The insulin-regulated glucose transporter isotype Glut4 expressed only in muscle and adipose cells is sequestered in a specific secretory vesicle. These vesicles harbor another major protein, referred to as vp165 (for vesicle protein of 165 kDa), that like Glut4 redistributes to the plasma membrane in response to insulin. We describe here the cloning of vp165 and show that it is a novel member of the family of zinc-dependent membrane aminopeptidases, with the typical large extracellular catalytic domain and single transmembrane domain but with a unique extended cytoplasmic domain. The latter contains two dileucine motifs, which may be critical for the specific trafficking of vp165, since this has been shown to be the case for this motif in Glut4. However, the tissue distribution of vp165 is much wider than that of Glut4; consequently, vp165 may also function in processes unrelated to insulin action and may serve as a ubiquitous marker for a specialized regulated secretory vesicle.

Adipose and muscle cells contain a specific type of glucose transporter Glut4 (1). As determined by immunoelectron microscopy, this isoform is almost exclusively found in intracellular vesicular and tubular structures and the trans-Golgi reticulum in untreated cells (2, 3). Insulin treatment leads to the rapid redistribution of Glut4 from this intracellular pool to the cell surface and thus to a large increase in glucose transport (reviewed in Refs. 4 and 5). An enhanced rate of fusion of the Glut4-containing vesicles with the plasma membrane is most likely largely responsible for this effect (6).

Unique Glut4 vesicles have been isolated from the low density microsomal fraction of adipocytes and skeletal muscle cells by immunoadsorption and by gel filtration chromatography and sucrose gradient centrifugation, and further characterized (3, 7–10). Members of the VAMP and SCAMP family, proteins sequestered in a specific secretory vesicle. These vesicles likely largely responsible for this effect (6).

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### EXPERIMENTAL PROCEDURES

### Cloning of vp165 cDNA

An oligonucleotide probe of low degeneracy for screening the cDNA library was obtained by amplifying the DNA encoding one of the known peptides (17, 18). The tryptic peptide IDQVYHALDTTVK (8) (residues 386–399) was used to design short degenerate primers (underlined amino acids): sense primer, 5'-CGGAATTCACTGAYCARGNTNA-3'; antisense primer, 5'-CGGGATCTTACNTGCTGGRTC-3' (N = A, T, or C; H = A, T, or C; R = A or G; Y = T or C). The template in the first PCR was rat genomic DNA (0.3 μg/50 μl reaction). Colonies containing the desired 54-bp PCR product were selected by hybridization at 37 °C with a 2:1 mix (3.3 pmol/ml) of digoxigenin-11-ddUTP-labeled degenerate oligonucleotides derived from the middle portion of the peptide (5'-GNTAYCGAYCNGTAYCAY-3' and 5'-GNTAYCAYGNCYTTRGAYAC-3'). Washes were done in 2× SSC, 0.1% SDS two times for 10 min at 22 °C and once for 15 min at 45 °C. Detection was by chemiluminescence with an alkaline phosphatase-conjugated antidigoxigenin antibody and Lumiphos according to the manufacturer's instructions (Genius system, Boehringer Mannheim). An oligonucleotide probe was synthesized based on the unique sequence thus obtained (5'-GARYCARGNTACCTGCTTCCA-3'), 3'end labeled with digoxigenin-11-ddUTP, and used for screening an oligo(dT)-primed rat adipose tissue cDNA library in a ZAP II (kindly provided by Dr. Vincent C. Manganelli, National Institutes of Health). Hybridization was done for 3 h at 43 °C in 6× SSC, 5° Denhardt's, 0.05% sodium pyrophosphate, 0.1% SDS containing the labeled oligonucleotide at a concentration of 10 pmol/ml, followed by washes in 2× SSC, 0.1% SDS, three times for 10 min at 22 °C and once for 10 min at 48–51 °C. Detection was by chemiluminescence as described above. The insert of one positive clone was subcloned into pBluescript SKII+ and sequenced on both strands ( nt 854-2447 in cDNA).

The 5'end of the cDNA was obtained by 5'-RACE using the 5'-RACE system from Life Technologies, Inc. Total RNA (1 μg) purified from rat epididymal fat pads with Trisolv (Biotec Laboratories, Inc.) was reverse transcribed with the degenerate antisense primer given above at 4 μl. The dCPT-tailed cDNA (2/5 of the total) was used in an amplification reaction with the specific primer 5'-AGGTTCAAACTGAGTT-3' (nt 939–959) and the anchor primer under conditions optimal for the U17a DNA polymerase, which has an inherent 3' to 5' exonuclease activity.

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5'-exonuclease proofreading activity (Perkin-Elmer Corp.). After an initial denaturation at 94°C for 5 min and addition of the polymerase, 35 cycles were run with denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min 30 s, followed by a final extension at 72°C for 10 min. The predominant PCR product at 1 kb was gel purified and reamplified with the nested primer 5'-ATCTGTTGATGAGCAC-GCG-3' (nt 896–914) and a universal anchor primer. The PCR product was subcloned into pCR-Script SK (+) (Stratagene) and sequenced (nt 1–913).

The 3'-end was obtained in two steps by the 3'-RACE method (17). In the first step a degenerate primer derived from one of the tryptic peptides (residues 894–905), which was missing in the sequence so far obtained, was attached to an adaptor (5'-GGGACCCGCTGACTGACTTG- TACTTCTACAGCTG-3') and used at 2 μM to reverse transcribe 0.5 μg of poly(A) RNA purified from isolated rat adipocytes using the FastTrack mRNA isolation kit (Invitrogen). The cDNA purified with GlassMAX (Life Technologies, Inc.) was amplified with the sense primer 5'-GAATCTCCTATGTTTCTGGTGAG-3' (nt 2224–2248) and the adaptor primer 5'-GGCCACCGCTGACTGACATAG-3' under conditions used for the amplification in the 5'-RACE, but with annealing at 60°C and extension at 72°C for only 1 min. The predominant product at 600 bp was subcloned and sequenced (nt 2224–2271). The most 3'-end was then obtained as described above except with an oligo(dT)-adapter primer used at 4 μM for the reverse transcription (5'-GGGACCCGCTGACTGACTTG- TACTTCTACAGCTG-3') and the adaptor primer 5'-GGCCACCGCTGACTGACATAG-3' were used. A smaller aliquot (1/50) of the first amplification reaction was reamplified with a nested sense primer 5'-GGCTGTTTGTCTCTTCTTAGC-3' (nt 2688–2708) and the adaptor primer. The PCR conditions were as described for the first 3'-RACE step. The major product at 500 bp was subcloned and sequenced (nt 2688–3171). All cDNAs were sequenced on both strands by the dideoxynucleotide method with the Sequenase Kit (U. S. Biochemical Corp.). Sequences analyses were done with the software provided in the BIONET Time Sharing Service from IntelliGenetics Inc.

**Generation of Antibodies**

The N-terminal segment (amino acids 2–109) of vp165 was expressed as a glutathione S-transferase fusion protein (Pharmacia Biotech Inc.). A PCR product was generated from vp165 cDNA with the primers 5'-GGGACCCGCTGACTGACTTG-3' and a 3'-end was then obtained as described above except with an oligo(dT)-adapter primer used at 4 μM for the reverse transcription (5'-GGGACCCGCTGACTGACTTG- TACTTCTACAGCTG-3') and the adaptor primer 5'-GGCCACCGCTGACTGACATAG-3' were used. A smaller aliquot (1/50) of the major product at 500 bp was subcloned and sequenced (nt 2688–3171). All cDNAs were sequenced on both strands by the dideoxynucleotide method with the Sequenase Kit (U. S. Biochemical Corp.). Sequences analyses were done with the software provided in the BIONET Time Sharing Service from IntelliGenetics Inc.

**Protease Treatment**

LDM were prepared from basal rat adipocytes as described (12) and washed once with and resuspended in 255 mM sucrose, 20 mM Hepes, 1 mM EDTA, pH 7.4. LDM, at 1.2 mg/ml, were treated with 100 μg/ml proteinase K at room temperature in the absence and presence of 0.5% C2H5OH. All aliquots were removed before proteinase addition (0 time) and at 5 and 30 min thereafter, and the reaction was stopped by addition of phenylmethylsulfonyl fluoride to 2 mM. SDS samples were prepared, and aliquots containing 15 and 25 μg of LDM were immunoblotted with affinity-purified antibodies against the N and C termini of vp165 (at 10 μg/ml), respectively, with detection by chemiluminescence (ECL reagent, Amersham) as described (8), except that all the washes were done in Tris-buffered saline, 0.3% Tween 20.

**Immunoprecipitations and Aminopeptidase Assays**

Glut4 vesicles were isolated from rat adipocytes as described (8), except that the final washes were with 255 mM sucrose, 20 mM Hepes, pH 7.4, and 100 μM NaCl. The vesicles were solubilized in this buffer plus 0.5% C2H5OH and brij 35 and 100 μM EDTA and the supernatant was removed to yield the soluble fraction. Aliquots (300 μl) corresponding to the vesicles derived from 1/5 of the adipocytes from one rat (about 3 × 10^6 cells) were incubated with the affinity-purified antibodies against the N-terminal portion of vp165 or nonspecific rabbit IgG (Sigma) at 67 μg/ml in solubilization buffer for 2 h on ice. The immunocomplexes were collected by mixing with 15 μl of protein A-Sepharose for 90 min at 4°C. After pelleting the beads, the supernatant was removed to yield the soluble fraction. The beads were washed twice in the solubilization buffer and resuspended in 300 μl of this buffer. The fractions were snap-frozen in liquid nitrogen in small aliquots and stored at −70°C until assay for aminopeptidase activity. Equivalent amounts of each fraction, as well as solubilized Glut4 vesicles from which the immunoprecipitates were derived (corresponding to approximately 6 × 10^5 or 3 × 10^5 adipocytes), were diluted into 100 μl of solubilization buffer and added to 400 μl of 2 mM aminoacyl β-naphthylamide (Sigma or Fluka), 125 mM Tris-Cl, pH 7.0, at 25°C. The fluorescence of the liberated β-naphthylamide at excitation 340 nm and emission 410 nm was recorded over a 10-min incubation period at 25°C. The rate of cleavage was calculated from the linear slope of the graph as nmol/min using commercially available β-naphthylamide (Sigma) as a standard.

**Northern Blotting and Immunodetection**

vp165 mRNA — A single-stranded digoxigenin-11-dUTP-labeled PCR product was generated as described (21). The primers were 5'-GGGACCCGCTGACTGACTTG-3' (nt 2224–2248) and 5'-ATCTGACTCCTGGAGTGCAGC-3' (nt 2267–2283); the template was vp165 cDNA. The amplification reaction with AmpliTaq (Perkin Elmer) was done with an initial denaturation at 94°C for 5 min, 50 cycles with denaturation at 94°C for 1 min, annealing at 55°C for the first five cycles and 60°C for the next 45 cycles for 1 min, and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. The PCR product was gel purified and extracted with SpinBind (FMC BioProducts). This product (50 ng) was used in the second amplification reaction, in which digoxigenin-11-dUTP-label replaced 30% of the dTTP. A rat multiple tissue Northern blot (Clontech) was prehybridized for 3–4 h and hybridized at 58°C for 20 h in 5 × SSC, 50% formamide, 0.1% SDS, 0.1% N-laurylsarcosine, 2% blocking reagent (Boehringer Mannheim), 10% sodium dodecyl sulfate, and 50 ng of probe in 10 ml for hybridization. Washes were done twice for 15 min in 2 × SSPE, 0.1% SDS and once in 0.1 × SSPE, 0.1% SDS at room temperature and twice for 30 min in 0.1 × SSPE, 0.1% SDS at 65°C. Detection was by chemiluminescence (Genius system, Boehringer Mannheim).

**RESULTS AND DISCUSSION**

Primary Structure and Membrane Topology of vp165 — The cDNA for vp165 was cloned from rat adipocytes based on peptide sequences obtained from the purified protein (8) (Fig. 1). It contains a 2748-bp open reading frame encoding a protein with 916 amino acids and a calculated molecular mass of 104,575 Da. The hydropathy plot shows the characteristics of a type II integral membrane protein (22), with a single 22-amino acid potential transmembrane domain following a short 109-amino acid hydrophilic N-terminal segment (Fig. 2a). The results obtained from immunoblotting protease-treated low density microsomal membranes of rat adipocytes with antibodies
against the N and C termini of vp165 are consistent with the predicted membrane topology (Fig. 2). Upon digestion of intact membranes with proteinase K, vp165 shifted to a lower molecular weight and was only recognized by the antibody against the C terminus. The shift in mass corresponds to the size of the predicted cytoplasmic portion of vp165, which is 12.5 kDa. When the membrane proteins were solubilized prior to protease treatment, vp165 was completely digested. Thus, the N-terminal domain was degraded by the protease and therefore protrudes from the vesicles, whereas the remainder of the protein was protected from digestion in the intact vesicles and is therefore transmembrane and intralumenal (Fig. 3).

The predicted 105-kDa molecular mass for vp165 is less than the apparent 165-kDa size of the protein on SDS-polyacrylamide gels. This difference is at least in part due to glycosylation of the protein. The amino acid sequence contains 16 potential extracellular N-glycosylation sites (marked with an asterisk in Fig. 1), and treatment of GluT4 vesicle proteins with peptide N-glycosidase F caused a decrease in the mass of vp165 to 140 kDa (data not shown).

vp165 as an Aminopeptidase—Comparison of the predicted amino acid sequence of vp165 with those in the sequence data banks revealed that the large 785-amino acid C-terminal portion of vp165 contained regions with high homology to domains conserved between the mammalian aminopeptidases A and N (23, 24), aminopeptidase yscII from Saccharomyces cerevisiae (25), and aminopeptidase N from Lactococcus lactis (26), as well as the recently described thyrotropin-releasing hormone degrading enzyme from rat (27). The similarity is highest in a stretch of 349 amino acids, where the overall identity to vp165 ranges between 36% for thyrotropin releasing hormone degrading enzyme and 45% for aminopeptidase yscII (Fig. 3). Specific sequences in this domain are identical, including the zinc-binding domain with the sequence I461IAHELAHQWFL483WLNEGF characteristic of this group of aminopeptidases (28).

Because of this similarity, we assayed detergent-solubilized GluT4 vesicles for aminopeptidase activity using as substrates the aminoacyl \( \beta \)-naphthylamides derived from all 20 amino acids, except cysteine. The leucine substrate was hydrolyzed most efficiently, followed by lysine at 58%, methionine at 44%, alanine at 28%, and arginine at 23% of the rate for leucine; the other substrates were cleaved at less than 7% of the rate for leucine (Table I and data not shown).

To determine whether the vesicular aminopeptidase activity was due to vp165, we immunoprecipitated vp165 from detergent-solubilized GluT4 vesicles with an antibody against its N terminus and assayed the immunoprecipitate and the depleted supernatant for activity against aminoacyl \( \beta \)-naphthylamide substrates (Table I). For each substrate, approximately 85% of activity was recovered in the immunoprecipitate.
the total activity associated with GluT4 vesicles was recovered in the vp165 immunoprecipitate. The activities in the immunoprecipitates and depleted supernatants correlated with the distribution of vp165 protein as assessed by immunoblotting the same samples for vp165 (data not shown). From these data, we conclude that vp165 is an aminopeptidase and that it accounts for all the aminopeptidase activity found in the GluT4 vesicles. Its substrate preference is distinct from the mammalian aminopeptidases A and N, which are most active against glutamyl and alanyl substrates, respectively (29).

Comparison of vp165 and Glut4—vp165 is sequestered in the same intracellular vesicular compartment as Glut4 in unstimulated adipocytes (8, 15). In contrast, the mammalian membrane aminopeptidases homologous to vp165 are predominantly localized at the cell surface (23, 24, 27). The explanation for this difference may lie in the structure of the N-terminal cytoplasmic domain of vp165. This domain is longer than in the other aminopeptidases (Fig. 3a), and it contains two double leucines (positions 53/54 and 76/77), which are both preceded by highly acidic regions (positions 40–48 and 60–71). Each of these acidic regions contains one tyrosine (positions 46 and 70), which is a potential site for phosphorylation. Notably, the same two motifs are found in the C-terminal cytoplasmic domain of Glut4, which contains a dileucine followed by an acidic region with a tyrosine (R\textsubscript{485}TPS\textsubscript{487}LEQEVR\textsubscript{501}KPS\textsubscript{506}TELEY\textsubscript{512}GP\textsubscript{517}DEND). In the case of Glut4, the C-terminal segment has been found to be sufficient to direct Glut4 to an intracellular location in transfected fibroblasts and L6 myoblasts (30–32). Moreover, the dileucine motif within this segment is necessary (30–32) but not sufficient (32) for this localization. Thus, the acidic region, as well as the dileucine, may be signals in the trafficking of vp165 and Glut4.

Apart from the two tyrosines, there are other potential sites for regulation by phosphorylation present in the cytoplasmic tail of vp165. Several serines and threonines are found in recognition motifs for protein kinase A (positions 51, 86, 91, 98), protein kinase C (positions 51, 73, 86, 91, 98, 107), casein kinase I (positions 5, 19, 73), and casein kinase II (position 19) (33). Glut4 has only one major site of phosphorylation in vivo; this is serine 488, which is adjacent to its dileucine (see above) and is in motifs specific for both protein kinase A and casein kinase II. Although the extent of phosphorylation in Glut4 in the plasma membrane decreases in response to insulin and increases upon stimulation with a β-adrenergic agent, the functional significance of these changes is not known (34).

Tissue Distribution of vp165—The tissue distribution of vp165 was determined by both Northern blotting and by immunoblotting. Northern blots were performed using probes derived from the N- and C-terminal regions of vp165 with little homology to the other aminopeptidases (Fig. 4a and data not shown). vp165 mRNA was present at comparable levels in all the tissues examined, with the exception of liver, where the amount was lower. Immunoblots were done with the antibodies against the unique N-terminal cytoplasmic domain, as well as with ones against the C-terminal peptide of vp165, on immunoprecipitates derived from whole tissue homogenates with the N-terminal antibodies (Fig. 4b and data not shown). In addition the whole tissue homogenates were blotted directly with the antibodies against the N-terminal domain (data not shown). All the immunoblots yielded the same result: vp165 protein was present at comparable levels in all the tissues, except for liver, which contained a low amount. This tissue distribution differs markedly from that reported previously by Kandror and Pilch (15), who found vp165 (designated gp160 by them) to be present only in fat, heart, and muscle but not in brain, lung, kidney, spleen, or liver. We have no explanation for this discrepancy but note that they determined the tissue distribution only by immunoblotting a crude membrane fraction with antibodies against a short peptide (residues 630–642 in Fig. 1).

The size of the vp165 mRNA was approximately 12.5 kb, which is much larger than the cloned cDNA (3.2 kb). This result was unexpected, since the cDNA ends in a poly(A) sequence. A likely explanation is that there is a less abundant, shorter mRNA that gave rise to the oligo(dT)-primed cDNA. Consistent with this hypothesis is the fact that 22 bp upstream from the poly(A) tail in the cDNA there is an inefficiently used polyadenylation signal, AAATACA (35). The size of the vp165 protein was 165 kDa in all tissues, except brain, where it was 140 kDa. Most likely, this is due to a difference in glycosylation of the brain protein.

The tissue distribution of vp165 differs markedly from that of Glut4, which is restricted to skeletal muscle, heart, and brain protein.
Comparison of vp165 with homologous aminopeptidases. a, schematic illustration of the overall structure for vp165, aminopeptidase N from rat (AMPN rat), aminopeptidase A from human (AMPA human), thyrotropin-releasing hormone degrading enzyme from rat (TRH DE rat), aminopeptidase yscII from S. cerevisiae (yscII yeast), and aminopeptidase N from L. lactis (AMPN lacto). The arrows define the location of the zinc-binding motif in all of the sequences and the two unique double leucines in vp165. The lengths of the boxes are proportional to the sequence lengths.
The aminopeptidase activities towards the aminoacyl \( \beta \)-naphthylamides, in nmoles per min, are given for solubilized GluT4 vesicles, the immunoprecipitates from these (anti-vp165 and control IgG beads), and the supernatants after immunodepletion (anti-vp165 and control IgG Sup). The values are those for the fractions derived from approximately \( 6 \times 10^6 \) adipocytes.

| GluT4 vesicles | Anti-vp165 | Control IgG |
|----------------|------------|-------------|
| **Sup**       | **Beads**  | **Sup**     | **Beads**  |
| L-Leu         | 408        | 57          | 350        | 384        | 8           |
| L-Lys         | 235        | 28          | 195        | 239        | 6           |
| L-Met         | 179        | 21          | 155        | 186        | 6           |
| L-Ala         | 116        | 15          | 105        | 99         | 3           |
| L-Arg         | 94         | 14          | 80         | 80         | 3           |

Fig. 4. Tissue distribution of vp165 mRNA and protein. a, Northern blot. Poly(A)\(^\ast\) RNA from various tissues (2 \( \mu \mathrm{g} \) for each) was probed with a single-stranded digoxigenin-11-dUTP-labeled PCR product corresponding to nucleotides 2224–2783 in vp165 cDNA. RNA markers in kb are indicated on the right. b, Immunoblot. vp165 immunoprecipitates derived from 375 \( \mu \mathrm{g} \) of total protein for each tissue were blotted with the antibody raised against the N-terminal domain of vp165. As a control, the precipitate from heart with unspecific rabbit IgG is shown (Heart IgG). Molecular mass markers in kDa are indicated on the right.

Table I

| Aminopeptidase activities |
|---------------------------|
| GluT4 vesicles            |
| Anti-vp165                |
| Control IgG               |
| **Sup**                   |
| **Beads**                 |
| **Sup**                   |
| **Beads**                 |
| L-Leu                     | 408 | 57 | 350 |
| L-Lys                     | 235 | 28 | 195 |
| L-Met                     | 179 | 21 | 155 |
| L-Ala                     | 116 | 15 | 105 |
| L-Arg                     | 94  | 14 | 80  |

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proportional to the lengths of the amino acid sequences. a, alignment of the amino acid sequences in the region most conserved between the aminopeptidases. Abbreviations are as stated above. The numbers on the left of each line give the position of the first amino acid residue in the respective sequences. The alignment was initially done with GENALIGN (BIONET Time Sharing Service, IntelliGenetics Inc.) and was subsequently maximized by eye. Gaps (indicated by a dot) have been introduced for optimal alignment. Amino acid residues identical for at least four of the aligned sequences are in gray. The numbers at the end of each sequence indicate the % identity of the respective sequence with vp165.

2 One possible physiological function of vp165 is to process peptide hormones; this has been found for the homologous mammalian aminopeptidases (37–39). Insulin could thus modulate the activities of other peptide hormones and thereby broaden its own spectrum of action by bringing vp165 to its substrate(s) at the cell surface. Alternatively insulin could cause the release of a peptide hormone processed by vp165 in vesicles. A defect in insulin-stimulated exocytosis of GluT4 vesicles, proposed to occur in skeletal muscle of insulin-resistant individuals (5), could thus cause a myriad of abnormalities now unexplained by the direct hormone effects (40).
Insulin-regulated Membrane Aminopeptidase in GluT4 Vesicles

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