A Cell-Specific Transgenic Approach in *Xenopus* Reveals the Importance of a Functional p24 System for a Secretory Cell

Gerrit Bouw, Rick Van Huizen, Eric J.R. Jansen, and Gerard J.M. Martens*

Department of Molecular Animal Physiology, Nijmegen Center for Molecular Life Sciences, University of Nijmegen, 6525 GA Nijmegen, The Netherlands

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The p24α, -β, -γ, and -δ proteins are major multimeric constituents of cycling endoplasmic reticulum-Golgi transport vesicles and are thought to be involved in protein transport through the early secretory pathway. In this study, we targeted transgene overexpression of p24α, specifically to the *Xenopus* intermediate pituitary melanotrope cell that is involved in background adaptation of the animal and produces high levels of its major secretory cargo proopiomelanocortin (POMC). The transgene product effectively displaced the endogenous p24 proteins, resulting in a melanotrope cell p24 system that consisted predominantly of the transgene p24α protein. Despite the severely distorted p24 machinery, the subcellular structures as well as the level of POMC synthesis were normal in these cells. However, the number and pigment content of skin melanophores were reduced, impairing the ability of the transgenic animal to fully adapt to a black background. This physiological effect was likely caused by the affected profile of POMC-derived peptides observed in the transgenic melanotrope cells. Together, our results suggest that in the early secretory pathway an intact p24 system is essential for efficient secretory cargo transport or for supplying cargo carriers with the correct protein machinery to allow proper secretory protein processing.

INTRODUCTION

Transport of cargo proteins through the early secretory pathway involves cargo selection, transport vesicle formation, quality control to recycle misfolded cargo, and cycling of the COPI- and COPII-coated vesicles between the endoplasmic reticulum (ER) and Golgi (Barlowe, 2000). One of the major constituents of the transport vesicles is the p24 family of type I transmembrane proteins that can be classified into four main subfamilies, designated p24α, -β, -γ, and -δ (Schimmöller et al., 1995; Stamnes et al., 1995; Sohn et al., 1996; Nickel et al., 1997; Dominguez et al., 1998). The p24 proteins share a number of structural characteristics, such as a relatively large luminal putative cargo-binding domain, a coiled-coil region thought to be involved in the formation of multimeric p24 complexes, a transmembrane region, and a short cytoplasmic tail containing COPI- and COPII-binding motifs that are used for p24 traveling from the ER to the Golgi and back (for review, see Kaiser, 2000). In yeast and mammalian cells, p24 proteins form functional heterotetrameric complexes containing one representative of each subfamily, whereby the composition of the complex may differ in various cell types (Dominguez et al., 1998; Füllekrug et al., 1999; Marzioch et al., 1999; Ciufò and Boyd, 2000; Emery et al., 2000; Belden and Barlowe, 2001). Furthermore, the stability of the p24 members seems to be compromised when cells are deficient in the expression of a single p24 protein (Marzioch et al., 1999; Denzel et al., 2000). Recent evidence suggests a complex and dynamic p24 system of mostly monomers and homo-/heterodimers and that the degree of oligomerization constantly alters and largely depends on the subcellular localizations of the p24 subfamily members (Jenne et al., 2002).

The p24 proteins have been suggested to play a key role in cargo-selective protein transport at the ER/Golgi interface (Kaiser, 2000). For the elusive mechanism of action of p24, a number of functional models have been proposed, including a role as cargo receptor, membrane organizer, or regulator of vesicle budding, as well as in the ER quality control system, or excluding ER resident proteins from the vesicular lumen (Schimmöller et al., 1995; Elrod Erickson and Kaiser, 1996; Rojo et al., 1997; Bremsner et al., 1999; Lavoie et al., 1999; Wen and Greenwald, 1999; Denzel et al., 2000; Kaiser, 2000; Muñiz et al., 2000; Springer et al., 2000; Belden and Barlowe, 2001). Defining the importance of a functional p24 system for proper cell physiology has however turned out to be difficult. For instance, deletion of all p24 proteins resulted in viable yeast (Marzioch et al., 1999; Springer et al., 2000), whereas genetic ablation of a single p24 family member caused early lethality in mice (Denzel et al., 2000). To investigate the significance of the p24 system in a highly specialized secretory cell, we decided to use a physiological model (background adaptation of the South-African clawed frog, *Xenopus laevis*) with a well-defined secretory cell (the intermediate pituitary melanotrope cell) and its single major soluble cargo protein proopiomelanocortin (POMC) (Roubos, 1997). In the trans-Golgi network/mature secretory granules of *Xenopus* melanotrope cells, endoproteolytic cleavage of POMC results in a number of bioactive peptides, including α-melanophore-stimulating hormone (α-MSH). This hormone mediates adaptation of the animal to a black background by causing dispersion of melanin pigment granules (melanosomes) in skin melanophores. On a black back-
ground, the melanotrope cell is dedicated to produce vast amounts of POMC such that this prohormone represents ~80% of all newly synthesized melanotrope proteins. On a white background, POMC mRNA levels are decreased ~30-fold (Holthuis et al., 1995a) and α-MSH secretion from the melanotropes into the bloodstream is inhibited by neurons of hypothalamic origin that directly innervate the cells (Jenks et al., 1993; Tuinthof et al., 1994), leading to melano-some aggregation and, consequently, pallor of the skin. Placing Xenopus on a black or a white background therefore allows physiological manipulation of the biosynthetic and secretory activities of the melanotrope cell. Using a differen-tial screening approach, we have identified a number of proteins coexpressed with POMC and thus differentially expressed in the melanotrope cells of black- and white-adapted Xenopus, including the POMC cleavage enzyme prohormone convertase PC2 and a member of the p24 family, namely, Xp24α (Holthuis et al., 1995b). Subsequent ex-tensive cDNA library screening resulted in the identification of all members of the p24 family that are expressed in the Xenopus melanotrope cell (Xp24α, -β1, -γ2, and -δ1, 2) (Röt-ter et al., 2002). Of these, Xp24α, -β1, -γ2, and -δ2 constitute the major representatives and are highly up-regulated with POMC in the melanotropes during black background adaptation (at least 20-fold), whereas the two low-abundant ones (Xp24γ and -δ4) are not or only slightly induced (Kuiper et al., 2001; Rötter et al., 2002). The coordinate and induced expression of a selective set of Xenopus p24 proteins (Xp24α, -β1, -γ2, and -δ3) in the melanotrope cell suggests that these p24 members are somehow involved in POMC biosynthesis. To explore the importance of p24 in the Xenopus melanotrope cell, we combined the unique properties of this cell with the technique of stable Xenopus transgenesis by using a Xenopus POMC gene promoter fragment to target transgene expression specifically to the melanotrope cell, leaving the integrity of the regulation by the hypothalamic neurons intact. For transgenic overexpression, we selected one of the Xenopus melanotrope p24 proteins coexpressed with POMC, namely, the Xp24α protein, and fused it to the N terminus of the green fluorescent protein (GFP). Here, we report the effect of this transgenic manipulation of the endogenous p24 system on the functioning of the Xenopus melanotrope cells.

**Materials and Methods**

**Animals**

X. laevis were reared in the Central Animal Facility of the University of Nijmegen (Nijmegen, The Netherlands). For the transgenesis experiments, female X. laevis were obtained directly from South Africa. For background adaptation, the animals were kept in either white or black containers under constant illumination for at least 3 wk. All animal experiments were carried out in accordance with the European Communities Council Directive 86/609/EEC for animal welfare, and permit TRC 99/15072 to generate and house transgenic Xenopus.

**Antibodies**

The rabbit polyclonal antibodies against portions of the luminal and C-terminal regions of Xp24α (anti-1262N and anti-1262C, respectively), against part of the luminal region of Xp24α (anti-RH6), and against a region in the luminal part of Xp24α, have been described previously (Kuiper et al., 2001; Rötter et al., 2002). A polyclonal antibody to human p24α (p24α) was kindly provided by Dr. I. Schulz (University of the Saarland, Heidelberg; Germany; Dominguez et al., 1998), against recombinant mature human PC2 by Dr. W.J.M. Van de Ven (University of Leuven, Belgium; Van Horsens et al., 1996), to GFP by Dr. J. Fransen (Cuppen et al., 1999), and against Xenopus POMC (ST62, recognizing only the precursor form) by Dr. S. Tanaka (Shizuoka University, Hamamatsu, Japan; Bergs et al., 1997).

**Generation of Xenopus Transgenic for Xp24α-GFP**

A linear 2166bp-base pair SalI/Nari DNA fragment encoding the Xenopus p24α protein with the enhanced GFP protein fused in frame to its C terminus (Xp24α-GFP fusion protein) and cloned behind a 520-bp fragment of Xenopus POMC gene a promoter (pPOMC: Jansen et al., 2002) fragment (construct pPOMC-Xp24α-GFP) was used for stable Xenopus transgenesis (Kroll and Amaya, 1996; Sparrow et al., 2000). A number of injection reactions resulted in animals transgenic for Xp24α-GFP and expressing the fusion protein at various levels (animals 115 and 125 with moderate and 124 with high expres-sion levels). The number of integration sites and integrated copies of the transgene were determined by Southern blot analysis of genomic DNA iso-lated (Rebel et al., 2003) in three sites of integration, and ~25, 2, and ~20 integrated copies of the transgene in animals 115, 125, and 124, respectively. To generate F1 offspring, the testes of male transgenic Xenopus frogs were isolated and for in vitro fertilization pieces of testis were incubated with eggs harvested from wild-type Xenopus females.

**Microscopy**

For ultrastructural analysis, electron microscopy was performed as described previously (de Rijk et al., 1990). Ultrastructural (immunolocalization studies were performed on neurointermediate lobes (NILs) of wild-type Xenopus and #124 and #224 transgenic animals expressing Xp24α-GFP at high levels. Entire lobes were fixed for 1 h at room temperature in 2% paraformaldehyde + 0.01% glutaraldehyde in PHEM buffer (50 mM MgCl2, 70 mM KCl, 10 mM EGTA, 20 mM HEPS, 60 mM PIPES, pH 6.8). Fixed tissue was stored in 1% paraformaldehyde in 0.1 M phosphate buffer until use. Ultrathin cryosectioning was performed as described previously (Fransen et al., 1985; Schweizer et al., 1994). Sections were incubated with an antiserum against the recombinant green fluorescent protein at a 1:100 dilution followed by protein A complexed with 10-nm gold (Fransen et al., 1985). Electron microscopy experiments using the anti-Xp24α antibodies were not successful. Electron microscopy was performed using a JEOL 1010 electron microscope operating at 80 kV. For confocal microscopy, brains with the pituitaries attached were dissected and fixed in 4% paraformaldehyde in phosphate-buffered saline. After cryo-protection in 30% sucrose-phosphate-buffered saline, sagittal 20-μm cryosections were mounted on poly-l-lysine-coated slides, dried for 2 h at 45°C, and studied with an MRC 1024 confocal laser scanning microscope (Bio-Rad, Hercules, CA). To examine direct fluorescence as a result of GFP fusion protein expression, cryosections were directly viewed under a Leica DM RA fluorescent microscope and photographed were taken with a Cohu high-performance charge-coupled device camera using the Leica Q Fluoro software. Immunohistochemistry for POMC and α-MSH was performed as described previously (Jansen et al., 2002). For light microscopy analysis of the webs and melanophores of wild-type and transgenic animals, webs were cut out, mounted on slides, and coverslipped. Digital images were obtained using a Leica MZ Fl III microscope mounted with a DC200 digital color camera. Equal integration intervals and magnifications were used to capture images with Leica DC viewer software.

**Western Blot Analysis**

Western blot analysis was performed as described previously (Kuiper et al., 2000) for quantification, detection was performed using a Bio-Imaging system, and signals were analyzed using the Labworks 4.0 program (UVP Biol imaging systems, Cambridge, United Kingdom).

**Pulse and Pulse-Chase Analysis**

For metabolic labeling, NILs from wild-type and transgenic Xenopus were preincubated for 30 min, pulse labeled in the presence of 5 μCi/ml Tran 35S-label (ICN Radiochemicals) and chased with 0.5 mM unlabeled methionine, and against as described previously (Braks and Martens, 1994). Parts of the lysates and incubation media were analyzed directly on SDS-PAGE, while the remainder was used for immunoprecipitation, western blot and/or high-performance liquid chromatography (HPLC) analysis.

**Immunoprecipitation Analysis**

For immunoprecipitation analysis, NILs were lysed with lysis buffer to 1 ml, and supplemented with SDS (final concentration of 0.07%) and the respective antibodies. Precipitation was performed overnight at 4°C while rotating the samples. Immune complexes were precipitated with protein A-Sepharose (Amersham Biosciences, Fiscatay, NJ) and resolved by SDS-PAGE. Radiolabeled proteins were detected using autoradiography at ~70°C or a Phosphomager (Personal FX; Bio-Rad).

**HPLC Analysis**

For the separation of the small newly synthesized end products of POMC processing, radiolabeled NIL lysates were subjected to HPLC analysis as described previously (Martens et al., 1982a).
RESULTS

Generation of Xenopus Transgenic for Xp24\(_{\alpha}\)-GFP

To generate Xenopus transgenic for the Xenopus p24\(_{\alpha}\) protein with GFP fused to its C terminus (Xp24\(_{\alpha}\)-GFP), we first made a DNA construct (pPOMC-Xp24\(_{\alpha}\)-GFP) containing a 529-base pair Xenopus POMC gene promoter fragment in front of the sequence encoding the fusion protein. The GFP moiety was fused to the C terminus of the Xp24\(_{\alpha}\)-protein to avoid interference with a possible binding of cargo to the N-terminal loop domain of Xp24\(_{\alpha}\). The pPOMC-Xp24\(_{\alpha}\)-GFP DNA was mixed with Xenopus sperm nuclei and the mixture was microinjected into unfertilized Xenopus eggs. The different levels of expression of the fusion protein among the various transgenic animals could be readily and directly established by visual inspection of the living embryos under a fluorescence microscope (Figure 1A). Lifting the brain of the transgenic animal showed that the expression of the Xp24\(_{\alpha}\)-GFP fusion protein was restricted to cells located in the intermediate lobe of the pituitary, and no fluorescence was observed in the anterior lobe of the pituitary or in any other brain structures (Figure 1B). An immunocytochemical analysis revealed that the fusion protein was coexpressed in the melanotrope cells with POMC and \(\alpha\)-MSH (data not shown). Adaptation of the transgenic animals to a black or a white background resulted in high and low levels of fluorescence in the intermediate pituitary, respectively (Figure 1C), suggesting that the level of Xp24\(_{\alpha}\)-GFP transgene expression was dependent on the color of the background of the animal and coregulated with POMC expression. Thus, the 529-base pairs of Xenopus POMC gene promoter fragment was sufficient to drive melanotrope cell-specific expression of the transgene and give different levels of transgene expression depending on background color.

Steady-State p24 Protein Levels in the Pituitary of Xenopus Transgenic for Xp24\(_{\alpha}\)-GFP

From the pituitary (consisting of the pars nervosa and the anterior and intermediate lobes), the anterior part can be dissected but the pars nervosa (biosynthetically not active nerve terminals of hypothalamic origin) is intimately associated with the intermediate pituitary (the neuroendocrine nerve terminals of hypothalamic origin) is intimately associated with the intermediate pituitary (the neuroendocrine nerve terminals of hypothalamic origin). For our studies, we therefore used the anterior lobe (AL) and NIL (pars nervosa plus intermediate lobe) of the pituitary. Western blot analysis of p24 steady-state protein levels was performed on lysates of NILs and ALs of wild-type and transgenic Xenopus. NILs were pulse labeled for 1 h, part of the total cell lysates was analyzed directly on SDS-PAGE, and radiolabeled proteins were visualized by fluorography.

Figure 1. Xp24\(_{\alpha}\)-GFP transgene expression is specific to Xenopus intermediate pituitary and dependent on background color. (A) Pituitary-specific fluorescence in transgenic Xenopus embryos. Shown are living stage 45 embryos, whereby the arrows indicate the locations of the pituitaries with various levels of transgene expression. Fluorescent pituitaries expressing the transgene fusion product could be detected from stage 25 onwards. Bar, 0.4 mm. (B) Fluorescence is specific to the intermediate pituitary of transgenic Xenopus. Ventrocaudal view on the brain that was lifted to reveal the bright fluorescence caused by the Xp24\(_{\alpha}\)-GFP fusion protein and observed in the intermediate lobe (IL), but not in the AL, of the pituitary of a black-adapted transgenic frog of 6 mo. Bar, 0.5 mm. (C) Fluorescence in the intermediate lobe of black- (BA) and white- (WA) adapted transgenic (tr) Xenopus. Ventrocaudal view with the anterior part of the pituitary removed. Bar, 0.5 mm. (D) Western blot analysis of p24 protein expression in the NIL and AL of black-adapted wild-type (wt) and transgenic (tr) Xenopus. (E) Western blot analysis of p24 protein expression in the NIL of black- and white-adapted wild-type and transgenic Xenopus using the p24\(_{\alpha}\)-\(\beta\) antibody mix. (F) Newly synthesized proteins produced in NILs of BA and WA wild-type and transgenic Xenopus. NILs were pulse labeled for 1 h, part of the total cell lysates was analyzed directly on SDS-PAGE, and radiolabeled proteins were visualized by fluorography.
recognizes endogenous Xp24δ and Δ24 with comparable affinities (Kuiper et al., 2000). With this antibody, we detected ~8 and ~3 times more of the ~24-kDa Xp24δ protein than ~23-kDa Xp24δ in the wild-type NIL and AL, respectively (Figure 1D, top). However, the C-terminally directed antibody 1262C hardly recognized the Xp24δ-GFP fusion protein (Figure 1D, compare lanes 2 of top and bottom panels), presumably because of the fusion of GFP to the C terminus of Xp24δ. For the simultaneous detection of the transgene and endogenous Xp24δ products, we therefore used in all subsequent experiments a mixture of anti-Xp24δ and anti-Xp24β antibodies (RH6 and 1262N, respectively), each directed against a portion of the respective N-terminal region and specifically recognizing the corresponding Xp24δ protein. This antibody mix showed in the wild-type NIL about equal amounts of the endogenous Xp24δ and Xp24β proteins (Figure 1D, bottom, lane 1). In the transgenic NIL, the antibodies revealed an additional product of ~52 kDa, presumably corresponding to the transgene Xp24δ-GFP fusion protein (~24 kDa for Xp24δ, and ~28 kDa for GFP) (Figure 1D, bottom, lane 2). The fusion protein was found only in the NIL and not AL (Figure 1D, bottom), again indicating that the expression of the transgene product is melanocyte cell specific. In the transgenic cells, the fusion protein was about ~15-fold higher in black than in white animals (Figure 1E), in line with the data obtained by direct fluorescence analysis (Figure 1C). Furthermore, metabolic labeling of wild-type and transgenic NILs revealed an approximately ninefold higher level of newly synthesized Xp24δ-GFP fusion protein in black- than in white-adapted #124 transgenic animals, similar to the ~10-fold difference in radio labeled POMC precursor levels (Figure 1F). Having established that transgene expression is coregulated with POMC and specific for the melanocyte cells, we then wondered what the effect of the overexpression of the Xp24δ-GFP fusion protein would be on the levels of the endogenous p24 proteins. For this and subsequent analyses, two male transgenic Xenopus that differed in Xp24δ-GFP expression levels (animals 124 and 125) were selected and used to generate F1 offspring by in vitro fertilization. Western blot analysis revealed that the expression of the transgene product was approximately fourfold higher in #124 than in #125 transgenic melanocyte cells (Figure 2A). The overexpression of the Xp24δ-GFP fusion protein resulted in reduced levels of the endogenous Xp24δ and Δ24 proteins in #125 cells (~40 and ~54% reduction, respectively), whereas in #124 cells the two endogenous Xp24δ proteins were even nearly completely displaced (~87 and ~95% reduction of Xp24δ and Δ24, respectively) (Figure 2A). These findings indicate that high levels of the transgene product cause low levels of the endogenous Xp24δ proteins. To examine whether the degree of competition between the exogenous and endogenous Xp24δ proteins was correlated with the level of newly synthesized Xp24δ-GFP produced in the #124 and #125 transgenic NILs, we performed metabolic cell-labeling experiments. Direct SDS-PAGE analysis of newly synthesized NIL proteins revealed an ~52-kDa radiolabeled product in #124 transgenic but not in #125 transgenic or wild-type cells (Figure 2B, left). The ~52-kDa product comigrated with a radiolabeled protein immunoprecipitated with the anti-Δ24/Δ6 antibody mix from newly synthesized proteins produced by transgenic NILs (Figure 2B), indicating that it represents the newly synthesized Xp24δ-GFP fusion protein. The #125 melanocyte cells produced approximately fivefold and the #124 cells at least 15-fold more newly synthesized transgene Xp24δ-GFP product than newly synthesized endogenous Xp24δ protein. The lower level of immunoprecipitated newly synthesized endogenous Xp24δ in the #125 cells was likely due to the high amount of competing radiolabeled transgene Δ24 fusion product, because in cells from the independent line 115 with less transgene expression, the amount of immunoprecipitated endogenous Xp24δ was not affected (Figure 2B, right). Therefore, the biosynthesis of the endogenous Xp24δ and Δ24 proteins does not seem to be affected by the transgene expression. Together, the above-mentioned findings indicate that in the #124 melanocyte cells the high level of Xp24δ-GFP protein biosynthesis resulted in lower amounts of the endogenous Xp24δ and Δ24 proteins than in the #125 cells and, thus, that the level of transgene expression is correlated with the degree of displacement of the endogenous Xp24δ proteins by the exogenous fusion product.

We next examined what the consequences of the expression of the Xp24δ-GFP protein were on the steady-state expression levels of the major endogenous Xenopus melanotrope p24 members other than the Xp24δ proteins. Overexpression of the fusion protein in #124 transgenic melanocyte cells led to a more than fivefold reduction in the amounts of the endogenous Xp24βα-β2, -γ1, and -γ2 proteins, whereas these levels were essentially unchanged in the #125 cells (Figure 2C). The level of expression of the transgene product therefore seems to determine the degree of displacement not only of the endogenous Xp24δ proteins but also of the other endogenous p24 members. Together, we conclude that in the #124 transgenic melanocyte cells the exogenous Xp24δ-GFP fusion protein caused a drastic reduction in the amounts of the endogenous p24 members, resulting in a p24 system predominantly consisting of the transgene product.

Microscopy Analyses of Xenopus Melanocyte Cells Transgenic for Xp24δ-GFP

In transfected mammalian cells in culture, overexpression of p24δ (p23) or p24β (p24) caused the induction of an expansion of smooth ER membranes (Rojo et al., 1997; Blum et al., 1999). We therefore wondered what in the #124 transgenic Xenopus melanocyte cells the effect of the overexpression of the Xp24δ-GFP protein would be on the morphology of subcellular structures. Electron microscopy analyses were performed on intermediate pituitaries of both black- and white-adapted wild-type and #124 transgenic animals. Despite the severely affected p24 system in the #124 transgenic melanocyte cells, at the ultrastructural level no gross morphological differences were observed between the wild-type and transgenic cells (Figure 3A). As expected, the melanocyte cells of black-adapted animals showed extensive ER structures as these cells are highly active in synthesizing large amounts of POMC. The melanotropes of white-adapted animals showed virtually no ER structures but many storage granules, reflecting their biosynthetic and secretory inactivity (Figure 3A). We can thus conclude that the structural changes occurring in the melanocyte cells during background adaptation of the animal are similar in the wild-type and #124 transgenic cells, and consistent with previous electron microscopy studies on wild-type Xenopus melanocyte cells (Weatherhead et al., 1971; de Rijk et al., 1990). The Xp24δ-GFP fusion protein was found to be capable of reaching the Golgi, because confocal microscopy on the transgenic melanocyte cells revealed that both ER- and Golgi regions displayed fluorescence (our unpublished data), and immunoelectron microscopy confirmed that the Xp24δ-GFP fusion protein was localized to structures that resemble the ER and the Golgi (Figure 3B). These results are in line with previous findings showing that endogenous Xp24δ localizes to the ER and the Golgi in wild-type Xen-
pus melanotrope cells (Kuiper et al., 2001) and that p24 proteins shuttle between the ER and the Golgi (Sohn et al., 1996; Barlowe, 1998; Dominguez et al., 1998). Together, these observations suggest that the overexpression of the transgene product was to such an extent that in the transgenic cells the early secretory pathway was not destroyed and that the transgene product was localized to the proper secretory pathway subcompartments.

**Background Adaptation of Xenopus Transgenic for Xp24δ-GFP**

In *Xenopus*, the intermediate pituitary melanotrope cells to which we specifically targeted transgene expression are involved in the process of background adaptation (Jenks et al., 1977). This fact, together with the disrupted p24 machinery in the #124 *Xenopus* transgenic melanotrope cells, prompted us to examine the physiological consequence of this situation for background adaptation of the transgenic animal. After their metamorphosis, animals were placed on a black background for four months and thus the melanotrope cells were biosynthetically very active during a relatively long time period. As expected, wild-type *Xenopus* were black and contained many completely dispersed pigment-filled granules in the dermal melanophores of their webs. After the long adaptation to a black background, the skin color of the #124 transgenic animal was lighter than those of wild-type and #125 animals. On closer inspection of the webs, only in the vicinity of blood vessels were pigment-containing web melanophores observed, and the number and sizes of melanophores were clearly reduced in the #124 transgenic animal (approximately five- and threefold reduction, respectively) (Figure 4). These results indicate that the transgenic manipulation of the p24 system exclusively in the

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**Figure 2.** Xp24δ-GFP protein levels in transgenic *Xenopus* intermediate pituitary determine the degree of displacement of the endogenous p24 proteins. (A) Western blot analysis of Xp24δ protein expression in the NIL of wild-type (wt) and transgenic (#125 and #124) *Xenopus* by using an anti-Xp24δ/-δ, antibody mix. (B) Newly synthesized proteins produced in NILs of wild-type and transgenic *Xenopus*. NILs were pulse labeled for 1 h, and parts of the total cell lysates were analyzed directly on SDS-PAGE (left) or immunoprecipitated using an anti-Xp24δ/-δ, antibody mix followed by resolving the immunoprecipitates on SDS-PAGE (right). Radiolabeled proteins were visualized by fluorography. Asterisks indicate POMC- and PC2-related proteins binding nonspecifically to the antibodies. (C) Western blot analysis of Xp24α-, β, and γ protein expression in the NIL of wild-type and transgenic *Xenopus*. 

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Xenopus melanotrope cells led to a physiological effect regarding morphological changes in skin melanophores.

**Steady-State Protein levels of POMC and PC2 in Xenopus Melanotrope Cells Transgenic for Xp246-GFP**

Because the process of background adaptation is mediated by α-MSH, a cleavage product of POMC, we next examined by Western blot analysis whether the altered p24 system had affected the steady-state level of the 37-kDa POMC precursor in the transgenic melanotrope cells. No differences in POMC levels were observed between wild-type and #124 transgenic NILs of black-adapted animals (Figure 5). On white-background adaptation of the transgenic animals, the amount of the POMC protein decreased to similar levels as observed in wild-type melanotrope cells of white animals (at least 10-fold reduction; Figure 5). Likewise, in the #124 transgenic cells of black-adapted animals, the steady-state amounts of both the proenzyme and mature forms of the POMC cleavage enzyme PC2 (75-kDa proPC2 and 69-kDa PC2, respectively) were not affected when compared with those in the wild-type situation. Furthermore, in both the inactive wild-type and transgenic cells of white animals, the expression of the proPC2 protein was greatly reduced (at least 15-fold), whereas the amount of mature PC2 remained essentially the same as in black-adapted animals (Figure 5). These results indicate that the steady-state POMC and proPC2 protein levels, and the changes in these levels in the transgenic NILs of black-adapted animals (Figure 5). On white-background adaptation of the transgenic animals, the amount of the POMC protein decreased to similar levels as observed in wild-type melanotrope cells of white animals (at least 10-fold reduction; Figure 5). Likewise, in the #124 transgenic cells of black-adapted animals, the steady-state amounts of both the proenzyme and mature forms of the POMC cleavage enzyme PC2 (75-kDa proPC2 and 69-kDa PC2, respectively) were not affected when compared with those in the wild-type situation. Furthermore, in both the inactive wild-type and transgenic cells of white animals, the expression of the proPC2 protein was greatly reduced (at least 15-fold), whereas the amount of mature PC2 remained essentially the same as in black-adapted animals (Figure 5). These results indicate that the steady-state POMC and proPC2 protein levels, and the changes in these levels induced by the process of background adaptation were not affected by the introduction of the transgene into the melanotrope cells.

**Biosynthesis and Processing of Newly Synthesized POMC and proPC2 in Xenopus Melanotrope Cells Transgenic for Xp246-GFP**

We next studied the dynamics of protein synthesis by performing in vitro pulse- and pulse-chase analyses of newly synthesized proteins produced in wild-type and transgenic NILs. Because besides the melanotrope cells, the Xenopus NIL consists of nerve terminals of hypothalamic origin that are biosynthetically not active (the pars nervosa), the radio-labeled proteins are synthesized by the melanotropes. During the 10-min pulse incubation of wild-type NILs, the 37-kDa POMC precursor protein was clearly the major newly synthesized protein (Figure 6, A and B, lane 1). During the subsequent 1.5-h and 2.5-h chase incubations, 37-kDa POMC was gradually processed to an 18-kDa cleavage product (Figure 6A, top left and B, lane 2). This product represents the N-terminal portion of 37-kDa POMC, is generated by the first endoproteolytic cleavage step during POMC processing and contains the only N-linked glycosylation site present in the POMC molecule (Martens, 1986). The amount of the 18-kDa POMC protein was lower for the 2.5-h than for the 1.5-h time point, because during the chase period this newly synthesized product is processed further (to γ-MSH; Martens et al., 1982b) (Figure 6A, top left). During the 10-min pulse, the POMC cleavage enzyme PC2 was synthesized as a 75-kDa proenzyme form that in the course of the subsequent 1.5-h and 2.5-h chase incubations was processed to a 69-kDa mature form of PC2 that represents the end product of proPC2 processing (Figure 6A, top right, and B, lanes 1 and 2). Within the time frame of these pulse-chase experiments, virtually no newly synthesized 18-kDa POMC and mature PC2 was released into the incubation medium (<10% of the cellular content). In the #124 transgenic melanotrope cells, similar amounts of 37-kDa POMC were synthesized during the 10-min pulse incubation as in wild-type cells. However, the amounts of 18-kDa POMC that were produced in the transgenic cells after 1.5 h and 2.5 h of chase were less than those synthesized in the wild-type cells (Figure 6A). Moreover, reloading of the samples on a higher-percentage polyacrylamide gel revealed that a substantial portion of the newly synthesized 18-kDa product produced in the #124 transgenic cells migrated slower than the majority of 18-kDa POMC synthesized in the wild-type cells (Figure 6C). We refer to this slower-migrating product as 18-kDa POMC* and the normal product as 18-kDa POMC without an asterisk. The nature of the difference between the two 18-kDa POMC products is presently unknown. During the
POMC and PC2 protein levels are similar in the inter-
transgenic than in the wild-type cells (Figure 6A). Newly synthesized proPC2 into mature PC2 was lower in the
similar to that synthesized by the wild-type cells. In contrast,
as for the reduced rate of 37-kDa POMC processing, during
transgenic melanotrope cells, des-N-alpha-acetyl-alpha-MSH is the major form of
and its acetylation occurs just before release, thereby
making the acetylated form (alpha-MSH) the released (and more
bioactive) product (Martens et al., 1981). HPLC analysis re-
vealed that, after a 10-min pulse/2.5-h chase and relative to
the peptides produced in wild-type melanotrope cells, the
amounts of the small POMC cleavage products (des-N-alpha-
acetyl-alpha-MSH, CLIPs, and endorphins) were reduced in the
#124 cells (Figure 6E). Thus, besides the production of a
lower amount of 18-kDa POMC and the additional form of
the 18-kDa POMC cleavage product (18-kDa POMC*), re-
duced amounts of the POMC-derived peptides were synthe-
sized in the #124 transgenic cells.

DISCUSSION
The type 1 transmembrane p24 proteins are abundantly
present in ER- and Golgi-derived transport vesicles and are
therefore thought to play an important role in some aspect of
cargo-selective transport through the early secretory path-
way. The complex and dynamic behavior of this protein
family has hampered functional analyses. In this study, we
used the X. laevis intermediate pituitary melanotrope cell
with one major secretory cargo protein (the prohormone
POMC) and melanotrope cell-specific transgene expression
of a GFP-tagged Xenopus p24 family member as a model to
explore the importance of a functional p24 complex for a
highly specialized secretory cell. Of the four abundant melano-
ntrope p24 members upregulated with POMC (Xp24alpha,-beta,
-gamma, and -delta) and thus likely somehow involved in the bio-
synthesis of the prohormone, Xp24delta was chosen for trans-
genetic expression. The microscopy analyses revealed that
the GFP-tag did not prevent the Xp24delta-GFP fusion protein
from reaching the Golgi. The two selected, independent
transgenic lines 125 and 124 displayed moderate and high
expression levels of the Xp24delta-GFP fusion protein, respec-
tively. From the Western blot and biosynthetic studies on the
Xenopus p24 proteins, we conclude that the level of newly
synthesized Xp24delta-GFP produced in the transgenic cells
determined the degree of displacement of the endogenous
Xp24delta and -delta proteins by the fusion protein. Thus, high
levels of newly synthesized fusion protein, as produced in the
#124 transgenic melanotrope cells, caused the near-ab-
sence of endogenous Xp24delta and -delta. Due to the lower level
of transgene expression in the #125 cells, substantial
amounts of the endogenous Xp24delta proteins were still
present, albeit at lower steady-state levels than in the wild-
type cells. In the #124 cells, the high level of Xp24delta-GFP
effectively displaced not only the endogenous Xp24delta pro-
teins, but also the normally abundant Xp24alpha,-beta, and -gamma
family members such that the resulting p24 system consisted
mainly of the transgene product. It therefore seems that the
number of ER/Golgi subcompartments that can harbor p24
proteins is limited and that the relative amounts of the
various newly synthesized p24 family members expressed
in a cell determine the final composition of the p24 machin-
ery in the early secretory pathway (by a "displacement
effect"). In transiently transfected cells in culture, overex-
pression of a single p24 member resulted in aberrant ER
structures (Rojo et al., 2000). Because our ultrastructural
analysis did not reveal gross morphological changes, the level of transgene expression may have been relatively less than the amount of exogenous p24 produced in the transduced cells and thus to an extent that did not destroy the early secretory pathway in the transgenic Xenopus melanotrope cells.

Of special interest was that the number and sizes of the melanophores in the skin of the #124 transgenic animals were clearly reduced and as a result, these animals were not able to fully adapt to a black background. Because in Xenopus the intermediate pituitary melanotrope cells regulate skin melanophores, the phenotype of the #124 animal urged us to investigate in detail the functioning of the transgenic melanotropes. From the Western blot analyses of POMC and the POMC cleavage enzyme PC2, it seemed that the steady-state levels of these proteins were similar in the wild-type and transgenic melanotrope cells. We then examined the dynamics of protein biosynthesis and for this study we focused on the major newly synthesized secretory cargo proteins (POMC, its well-defined processing products and PC2). The results of the in vitro metabolic cell labeling studies suggested that in the #124 transgenic melanotrope cells the distortion of the endogenous p24 complex did not affect the

level of POMC and proPC2 biosynthesis. However, relative to the wild-type melanotrope cells, the transgenic cells produced lower amounts of newly synthesized 18-kDa POMC, of the newly synthesized peptides derived from POMC (des-N-acetyl-α-MSH, CLIPs, and endorphins) and of newly synthesized mature PC2. This effect may have been caused by a lower rate of transport of newly synthesized POMC and proPC2 through the secretory pathway, resulting in a lower rate of precursor protein processing and the observed reduced amounts of the newly synthesized precursor-derived peptides produced within the time frame of the pulse-chase experiments. Alternatively, the distorted p24 system may have exerted a more direct effect on the POMC processing event itself, e.g., because it failed to provide the proper processing conditions in the various secretory pathway subcompartments. In considering such a role in processing, a recently proposed model for ER-to-Golgi cargo transport is of special interest (Mironov et al., 2003). According to this model, which was based on the results of high-resolution morphological studies, secretory proteins would exit the ER by bulk flow in large transport carriers emerging from specialized ER exit sites, and this process would not involve budding and fusion of COPII-coated vesicles. In adjacent
COPII-coated exit sites, a specific set of “machinery proteins” would be recruited and subsequently incorporated into the outgoing secretory cargo-containing carrier, e.g., for providing the correct luminal environment in the carrier (Mironov et al., 2003). Because of their well-established ability to bind COPII (Fiedler et al., 1996; Nickel et al., 1997; Dominguez et al., 1998), p24 proteins may be involved in the COPII-dependent targeting of the machinery proteins to the secretory cargo transport carriers. In view of the results from our transgenic studies, the proteins recruited by p24/COPII could include components of the biosynthetic machinery that are needed for proper prohormone processing. Thus, in the #124 transgenic *Xenopus* melanotrope cells with the severely distorted p24 system, the set of machinery proteins incorporated into the outgoing POMC-containing carriers may be incomplete and these cells would therefore lack a fully functional POMC processing system.

The observation that the #124 pigment-containing skin cells were found only in the vicinity of blood vessels suggests that these transgenic animals have a shortage of the factor(s) responsible for the signaling to these cells. The reduced size and pigment content of the melanophores may be attributed to the lower amount of intermediate pituitary α-MSH, the POMC-derived peptide with a well-established role in background adaptation of amphibians by causing both the dispersion and synthesis of melanin in dermal melanophores (Hadley et al., 1981). An intriguing explanation for the lower number of skin melanophores in the #124 animal concerns the 18-kDa POMC cleavage product. The N-terminal 52 amino acids of the mammalian counterpart of *Xenopus* 18-kDa POMC (16-kDa POMC, also named pro-γ-MSH), resulting from a postsecretional cleavage of 16-kDa POMC by a serine protease localized on the target cell membrane, has been found to act as a growth factor (Bicknell et al., 2001). Hence, a deficit in normal melanotrope 18-kDa POMC may have resulted in insufficient mitogenic activity to produce normal quantities of skin melanophores in the #124 transgenic animal.

Together, our results are most consistent with a role for p24 in the transport of newly synthesized secretory cargo proteins through the early stages of the secretory pathway or in the processing of secretory cargo by recruiting the proper components of the biosynthetic machinery into ER-to-Golgi cargo transport carriers. Furthermore, our transgenic approach in a physiological context has shown that distortion of the complex p24 system results in an affected profile of prohormone-derived bioactive peptides with the eventual consequence at the level of the target cell of the secretory signals.

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