ORIGINAL PAPER

Localization of the small monomeric GTPases Rab3D and Rab3A in the AtT-20 rat pituitary cell line

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SUMMARY

We investigated the cellular localization of the small GTPases Rab3D and Rab3A in AtT-20 cells treated with the drug Brefeldin A. Brefeldin A induces the redistribution of the Golgi complex into the endoplasmic reticulum and tubulation of endosomes. However, in Brefeldin A-treated wild-type AtT-20 cells, both Rab3D and Rab3A retained their distribution, indicating that they belong to a non-endosomal, post-Golgi compartment. Immunoelectron microscopy experiments indicated that both Rab3D and Rab3A localized to the ACTH-containing, large dense core granules. In contrast, in cell clones overexpressing a mutated form of Rab3D (Rab3D N135I), Rab3A did not localize to the dense core granules. Moreover, since our previous results showed that overexpression of Rab3D N135I severely impaired regulated ACTH secretion in AtT-20 cells, we sought to determine whether the impairment could depend on a redistribution of two key components of the regulated exocytosis machinery, synaptotagmin and SNAP-25. As far as synaptotagmin was concerned, in cell clones overexpressing Rab3D N135I, the protein did not localize close to the plasma membrane, in agreement with the previously reported defective docking of dense core granules to the plasma membrane. Immunofluorescence experiments showed that SNAP-25 did not change its localization in these cell clones. All in all, our findings strengthen the notion that both Rab3D and Rab3A are associated with the dense core granule compartment of AtT-20 cells, and that the impairment in the ACTH secretion caused by overexpression of a mutated Rab3D form is likely to be due to a lacking of granule docking to the plasma membrane, possibly because Rab3A fails to associate with the granules.

INTRODUCTION

Rab proteins are low molecular weight GTP-ases belonging to the Ras superfamily that are thought to play a key role in the regulation of vesicle trafficking along exocytotic and endocytotic pathways in eukaryotes, where they regulate multiple, distinct steps of intracellular transport (Martinez and Goud, 1998; Chavrier and Goud, 1999; Armstrong, 2000). At present, almost 40 Rab proteins have been identified and found to be associated with specific subcellular compartments (Martinez and Goud, 1998; Masuda *et al.*, 2000; Yoshie *et al.*, 2000).

Among Rab proteins, the four members of the Rab3 subfamily (Rab3A, Rab3B, Rab3C, Rab3D)

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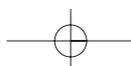
have been found to be selectively expressed in regulated secretion-competent cells, and to be associated with synaptic vesicles or secretory granules, suggesting that they are involved in stimulus-secretion coupling (see Ybe *et al.*, 2000; Moyer and Balch, 2001; Zerial and McBride, 2001 for updated reviews on this issue). Rab3A and –C are primarily expressed in neuronal and neuroendocrine cells, while Rab3B and –D are more abundant outside the nervous system (Baldini *et al.*, 1992; Fischer von Mollard *et al.*, 1994; Novick and Zerial, 1997; Al-Matubsi *et al.*, 1999).

We have recently reported that in wild-type AtT-20 rat pituitary cells, Rab3D localizes to ACTH-containing dense core granules and that in a cell clone overexpressing a mutated Rab3D protein (Rab3D N135I) there is a marked impairment of regulated secretion of ACTH (Baldini *et al.*, 1998). However, our immunofluorescence analysis results also showed a diffuse distribution of Rab3D immunoreactivity (Martelli *et al.*, 1995; Baldini *et al.*, 1998). Therefore, we reasoned that Rab3D, other than in dense core granules, may also be present in other cellular districts. Since Rab family members such as Rab33B, Rab6, Rab 4, Rab 5, Rab11 have been reported to be associated with either the Golgi apparatus or the endosomal compartment, respectively (Zheng *et al.*, 1998; Trischler *et al.*, 1999), we investigated by means of the drug Brefeldin A whether or not also Rab3D and/or Rab3A could be present in these domains of AtT-20 cells. It is well established that Brefeldin A induces a redistribution of the Golgi proteins into the endoplasmic reticulum (Lippincott-Schwarz *et al.*, 1989) and fusion of the endosomal compartment and the Trans-Golgi Network into tubular structures (Lippincott-Schwarz *et al.*, 1991). To label the Golgi apparatus we employed an antibody to the 58-kDa Golgi protein. Such a protein binds the Golgi apparatus to microtubules and redistributes to the endosplamic reticulum after treatment with Brefeldin A (Bloom and Brashear, 1989; Ktistakis *et al.*, 1991).

Moreover, as a step towards the comprehension of the mechanisms through which Rab3D controls regulated ACTH secretion, we have studied the effect of overexpressing the mutated Rab3D N135I on the distribution of two proteins which are considered to be critical for regulated exocytosis, such as synaptotagmin, which has been reported to be associated with dense core granules (Walch-Solimena *et al.*, 1993; Papini *et al.*, 1995) and SNAP-25, a component of the t-SNARE complex (Sadoul *et al.*,1995).

MATERIALS AND METHODS

Cell culture

AtT-20 cells (both wild-type or overexpressing mutated Rab3D) were cultured in DME supplemented with 10% fetal calf serum. To label the endosomal compartment, the procedure described by Mundigl *et al.* (1993) was followed. Briefly, cells grown on poly-L-lysine-coated coverslips for at least 24 h, were incubated for 1 h at 37°C in the presence of 2.5 μg/ml of Texas red-conjugated human transferrin (Molecular Probes, Eugene, OR). Samples were then incubated for an additional 40 min at 37°C in the presence of the fungal metabolite, Brefeldin A (10 μg/ml, Sigma Chemical Co, St. Louis, MO). Addition of an equivalent amount of solvent (ethanol) was without effect.

The AtT-20 cell clones overexpressing mutated Rab3D N135I (clones A3C1 and A3C4) were as previously reported. This mutation impairs the GTP binding capability of Rab3D (Baldini *et al.*, 1998).

Immunofluorescent staining

For immunofluorescent staining, AtT-20 cells, grown on coverslips, were briefly washed with phosphate buffered saline (PBS), then fixed for 20 min at room temperature in 4% freshly-prepared paraformaldehyde in PBS. Samples were permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature and washed three times in PBS. They were then incubated with the following primary antibodies, all diluted in PBS containing 3% bovine serum albumin (BSA) and 2% normal goat serum (NGS): monoclonal antibody to Golgi 58-kDa protein (1:50, Sigma); affinity-purified rabbit antibody to Rab3D (1:200, see Martelli *et al.*, 1995); monoclonal antibody to SNAP-25 (1:1000, Sternberg Monoclonals Inc., Baltimore, MD); monoclonal antibody to synaptotagmin (1:100, clone SY130, Stressgen Biotechnologies Corp., Victoria, B.C., Canada); monoclonal antibody to Rab3A (Matteoli *et al.*, 1991) (used 1:100, a kind gift from Dr. R. Jahn).

After several washes in PBS, samples were reacted with the appropriate secondary antibodies (diluted 1:200 in PBS, 3% BSA, 2% NGS), i.e. Cy-3-conjugated anti-mouse IgG or fluorescein isothyocyanate-conjugated anti-rabbit IgG (both from Sigma).

Slides were observed and photographed using a Zeiss Axiophot epifluorescence microscope.

Immunoelectron microscopy

Immunogold labelling of agarose embedded AtT-20 cells was performed as reported, using the broken cell method (Baldini *et al*., 1998). Briefly, preconfluent cells were scraped in ice-cold homogenization buffer containing 250 mM sucrose, 25 mM KCl, 2 mM EGTA, and 10 mM sodium phosphate, pH 6.5. Cells were lightly homogenized by a passage through a 20-gauge needle fitted to a 5-ml syringe. Cells were fixed by addition of 15 vol of ice-cold fixative solution containing 300 mM sucrose, 3% freshly prepared paraformaldehyde, 0.25% glutaraldehyde, and 5 mM sodium phosphate, pH 6.5. Cells were kept in the fixative solution for 30 min, pelleted by centrifugation at 1,000 g for 10 min, and then resuspended in 4 vol of 120 mM sodium phosphate, pH 7.5. The entire procedure was carried out at 4°C. Broken cells were agarose-embedded as described elsewhere (De Camilli *et al*., 1983). Incubations of agarose-embedded samples with either polyclonal antibody to Rab3D (1:20) or monoclonal antibody to Rab3A (1:25) was done as described (Jena *et al*., 1994). For gold immunolabeling, 10 nm colloidal gold-conjugated anti-rabbit IgG were used to detect Rab3D, while for Rab3A detection 5 nm colloidal gold-conjugated anti-mouse IgG were used (both diluted 1:25, from Biocell International, Cardiff, UK).

RESULTS

To better define the localization of both Rab3D and Rab3A in AtT-20 cells, we used the fungal metabolite, Brefeldin A. Antibody to 58-kDa protein labeled the Golgi complex in control AtT-20 cells (Fig. 1A). After addition of 10 μg/ml Brefeldin A for 40 min at 37°C, the 58-kDa polypeptide immunofluorescent staining changed into a finer and more diffuse pattern, indicating that a disruption of the Golgi apparatus occurred (Fig. 1B). We next analyzed the effects of Brefeldin A on the morphology of the endosomal compartment and on Rab3D distribution by double immunofluorescence experiments. In wild-type AtT-20 cells, Rab3D displayed its characteristic diffuse distribution (Fig. 1C). AtT-20 cells were incubated with iron-bound, Texas Red-conjugated transferrin for 1 h at 37°C, to label the endosomal compartment. Transferrin was distributed over the entire cellular body decorating the plasma membrane and accumulated at the perinuclear region (Fig. 1D). Cells were first incubated with Texas Red-conjugated transfer-

rin and then incubated for 40 min at 37°C in the presence of Brefeldin A and immunostained for Rab3D. The drug induced a segregation of the transferrin fluorescence in a perinuclear region from which some long tubular structures extended to the cell periphery (Fig. 1E). Compare the transferrin distribution without (Fig. 1D) and with (Fig. 1F) incubation with Brefeldin A. Instead, the punctate fluorescent distribution of Rab3D was unchanged (compare Fig. 1E, Rab3D with Brefeldin A incubation; with Fig. 1C untreated AtT-20 cells immunostained for Rab3D) and the Rab3D-labeled compartment did not form tubules. After addition of Brefeldin A, also Rab3A did not change its punctate fluorescent distribution at the cell body and its characteristic accumulation at the tips of the processes (compare Fig. 1G and Fig. 1H).

We next examined, by immunofluorescent staining, the cellular distribution of Rab3D in wild-type AtT-20 cells as compared with clones overexpressing mutated Rab3D. In wild-type cells, Rab3D displayed the characteristic diffuse and punctate fluorescence (Martelli *et al*., 1995; Baldini *et al*., 1998) which was also present in the tips of the processes (Fig. 2A and B), whereas, in both A3C1 and A3C4 cell clones, the immunostaining was more homogeneously distributed, less punctated, and absent from the tips (Fig. 2, panels C, D, and E). A merging of the phase contrast and of immunofluorescent staining confirmed that the tips of clone A3C4 were indeed devoid of staining (Fig. 2, panel G).

We next performed immunoelectron microscopy on agarose-embedded, broken AtT-20 cells (both wild-type and overexpressing Rab3D N135I), double-labeled for Rab3D and Rab3A. As shown in Fig. 3 (panel A) both Rab3D and Rab3A were localized to dense core granules present at the tips of wild-type AtT-20 cells. Interestingly, in cells overexpressing mutated Rab3D (clone A3C1), the amount of Rab3D bound to dense core granules was reduced and no Rab3A was found on these organelles (Fig. 3, panel B). Similar results were obtained with the A3C4 clone (data not shown).

To further strengthen this conclusion, we immunostained for Rab3A both wild type AtT-20 cells and clones A3C1 and A3C4. As presented in Fig. 4 (panel A), in wild-type AtT-20 cells Rab3A was located both in the cell body and at the tips. In contrast, in both clone A3C1 (panel B) and A3C4 (panel C), the tips were devoid of immunoreactivity.

As far as synaptotagmin distribution was concerned, it should be stressed that in wild-type AtT-
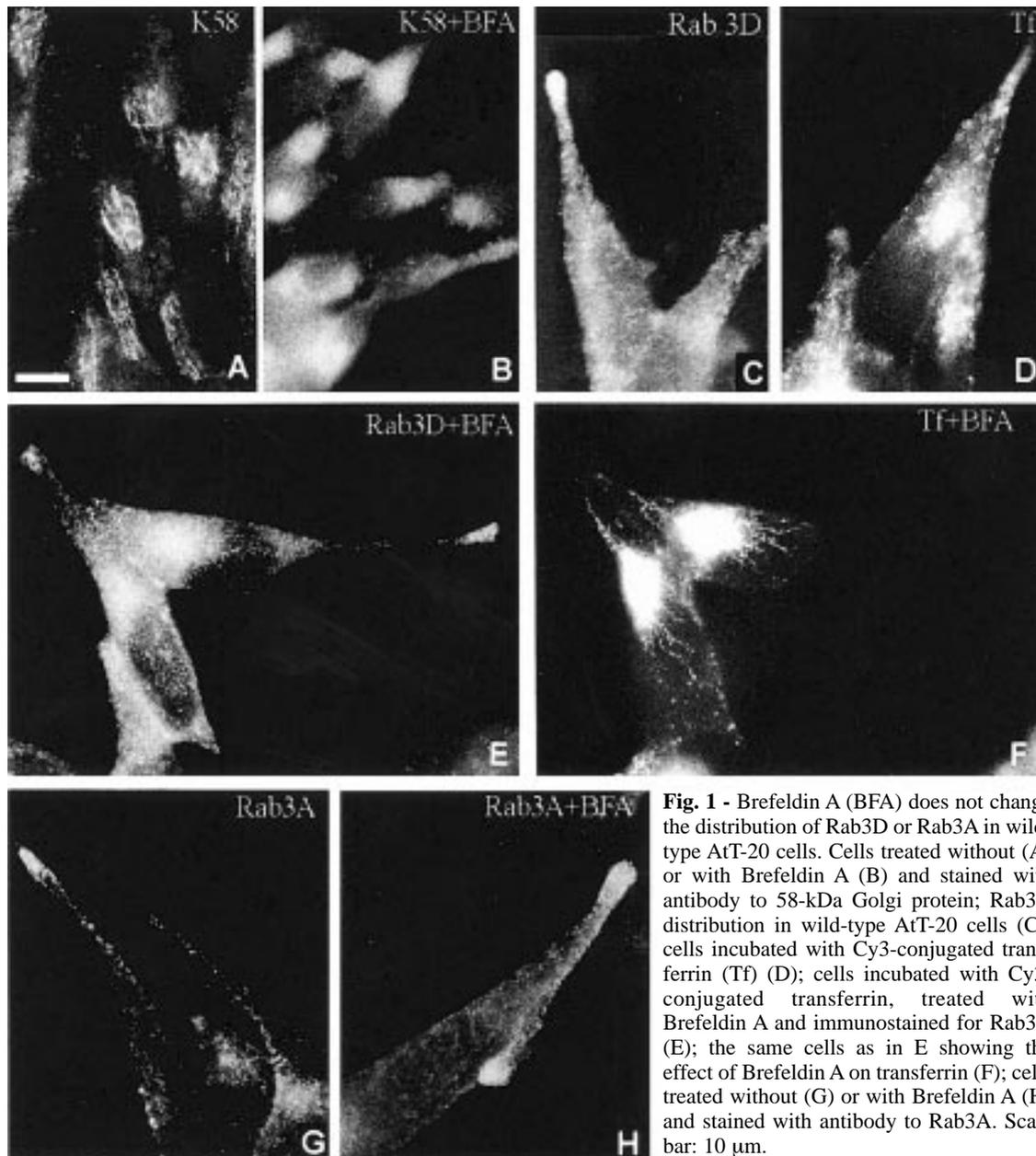
Fig. 1 - Brefeldin A (BFA) does not change the distribution of Rab3D or Rab3A in wild-type AtT-20 cells. Cells treated without (A) or with Brefeldin A (B) and stained with antibody to 58-kDa Golgi protein; Rab3D distribution in wild-type AtT-20 cells (C); cells incubated with Cy3-conjugated transferrin (Tf) (D); cells incubated with Cy3-conjugated transferrin, treated with Brefeldin A and immunostained for Rab3D (E); the same cells as in E showing the effect of Brefeldin A on transferrin (F); cells treated without (G) or with Brefeldin A (H) and stained with antibody to Rab3A. Scale bar: 10 μm.

20 cells, the immunoreactivity, besides a diffuse distribution, showed in some cases an accumulation near the plasma membrane, with an aspect reminiscent of beads-on-a string (Fig. 5A). In A3C1 cells, we never saw such an accumulation, consistent with the impaired docking of dense core granules to the plasma membrane (Fig. 5B). Similar results were obtained with the A3C4 clone (Fig. 5C).

When A3C1 cells were immunostained for SNAP-25, we found that the immunoreactivity accumulated at the cell periphery similarly to wild-type AtT-20 cells

(Fig. 5, compare E with D). Therefore, overexpression of Rab3D N135I did not prevent targeting of a t-SNARE component to the plasma membrane. Similar data were obtained with the A3C4 clone (Fig. 5F).

DISCUSSION

A key step in the understanding of the functions played by Rab proteins is to define the subcellular
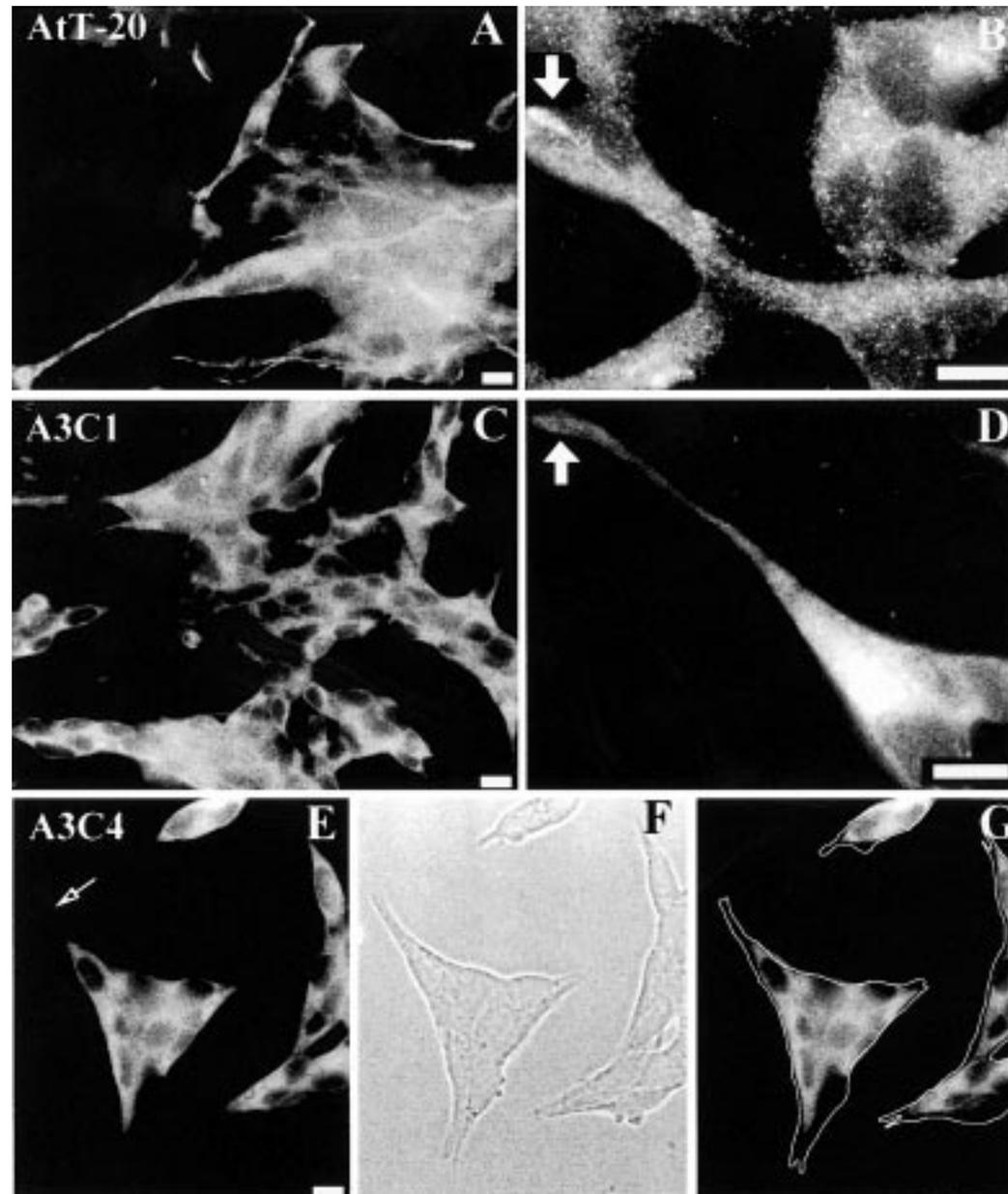
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Fig. 2 - Comparison of Rab3D distribution between wild-type AtT-20 cells (A, B) and cell clones overexpressing Rab3D N135I: A3C1(C, D) and A3C4 (E, F, G). The low magnification pictures (A, C, E) show that Rab3D immunostaining of cell clones is more homogeneously distributed and absent from the tips. Both A3C1 and A3C4 cells have tips but they are not stained and in the cell body the fluorescence is not as punctate (D) as in wild-type AtT-20 cells (B). Arrows indicate cell tips. Note that in wild-type AtT-20 cells (D) the tips are heavily immunostained by antibody to Rab3D, whereas in cell clones overexpressing mutated Rab3D tips are either barely stained (D) or completely unstained (E) . Panel G shows a merging of panel F with panel E to emphasize the fact that there is no fluorescent staining at the tips. Scale bar: 10 μm.

domains to which these GTPases are associated. In the present investigation, we first employed the fungal toxin, Brefeldin A, to determine whether or not Rab3D and/or Rab3A localize to either the Golgi apparatus or the early endosomal compartment. Indeed, Brefeldin A is known to induce major structural changes in cellular compartments involved in constitutive secretion and recycling,
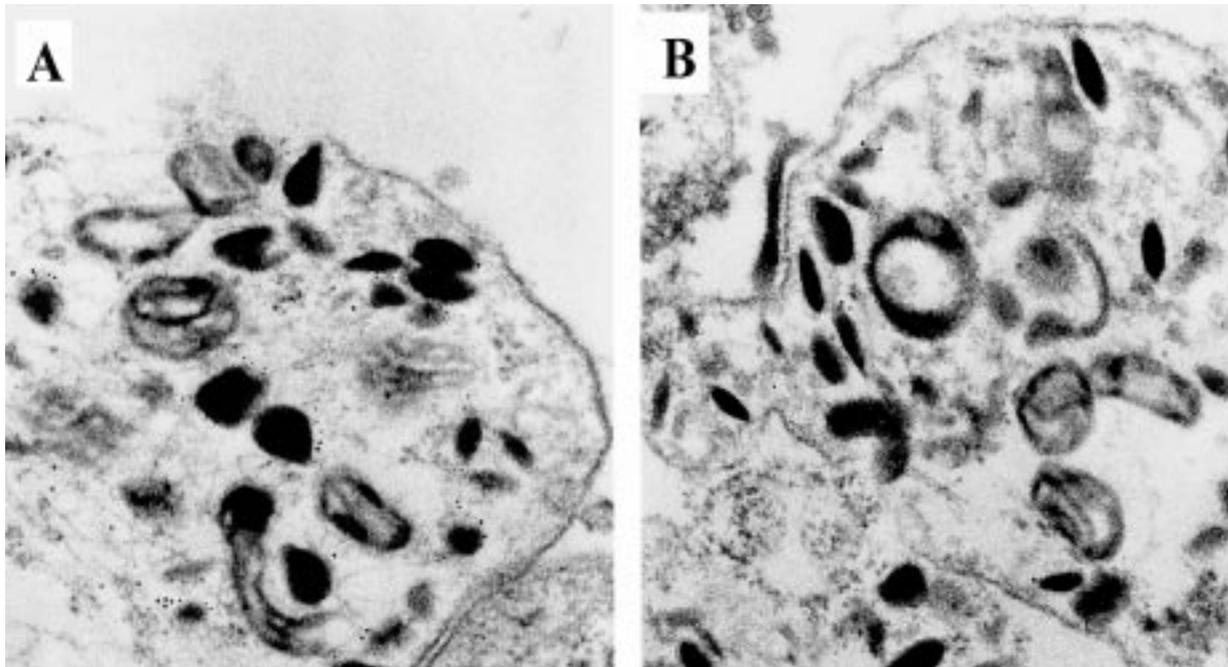
Fig. 3 - Ultrastructural analysis of Rab3D (10 nm gold) and Rab3A (5 nm gold) distribution in broken, wild-type AtT-20 (A) and A3C1 clone cells (B). In both of these pictures a portion of a cell tip in shown. Note that in wild-type cells both Rab3A and Rab3D immunoreactivity is associated with the granules with no labeling at the plasma membrane of the cell. In contrast, in A3C1 cells there is lower immunoreactivity for Rab3D and Rab3A is not labeled at all. Scale bar: 100 nm.

whereas the morphology of late regulated secretory organelles is preserved (Matteoli *et al*., 1991). However, our results indicated that in Brefeldin A-treated AtT-20 cells, both Rab3D and Rab3A retained their characteristic diffuse fluorescence, and did not form tubules. On the contrary, the fluorescent signal given by either Texas Red-conjugated transferrin or the 58-kDa Golgi protein changed in a dramatic manner, indicating the efficacy of the Brefeldin A treatment. These results suggest that, in AtT-20 cells, both Rab3D and Rab3A are present in a post-Golgi, non-endosomal compartment. Thus, Rab3D conceivably associates only with the dense core granules of AtT-20 cells (Baldini *et al*., 1998), even though at present we do not know whether Rab3D is a component of the more mature granules, or is also present in granules that leave the Golgi and in secretory vesicles at an intermediate stage of maturation.

Our findings are in agreement with the data reported by Mundigl *et al*. (1993) who did not observe a redistribution of Rab3A in Brefeldin A-exposed cultured hippocampal neurons. However, others have recently demonstrated that Rab3A is associated with an endosomal compartment in bovine chromaffin cells in primary culture (Slembrouck *et al*., 1999). It is conceivable that these discrepancies are due to the different cell systems employed in these investigations. Alternatively, the existence of a Brefeldin A-insensitive endosomal compartment could not be ruled out, although at present such a compartment has not yet been identified (e.g. Blagoveshchenskaya and Cutler, 2000).

It is interesting that in AtT-20 cells, also Rab3A was found to be associated with dense core granules. It was originally thought that Rab3A was a component of synaptic vesicles (Sudhof and Jahn, 1991; Chou and Jahn, 2000). However, a number of more recent reports have indicated that Rab3A is also present in the dense core granules of various cell lines, including PC12 and insulin-secreting HIT-T15 or INS-1 cells (e.g. Darchen *et al*., 1995; Regazzi *et al*., 1996; Iezzi *et al*., 1999; Chung *et al*., 1999). Our previous results (Martelli *et al*., 1995; Baldini *et al*., 1998) have demonstrated that in wild-type AtT-20 cells, Rab3D and Rab3A did not for the most part co-localize, their immunofluorescent patterns being different. From the data presented in this paper we can infer that the two proteins co-localize at the dense core granules but not in other cell
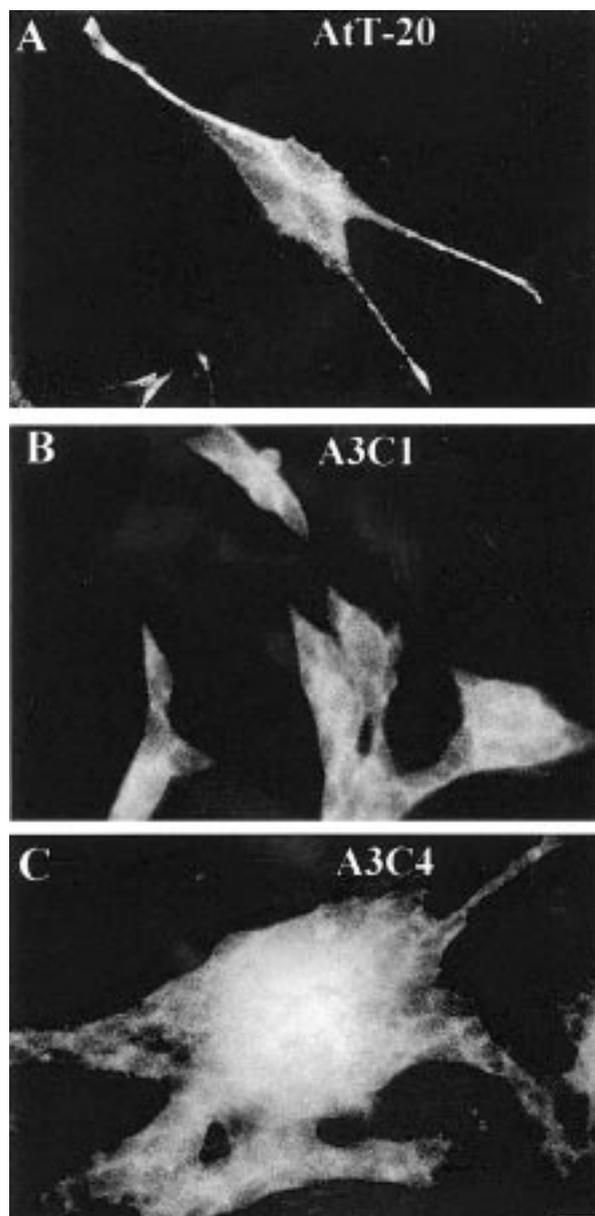
Fig. 4 - Immunofluorescence staining for Rab3A in wild-type AtT-20 cells and cells clones overexpressing mutated Rab3D. A: wild-type AtT-20 cells; B: clone A3C1; C: clone A3C4. Note the absence of Rab3A immunoreactivity at the tips of the processes in the two cell clones. Bar: 10 μm.

domains, even though their behavior in response to Brefeldin A was similar.

To further explore the mechanisms by which Rab3D controls regulated secretion in AtT-20 cells, we overexpressed a mutated form of Rab3D. In cell clones overexpressing this form, we observed a reduction in the Rab3D immunoreactivity present on dense core granules. Moreover, Rab3A was not found associated with the dense core granules. This may indicate that a sort of cross-talk exists between Rab3A and Rab3D, and that a functional Rab3D is necessary for a correct association of Rab3A with dense core granules. The absence of Rab3A from dense core granules of A3C1 cells, as demonstrated by immunoelectron microscopy, is in agreement with our own previous findings obtained by Western blotting analysis that showed a relative inability of Rab3A to bind to organelles of A3C1 cells (Baldini *et al.*, 1998). It should be pointed out that in the clones overexpressing mutated Rab3D, very low levels of Rab3D were seen by immunoelectron microscopy on dense core granules. In contrast, the same cells were positive by immunofluorescence. This may be another indication that in these clones most of the Rab3D (both the endogenous and the mutated form) is present in a soluble form and not associated with secretory granules, given that our antibody to Rab3D recognizes both the wild-type and the mutated form of the protein. Conceivably, the soluble form of Rab3D is lost when cells are "broken" for immunoelectron microscopy, whereas it is retained in cells fixed for immunofluorescent staining. At present, it is not clear how the mutated form of Rab3D could impair the association of wild-type Rab3D with the granules.

Fusion of dense core granules is thought to occur prevalently at the tips of AtT-20 cells, as dense core granules as well as synaptic-like microvesicles are accumulated there (Matsuuchi *et al.*, 1988; Rivas and Moore, 1989; Ngsee *et al.*, 1993). In wild-type AtT-20 cells, we have found that dense core granules are clustered very near the plasmalemma at the cell body indicating that the sites for docking/fusion of granules are distributed along the entire cell surface (Baldini *et al.*, 1998). We reasoned that overexpression of mutated Rab3D N135I might disrupt these sites and impair the ability of the granules to dock to the plasma membrane. SNAP-25 is a t-SNARE component thought to be involved in docking of hormone-containing granules (Sadoul *et al.*, 1995). However, we did not find any significant differences in the distribution of SNAP-25 between wild-type AtT-20 cells and cell clones overexpressing a mutated Rab3D form. Therefore, it is tempting to speculate that the impairment of granule docking we observed in A3C1 cells is dependent on the failure of Rab3A to associate with the membrane of large dense core granules. In agreement with a recent model proposed for neuro-exocytosis (Wang
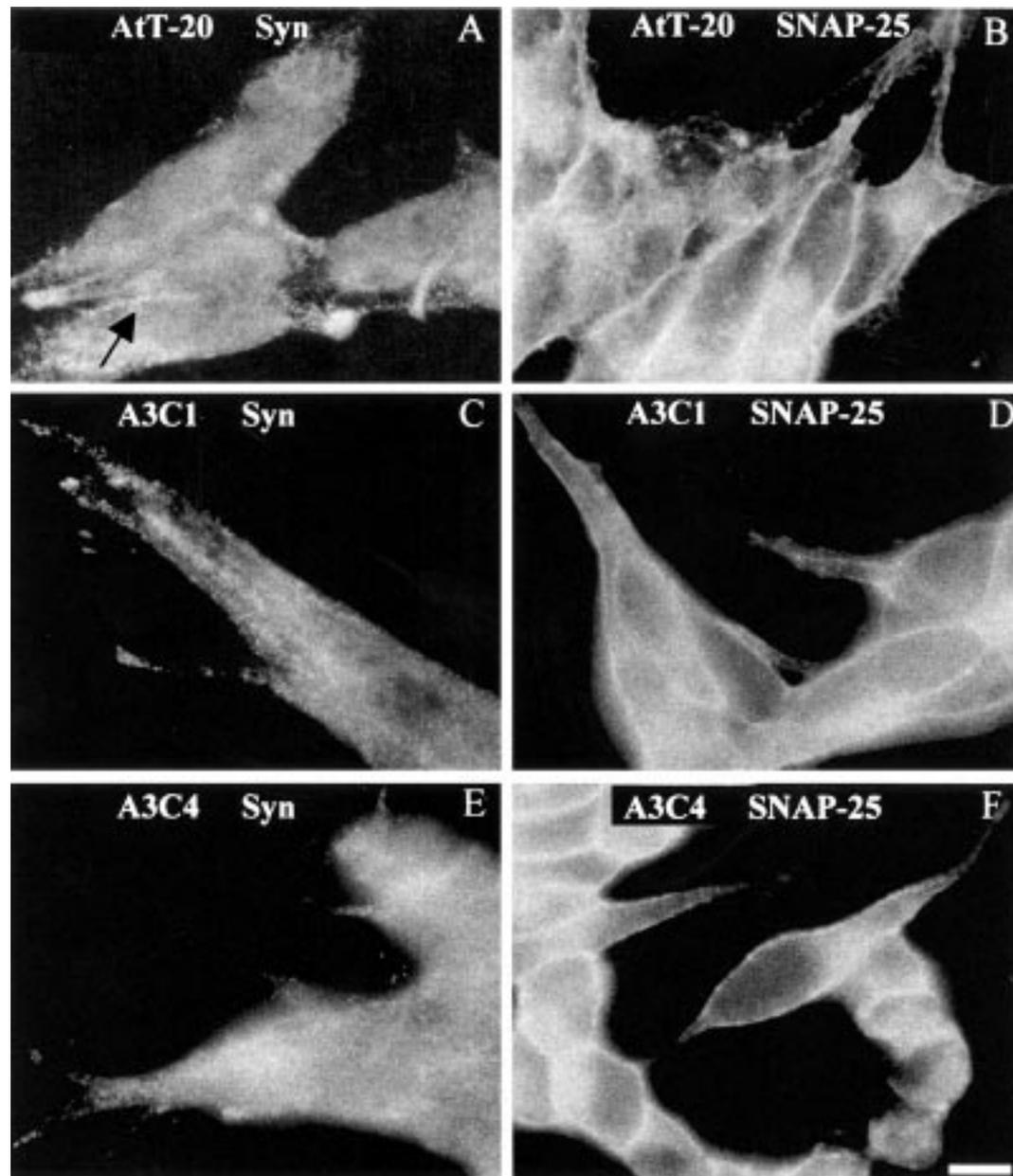
Fig. 5 - Distribution of synaptotagmin (Syn, A,C,E) and SNAP-25 (B,D,F) in wild-type AtT-20 cells and cell clones overexpressing a mutated Rab3D. Wild-type cells (A, B), A3C1 (C,D), and A3C4 (E,F) cells were immunostained with a monoclonal antibody to synaptotagmin. In A the arrow points to the characteristic "beads on a string" distribution of synaptotagmin. Scale bar: 10 μm.

et al., 1998), the dense core granules would be clamped by the interaction of Rab3A-GTP on their surface with the protein referred to as RIM (for Rab-Interacting Molecule), which is localized at the plasma membrane close to L-type Ca^{2+} channels (Iezzi et al., 2000). The absence of Rab3A from dense core granules would prevent the interaction with RIM, thus resulting in a failure of docking. Alternatively, it may be that in the absence of Rab3A, the dense core granules fail to interact with yet another putative effector of Rab3A, that is rabphilin-3A (Schluter et al., 1999). This protein has been reported to be a component of dense core granules of bovine chromaffin cells and is thought to be an

enhancer of regulated exocytosis (Chung *et al.*, 1995). However, it should be recalled that in AtT-20 cells, rabphilin-3A is expressed at very low levels (G. Tabellini, unpublished experiments). Therefore, other effectors of Rab3A, which are more abundant in endocrine cells such as the recently described granuphilin, could be involved (Wang *et al.*, 1999).

It should be emphasized that we have recently reported that in PC-12 cells, overexpressed Rab3A N135I still associates with dense core granules but is capable of impairing docking (Martelli *et al.*, 2000). Therefore, the results presented here likely do not reflect a universal phenomenon, even though others have shown that in PC-12 cells the overexpressed N135I mutated form of Rab3B failed to correctly bind to organelle membranes (Weber *et al.*, 1996).

We have previously shown that the amount of synaptotagmin, Vamp2/synaptobrevin 2, and SNAP-25, are similar in wild-type AtT-20 cells and in cell clones overexpressing Rab3D N135I (Baldini *et al.*, 1998). However, we did not rule out the possibility that these proteins, which are important for regulated secretion, changed their localization. The results presented in this manuscript clearly demonstrated that SNAP-25 did not change its location in clones overexpressing mutated Rab3D. Thus, the impairment in the ACTH secretion that we measured in AtT-20 cells overexpressing mutated Rab3D (Baldini *et al.*, 1998) cannot be explained by changes in the expression and/or localization of this key component of the secretory machinery.

On the other hand, the fact the synaptotagmin immunofluorescent distribution was different in both A3C1 and A3C4 cell clones when compared to controls, is conceivably due to the fact that in these cells the dense core granules are not docked to the plasma membrane.

Taken together, we feel that these results will provide an important basis for further investigations aimed at a complete understanding of the mechanisms regulated by Rab3D and Rab3A that lead to granule docking at the plasma membrane.

ACKNOWLEDGEMENTS

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