Sorting of the vasopressin prohormone into the regulated secretory pathway

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Abstract The sorting of soluble proteins into the regulated secretory pathway (RSP) involves aggregation, but whether an additional sorting domain is also required is not clear. By fusing vasopressin prohormone (proVP) fragments to green fluorescent protein (eGFP) we have determined whether a sorting domain can function independently of the aggregative neurophysin domain. Although eGFP itself can be immunolocalised in the RSP of Neuro2A and AtT20 cells, most of the protein enters the constitutive pathway, and is found in the culture medium. In contrast, the N-terminal 27 residues of proVP promote residence in the RSP. Furthermore, only the processed form of this fusion was secreted when stimulated. We suggest a sorting mechanism based on the recognition of a sorting motif, the efficiency of which is enhanced by neurophysin aggregation.

Key words: Vasopressin; Secretory pathway; Protein sorting

1. Introduction

The trafficking of secretory proteins through the regulated secretory pathway (RSP) has been studied extensively during the last decade (reviewed in [1,2]), but a satisfactory description of the sorting mechanism remains elusive. Regulated secretory proteins aggregate once they encounter the acidic milieu of the *trans*-Golgi network (TGN). This is inherent in many, if not all, regulated secretory proteins. Once aggregated, the secretory proteins form a dense core characteristic of secretory vesicles of the RSP. It might be that the aggregate is enough to induce granule formation, as proposed in the sorting-by-retention hypothesis [1]. Further exclusion of non-regulated secretory proteins is achieved during maturation of the dense core granule in which the non-aggregative, soluble proteins are diverted into the constitutive secretory pathway. Importantly, this model allows for the entry of non-regulated secretory proteins into the RSP, and does not require association with a sorting receptor or the secretory granule membrane. On the other hand it might be that the aggregate, once formed, or during formation in the TGN, is being recognised by sorting receptors for entry into the regulated secretory vesicle [3]. The nature of sorting domains is still unclear, since all the sorting domains found so far do not display an obvious similarity. How aggregation is regulated intra- or intermolecularly is only clear in the case of the vasopressin (VP) prohormone. This gives an opportunity to study the presence of a sorting domain separate from the aggregation, which relies on an intact VP prohormone.

The VP prohormone consists of three domains: the nonapeptide hormone VP, neurophysin (NP, 93 residues) and a C-terminal glycopeptide (GP, 39 residues) (Fig. 1). This prohormone is closely related to the oxytocin (OT) prohormone which looks identical in organisation, having a hormone domain and a NP domain, but not a GP domain. The OT hormone only differs in two amino acids and the OT-associated NP domain is 96% identical to VP-associated NP [4]. NP has the capacity to oligomerise into dimers and high-order complexes and this is greatly facilitated by hormone association to NP (reviewed in [5]). These properties of NP are especially favoured by TGN conditions. Therefore, prohormone aggregation is either under the control of the hormone binding or directly triggered by encountering the TGN milieu. In fact, the hormone binding takes place in the endoplasmic reticulum (ER), where the folding of the prohormone and the correct disulfide linkage pattern (of eight disulfides) is safeguarded by the hormone–NP binding [6,7]. It has been proposed recently that the hormone domain acts as an internal chaperone for the folding of the whole prohormone [8]. Following on from this, it appears that the aggregation of NP in the secretory pathway is solely regulated by the solvent conditions in the TGN. Thus, we reasoned it may be possible, for the first time, to dissect aggregation from a putative sorting-by-recognition mechanism by fusing truncated fragments of VP prohormone to a fluorescent tag (the enhanced form of green fluorescent protein, eGFP) which can be followed through the secretory pathway. Therefore, we fused eGFP to various truncated VP prohormone fragments, in which regions of the NP domain were eliminated (Fig. 1). We found that sorting was achieved by only a small N-terminal domain of the VP prohormone of 27 amino acids.

2. Materials and methods

2.1. Constructs

Fusions of N-terminal fragments of the VP preprohormone with eGFP were generated. The cDNAs encoding the wild-type rat VP preprohormone (pWT-cDNA/AmpI) or the preprohormone in which the VP domain is deleted (pΔVP-cDNA/AmpI) were inserted into the *Eco*RV site of pcDNA/AmpI (Invitrogen) [9,10]. Deletion fragments were generated from these constructs using different restriction sites

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Abbreviations: eGFP, enhanced form of green fluorescent protein; ER, endoplasmic reticulum; GFP, green fluorescent protein; GP, glycopeptide; NP, neurophysin; OT, oxytocin; RSP, regulated secretory pathway; SP, signal peptide; TGN, *trans*-Golgi network; VP, vasopressin

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and inserted in frame with the eGFP coding sequence in pEGFP-N1 (Clontech). The following fusion plasmids were generated: (a) pVP121-eGFP-N1, a *Hin*dIII-*Sma*I fragment of the preproVP cDNA was inserted into the corresponding sites of pEGFP-N1. The corresponding fusion is called VP121-eGFP; (b) pVP27-eGFP-N1, a *Hin*dIII-*Bgl*I fragment of the preproVP cDNA was inserted into a *Hin*dIII-*Pst*I-digested pEGFP-N1, where the *Bgl*I and *Pst*I ends were blunt-ligated. The corresponding fusion is called VP27-eGFP; (c) pVP10-GFP-N1, a *Hin*dIII-*Fok*I fragment of the preproVP cDNA was inserted into a *Hin*dIII-*Acc*I-digested pEGFP-N1, where the *Fok*I and *Acc*I ends were blunt-ligated. The corresponding fusion is called VP10-eGFP; (d) p(ΔVP)NP15-GFP-N1, a *Hin*dIII-*Bgl*I fragment of pΔVP-cDNA/AmpI was inserted into a *Hin*dIII-*Pst*I-digested pEGFP-N1, where the *Bgl*I and *Pst*I ends were blunt-ligated. The corresponding fusion is called [ΔVP]NP15-eGFP. All generated fusions were checked by sequencing.

2.2. Cell culture and transfection

Mouse Neuro2A neuroblastoma cells and mouse anterior pituitary AtT20 cells (kindly provided by Prof. J.P.H. Burbach, Utrecht, The Netherlands) were cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal bovine serum, non-essential amino acids and 200 IU/ml pentamycin/streptomycin (medium and all supplements from Life Technologies). Cells were transfected by calcium precipitation [11] or liposome-mediated transfection using Escort™ (Sigma) or Fugene6 (Roche Molecular Biochemical). After transfection, cells were either analysed over the following 2 or 3 days or further selected for stable expression with 500 μg/ml gentamicin (G418, Life Technologies).

2.3. Immunofluorescence microscopy

Cells were plated onto 22 mm diameter glass coverslips and fixed with 4% (w/v) paraformaldehyde at a density of approximately 70%. For immunostaining, the cells were first permeabilised with 0.1% (v/v) Triton X-100 for 4 min, then subjected to a 1 h incubation at room temperature with 1% (w/v) bovine serum albumin in phosphate-buffered saline (PBS, pH 7.4) and then incubated for 2 h at room temperature with primary antibody: anti-chromogranin (Research Diagnostics Inc.), then washed with PBS and further incubated for another 2 h at room temperature with secondary antibody: TRITC-labelled anti-rabbit antibody (Vector Laboratories). After several washes with PBS, stained cells were embedded in Vectashield (Vector Laboratories) and analysed using a Leitz confocal laser scanning microscope and analysis software package.

2.4. Immunoprecipitation

Cells were treated and analysed as described previously [12]. In short, cells were labelled overnight for 16–18 h with 2.5 μl (0.74 MBq) [^{35}S]Met or [^{35}S]Cys (ICN Biochemicals). To assess the steady-state distribution of a fusion, the cells and media were harvested directly after the overnight labelling and immunoprecipitated as described [12]. To measure regulated secretion, the cells were washed twice with PBS and subsequently incubated in either basal secretion medium containing 125 mM NaCl, 4.8 mM KCl, 1.8 mM $CaCl_2$, 1.4 $MgCl_2$, 10 mM glucose, 25 mM HEPES (pH 7.4), or stimulus secretion medium which is basal secretion medium with $CaCl_2$ replaced by 3 mM $BaCl_2$. After 10 min incubation at 37°C in a CO_2 incubator (5% v/v CO_2) cells and medium were harvested and immunoprecipitated as described [12] with the only differences that the following primary antibodies were used: 1:500 anti-NP (ICN Biochemicals) or 1:500 anti-GFP polyclonal (Clontech), and that in case of the GFP immunoprecipitation the protein A Sepharose beads were washed four times with lysis buffer containing 0.1% (v/v) Triton X-100.

2.5. Western blotting and secretion experiments

Cells were plated as for immunoprecipitation. For secretion, cells were grown to a density of 80%, washed two times with PBS and subjected to either 1 ml/well basal secretion medium (as above) or 1 ml/well stimulus secretion medium (as above). The secretion media were dialysed against 2 l of distilled H_2O overnight at 4°C and subsequently dried in vacuo using a Speedvac (Savant). For analysis, cell and medium pellets were boiled in Tris–glycine sample buffer and loaded onto a 13.5 or 15% (w/v) SDS–PAGE Tris–glycine (pH 8.8) gel system and transferred onto Immobilon-P transfer membranes (Millipore). After transfer the blots were equilibrated in TBST (20 mM Tris–HCl (pH 7.4), 137 mM NaCl, 0.1% (v/v) Triton X-100) for 15 min and then blocked with TBSAT (5% (w/v) BSA in TBST) for 1 h at room temperature. Subsequently, the blots were incubated for 1 h at room temperature in TBSAT with primary antibody: anti-eGFP (Living Colors Peptide antibody, Clontech) or anti-VP (86/3-F, kindly given by Sonia Birkett) [13]. The blots were washed several times in TBST and subsequently incubated with a horseradish peroxidase-linked anti-rabbit antibody (Amersham) in TBSAT for 1 h at room temperature. The blots were washed again several times in TBST and finally the blots were incubated with a 1:1 mixture of ECL reagents 1 and 2 (ECL kit from Amersham, RPN2109) for exactly 1 min at room temperature. Several exposures were taken from the blots and the films were developed and analysed.

3. Results

3.1. Introducing eGFP into the secretory pathway

Native GFP is a cytosolic protein in jellyfish (*Aequorea victoria*) and when the enhanced form (eGFP) is expressed in eukaryotic cell lines it also accumulates in the cytosol (Fig. 2) [14,15]. GFP has been fused with several targeting sequences recently to show that cellular location is not affected by the eGFP tag [17,18]. However, eGFP might be targeted to any organelle within the secretory pathway by cryptic sequences. Therefore, eGFP was fused with the signal peptide of the VP preprohormone (SP-eGFP). The signal peptide will deliver eGFP into the ER and since proper cleavage of the signal peptide is directed by the structural information within the signal peptide itself [16], eGFP would be able to follow its own route through the secretory pathway. After heterologous expression in the neuroendocrine Neuro2A and AtT20 cell lines, immunolocalisation revealed green fluores-

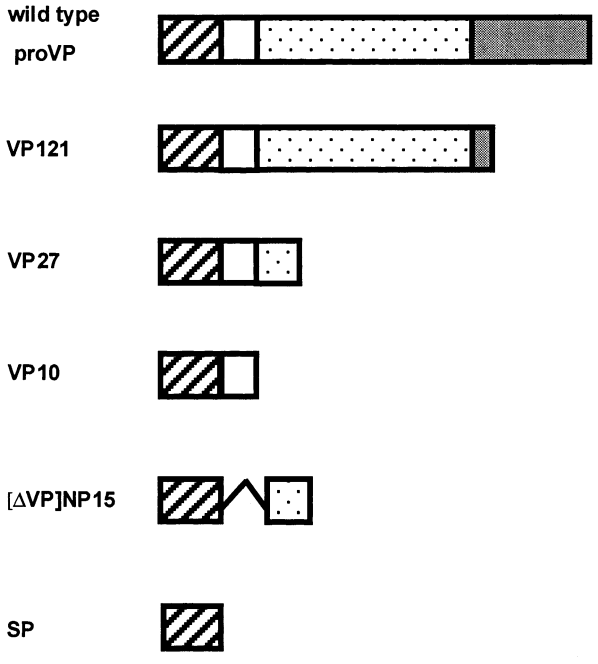

Fig. 1. Schematic overview of the various eGFP fusions. Shown are all the fusions used in this study compared with the wild-type VP preprohormone with the signal peptide as a striped bar, the VP domain in white, the NP domain in dotted, and the GP domain in grey. The number after the fragment name 'VP' indicates the number of N-terminal proVP residues, whereas the number after the fragment name 'NP' indicates the number of N-terminal NP residues.

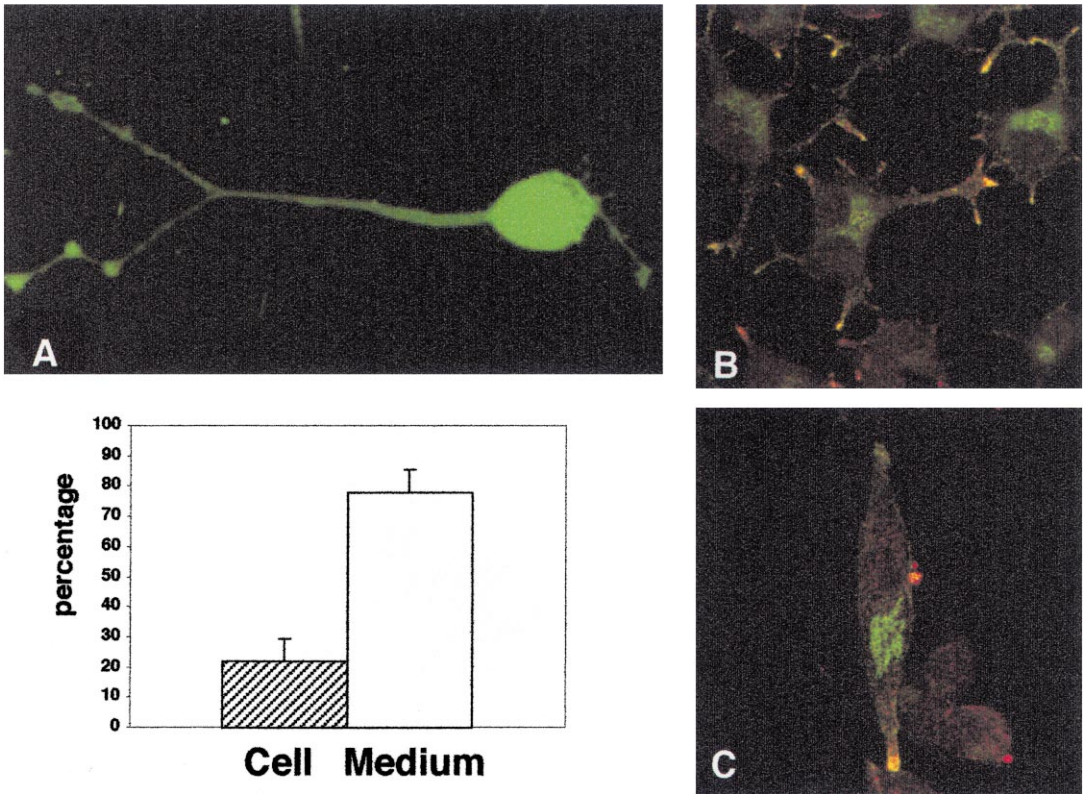

Fig. 2. Heterologous expression of SP-eGFP. Expression of eGFP in Neuro2A cells shows expression in the cytosol (A). SP-eGFP expressed in Neuro2A (B) and AtT20 (C) cells is detected in a granular fashion and colocalised with red anti-chromogranin staining. When cell and culture medium were analysed by immunoprecipitation using a polyclonal anti-GFP antiserum eGFP was predominantly found in the culture medium after overnight labelling (histogram).

cent granules that colocalised with chromogranin, an established soluble marker of the RSP (Fig. 2). Since immunolocalisation only allows the observation of the intracellular portion of a secretory protein it was necessary to analyse the distribution of eGFP inside and outside SP-eGFP-expressing cells by immunoprecipitation. This demonstrated that after an overnight labelling, which can be regarded as an assessment of the steady-state distribution, eGFP introduced into the secretory pathway is mainly found outside the cell in the culture medium (Fig. 2). Only 20–26% of eGFP expressed as SP-eGFP was retained inside Neuro2A and AtT20 cells. The remaining 74–80% was released into the culture medium, which is typical of a predominantly constitutively secreted protein. A constitutive secretory protein (ovalbumin) was similarly distributed in the cell and medium (data not shown).

3.2. *Heterologous expression of eGFP fused to truncated fragments of proVP*

To address the question of whether a regulated secretory protein like proVP harbours a sorting signal for delivery into

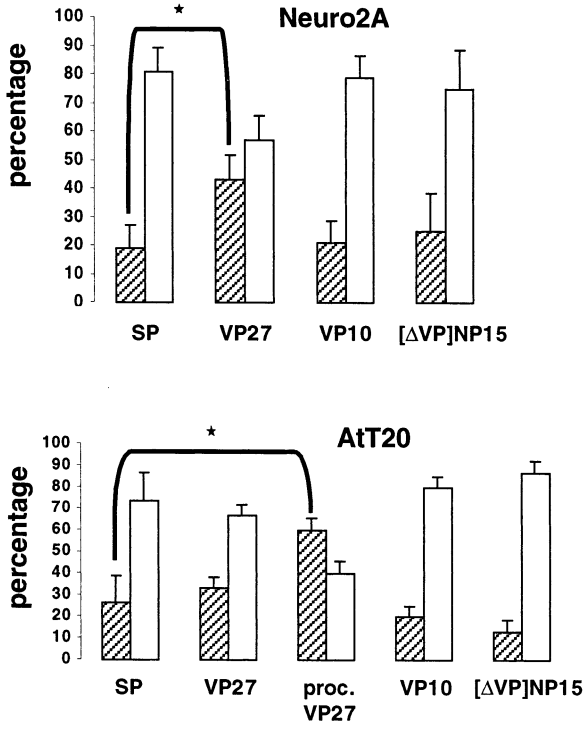

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Fig. 3. Distribution of VP-eGFP fusions over cell and medium. Shown is the result of immunoprecipitation analysis after overnight labelling of cell (striped) and culture medium (white) of Neuro2A and AtT20 cells expressing the different VP-eGFP fusions, which are abbreviated to their respective VP domains fused to eGFP (SP, VP27, VP10, [ΔVP]NP15). In AtT20 cells the VP27-eGFP fusion was also found as a smaller, processed form (proc. VP27). Statistical analysis was performed using ANOVA or Student's *t*-test ($P < 0.05$).

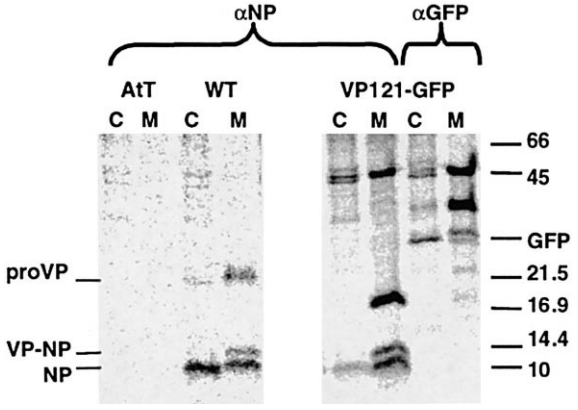

Fig. 4. Expression and distribution of VP121-eGFP over cell and medium. Distribution over cell and medium of VP121-eGFP in AtT20 cells (C) and culture medium (M) was assessed by immunoprecipitation. Using an anti-NP antiserum (αNP) one band corresponding to NP is found inside the cell of wild-type prohormone-expressing cells (WT), whereas three bands are found in the culture medium corresponding to prohormone, partially processed VP-NP and fully processed NP. These bands were identified as such earlier [12]. In VP121-eGFP-expressing cells a similar pattern is found with the anti-NP antiserum, but in addition also a band corresponding to the full-length fusion (43 kDa) is detected at 45 kDa which is also detected by an anti-eGFP antiserum (αGFP). The band running at around 16.9 kDa corresponds to the shorter VP-NP-GP_{1-14} (17 kDa). The anti-eGFP antiserum detected at least three bands in cell and medium of which the first one corresponds to the full-length fusion (45 kDa), the second one corresponds to GP_{1-14}-eGFP (33 kDa) and the third one corresponds to eGFP (26 kDa).

the RSP, various fusions with eGFP were constructed (Fig. 1). Immunolocalisation of all fusions looked identical with regard to their colocalisation with chromogranin and identical to SP-eGFP as shown in Fig. 2B,C. However, as demonstrated with the SP-eGFP fusion, this does not necessarily indicate sorting into the RSP. Further analysis by immunoprecipitation of the relative distribution of steady-state eGFP levels in the cell and in medium revealed that the fusions VP10-eGFP (VP domain fused to eGFP) and [ΔVP]NP15-eGFP were mainly released into the culture medium similar to SP-eGFP (Fig. 3). In contrast, two fusions were found more inside the cell – VP121-eGFP and VP27-eGFP (Figs. 3 and 4). The fusion VP121-eGFP is the longest fusion used in this study, and incorporates the VP domain, the NP domain and 14 residues of the C-terminal glycopeptide (Fig. 1). Immunoprecipitation with an anti-NP antiserum demonstrated the presence of fully processed NP, identical to that of the wild-type VP prohormone (Fig. 4). More importantly, the shorter fusion VP27-eGFP, consisting of the VP domain and the first 14 residues of the NP domain, is also mainly localised inside the cell (Fig. 3). In addition, in AtT20 cells, VP27-eGFP is found to be processed into two forms: one form migrating at the same level as (SDS–PAGE) or slightly above (Tris–Tricine–PAGE) standard eGFP, and one form migrating at approximately 30 kDa, corresponding to the size of the full-length fusion, and with the single eGFP-immunoreactive form detected in Neuro2A cells (Fig. 5). These two bands were always detected in AtT20 cells whether heterologous or homologous stable cell lines were analysed. These data suggest a higher efficiency of processing in AtT20 cells compared to Neuro2A cells and, indeed, the prohormone convertases PC1/3 and PC2, which are regarded as the main candidates for cleaving the dibasic site between VP and NP in the VP prohormone, are expressed abundantly in AtT20 cells, but are virtually absent in Neuro2A cells [17,18]. Probing with an antibody recognising VP further confirmed that only the upper band contains the VP domain, whereas the lower band was not detected (Fig. 5). Analysis of the distribution of the two forms over cell and medium showed that the full-length fusion was released in a constitutive fashion whereas the processed form was retained in the cell (Fig. 3). To assess whether the processed form was secreted in a regulated fashion, a secretion stimulus was applied for 10 min and secretion of eGFP- and VP-related forms were measured by Western blotting (Fig. 6). Our secretion paradigm proves to be an adequate model to compare the secretion of different fusions, because the secretion of NP derived from wild-type VP prohormone is released effectively by this paradigm (Fig. 6A). Furthermore, the fact that AtT20 cells harbour predominantly the fully processed NP after overnight labelling indicates strongly that there is an efficient sorting and storage of VP prohormone (product). When this paradigm is applied to SP-eGFP-expressing AtT20 cells, there is a small amount of stimulated release (Fig. 6B), but more importantly, when applied to VP27-eGFP-expressing AtT20 cells, there is almost exclusively release of the processed fusion (Fig. 6C).

These data confirm that the unprocessed form of VP27-

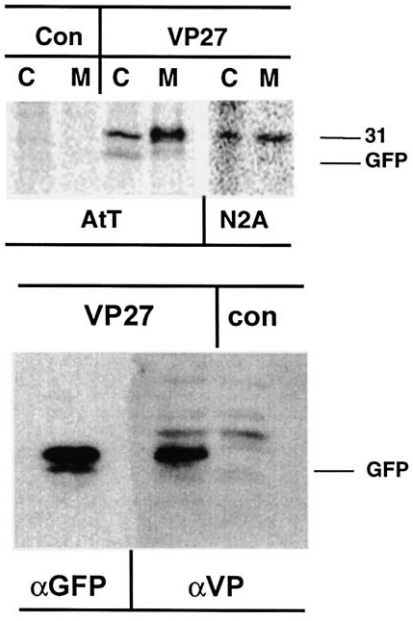

Fig. 5. Processing of VP27-eGFP in AtT20 cells. The top panel shows processing of VP27-eGFP in AtT20 cells (AtT, lanes 1–4), but not in Neuro2A cells (N2A, lanes 5–6). In both cell (C) and medium (M) two bands are immunoprecipitated with an anti-GFP antiserum (αGFP) in VP27-eGFP-expressing AtT20 cells (VP27, lanes 3–4), whereas in VP27-eGFP-expressing Neuro2A cells (VP27, lanes 5–6) only one band appears corresponding to 31 kDa, the expected size of the full-length fusion. Control cells and medium of normal AtT20 cells do not show any immunoreactive bands (con, lanes 1–2). In the bottom panel Western blotting analysis of VP27-eGFP-expressing AtT20 cells shows that an anti-GFP antiserum (lane 1) detected two bands, whereas an anti-VP antiserum (αVP) detected only the upper band corresponding to the full-length fusion. The third lane shows the non-specific bands found with anti-VP antiserum in control AtT20 cells (con). Equal amounts of all cell samples were loaded onto gel for the Western blot: the equivalent of 10% of cells from a 3.5 cm well (6 well plate).

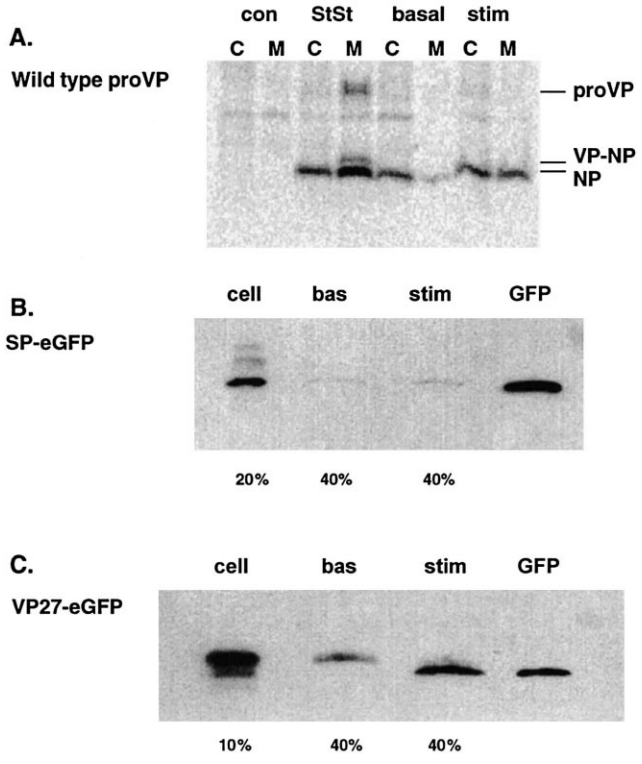

Fig. 6. Regulated secretion of wild-type VP prohormone, SP-eGFP and VP27-eGFP. A: Wild-type VP prohormone-expressing AtT20 cells (C) and medium (M) were analysed for the steady-state distribution of NP-related prohormone material (StSt, lanes 3–4) and regulated secretion (lanes 5–6 = basal release, lanes 7–8 = stimulated release) and compared to normal, control AtT20 cells (lanes 1–2, con) as assessed by immunoprecipitation with an anti-NP antiserum. For the secretion protocol see Section 2. B: The regulated secretion of SP-eGFP-expressing AtT20 cells was analysed by Western blotting using an anti-GFP antiserum. The same secretion protocol was used as in A for basal (bas, lane 2) and stimulated (stim, lane 3) release. As a control is shown the intracellular content (lane 1, cell) and the migration position of standard GFP (GFP, lane 4). C: The regulated secretion of VP27-eGFP fusion was analysed identical to the SP-eGFP fusion analysis in A and B, where 'cell' is the intracellular content, 'bas' is basal release, 'stim' is stimulated release and 'GFP' indicates the migration position of standard GFP as assessed by Western blotting using an anti-GFP antiserum. Under each lane of the Western blots in B and C is indicated in percentages how much of each sample was loaded.

eGFP is predominantly directed to the constitutive pathway, whereas the processed VP27-eGFP form is targeted to the regulated secretory pathway.

4. Discussion

We have demonstrated that a short linear signal from a prohormone can direct the sorting of a heterologous protein into the RSP.

We were initially surprised to find that the non-secretory protein like eGFP could be found in the RSP, once directed into the ER by a signal peptide. GFP/enhanced GFP has been used several times in sorting studies, but it has never been shown how it travels through the secretory pathway on its own. Since 80% of the signal peptide-eGFP was found outside the cell, there appears to be no problem with the trafficking of the signal peptide-eGFP fusion. Relying on immunoprecipitation data alone we simply would have concluded that the signal peptide-eGFP fusion behaves like a constitutively secreted protein. However, the strong fluorescence of eGFP made it possible to localise it in granules of the RSP as well. Thus, regarding the immunoprecipitation data, this indicates that a small proportion of the expressed signal peptide-eGFP is found in the RSP. Since such an analysis has not been carried out before it adds two important findings to the knowledge about sorting into the RSP: (1) eGFP behaves like a constitutively secreted protein and is therefore a legitimate marker protein for any study relating to the trafficking through the secretory pathway starting from the ER; (2) more importantly, a non-secretory protein can inefficiently enter the RSP, most likely through leakage as proposed by Arvan and Castle [1]. Their sorting-by-retention hypothesis allows for 'leakage' of non-regulated secretory proteins into the RSP which does not require any recognition process during sorting [1].

However, we went on to show that fusions of regions of the VP prohormone with eGFP could result in a higher extent of sorting. Leaving the NP domain intact results in full processing of the prohormone, as demonstrated by the analysis of the fusion VP121-eGFP, whereas partial or complete deletion of the NP domain leads to incomplete processing and sorting, or even missorting. Only the processed derivative of fusion VP27-eGFP was retained in the RSP.

Since only VP27-eGFP was found to be sorted into the RSP and not the separate domains of the VP27 fragment (VP10 and [ΔVP]NP15 fused to eGFP), we have demonstrated that it is crucial to have both the VP domain and the N-terminus of the NP domain to achieve sorting. This could mean that the enzymes that recognise the processing site between VP and NP (Fig. 1) are involved in sorting. However, VP27-eGFP is still sorted into the RSP of Neuro2A cells, which virtually lack prohormone convertases. It is unlikely, therefore, that the prohormone convertases are involved in specific retention. The explanation for the exclusive sorting of VP27-eGFP only may be found in the ample knowledge of the physicochemical properties of the VP precursor. In the crystal structures of the hormone–NP complexes, the N-terminal portion of NP is disordered, without a defined structure. However, when analysed in the prohormone setting, with the N-terminal portion of NP covalently linked to the hormone, it retains a defined secondary structure [19,20]. Therefore, only in the prohormone setting, as in VP27-GFP, can the N-terminus of NP (residues 1–15) retain a specific structure, which could be required for sorting-recognition.

Taking the processing in AtT20 cells as a measure of sorting into the RSP, it appears that only a relatively small proportion of the VP27-eGFP fusion is sorted and processed, suggesting that the mechanism of sorting by recognition is, on its own, rather inefficient. Since we have shown that sorting by recognition is utilised as a means to retain a prohormone inside the regulated secretory granules, it is likely that adding the aggregative property to this would increase the retention of the prohormone to required efficiency. Only the vast structural and physicochemical knowledge of the VP/OT precursor (domains) [4,5] and previous work [6,10,12] makes it possible to dissect the aggregative and sorting properties of this prohormone.

In summary, we have identified a sorting domain in the VP prohormone, which resides within the N-terminal 27 residues. Physicochemical data indicate that it is largely located on the

surface of the prohormone. This study shows, for the first time, that sorting by recognition, on its own, is an inefficient mechanism of sorting and also for the first time, that sorting by retention can cause visible leakage of non-secretory proteins into the RSP as anticipated [1]. Our data suggest that a combinatorial mechanism of aggregate sorting by recognition and sorting by retention is responsible for a complete, efficient sorting into the RSP.

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