Manipulating sorting signals to generate co-expression of somatostatin and eGFP in the regulated secretory pathway from a monocistronic construct

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Abstract

Targeted overexpression of biologically active peptides represents a powerful approach to the functional dissection of neuroendocrine systems. However, the requirement to generate separate, biologically active and reporter molecules necessitates the use of internal ribosome entry site (IRES) technology, which often results in preferential translation of the second cistron. We report here a novel approach in which the proteolytic processing machinery of the regulated secretory pathway (RSP) has been exploited to generate multiple mature proteins from a monocistronic construct that encodes a single precursor. This was achieved by duplication of the pre-pro cleavage sites in pre-prosomatostatin cDNA. The duplicated site included 10 flanking amino acids on either side of the Gly-Ala cleavage position. This enabled the incorporation of a foreign protein-coding sequence (in this case, enhanced green fluorescent protein (eGFP)) between these sites. The pre-eGFP-prosomatostatin (PEPS) construct generated co-localized expression of fully processed eGFP and somatostatin to the RSP of transiently transfected AtT20 cells. This approach represents an advance upon bicistronic and other extant approaches to the targeting of multiple, biologically active proteins to neuroendocrine systems, and, importantly, permits the co-expression of fluorescent markers with biologically active neuropeptides. In this study, our demonstration of the fusion of the first 10 amino acids of the prosomatostatin sequence to the N-terminus of eGFP shows that this putative sorting sequence is sufficient to direct expression to the RSP.

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Introduction

Neuropeptide gene promoters are increasingly being used in transgenic studies of gene expression and function in neuroendocrine systems (reviewed in Wells & Carter 2001). In this context, the necessity to distinguish between endogenous and transgene-derived products is routinely overcome by the expression of a fusion protein, in which a fluorescent marker is fused to the carboxyl terminal of the protein of study. This approach has been used successfully to trace processing, packaging and trafficking of a variety of neuropeptides (e.g. Burke *et al.* 1997, Magoulas *et al.* 2000, Han *et al.* 2002,

Zhang *et al.* 2002). However, this technique is not always appropriate for the identification of small peptide products, particularly when biological activity is required. Conversely, generation of an amino terminal fusion protein is equally inappropriate, since this is likely to interfere with targeting to the endoplasmic reticulum (ER).

An alternative approach is to utilize technologies developed to generate co-expression of two separate products from one transgene. In the most established method, a viral internal ribosome entry site (IRES) is used to drive bicistronic expression (Houdebine & Attal 1999). A drawback of this strategy is that the two transgene products are not

Figure 1 The construction of the novel PEPS transgene. The PEPS construct was generated by PCR products derived from the preprosomatostatin cDNA (A). The 124 bp N-terminal sequence (B) was cloned into the 342 bp C-terminal sequence (within the pGEM-T vector) (D) via HindIII and BglII restriction sites, to yield a 466 bp sequence (E). Finally, the 729 bp eGFP sequence (C) was ligated into the 466 bp sequence via BglII and BamHI restriction sites, to yield the final 1195 bp sequence (F). The duplicated cleavage sites included 10 flanking amino acids on either side of the Gly-Ala cleavage position (G). This final sequence of approximately 1.2 kb was cloned into the mammalian expression vector, pcDNA 3.1 myc'hisA(+) (5.7 kb), for transient transfection of AtT20 cells.

always expressed equally, possibly as a result of variability in ribosome recruitment of biologically active and reporter molecules (Houdebine & Attal 1999). In a second system, a bidirectional cassette, consisting of two cytomegalovirus promoters orientated in opposite directions, drives expression of both products (Jerecic *et al.* 1999), but this approach has not been widely tested. We have now developed a novel strategy in which a biologically active neurohormone and a non-interfering fluorescent reporter are generated from a monocistronic construct.

Like other hypophysiotropic hormones, the neuropeptide somatostatin is produced as a pre-propeptide (Shen *et al.* 1982, Funckes *et al.* 1983, Goodman *et al.* 1983). Targeting of somatostatin to the regulated secretory pathway (RSP) is believed to be determined by sorting signals contained within the pre-pro sequence (Kelly 1985, Sevarino *et al.* 1989). The pre-region directs entry of the full-length precursor into the ER, with concomitant removal of the pre-sequence (Chu *et al.* 1990). Mutation and substitution studies suggest that a sorting signal within the first 10 amino-acid residues of the pro-region, which is highly homologous between species (Argos *et al.* 1983), directs prosomatostatin to the RSP

(Sevarino & Stork 1991), further processing to somatostatin-14 and somatostatin-28 occurring in the Golgi apparatus (Lepage-Lezin *et al.* 1991). We have now utilized these putative sorting signals to target expression of both a fluorescent reporter protein and fully processed somatostatin to the RSP.

In this study, a transgene construct has been generated in which the endogenous pre-pro cleavage site in a somatostatin cDNA has been duplicated and an enhanced green fluorescent protein (eGFP) sequence cloned between these two sites (Fig. 1). The upstream pre-pro cleavage site included a 10-amino-acid sequence of the pro-peptide fused to the amino-terminal of eGFP, and the second site (downstream of eGFP) included the last 10 amino acids of the pre-sequence, plus the entire pro-somatostatin sequence. Thus, expression of the pre-eGFP-prosomatostatin (PEPS) construct should give rise to the pre-peptide, the eGFP reporter (bearing 10 amino acids of the pro-sequence at the amino terminal and 10 amino acids of the pre-sequence at the carboxyl terminal) and prosomatostatin.

GFP was used as a reporter in this study, as it has previously been targeted to most cell organelles, including the ER (Miyawaki *et al.* 1997), Golgi apparatus (Presley *et al.* 1997), and secretory

vesicles (Kaether & Gerdes 1995). Enhanced GFP (eGFP) was selected, as it produces a more robust fluorescent signal and has previously been expressed in neuroendocrine cells (Young *et al.* 1999, de Bree *et al.* 2000, Balthasar *et al.* 2003), without disrupting secretory function (Balthasar *et al.* 2003). The subcellular pattern of eGFP and somatostatin expression generated by this PEPS construct was compared with that produced by a somatostatin-IRES-eGFP construct after transient transfection of AtT20 cells. This pituitary-derived cell line is routinely used to analyse the processing of peptide hormones in both the regulated and constitutive secretory pathways (Sevarino *et al.* 1989, Sevarino & Stork 1991, de Bree *et al.* 2000). A preliminary report of sections of this work has previously been communicated (Davies *et al.* 2002).

Materials and Methods

Construction of the PEPS transgene

First-strand cDNA was synthesized from rat hypothalamic total RNA with the SuperScript Preamplification System (Invitrogen, Paisley, UK). Oligonucleotide primers (forward primer: ATGCTGTCCTGCCGTCTCCAGTGC; reverse primer: GCTAACAGGATGTGAATGTCTTCC) were used to generate the somatostatin cDNA (351 bp) by PCR. The cDNA sequence was subsequently cloned into pGEM-T Vector (Promega) for use in subsequent cloning steps. The cDNA sequence was verified by sequencing on both strands (Lark DNA Technologies, Saffron Waldon, UK).

Oligonucleotide primers were designed to generate three sequences from the somatostatin-pGEM-T template (Fig. 1a): (1) an N-terminal prepro-somatostatin sequence (124 bp), flanked by HindIII and BglII restriction sites (Fig. 1b) generated with 5'-CAAAGCTTGGCAGGGGAGATGCTGTCC TGCTGTCTCTGCCGTCTCCAGTG-3' (forward primer) and 5'-CAAGATCTAACTGACGGAG TCTGGGGTCCG-3' (reverse primer); (2) a C-terminal prepro-somatostatin sequence (342 bp), flanked at the N-terminal by HindIII-BglII-BamHI and at the C-terminal by XbaI restriction sites (Fig. 1d), generated with 5'-GGTAAGCTTACGAGATCTCTTGGATCCATCGTCCTGGC TTTGGGCGG-3' (forward primer) and 5'-CATCTAGACTAACAGGATGTGAATGTCTTCCAGAAG-3' (reverse primer); (3) a sequence

comprising the eGFP reporter molecule (pEGFP-N1, BD Clontech, Palo Alto, CA, USA), flanked by BglII and BamHI restriction sites (729 bp) (Fig. 1c), generated with 5'-CAAGATCTATGGTGAGCAAGGGCAGGAGC-3' (forward primer) and 5'-CAGGATCCCTTGACAGCTCGTCCATGCCG-3' (reverse primer). The 124 bp N-terminal sequence (Fig. 1b) was cloned into the 342 bp C-terminal sequence within the pGEM-T vector (Fig. 1d) via HindIII and BglII restriction sites, to yield a 466 bp sequence (Fig. 1e). Finally, the 729 bp eGFP sequence (Fig. 1c) was ligated into the 466 bp sequence via BglII and BamHI restriction sites, to yield the final 1195 bp PEPS sequence (Fig. 1f), in which eGFP was incorporated between duplicated pre-pro cleavage sites. Each site included 10 flanking amino acids on either side of the Gly-Ala cleavage position. This was subsequently cloned into the mammalian expression vector, pcDNA 3.1 myc⁺hisA(+) (Invitrogen, 5.7 kb). This resulted in a 6.9 kb plasmid, the size of which was confirmed by restriction digest by HindIII and XbaI enzymes, the PEPS sequence being confirmed by DNA sequencing (Lark DNA Technologies).

Construction of somatostatin-pIRES2-eGFP transgene

With somatostatin-pGEM-T as template (generated using the previously described somatostatin cDNA) (Fig. 1a), somatostatin was cloned into the multiple cloning site (MCS) of the pIRES2-eGFP vector (BD Clontech), via BglII and XhoI restriction sites after amplification by PCR (primers 5'-CAAGATCTGGCAGGGGAGATGCTGTCC TGCCGTCTCCAGTG-3' (forward primer) and 5'-CACTCGAGCTAACAGGATGTGAATGCTTCCAGAAG-3' (reverse primer)). This generated a 5.7 kb plasmid, the somatostatin sequence region being verified by DNA sequencing (Lark DNA Technologies).

Cell culture and transgene transfection

All cell-culture work was carried out under aseptic conditions in a laminar flow hood. Unless stated otherwise, cells were cultured in 75 cm² tissue culture flasks and incubated at 37 °C in humidified air containing 5% CO₂. Frozen cells were removed from liquid nitrogen storage and thawed at room

temperature for 1 min, followed by incubation in a 37 °C water bath for 1 min. Cells were resuspended in 10 ml culture medium (Dulbecco's Modified Eagle's Medium (DMEM) with Glutamax-1 (4500 mg/l glucose; Gibco, (Invitrogen) Paisley, UK) supplemented with 10% heat-inactivated foetal calf serum (FCS) (Sigma), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Gibco); complete cell medium), and pelleted by centrifugation at 150 *g* (Lab-4000, Heraeus Sepatech, Germany) for 10 min at room temperature. The supernatant was discarded, and the pellet was resuspended in 20 ml complete cell medium and placed in a 75 cm² tissue-culture flask.

After cell growth, the flasks were rinsed with PBS (Gibco) and incubated in trypsin-EDTA solution (500 g/ml trypsin and 200 g/ml EDTA in PBS) until detached. Cell-culture medium was added and cells pelleted by centrifugation at 150 *g* for 5 min at room temperature. The supernatant was discarded and the pellet resuspended in cell culture medium for distribution into culture flasks. The population of viable cells was determined by diluting a known volume of cells in culture medium (containing 0.4% (w/v) trypan blue (Sigma)) and counting the cells in a haemocytometer.

PEPS and somatostatin-IRES-EGFP transgenes together with negative (no DNA) and positive (eGFP-N1; Clontech) controls were transiently transfected by a calcium phosphate precipitation into AtT20 cells. Prior to transfection, cells were grown on untreated sterile glass cover slips (BDH, Poole, UK) in six-well plates. A volume of 500 µl HEPES-buffered saline (HEBS), pH 7.05, containing circular DNA (32–35 µg) and CaCl₂ (30 µl of 2 M, Sigma) was added to the cells and incubated for 20–96 h at 37 °C in humidified air containing 5% CO₂.

Immunocytochemical analysis of transgene expression

When almost confluent, the cells were washed with PBS (pH 7.4) and fixed in 4% (w/v) paraformaldehyde (in PBS) for 30 min. After three washes with PBS, the cells were permeabilized with 0.1% (v/v) Triton X-100/PBS for 3–4 min, and washed four times in 1% PBS. Nonspecific protein binding was blocked with 1% (w/v) BSA/PBS for 20 min at room temperature, prior to incubating with primary antibody (in 1% BSA/PBS; 50 µl/cover

slip) overnight at 4 °C in a moistened container. The primary antibody used for localization of somatostatin was a 1:1000 dilution of rabbit antisomatostatin (Peninsula Laboratories, San Carlos, CA, USA; cat. no. IHC 8001), which only binds fully processed somatostatin-28 and -14. The primary antibody for chromogranin was a 1:200 dilution of rabbit antichromogranin (Research Diagnostics, Inc., Flanders, NJ, USA; cat. no. RDI-PRO 11422). After overnight incubation, the cells were washed three times with 1% PBS prior to incubation with secondary antibody (in 1% BSA/PBS; 50 µl/cover slip). Cyan³-conjugated antirabbit antibody (1:100 dilution) and Texas Red isothiocyanate (TRITC)-conjugated antirabbit immunoglobulin G (Sigma) antibody (1:100 dilution) were used for detection of somatostatin and chromogranin respectively. After 2-h incubation at 4 °C, cover slips were washed in PBS and mounted onto a glass slide with VectaShield mounting medium (Vector Labs, Burlingame, CA, USA).

Cells were analysed by a Leica TCS SP scanning confocal laser microscope equipped with a Kr/Ar laser and 63X oil immersion objective. For the detection of EGFP, a filter set was used with an excitation filter of 510 nm and emission filter of 530 nm. TRITC and Cy³ fluorescence was visualized with a filter set with an excitation filter of 568 nm and emission filter of 590 nm. All images were processed with Leica software for 2-D analysis.

Western blot analysis of transgene product secretion

To measure regulated secretion, transfected cells were washed twice with PBS and incubated in either basal secretion medium (125 mM NaCl, 4.8 mM KCl, 1.8 mM CaCl₂, 1.4 mM MgCl₂, 10 mM glucose and 25 mM HEPES, pH 7.4), or stimulus secretion medium (basal secretion medium with CaCl₂ replaced by 3 mM BaCl₂) (Dittie & Tooze 1995) at 37 °C with 5% CO₂. After 60 min, the medium was collected and vacuum-dried overnight. The remaining cells were lysed with cell-lysis buffer (Promega), collected and stored overnight at 20 °C.

For Western blot analysis, lyophilized secretion media were resuspended in 30 µl sterile H₂O at 37 °C for 30 min. Secretion media or cell lysates (10 µl) were incubated with 15 µl Tris-glycine

loading buffer for 15 min at 90 °C. An amount of 10 µg cell lysates (measured by Bradford assay), or 10 µl secretion media was loaded onto a SDS-PAGE Tris-glycine gel system and resolved at 15 A for 2–3 h. It should be noted that, due to the low protein content (below the Bradford assay detection threshold), the total protein content of secretion media could not be quantified. Protein was subsequently transferred onto Immobilon-P transfer membranes (Millipore) by the Trans-Blot system (BioRad). The membranes were equilibrated in Tris-buffered saline plus 0.1% Tween 20 (TBST) for 15 min and then blocked with TBSAT (5% (w/v) BSA in TBST) overnight at 4 °C. Membranes were incubated overnight at 4 °C in TBSAT with primary antibody (anti-eGFP (1:100 dilution), BD Clontech; cat. no. 8367). The membranes were washed in TBST and incubated with a peroxidase-conjugated secondary antibody (anti-mouse IgG-peroxidase conjugate (1:8000 dilution in TBSAT), Sigma) for 1.5 h at room temperature. Membranes were washed again in TBST and incubated with chemiluminescence detection reagents (ECL kit; Amersham) prior to autoradiography. Repeated Western blot analysis was performed on duplicate transfection experiments.

Results

eGFP reporter expression in AtT20 cells

Transfection of AtT20 cells with the conventional somatostatin-IRES-eGFP transgene generated non-granular, cytoplasmic eGFP expression, with diffuse distribution throughout the cell (Fig. 2A (white arrow), H and I). In contrast, transfection with the PEPS construct generated granular distribution of eGFP at the distal ends of the neurite-like processes (Fig. 2B (white arrows), K and L). A dense region of extranuclear fluorescence, thought to correspond with the ER and/or the trans-Golgi network (TGN), was also observed (Fig. 2B (white arrowhead), K and L). As expected, a diffuse non-granular cytoplasmic distribution of eGFP was also observed in AtT20 cells transfected with the control eGFP-N1 construct (Fig. 2C; white arrows).

Subcellular localization of eGFP

Given the distinctly granular appearance of eGFP fluorescence after transfection with the PEPS

construct (Fig. 2B), we investigated whether eGFP expression was co-localized with chromogranin, a secretory granule protein. Analysis of chromogranin localization in untransfected AtT20 cells showed the expected granular expression, most prominent at the distal ends of the neurite-like processes (Fig. 2F). After transfection with the PEPS construct, eGFP and chromogranin expression was found to be co-localized in the secretory granules (Fig. 2E; yellow fluorescence and yellow arrow) with noticeable co-localization in the TGN. In contrast, there was no observable co-localization after transfection with the somatostatin-IRES-eGFP construct (Fig. 2D).

Co-localization of eGFP and somatostatin

Double-fluorescence immunohistochemistry was then performed on transfected AtT20 cells to determine whether transgene-derived somatostatin was co-localized with eGFP in the RSP. The somatostatin-IRES-eGFP construct produced differential intracellular distribution of somatostatin (red fluorescence) and eGFP (green fluorescence) (Fig. 2G–I). Somatostatin expression was distinctly granular in appearance, with the highest concentrations of expression being seen adjacent to the nucleus and in the distal ends of neurite-like processes (Fig. 2G and I). However, eGFP was not co-localized with somatostatin, being distributed throughout the cytoplasm in a non-granular fashion (Fig. 2H and I). In contrast, the PEPS construct generated granular expression of both somatostatin (Fig. 2J and L; red fluorescence) and eGFP (Fig. 2K and L; green fluorescence). The co-localization of both transgene products was confirmed by the presence of yellow fluorescence, which was particularly prominent in the TGN and the distal ends of the neurite-like processes (Fig. 2L). The low level of endogenous somatostatin expression that is apparent in untransfected AtT20 cells is illustrated in Fig. 2I (inset).

Western blot analysis of eGFP secretion from AtT20 cells

To confirm whether eGFP in transfected AtT20 cells is under secretory-type regulation, Western analysis of eGFP was performed on cell lysates and incubation media under basal and stimulated conditions. Lysates of AtT20 cells transfected with

Cell lysates

Figure 3 Western blot analysis of eGFP in cell lysates for AtT20 cells transiently transfected with the somatostatin-IRES-eGFP (lanes 1 and 2) and the PEPS transgene (lanes 3 and 4), after basal (lanes 1 and 3) and stimulated (lanes 2 and 4) secretion. Lane 5 contains a molecular weight protein marker (BioRad Polypeptide SDS-PAGE standard, cat. no.161-0326).

the somatostatin-IRES-eGFP transgene showed a 27 kDa protein band (Fig. 3, lanes 1 and 2) corresponding to the molecular mass of eGFP. The intensity of this band was not affected by incubation in stimulation medium (Fig. 3, lane 2).

AtT20 cells transfected with the PEPS construct also expressed a 27 kDa product, corresponding to eGFP (Fig. 3, lanes 3 and 4), the intensity of expression being higher in cells incubated in stimulation secretion medium (Fig. 3, lane 4). A number of larger molecular mass bands (major bands of approximately 55 and 70 kDa), possibly corresponding to intermediate processing products (e.g. pre-eGFP-prosomatostatin, eGFP-pro, eGFP-prosomatostatin), were also observable in cell lysates after transfection with the PEPS construct (Fig. 3, lanes 3 and 4).

The amount of protein recovered from the secretion media was too low to be measured accurately by Bradford assay. We were, therefore, unable to perform a quantitative analysis. Despite these limitations, Western blot analysis of the secretion media of PEPS-transfected AtT20 cells demonstrated the absence of eGFP in the basal secretion medium and the presence of only fully processed eGFP in the stimulation medium, indicating that the reporter molecule was secreted in a regulated manner (data not shown).

Discussion

Targeted overexpression of biologically active peptides represents a powerful approach to the functional dissection of neuroendocrine systems.

Figure 2 Confocal microscopic analysis of transgene product expression patterns *in vitro*. AtT20 cells were transiently transfected with either somatostatin-IRES-eGFP (A, D, G–I), PEPS (B, E, J–L) or eGFP-N1 (C) constructs. Fluorescence for eGFP (green) is shown either alone (A–C, H, K), or in combination with either chromogranin (red; D, E) or somatostatin (red; I, L). Single fluorescent images of either chromogranin (red; F) or somatostatin (red; G, J) are shown. Endogenous somatostatin expression is shown (red; I, inset). The yellow fluorescent signal in double fluorescent images indicates the distribution of co-localization of eGFP with either chromogranin (E) or somatostatin (L). White arrows indicate either diffuse (A and C) or punctate (B) eGFP fluorescence. White arrowhead (B) indicates eGFP expression in either trans-Golgi network or endoplasmic reticulum. The absence (D) or presence (E) of co-localization of eGFP with chromogranin is indicated by red or yellow arrows respectively.

However, in order to validate this approach, co-expression of a non-interfering reporter molecule (such as eGFP) in the same cellular compartment is required.

Endogenous neuropeptides are secreted from neuroendocrine cells via either the constitutive, or regulated secretory pathways (CSP or RSP). In the CSP, protein products are continuously delivered to the plasma membrane in small vesicles by an unregulated, calcium-independent mechanism. In the RSP, polypeptide products are transported in larger, dense-core vesicles, and secreted by stimulus-regulated, calcium-dependent exocytosis. Sorting signals that generate preferential targeting to the RSP are thought to reside in either the pre-(NPY) (Meskini *et al.* 2001) or pro- (somatostatin) (Sevarino & Stork 1991) hormone sequences. We have used this putative sorting signal in prosomatostatin to develop a novel approach in which biologically active somatostatin and an eGFP reporter are targeted simultaneously to the RSP. The incorporation of an eGFP sequence between duplicated pre-pro cleavage sites in a pre-prosomatostatin cDNA also enables this PEPS construct to generate both transgene products from a monocistronic construct. In this study, the expression patterns of somatostatin and eGFP in AtT20 cells after transient transfection of the PEPS construct have been compared with the expression produced by a conventional bicistronic IRES transgene.

Transient transfection of AtT20 cells with the bicistronic somatostatin-IRES-eGFP transgene generated a different expression pattern for the two transgene products. Fully processed somatostatin appeared to be confined to the RSP, being located within the TGN and the secretory granules, and these granules being especially prominent at the distal ends of the neurite-like processes. In contrast, this transgene generated a nuclear and cytoplasmic expression pattern for eGFP, similar to that observed with the unmodified eGFP-N1 vector. The absence of either significant eGFP expression in the TGN (Fig. 2H and I), or co-localization of eGFP with chromogranin (Fig. 2D), confirms that the bicistronic IRES construct did not target eGFP to the ER or the secretory apparatus. This is to be expected as the signal peptide and sorting signals that direct somatostatin firstly to the ER and hence to the RSP are not contained in the wild-type form of the eGFP protein. Nevertheless, the bicistronic

IRES construct clearly resulted in the expression of both transgene products, as has previously been demonstrated in peptidergic neurons *in vivo* with an oxytocin-IRES-eGFP construct (Young *et al.* 1999). Although, in the present study, this approach appeared to generate approximately equal expression of eGFP and somatostatin, it is now established that IRES sequences can favourably promote the translation of the second cistron (Houdebine & Attal 1999). Consequently, it is not possible to use the expression of a fluorescent reporter to make any quantitative assessment of the expression of the product translated from the alternative cistron.

In order to overcome this problem, we have devised a new approach in which two transgene products should result from a monocistronic construct. In addition, the novel PEPS construct, unlike the bicistronic transgene, produces co-localized expression of somatostatin and eGFP. Two lines of evidence indicate that this expression was specific for the RSP.

Firstly, transfection with the PEPS construct produced prominent granular expression of both transgene products, somatostatin and eGFP being co-localized in the ER and TGN, and at the distal ends of the neurite-like processes. Double immunofluorescence confirmed that eGFP was co-localized with the secretory granule-specific protein, chromogranin. Secondly, as shown here for the IRES-containing construct and previously with a somatostatin cDNA construct (Sevarino *et al.* 1987), the somatostatin peptide encoded by the PEPS transgene is subject to appropriate processing and transport in AtT20 cells. The antisomatostatin antibody used here for the immunocytochemical localization of somatostatin recognizes only fully processed somatostatin (somatostatin-28 or somatostatin-14), with no cross-reactivity to pro-somatostatin. The presence of several higher molecular mass forms of eGFP in our Western blot analysis of cell lysates indicates that the PEPS products are subject to a processing mechanism not seen with the IRES-containing construct. These higher molecular mass forms of eGFP probably correspond to either intact PEPS or partially processed protein (e.g. pre-eGFP, eGFP-pro or eGFP-prosomatostatin).

Thus, the functionality of the cleavage sites necessary for correct proteolytic processing (Brakch *et al.* 1994) of the pro-hormone sequence is

conserved within the PEPS construct, even with the incorporation of a large (27 kDa) reporter between the duplicated cleavage sites. This study confirms that, in contrast to the IRES-containing transgene, the monocistronic PEPS construct directs expression of both somatostatin and eGFP to the RSP. It should be noted that eGFP production after transfection with the PEPS construct was lower than that achieved with the IRES approach (Fig. 3). Apart from possible differences in transfection efficiency, this may reflect preferential translation of the second cistron, as is known to occur with IRES constructs (Houdebine & Attal 1999), and which, in this case, generates eGFP.

The findings of the present study also contribute to our understanding of the subcellular targeting of secretory proteins. Targeting to the RSP is believed to occur at, or just distal to, the TGN, during the formation of immature secretory granules (Farquhar 1985), but the precise mechanism by which this occurs differs between neuropeptides. The targeting of pro-opiomelanocortin (POMC) is the best characterized of these mechanisms. The amino-terminal of POMC is thought to contain a disulphide bond-constrained amphipathic hairpin loop (Cool *et al.* 1995), recognized by a sorting receptor in the TGN, the membrane-associated carboxypeptidase E (Zhang *et al.* 1999). In contrast, the sorting signal in the amino-terminal of prosomatostatin is less well characterized. It was initially thought that the first 10 residues of the amino-terminal pro-sequence are crucial for sorting (Sevarino & Stork 1991), forming a degenerate amphipathic α -helix (Kizer & Tropsha 1991). More recently, it has been suggested that four side-chain residues, Leu⁷, Phe¹⁰, Leu¹¹ and Leu¹⁵, form a contiguous hydrophobic patch on the helix surface, Leu⁷ and Leu¹¹ being critical for entry into the RSP (Mouchantaf *et al.* 2001). Our results corroborate the original mutation and substitution studies (Sevarino & Stork 1991) suggesting that the first 10 amino acids of the pro-sequence are sufficient to target the fused eGFP to the RSP. However, we cannot exclude the possibility that the last 10 amino acids of the pre-region, fused to the carboxyl terminal of eGFP, may make a functional contribution.

The generation of the monocistronic PEPS transgene represents a novel tool to investigate the role of somatostatin in neuroendocrine systems. The system has particular utility in heterogenous

neuronal populations that express endogenous somatostatin, and we are currently investigating the interaction of somatostatin with arcuate growth hormone-releasing factor (GRF) neurons, by expressing the PEPS construct in transgenic rats, under the control of the rat GRF promoter. The utility of this approach is not, however, restricted to the neuroendocrine interactions of somatostatin. The duplication of endopeptidase cleavage sites, and incorporation of fluorescent reporter molecules, should be applicable in any pre-propeptide system.

In addition, the monocistronic nature of the PEPS construct should, in theory, produce somatostatin and eGFP in a 1:1 stoichiometric ratio, raising the possibility that eGFP could be used as a surrogate marker of peptide secretion. However, the two transgene products may be subject to differential post-translational handling. For example, it is conceivable that the absence of the pro-hormone cleavage site in the eGFP product could influence ER-associated degradation and the rate of transportation in the RSP. Similarly, the secretion and degradation rates of the two products may be significantly different. Therefore, any attempt to quantify the ratio of the two secreted products in absolute terms would be difficult to interpret. That said, the extensive co-expression of eGFP and somatostatin in the secretory granules after transient transfection suggests that the two transgene products are not subjected to markedly different post-translational handling.

In summary, we have generated a novel approach to produce both a fully processed neuropeptide and a fluorescent reporter molecule in the RSP from a monocistronic construct. The genetic manipulation of the proposed sorting signal within the PEPS transgene confirms the biological functionality of the first 10 amino acids of the prosomatostatin sequence to direct expression to the RSP (Sevarino & Stork 1991).

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