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Nonneuronal expression of Rab3A: Induction during adipogenesis and association with different intracellular membranes than Rab3D

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ABSTRACT **Rab3A is a small GTP-binding protein expressed predominantly in brain and neuroendocrine cells, in which it is associated with synaptic and synaptic-like vesicles, respectively. Here we report that adult mouse fat cells and 3T3-L1 adipocytes also express Rab3A mRNA and protein. They do not express synaptophysin, an abundant protein in synaptic vesicles or synaptic-like vesicles. The amount of Rab3A mRNA and protein, like that of the highly homologous isoform Rab3D, increases severalfold during differentiation of 3T3-L1 fibroblasts into mature adipocytes. In fat cells, most Rab3D and Rab3A protein is bound to membrane, irrespective of insulin addition. Rab3A and Rab3D are localized in different subcellular compartments, since about half of the Rab3A, but none of the Rab3D, is associated with a low-density organelle(s). Rab3D and Rab3A may be involved in different pathways of regulated exocytosis in adipocytes. Moreover, in adipocytes Rab3A may define an exocytic organelle that is different from synaptic vesicles or synaptic-like microvesicles found in neuronal and endocrine cells.**

The small GTP-binding Rab proteins are localized to specific intracellular vesicles and organelles and are important for vesicular traffic (1). Many are expressed in all mammalian cells. Members of the Rab3 family, in contrast, are expressed only in certain types of cells and are specifically associated with organelles involved in regulated exocytosis (2–5). Rab3A is expressed in neuronal and neuroendocrine cells, where it is associated with synaptic and synaptic-like vesicles. Several studies indicate that Rab3A is involved at a late stage of neurotransmitter exocytosis, possibly at the stage of recruitment of vesicles at the presynaptic membrane (6).

Insulin stimulation of glucose uptake by adipocytes also involves regulated exocytosis (7). Adipocytes express two glucose transporter isoforms, Glut1 (8) and Glut4 (9). In nonstimulated adipocytes about half of the Glut1 and >98% of Glut4 are localized to an intracellular compartment often termed low-density microsomes. Addition of insulin causes most of the Glut4- and Glut1-containing vesicles to fuse with the plasma membrane, increasing the number of glucose transporters at the cell surface and therefore the velocity of hexose uptake (7). In permeabilized adipocytes, nonhydrolyzable analogs of GTP induce fusion of Glut4-containing membranes with the plasma membrane, similar to the effects of these compounds on other types of regulated exocytosis (10, 11). Since Rab3 proteins may be involved in regulated secretion, we have tried to identify the Rab3 isotype(s) involved in translocation of glucose transporters. Previously, we showed that Rab3D is predominantly expressed in adipocytes and is induced during adipogenesis and, thus, could be involved in such a process (12). To identify other members of the Rab3 family expressed in adipocytes and possibly associated with insulin-regulated secretory pathway(s), we screened a 3T3-L1 adipocyte cDNA library with a Rab3A cDNA probe. We found that all the isolated clones encoded Rab3A. Here we characterize Rab3A expression in mature adipocytes.

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EXPERIMENTAL PROCEDURES

Cell Culture. 3T3-L1 fibroblasts and AtT-20 pituitary tumor cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. 3T3-L1 fibroblasts were differentiated to adipocytes by an established protocol (13); prior to experimentation, cells were grown in DMEM without serum for 2 hr. When indicated, insulin was added to a final concentration of 160 nM. The RINm5F insulinoma cell lines were grown as described (14).

Screening of the 3T3-L1 Adipocyte cDNA Library and Northern Blot Analysis. The cDNA library has been described (12); 200,000 bacterial colonies were plated, grown, transferred to Hybond-N filters (Amersham), and screened at high stringency with a rat Rab3A cDNA (gift of A. Tavitian, Institut National de la Santé et de la Recherche Médicale, Unité 248, Faculté de Medecine, Paris) probe labeled with digoxigenin-11-dUTP (Boehringer Mannheim) according to the manufacturer's instructions. The preparation of poly(A)$^+$ RNA for Northern blot analysis and the techniques for preparation and labeling of cDNA probes have been described before (12).

Antibodies. A polyclonal rabbit serum (NCG) was generated that is specific for a synthetic peptide corresponding to the N-terminal residues of Rab3D. The peptide, Ala-Ser-Glu-Pro-Pro-Ala-Ser-Pro-Arg-Asp-Ala-Ala-Cys, was coupled to keyhole limpet hemocyanin and used for immunization of rabbits. To generate affinity-purified antibodies against Rab3D, the above peptide was coupled to SulphoLink gel (Pierce) and the antibodies were bound and eluted according to the manufacturer's instructions. The monoclonal antibody Cl42.2, specific for Rab3A (15), was a kind gift of R. Jahn (Yale University School of Medicine). The antibody against Glut4 has been described (10). Antibody SY 38 against synaptophysin was purchased from Boehringer Mannheim.

Transfection of COS-7 cells, Gel Electrophoresis, and Immunoblotting. COS-7 cells were transfected by the DEAE-dextran technique (16). Separation of proteins by SDS/PAGE, fluorography, immunoblotting, protein determinations, and densitometric scanning of the gels were performed as described (17).

Preparation of Detergent Extracts of Adipocytes and Sodium Carbonate Extractions. Each 10-cm diameter cell culture dish was washed twice with 2 ml of ice-cold phosphate-buffered saline. The cells were suspended in 1 ml of buffer A [50 mM Tris, pH 7.4/1 mM EDTA/150 mM NaCl/1% (vol/vol) Triton X-100/0.1% (wt/vol) SDS with leupeptin at 1.5 μg/ml, aprotinin at 6 μg/ml, and phenylmethanesulfonyl fluoride at 300 μg/ml]. After 10 min of incubation on ice, the

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cell extracts were centrifuged for 10 min at maximum speed in an Eppendorf centrifuge. The pellets were discarded, and the supernatants were immediately mixed with SDS/PAGE sample buffer and boiled for 5 min. An extract from fat cells was similarly prepared by utilizing dissected adult mouse (BALB/c strain) epididymal fat pads. Extraction of 3T3-L1 adipocytes with sodium carbonate was done according to a published protocol (18).

Equilibrium Sucrose Density Gradient Centrifugation. The 3T3-L1 adipocytes on four 10-cm plates were scraped into 2 ml of buffer B (10 mM Tris, pH 7.4/150 mM NaCl with leupeptin at 1.5 μg/ml, aprotinin at 6 μg/ml, and phenylmethanesulfonyl fluoride at 300 μg/ml). As above, the cells were homogenized and the suspension was clarified by centrifugation at 600 × *g* for 5 min. The supernatant was layered onto a 10-ml continuous 10–40% (wt/vol) sucrose gradient made up in 10 mM Tris, pH 7.4/1 mM EDTA. After centrifugation at 39,000 rpm for 16 hr at 4°C in a Beckman SW-41 rotor, fractions (0.923 ml each) were collected from the top. The pellet at the bottom was resuspended in the same volume of buffer B. The fractions were then analyzed by SDS/PAGE and Western blotting as above.

Polymerase Chain Reaction. Two degenerate oligodeoxynucleotides, encoding aa 21–28 and 300–308 of rat synaptophysin, respectively, were constructed (oligonucleotide 1, 5′-GGITTYRTIAARGTIYTISARTGG-3′; oligonucleotide 2, RWAIACRAACCAIRIRTTICCIMCCCA; I = deoxyinosine). They were used to amplify cDNAs homologous to synaptophysin from 3T3-L1 adipocyte poly(A)$^+$ RNA. Standard protocols were used for reverse transcription reactions (19). The single DNA band resulting from the amplification reaction was subcloned, and nine independent clones were sequenced.

RESULTS

We screened 200,000 colonies of our 3T3-L1 adipocyte cDNA library at high stringency with a digoxigenin-11-dUTP-labeled Rab3A cDNA probe. We isolated three positive clones, each 1.3 kb in length. The sequence of these clones was completely identical with the sequence of exons 1–5 of mouse Rab3A genomic DNA, the segments encoding the Rab3A polypeptide (20).

The Northern blot in Fig. 1 establishes that Rab3A mRNA is indeed expressed in 3T3-L1 adipocytes. Similar to Rab3D mRNA and Glut4 mRNA (Fig. 1 and ref. 12), the amount of Rab3A mRNA abruptly increases >5-fold between days 2 and 4 of differentiation of 3T3-L1 fibroblasts into adipocytes. In contrast, Rab5 mRNA is expressed in abundance in 3T3-L1 fibroblasts and its level increases no more than 2-fold during differentiation into adipocytes. As an additional control, the level of cytosolic mRNA encoding the 70-kDa heat shock protein (Hsp70) remains constant during differentiation (Fig. 1).

The Western blot in Fig. 2 shows that Rab3A protein is expressed in 3T3-L1 adipocytes (lane 5). The monoclonal antibody 42.2 used is specific for Rab3A (15), as no reacting protein was detected in COS-7 cells transfected with the pcDNA1 vector alone (lane 1) or with the pcDNA1 neo-Rab3D vector expressing Rab3D (lane 2). COS-7 cells transfected with the pcDNA1 vector expressing Rab3A do exhibit the expected protein of 25 kDa recognized by monoclonal antibody 42.2. A reactive protein of the same molecular mass is present in the neuroendocrine cell line AtT-20 (lane 4), which expresses Rab3A, and in 3T3-L1 adipocytes.

The Western blot in Fig. 3*A* shows that Rab3A protein is undetectable in 3T3-L1 fibroblasts at day 0 of differentiation (lane 4). Consistent with the blot in Fig. 2, Rab3A is expressed at day 8 of differentiation (lane 3) and also in adult mouse adipocytes (lane 5). It is also expressed in RINm5F insulinoma

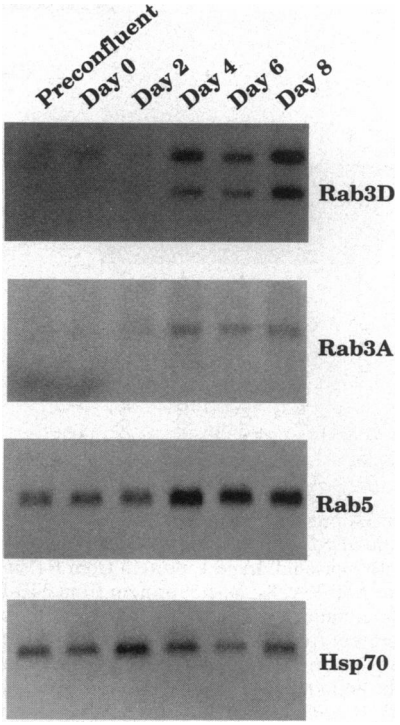

FIG. 1. Levels of Rab3A, Rab3D, Rab5, and Hsp70 mRNA during differentiation of 3T3-L1 fibroblasts into adipocytes. Each lane of the gel contained 0.5 μg of poly(A)$^+$ RNA isolated at the indicated days of the differentiation program of 3T3-L1 fibroblasts into adipocytes. The blot was hybridized with the mouse Rab3A cDNA, isolated from the 3T3-L1 adipocyte library, and excised with *Bam*HI and *Sph* I restriction enzymes from the pcDNA1 vector. The same blot was stripped and probed with Rab3D, Rab5, and Hsp70 cDNAs.

cells (lane 1) and in AtT-20 cells (lane 2). Since the level of Rab3A message and protein is substantially increased in fat cells compared with fibroblasts, Rab3A, like Rab3D, may be involved in an exocytic pathway which becomes important during the course of adipocyte differentiation.

Fig. 3*B* shows that adipocytes, in contrast to neuronal and endocrine cells, do not express synaptophysin, one of the most abundant integral proteins in synaptic vesicle membranes. Fig.

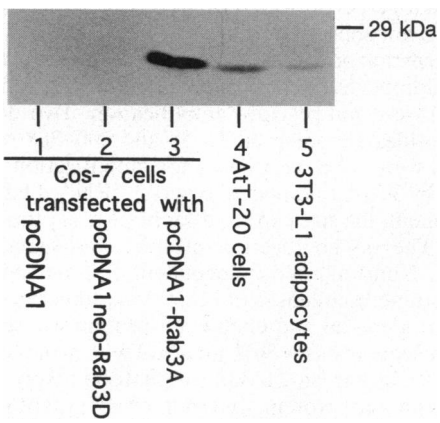

FIG. 2. Expression of Rab3A protein in 3T3-L1 adipocytes. Each lane of the SDS/polyacrylamide gel contained 40 μg of protein. Lane 1, COS-7 cells transfected with the pcDNA1 vector; lane 2, COS-7 cells transfected with the pcDNA1-neo vector containing Rab3D cDNA; lane 3, COS-7 cells transfected with the pcDNA1 vector containing Rab3A cDNA; lane 4, AtT-20 cells; lane 5, 3T3-L1 adipocytes at day 8 of differentiation. The blot was probed with antibody Cl42.2, which is specific for Rab3A, and bound antibody was visualized by enhanced chemiluminescence.

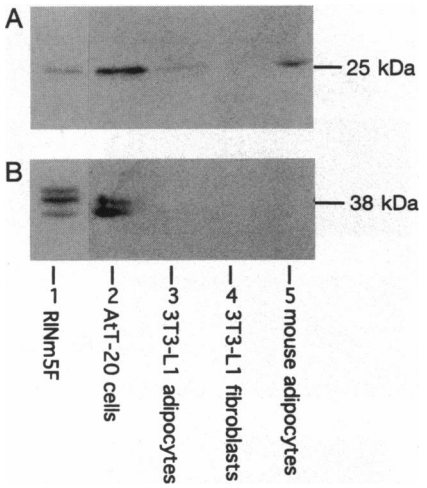

FIG. 3. Rab3A but not synaptophysin is expressed in adipocytes. Each lane of the SDS/polyacrylamide gel contained 20 μg of protein unless otherwise indicated. Lane 1, protein from RINm5F cells; lane 2, protein from AtT-20 cells; lane 3, protein from 3T3-L1 adipocytes at day 8 of differentiation; lane 4, protein from 3T3-L1 fibroblasts; lane 5, 100 μg of protein from adult mouse epididymal fat cells. The blot was cut at the position of the 29-kDa marker protein and probed for Rab3A with the antibody Cl42.2 (*A*), and for synaptophysin with antibody SY38 (*B*). Bound antibodies were visualized with the enhanced chemiluminescence assay.

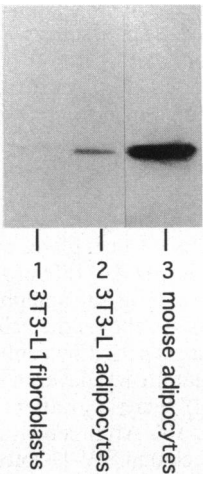

FIG. 4. Expression of Rab3D protein in mouse adipocytes and 3T3-L1 fibroblasts and adipocytes. Each lane of the SDS/polyacrylamide gel contained 20 μg of protein. Lane 1, 3T3-L1 fibroblasts isolated at the beginning of the adipocyte differentiation program; lane 2, 3T3-L1 adipocytes isolated at day 8 of adipocyte differentiation; lane 3, normal adult mouse fat pad. The blot was probed with affinity-purified NCG antibody, specific for Rab3D, and the bound antibody was visualized with the enhanced chemiluminescence assay.

3*B* depicts the same Western Blot as in Fig. 3*A*, cut above the 29-kDa marker protein and probed with a monoclonal antibody against synaptophysin. As expected from previous reports (21, 22), both RINm5F cells and AtT-20 cells express synaptophysin (Fig. 3*B*, lanes 1 and 2). There is a slight difference in the pattern of immunodetectable proteins in the two cell lines, probably due to different posttranslational modifications of synaptophysin. Neither 3T3-L1 adipocytes nor fat cells nor 3T3-L1 fibroblasts express detectable levels of synaptophysin (Fig. 3*B*, lanes 3–5). This experiment suggests that in fat cells Rab3A is not associated with a synaptic vesicle or synaptic-vesicle-like vesicle, as it is in neurons and neuroendocrine cells. This experiment also indicates that the Rab3A protein detected in mouse adipocytes (Fig. 3*A*, lane 5) is not simply due to contamination from synaptic vesicles in neuronal termini in fat tissue; if this were the case, synaptophysin should be detected together with Rab3A, as it is in extracts from brain tissue (data not shown).

To confirm that brain synaptophysin mRNA is not expressed in 3T3-L1 adipocytes, we used a reverse transcription followed by polymerase chain reaction amplification. Two degenerate oligonucleotides encoding aa 21–28 and 300–308 of rat synaptophysin were used as primers for amplification of cDNA templated by 3T3-L1 adipocyte poly(A)$^+$ RNA. Only a 0.9-kb DNA fragment, the size expected for brain synaptophysin, was obtained. The 0.9-kb reaction product was subcloned and sequenced. None of nine independent colonies with 0.9-kb inserts contained sequences of brain synaptophysin. The nine clones were identical and encoded a protein whose deduced amino acid sequence is ≈50% identical with synaptophysin in the region spanning aa 21–308 (unpublished work). Thus, a synaptophysin-like protein, but not brain synaptophysin, is expressed in adipocytes.

To determine the subcellular localization of Rab3D, we generated a polyclonal antibody, NCG, against a peptide corresponding to the N terminus of Rab3D. This sequence is unique to Rab3D. This antibody detects a polypeptide of the expected size, 26 kDa, in both 3T3-L1 adipocytes (Fig. 4, lane 2) and mouse adipocytes (lane 3). No Rab3D is detectable in 3T3-L1 fibroblasts (lane 1), and the increase in amount of Rab3D during differentiation of 3T3-L1 fibroblasts into adipocytes is at least 10-fold. The same 26-kDa polypeptide is detected by the NCG antibody in COS-7 cells transfected with plasmid pcDNA1-neo-Rab3D, whereas no specific protein is detectable in COS-7 cells transfected with the pcDNA1-neo vector alone (data not shown). As an additional control, all blots probed with the corresponding preimmune serum were negative (data not shown).

Fig. 5 shows that in fat cells both Rab3A and Rab3D are bound to membranes, whether or not the cells have been stimulated with insulin. In this study, 3T3-L1 adipocytes were exposed to insulin for 0, 2, 5, or 20 min, washed, and homogenized in a solution of sodium carbonate buffered at pH 11.5. This procedure dissociates all nonintegral proteins from membranes (18). Western blot analysis of the carbonate-soluble and -insoluble proteins shows that >90% of Rab3A and Rab3D is insoluble in sodium carbonate and therefore are integral membrane proteins. The fraction of Rab3A and Rab3D proteins in the carbonate pellet remains constant after insulin stimulation. Conversely, Rab3B in fat cells was shown to be mainly cytosolic, unattached to membranes (23). As a control for the specificity of the fractionation, Glut4, an integral membrane protein, is almost entirely in the sodium carbonate pellet before or after insulin treatment (Fig. 5*B*). Hsp70, in contrast, was entirely in the carbonate-soluble fraction, as expected.

Fig. 6 shows that, in unstimulated 3T3-L1 adipocytes, Rab3A and Rab3D are localized to different intracellular membranes. When membranes from 3T3-L1 adipocytes are subjected to equilibrium centrifugation in a sucrose density gradient, a substantial fraction, 48%, of Rab3A is recovered in a low-density fraction (<20% sucrose) that is almost completely devoid of Rab3D, containing <3% of the total amount of Rab3D (Fig. 6 *Upper*). No Glut4 is recovered in the low-density fractions that contain the bulk of the Rab3A protein (Fig. 6 *Lower*). Over 97% of Rab3D and Glut4, but only about half of the Rab3A, is recovered in denser fractions 9–15. Even in this region the profile of Rab3D is different from that of Rab3A. Nor is the profile of Glut4 similar to that of Rab3D, indicating that the majority of Glut4 and Rab3D molecules are localized to different organelles, at least in unstimulated cells. Most of the total cellular protein is recovered in the pellet of the gradient (Fig. 6 *Lower*), probably consisting mainly of denser organelles such as mitochondria and nuclei. The pellet did not contain any Glut4, Rab3D, or

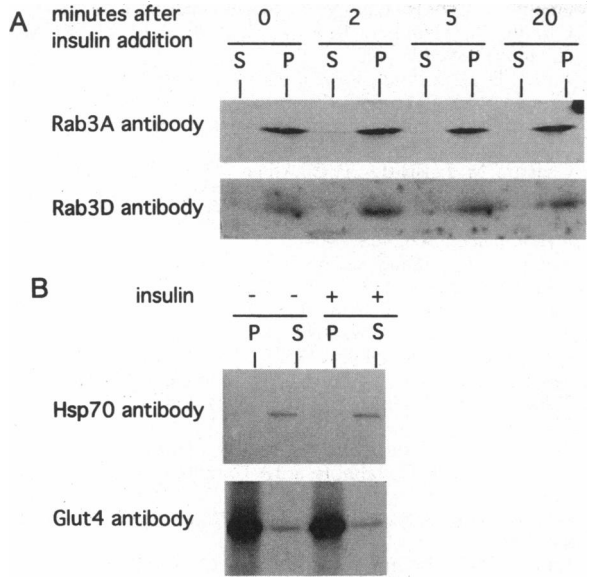

FIG. 5. Most Rab3A and Rab3D in 3T3-L1 adipocytes is membrane bound. (*A*) Rab3A and Rab3D. Unlabeled 3T3-L1 adipocytes were suspended in 100 mM sodium carbonate buffer, homogenized, and incubated on ice for 30 min. The homogenates (1 ml) were centrifuged at 100,000 rpm in a Beckman TLA100.2 rotor for 30 min. Fifty microliters of the pellets (resuspended to a final volume of 0.5 ml) and 50 μl of the 1-ml supernatants (lanes P and S, respectively) were mixed with an equal volume of SDS sample buffer and loaded on an SDS/polyacrylamide gel. Thus twice as much of each pellet, relative to the supernatant, was analyzed. Rab3A was detected as in the legend to Fig. 3. Rab3D was detected with the NCG antibody; bound antibody was detected with ^{125}I-labeled protein A followed by autoradiography. (*B*) Glut4 and Hsp70. 3T3-L1 adipocytes were labeled for 12 hr with [^{35}S]methionine and [^{35}S]cysteine. They were then left untreated (− insulin) or treated with insulin for 20 min (+ insulin). Extraction of the cells with sodium carbonate was as in *A*. Samples were then subjected to immunoprecipitation with antisera against Glut4 and Hsp70, and the immunoprecipitates were analyzed by SDS/PAGE followed by autoradiography.

Rab3A protein. The principal conclusions of this experiment are that Rab3A is localized to at least two principal membranous organelles in unstimulated adipocytes and that at least one of these, fractions 3–5, contains no Rab3D or Glut4. We conclude that Rab3A and Rab3D are localized to different intracellular membranous organelles and, thus, that adipocytes probably have at least two pathways for regulated exocytosis.

DISCUSSION

Rab3A but Not Synaptophysin Is Expressed in Adipocytes. Rab3A is a small GTP-binding protein expressed at very high levels in brain, where it is localized to synaptic vesicles. Rab3A is also expressed in endocrine cells such as those in the adrenal gland (3, 5), in β islet cells of the pancreas, and in cultured β islet cells and insulinomas (22). These cells also express other abundant synaptic vesicle proteins such as synaptophysin and SV2 (21, 24). In pancreatic β cells, synaptophysin and SV2 are localized to synaptic microvesicles which contain the neurotransmitter γ-aminobutyrate (22). These observations suggest that Rab3A is also associated with microvesicles similar in function to synaptic vesicles. However, it is controversial whether all Rab3A is associated with synaptic-like microvesicles (5) or with dense core granules (5, 14) and vesicles of exocrine secretory tissues (4, 25). In summary, it had been unclear whether Rab3A is expressed in nonneuronal or nonneuroendocrine cells, and whether Rab3A is associated with organelles other than synaptic vesicles or synaptic vesicle-like microvesicles.

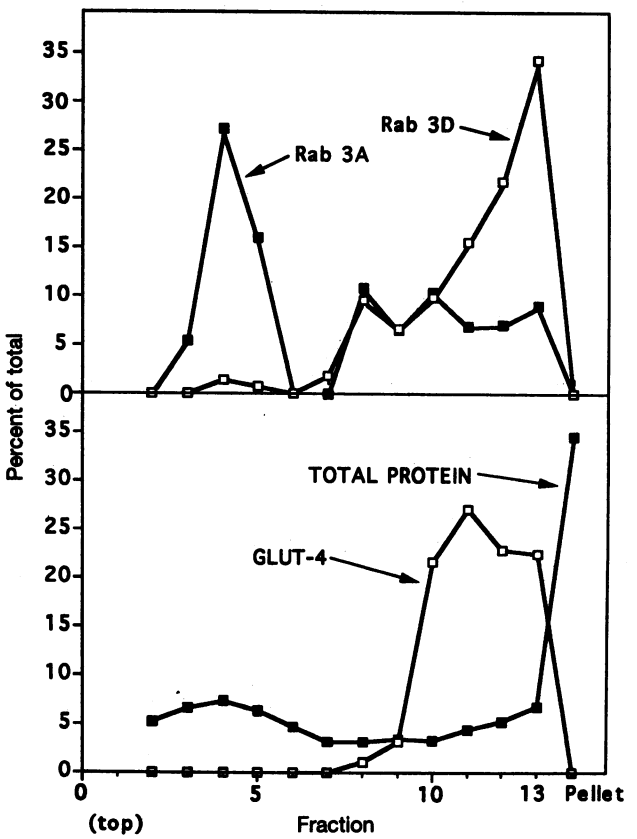

FIG. 6. Equilibrium sucrose density gradient analysis of 3T3-L1 adipocyte membranes: Localization of Rab3A and Rab3D. A postnuclear homogenate of unstimulated 3T3-L1 adipocytes was layered onto a 10-ml continuous 10–40% sucrose gradient. After centrifugation for 16 hr at 4°C in a Beckman SW-41 rotor, fractions (0.923 ml each) were collected from the top. The pellet at the bottom was resuspended in the same volume of buffer B. The fractions were then analyzed by SDS/PAGE and Western blotting as described in the legend to Fig. 5. Autoradiograms were quantified by densitometry, and the percentage of each protein in each fraction was calculated.

Principal conclusions of this paper are that Rab3A is expressed in adipocytes and that expression of both Rab3A mRNA and protein is induced during differentiation of 3T3-L1 fibroblasts to adipocytes. Three independent lines of evidence establish the expression of Rab3A in these cells: (*i*) isolation of Rab3A cDNA clones from a 3T3-L1 adipocyte cDNA library; (*ii*) identification of Rab3A mRNA by Northern blot analysis; and (*iii*) expression of Rab3A protein, as detected by a monoclonal antibody. This antibody is specific for Rab3A; it does not crossreact with Rab3B (15) or with Rab3D (this work).

Adipocytes are the only known type of cell in which Rab3A but not synaptophysin is expressed. Using a specific monoclonal antibody, we could not detect any synaptophysin in fat cells, whereas we could detect both synaptophysin and Rab3A in the two endocrine cell lines examined. Using a polymerase chain reaction-based approach, we detected in adipocytes an mRNA encoding a synaptophysin-like protein, but not brain synaptophysin. Another synaptic vesicle protein, synaptobrevin, or alternatively spliced variants of synaptobrevins are expressed in adipocytes and may be a component of adipocyte vesicles that contain Glut4 (26).

These considerations suggest that in fat cells Rab3A is associated with a cellular organelle which develops during adipogenesis and is different from synaptic or synaptic-like vesicles.

Adipocytes May Have Multiple Pathways of Regulated Exocytosis. We showed by equilibrium sucrose density gradi-

ent centrifugation that in adipocytes Rab3A and Rab3D are associated primarily with different intracellular membranes. Also Rab3A and Rab3D mRNA, and presumably also Rab3A and Rab3D proteins, are generally expressed in different kinds of cells and tissues. Thus, we believe that Rab3A and Rab3D are associated with different exocytic organelles and probably do not function sequentially in the same exocytic pathway. Glut4 is not found in the low-density adipocyte vesicles that contain the majority of Rab3A. Glut1 and Glut4 are localized to denser vesicles, and we do not know whether these vesicles also contain Rab3D or Rab3A. One possibility is that either Rab3D or Rab3A could regulate fusion of Glut4-containing vesicles and the other fusion of vesicles containing Glut1. Another possibility is that either Rab3D or Rab3A is involved in the insulin-regulated secretion of the adipocyte-specific protein adipsin, a serine protease (27), or other secreted proteins, such as the ob gene product (28).

After addition of insulin there was no detectable change in the amount of soluble Rab3A or Rab3D, but this does not mean that Rab3 proteins are not involved in insulin-regulated exocytosis. Although induction of neurotransmitter release causes solubilization of both Rab3A and Rab3C in purified synaptosomal preparations (29, 30), a Rab3 protein associated with zymogen-containing granules in the exocrine pancreas remains membrane-bound after induction of exocytosis (4). Changes in the fraction of soluble Rab3 proteins may be too fast or too subtle to be detected in intact cells.

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