Localization of p24 putative cargo receptors in the early secretory pathway depends on the biosynthetic activity of the cell

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Members of the p24 family of putative cargo receptors (subdivided into p24- α , - β , - γ and - δ) are localized in the intermediate- and *cis*-Golgi compartments of the early secretory pathway, and are thought to play an important role in protein transport. In the present study, we wondered what effect increased biosynthetic cell activity with resulting high levels of protein transport would have on the subcellular localization of p24. We examined p24 localization in *Xenopus* intermediate pituitary melanotrope cells, which in black- and white-adapted animals are biosynthetically highly active and virtually inactive respectively. In addition, p24 localization was studied in *Xenopus* anterior pituitary cells whose activity is not changed during background adaptation. Using organelle fractionation, we found that in the inactive melanotropes and moderately active anterior pituitary cells of white-adapted animals, the p24- α , - β , - γ and - δ proteins are all located in the Golgi compartment. In the highly active melanotropes, but

not in the anterior cells of black-adapted animals, the steady-state distribution of all four p24 members changed towards the intermediate compartment and subdomains of the endoplasmic reticulum (ER), most probably the ER exit sites. In the active melanotropes, the major cargo protein pro-opiomelanocortin was mostly localized to ER subdomains and partially co-localized with the p24 proteins. Furthermore, in the active cells, *in vitro* blocking of protein biosynthesis by cycloheximide or dispersion of the Golgi complex by brefeldin A led to a redistribution of the p24 proteins, indicating their involvement in ER-to-Golgi protein transport and extensive cycling in the early secretory pathway. We conclude that the subcellular localization of p24 proteins is dynamic and depends on the biosynthetic activity of the cell.

Key words: endoplasmic reticulum export, intermediate pituitary, p24 distribution, subcellular localization, *Xenopus laevis*.

INTRODUCTION

Proteins destined to leave the endoplasmic reticulum (ER) are transported to specialized regions, called ER exit sites [1,2], where they are packaged in coat protein (COP)II-coated vesicles [3–5]. These vesicles form the vesicular tubular clusters, which fuse to form the ER–Golgi intermediate compartment (ERGIC) [6,7], and are subsequently transported to the Golgi complex. The mechanism that underlies the inclusion of cargo in the ER-derived vesicles is still poorly understood, but several studies have pointed out that this is a selective process that may involve transmembrane proteins acting as cargo receptors [8–10]. These receptors, which continuously cycle between the ER and Golgi complex, thereby facilitating efficient ER-to-Golgi transport, may include ERGIC-53/p58 (which selectively and transiently interacts with glycoproteins) [7] and the p24 protein family (subdivided into p24- α , - β , - γ and - δ) [11]. The putative role of p24 proteins as cargo receptors was based on the finding that in yeast, deletion of certain members of the p24 family caused a selective defect in the transport of invertase and Gas1p (a yeast glycosylphosphatidylinositol-anchored protein), but left other cargo protein transport unaffected [12–14]. The recent finding that two of these members, Emp24p and Erv25p, which coexist in a heteromeric complex, can be directly cross-linked to the cargo protein Gas1p in ER-derived vesicles strongly supports the hypothesis that p24 proteins play a direct role in cargo inclusion at the level of the ER [15]. In addition, based on a genetic study in *Caenorhabditis elegans*, p24 proteins have been suggested to be involved in the quality control of newly synthesized proteins in the ER [16], and in mammalian cells they may play an important

role in vesicular transport as well [17]. Although p24 proteins are not essential for vesicular transport in yeast [18], they are necessary for mice viability [19].

In general, the steady-state localization of the p24 proteins has been found to be in the intermediate- and *cis*-Golgi compartments [11,17,20–23]. However, considering the role p24 proteins may play in cargo transport, one could imagine that p24 localization is different in biosynthetically active and inactive cells. To test this hypothesis, we have now investigated the subcellular localization of p24 family members in the melanotrope cell of the intermediate pituitary of the South-African clawed toad *Xenopus laevis*. This cell type has a number of interesting features. First, melanotrope cells are involved in the process of background adaptation of the animal, and their biosynthetic activity can therefore be manipulated in a physiological manner from virtually inactive (when the animal is adapted to a white background) to highly active (in a black-adapted animal). Changing the background colour of the animal from white to black leads to an enormous increase of cargo transport in the melanotrope cells. Secondly, the melanotrope cells are primarily focused on the biosynthesis and processing of the prohormone pro-opiomelanocortin (POMC), the precursor protein of α -melanophore stimulating hormone that is responsible for pigment dispersion in the skin. POMC is by far the major cargo protein in the melanotrope cells, representing approx. 80 % of all newly synthesized proteins, and thus most of the p24 proteins co-expressed in these cells are expected to be linked to POMC transport. Thirdly, the *Xenopus* intermediate pituitary can be easily dissected and consists of a homogeneous population of melanotrope cells. Together, these characteristics make the melanotrope cell an interesting physio-

Abbreviations used: ACTH, corticotropin; AL, anterior lobe; anti- δ_2 , affinity-purified rabbit polyclonal antibody against the luminal domain of *Xenopus* p24 δ_2 ; BFA, brefeldin A; COP, coat protein; endoH, endoglycosidase H; ER, endoplasmic reticulum; ERGIC, ER–Golgi intermediate compartment; NIL, neurointermediate lobe; PDI, protein disulphide isomerase; POMC, pro-opiomelanocortin; SgIII, secretogranin III.

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logical model system to study the subcellular localization of p24 proteins during different states of biosynthetic cell activity. We found that depending on the biosynthetic activity of the melanotrope cell the p24 family members redistribute between the *cis*- and pre-Golgi compartments.

MATERIALS AND METHODS

Animals

South-African clawed toads, *X. laevis*, were adapted to their background by keeping them in either white or black buckets under constant illumination for at least three weeks at 22 °C.

Antibodies

The affinity-purified rabbit polyclonal antibody against the luminal domain of *Xenopus* p24 δ_2 (anti- δ_2) and the C-terminally-directed p24 δ antibody have been described previously [24]. Two other rabbit polyclonal antibodies were raised against synthetic peptides that comprised the C-terminal part of *Xenopus* p24 α_3 (CRHLKSFEAKKL; where single-letter amino-acid notation has been used) and *Xenopus* p24 γ_3 (CFSDKRTTTTRVGS) respectively. Both peptides were coupled to keyhole limpet haemocyanin (Pierce, Rockford, IL, U.S.A.) via the cysteine residues and used for immunization as previously described [24]. The generation of the polyclonal rabbit anti-[rat corticotropin (ACTH)] and anti-[*Xenopus* secretogranin III (SgIII)] antibodies has been described previously [25,26]. The following antibodies were kindly provided by others: guinea pig polyclonal serum against the precursor of POMC (ST62; Dr S. Tanaka, Shizuoka University, Shizuoka, Japan) [27], rabbit polyclonal serum against human p24 β (Dr T. Nilsson, EMBL, Heidelberg, Germany) [11], bovine α/γ COP antibodies (Dr F. Wieland, EMBL, Heidelberg, Germany) [28], rat p58 antibodies (Dr J. Saraste, University of Bergen, Bergen, Norway) [29], yeast Sec23p antibodies (Dr R. Schekman, University of California, Berkeley, CA, U.S.A.), human calnexin antibodies (Dr J. Bergeron, McGill University, Montreal, Canada), murine ERp72 antibodies (Dr M. Green, St Louis University, St Louis, MO, U.S.A.) and bovine protein disulphide isomerase (PDI) antibodies (Dr N. Bulleid, University of Manchester, Manchester, U.K.) [30].

Subcellular fractionation of organelles from *Xenopus* pituitary tissues

Organelle fractionation of *Xenopus* pituitary tissues was performed using a 10–30 % linear iodixanol gradient (Optiprep™; Nycomed Pharma AS, Oslo, Norway) according to the manufacturer's instructions. In such a fractionation gradient, Golgi membranes can be expected to appear in the lowest-density fractions, whereas higher-density ER membranes migrate further into the gradient. Twenty-five neurointermediate lobes (NILs) or anterior lobes (ALs) were homogenized in 500 μ l of homogenization buffer (0.25 M sucrose, 1 mM EDTA and 10 mM Hepes, pH 7.4) in a glass/glass homogenizer (10 μ m inner space), and the homogenate was centrifuged at 3000 *g* for 10 min at 4 °C. When indicated, NILs were incubated in *Xenopus* culture medium (XL15; 67 % Leibovitz's-15 medium; Life Technologies BRL) in the presence of 50 μ g/ml cycloheximide for 1 h or 5 μ g/ml brefeldin A (BFA) for 2 h at 22 °C prior to homogenization. Following rehomogenization of the pellet in 500 μ l of homogenization buffer and centrifugation (3000 *g* for 10 min at 4 °C), supernatants were pooled and loaded on top of a preformed 11 ml 10–30 % iodixanol gradient and centrifuged for 1.5 h at 26000 *g* in an SW40 rotor (Beckman Instruments, Palo Alto,

CA, U.S.A.). Fractions (1 ml) were collected, with the first fraction being the top of the gradient. All fractions were analysed by enzymic assays, Western blotting or ELISA. For the gradients presented in Figure 5, the total volume of NIL homogenate loaded on the gradient was 500 μ l instead of 1 ml, which resulted in a relative shift of the fraction contents towards the top of the gradient. To allow comparisons between the various subcellular fractionation experiments, the distributions of α - and γ -COP were determined for each experiment.

Enzyme assays

The fractions obtained by subcellular fractionation were tested in enzyme assays for the presence of α -mannosidase II activity, which is found in the Golgi complex and to some extent in the lysosomes, and acid phosphatase (a lysosomal enzyme). α -Mannosidase II activity was measured according to Storrie and Madden [31], and the acid phosphatase assay was performed as described by Graham [32].

Immunodetection of marker proteins in the subcellular fractions

To localize marker proteins in the subcellular fractions, Western-blot analysis was performed. Gradient fractions were loaded on SDS/polyacrylamide gels and proteins were transferred on to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) by electroblotting. Blots were blocked for 1 h in blocking buffer [5 % (w/v) non-fat dried milk powder in PBS containing 1 % (v/v) Tween 20 and 0.3 % Triton X-100] and incubated overnight with specific antibody in blocking buffer. Bound antibodies were detected with peroxidase-conjugated goat anti-rabbit antibodies followed by chemiluminescence (Lumilight, Roche Diagnostics, Mannheim, Germany). In an alternative procedure, the subcellular fractions were sonicated for 10 s in the presence of 0.1 % Triton X-100, coated on microtitre plates and analysed by ELISA.

Deglycosylation with endoglycosidase H (endoH)

Proteins in the subcellular gradient fractions were deglycosylated using endoH (Roche Diagnostics, Mannheim, Germany), which cleaves glycoproteins that contain high-mannose N-glycan chains. Fractions were boiled for 10 min in 50 mM Na/citrate buffer (pH 5.5) containing 0.1 % SDS, supplemented with 0.5 % Nonidet P40, 40 μ g/ml soybean trypsin inhibitor and 40 μ M PMSF, and incubated in the presence or absence of 40 m-units/ml endoH for 18 h at 37 °C.

Primary culture of *Xenopus* melanotrope cells

To perform immunofluorescence localization studies on the melanotrope cells, a primary culture was made of NILs of *Xenopus* that were adapted to a black background. Lobes were dissected, washed several times in sterile XL15 medium, and transferred to Ringer's solution (112 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 15 mM Hepes and 2 mg/ml glucose, pH 7.4) containing 0.25 % (w/v) trypsin. After incubating for 45 min at 20 °C, trypsin activity was blocked by adding XL15 medium supplemented with 10 % (v/v) fetal-calf serum. The lobes were suspended by 15 passes through a siliconized Pasteur pipette, transferred to a syringe and filtered through a nylon filter (pore size of 60 μ m). Cells were collected by centrifugation, resuspended in a small volume of serum-free XL15 medium, and seeded on poly(L-lysine)-coated coverslips. After 1 h, XL15 medium containing 10 % (v/v) fetal-calf serum was added and the cells were cultured overnight at 22 °C before using them for experiments.

Immunofluorescence microscopy

Primary cultures of melanotrope cells were fixed in 2% (w/v) paraformaldehyde/XPBS, pH 7.4 [where XPBS corresponds to 67% (v/v) PBS] for 1 h on ice. All the subsequent steps were performed at 20 °C. The melanotropes were rinsed in XPBS, incubated in 100 mM glycine/XPBS for 30 min and permeabilized by three 5 min washes in 0.1% Triton X-100/XPBS. Antibody incubations were performed sequentially for 1 h in 0.1% Triton X-100/XPBS containing 2% (w/v) BSA. Texas Red-conjugated donkey anti-rabbit antibodies (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.) were used to visualize the first antibody-antigen complex. The second antibody-antigen complex (guinea pig anti-POMC) was visualized using FITC-conjugated donkey anti-guinea pig antibodies (1:300; Jackson ImmunoResearch Laboratories). Finally, coverslips were mounted in 10% (w/v) Mowiol (Calbiochem, La Jolla, CA, U.S.A.), 15% (v/v) glycerol, 2.5% (w/v) NaN₃ and 100 mM Tris/HCl (pH 8.5), and analysed with a DM RB/E microscope (Leica Instruments, Nussloch, Germany). Digital images were obtained using a confocal laser scanning microscope (MRC 1000; Bio-Rad).

RESULTS

Redistribution of p24 proteins from *cis*- to pre-Golgi compartments upon activation of *Xenopus* melanotropes

To establish the distribution of the p24 proteins in biosynthetically active and inactive cells, we performed subcellular fractionation on homogenates of NILs and ALs of pituitaries of black- and white-adapted *Xenopus*. In *Xenopus* adapted to a black background, the intermediate pituitary melanotrope cells are activated and start to produce high amounts of the pro-hormone POMC, whereas in white-adapted animals these cells are virtually inactive [33]. The biosynthetic activity of anterior pituitary cells is not influenced by changing the background of the animal, and these cells were used as a control. The subcellular fractionation was accomplished with an iodixanol density gradient, and Western-blot analysis was used to determine the migration of the p24 proteins on the gradient. We initially started our p24 studies with members of the p24 δ family, of which two (δ_1 and δ_2) are expressed in the *Xenopus* melanotrope cells [24]. In the gradient of the active melanotrope cells of black-adapted animals we found both δ_1 and δ_2 predominantly in fractions 2, 3 and 4, and to a much lesser extent in fraction 5 (Figure 1A). The ratio between δ_1 and δ_2 was always approx. 1:10, as was previously found in unfractionated NIL lysates [24]. Interestingly, in the analysis of the virtually inactive melanotropes of white-adapted animals, the p24 δ doublet was only found in fraction 2 (Figure 1A), again with a ratio similar to what was found for the unfractionated tissues [24]. For the moderately active cells of the ALs of both black- and white-adapted animals, the distribution of the p24 δ proteins was also restricted to fraction 2 (Figure 1A). Apparently, in less active secretory cells the p24 δ proteins have a restricted steady-state localization (fraction 2), whereas in the biosynthetically active melanotrope cells of black-adapted animals these proteins are distributed over a much broader range (fractions 2–5).

To establish which subcellular compartments are present in the various fractions of our gradient, we determined the composition of the fractions by performing both enzymic assays and Western-blot analysis. The luminal ER marker protein PDI [34,35] was restricted to fractions 7–10 (Figure 1B). The COPII subunit Sec23p, which is localized to the ER exit sites and the intermediate compartment [36,37], was found only in fraction 3,

Figure 1 Subcellular fractionation of *Xenopus* pituitary tissues in a 10–30% iodixanol gradient

NILs and ALs of pituitary glands of 20 animals were used for fractionation as described in the Materials and methods section. (A) Western-blot analysis of the p24 δ proteins in the organellar fractions of the NILs and ALs of black- and white-adapted (BA and WA respectively) *Xenopus* was performed using the C-terminally-directed p24 δ antibody. An equivalent of 0.5 NIL/AL was loaded on to the gel, except for the inactive NIL of white-adapted toads where an equivalent of 2.5 NILs was loaded. The data presented are representative examples of the results obtained from three independent experiments. (B) Western-blot analysis demonstrating the localization of the ER marker protein PDI and the Golgi marker α / γ COP in the gradient fractions of black-adapted *Xenopus* NIL homogenate. (C) Distribution of marker proteins in the gradient fractions of black-adapted *Xenopus* NIL homogenate indicating the position of the ER exit sites and the intermediate compartment (Sec23p, $\blacklozenge$; p58, $\blacktriangle$), determined by ELISA, and the Golgi apparatus (mannosidase II, $\bullet$), obtained by an enzyme assay. (D) Western-blot analysis of the neuroendocrine protein SgIII in fractions 1–11 (NIL of black-adapted *Xenopus*), showing the 61/63 kDa precursor form and the 48, 28 and 20 kDa processing products of SgIII.

and the intermediate compartment marker protein p58 [29] in fractions 3 and 4 (Figure 1C). Since the first two fractions of the gradient were positive for the Golgi-localized COPI subunits α -COP and γ -COP (Figure 1B), and displayed α -mannosidase II activity (Figure 1C), these fractions are likely to contain Golgi membranes. Moreover, using an antibody directed against the neuroendocrine secretory protein SgIII [26], we detected the 61/63 kDa precursor forms of this protein in fractions 1–10, whereas the first cleavage product of SgIII (48 kDa), which was found to be formed only after the precursor form is tyrosine-sulphated in the *trans*-Golgi [26], was most prominent in fractions

Figure 2 Subcellular distribution of p24- α_3 , - β_1 and - γ_3 in *Xenopus* NILs

Western-blot analysis of *Xenopus* p24- α_3 , - β_1 and - γ_3 in the first eight subcellular fractions of the NIL homogenates of black-adapted and white-adapted *Xenopus*. The remaining fractions, 9–13, were negative for the presence of these p24 proteins. An equivalent of 0.5 NIL was loaded on to the gel, except for the inactive NIL of white-adapted toads where an equivalent of 2.5 NILs was loaded. Antibodies were against the C-terminal region of *Xenopus* p24- α_3 or - γ_3 , or against the luminal part of human p24 β_1 . The data presented are representative examples of the results obtained from three independent experiments.

1 and 2, and to a lesser extent in fractions 7–10 (Figure 1D). Furthermore, other cleavage products of SgIII were found in fractions 1 and 2, fractions 8–11 (28 kDa), and fractions 8 and 9 (20 kDa; Figure 1D). Together, these findings indicate that the Golgi membranes migrate to fractions 1 and 2 of our gradient, whereas the high-density *trans*-Golgi network and/or the post-*trans*-Golgi network compartments (immature and mature secretory granules) are distributed in fractions 7–11 of our gradient. The positions of the marker proteins in the gradient of melanotrope cells of white-adapted animals or AL cells of black- or white-adapted animals were not different from those in the gradient obtained with melanotropes of black-adapted animals, and thus were not influenced by the biosynthetic activity of the cell. Together, these findings suggest that in the inactive intermediate pituitary cells and in the cells of the AL, the steady-state distribution of p24 δ is restricted to a single Golgi membrane-containing fraction, whereas in the biosynthetically active melanotrope cells approx. 60 % of the δ proteins has shifted to the higher density fractions that contain subcompartments of the ER (ER exit sites) and/or the intermediate compartment.

The members of the p24 family are assembled into hetero-oligomeric complexes [13,14,21,38,39], and influence the localization of each other [11]. We therefore investigated whether in the gradient, in addition to p24 δ , other p24 members expressed in the NIL were also redistributed to pre-Golgi fractions upon adaptation of the animal to a black background. To identify the *Xenopus* representatives of the p24- α , - β and - γ subfamilies that are expressed in the NIL, we screened an NIL cDNA library of black-adapted toads, and found p24 α_3 , p24 β_1 and p24 γ_3 (J. Rötter, R. P. Kuiper, G. Bouw and G. J. M. Martens, unpublished work). Western-blot analysis of the gradient fractions also analysed for p24 δ (see above) revealed that in the fractionated melanotrope cell homogenates from white-adapted *Xenopus*, the p24- α_3 , - β_1 and - γ_3 proteins had a distribution that was restricted to fraction 2, whereas in the analysis of the melanotropes of black-adapted animals approx. 60 % of these proteins migrated to fractions 3 and 4 (Figure 2), similar to what was observed for the p24 δ proteins (Figure 1A). Thus the dynamics in the distribution upon physiological activation of the melanotrope cells is similar for the members of the four p24 subfamilies.

Distribution of POMC in *Xenopus* pituitary cells

The major task of the melanotrope cells of the *Xenopus* intermediate pituitary is the production of the prohormone POMC. This is reflected by an increase in POMC mRNA levels of approx. 30-fold in the biosynthetically activated cells of black-

Figure 3 Distribution of the prohormone POMC in the subcellular fractions of *Xenopus* pituitary homogenates

Western-blot analysis of NIL homogenates of both black- and white-adapted (BA and WA respectively) *Xenopus*, and the AL of black-adapted toads. For immunolabelling of POMC, an antibody directed against ACTH was used. Blots of the white-adapted NIL and black-adapted AL were exposed for considerably longer than that of the black-adapted NIL. The data presented are representative examples of the results obtained from three independent experiments.

adapted animals, and the fact that POMC represents approx. 80 % of the total of newly synthesized proteins. Thus in these active melanotropes POMC is by far the major cargo protein to be transported through the secretory pathway. Since the p24 proteins have been proposed to fulfil an important role in cargo transport between the ER and Golgi complex, we analysed the steady-state localization of POMC in the pituitary cells and compared this localization with that of the p24 proteins. For immunodetection, we used an antibody raised against ACTH, one of the POMC-derived peptide hormones, recognizing POMC and several of its cleavage products. As expected, the amount of POMC that could be detected in the fractions of the NIL gradient of black-adapted animals was much higher than in that of white-adapted toads. Moreover, the former gradient showed a number of cleavage intermediates that were also recognized by the ACTH antibody. These POMC cleavage intermediates, which, as for the cleavage products of SgIII (Figure 1D), indicate the presence of late- and post-Golgi compartments, were found mainly in fractions 2 and 3 (23 and 16 kDa) and fractions 7–10 (< 16 kDa), and could not be observed in the gradient of the NIL of white-adapted animals (R. P. Kuiper, K. P. C. Janssen and G. J. M. Martens, unpublished work). In the gradient of active melanotropes, the 37 kDa precursor form of POMC was abundantly present in fractions 2–7, with highest amounts in fraction 5, whereas in the gradient of inactive melanotropes, POMC was found almost exclusively in fraction 2 (Figure 3). In cells of the AL, POMC had a somewhat broader distribution than in the melanotropes of white-adapted toads (fractions 2–4), but also in this case, POMC migrated predominantly to fraction

Figure 4 Double-immunofluorescence labelling of *Xenopus* p24 δ_2 and POMC in primary cultured pituitary cells

Melanotrope cells of the NIL and a number of POMC-producing cells of the AL of black-adapted *Xenopus* were used. The amount of POMC-producing cells in a cell suspension of the AL was approx. 10%. Antibodies used were anti- δ_2 and the anti-POMC antibody ST-62 that specifically recognizes the precursor form and not the cleavage products of POMC. Bar = 10 μ m.

2 (Figure 3). Thus the high rate of POMC biosynthesis in the melanotropes of black-adapted animals clearly results in a steady-state distribution of POMC that is different from that in the melanotropes of white-adapted animals and the cells of the AL, an observation similar to what was found for the p24 proteins. Although the composition of fractions 5 and 6 could not be determined with marker proteins, the POMC molecules present in these fractions, which are N-linked glycosylated, were sensitive to endoH (R. P. Kuiper and G. J. M. Martens, unpublished work), indicating that these POMC molecules did not pass the medial-Golgi [40]. On the basis of this observation, together with the finding that the *cis*- and medial-Golgi and the intermediate compartment were localized to fractions 1–4, we conclude that the major pool of POMC in fractions 5 and 6 may be located to the ER. It therefore appears that the high levels of POMC that are produced in the melanotrope cells of black-adapted animals can be found almost exclusively in the early compartments of the secretory pathway, with highest amounts in the ER. Although the distribution of POMC in the gradient of melanotropes of black-adapted animals does not completely overlap with that of the p24 proteins, there was a considerable amount of co-distribution of these proteins (Figures 1–3). To further investigate the degree of overlap in the localization of the p24 proteins and POMC, we performed double-labelling immunolocalization experiments with anti- δ_2 and anti-POMC antibodies (Figure 4). To circumvent any interference of POMC-derived cleavage products present in the late secretory pathway, for the immunodetection of POMC we used an antibody that recognizes only the precursor form of POMC. In the melanotropes of black-adapted toads, part of the POMC staining was present in distinct perinuclear structures that completely overlapped with the staining pattern of δ_2 , whereas a substantial amount of POMC could be detected as a diffuse staining throughout the melanotrope cell that was not overlapping with δ_2 (Figure 4). In the melanotropes of white-adapted animals, hardly any POMC could be detected, except for a limited number of cells that showed some POMC-staining in perinuclear structures (R. P. Kuiper and G. J. M.

Martens, unpublished work). This latter finding is in line with previous results showing that a small subpopulation of melanotropes of white-adapted animals is active [41]. In the POMC-producing corticotrope cells of the AL (approx. 10% of the total population of AL cells), the immunolabelling of POMC often showed a typical Golgi staining that, despite their co-migration on the density gradient, had only a limited overlap with δ_2 , and in some cases the staining patterns of the two proteins were totally different (Figure 4). In this respect it is important to note that the diffuse staining pattern of POMC that was observed throughout the active melanotrope cells was not found in the POMC-producing cells of the AL (Figure 4), suggesting that this diffuse pattern is not background staining, but represents POMC molecules localized to the ER. Thus a complete overlap between POMC and δ_2 was observed only in the perinuclear structures in the melanotropes of black-adapted toads. Together, these data indicate that in the biosynthetically active melanotrope cells of the NIL, the localization of POMC partly overlaps with that of the p24 proteins in early secretory pathway compartments, while an additional pool of POMC can be found in p24-negative subdomains of the ER.

Localization of p24 is linked to POMC biosynthesis

The observed differences in subcellular localization of the p24 proteins between the melanotropes of black- and white-adapted animals may be directly related to the very different biosynthetic activities of these cells. To explore this possibility, we investigated whether blocking protein synthesis would affect p24 localization in active melanotropes. NILs of black-adapted toads were incubated for 1 h in the presence of cycloheximide, and subjected to subcellular fractionation and Western-blot analysis. The distributions of α - and γ -COP were determined to establish the reproducibility among the various fractionation experiments (see the Materials and methods section). In this experiment α - and γ -COP were found to be restricted to fraction 1. Upon cyclo-

Figure 5 Effect of cycloheximide on the subcellular localization of p24- δ_1 and - δ_2 in biosynthetically active melanotrope cells

NILs of black-adapted *Xenopus* were incubated in the absence or presence of 50 μ g/ml cycloheximide for 1 h at 22 °C. The homogenates were subsequently subjected to subcellular fractionation and were analysed by Western blotting (the first five fractions are shown). The distributions of α - and γ -COP were determined to enable comparisons between the various subcellular fractionation experiments (see the Materials and methods section). Higher exposures revealed that POMC again migrated further into the gradient (as far as fraction 9) compared with the p24 δ proteins (fraction 1–5). The experiment was carried out in duplicate with similar results.

heximide treatment, the biosynthesis of POMC was decreased (R. P. Kuiper, G. Bouw, K. P. C. Janssen and G. J. M. Martens, unpublished work). Furthermore, at steady-state levels, POMC had shifted towards the top of the gradient (Figure 5). Thus cycloheximide treatment of biosynthetically active NILs resulted in a significant decrease of POMC levels in the higher-density ER-containing fractions. As shown in Figure 5, the p24 proteins δ_1 and δ_2 distributed in the gradient of the untreated NILs to fractions 1–5, with most immunoreactivity in fraction 2. Upon treatment of the biosynthetically active NILs with cycloheximide, δ_1 and δ_2 redistributed towards lower-density fractions, with the highest levels in fraction 1. Again, as was found in the case of the physiologically manipulated NILs (Figures 1 and 2), this redistribution of the δ proteins was accompanied by a similar shift in the distribution of γ_3 (R. P. Kuiper, K. P. C. Janssen and G. J. M. Martens, unpublished work). Thus blocking of protein synthesis in the active melanotropes results in a redistribution of the subcellular localization of the p24 proteins.

Effect of BFA on p24 localization in *Xenopus* melanotropes

The difference in the steady-state localization of the p24 proteins between active and inactive melanotrope cells could be the result of a true redistribution of p24 to other subcellular compartments or, alternatively, may reflect activity-dependent differences in p24 cycling behaviour. To be able to distinguish between these two possibilities, we tried to interfere with any continuous p24 cycling in the active melanotropes using the fungal metabolite BFA, and then analysed the subcellular distribution of the p24 proteins. BFA is known to block COPI-mediated transport, resulting in the dispersion of the Golgi complex and a block in forward transport [42,43]. Several cycling components of the ER–Golgi interface, including members of the p24 family, have been described to accumulate in structures localized to the periphery of the BFA-treated cell [17,21,22,29]. Therefore redistribution of p24 proteins after treatment with BFA serves as a good indication that these proteins are recycling from the Golgi apparatus [21]. In *Xenopus* melanotrope cells, BFA effectively inhibits transport and processing of POMC [44]. Immunofluorescence analysis of BFA-treated *Xenopus* melanotrope cells revealed a drastic redistribution of p24 δ_2 towards peripheral structures (Figure 6A), similar to what was found for other

Figure 6 Effect of BFA on the subcellular localization of p24- δ_1 and - δ_2 in biosynthetically active melanotrope cells

(A) Immunofluorescence analysis of primary cultured melanotrope cells of black-adapted *Xenopus* after a 2 h incubation with 5 μ g/ml BFA using anti- δ_2 . Bar = 5 μ m. (B) Western blot analysis of subcellular fractions of BFA-treated NILs; experimental conditions were the same as those described in (A). The distributions of α - and γ -COP were determined to enable comparisons between the various subcellular fractionation experiments. The experiment was carried out in duplicate with similar results.

members of the p24 family [17,21,22]. Moreover, subcellular fractionation of BFA-treated NILs revealed that the p24 δ proteins were predominantly present in fraction 2 of the gradient, clearly different from the broader distribution observed in untreated cells (compare Figures 6B and 1A). Thus upon BFA treatment the majority of the p24 δ proteins that originally appeared in the pre-Golgi compartments (fractions 3 and 4) redistributed towards lower-density structures, suggesting that this pool of p24 proteins is indeed actively cycling between the ER and Golgi complex.

DISCUSSION

We have analysed the steady-state subcellular distribution of members of the four subfamilies of p24 proteins (p24- α , - β , - γ and - δ) during different states of cellular biosynthetic activity. For the present study, we used the physiologically inducible POMC-producing melanotrope cells of the *Xenopus* intermediate pituitary. These cells regulate background adaptation of this animal and their biosynthetic activity can vary from virtually inactive (in white-adapted toads) to highly active (in black-adapted animals). The analysis of the two physiological states of the melanotropes revealed an interesting correlation between the subcellular sites of p24 localization and biosynthetic cell activity. Using subcellular fractionation, the p24 proteins of the biosynthetically active melanotropes distributed to fractions 2–5, including those containing the intermediate compartment and ER exit sites. In contrast, the p24 proteins of the inactive melanotrope cells were found in the low-density Golgi-containing fraction 2, similar to the situation for the non-induced, moderately active anterior pituitary cells that were used as a control. Furthermore, blocking of protein synthesis in the active melanotropes also caused the redistribution of the p24 proteins to lower-density fractions. From these results we conclude that the distribution of the p24 proteins varies between *cis*- and pre-Golgi

compartments, depending on the biosynthetic activity of the cells.

Melanotrope cell activation is accompanied by a drastic increase in the amount of the cargo protein POMC that has to be transported through the secretory pathway. This extremely high level of cargo transport requires an increase in the capacity of the whole transport machinery, which results in the co-ordinate up-regulation of the expression of a number of proteins, including members of the p24 family [24,45,46] (J. Rötter, R. P. Kuiper, G. Bouw and G. J. M. Martens, unpublished work). We therefore considered the possibility that the appearance of the higher-density p24-containing compartments in the biosynthetically active melanotropes could be caused simply by an increase in the amount of cargo leading to higher densities of these structures, rather than reflecting a true shift in the steady-state localization of the p24 proteins to other compartments. However, our subcellular fractionation data indicate that the localization of the p24 proteins changes relative to markers of the early secretory pathway, which suggests that the density of these compartments is not influenced by the high amounts of POMC produced in the active melanotropes. Earlier studies on the localization of p24 family members in transfected tumour cells in culture have revealed that overexpression of p24 proteins can result in the appearance of artificial membranous structures, especially when individual members are overexpressed [11,21,47,48]. We believe that the change in distribution of the p24 proteins observed in the present study is not caused by such a phenomenon, since the biosynthetic activation of the melanotrope cells is a physiological process that is necessary for efficient, high-level transport and processing of POMC. Together, we conclude that *Xenopus* melanotrope cell activation leads to the localization of p24 proteins in a broad spectrum of compartments, including the *cis*-Golgi, the intermediate compartment and, most probably, the ER exit sites.

Similar to what was observed for the p24 proteins, the subcellular localization of POMC was found to be dependent on the biosynthetic activity of the cell. In the gradient containing the NIL compartments of white-adapted toads, POMC co-migrated with the p24 proteins to the low-density fraction 2. In the subcellular fractions containing the highly active NIL compartments of black-adapted toads, however, only low amounts of the prohormone were localized to the low-density fraction, while the majority was found in a number of higher-density fractions. Hence, both the p24 family members and the cargo protein POMC are distributed over a broader range of subcellular compartments when the melanotrope cells become active. However, POMC and the p24 proteins were not completely overlapping in the active melanotrope cells, since a substantial amount of POMC migrated further into the density gradient (mainly to fraction 5; Figure 3). This finding is in line with our immunofluorescence data with primary melanotrope cells in culture, showing that structures containing p24 δ_2 were always positive for POMC, while a substantial amount of POMC did not co-localize with δ_2 and was present as a diffuse staining throughout the melanotrope cells. Thus two pools of POMC could be identified in the melanotrope cells of black-adapted toads. The first pool, which distributed to fractions 2–4 of the density gradient in Figure 3 and co-localized with the p24 proteins, is probably present in the ER exit sites, and the intermediate- and *cis*-Golgi compartments. The second, p24-negative pool of POMC is, although only partially overlapping with the ER marker PDI, most probably localized to (subdomains of) the ER, since (1) it was sensitive to endoH and thus did not pass the medial-Golgi, and (2) migrated to fractions 5–7 (in Figure 3), while the Golgi and intermediate compartments are in the first

four fractions of the gradient. Together, these data indicate that the high expression of POMC in the melanotropes of black-adapted animals leads to a steady-state localization of this prohormone in the early compartments of the secretory pathway. The p24-negative pool of POMC may represent freshly made molecules that just entered the ER lumen, which may suggest that in these cells ER exit is a rate-limiting step in POMC biosynthesis.

The very high level of POMC biosynthesis in the melanotropes of black-adapted toads requires a highly active and efficient ER-to-Golgi transport machinery. Our observation that the p24 proteins have a broader subcellular distribution in the highly active melanotrope cells may be a direct result of this increased vesicular transport. This assumption was confirmed by our experiments with BFA, a metabolite that is known to interfere with the cycling of components between the ER and Golgi complex [21,49]. BFA treatment of active melanotropes led to a redistribution of pre-Golgi-localized p24 molecules towards low-density Golgi-like peripheral structures. Thus the observed change in steady-state p24 localization actually indicates that the dynamics of p24 cycling has changed, and that in the highly active melanotropes the time period that the p24 proteins reside in the ER and intermediate compartment is longer than in less active cells.

The hypothesis that p24 proteins play a role in protein transport has been well accepted [50]. Our finding that the sites of localization of p24 proteins in the secretory pathway of *Xenopus* melanotropes are linked to the biosynthetic activity of the cell is in line with this hypothesis. How can this notion be correlated to existing models for p24 function? The ability of some members of the p24 family to interact with coatomer and their enrichment in COPI-coated vesicles led to the hypothesis that p24 proteins could act as coatomer receptors, driving and regulating the formation of COPI-coated vesicles [20,51,52]. However, the pre-Golgi compartments that accumulate high amounts of p24 proteins in the biosynthetically active *Xenopus* melanotropes do not contain COPI, suggesting that an additional role for the p24 proteins may exist in these compartments. Several functional models have been proposed in which the p24 proteins fulfil a regulatory role during the inclusion of cargo in transport vesicles at the ER membrane. For instance, p24 proteins could act as cargo receptors, directly interacting with cargo and thereby facilitating cargo inclusion in COPII-coated vesicles at the ER membrane [12,15]. Furthermore, since in yeast the p24 proteins are not essential for COP-mediated vesicular transport in the early secretory pathway, they may be indirectly involved in a selection mechanism during vesicle formation through the active exclusion of misfolded cargo proteins (as part of the quality control mechanism) or ER resident proteins [16,18,50,53]. Finally, p24 proteins have been proposed to delay the budding process, enabling correct packaging of cargo in vesicle buds, or to create membrane rafts that define nucleation sites for the generation of vesicles and tubules [47,50,54]. All of these models have in common that the p24 proteins would control the selectivity during cargo packaging at the ER membrane. In the biosynthetically active melanotropes of black-adapted *Xenopus*, the extremely high level of POMC biosynthesis may increase the need for regulation at this stage, which would explain the shift in steady-state distribution of the p24 proteins in these cells during the process of background adaptation of the animal.

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