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Rab11, a small GTPase associated with both constitutive and regulated secretory pathways in PC12 cells

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A specific polyclonal antibody was used to investigate the subcellular distribution of the small GTPase, rab11p, in the neuroendocrine cell line, PC12. We took advantage of a previously described pulse-chase protocol based on sulfation [1] to examine the distribution of rab11 along the secretory pathway. Using the rab11 antiserum, but not serum depleted of rab11 antibodies, we were able to specifically immunoisolate markers of the constitutive and the regulated secretory pathway in the trans-Golgi network (TGN) as well as after their exit from this compartment (constitutive secretory vesicles, immature, and mature secretory granules). We therefore conclude that rab11p is associated with the TGN and with TGN-derived vesicles of both the constitutive and the regulated secretory pathway in PC12 cells.

Small GTP-binding protein; Secretion; Immunoisolation

1. INTRODUCTION

Small GTPases of the rab-family have been shown to play a critical role in vesicular traffic in eucaryotic cells [2]. Since their discovery the number of rab-proteins has increased such that the family now comprises more than 30 members [3]. The differential distribution of the rab-proteins to distinct intracellular compartments suggested that different members of the family might convey specificity to different intracellular pathways possibly by providing the vesicles with a proof-reading mechanism to ensure correct targeting [4]. However, the precise function of rab proteins in this complex process is not yet understood. We have investigated the subcellular distribution of rab11p [5], which although ubiquitously expressed, has been reported to be more abundant in tissues with a high level of secretion [6]. This observation suggested that rab11p might play a role in the exocytic pathway. All cells secrete some proteins in a constitutive fashion. Some cell-types are known to exhibit an additional, triggered form of secretion [7]. Proteins destined for this regulated secretory pathway are sorted from constitutively secreted proteins in the TGN where they are packaged into special organelles, referred to as regulated secretory granules, that subsequently undergo a process of maturation [8]. The mature secretory granules are stored in the cytoplasm and do not release their content unless a specific signal induces their fusion with the plasma-membrane [9]. We chose the neuroendocrine cell-line PC12 to localize rab11p, as both the constitutive and the regulated secretory pathway have been well described for this cell-line [1]. Using an approach based on immunoisolation of well-characterized sulfate-labeled markers [10], we were able to show that rab11p is associated with both the constitutive and the regulated secretory pathway in PC12 cells.

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2. MATERIALS AND METHODS

2.1. *Preparation of antisera against rab11p*

The polyclonal antibody 121 was raised against recombinant rab11p expressed in *E. coli* BL21(DE3) using the pET8c expression system [11]. Expression and purification of the rab-protein was essentially as previously described in Lombardi et al. [12] and the purity was monitored by SDS-PAGE.

2.2. *Preparation of an affinity-purified and an immunodepleted antiserum fraction*

In order to generate an affinity-purified antibody and a fraction of the antiserum that was depleted of rab11-specific antibodies, 2 ml of the antiserum 121 were incubated for 2 h at 4°C with 2 ml of an affinity resin (MS2-polymerase-rab11-fusion-protein coupled to a CNBr-activated Sepharose-4B column) as previously described [13]. The flow-through of this column was collected and the specific antibodies were eluted from the column as previously described [13]. After reequilibration of the column, the flow-through was passed a second time over the column. The relative antibody-concentration of the collected flow through of this second passage, referred to below as 'flow through' (FT) was analyzed by measurement of the OD at 280 nm and by immunofluorescence on fixed cells. As expected, only a minor fraction of the antibody bound to the affinity-purification column.

2.3. *Immunoprecipitation of in vitro translated protein*

In vitro translated protein was prepared essentially as previously described [14]. For immunoprecipitation under native conditions 10 μl of the antiserum was incubated for 30 min at room temperature with 20 μl of 10% w/v Protein A-Sepharose (Pharmacia) equilibrated in IP-buffer (10mM Tris pH 7.3, 150 mM NaCl, 1 mM EDTA). The unbound serum was removed, the beads were washed three times with IP-buffer and incubated for 1 h at room temperature with the in vitro translation reaction in a final volume of 150 μl. The beads were washed

twice with IP-buffer supplemented to final concentration of 1% Triton X-100 and twice with 10 mM HEPES, before resuspension in SDS sample buffer. The antibody–Protein A-Sepharose coupling for immunoprecipitations under denaturing conditions was carried out in buffer A (0.2% v/v Triton X-100, 10 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA). After several washes in buffer A the beads were resuspended in buffer B (20 μl 2% Triton X-100, 0.4% SDS, 50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA). In vitro translation reactions were denatured in 4% SDS and then brought to the same conditions as buffer B. The denatured proteins were incubated for 2 h at room temperature with the antibody–bead complex. The beads were washed twice in buffer A, once in 10 mM Tris pH 7.5 and the precipitated proteins were lysed in SDS sample buffer, analyzed by SDS-PAGE and fluorography.

2.4. *Cell culture*

PC12 cells (clone 251, [15], originally obtained from Dr. H. Thoenen, Martinsried, Munich) were grown in DMEM supplemented with 10% horse serum and 5% fetal calf serum (growth medium) as previously described [1].

2.5. *[^{35}S]sulfate labelling of PC12 cells*

PC12 cells were pulse labeled and pulse labeled and chased (as indicated in the figure legends) with [^{35}S]sulfate exactly as described in Tooze et al, 1990 [1].

2.6. *EXPRE^{35}S^{35}S labeling of PC12 cells*

PC12 cells were incubated for 30 min at 37°C in methionine–cysteine-free medium (DMEM lacking methionine and cysteine plus 1% dialyzed horse serum and 0,5% dialyzed fetal calf serum) in order to deplete endogenous methionine and cysteine. Cells were then either pulsed with EXPRE^{35}S^{35}S (L-[^{35}S]methionine and L-[^{35}S]cysteine; spec. activity: 1140 Ci/mmol. Amersham) or pulsed and then chased with growth medium supplemented with 1% of the level of normal methionine and cysteine as described in the figure legends. To chase the label and in long-term (16 h) labeling incubations, the typically 1 to 2 mCi of EXPRE^{35}S^{35}S label was used per 15 cm dish.

2.7. *Subcellular fractionation of PC12 cells*

CSV and ISG were separated from the TGN by velocity and equilibrium sucrose gradient centrifugation as previously described [1,10]. Fractions 2–4 or 5–7 of the velocity gradient, containing the bulk of CSV and ISG, or MSG respectively were subjected to equilibrium sucrose gradient centrifugation. The fractions from the velocity and the equilibrium gradient were analysed by SDS-PAGE and fluorography. For some experiments a total membrane fraction was prepared from a PNS as follows: 1.0 ml of a PNS was loaded on top of a 0.3 M Sucrose-cushion (0.4 ml) and centrifuged for 30 min at 45,000 rpm in a TLS 5,55 rotor in a TL 100-ultracentrifuge (Beckmann). The supernatant was removed and the pellet was taken up either in homogenization buffer, immunoisolation buffer, IEF sample buffer or SDS-sample buffer. For the determination of relative protein concentrations, a fixed volume of each fraction, derived from metabolically labeled cells, was applied to a Whatman 3MM 2.5 cm filter, precipitated with 10% TCA for 10 min at 4°C, washed with 5% TCA at 95°C for 5 min and rinsed for 5 min at 4°C with ethanol/ether (1:1). TCA-precipitated counts were analysed by scintillation counting (Wallac1410 Liquid Scintillation Counter, Pharmacia)

2.8. *Immunoblotting*

For Western blot analysis, proteins were separated either by 12.5% SDS-PAGE or by high resolution 2D gel electrophoresis and subsequently transferred onto nitrocellulose filters. Filters were blocked for 16h at 4°C with PBS/5% milk powder, 0.2% Tween 20. Incubations with primary antibody were for 2 h at room temperature in PBS/5% milk, 0.2% Tween 20. After extensive washing with the same buffer, membranes were incubated with 0.138 μCi/ml ^{125}I-labeled Protein A (spec. activity: 8.91 μCi/μg; DuPont) in PBS/5% milk, 0.2% Tween 20 for 1 h at room temperature, washed several times with PBS/0.2% Tween 20, dried and exposed at −80°C. Subsequently immunoblots were quantitated by analysis with a PhosphorImager and the ImageQuant Softwarepacket (Molecular Dynamics).

2.9. *Immunoisolation*

Staphylococcus aureus cells (Staph A) were formalin-fixed and heat-inactivated by Kessler's procedure [16]. The thawed cells were equilibrated at a concentration of 10% (w/v) in immunoisolation buffer (IB, 50 mM Tris, pH 7.5; 150 mM NaCl and 2 mM $MgCl_2$) supplemented with 0.5% BSA (lyophilized, fatty acid-free BSA, Sigma) by three washing steps (cycles of resuspension and centrifugation for 20 s at 10,000 rpm approx. (8800×*g*) in an Eppendorf centrifuge). The following incubations and washing steps were all carried out at 4°C on a rotating wheel. With the exception of the samples prepared for electron microscopy, 200 μl washed Staph A was incubated prior to antibody coupling for 2h with 200 μl of a cold PNS, prepared from a 15 cm dish of 80% confluent cells. The 'precoated' Staph A was incubated for 16 h with 20 μl of antiserum 121 or with the immunodepleted antiserum ('flow through'). Excess antibody was removed by three washing steps with IB/0.5% BSA and the Staph A-antibody complex was incubated for 2 h with 200 μl of a labeled PNS, obtained from a 15 cm dish of 80% confluent cells. For the immunoisolation from subcellular fractions, the volume of the input was normalized to the amount of antigen quantitated by immunoblotting of the respective fractions. All immunoisolation experiments were carried out in excess of antibody-Staph A complex. Unbound material was removed by two washing steps with IB/BSA followed by two washes with IB. The pellet was resuspended in IEF sample buffer or SDS sample buffer for biochemical analysis. When indicated, heat stable fractions were obtained by boiling samples for 5 min at 95°C and pelleting the denatured protein for 5 min at 13.000 rpm in an Eppendorf centrifuge.

2.10. *Electron microscopy*

For analysis by electron microscopy, the Staph A pellet with the bound immunoisolated material was fixed for 30 min with 0.5% glutaraldehyde. The pellet was gently washed with 0.1 M cacodylate buffer and processed for electron microscopy as previously described [17]. Ultrathin sections were cut and the ratio of dense core granules per Staph A was determined by counting at random 15 fields containing a total of 180–200 profiles of Staph A particles.

2.11. *Two-dimensional (2D) gel electrophoresis, transfer to nitrocellulose blots and [α-^{32}P]GTP overlay*

A combination of isoelectric focusing (IEF) and SDS-PAGE was used to resolve proteins in two dimensions essentially as described previously [18]. [α-^{32}P]GTP overlays were performed as described recently [19,20]. [α-^{32}P] GTP binding was visualized by autoradiography (12–24 h, −80°C) using Kodak X-OMAT film with an intensifying screen.

3. RESULTS

3.1. *Characterization of the antibody 121*

In order to investigate the localization of the small GTPase rab11p, we raised antibodies against the full length recombinant rab11 protein expressed in bacterial cells. The specificity of the antiserum 121 was analysed by immunoprecipitation under native and denaturing conditions with in vitro translated, [^{35}S]methionine labeled rab11p. To test for cross reactivity with other proteins of the rab-family, we used in vitro translated, [^{35}S]methionine-labeled rab5p as a control. Fig. 1 shows that the antiserum 121 specifically recognizes rab11p under both native and denaturing conditions but does

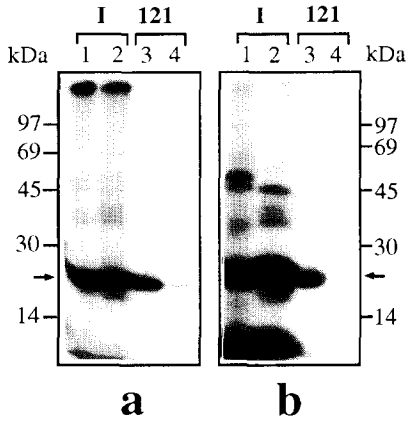

Fig. 1. Immunoprecipitation of in vitro translated rab11p. In vitro transcribed rab11- or rab5-mRNA was translated in reticulocyte lysate in the presence of [^{35}S]methionine. Antiserum 121 was coupled to protein A-Sepharose and incubated either (a) under denaturing conditions (in the presence of SDS), or (b) under native conditions with untreated in vitro translation reactions (lanes 1 and 3, rab11p; lanes 2 and 4, rab5p). The immunoprecipitated proteins were resuspended in SDS sample buffer and analysed by 12.5% SDS-PAGE followed by fluorography. I, starting material; 121, immunoprecipitated fraction.

not immunoprecipitate rab5p. From these data it can be concluded that the antiserum raised against rab11p does not recognize the regions that are highly conserved within the rab-family. Furthermore this antibody recognizes only one spot on a blot of a two-dimensionally resolved immunoisolated fraction that was enriched for several GTP-binding proteins (see below and Fig. 5c and d).

3.2. *Biochemical localization of rab11p on PC12 cells in TGN and post TGN vesicles*

Intracellular labelling obtained by indirect immunofluorescence as well as immunogold labelling on cryosections of different cell types suggested that at least part of the endogenous rab11p-pool might be associated with vesicular structures in the area of the trans-Golgi network (TGN) as well as in the cell periphery (data not shown). However, due to the low level of labeling on cryosections, we were not able to draw solid conclusions from our morphological data. Therefore we decided to use a biochemical approach in order to examine the localization of rab11p. Previously it has been shown that rab11p is highly expressed in secretory tissues [6]. We chose the well-studied secretory cell line PC12 (a rat cell line derived from a pheochromocytoma) to analyze the distribution of rab11p. A system based on this cell line has been described that allows the separation and resolution of at least two different types of secretory vesicles from each other and from their donor compartment, the TGN [1]. Briefly, following a velocity centrifugation of a post-nuclear supernatant (PNS) over a linear sucrose gradient, TGN-derived membranes sediment as a peak in the denser part of the gradient, whereas post-TGN vesicles migrate towards the top. By immunoblotting with antisera 121, rab11p was found in both the fractions containing TGN-derived membranes (Fig. 2; fractions 9, 10 and 11) and the fractions containing post-TGN vesicles (Fig. 2; fractions 2 and 3). This result did not merely reflect the protein distribution over the gradient, as for each fraction an equal amount of TCA-precipitable proteins was separated by SDS-PAGE and quantitatively analysed.

3.3. *Immunoisolation of rab11p-positive membranes from PC12 cells*

To further characterize the vesicles with which rab11p is associated, we decided to make use of a system that is based on sulfation of proteins, a post-translational modification occurring in the trans-Golgi network [10]. In PC12 cells, two of the three major sulfated proteins, Secretogranin I and II have been shown to be sorted efficiently into secretory granules, whereas a third sulfated protein, a heparansulfate proteoglycan, is constitutively secreted [1]. Using pulse-chase experiments of [^{35}S]sulfate-labeled PC12 cells, markers of the constitutive and the regulated secretory pathway can therefore be followed upon exit from the TGN. After velocity centrifugation (described above) a second centrifugation step allows the separation of constitutive secretory vesicles from immature secretory granules. A

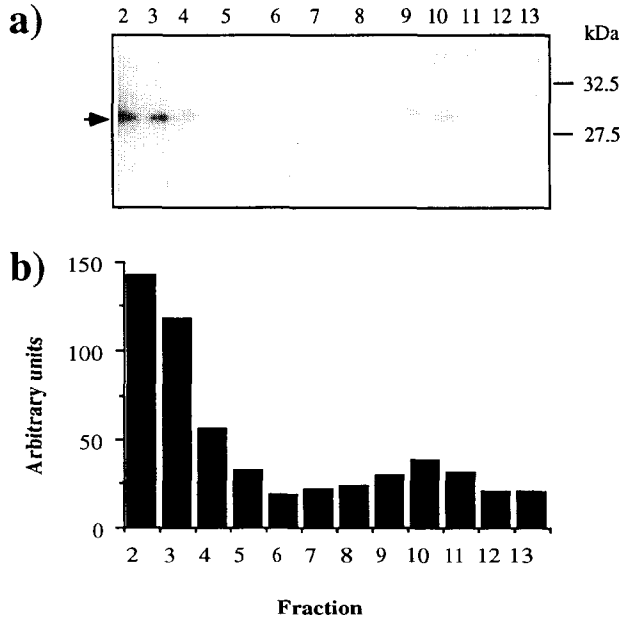

Fig. 2. Distribution of rab11p on a gradient separating TGN-derived vesicles from the TGN. PC12 cells were metabolically labeled for 16 h with [^{35}S]methionine. A PNS was prepared from these cells and subjected to velocity sucrose centrifugation. Fractions 2–13 were TCA-precipitated and an equal number of TCA-precipitable counts was subjected to 12.5% SDS-PAGE. Proteins were transferred onto nitrocellulose filters and blotted with affinity-purified antibody 121. Bound antibodies were detected with ^{125}I-labeled protein A and subsequently visualized by autoradiography (a). Quantitation was done by analysis with a PhosphorImager and the ImageQuant Software packet (b). An arrow indicates the position of rab11p.

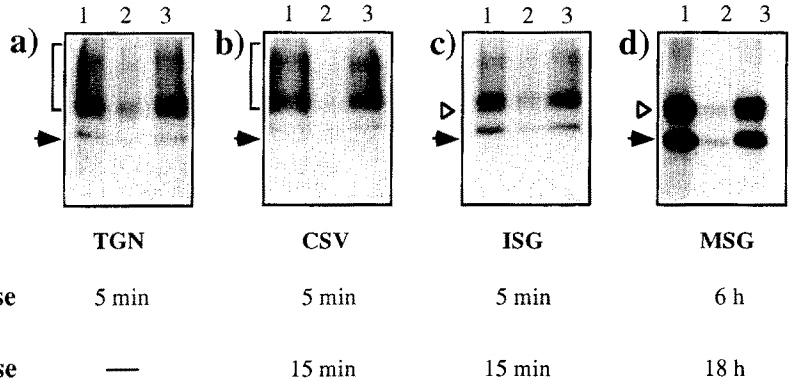

Fig. 3. Immunoisolation of membrane vesicles from sulfate-labeled PC12 cells. PC12 cells were labeled with [^{35}S]sulfate either by (a) a 5 min pulse, (b,c) a 5 min pulse followed by a 15 min chase, or (d) 6 h labeling followed by 18 h of chase to label proteins at different stages of the exocytic pathway (TGN, trans-Golgi network; CSV, constitutive secretory vesicles; ISG, immature secretory granules; MSG, mature secretory granules). The PNS from each of the three conditions was subjected to velocity sucrose gradient centrifugation. Fractions 9 and 10 containing the peak of the labeling in condition (a) were kept on ice and used later for immunoisolation (TGN). Fractions containing the peak of the labeled proteins of conditions (b,c) and (d) were collected into two separate pools (fractions 2–4 and fractions 5–7 respectively) and submitted to equilibrium sucrose gradient centrifugation for further separation. Fractions from the equilibrium gradient of condition (b/c) containing either (b) the bulk of the hsPG, the constitutive marker (fractions 5 and 6), or (c) the regulated secretory proteins, SgI and SgII (fractions 8 and 9) were pooled and used as starting material for two immunoisolations (CSV, ISG). Fractions from the bottom of equilibrium gradient (d) containing long-term labeled secretogranins SgI and SgII (fractions 11 and 12) were used for immunoisolation of MSG. For each immunoisolation an equal volume of starting material was incubated with precoated Staph A-antibody 121 complex (3), or incubated with precoated Staph A alone (2) or kept on ice and acetone-precipitated (1). Immunoisolated material was analysed in parallel with the starting material by 7.5% SDS-PAGE and subsequent fluorography. Brackets indicate the heparansulfate proteoglycan (hsPG). Filled arrowheads indicate secretogranin II (SgII), whereas empty arrowheads indicate the position of secretogranin I (SgI).

long term incubation with [^{35}S]sulfate followed by an extended chase period (18 h) restricts the labeling to mature secretory granules that can be separated from less dense vesicles (including constitutive secretory vesicles and immature secretory granules) by the same centrifugation steps. Starting from four different pools of gradient fractions, containing either the TGN (as identified by a 5-min sulfate pulse), constitutive secretory vesicles and immature secretory granules both labeled by a 5 min sulfate pulse followed by a 15 min chase or mature secretory granules (labeled by a 6 h sulfate-labeling followed by 18 h chase), we asked whether the antibody 121 could specifically immunoisolate any of these labeled compartments. Surprisingly, we were able to immunoisolate all four compartments with high efficiency (Fig. 3a–d). The specificity of this result was shown by several criteria. First, Staph A alone without coupled antiserum did not reveal any significant ability to bind labeled vesicles (Fig. 3, lane 2). Second, a similar immunoisolation experiment starting from a membrane fraction prepared from a PNS of metabolically pulse-labeled or pulse-labeled and chased cells showed that rab11p is not associated with the early secretory pathway (Fig. 4); secretogranin I and II labeled by a 5 minute [^{35}S]methionine pulse (which should identify the proteins while they are still present in the endoplasmic reticulum) could not be efficiently immunoisolated (lanes 1 and 3) whereas after 20 min of chase the same proteins appeared to be isolated with high efficiency in a later stage of the secretory pathway (lanes 2 and 4). The apparent shift in molecular weight of SgI confirmed that the marker proteins had indeed reached a late Golgi or post-Golgi compartment [21]. Finally, a fraction of the antiserum that had been previously depleted by affinity absorption of rab11p-specific antibodies (the flow

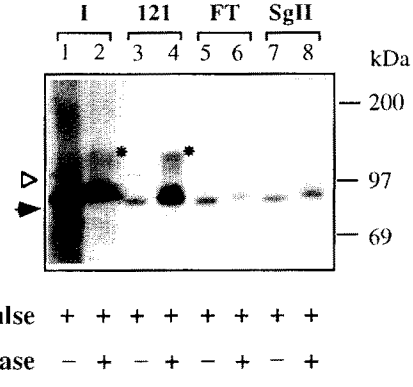

Fig. 4. Immunoisolation of membrane vesicles from metabolically-labeled PC12 cells. PC12 cells were either pulse-labeled for 5 min with [^{35}S]methionine (lanes 1,3,5,7) or pulse-labeled (5 min) and chased for 20 min (lanes 2,4,6,8). A total membrane fraction was prepared from the PNS obtained from each of the conditions and equal parts were either incubated with precoated Staph A that was coupled with anti rab11p antiserum (121), with a fraction of the antiserum that was depleted of specific antibodies (FT, flow through), with an antiserum raised against secretogranin II (SgII), a luminal antigen, or simply left on ice (I, input). A heat-stable fraction was obtained of the immunoisolated material and the input-fraction and analyzed by 7.5% SDS-PAGE followed by fluorography. The heat stable proteins secretogranin I and II are indicated with an empty (SgI) and a filled (SgII) arrowhead. The asterisk marks the higher molecular weight form of SgI characteristic of the trans-Golgi form as opposed to the rough ER form (filled arrowhead)

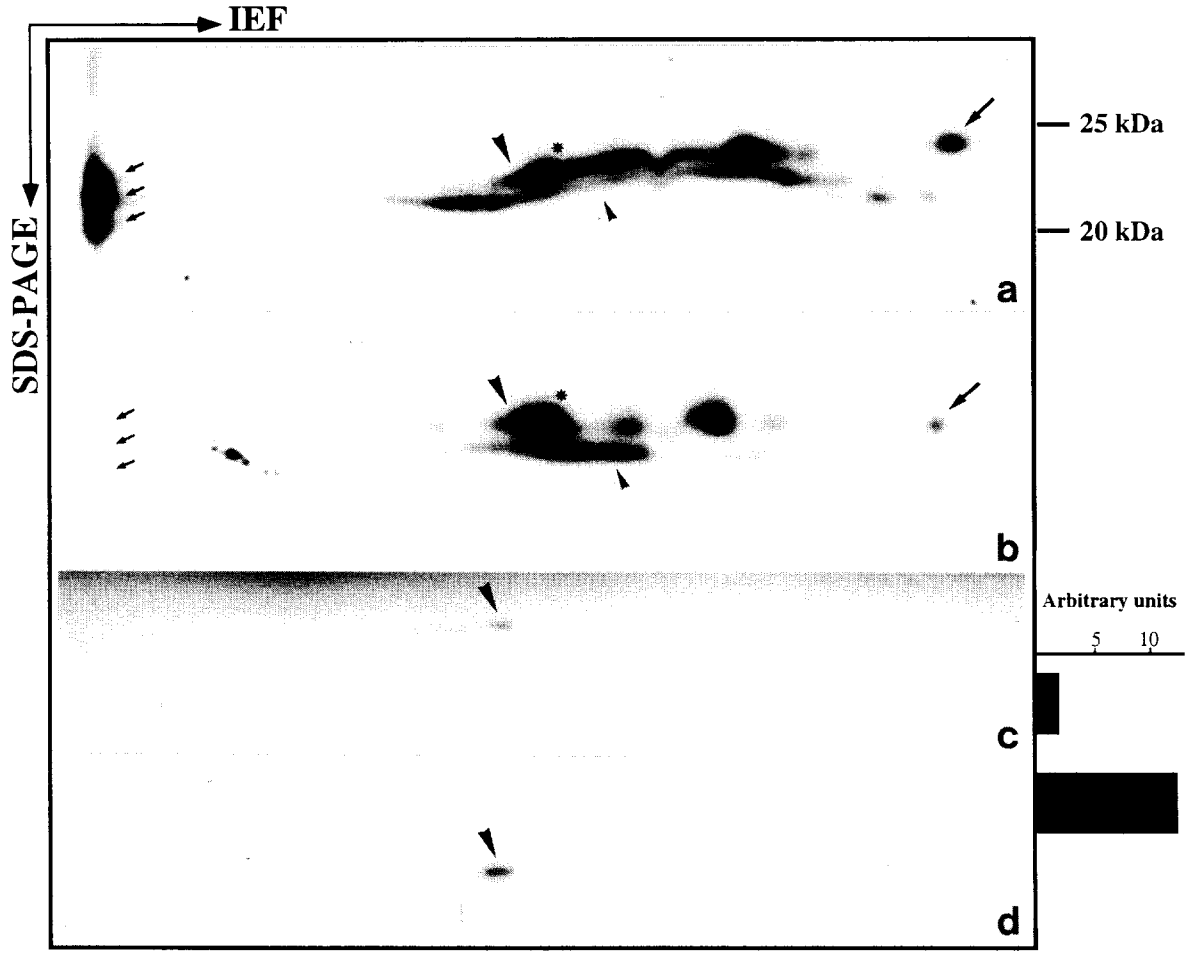

Fig. 5. 2D gel analysis of the immunoisolation by [^{32}P]GTP overlay (a and b) and [^{125}I]Protein A-immunoblot (c and d). (a) and (b): A PNS was prepared from PC12 cells and a total membrane fraction was obtained. Equal parts of the membrane fraction were incubated with precoated Staph A coupled with antiserum 121 and lysed in IEF sample buffer. Immunoisolated material (b) was solubilised in IEF sample buffer and analysed in parallel with the starting material (a) by 2D gel analysis followed by [^{32}P]GTP overlay and subsequent autoradiography. (c) and (d). PC12 cells were labeled for 16 h with [^{35}S]methionine and a membrane fraction from a PNS was prepared. An aliquot of the membrane fraction was incubated with precoated Staph A coupled with antiserum 121 whereas another part was lysed in IEF sample buffer. Bound material was solubilised in IEF-buffer and an equal amount of TCA-precipitable counts of both the membrane fraction (c) and the immunoisolated fraction (d) were analysed by 2D gel analysis. Separated proteins were transferred to nitrocellulose filters and immunoblotted with affinity-purified antiserum 121. Bound antibodies were detected by [^{125}I]Protein A and quantitation (see bar-diagram) was done with a PhosphorImager. Large arrows indicate the position of rab11p; small arrows indicate the rab5p-isoforms; large arrowheads indicate rab3p and small arrowheads indicate rab6p. The asterisk marks a ubiquitous unidentified GTP-binding protein.

through from the affinity column), only displayed a low level of background signal (Fig. 4, lanes 5 and 6). Furthermore, the observed signal could not be accounted for by non specific binding after lysis of the compartments, as an immunoisolation with an antibody raised against secretogranin II (a luminal antigen) gave a very low signal (Fig. 4, lanes 7 and 8). Therefore we conclude that rab11p is indeed associated with the TGN and TGN-derived vesicles comprising both constitutive secretory vesicles as well as immature and mature secretory granules.

3.4. *Two-dimensional gel analysis of immunoisolated membrane fractions*

Analysis of the immunoisolated fraction by [^{32}P]GTP overlay of two-dimensional gels showed a simplified pattern compared to the membrane fraction used as starting material (Fig. 5b and a). In particular, the signal of the rab5p-isoforms [19], that have previously been localized to the early endocytic pathway [22], were dramatically decreased in the immunoisolate. In contrast a few GTP-binding proteins appeared to be enriched in the immunoisolated fraction. One of these proteins might correspond to rab6p which has been localized previously to the TGN [23,24] and to a post-Golgi vesicle [25]. Interestingly the signal corresponding to rab3p [19] was decreased after the immunoisolation. The detection of rab11p in [^{32}P]GTP-overlays of 2D gels proved problematic as overexpression studies showed that rab11p is poorly resolved from a yet unidentified,

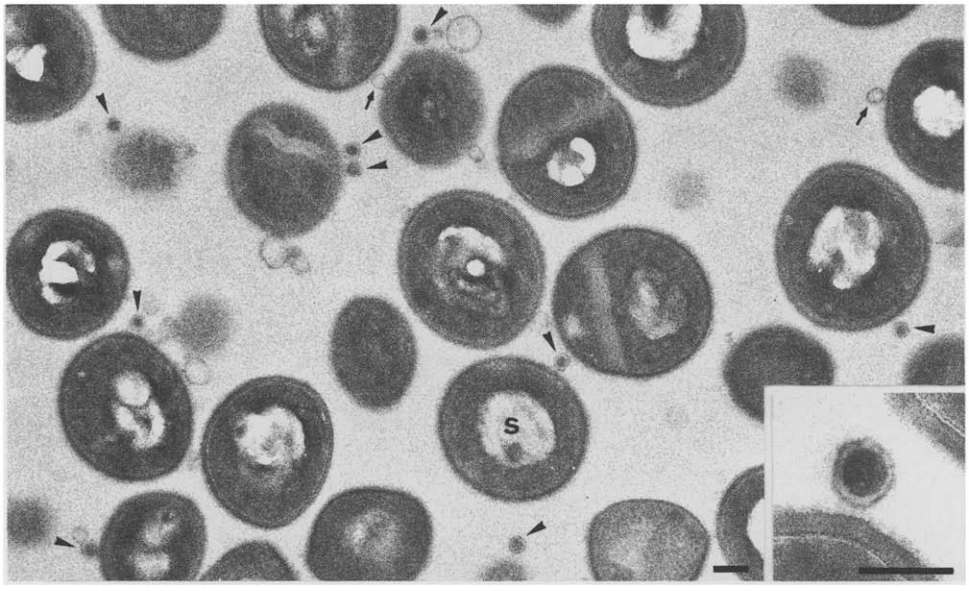

Fig. 6. Electron microscopic analysis of the immunoisolated fraction. Anti rab11 antiserum adsorbed to Staph A was incubated with a total membrane fraction from PC12 cells as described in section 2. The Staph A with associated immunoisolated material was then washed, fixed, and processed for electron microscopy. The material associated with the Staph A particles (s) comprises uncoated electron lucent vesicles (arrows) and vesicles with an electron dense content (arrowheads). The latter have the characteristic morphology of secretory granules as shown at higher magnification in the inset. Bars, 200 nm.

prominent spot (*), that is found in every membrane fraction [19]. Therefore we investigated the enrichment of rab11p in the immunoisolate by immunoblotting. A PNS was obtained from metabolically labeled PC12 cells and the membrane fraction prepared from this PNS was used as a starting material in an immunoisolation experiment. We then compared equal amounts of TCA-precipitable counts of the starting material and the immunoisolate by immunodetection on a Western blot from a 2D gel. Only one spot was revealed by the anti rab11p antibody which, as indicated in previous experiments with overexpressed rab11p-protein (results not shown), lay in the same position as the prominent ubiquitous GTP-binding protein (spot (*)) (Fig. 5c and d). Quantitation of the immunoblots showed a 6.5-fold enrichment of rab11p in the immunoisolate (Fig. 5, barchart), consistent with the conclusion that our data reflect a specific interaction of the antiserum with rab11p. The fact that only one spot is detected by the antibody in the immunoisolated fraction shows that the enrichment observed for other GTP-binding proteins in this fraction (Fig. 5b) is not due to a crossreactivity of the antiserum with these proteins and shows the specificity of the immunoisolation procedure.

3.5. *Electron microscopy of the immunoisolated membrane fraction*

To determine morphologically what type of structures were specifically immunoisolated by the antisera 121 we performed electron microscopy after immunoiCsolation (Fig. 6). Apart from dense-core granules of mature appearance, some granules with an irregular shape typical for immature granules were also detected in the preparation. Structures reminiscent of condensing vacuoles were also observed (data not shown), in agreement with our biochemical finding of rab11p on the TGN membranes. Furthermore other smaller vesicular profiles of translucent appearance were observed (Fig. 6) which might correspond to constitutive secretory vesicles or to tubular structures of the TGN. We quantitated the number of dense-cored secretory granules per Staph A to evaluate the specificity of the immunoisolation. Comparison of fractions immunoisolated using the complete antiserum or the antiserum depleted of rab11p-specific antibodies showed a 7.4-fold higher density of dense-core granules per Staph A in the immunoisolated fraction obtained with complete antiserum.

4. DISCUSSION

The localization of proteins of the rab-family by conventional methods such as indirect immunofluorescence and immunogold labeling has often proved to be problematic. Many antibodies raised against this class of proteins react poorly with the endogenous protein on cells processed for immunoelectron microscopy, so that the specific labeling is barely discernible. In our attempts to localize rab11p, we therefore adopted an approach based on immunoisolation of pulse-chase labeled markers with a polyclonal antibody that specifically recognized the native form of rab11p. To our knowledge this is the first time a member of the rab-family has been localized using this technique. We have excluded possible crossreactivity of the antibody with

other members of the rab-family by showing that this antibody only reacts with a single protein on a two-dimensionally resolved immunoisolated fraction although this fraction was enriched for several GTP-binding proteins. Amongst these proteins, rab11p was shown to be enriched 6.5-fold.

Using a combination of immunoisolation experiments and different metabolic labeling procedures of known secretory proteins in PC12 cells, we were able to identify the subcellular distribution of rab11p. Two secretory proteins, SgI and SgII, labeled by a short pulse of [^{35}S]methionine at the site of protein synthesis could not be specifically detected (in significant amounts) in an immunoisolated fraction. However, if the labeled proteins were chased to a late secretory compartment (TGN and post-TGN), the signal detected in the specific immunoisolated fraction was significantly higher than in control samples. The same proteins labeled with [^{35}S]sulfate were already detectable in the immunoisolate at the earliest measurable time point of sulfate incorporation, that is in the TGN. Furthermore, these labeled proteins could be immunoisolated after their exit from the TGN and appearance in secretory vesicles. Therefore, our data show that rab11p is associated with both the TGN and TGN-derived vesicles.

Both a constitutively secreted heparansulfate proteoglycan (hspG) and two granule specific proteins (SgI and SgII) can be efficiently labeled with [^{35}S]sulfate in the TGN in PC12 cells. Using these proteins as markers, we were able to show that rab11p is associated with both constitutive secretory vesicles and regulated secretory granules. It is unlikely that the rab11p found on the secretory granules is a result of a missorting of rab11p to the regulated secretory pathway as we find no other known TGN-localized rab-protein, such as rab8p, on regulated secretory granules (data not shown). Furthermore, the efficiency of the immunoisolation suggests that rab11p is associated with a large proportion of the mature secretory granules. The presence of rab11p on both constitutive and regulated secretory pathways and its tissue distribution make it unlikely that the function of rab11p is involved in a process that is restricted to the regulated secretory pathway. Indeed, the existence of a highly homologous protein in yeast, ypt3 [5,26] suggests that this protein even plays a role in a lower eucaryote for which no regulated secretion has been described so far. Recently it has been postulated that both constitutive and regulated secretion are based on the same principles with the exception of an inhibitory factor, restricted to the regulated secretory pathway and neutralized only in response to an extracellular trigger [27]. Our results on the localization of a rab-protein to both constitutive secretory vesicles and regulated secretory granules strengthens the idea of a basic similarity between constitutive and regulated secretion. What might be the function of rab11p? Members of the ypt/rab family have been postulated to be involved in the targeting of transport vesicles to different subcellular compartments [2,4,28]. In this context, we suggest a putative role for rab11p in a specific recognition process at the plasma membrane, as a common destination for both the constitutive and the regulated secretory pathway. This raises the possibility that other organelles that are targeted to the plasma membrane might also utilize rab11p. In this context it is interesting to note that a homologue of rab11p, ora3 [29], (96.9% identity with rab11p [5] over the sequence spanning region 1 to region 4; Urbé and Parton, in preparation), although ubiquitously expressed [29], is associated with cholinergic synaptic vesicles derived from the electric organ of the marine ray [30]. Further work will be required to determine other possible locations of rab11p and to ascertain its function.

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