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# COP-binding sites in p24$\delta_2$ are necessary for proper secretory cargo biosynthesis

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ABSTRACT

The p24 family is thought to be somehow involved in endoplasmic reticulum-to-Golgi protein transport, and its members are major constituents of transport vesicles and bind to the vesicle coat protein complexes COPI and COPII. A subset of the p24 proteins (p24$\alpha_3$, -$\beta_1$, -$\gamma_3$ and -$\delta_2$) is upregulated when *Xenopus laevis* intermediate pituitary melanotrope cells are physiologically activated to produce vast amounts of their major secretory cargo, the prohormone proopiomelanocortin (POMC). To investigate the role of the COP-binding motifs of p24 proteins in POMC biosynthesis, we here generated and analysed *Xenopus* with stable, melanotrope cell-specific transgene expression of p24$\delta_2$-GFP mutated in its COPI- or COPII-binding motif. In contrast to what has been found previously for wild-type (wt) p24$\delta_2$-GFP, the p24$\delta_2$ mutations prevented the Golgi localisation of the transgene products and caused a reduced rate of POMC cleavage, but did not lead to a reduction of the endogenous p24 proteins nor to aberrations in POMC glycosylation and sulphation. We conclude that p24$\delta_2$ requires the presence of the COPI- and COPII-binding sites to allow proper POMC processing. Thus, the p24 proteins fulfil their role in secretory protein biosynthesis via COPI- or COPII-coated transport vesicles.



## 1. Introduction

The secretory pathway comprises a collection of membrane-bounded organelles with specialised functions in the biosynthesis of proteins destined for the plasma membrane or the extracellular space (in this report referred to as secretory cargo). Proteins enter the secretory pathway in the endoplasmic reticulum (ER), where they are properly folded and can undergo initial posttranslational modifications, such as N-glycosylation. Following selective packaging into transport vesicles, secretory cargo is carried to the Golgi apparatus and beyond to subsequent compartments in which the cargo molecules undergo further posttranslational processing necessary for their biological activity (e.g. N-glycan maturation, sulphation and proteolytic cleavage) (Palade, 1975). The various vesicular transport steps in the secretory pathway operate both in the anterograde direction (to move secretory cargo and bring machinery proteins to their correct subcompartments) and in the retrograde direction (to retrieve escaped machinery proteins and thus preserve compartmental identity). Transport vesicles coated with the COPII–protein complexes mediate anterograde ER-to-Golgi transport (Barlowe et al., 1994), whereas COPI-coated vesicles serve retrograde intra-Golgi and Golgi-to-ER transport processes (Cosson and Letourneur, 1994, Letourneur et al., 1994).

Members of the p24 family of ~24K type-I transmembrane proteins (subdivided into subfamilies p24α, -β, -γ and -δ; Dominguez et al., 1998) are abundant constituents of both COPI- and COPII-coated vesicles, as well as of ER and *cis*-Golgi membranes (Bell et al., 2001; Blum et al., 1996, 1999; Dominguez et al., 1998; Füllekrug et al., 1999; Kuiper et al., 2001; Rojo et al., 1997; Schimmöller et al., 1995; Sohn et al., 1996; Stamnes et al., 1995; Wada et al., 1991), and cycle constantly between these membranes (Blum et al., 1999; Füllekrug et al., 1999; Gommel et al., 1999; Kuiper et al., 2001; Nickel et al., 1997). The p24 proteins bind the COPI- and COPII-coat complexes through motifs in their cytoplasmic tails (Béthune et al., 2006; Contreras et al., 2004; Dominguez et al., 1998; Sohn et al., 1996). COPII-binding takes place via a double-phenylalanine (FF) motif that mediates binding to the Sec23-subunit (Dominguez et al., 1998). COPI-binding has initially been proposed to occur via the dilysine (KK) motif (Dominguez et al., 1998; Sohn et al., 1996) that resembles the COPI-binding ER-retrieval motif K(X)KXX (Cosson and Letourneur, 1994; Jackson et al., 1990), although the binding appeared to be modulated by other residues, including the FF-motif (Dominguez et al., 1998; Fiedler et al., 1996; Sohn et al., 1996). Recently, Béthune et al. (2006) showed that only the p24α-subfamily members contain the canonical K(X)KXX-motif for COPI-binding and discovered a novel COPI-binding motif (FFXXBBX$_n$, $n \geq 2$; B indicates a basic residue) that is present in all members of the p24 family (including p24α).

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The p24 proteins play an important but poorly understood role in selective transport processes in the early secretory pathway (reviewed in Carney and Bowen, 2004). Initially, p24 proteins have been proposed to act as receptors for specific sets of secretory cargo molecules (the cargo receptor model) (Belden and Barlowe, 1996; Marzioch et al., 1999; Muñiz et al., 2000; Rojo et al., 1997; Schimmöller et al., 1995; Stamnes et al., 1995). Additional roles have been suggested in the biogenesis and functioning of transport vesicles, in the organisation of secretory pathway membranes (Emery et al., 2003; Gommel et al., 1999; Lavoie et al., 1999; Rojo et al., 1997, 2000) and in the supply of machinery cargo to subcompartments of the secretory pathway (Strating et al., 2007).

For our studies on p24, we employ a well-defined, physiologically relevant model system, namely the melanotrope cells in the intermediate pituitary of the amphibian *Xenopus laevis*. The melanotrope cells are neuroendocrine cells strictly regulated by inhibitory and stimulatory neurons of hypothalamic origin. The activity of the melanotrope cells can be influenced by placing the frogs on a white or black background (inactive and highly active melanotrope cells, respectively). Activated melanotrope cells are professional secretory cells that produce vast amounts of the prohormone proopiomelanocortin (POMC). POMC is proteolytically processed to a number of bioactive peptides among which α-melanophore-stimulating hormone (α-MSH), which mediates background adaptation of the animal (reviewed by Kuiper and Martens, 2000). A member of each of the four-p24 subfamilies (p24$\alpha_3$ also named GMP25iso; p24$\beta_1$ (p24), p24$\gamma_3$ (p27) and p24$\delta_2$ (p23iso)) is coordinately expressed with POMC, suggesting a role in the biosynthesis of POMC (Rötter et al., 2002). We previously generated four separate lines of transgenic *Xenopus* each stably expressing one of these four p24 proteins specifically and inducibly in the melanotrope cells to investigate the function of p24 proteins in POMC biosynthesis. We found disparate effects for each of the four-p24 proteins on the transport, processing rate, glycosylation and sulphation of POMC (Strating et al., 2007, 2009). In the p24$\delta_2$-transgenic melanotrope cells, we observed altered POMC glycosylation and sulphation patterns. Since p24 proteins bind COPI and COPII (Béthune et al., 2006; Contreras et al., 2004; Dominguez et al., 1998; Sohn et al., 1996), and are required for the formation of – at least – COPI-vesicles (Bremser et al., 1999), we wondered to what extent the binding of COPI and COPII is important for the role of p24$\delta_2$ in the process of secretory protein biosynthesis.

Here, we mutated the COPI-binding motif KK or the COPII-binding motif FF in the cytoplasmic tails of p24$\delta_2$ and transgenically expressed the p24$\delta_2$ mutants specifically in the melanotrope cells of *Xenopus*. We report that each of the mutations resulted in a melanotrope cell phenotype that in various aspects is distinct from the previously observed effects caused by the wild-type (wt; nonmutated) p24$\delta_2$-transgene product (Strating et al., 2007), illustrating the specificity of the effects and thus the applicability of our transgenic approach in an in vivo context to study the functioning of p24.

## 2. Materials and methods

### 2.1. Animals

South African claw-toed frogs *X. laevis* were bred and reared in the Central Animal Facility of the Radboud University (Nijmegen, The Netherlands). Animals were adapted to a black background for 3–6 weeks. All animal experiments were carried out in accordance with the European Communities Council Directive 86/609/EEC for animal welfare, permit RBD0166(H10) to generate and house transgenic *Xenopus* and permits RU-DEC 2003–53 and 2007–027 from the animal experiment committee of the Radboud University for the use of *X. laevis* frogs.

### 2.2. Antibodies

The rabbit polyclonal antibodies against a region in the lumenal parts of the *X. laevis* p24$\alpha_3$ protein and against the C-terminal regions of *X. laevis* p24$\delta_2$ and APP have been described previously (Collin et al., 2005; Kuiper et al., 2000; Rötter et al., 2002). Rabbit polyclonal antibodies against mammalian p24 (p24$\beta_1$ (Frieda)), rat p24$\gamma_{3/4}$, GFP (Cuppen et al., 1999), *X. laevis* POMC (ST62, recognising only the precursor form), recombinant mature human PC2 and the monoclonal anti-tubulin antibody E7 were obtained from Drs F. Wieland (BZH, Heidelberg, Germany) (Jenne et al., 2002), T. Nilsson (Göteborgs University, Göteborg, Sweden) (Dominguez et al., 1998), J. Fransen (NCMLS, Nijmegen, The Netherlands), S. Tanaka (Shizuoka University, Japan), W.J.M. Van de Ven (University of Leuven, Belgium) and B. Wieringa (NCMLS, Nijmegen, The Netherlands) (Chu and Klymkowsky, 1989), respectively.

### 2.3. Generation of transgenic X. laevis

Constructs pPOMC-p24$\delta_2$ FF/AA-GFP and pPOMC-p24$\delta_2$ KK/SS-GFP (Fig. 1B) were used to generate *X. laevis* transgenic for p24$\delta_2$ FF/AA-GFP and p24$\delta_2$ KK/SS-GFP, respectively. Stable *Xenopus* transgenesis to produce $F_0$ animals and in vitro fertilisation for obtaining $F_1$ transgenic animals were performed as described previously (Strating et al., 2007). Western blot analysis revealed that the expression levels of the p24$\delta_2$ FF/AA-transgene product in line #226 (∼3.0-fold) and of the p24$\delta_2$ KK/SS-transgene product in line #241 (∼10.6-fold) were lower than that of the previously described wt p24$\delta_2$-transgene product in line #224 (Strating et al., 2007).

### 2.4. Western blot analysis

Quantitative Western blot analysis was performed using standard procedures. The proteins on the blot were visualised using peroxidise-conjugated secondary antibodies and chemoluminescence. The proteins on the blot were detected with a BioChemi imaging system and non-saturated recordings were quantified using the Labworks 4.0 program (UVP BioImaging systems, Cambridge, United Kingdom), except that for the quantification of APP the secondary antibody was IRDye800CW-conjugated goat-anti-rabbit (LI-COR, Lincoln, NE, USA). The proteins on the blot were detected on an Odyssey infrared imaging system (LI-COR, Lincoln, NE, USA) and quantified using the Odyssey v1.2 program (LI-COR, Lincoln, NE, USA).

To remove N-linked glycogroups, protein homogenates were boiled in 6 mM HEPES/0.06% SDS pH 7.4 for 10 min, cooled to room temperature, 1 μl 12.5% NP40/2.5 mM phenylmethylsulphonyl fluoride (PMSF)/0.25 mg/ml trypsin inhibitor and 1 U peptidyl N-glycosidase F (Roche) were added, and the samples were incubated overnight at 37 °C. For the selective removal of high-mannose glycogroups, protein homogenates were boiled in 85 mM sodium acetate pH 5.5/0.04% SDS/0.08% β-mercaptoethanol/1 mM PMSF for 10 min, cooled to room temperature, 1 μl 5% Triton X-100 and 5 mU endoglycosidase H (Roche) were added, and the samples were incubated overnight at 37 °C. After deglycosylation, the samples were processed for Western blotting and analysed as described above.

### 2.5. MALDI-TOF MS

To examine the POMC-derived peptides, matrix-assisted laser-desorption-ionisation time-of-flight mass spectrometry (MALDI-TOF MS) was performed as described previously (Strating et al., 2007).