Pantophysin is a Phosphoprotein Component of Adipocyte Transport Vesicles and Associates with GLUT4-containing Vesicles*

(Received for publication, April 19, 1999, and in revised form, October 19, 1999)

Cydney C. Brooksb, Philipp E. Schererb, Kelly Clevelandb, Jennifer L. Whittemorec, Harvey F. Lodishd, and Bentley Cheatham§

From the §Research Division, Joslin Diabetes Center and Department of Medicine, Harvard Medical School, Boston, Massachusetts 02215, the Research Division, Albert Einstein College of Medicine, New York, New York 10461, and ¶The Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142

Pantophysin, a protein related to the neuroendocrine-specific synaptophysin, recently has been identified in non-neuronal tissues. In the present study, Northern blots showed that pantophysin mRNA was abundant in adipose tissue and increased during adipogenesis of 3T3-L1 cells. Immunoblot analysis of subcellular fractions showed pantophysin present exclusively in membrane fractions and relatively evenly distributed in the plasma membrane and internal membrane fractions. Sucrose gradient ultracentrifugation demonstrated that pantophysin and GLUT4 exhibited overlapping distribution profiles. Furthermore, immunopurified GLUT4 vesicles contained pantophysin, and both GLUT4 and pantophysin were depleted from this vesicle population following treatment with insulin. Additionally, a subpopulation of immunopurified pantophysin vesicles contained insulin-responsive GLUT4. Consistent with the interaction of pantophysin with vesicle-associated membrane protein 2 in neuroendocrine tissues, pantophysin associated with vesicle-associated membrane protein 2 in adipocytes. Furthermore, in 32Porthophosphate-labeled cells, pantophysin was phosphorylated in the basal state. This phosphorylation was unchanged in response to insulin; however, insulin stimulated the phosphorylation of a 77-kDa protein associated with α-pantophysin immunoprecipitates. Although the functional role of pantophysin in vesicle trafficking is unclear, its presence on GLUT4 vesicles is consistent with the emerging role of soluble N-ethylmaleimide-sensitive protein receptor (SNARE) factor complex and related proteins in regulated vesicle transport in adipocytes. In addition, pantophysin may provide a marker for the analysis of other vesicles in adipocytes.

Adipocytes undergo agonist-stimulated exocytosis that is best characterized by the insulin-stimulated translocation of the GLUT4 glucose transporter from a unique intracellular vesicle pool to the plasma membrane (PM)1 (1, 2). Interestingly, however, adipocytes secrete a variety of other proteins such as adipsin, complement factors C3 and B, the complement-related protein Acrp30, tumor necrosis factor-α, leptin, and others (3–6). Despite the relatively large volume of vesicle traffic in adipocytes, the pathways and molecular components governing exocytosis of these factors remain undescribed. The transport and recycling of vesicles have been studied in depth using yeast, neuronal and neuroendocrine systems to identify molecular components involved in protein trafficking pathways, and molecular mechanisms of vesicle docking and fusion (7–9). With respect to the insulin-stimulated translocation of GLUT4 in adipocytes, several SNARE complex proteins recently have been shown to be functionally involved in the docking of GLUT4 vesicles. These include the v-SNARE VAMP2 and the t-SNAREs syntaxin-4 and synaptotagmin-5 (10–15). Potential modulators of SNARE complex formation also have been identified in adipocytes and these include several members of the Rab family of small GTP-binding proteins, members of the Munc18 family of proteins, and synaptotagmin-5 (16–22). Adipocytes also express the N-ethylmaleimide-sensitive fusion protein and α-synaptotagmin-associated proteins involved in catalyzing vesicle membrane fusion events (23).

Another potential modulator of SNARE complex formation is the integral membrane protein synaptophysin. Synaptophysin is a neuroendocrine-specific protein and is characterized by its association with synaptic vesicles as well as small vesicles in these cell types (24, 25). The in vivo function of synaptophysin is unclear; however, in vitro studies suggest that synaptophysin may play a role in regulating interactions of SNARE complex proteins (26). Homozygous deletion of the synaptophysin gene in mice caused no identifiable phenotype; synaptic vesicles appeared to traffic normally in these animals (27, 28), suggesting that related proteins may function in the place of the deleted synaptophysin. Indeed, synaptophysin-related molecules have been described (29–32).

Recently, the cDNA for pantophysin, a protein with sequence similarity to synaptophysin, was cloned from non-neuroendocrine cells (31, 33). Pantophysin displays only a 43% overall amino acid sequence identity to synaptophysin. The primary structure of pantophysin features four putative transmembrane domains similar to those of synaptophysin; however, pantophysin differs from synaptophysin in the cytoplasmic N- and C-terminal domains. Pantophysin mRNA and protein have been shown to be expressed in a variety of tissues and cell lines. Immunofluorescent microscopy of cultured cells showed colocalization of endogenous pantophysin with secretory carrier
membrane proteins and the v-SNARE cellubrevin (33). Based on these studies, pantophysin is proposed to be associated with small cytoplasmic transport vesicles; however, very little is known about the function of pantophysin in vesicle trafficking and fusion events.

The aim of the present investigation was to characterize pantophysin in adipocytes and to explore the possibility that pantophysin may be used as a marker and potential tool for analysis of transport vesicles in adipocytes. As a model of SNARE complex regulation in GLUT4 translocation emerges, the relation of endogenous pantophysin to GLUT4-containing vesicles and the involvement of pantophysin in the trafficking of GLUT4 is of interest. Moreover, the relation of pantophysin to the transport of other vesicles in adipocytes is of importance to the characterization of regulated vesicle trafficking processes in these cells.

**EXPERIMENTAL PROCEDURES**

**Materials—**All tissue culture reagents were purchased from Life Technologies, Inc. with the exception of calf serum, which was purchased from HyClone (Logan, UT). Human insulin was purchased from Roche Diagnostics, Proteolysis/acrylamide/bis-acrylamide solution was from National Diagnostics (Atlanta, GA), bovine serum albumin was from Intergen Company (Purchase, NY), and all other chemicals were from Sigma or Fisher unless otherwise noted.

**Cell Culture and Preparation of 3T3-L1 Cell Lysates—**3T3-L1 fibroblasts were maintained in DMEM containing 10% calf serum (34). Following growth to confluence, adipogenesis was induced with DMEM containing 10% fetal bovine serum, dexamethasone, isobutylmethyl-xanthine, and insulin as described (35). For differentiation studies, cells were harvested at 2-day intervals throughout adipogenesis and were lysed in PBS, 2% Triton X-100, 10 μg/ml apomelin, 1 μg/ml leupeptin, and 2 mM PMSF. Protein concentrations were determined by Bradford analysis (Bio-Rad).

**Cloning of Pantophysin mRNA—**Human and rat synaptophysin and rat synaptotagmin protein sequences as well as the translation product of the synaptophysin-related h-sp1 (GenBank™ Accession number S72481) were aligned using the Jotun Hein algorithm in the DNASTAR MegAlign program. Degenerate oligonucleotide primers were designed based on peptides in two of the four highly conserved transmembrane regions of synaptophysin. The primer PANS5, 5′-GGI TTC/CTT (G/A)AA(A/G)AA/AA(T/C)TG-3′ (bases in parenthesis represent degenerate bases; I, inosine), encodes the peptide W(V/G)GN(L/A)WFV(F/Y) located in the fourth transmembrane domain. These primers were used in RT-PCR with mRNA isolated from differentiated 3T3-L1 adipocytes as described below. The reverse transcription was performed using superscript reverse transcriptase (Life Technologies, Inc.) according to manufacturer’s instructions. The PCR cycling profile was: 94 °C for 3 min, 45 °C for 1 min, 72 °C for 2 min followed by 29 cycles of 94 °C for 1 min, 45 °C for 1 min, and 72 °C for 2 min. The 600-bp product of RT-PCR was subcloned into pCRII (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions for dideoxy chain termination sequence determination using T7 polymerase (Sequenase version 2.0, United States Biochemical, Cleveland, OH) (36). This 600-bp fragment was used as a hybridization probe to isolate full-length clones from a cDNA library made from differentiated 3T3-L1 adipocytes (17). Five independent clones were isolated, and their sequence was determined. To obtain a complete cDNA, 5′-rapid amplification of cDNA ends analysis kit according to manufacturer’s instructions (CLONTECH, Palo Alto, CA).

**RNA Isolation and Northern Blotting—**Poly(A)+ mRNA was isolated from 3T3-L1 cells during differentiation to adipocytes at Day 0 (fibroblast stage) and Day 8 (fully differentiated adipocytes) and from murine tissues as described (17). RNA (1 μg) was separated by electrophoresis in 1.0% formaldehyde-agarose denaturing gels and transferred to Bio-Probe membranes (ICN Pharmaceuticals, Costa Mesa, CA). The 600-bp fragment of the pantophysin cDNA obtained by RT-PCR or the Msc23 cDNA (37) (kind gift of Dr. David Shaywitz) was 32P-labeled by random priming and used at 2 × 106 cpm/ml in hybridizations overnight at 42 °C in 50% formamide; 5% SSC (1 × 150 mM NaCl, 15 mM sodium citrate, pH 7.0; 25 mM sodium phosphate, pH 7.0; 10× Denhardt’s solution; 5 mM EDTA; 1% SDS; 0.1 mg/ml poly(A)). Membranes were washed in 2× SSC, 0.1% SDS and in 0.1× SSC, 0.1% SDS at 50 °C prior to autoradiography.

**SDS-PAGE, Antibodies, and Immunoblotting—**Triton-soluble lysates and subcellular membrane fractions were adjusted to equal protein concentrations by dilution with HES (20 mM HEPES, pH 7.4; 250 mM NaCl; 1 mM EDTA; 10 μg/ml apomelin; 1 μg/ml leupeptin; 2 mM PMSF) and solubilized in SDS-PAGE sample buffer. Equal amounts of proteins were separated by SDS-PAGE, electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell), blocked in Tris-buffered saline, 0.5% Tween-20, 3% BSA and analyzed by immunoblotting with α-Glut4, α-pantophysin, α-VAMP2, or α-insulin-responsive aminopeptidase (IRAP). Rabbit polyclonal antibodies were raised (Co- vance, Denver, PA) against synthetic peptides corresponding to the 12 C-terminal amino acids of human GLUT4, the 16 N-terminal amino acids of murine VAMP2, or the 14 N-terminal amino acids of VAMP3 and against GST fusion proteins coding for either the 26 C-terminal amino acids of murine pantophysin or the 80 N-terminal amino acids of murine IRAP. These antisera were used at 1:100 to 1:200 dilution and incubated for 60 min at room temperature. Membranes were washed in Tris-buffered saline, 0.5% Tween-20, and immunoreactive protein was detected by incubation with 125I-labeled protein A (NEN Life Science Products) followed by autoradiography (BioMax MR, Eastman Kodak Co.) for 18–48 h at ~80 °C with an intensifying screen. Intensities of bands on autoradiographs were measured using a Molecular Dynamics densitometer.

**Immunoprecipitation—**Fully differentiated 3T3-L1 adipocytes were starved overnight in DMEM, 0.1% BSA and incubated in the absence or presence of 100 nM insulin for 10 min. Cells were washed with PBS and lysed in harvest buffer (20 mM HEPES, pH 7.4; 1% Triton X-100, 150 mM NaCl; 10 μg/ml apomelin; 1 μg/ml leupeptin; 2 mM PMSF). Triton-soluble material was incubated with α-pantophysin overnight followed by the addition of protein A-Sepharose beads (Amersham Pharmacia Biotech). Immunoprecipitates were washed twice with harvest buffer, solubilized in SDS-PAGE sample buffer, and analyzed by SDS-PAGE and immunoblotting.

**Subcellular Fractionation of 3T3-L1 Adipocytes—**3T3-L1 adipocytes were starved overnight in DMEM, 0.1% BSA and incubated in the absence or presence of 100 nM insulin for 10 min. Cells were washed with PBS and harvested in HES buffer and homogenized using 26 strokes of a Dounce homogenizer. Following the removal of the fat layer, the supernatant from a 10-min 16,000 × g centrifugation was centrifuged at 48,000 × g for 30 min to obtain a high density microsomal (HDM) pellet, which was resuspended in HES buffer. The 48,000 × g supernatant was centrifuged at 210,000 × g for 50 min to obtain a low density membrane (LDM) pellet, which was resuspended in HES buffer. The 16,000 × g pellet was washed with HES buffer, resuspended in HES buffer, layered over a 1.12 u sucrose cushion, and centrifuged at 100,000 × g for 60 min. The PM at the interface was removed, collected by centrifugation, and resuspended in HES buffer.

**Endoglycosidase Treatment—**Whole cell lysates or membrane protein samples were boiled in 0.5% SDS for 3 min. Samples were diluted with PBS and harvested in HES buffer and homogenized using 26 strokes of a Dounce homogenizer. Following removal of the fat layer, the supernatant from a 10-min 16,000 × g centrifugation was centrifuged at 48,000 × g for 30 min to obtain a high density microsomal (HDM) pellet, which was resuspended in HES buffer. The 48,000 × g supernatant was centrifuged at 210,000 × g for 50 min to obtain a low density membrane (LDM) pellet, which was resuspended in HES buffer. The 16,000 × g pellet was washed with HES buffer, resuspended in HES buffer, layered over a 1.12 u sucrose cushion, and centrifuged at 100,000 × g for 60 min. The PM at the interface was removed, collected by centrifugation, and resuspended in HES buffer.

**Sucrose Gradient Centrifugation—**Sucrose velocity and equilibrium density gradient centrifugations were carried out essentially as described (38). Briefly, 3T3-L1 adipocytes were homogenized, and LDM was prepared from control or insulin-treated cells as described above for subcellular fractionation. The LDM pellet was resuspended in buffer containing 10 mM HEPES, pH 7.4; 1 mM EDTA; 5% sucrose. For velocity gradient centrifugation, the resuspended LDM was layered on top of a 10–50% continuous sucrose gradient and centrifuged at 4 °C for 55 min at 150,000 × g. For equilibrium density centrifugation, the resuspended LDM was layered onto a 10–50% continuous sucrose gradient and centrifuged at 4 °C for 16 h at 150,000 × g. Fractions were collected from the bottom of the gradients and subjected to protein determination, SDS-PAGE, and immunoblotting.

**Immunopurification of Vesicles—**Fully differentiated 3T3-L1 adipocytes were starved overnight in DMEM, 0.1% BSA and incubated in the absence or presence of 100 nM insulin for 10 min. Cells were washed with PBS and harvested in HES buffer and homogenized using 26 strokes of a Dounce homogenizer. Following removal of the fat layer, the supernatant from a 10-min 16,000 × g centrifugation was centrifuged at 48,000 × g for 30 min. The fat cake was removed and the supernatant, containing LDMS, was adjusted to 150 mM NaCl. Following preclearing of the LDMS fraction with protein A-Trisacryl beads (Pierce), samples were incubated with antibodies...
Northern blot analysis of poly(A)
differentiation from fibroblasts to adipocytes. In addition,
thovanadate; 100 mM NaF; 10 mM sodium pyrophosphate. The lysates
autoradiography. Separated by SDS-PAGE. The gels were dried and subjected to
increased 1.7-fold (2.1 kilobase pantophysin mRNA in-
differentiated adipocytes) during differentiation, and Northern blot
analysis was performed using the32P-labeled 600-bp fragment
hybridization probe to isolate a full-length clone from a cDNA
murine tissues indicated that pantophysin mRNA was abun-
ded eight amino acids (GF(V/I)KVL(Q/E)W) located in the
predicted start site. The sequence coding for the amino acids "MASKAN-
MVRQRFSRLSQR" previously reported for the mouse panto-
tophysin recognized a broad band that exhibited a differentiation-
dependent alteration in electrophoretic mobility (predicted
MW = 28,000) (Fig. 2A). The diffuse pattern of migration is
consistent with N-linked glycosylation and variable processing.
A similar observation has been made for synaptophysin, and
both proteins contain a potential N-glycosylation site in the first intravesicular loop (33). To verify glycosylation of panto-
physin, protein samples were treated with N-glycosidase F,
which cleaves asparagine-linked glycans (40). Immunoblot
analysis revealed that treatment with N-glycosidase F resulted
in collapse of the broad pantophysin band to a single immuno-
reactive species migrating at the predicted molecular weight
and that steady-state protein levels remained relatively con-
stant throughout adipogenesis of 3T3-L1 cells (Fig. 2B).

Subcellular Distribution of Pantophysin in 3T3-L1 Adipo-
cytes—To determine the subcellular distribution of pantophy-
sin in adipocytes and whether this distribution was altered by
insulin, PM, HDM, and LDM fractions were prepared by dif-
ferential centrifugation of control or insulin-treated 3T3-L1
adipocytes. For precise measurement of pantophysin, each sub-
cellular membrane fraction was treated with N-glycosidase F
to produce the single migrating species of pantophysin. Immuno-
blots of these fractions using α-pantophysin showed that in the
basal state pantophysin was localized to the PM, LDM, and
HDM fractions. Pantophysin was not observed in the cytosolic
fraction (data not shown). As shown in Fig. 3, insulin stimu-
lated the redistribution of pantophysin from internal mem-
brane fractions to the PM by a modest but significant 1.4-fold
(40) in insulin-treated samples, much less than the insulin-
stimulated recruitment of GLUT4 to the PM (3–5-fold). Similar
results for the insulin-stimulated redistribution of pantophysin
were obtained from experiments using freshly isolated rat ep-
idydalm adipocytes (data not shown).

Equilibrium and Velocity Gradient Centrifugation—To fur-
ther characterize pantophysin-containing vesicles, the LDM

RESULTS
Cloning of Pantophysin cDNA—Synaptophysin contains four
membrane-spanning regions and cytoplasmic N and C termini
(39). Human and rat synaptophysin, rat synaptoporin, and a
synaptophysin-like protein h-Sp1 were aligned, and DNA se-
quences encoding the highly conserved transmembrane do-
 mains were chosen as targets for degenerate oligonucleotide
primers in RT-PCR using mRNA isolated from fully differenti-
ated 3T3-L1 adipocytes as template. The upstream primer en-
coded eight amino acids (GF(V/I)KVL(Q/E)W) located in the
transmembrane domain and the downstream primer en-
coded the peptide W(V/G)NI/L/A(WF/V/F/Y) located in the
fourth transmembrane domain. After RT-PCR, a fragment of
the predicted size (600 bp) was obtained and was used as a
hybridization probe to isolate a full-length clone from a cDNA
library made from differentiated 3T3-L1 adipocytes (17). Five
independent clones were isolated; their sequences were deter-
mined and shown to correspond to the previously identified
protein pantophysin (31, 33). In all clones isolated, the se-
dence at the 5'-end of the clone began with a methionine
codon. To determine whether this methionine codon corre-
sponded to the start of translation, the 5'-end of the clone was
completed using 5'-rapid amplification of cDNA ends as de-
scribed under "Experimental Procedures." The additional 5'-
sequence (42 nucleotides) was analyzed and confirmed the
methionine identified in the original clone as the translational
start site. The sequence coding for the amino acids "MASKAN-
MVRQRFSRLSQR" previously reported for the mouse panto-
tophysin was not obtained (31). This difference may re-
fect a tissue-specific alternate transcriptional start site;
however, the possibility cannot be excluded that the mRNA
coding for these additional N-terminal amino acids is expressed
at low levels in adipocytes.

Pantophysin mRNA and Protein Expression—To begin to
characterize the distribution and function of pantophysin in
adipocytes, differentiation-dependent expression of pantophysin
mRNA was examined. Poly(A)+ RNA was isolated from
3T3-L1 cells at Day 0 (fibroblast stage) and Day 8 (fully differ-
entiated adipocytes) during differentiation, and Northern blot
analysis was performed using the32P-labeled 600-bp fragment
obtained by RT-PCR as probe. As indicated in Fig. 1A, the
steady-state level of the 2.1 kilobase pantophysin mRNA in-
creased 1.7-fold (± 0.3) between Day 0 and Day 8 during
differentiation from fibroblasts to adipocytes. In addition,
Northern blot analysis of poly(A)+ RNA prepared from various
murine tissues indicated that pantophysin mRNA was abun-
dantly expressed in adipose compared with the other tissues
examined (Fig. 1B).

To assess the expression of pantophysin protein throughout
differentiation, Triton-soluble protein lysates were immuno-
blotted with a rabbit polyclonal antibody directed against the
C-terminal 26 amino acids of murine pantophysin. The α-
pantophysin recognized a broad band that exhibited a differen-
tiation-dependent alteration in electrophoretic mobility (predicted
MW = 28,000) (Fig. 2A). A diffuse pattern of migration is
consistent with N-linked glycosylation and variable processing.

A similar observation has been made for synaptophysin, and
both proteins contain a potential N-glycosylation site in the first intravesicular loop (33). To verify glycosylation of panto-
physin, protein samples were treated with N-glycosidase F,
which cleaves asparagine-linked glycans (40). Immunoblot
analysis revealed that treatment with N-glycosidase F resulted
in collapse of the broad pantophysin band to a single immuno-
reactive species migrating at the predicted molecular weight
and that steady-state protein levels remained relatively con-
stant throughout adipogenesis of 3T3-L1 cells (Fig. 2B).

Subcellular Distribution of Pantophysin in 3T3-L1 Adipo-
cytes—To determine the subcellular distribution of pantophy-
sin in adipocytes and whether this distribution was altered by
insulin, PM, HDM, and LDM fractions were prepared by dif-
ferential centrifugation of control or insulin-treated 3T3-L1
adipocytes. For precise measurement of pantophysin, each sub-
cellular membrane fraction was treated with N-glycosidase F
to produce the single migrating species of pantophysin. Immuno-
blots of these fractions using α-pantophysin showed that in the
basal state pantophysin was localized to the PM, LDM, and
HDM fractions. Pantophysin was not observed in the cytosolic
fraction (data not shown). As shown in Fig. 3, insulin stimu-
lated the redistribution of pantophysin from internal mem-
brane fractions to the PM by a modest but significant 1.4-fold
(40) in insulin-treated samples, much less than the insulin-
stimulated recruitment of GLUT4 to the PM (3–5-fold). Similar
results for the insulin-stimulated redistribution of pantophysin
were obtained from experiments using freshly isolated rat ep-
idydalm adipocytes (data not shown).

Equilibrium and Velocity Gradient Centrifugation—To fur-
ther characterize pantophysin-containing vesicles, the LDM
fraction of 3T3-L1 adipocytes was analyzed by velocity and equilibrium density gradient ultracentrifugation. For separation of the vesicles based on their relative size, LDM pellets from 3T3-L1 adipocytes were resuspended and separated on a continuous 10–30% sucrose velocity gradient as described under “Experimental Procedures.” Fractions from the velocity gradient were taken from the bottom to the top and subjected to protein determination and analyzed by immunoblotting for pantophysin and GLUT4. Pantophysin exhibited a broad distribution profile indicative of variably sized vesicles; GLUT4 showed a more distinct peak; however, considerable overlap between the two vesicle populations was observed (Fig. 4A). Further analysis of these vesicle populations by equilibrium density ultracentrifugation showed nearly identical distribution profiles for pantophysin and GLUT4, suggesting that pantophysin- and GLUT4-containing vesicles within the LDM are of similar buoyant densities (Fig. 4B).

The effect of insulin on the distribution of pantophysin- and GLUT4-containing vesicles was examined using equilibrium density centrifugation of the LDM fractions isolated from control or insulin-treated cells. This analysis indicated that insulin stimulated the redistribution of GLUT4 out of the LDM compartment as expected (Fig. 5A). In addition, insulin stimulated a redistribution of pantophysin out of the LDM (Fig. 5B). This is in agreement with the immunoblot analysis of subcellular fractions from 3T3-L1 adipocytes (Fig. 3); however, a more pronounced insulin effect on pantophysin redistribution is consistently observed with the gradient centrifugation analysis. Total protein content in the gradient fractions obtained from the LDM did not change in response to insulin (data not shown).

**Immunopurification of GLUT4 and Pantophysin-containing Vesicles—Subcellular fractionation and sucrose gradient centri**

**FIG. 3. Subcellular distribution of pantophysin in 3T3-L1 adipocytes.** A, membrane fractions treated with N-glycosidase F and immunoblotted with α-pantophysin. B, untreated membrane fractions immunoblotted with α-GLUT4. Lower panels in A and B represent immunoreactive material quantified by densitometry and expressed as a percent of the total pantophysin or GLUT4 signal. Membrane fractions were prepared as described under “Experimental Procedures” from fully differentiated 3T3-L1 adipocytes incubated in DMEM, 0.1% BSA overnight and treated for 10 min in the absence (−) or presence (+) of 100 nM insulin. Ten μg of protein from each of these fractions were untreated (B) or treated with N-glycosidase F (A) as described under “Experimental Procedures.” Samples were solubilized in SDS-PAGE sample buffer, separated on 10% SDS-PAGE, and electrophoretically transferred to nitrocellulose membranes for immunoblotting (IB) with either α-pantophysin or α-GLUT4. Immunoreactive material was detected by 125I-labeled protein A and subsequent autoradiography. The bracket indicates glycosylated pantophysin. The arrow indicates the 28-kDa pantophysin species.

**FIG. 2. Expression of pantophysin protein during differentiation of 3T3-L1 fibroblasts to adipocytes.** Cell extracts prepared at two day intervals during differentiation were incubated in the absence (A) or presence (B) of N-glycosidase F as described under “Experimental Procedures.” Ten (A) or five μg (B) of total protein were solubilized in SDS-PAGE sample buffer, separated by 10% SDS-PAGE, and electrophoretically transferred to nitrocellulose membranes for immunoblotting (IB) with α-pantophysin. Immunoreactive material was detected by 125I-labeled protein A and subsequent autoradiography. The bracket indicates glycosylated pantophysin. The arrow indicates the 28-kDa pantophysin species.

**Association of Pantophysin with VAMP2—Synaptophysin has been shown by co-immunoprecipitation and cross-linking experiments to associate with the v-SNARE VAMP2 (26, 44, 45). This interaction appears to occur through the cytoplasmic N terminus of VAMP2 (45). To determine whether VAMP2 associates with pantophysin, α-pantophysin immunoprecipitates were prepared from fully differentiated 3T3-L1 cells and immunoblotted with α-VAMP2 or α-VAMP3. As shown in Fig. 7, VAMP2 was associated with α-pantophysin but not with pre-immune immunoprecipitates. VAMP3 was not detected in these α-pantophysin immunoprecipitates. Pantophysin also was observed in α-VAMP2 immunoprecipitates (data not shown). In addition, these interactions did not appear to be affected by insulin.**

**Pantophysin and Insulin-mediated Translocation of**
GLUT4—The association of pantophysin with GLUT4 vesicles and its interaction with VAMP2 suggest a potential role for this protein in insulin-regulated translocation of GLUT4. Although the exact role of synaptophysin is unclear, the cytoplasmic C-terminal portion of the protein contains motifs that are suggestive of a possible role in the function of the protein (46). These motifs include potential endocytic signals and serine phosphorylation sites, and a tyrosine-rich domain (47–51). The C terminus of pantophysin lacks many of these motifs present in synaptophysin; however, to test for the involvement of the C terminus of pantophysin in GLUT4 translocation, streptolysin-O-permeabilized 3T3-L1 adipocytes were used. Permeabilized adipocytes pre-incubated with either GST or GST fused to the C-terminal cytoplasmic domain of pantophysin (GST-panto-CT) were stimulated with insulin, and translocation of GLUT4 to the PM was assessed by immunofluorescence staining of PM sheets. Incubation with GST-panto-CT (25 \mu M) had no effect on GLUT4 translocation either in the basal state or in response to insulin (data not shown).

Phosphorylation of Pantophysin—Synaptophysin has been shown to be phosphorylated in vitro on serine residues by Ca²⁺/calmodulin-dependent protein kinase II and on tyrosine residues by pp60c-src (49, 51, 52). To determine whether pantophysin is phosphorylated in adipocytes, 3T3-L1 adipocytes were labeled in vivo with [32P]orthophosphate. Lysates from control and insulin-treated 32P-labeled cells were prepared and

---

**FIG. 4. Sucrose gradient ultracentrifugation of LDM fraction of 3T3-L1 adipocytes.** A, separation of vesicles in LDM fraction by velocity gradient centrifugation. B, separation of vesicles in LDM fraction by equilibrium density gradient centrifugation. Lower panels in A and B represent immunoreactive material (pantophysin ● or GLUT4 ○) quantified by densitometry and expressed as percent of the total pantophysin or GLUT4 signal or amount of protein (A) expressed as A₅₉₅ units in each fraction. LDM fractions from fully differentiated 3T3-L1 adipocytes were prepared and resuspended as described under “Experimental Procedures.” For velocity gradient centrifugation (A), the LDM fraction was layered on top of a 10–30% continuous sucrose gradient and centrifuged at 4 °C for 55 min at 150,000 × g. For equilibrium density centrifugation (B), the resuspended LDM was layered onto a 10–50% continuous sucrose gradient and centrifuged at 4 °C for 16 h at 150,000 × g. Fractions were collected from the bottom of the gradients and subjected to protein determination, 10% SDS-PAGE, and immunoblotting with either α-pantophysin (Pant) or α-GLUT4. Results are representative of three or more independent experiments.

**FIG. 5. Equilibrium density gradient centrifugation of LDM fraction of 3T3-L1 adipocytes in the basal state and in response to insulin.** Resuspended LDM fractions prepared from fully differentiated 3T3-L1 adipocytes incubated for 10 min in the absence (Control) or presence of 100 nM insulin were layered onto a 10–50% continuous sucrose gradient and centrifuged at 4 °C for 16 h at 150,000 × g. Fractions were collected from the bottom of the gradients and subjected to protein determination, 10% SDS-PAGE, and immunoblotting with either α-GLUT4 (A) or α-pantophysin (B). Lower panels in A and B represent immunoreactive material from control (●) or insulin-treated (○) cells quantified by densitometry and expressed in arbitrary units (a.u.). Results are representative of three or more independent experiments.
Adipocytes have a large volume of intracellular vesicle traffic and undergo regulated exocytosis. However, with the exception of the insulin-stimulated redistribution of GLUT4 from an intracellular vesicle pool to the PM, the process of vesicular transport in these cells is poorly understood. The present study begins to characterize pantophysin in 3T3-L1 adipocytes, and to address the hypothesis that this ubiquitously expressed integral membrane protein may be a useful marker and functional component of adipocyte transport vesicles.

Northern and Western blot analysis of 3T3-L1 adipocyte RNA and Triton-soluble lysates, respectively, revealed the expression of a 2.1-kilobase mRNA and protein of the predicted size, 28 kDa, in 3T3-L1 adipocytes. The steady-state levels of the pantophysin transcript show a modest increase during adipogenesis of 3T3-L1 cells. Moreover, adipose tissue exhibited the highest level of pantophysin mRNA on tissue Northern blots.

Immunoblotting of cellular lysates prepared during adipogenesis with \( \alpha \)-pantophysin showed that the steady-state level of pantophysin protein remains relatively constant during differentiation. These data, coupled with the increase in pantophysin mRNA during adipogenesis, suggest potential differentiation-dependent changes in mRNA and/or protein turnover events. The broad band of \( \alpha \)-pantophysin-reactive material is consistent with modification by glycosylation. The related protein synaptophysin is glycosylated in vivo (25), and pantophysin and synaptophysin both contain a putative N-glycosylation site in the first intravesicular loop of the proteins (33). Indeed, glycosidase treatment of total cell lysates and of adipocyte membrane fractions prepared by differential centrifugation showed that pantophysin was glycosylated in 3T3-L1 cells. Moreover, glycosylated pantophysin exhibited a differentiation-dependent alteration of its migration pattern on SDS-PAGE suggesting differential processing of the oligosaccharide moiety.

Synaptophysin has been localized to synaptic vesicles and small vesicles in neuroendocrine tissue (24, 25), and pantophysin...
Pantophysin and GLUT4 Vesicles

Pantophysin has been localized to small cytoplasmic trafficking vesicles (33). In the present study, three biochemical analyses were used to study the distribution of pantophysin in 3T3-L1 adipocytes, and each showed an overlap of pantophysin and the insulin-sensitive glucose transporter GLUT4. First, subcellular fractionation of 3T3-L1 adipocytes by differential centrifugation was used to localize pantophysin in these cells; in the basal state pantophysin was localized to the PM, LDM, and HDM fractions but was undetectable in the cytoplasm, consistent with the presence of transmembrane domains. Immunoblotting of equal amounts of protein from these subcellular membrane fractions indicated that pantophysin appears to be relatively evenly distributed among these compartments.

Second, the distribution of pantophysin in cultured adipocytes was examined using sucrose gradient ultracentrifugation of LDM preparations. Fractions collected from both velocity and equilibrium density gradient centrifugation revealed that pantophysin and GLUT4 reside in vesicles of overlapping size and almost identical density. Similar experiments examining the distribution of synaptophysin in vesicle preparations of neuronal or neuroendocrine cells have shown a broad profile in both velocity and equilibrium gradient centrifugation (53, 54). Interestingly, in this study pantophysin was associated with LDM-derived vesicles of distinct density that overlap entirely with GLUT4-containing vesicles.

Analysis by both subcellular fractionation and sucrose gradient centrifugation showed that pantophysin and GLUT4 reside in overlapping membrane compartments. To determine whether these proteins localized to the same vesicle population, a third method was employed. Immunopurification of LDM-derived vesicles demonstrated that pantophysin and GLUT4 were present on the same vesicles: GLUT4 IPVs contained pantophysin, and pantophysin IPVs contained GLUT4. In addition, the three biochemical analyses employed here also showed that, as is the case for GLUT4, insulin stimulates a portion of pantophysin to redistribute from internal membranes to the PM. Subcellular fractionation experiments in this study showed as much as an 8-fold increase in PM-associated GLUT4 in response to insulin. Insulin also stimulated a redistribution of pantophysin from intracellular membrane compartments to the PM. However, the fraction of pantophysin moving to the PM (1.4-fold increase) following treatment with insulin was much less than that for GLUT4. Consistent with these observations, equilibrium density gradient ultracentrifugation also showed a depletion of both GLUT4 and pantophysin from the LDM fraction in response to insulin. Furthermore, immunopurification of vesicles from the LDM fraction with α-GLUT4 and α-pantophysin showed depletion of both vesicle pools from this fraction in response to insulin. Insulin induced approximately a 66 ± 10% depletion of GLUT4 and a 41 ± 2% depletion of pantophysin associated with the GLUT4 IPVs. Pantophysin IPVs exhibited approximately a 21 ± 8% and 17 ± 5% depletion of GLUT4 and pantophysin, respectively, in response to insulin. Finally, only 34% of the total LDM-derived pantophysin is associated with GLUT4 IPVs in the same compartment. The above data suggest that a subpopulation of the total pantophysin is present on GLUT4 vesicles and that this population traffics out of the LDM in response to insulin.

Our findings are consistent with the hypothesis that pantophysin is a component on a variety of adipocyte transport vesicles and that GLUT4 is localized to only a subpopulation of pantophysin-containing vesicles. Thus, the translocation of pantophysin observed following insulin stimulation would be a response of only a subpopulation of pantophysin-containing vesicles and would not be expected to be as dramatic as that observed for the translocation of GLUT4. One also might speculate that if pantophysin is present on all vesicles undergoing regulated transport, within the LDM for example, then insulin-stimulated vesicle transport represents only a minor component of the total population of these vesicles and that this subpopulation is composed primarily of GLUT4-containing vesicles within this compartment.

The v-SNARE VAMP2 has been shown, in various systems, to be involved in targeting intracellular transport vesicles (7–9). In adipocytes, both cleavage of VAMP2 by clostridial neurotoxins and addition of the cytoplasmic domain of VAMP2 blocked GLUT4 translocation to the PM in response to insulin (11, 12, 55). In neuronal systems, an association between VAMP2 and synaptophysin has been observed by co-immunoprecipitation and cross-linking experiments (26, 44, 45). In isolated synaptosomes it appears there are two pools of VAMP2, one complexed with syntaxin-1 (forming the SNARE complex) and one complexed with synaptophysin. When complexed with synaptophysin, VAMP2 appears to be unavailable for interaction with the t-SNAREs syntaxin-1 and synaposome-associated protein-25 (26). These data suggest that synaptophysin may play a role in regulation of SNARE complex formation. In the present study pantophysin was shown to associate with VAMP2 in α-pantophysin immunoprecipitates. This protein-protein interaction occurred despite the lack of significant overall sequence identity (43%) and many of the sequence motifs present in the C-terminal tail of synaptophysin (31, 33). A low affinity interaction of synaptophysin with VAMP3 has been reported (26); however, VAMP3 was not detected in α-pantophysin immunoprecipitates. This difference may reflect the difference in experimental methods or in the primary structures of synaptophysin and pantophysin. At this point the exact nature of the protein-protein interaction of pantophysin with VAMP2 and the role this interaction may play in vesicle trafficking requires additional characterization.

The function of pantophysin in vesicle trafficking remains unclear. The in vivo function of the related synaptophysin also is unclear; however, in vitro studies suggest that synaptophysin may play a role in regulating interactions of SNARE complex proteins (see above). Furthermore, synaptophysin forms hexameric homo-oligomers in vitro and may be involved in formation of pores for vesicle fusion (56). Ablation of the synaptophysin gene in mice does not disrupt vesicle trafficking, and other proteins of the synaptophysin family may carry out the function of synaptophysin in its absence (27, 28). Synaptophysin is detected only in neuroendocrine tissues, and in other cell types pantophysin may function in an analogous manner to synaptophysin. Along these lines, to attempt to disrupt a potential function of pantophysin in GLUT4 vesicle trafficking, permeabilized adipocytes were incubated with the C-terminal cytosolic domain of pantophysin expressed as a GST fusion protein. GST-panto-CT had no effect on the ability of insulin to stimulate GLUT4 translocation. It is possible that if pantophysin does participate in the trafficking of GLUT4 vesicles, this experimental approach may be unable to define such a role. Alternatively, perhaps higher concentrations of the fusion protein are required, and/or the N-terminal cytoplasmic domain plays a role independently or in conjunction with other regions of the protein.

Synaptophysin has been shown to be phosphorylated in vitro by several kinases (48, 49, 51, 52). In this study, pantophysin appeared as a phosphoprotein in [32P]orthophosphate-labeled adipocytes in the basal state, and this phosphorylation was unchanged in response to insulin. This labeling did not appear to be a result of tyrosine phosphorylation (data not shown) suggesting phosphorylation on serine/threonine residues.
Throughout the membrane spanning regions of the proteins, the primary structure of pantophysin is similar to that of synaptophysin. Synaptophysin, however, contains a C-terminal cytoplasmic region containing multiple copies of a tyrosine-rich domain that is absent in pantophysin (33). The lack of tyrosine phosphorylation observed here is consistent with the absence of this tyrosine-rich domain in pantophysin. In addition to undergoing tyrosine phosphorylation, synaptophysin is phosphorylated in vitro on serine residues by calmodulin kinase II (51). It will be of interest to determine which kinase(s) phosphorylates pantophysin and whether its phosphorylation state is related to regulation of function. In addition, despite no observable change in phosphorylation of pantophysin in response to insulin, there was a 77-kDa phosphoprotein associated with pantophysin in α-pantophysin immunoprecipitates. This protein did not exhibit phosphorylation on tyrosine (data not shown). At present the identity of this phosphoprotein is unknown.

This study provides the first characterization of pantophysin in adipocytes. Pantophysin is an abundant protein in adipocytes. Pantophysin is a resident protein in GLUT4-containing vesicles and this overlap with those containing GLUT4. Furthermore, pantophysin is a resident protein in GLUT4-containing vesicles and this population of pantophysin-associated vesicles exhibits agonist-stimulated regulation. In addition to residing on GLUT4 vesicles, pantophysin may also be a useful marker for following other vesicle transport events, as well as for the enrichment and purification of adipocyte vesicles. Numerous questions regarding the molecular interactions and the specific function of pantophysin in regulated and constitutive exocytic pathways remain to be addressed.

Acknowledgments—We thank Dr. Derek Brazil for the rat brain lysate, Lois Wang for preparation of GST and GST-VAMP2 proteins, Dr. David Shaywitz for the mSec23 cDNA, and David Hirsch for assistance in isolation of the pantophysin cDNA.

REFERENCES
1. Cushman, S. W., and Wardzala, L. J. (1980) J. Biol. Chem. 255, 4758–4762
2. James, D. E., Brown, R., Navarro, J., and Pilch, P. F. (1988) Nature 333, 183–185
3. Cook, K. S., Min, H. Y., Johnson, D., Chaplinsky, R. J., Flier, J. S., Hunt, C. R., and Spiegelman, B. M. (1987) Biochim. Biophys. Acta 921, 420–425
4. Kitagawa, K., Rosen, B. S., Spiegelman, B. M., Lienhard, G. E., and Tanner, L. I. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 6179–6184
5. Wiedenmann, B., and Franke, W. W. (1985) Cell 41, 239–248