Research paper

p24 proteins from the same subfamily are functionally nonredundant

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ABSTRACT

The p24 proteins function in early secretory pathway transport processes, but their exact role is unclear. In physiologically activated Xenopus melanotrope cells, a representative of each p24 subfamily (p24α2, -β1, -γ3, -δ3) is upregulated coordinately with the major melanotrope cargo, proopiomelanocortin (POMC), whereas two other p24s (p24γ2 and -δ1) are also expressed, but not coordinately with POMC. Using melanotrope-specific transgene expression, we here find that the roles of both p24γ2 and p24δ1 in the transport, glycosylation, sulfation and cleavage of POMC are different from those of their upregulated subfamily relatives (p24γ3 and p24δ3 respectively). Thus, even p24 proteins from the same subfamily have distinct functions in secretory cargo biosynthesis.

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1. Introduction

The type-I transmembrane proteins of the p24 family (α, β, γ, δ subfamilies) play an important but poorly understood role in the selective transport processes at the endoplasmic reticulum (ER)-Golgi interface (reviewed in [1,2]). The p24 proteins are abundantly present in early secretory pathway membranes and cycle continuously between these compartments (reviewed in [3,4]). Proposed functions range from a role as receptor for specific secretory cargoes or as machinery for COPI vesicle budding, to the supply of machinery proteins to secretory pathway subcompartments (reviewed in [2]).

We use the Xenopus laevis intermediate pituitary melanotrope cells as a physiologically relevant, inducible cell model to study the p24 proteins. When the frogs are on a black background, the melanotrope cells are activated, and in this state, we find that the major secretory cargo protein proopiomelanocortin (POMC), whereas two other p24s (p24γ2 and -δ1) are also expressed, but not coordinately with POMC. Using melanotrope-specific transgene expression, we here find that the roles of both p24γ2 and p24δ1 in the transport, glycosylation, sulfation and cleavage of POMC are different from those of their upregulated subfamily relatives (p24γ3 and p24δ3 respectively). Thus, even p24 proteins from the same subfamily have distinct functions in secretory cargo biosynthesis.

2. Materials and methods

2.1. Animals

Animal experiments were carried out at the Central Animal Facility of the Radboud University in accordance with European, national and institutional guidelines and permits 86/609/EEC, RBD0166(H10), and RU-DEC 2003-53 and 2007-027.

2.2. Antibodies

Rabbit polyclonal antibodies against Xenopus p24α3, p24γ2, p24δ1, p24δ2 and APP have been described [6–8]. Polyclonal antibodies against p24/p24β1 [9], p24γ3/4 [10], green fluorescent protein (GFP), Xenopus 37K POMC [11] and PC2 [12], and the monoclonal anti-tubulin antibody [13] were obtained from colleagues.

2.3. Generation of X. laevis transgenic for p24γ2 or p24δ1

To generate F0 Xenopus transgenic for p24γ2 or p24δ1, the line-arised DNA-constructs pPOMC-p24γ2-GFP or pPOMC-p24δ1-GFP
(Fig. 1A) were mixed with wild-type (wt) Xenopus sperm nuclei and microinjected into unfertilised wt Xenopus eggs. F1 offspring was generated from a number of F0 animals by in vitro fertilisation (IVF). Details of the Xenopus transgenesis procedure have been described before [14,15]. The expression levels (assessed by Western blotting for GFP) of the p24γ2- and p24δ1-transgene products in the transgenic lines #25 (p24γ2) and #252 (p24δ1) were lower than those of the p24γ3-transgene product in the previously described line #222 [15] (1.8-fold and 1.7-fold, respectively) and the p24δ2-transgene product in line #224 [14] (3.2-fold and 3.0-fold, respectively).

2.4. Analysis of steady-state and newly synthesised proteins

Western blot analysis and protein quantification relative to tubulin, MALDI-TOF MS mass spectrometry, and metabolic cell labelling experiments were performed as described [14,15].

2.5. Microscopy analyses

Cryosectioning, immunostaining and imaging of frog brains with the pituitaries attached, isolation and imaging of individual transgenic melanotrope cells, and high-pressure freezing and sample processing for (immuno) electron microscopy analysis were performed as described [14,15].

2.6. Quantification & statistics

Statistical evaluation was performed using unpaired two-tailed t-tests, applying Welch’s correction where appropriate, as described [15]. Means were considered significantly different when p < 0.05.

3. Results

3.1. Generation of X. laevis transgenic for p24γ2 or p24δ1

We generated two independent X. laevis lines with melanotrope-specific stable transgene expression of the p24γ2-protein fused to GFP (p24γ2-GFP lines #25 and #118) and seven independent lines transgenic for p24δ1-GFP (#179, #181, #182, #183, #184, #252, #290) (Fig. 1A). Transgene expression specifically in the intermediate pituitary could be observed in living tadpoles (Supplementary Fig. S1A), in the dissection pituitary of adult frogs (not shown), and in sagittal brain-pituitary sections of adult animals (Supplementary Fig. S1B). This is in agreement with the observation that the expression of transgenes under the control of the POMC promoter occurs only in the melanotrope cells of the intermediate pituitary, and not in other POMC-expressing cells in the anterior pituitary or other parts of the brain of adult Xenopus [16].

The p24γ2-GFP protein displayed a reticular staining typical for the ER, whereas p24δ1-GFP showed a clear localisation to perinuclear Golgi-like structures along with a more diffuse staining (probably ER) (Fig. 1B). Apparently, the GFP-tag did not hamper the transport of the transgene product out of the ER. Western blot analysis revealed that lines #25 and #252 were the strongest expressing lines for the p24γ2-GFP and the p24δ1-GFP protein, respectively (data not shown), and that their transgene expression levels were similar (Fig. 1C). The other p24γ2- and p24δ1-transgenic lines showed similar characteristics as lines #25 and #252, respectively, and we therefore decided to focus our analysis on the latter two lines. In the transgenic melanotrope cells (tmcs) of lines #25 and #252, the levels of the endogenous p24 proteins were not significantly changed (Fig. 1C), indicating that the transgene expression of p24γ2 or p24δ1 did not affect the endogenous p24 system. This lack of effect is not due to the level of transgene expression per se. For instance, the level of p24γ2 transgene expression is relatively low (e.g., lower than that of p24γ3 or p24δ1), yet the endogenous p24 protein levels are severely affected in the tmcs [14]. Furthermore, for unknown reasons it appears that each p24 family member has its specific level of transgene expression in the tmcs.

3.2. Steady-state levels of secretory cargo proteins

Western blot analysis showed that the steady-state levels of the soluble secretory cargo proteins POMC and its processing enzyme prohormone convertase PC2, and the transmembrane cargo amyloid-β precursor protein (APP) were not affected in the

Fig. 1. Generation and expression analysis of transgenic Xenopus melanotrope cells. (A) Schematic depiction of the injection fragments pPOMC-p24γ2-GFP and pPOMC-p24δ1-GFP including a Xenopus POMC gene promoter fragment (pPOMC) [16] for melanotrope-specific transgene expression and the protein-coding sequences of p24γ2-GFP or p24δ1-GFP. (B) Localisation of the p24γ2-GFP and p24δ1-GFP transgene products by confocal microscopy in live melanotrope cells transgenic for p24γ2; (line #25) or p24δ1; (line #252). Bars: 5 μm. (C) Western blot analysis of neurointermediate lobe (NIL) lysates from wild-type frogs (wt) and frogs transgenic for p24γ2 (#25) or p24δ1; (#252) using antibodies against GFP or p24s. Shown are representative examples of at least three blots, each from individual animals. Tubulin was used as a control for equal loading.

Fig. 2. Steady-state levels of secretory cargo proteins. Western blot analysis of neurointermediate lobe (NIL) lysates from wild-type frogs (wt) and transgenic (p24γ2; #25; p24δ1; #252) frogs using antibodies against POMC, PC2 and APP. Shown are representative examples of at least three blots, each from individual animals. Tubulin was used as a control for equal loading.
p24γ2- and p24δ1-tmcs (Fig. 2). MALDI-TOF mass spectrometry revealed that the production of the POMC-derived peptides des-N-α-acetyl-α-MSH (the nonacetylated form of α-MSH), α-MSH, γ1-MSH, β-MSH and two corticotropin-like intermediate lobe peptides (CLIP A and B) was unaffected in the p24γ2- and p24δ1-tmcs (not shown), indicating that the p24γ2- and the p24δ1-tmcs converted POMC into the same set of bioactive peptides as wt cells.

3.3. Biosynthesis and posttranslational processing of newly synthesised POMC

The intermediate lobe of the pituitary consists of the neuroendocrine melanotrope cells and is intimately associated with the pars nervosa (biosynthetically inactive nerve terminals of hypothalamic origin), together making up the neurointermediate lobe (NIL) of the pituitary. We used the NIL to examine the biosynthesis of POMC in the tmcs. Metabolic pulse-labelling experiments revealed that the levels of newly synthesised 37K POMC and 75K pro-PC2 produced in the tmcs were not significantly changed (Fig. 3A and C). Following a pulse/chase period, a small increase of newly synthesised 37K POMC was observed in both the p24γ2- and the p24δ1-tmcs (1.3–1.5-fold), although this increase was only significant in the p24γ2-tmcs (Fig. 3B and D).

![Fig. 3. Biosynthesis and processing of POMC. (A–E) Neurointermediate lobes (NILs) from wild-type (wt) and transgenic (p24γ2 #25; p24δ1 #252) frogs were pulse labelled with [35S]-Met/Cys for 30 min and chased for the indicated time periods. Newly synthesised proteins extracted from the NILs (Cells; 5% of extract) or secreted into the incubation medium (Media; 20%) were visualised by autoradiography. Analyses are based on two (A) or at least four (B) independent experiments and representative autoradiograms are shown. (A) NILs were pulsed for 30 min (B) NILs were pulsed for 30 min and chased for 3 h. (C) The amount of newly synthesised 37K POMC produced during the 30 min pulse period (panel A) was quantified and is shown relative to the wt cells (wt n = 6; p24γ2 n = 6; p24δ1 n = 6). (D) The amount of newly synthesised 37K POMC remaining after the chase period (panel B) was quantified and is shown relative to the wt cells (wt n = 6; p24γ2 n = 6; p24δ1 n = 6). (E) The amounts of newly synthesised 18K and 18K* POMC after the 3-hr chase period (panel B) were quantified and are shown relative to wt 18K POMC (wt n = 6; p24γ2 n = 6; p24δ1 n = 6). Indicated are the 18K/18K* ratios and their statistical evaluations. Data are shown as means +/- SEM. n.s., not significant (i.e., p > 0.05); *, p < 0.05.

The proteolytic cleavage of 37K POMC yields an N-glycosylated ~18K POMC product corresponding to the N-terminal part of the prohormone [17]. Two forms of 18K POMC are produced in wt melanotropes, namely a major 18K POMC product (~70%) and a minor 18K* POMC product (~30%) (Fig. 3B). In the p24γ2-tmcs, the amount of 18K POMC was not affected, whereas the amount of 18K* POMC was only slightly increased (Fig. 3B and D), suggesting

![Fig. 4. Sulphation of newly synthesised POMC. Neurointermediate lobes (NILs) from wild-type (wt) and transgenic (p24γ2 #25; p24δ1 #252) frogs were pulse labelled with [35S]-sulphate and [3H]-lysine for 15 min. The amount of [35S]SO4 and [3H]-lysine incorporated into newly-synthesised 37K POMC was determined, and is shown relative to wt NILs and as means +/- SEM (wt n = 12; #25 n = 3; #252 n = 6). n.s., not significant (i.e., p > 0.05); ***, p < 0.001.
a minor alteration in Golgi-based glycomaturation. In the p24\textsubscript{d1}-tmcs, the amounts of both 18K and 18K* POMC were unaffected (Fig. 3B and E). Deglycosylation with peptidyl N-glycosidase F (PNGaseF) caused a shift of both the 18K and 18K* POMC products to a product of \( \sim 15.5K \) (data not shown), indicating that in wt cells and in the tmcs the 18K and 18K* POMC products differ in their glycoforms and not in their protein backbone.

In addition to N-glycosylation, POMC is also sulphated in the Golgi [18]. To examine the degree of sulphation of newly synthesised POMC, we labelled the cells with both 3H-lysine (to quantify the total amount of newly synthesised POMC) and 35S-sulphate (to quantify the level of sulphation). POMC sulphation was \( \sim 2 \)-fold increased in the p24\textsubscript{g2}-tmcs, but not affected in the p24\textsubscript{d1}-tmcs (Fig. 4).

3.4. Electron and immuno-electron microscopy analysis

To examine whether the expression of the p24\textsubscript{g2}- or the p24\textsubscript{d1}-transgene product had led to any ultrastructural changes in the tmcs, we studied the cells by (immuno-)electron microscopy (EM). Like in wt cells, the rough ER was well developed in the tmcs, and the Golgi had a normal ultrastructure with stacked cisternae and Golgi ribbons of \( \sim \sim 1 \mu \text{m} \) (Fig. 5A). Immuno-EM revealed that in wt and the tmcs POMC was localised mainly to the Golgi apparatus and dense-core secretory granules (Fig. 5B).

4. Discussion

We previously examined POMC biosynthesis in melanotrope cells transgenic for p24\textsubscript{g2} or p24\textsubscript{g3}, two p24 proteins upregulated together with the prohormone [14,15]. Through our present studies we are now in the unique position to compare the roles of these two upregulated p24 proteins in secretory cargo biosynthesis with those of the not coregulated p24\textsubscript{g2} and p24\textsubscript{d1} proteins. Whereas the p24\textsubscript{g2}- and p24\textsubscript{g3}-transgene products were localised differently (to the ER and perinuclear Golgi-like structures, respectively), the p24\textsubscript{d1}-transgene products were localised similarly (in perinuclear Golgi-like structures and more diffuse throughout the cell). These localisations are in line with those of their endogenous counterparts in the ER-Golgi area (reviewed in [3]). The p24\textsubscript{g2}, p24\textsubscript{g3}- and p24\textsubscript{d1}-transgene products did not affect the steady-state levels of the endogenous p24 proteins, whereas the p24\textsubscript{d2}-transgene product efficiently displaced all endogenous p24 proteins (70–90% displacement) [14]. Apparently, factors other than the localisation or expression level of the transgene product determine the extent of the displacement of the endogenous p24s. In contrast to p24\textsubscript{g3} [15], the p24\textsubscript{g2}-transgene product did not affect the transport of secretory cargo. However, the p24\textsubscript{g2}-tmcs showed a clear increase in POMC sulphation but not glycosylation, whereas the p24\textsubscript{g3}-tmcs display a normal sulphation but a clearly affected glycosylation of the prohormone. Both POMC sulphation and glycosylation is severely affected in the p24\textsubscript{d2}-tmcs [14], but unaffected in the p24\textsubscript{d1}-tmcs. Finally, like the p24\textsubscript{g2} and p24\textsubscript{g3}-tmcs [14,15], the p24\textsubscript{g2}- and p24\textsubscript{d1}-tmcs had a normal ultrastructure.

5. Conclusions

Our results demonstrate that, despite their structural relationship, the effects of the two p24\textsubscript{g} subfamily members on POMC biosynthesis are clearly different. Likewise, the phenotypes of the melanotrope cells transgenic for the two structurally related p24 subfamily members display variations in POMC transport and processing. We here thus show for the first time that in vertebrates not only members of different p24 subfamilies, but also members of the same p24 subfamily have non-redundant roles in secretory

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**Fig. 5.** Electron microscopy analysis. Electron microscopy analysis of neurointermediate lobes (NILs) from wild-type and transgenic (p24\textsubscript{g2} #25; p24\textsubscript{d1} #252) melanotrope cells. (A–C) Ultrastructural electron microscopy analysis. (D–F) Immuno-electron microscopy analysis using an anti-POMC antiserum. Dotted lines highlight the outline of the Golgi. In the wt as well as in the transgenic cells, the Golgi apparatus is present as a ribbon (length \( \geq 1 \mu \text{m} \)) of stacked cisternae. POMC immunostaining was mainly found in the Golgi area in dense-core secretory granules. er, rough endoplasmic reticulum; g, Golgi; l, lysosome; m, mitochondrion; n, nucleus; pm, plasma membrane; arrowheads, dense-core immature secretory granules. Scale bars equal 1 \( \mu \text{m} \) (A–C); 500 nm (D–F).
cargo biosynthesis. Altogether, the results from the present and our previous p24 transgenesis experiments [14,15] reveal that each of the GFP-tagged p24 proteins causes a specific phenotype. At present it is unclear whether the GFP-tagged transgene products can rescue loss-of-function phenotypes. In contrast to the situation in yeast [19], our findings imply that a particular vertebrate p24 cannot be simply substituted by another member of the same subfamily. Possibly, minor differences in the characteristics of closely related p24 proteins, such as differences in coatomer binding affinity [20], may provide a basis for the functional non-redundancy of p24 subfamily members in secretory cargo biosynthesis. On the basis of our findings, we speculate that each p24 protein is involved in providing the proper microenvironment in a specific subcompartment of the early secretory pathway, such that the various p24s together allow efficient and correct secretory cargo transport and processing.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.bioch.2010.11.007.

References
