Diffuse vesicular distribution of Rab3D in the polarized neuroendocrine cell line AtT-20

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Abstract **The neuroendocrine cell line AtT-20 has two types of
storage vesicles: dense core granules and synaptic vesicles, both
sequestered at the tip of the processes. Here we show that Rab3D
protein, which is abundant in fat cells, is also expressed in AtT-20
cells. Differently from Rab3A, which is localized in secretory
vesicles accumulated at the tips, Rab3D has a diffuse vesicular
distribution in the cytoplasm of the cell body, the processes and
the tips. In AtT-20 cells, Rab3D may define a regulated secretory
pathway which functions independently from cell polarity.**

Key words: Low molecular weight GTP-binding protein;
Regulated secretion; AtT-20 endocrine cell line

1. Introduction

Members of the small GTPase protein family called Rab3
have been functionally implicated in regulated exocytotic path-
ways [1]. Rab3A and Rab3C are concentrated in synaptic ves-
icles and dissociate from the vesicle membranes after stimula-
tion of Ca^{2+}-dependent neurotransmitter release [2-4]. These
experiments indicate that both Rab3A and Rab3C function in
vesicle docking or exocytosis. Moreover, studies of transgenic
mice lacking Rab3A revealed an impairment in the recruitment
of synaptic vesicles to release sites [5]. The finding that a point
mutation which constitutively activates Rab3A depresses Ca^{2+}
evoked secretion from endocrine cells [6,7] also supports the
notion that Rab3A is involved at a late stage of the secretory
process.

Fat cells express two members of the Rab3 protein family,
Rab3A and Rab3D [8,9]. Both Rab3A and Rab3D message
and protein levels increase during adipogenesis, suggesting that
both proteins function in insulin-stimulated exocytotic proc-
esses characteristic of mature adipocytes, such as externaliza-
tion of Glut4-containing vesicles [10] and secretion of adipsin
[11]. Fat cells also express Rab3B and Rab3C [12]. It is not clear
why secretory cells express multiple Rab3 isoforms, and
whether each Rab3 isoform is associated to a specific exocytotic
organelle.

AtT-20 cells have at least two distinct storage vesicles, synap-
tic vesicles and dense core granules, both packaged at the tips
of cellular processes [13] where Rab3A is segregated [14]. To
gain some insight into the possible role of multiple Rab3

isoforms in secretory cells, we investigated whether Rab3D is
present in AtT-20 cells and if it is associated with secretory
vesicles clustered at the tip of the processes. We find that
Rab3D protein is indeed expressed in AtT-20 cells. However,
Rab3D has a diffuse vesicular distribution in the cytoplasm of
the entire cell. In AtT-20 cells, Rab3D may either be a protein
component of storage vesicles en route to their final release site
or define a new class of storage vesicles.

2. Experimental

2.1. Materials
Monoclonal antibodies specific for Rab3A (CL42.2) were provided
by Dr. R. Jahn. A polyclonal antibody against the amino-terminal
region of Rab3D (NCG) was generated and affinity purified as de-
scribed before [9]. Fluorescein-coupled goat anti-rabbit IgG or Cy3-
coupled anti-mouse IgG were obtained from Jackson Immunoresearch.

2.2. Cell culture
AtT-20 cells, 3T3-L1 fibroblasts, Cos7, and RINm5F cells were
grown as described previously [9]. Cos7 cells were transfected according
to a DEAE-Dextran method [15] with pcDNA1/neo containing Rab3D
cDNA [9]. Immunofluorescence was performed 48-72 h after transfec-
tion. The transfection efficiency was 10%, assessed by parallel transfec-
tion of vector pcDNA1 containing the β-galactosidase cDNA (a gift
from H. Lin) followed by in situ staining in the presence of the chromo-
genic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside [16].

2.3. Immunofluorescence
44-72 h after transfection, Cos7 cells grown on coverslips were
washed three times with phosphate-buffered saline (PBS), at pH 7.4.
Cos7 cells were then fixed successively in methanol (-20°C, 5 min) and
acetone (-20°C, 2 min). The samples were air-dried and washed twice
with PBS, pH. 7.4. Coverslips were incubated for 1 h at 37°C in PBS
containing 4% w/v bovine serum albumin and 5% w/v normal goat
serum (buffer A) to block nonspecific binding. For Rab3D staining
experiments the affinity-purified rabbit polyclonal antibody raised
against Rab3D was diluted in buffer A at a final concentration of 0.35
µg/ml. To specifically block Rab3D immunostaining in control experi-
ments, the Rab3D antibody in buffer A was pre-incubated at room
temperature for 2 h together with 50 µg/ml of the peptide Ala-Ser-Glu-
Pro-Pro-Ala-Ser-Pro-Arg-Asp-Ala-Ala-Cys against which the anti-
body was originally raised. Coverslips were pre-incubated either with
the antibody or the antibody and the peptide in buffer A overnight at
4°C. Then samples were washed three times with PBS and incubated
for 1 h at 37°C in solution A containing 30 µg/ml goat anti-rabbit IgG
coupled to fluorescein. The samples were washed three times with PBS,
dehydrated in ascending alcohols, and mounted with glycerol contain-
ing 1,4-diazabicyclo(2.2.2)octane to retard fading during microscopy.
AtT-20 cells were fixed for 20 min at room temperature in freshly made
PBS containing 4% w/v paraformaldehyde. AtT-20 cells were then
permeabilized with PBS solution containing 0.2% v/v Triton X-100 for
10 min at room temperature and washed three times with PBS. Cov-
erslips were treated with buffer A as described before and then incu-

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bated for 1 h at 37°C with 1 : 1000 dilution of the monoclonal antibody Cl42.2 specific for Rab3A. After three washes in PBS, the samples were incubated for 1 h at 37°C with goat anti-mouse IgG coupled to a Cy3-cyanine dye at a final concentration of 30 μg/ml. The coverslips were then washed three times with PBS and stained for Rab3D as described above.

For AtT-20 cells, fluorescence micrographs were taken using rhodamine (for Cy3-cyanine) or fluorescein conditions with a 63 × objective, immersed in oil, on a Zeiss Axiophot fluorescence microscopy. For Cos7 cells, micrographs were taken with a 20 × objective.

2.4. Sodium carbonate extraction, gel electrophoresis and immunoblotting

Extraction of AtT-20 cells with sodium carbonate was conducted according to a published protocol [17]. Separation of proteins by SDS-PAGE, fluorography, immunoblotting and protein determination were performed as described [9].

2.5. Equilibrium sucrose density gradients

We followed the same procedure as described before for adipocytes [9]. The continuous 10–40% w/v sucrose gradient, made up in 10 ml 10 mM Tris buffer, pH 7.4/1 mM EDTA, was loaded with 2 ml of postnuclear supernatants derived from two 10 cm plates of 40–60% confluent AtT-20 cells. Samples were centrifuged for 16 h at 4°C in a Beckman SW-41 rotor.

3. Results

The antibody raised against the amino-terminal domain of Rab3D has been previously characterized [9]. Fig. 1 shows that in AtT-20 cells the Rab3D antibody detects a single protein (lane 4) with the same gel mobility as Rab3D in adult mouse tissue (lane 5) or in 3T3-L1 cells, but not before they have differentiated into adipocytes (lanes 1 and 2). The same blot stained with pre-immune serum was negative (not shown). The single Rab3D species detected in AtT-20 cells has the same molecular weight as recombinant Rab3D expressed in Cos7 cells. (not shown). From this experiment we conclude that Rab3D protein is expressed in AtT-20 cells. Another neuroendocrine cell line, the insulin-secreting RINm5F cells, also expresses Rab3D (lane 3).

In adipocytes Rab3D is primarily bound to membranes [9]. Fig. 2 shows that this is also the case in AtT-20 cells. AtT-20 cells were homogenized in sodium carbonate buffer at pH 11.5; the pellet and the supernatant fractions recovered after centrifugation (lanes 1 and 2, respectively), were analyzed for their Rab3D and Rab3A content by Western blotting. Both synaptophysin, an integral membrane protein abundant in synaptic vesicles, and the majority of Rab3A are recovered in the pellet
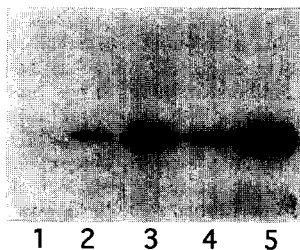

Fig. 1. Expression of Rab3D protein in AtT-20 cells. Each lane of the SDS-PAGE gel contained 20 μg protein. Lanes: 1, 3T3-L1 fibroblasts isolated at the beginning of the differentiation program; 2, 3T3-L1 adipocytes isolated at day 8 of the differentiation program; 3, RINm5F cells; 4, AtT-20 cells; 5, adult mouse fat pad. The blot was probed with affinity-purified NCG antibody, specific for Rab3D. The bound antibody is visualized as described [9].
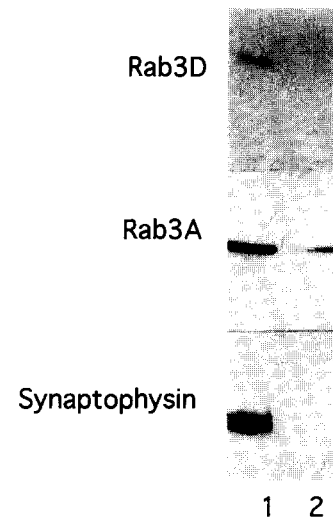

Fig. 2. Most Rab3D in AtT-20 cells is membrane bound. A 10 cm tissue culture dish of AtT-20 cells was washed twice in PBS and once in 150 mM NaCl at 4°C. Cells were scraped and suspended in 1 ml ice-cold 100 mM sodium carbonate, pH 11.5. After 30 min of incubation at 4°C, the cell homogenate was centrifuged at 100 000 rpm in a Beckman TL100.2 rotor for 30 min at 4°C and the pellet was resuspended in 1 ml of a buffer containing 50 mM Tris, pH 7.4/150 mM NaCl/1 mM EDTA 1% (v/v) Triton X-100/0.1% (w/v SDS, with leupeptin at 1.5 μg/ml, aprotinin at 6 μg/ml and phenylmethanesulphonylfluoride at 300 μg/ml. 50 μl of pellet and supernatant (lanes 1 and 2, respectively) were added to equal volumes of SDS-PAGE sample buffer and loaded on an SDS-PAGE gel. Rab3D was detected as in the legend to Fig. 1. Rab3A and synaptophysin were detected with the monoclonal antibodies Cl42.2 and SY38, respectively. Bound antibodies were visualized with the enhanced chemiluminescence assay [9].

fraction. Only 10–30% of total Rab3A protein was detectable in the supernatant fraction in agreement with previous reports describing a pool of soluble Rab3A in brain [18]. Rab3D protein is detectable only in the carbonate pellet. These results indicate that both Rab3A and Rab3D are mainly membrane-attached in AtT-20 cells.

Fig. 3 demonstrates that the affinity-purified Rab3D antibody specifically detects the recombinant protein by immunofluorescence. Approx. 10% of the Cos7 cell population transfected with the vector pcDNA1neo-Rab3D cDNA were stained with the Rab3D antibody (panel a). This number correlates well with the expected efficiency of Cos7 cell transfection detected using the β-galactosidase gene activity assay. Immunostaining was negative when transfected cells were incubated with a solution containing both the antibody against Rab3D and the amino-terminal peptide initially used for immunizing the rabbits (panel c). Mock-transfected cells were also negative (not shown). As an additional control, we determined by Western blot analysis that the Cos7 cell population transfected with pcDNA1neo-Rab3D cDNA indeed expressed Rab3D protein, differently from mock-transfected Cos7 cells. In transfected Cos7 cells, Rab3D gives a reticular fluorescence concentrated in the perinuclear region (panel a) suggesting a localization in the endoplasmic-reticulum/Golgi compartment.

To compare directly the distribution of Rab3D with that of Rab3A containing vesicles in AtT-20 cells, we performed double label immunofluorescence microscopy as shown in Fig. 4. Rab3D appears as a punctuate fluorescence distributed

throughout the entire cell, with some local concentration in the perinuclear region as well as in the processes (panel a). Immunofluorescence was negative when the affinity-purified Rab3D antibody was used in the presence of the immunizing peptide as a competitor (not shown). The punctuate immunofluorescence of Rab3D in AtT-20 cells is clearly different from the staining pattern observed in Rab3D-transfected Cos7. Since AtT-20 cells natively express Rab3D, the protein may be sorted into an exo-endocytotic compartment specific to secretory cells. As described by others, Rab3A generates a punctuate fluorescence accumulated at the tip of the processes (panel b). Additional weak Rab3A staining is detectable in the cytoplasm of the cell body and the processes. There is little or no overlap with Rab3D. The strikingly different distribution of Rab3A and Rab3D as detected by immunofluorescence staining indicates that these two isoforms are mostly localized in different membranes and that Rab3A, but not Rab3D, is accumulated at the tips of the processes, the location of dense core granules and synaptic vesicles.

To further characterize the Rab3D and Rab3A distributions in AtT-20 cells, a postnuclear supernatant from AtT-20 cells was layered on top of a continuous sucrose density gradient. After centrifugation fractions were analyzed for their Rab3A and Rab3D content by Western blot analysis (Fig. 5). Most of Rab3A immunoreactivity is concentrated in fractions 4–8. This 'light density' fraction may include Rab3A associated with synaptic vesicles as well as soluble Rab3A. Less than 5% of Rab3A is recovered in fractions 11–14. This additional pool probably corresponds to plasma membrane or dense core granules. In contrast, as resolved by sucrose density gradient centrifugation,

approx. 85% of Rab3D is recovered in fractions 2–4, and the remaining 15% is concentrated at the bottom of the gradient. The profile of Rab3D does not coincide with that of Rab3A in the gradient confirming that Rab3A and Rab3D are localized in different vesicles in AtT-20 cells, as well as in fat cells. Since all of Rab3D protein is membrane-bound in AtT-20 cells, the Rab3D protein recovered in fractions 2–4 belongs to a light density membrane compartment. Moreover, the presence of two distinct peaks of Rab3D after sucrose density centrifugation indicates that the protein is associated with more than one membrane compartment in AtT-20 cells.

4. Discussion

In AtT-20 cells Rab3D is membrane-bound and is associated with different cellular compartments than Rab3A. This last conclusion is based on observations using two different experimental approaches: (a) double immunofluorescence labeling demonstrating that the majority of Rab3D immunostaining did not co-localize with that of Rab3A; (b) fractionation of AtT-20 cell homogenate by sucrose density gradient centrifugation showing that the profiles of Rab3D and Rab3A immunoreactivity did not coincide. Interestingly, the majority of Rab3D protein migrates in the gradient with a light vesicular fraction of density less than 20% w/v sucrose. Over 97% of the Rab3D protein of fat cells postnuclear supernatants is associated with a higher density compartment (more than 30% w/v sucrose) [9]. Therefore, in fat cells and neuroendocrine cells, Rab3D protein is localized in populations of vesicles with different buoyant densities. In fat cells, differently than in neuronal and neuro-
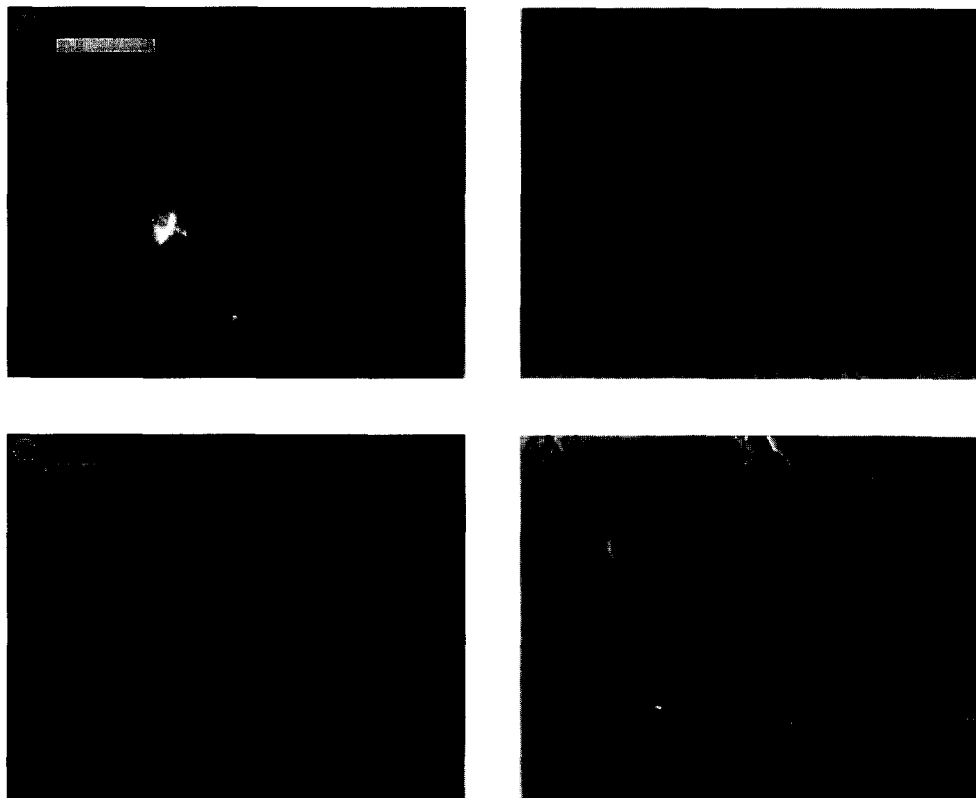

Fig. 3. Immunofluorescence analysis of Cos7 cells expressing recombinant Rab3D. (a) Cos7 cells transfected with pcDNA1neo-Rab3D stained with affinity-purified Rab3D NCG antibody, specific for Rab3D; (b) phase contrast of the same field; (c) a representative field of Cos7 cells stained with the NCG antibody in the presence of 50 μg/ml of the immunizing peptide as a competitor; (d) phase contrast of the same field. Scale bar 50 μm.
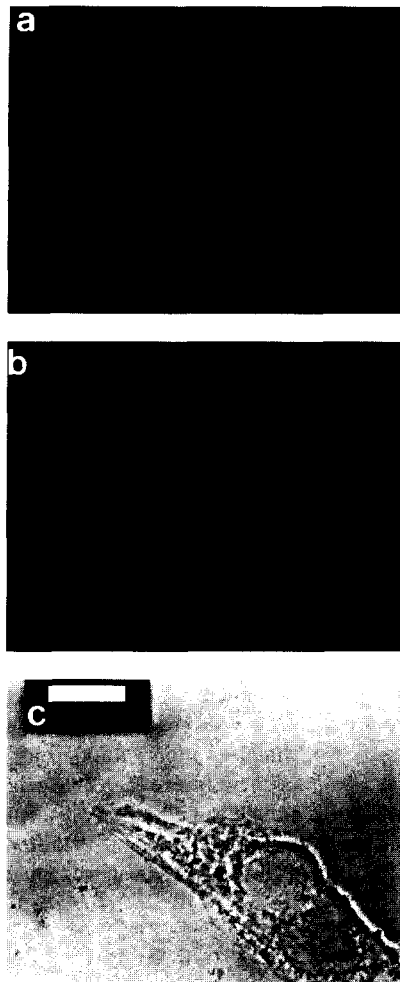
Fig. 4. Comparison of the distribution of Rab3D and Rab3A in AtT-20 cells demonstrated by double immunofluorescence. AtT-20 cells were labeled with Rab3D NCG affinity-purified antibody (a) and Rab3A monoclonal antibody Cl 42.2 (b). A phase contrast of the same field is shown in (c). Scale bar 15.8 μm.

endocrine cells, Rab3A is not localized to synaptic or synaptic-like vesicles [9]. Together these observations suggest that Rab3D, as well as Rab3A, is associated with heterogeneous vesicles in adipocytes and AtT-20 cells. These Rab3 isoforms can be used as unique markers to compare exocytotic organelles with different protein composition or physico-chemical characteristics in adipocytes and AtT-20 cells.

Importantly, in AtT-20 cells Rab3D is not segregated at the tip of neurite-like processes where synaptic-like microvesicles and dense core granules are clustered [13]. As expected, Rab3A, an integral membrane component of synaptic vesicles, is mainly localized at the tip of these processes [14].

In contrast to Rab3D, all the other known members of the Rab3 family are spatially segregated; in neurons, Rab3A and Rab3C are localized to synaptic vesicles at the periphery of axonal processes [3,19,20] and Rab3B is targeted to the apical pole of liver, intestine and kidney cells [21]. The polarized distribution of these Rab3 isoforms would reflect their physiological role in regulated exocytosis of neurotrasmitters, hormones or factors which have to be released at a specific site. However, other regulated secretory events such as insulin-induced externalization of Glut4 in fat cells occur over the entire cell surface [10]. The diffuse distribution of Rab3D in AtT-20 cells suggests the existence of a distinct class of storage vesicles, different from dense core granules and synaptic-like microvesicles. If Rab3A and Rab3D function at identical steps in different exocytotic pathways, Rab3D may be selectively involved in docking to the cell surface exocytotic vesicles which are not to be sequestered at the tip of the processes and will eventually fuse with the entire plasma membrane.

Clearly, other possibilities exist: in AtT-20 cells Rab3D may be associated with secretory vesicles at an intermediate stage of their maturation, while mature dense core granules or synaptic vesicles are sequestered at the tip of the processes.

Glut4 does not co-localize either with dense core granules or synaptic-like microvesicles when expressed in PC12 cells and may belong to a distinct class of storage vesicles shared by many cell types including fibroblasts and mature adipocytes [22]. However, at least some Glut4 and Rab3D protein may belong to the same vesicles population in fat cells as well as in Glut4-transfected neuroendocrine cells. Since the majority of
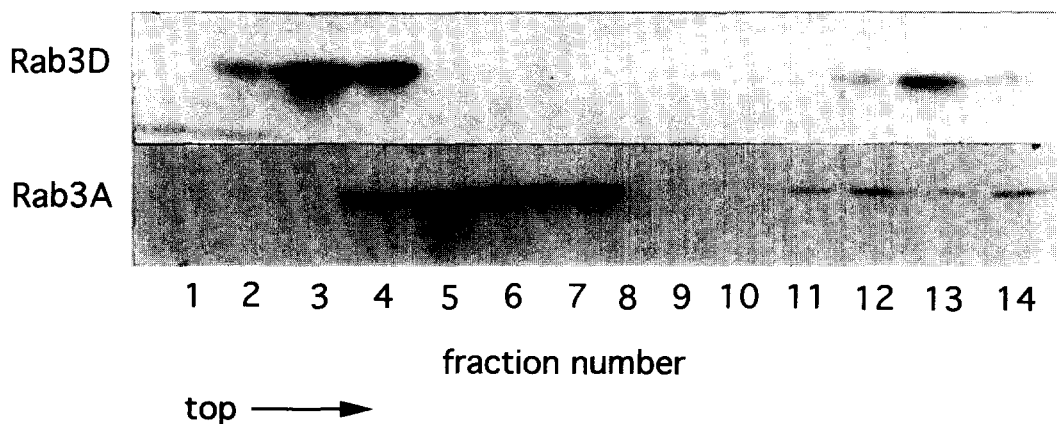

Fig. 5. Analysis of Rab3A and Rab3D distribution by sucrose density gradient centrifugation. 2 ml of a postnuclear homogenate of AtT-20 cells grown in two 10 cm plates was layered onto a 10 ml continuous sucrose density gradient. After centrifugation for 16 h at 4°C in a Beckman SW-41 rotor, fractions were collected from the top. The fractions were then analyzed for their content of Rab3D and Rab3A as described in the legend to Fig. 2. The pellet at the bottom did not contain detectable amounts of Rab3D or Rab3A.

Rab3D is bound to a characteristic low density fraction in AtT-20 cells, this cell line represents an attractive model to investigate this possibility.

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