areas of interest, it is interesting that in addition to the interaction with GLUT4 described here Daxx interacts with the centromeric protein CENP-C during interphase (7). In fact, the interaction between CENP-C and Daxx and the recently demonstrated binding of Daxx to SUMO makes it more comprehensible that the first reported member of the SUMO family, the Saccharomyces cerevisiae SMT3, was cloned in a screen for suppressors of a temperature-sensitive allele of MIF2, a gene encoding an homologue of the mammalian CENP-C (35).
One of the most relevant findings of the recent analysis of functional protein complexes in *S. cerevisiae* (33, 36) is that the same protein is often integrated in different complexes, a finding that suggests that the activity of a protein may depend on the protein complex in which it is incorporated.

In summary, we have shown here that Daxx interacts specifically with GLUT4 both *in vitro* and *in vivo* and that both proteins are conjugated to SUMO1. SUMO1 is known to enhance protein stability and to be an address for protein targeting. While SUMO 1 enhances GLUT4 stability (5) it is not known if this effect is produced through the regulation of GLUT4 trafficking. The ability of GLUT4 to interact with Daxx appears to be transient. This ability extends the number of SUMO targets that interact with Daxx.

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**Putative Daxx-Interacting Binding Domains**

| A | B | C |
|---|---|---|
| H-Glut4 | --- | --- |
| H-CENP | --- | --- |
| H-ASK | --- | --- |
| H-PML | --- | --- |
| H-ETS | --- | --- |
| H-Fbx | --- | --- |

**Fig. 10.** Binding domains in Daxx protein targets. The C-domain of GLUT4 was aligned with the sequences of six other Daxx-binding proteins. Putative Daxx binding domains are divided into three subdomains: A, B, and C. Identical and conserved residues are black and gray boxed, respectively.

GLUT4 and Daxx, Interaction and Sumolation
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