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A Signal Sequence Is Sufficient for Green Fluorescent Protein to Be Routed to Regulated Secretory Granules*

RAJAÂ EL MESKINI†, LIXIAN JIN†, RUTH MARX, ANGELA BRUZZANITI, JONGHO LEE, RONALD B. EMESON, AND RICHARD E. MAINS

Department of Neuroscience (R.E.M., L.J., R.M., A.B., R.E.M.), The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205; and the Department of Pharmacology (J.L., R.B.E.), Vanderbilt University School of Medicine, Nashville, Tennessee 37233

ABSTRACT

To investigate trafficking in neuroendocrine cells, green fluorescent protein (GFP) tags were fused to various portions of the preproneuropeptide Y (NPY) precursor. Two neuroendocrine cell lines, AtT-20 corticotrope tumor cells and PC-12 pheochromocytoma cells, along with primary anterior pituitary cells, were examined. Expression of chimeric constructs did not disrupt trafficking or regulated secretion of endogenous ACTH and prohormone convertase 1 in AtT-20 cells. Western blot and immunocytochemical analyses demonstrated that the chimeric constructs remained intact, as long as the Lys-Arg cleavage site within preproNPY was deleted. GFP was stored in, and released from, regulated granules in cells expressing half of the NPY precursor fused to GFP, and also in cells in which only the signal sequence of preproNPY was fused to GFP. Thus, in neuroendocrine cells, entering the lumen of the secretory pathway is sufficient to target GFP to regulated secretory granules. (*Endocrinology* **142:** 864–873, 2001)

THE USE OF green fluorescent protein (GFP) epitope tagging to study protein routing and function has yielded a great deal of information, for example, in the study of membrane protein routing from the endoplasmic reticulum to the Golgi and subsequently to the cell surface or to various intracellular organelles (1, 2). To date, soluble GFP-tagged molecules have been used largely as a tool to visualize the movement of large dense core vesicles (LDCVs) before secretion, focusing on when and how the LDCVs become immobilized, rather than addressing the question of how soluble proteins are routed within the secretory pathway. The studies of routing using GFP fused to secreted proteins and peptides have yielded mixed results. For example, when fused with GFP, chromogranin B, proneuropeptide Y (NPY), provasopressin, brain-derived neurotrophic factor, and atrial natriuretic peptide were localized to LDCVs in several cell lines and primary neuronal cells (3–9). However, proinsulin-GFP constructs were very poorly targeted to LDCVs in INS-1 β-cells (10).

The driving or controlling elements in intracellular trafficking of soluble proteins within the secretory pathway are not yet clear. When fused with a soluble LDCV protein such as GH, constitutively secreted marker proteins in mammalian cells are rerouted to LDCVs, suggesting that a positive signal is required for entry of soluble peptides and proteins into large dense core vesicles (11, 12). However, LDCVs may function as the default pathway in professional secretory cells, where 10–75% of the newly made proteins are directed to LDCVs (13–15). Much of the control of LDCV contents may occur with the maturation of immature secretory granules, when specific content proteins are selectively removed, leaving behind the mature LDCV (16, 17). These disparate views have been the subject of several recent reviews (13, 18–20).

Sorting of soluble proteins between the constitutive and the regulated pathways is clearly complex, and there is substantial evidence for cell-type specificity in the routing of soluble proteins to LDCVs, regardless of the level of expression. For example, amylase is a normal LDCV constituent in exocrine pancreatic cells, and is trafficked to LDCVs when transfected into exocrine pancreatic cell lines but is constitutively secreted in transfected endocrine cell lines (21). Similarly, anglerfish somatostatin II resides in LDCVs in the anglerfish, but is constitutively secreted from transfected mammalian endocrine cells (22). Cell type specificity may explain some of the contradictory results using portions of the amino terminal of the POMC molecule to study routing in various endocrine and neuronal cell lines (12, 23, 24). Cell specificity of protein sorting extends beyond cell lines to primary cultures, as the same constructs can be handled quite differently in primary endocrine and neuronal cells (25, 26).

In this work, we have used preproneuropeptide Y fusions with GFP to explore routing of the chimeric proteins in AtT-20 cells, PC-12 cells, and primary pituitary cells. Specifically, we wanted to examine the regions of the preproNPY structure essential for targeting to LDCVs. Surprisingly, appending the NPY signal peptide was sufficient to yield GFP storage in LDCVs that underwent stimulated release. This startling answer was found initially in AtT-20 mouse corticotrope tumor cells and then extended to PC-12 cells and primary anterior pituitary cells in culture.

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Address all correspondence and requests for reprints to: Richard E. Mains, Department of Neuroscience, The University of Connecticut Health Center, 263 Farmington Avenue, MC3401, Farmington, Connecticut 06030-3401. E-mail: mains@uchc.edu.
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† These authors contributed equally to this work

Materials and Methods

Constructs

Full-length human prepro-NPY (1–97), signal peptide-NPY (1–66) and signal peptide (1–28) were obtained by PCR amplification with specific oligonucleotide primers incorporating *Hin*dIII and *AgeI* restriction endonuclease sites. The PCR products were digested with *Hin*dIII and *AgeI* and subcloned into the same sites of the pEGFP-N1 expression vector (CLONTECH Laboratories, Inc.). All fusion constructs were sequenced before use.

The resultant peptide precursors are diagramed in Fig. 1. Adenoviral vectors expressing prepro-NPY-GFP were constructed by subcloning the prepro-NPY-GFP fragment (*Hin*dIII–*Xba*I) into the pAdLox.HTM shuttle vector. Then hEK-293 cells stably expressing Cre8 were used to make recombinant virus as described (27).

Expression of GFP chimeras

AtT-20 cells were stably transfected using lipofectin (Life Technologies, Inc., Gaithersburg, MD) and selected with 0.5 mg/ml G-418 (Life Technologies, Inc.) as described (28). At least two clonal lines were studied for each construct with identical results. Primary pituitary cells were prepared as described (26), and vectors were transiently transfected using lipofectamine (Life Technologies, Inc.) or GenePorter (Gene Therapy Systems) using the manufacturer's protocols; in our hands, GenePorter gave far higher transfection rates with negligible cytotoxicity. Adenoviral vectors were introduced into pituitary cells and cell lines as described (25).

Studies of stimulated and basal secretion

AtT-20 cells (nontransfected and stably transfected lines) were examined for peptide and protein secretion using a series of washes in basal medium containing albumin and lima bean trypsin inhibitor, followed by an identical collection period in medium containing 1 mM $BaCl_2$ as a general secretagogue; for AtT-20 cells, previous experiments established similar results with these cells using cAMP derivatives, phorbol esters, and CRH (29). Medium and cell extracts were analyzed for secretion of ACTH and NPY by RIA (28, 30), peptidylglycine a-hydroxylating monooxygenase (PHM) by enzyme assay (31), and GFP, NPY-fusions and prohormone convertase 1 (PC1) by Western blot analyses (32). The GFP monoclonal antibody was from CLONTECH Laboratories, Inc. Secretion from PC-12 cells was stimulated using 50 mM KCl or 1 mM phorbol myristate acetate, whereas primary anterior pituitary cells were stimulated with 1 mM $BaCl_2$ or with 1 mM phorbol myristate acetate with similar results (26). Subcellular fractionation was performed essentially as described (33).

Immunocytochemistry

Cells were either observed live or fixed using 4% paraformaldehyde at 37 C followed by permeabilization and staining with specific antisera for ACTH, NPY, PC1 (28), or GFP (CLONTECH Laboratories, Inc.). With intrinsic GFP fluorescence there was negligible background, and all pictures of GFP were done by observing the GFP fluorescence with a fluorescein excitation/emission filter set; the results obtained with the GFP monoclonal antibody were similar, but with a higher background. Double immunofluorescence was performed as described (28). Cells were photographed using a Princeton Micromax or a Hamamatsu Orca camera and a Carl Zeiss Axioskop microscope.

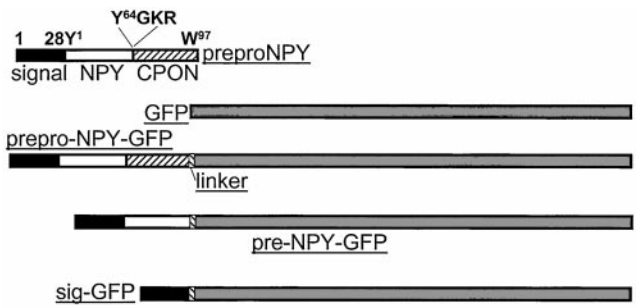

FIG. 1. Constructs expressed in this work. PreproNPY and native (cytosolic) GFP, plus the three chimeric proteins expressed in this work are diagrammed. For each chimera, the amino acid sequence -PVAT- is added between the NPY-related sequence and the GFP sequence, which begins MVSKGEEL. When the sequences are analyzed by the SignalP V2.0 server (http://genome.cbs.dtu.dk/services/SignalP-2.0/) (60, 61), all the NPY constructs are predicted to have the same signal peptide cleavage site with ≥99.5% likelihood (64–66).

Results

Comparisons among ACTH, NPY, and GFP in transfected AtT-20 cells

As expected from earlier studies, the immunocytochemical patterns for endogenous ACTH and exogenous prepro-NPY were very similar (Fig. 2, A and C). There is substantial staining in the TGN area and at the tips of the cellular processes, where the secretory granules accumulate (34), and also in vesicular structures distributed throughout the cells. There is a marked preponderance of NPY at the tips, as also seen with endogenous ACTH (*arrows*, Fig. 2, A and C) (28). Cells transfected with GFP show fluorescence distributed throughout the cytosol (Fig. 2B). Expression of exogenous GFP did not alter endogenous ACTH staining (34, 35). As seen in the combined image, native GFP and ACTH do not colocalize (Fig. 2D).

The signal sequence of preproNPY is sufficient for GFP to localize to secretory granules

Lang *et al.* (3) established that full-length preproNPY fused to GFP yielded green fluorescence in vesicular structures in PC-12 cells, and that release could be stimulated by depolarization in a Ca^{2+}-dependent manner. We established stable AtT-20 cell lines with full-length preproNPY fused to GFP and found similar results (not shown). However, Western blot analysis demonstrated that significant cleavage of the NPY region from the rest of the GFP fusion protein occurred in AtT-20 cells, as expected (28, 30) (not shown). Because different regions of a peptide precursor can be routed to distinct sets of secretory granules (36, 37), we reasoned that GFP localization and secretion might not accurately mimic NPY storage and secretion. To avoid this problem, the NH_2-terminal half of the NPY precursor was used, with the cleavage site within proNPY removed (pre-NPY-GFP; Fig. 1). There was still good trafficking of the chimeric NPY-GFP protein to secretory granules, as judged by GFP and NPY immunostaining at the light microscopic level (Fig. 3, A and B, C, D). There was good colocalization of the GFP, NPY, and ACTH images, with an accumulation of staining at the tips of processes (Fig. 3, C and D, *arrows*). The NPY-GFP construct does collect in the TGN area more markedly than does ACTH (Fig. 3, C and D, *asterisks*). Because pre-NPY-GFP was still targeted to LDCVs, we directly appended the signal sequence onto GFP (sig-GFP; Fig. 1). Even without the NPY peptide, there was significant routing of GFP to the same sites as ACTH (Fig. 3, E and F). GFP and ACTH accumulated at the tips of processes (*arrows*) and some accumulation of GFP in the TGN area was observed (*asterisks*).

Is the GFP still attached to NPY in the cell lines?

Western blot analyses were performed on all the AtT-20 stable cell lines to determine whether the GFP were still attached to the NPY in the secretory pathway, and whether

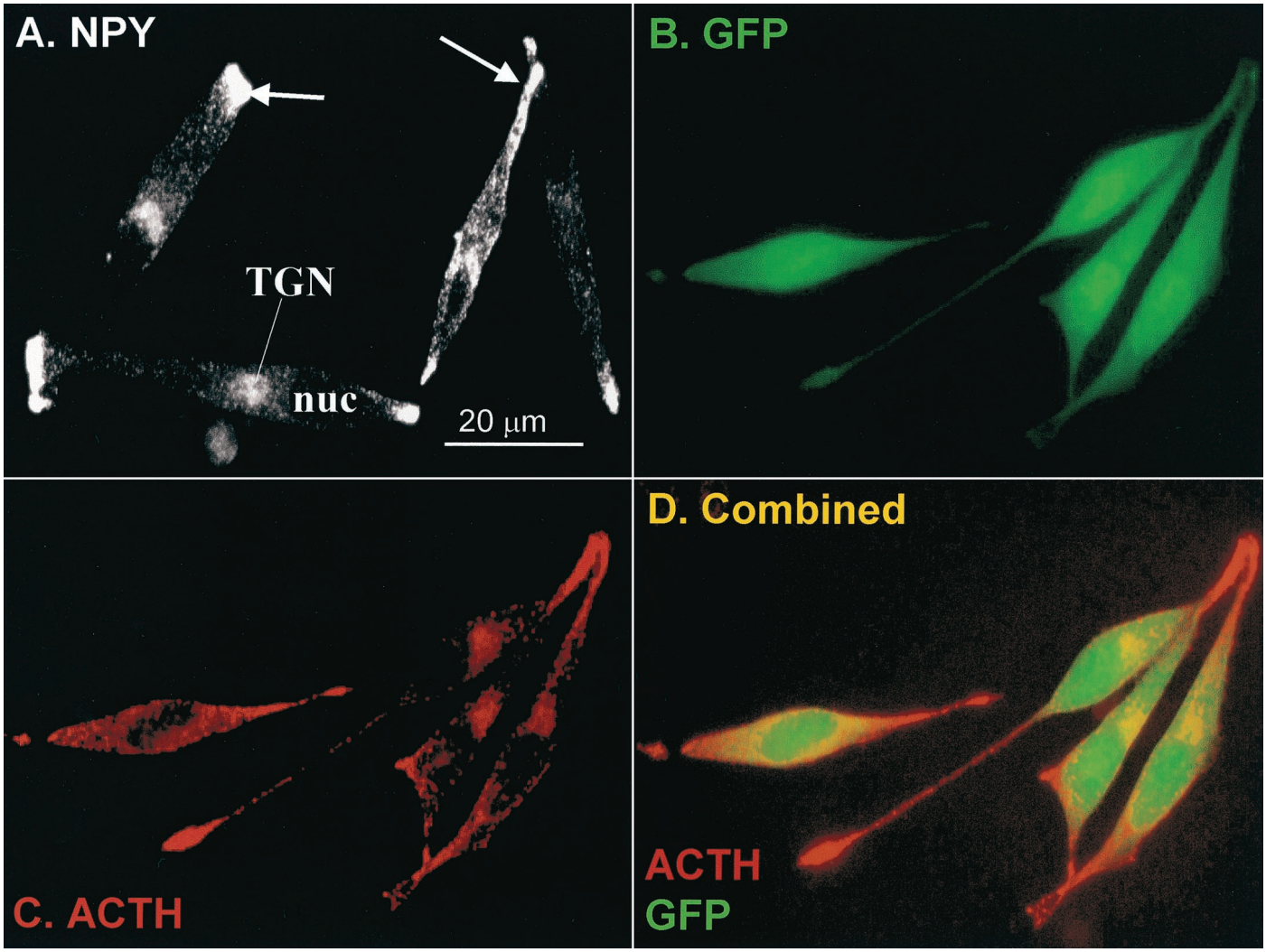

FIG. 2. Expression of preproNPY and native GFP in AtT-20 cells: immunocytochemistry. A, AtT-20 cells stably expressing preproNPY were stained for NPY. B–D, AtT-20 cells stably expressing native GFP were visualized directly for GFP (B) and immunostained for ACTH (C); merged images of GFP and ACTH (D).

the GFP in the secretory pathway stayed intact (Fig. 4). When extracts of AtT-20 cells expressing the pre-NPY-GFP fusion protein were examined with either the NPY or the GFP antibodies, a similar band at the expected molecular weight of 33 kDa was obtained. Cells expressing the preproNPY complementary DNA (as in Fig. 2A) did not produce a band detectable by Western blot analysis (not shown), as expected given the small size of the peptide and its failure to bind to PVDF membranes (28). The major GFP-positive product in extracts from cells expressing GFP and signal-GFP was the same size, about 28 kDa, as expected if the signal peptide is efficiently removed, and the resulting protein is stable. As noted above, much of the NPY is cleaved from the GFP in AtT-20 cells expressing prepro-NPY-GFP, so that construct was not studied further.

Regulated secretion by cells expressing the various GFP and NPY constructs: ACTH

To test whether regulated secretion from the various AtT-20 cell lines were normal or might be impaired, cells were washed in basal medium (complete serum-free medium) and then exposed to that medium for three successive periods, with 1 mM $BaCl_2$ included as a secretagogue in the third collection period (29) (Fig. 5). Previous work demonstrated that $BaCl_2$, isoproterenol, CRH, cAMP analogs, and phorbol esters all stimulated secretion from these cells under these conditions. LDCV are the only organelles known to secrete proteins in a calcium-dependent manner and thus to be responsive to $BaCl_2$. ACTH RIAs showed that all of the cell lines gave a 5-fold or better stimulation of ACTH release under these conditions.

Regulated secretion by cells expressing the various GFP and NPY constructs: GFP and NPY

When all the cell lines are compared simultaneously under the basal-stimulated secretion paradigm (Fig. 6), it becomes clear that GFP is secreted from both the pre-NPY-GFP and the signal-GFP cells in a strongly regulated manner. To monitor secretagogue responsiveness in all of the cell lines, we examined prohormone convertase 1 (PC1) secretion; PC1 is

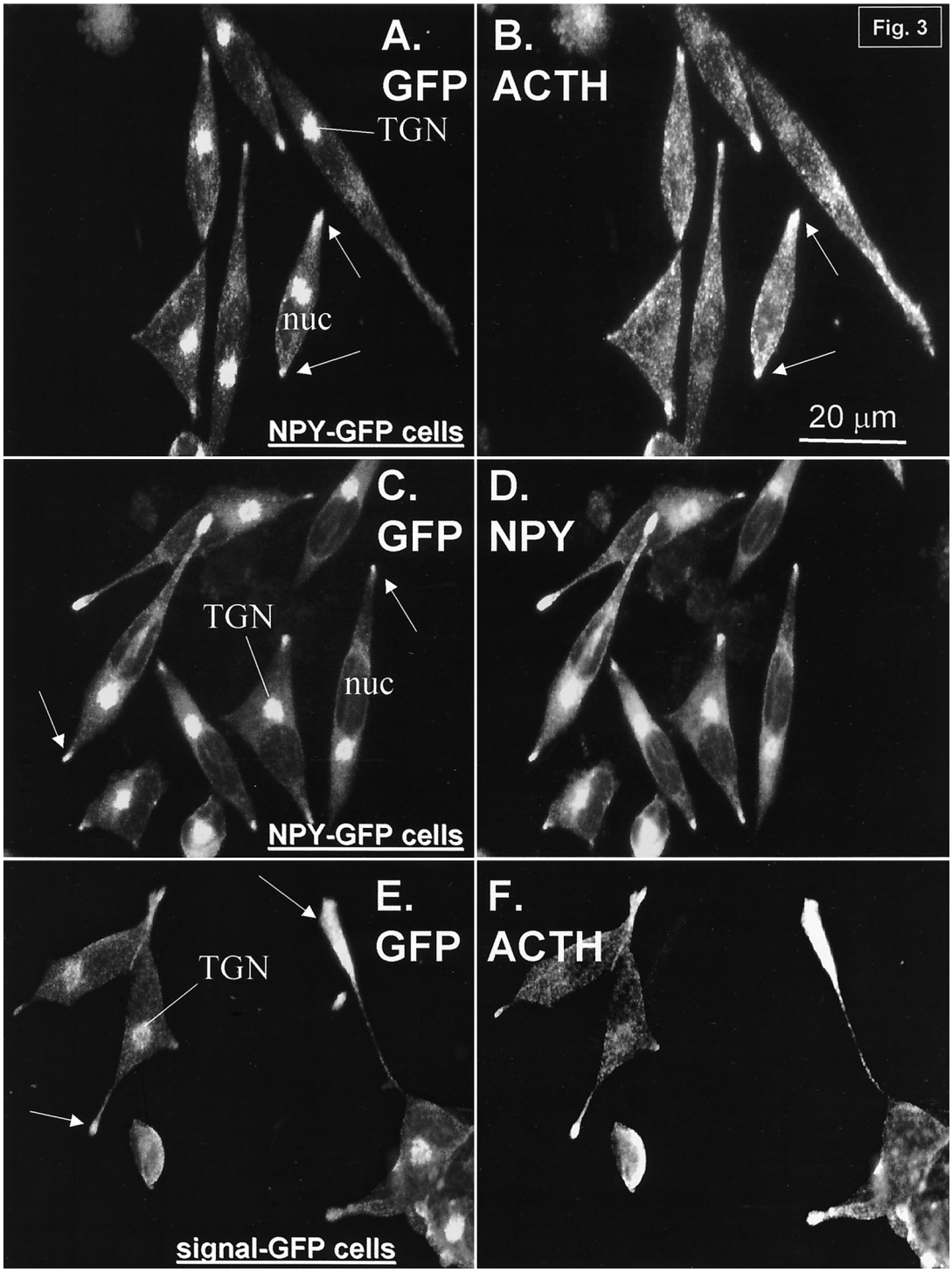

FIG. 3. Expression of pre-NPY-GFP and signal-GFP in AtT-20 cells: immunocytochemistry. AtT-20 cells stably expressing (A–D) pre-NPY-GFP and (E and F) signal-GFP were examined. A and B show the GFP and ACTH images for the same cells, whereas C and D show the GFP and NPY images for a second set of AtT-20 cells. E and F show GFP and ACTH from signal-GFP AtT-20 cells. *Arrows* mark the tips of cellular processes; *asterisks* mark the TGN area.

the processing enzyme responsible for POMC cleavage in these cells (38). All of the cell lines secreted mature the COOH-terminally truncated 65 kDa PC1 similarly in response to stimulation (Fig. 6, *top panel*) in a manner indistinguishable from nontransfected cells. Secreted NPY was detected by RIA from cells expressing native prepro-NPY and the pre-NPY-GFP fusion protein (Fig. 6, *bottom panel*); in both cases, addition of secretagogue stimulated NPY secretion approximately 6-fold.

Importantly, stimulatable GFP release was seen both from pre-NPY-GFP and signal-GFP cell lines (Fig. 6, *middle panel*), demonstrating by this functional criterion that the GFP from

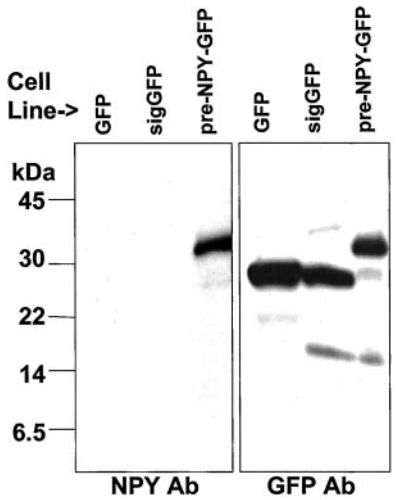

FIG. 4. Expression of GFP, signal-GFP and Pre-NPY-GFP in AtT-20 cells: Western blot analyses. AtT-20 cells stably expressing native GFP, signal-GFP, and pre-NPY-GFP were analyzed. Equal protein loadings (10 mg protein per lane) were fractionated on a 12% gel and analyzed using the NPY or GFP antisera. Similar results were obtained in four additional experiments of this type.

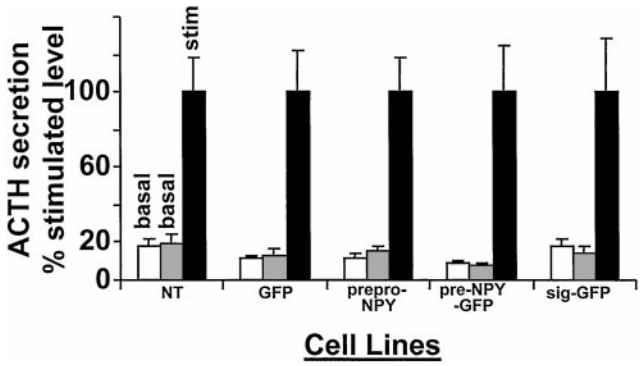

FIG. 5. Effect of stable transfection on regulated ACTH secretion. AtT-20 cell lines stably expressing the indicated constructs were examined in the basal-basal-stimulated (stim) paradigm, and the maximal secretion for each cell line was set to 100% for comparisons among the cell lines. Assays were performed in triplicate and the whole experiment was repeated twice with similar results. NT, Nontransfected.

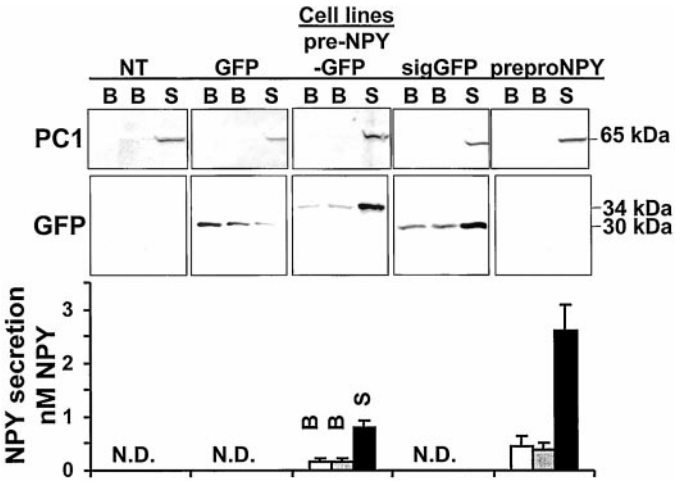

FIG. 6. GFP, PC1, and NPY stimulated secretion. AtT-20 cell lines stably expressing the indicated constructs were examined in the basal-basal-stimulated (stim) paradigm, and aliquots of the medium were subjected to Western blot analysis for PC1 and GFP, and to NPY RIA. Similar results were obtained in three or more additional experiments for each cell type. NT, Nontransfected.

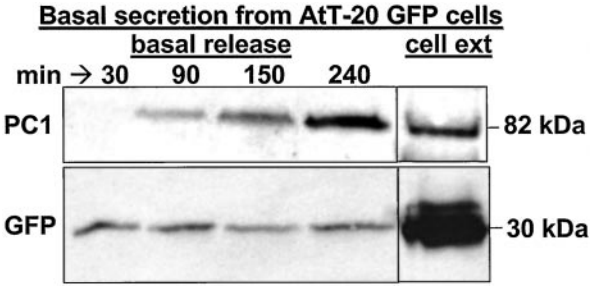

FIG. 7. Appearance of GFP and PC1 in basal medium. A single 35-mm well of AtT-20 cells stably expressing native GFP was washed as if to perform a test of stimulated secretion, but instead was fed with 3 ml of medium. Aliquots of the medium were then removed at the indicated times, with minimal disturbance of the cells. Aliquots corresponding to 1/8 of the spent medium and 1/50 of the cell extracts were separated on a 12% gel and subjected to Western blot analysis. Similar results were obtained in two additional experiments of this type.

the signal-GFP construct was transported to regulated secretory granules. Although GFP was detected in the medium from GFP cells, appearance of GFP was not stimulatable and, as demonstrated below, represents a very minor manifestation of the feeding effect seen for other cytosolic proteins such as lactate dehydrogenase (39). The magnitude of the stimulation was greater for cells expressing pre-NPY-GFP (4- to 5-fold) than for cells expressing signal-GFP (2- to 3-fold).

Secretion by cells expressing the native GFP construct: GFP and PC1

Our experiments unexpectedly showed some release of GFP from cells expressing native GFP (Fig. 6). A literature search revealed that up to 15% of the cytosolic lactate dehydrogenase can be released as a burst from healthy cultures subjected to a sham wash (39). Hence, additional experiments were performed to investigate the unexpected release of GFP (Fig. 7). Cells expressing the cytosolic GFP construct do demonstrate burst release of a small fraction of their total content of GFP; GFP released from the cells expressing cytosolic GFP does not accumulate over time as expected for true basal secretion (Fig. 7, *bottom*). PC1 was examined as a representative secretory protein (Fig. 7, *top*). There is a progressive basal accumulation of the larger 82-kDa form of PC1 in the medium, as expected for basal secretion. A much higher percentage of the cellular content of PC1 than GFP is released from the cells (Fig. 7, *right*). The 240 min PC1 band in the medium is more intense than the PC band in the chosen aliquot of cell extract. For GFP, the 240-min GFP band in the medium is much less intense than the GFP band in the same aliquot of cell extract. Thus, the basal appearance of GFP in the medium is negligible compared with authentic basal secretion of PC1, and does not display the time dependence expected for progressive secretion from the regulated secretory pathway.

Expression of pre-NPY-GFP and signal-GFP in PC-12 and anterior pituitary cells: immunocytochemistry

To determine whether the trafficking of pre-NPY-GFP and signal-GFP to LDCVs might be a peculiarity of the AtT-20 mouse corticotrope tumor cells, additional cell types were examined. When the pre-NPY-GFP fusion protein was expressed in PC-12 pheochromocytoma cells using an adenoviral construct, the NPY and GFP both collected at the tips of cellular processes (Fig. 8, A and B), as reported in PC-12 cells for the prepro-NPY-GFP fusion protein (3). GFP and NPY staining was also evident in the TGN area (*asterisks*). Thus, the signal sequence plus the 38 residues of NPY were sufficient to allow GFP to accumulate at the tips of cellular processes. In fact, when the mature NPY peptide was entirely deleted, the transfected signal-GFP construct also showed marked accumulation of GFP at the tips of cellular processes in PC-12 cells (Fig. 8G).

Endocrine cells of the anterior pituitary synthesize and store large amounts of hormone for release in response to stimulation. Because primary pituitary cells can be maintained in culture for long periods of time in a functional state (26), such cultures are an ideal way to test the universality of our findings. The pre-NPY-GFP adenoviral construct yielded GFP and NPY staining in morphologically identifiable se-

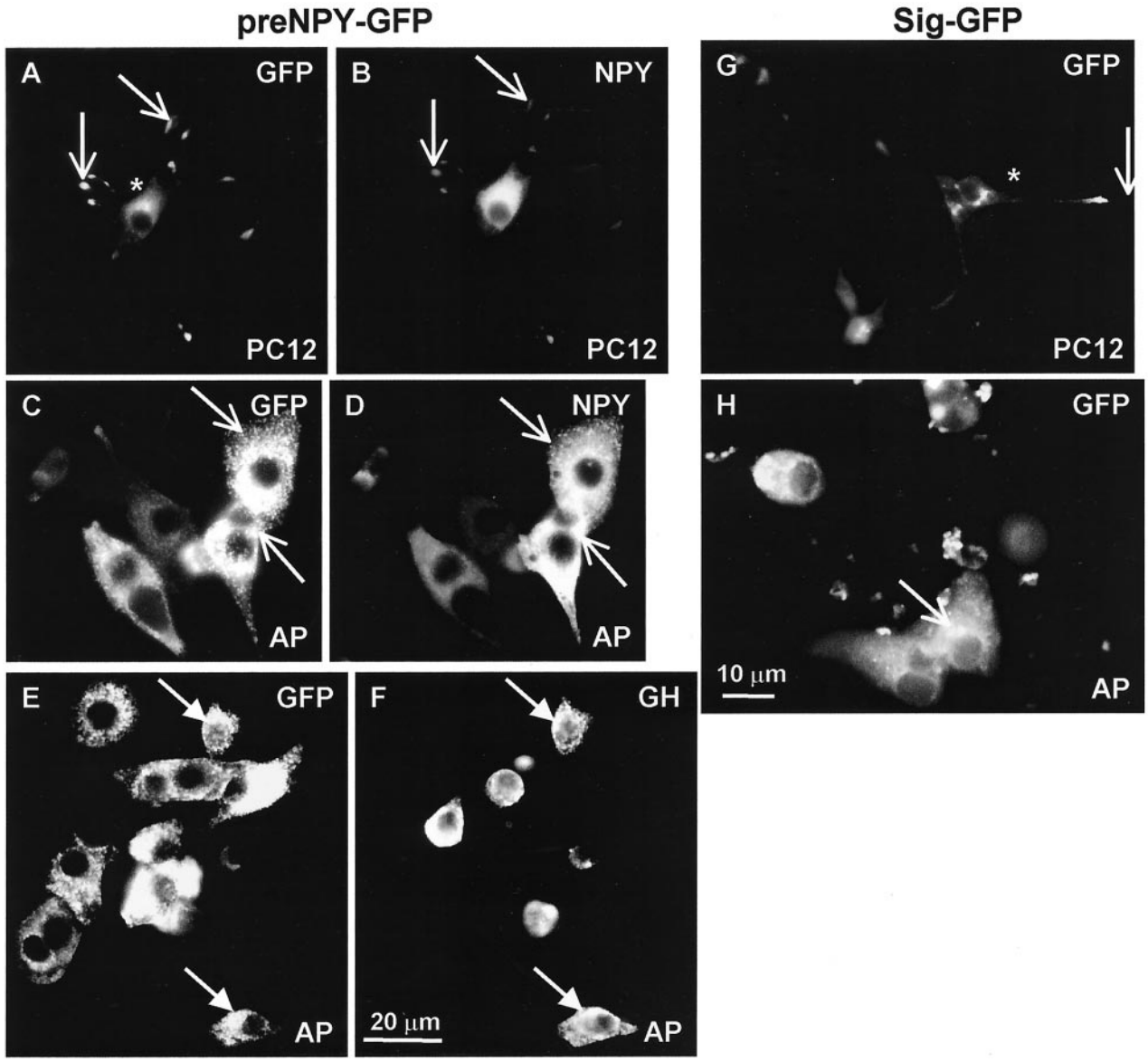

FIG. 8. Pre-NPY-GFP and signal GFP in PC-12 and anterior pituitary cells: immunocytochemistry. Adenoviral infection was used to express pre-NPY-GFP in PC-12 cells (A, B) and in primary anterior pituitary cells (C–F); GenePorter transfection was used to express signal-GFP in PC-12 cells and in primary anterior pituitary cells (G and H). Cells were visualized 2 days after infection or transfection. A and B, PC-12 cells visualized for GFP using intrinsic fluorescence, and immunostained for NPY. C and D, Primary anterior pituitary cells visualized for GFP using intrinsic fluorescence, and immunostained for NPY; *arrows* indicate areas of vesicular staining. E and F, Primary anterior pituitary cells visualized for GFP using intrinsic fluorescence, and immunostained for GH; *arrows* indicate areas of vesicular staining for both GFP and GH. GFP fluorescence is shown in G and H. *Arrows* mark the tips of cellular processes in PC12 cells and regions of secretory granule accumulation in primary anterior pituitary cells; *asterisks* mark the TGN area.

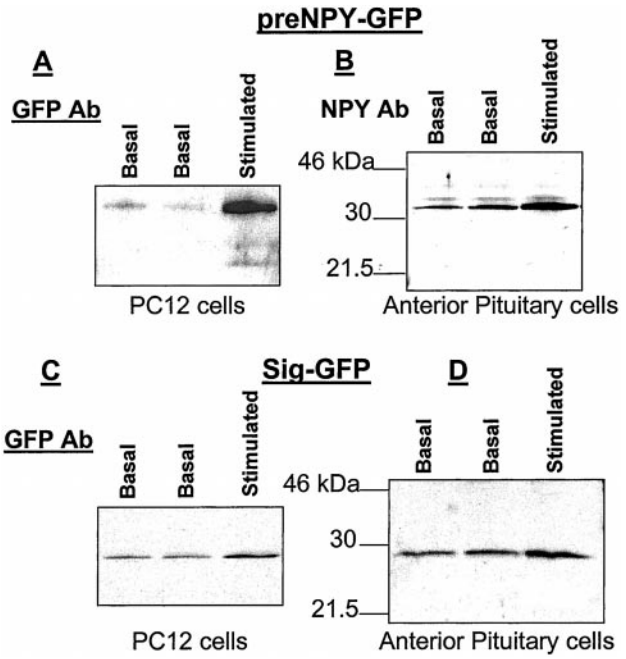

FIG. 9. Pre-NPY-GFP and signal-GFP in PC-12 and anterior pituitary cells: regulated secretion. PC-12 and primary anterior pituitary cells were infected and transfected as in Fig. 8, and after 2 days were subjected to the basal-basal-stimulated secretion paradigm. Secretion from PC-12 cells was stimulated with 1 mM phorbol myristate acetate for 30 min collections (A and C); similar patterns of stimulation were seen using other time periods and other stimuli. Anterior pituitary cells were stimulated with 1 mM $BaCl_2$ (B, D); similar results were seen using phorbol myristate acetate stimulation (26).

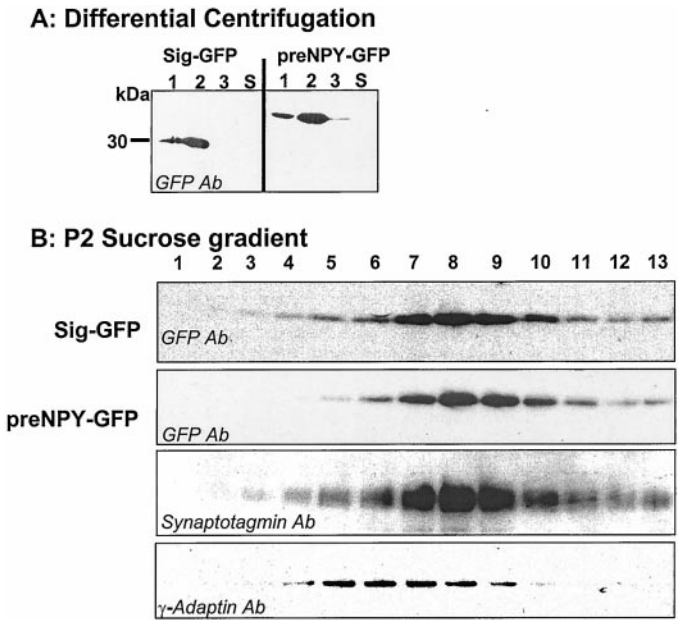

FIG. 10. Pre-NPY-GFP and signal-GFP in PC-12 cells: subcellular fractionation. PC-12 cells were infected and transfected as in Fig. 8, and then subjected to subcellular fractionation using differential centrifugation (A) and sucrose gradient separation (B) by standard techniques (33). GFP was visualized with the GFP monoclonal antibody, and synaptotagmin and γ-adaptin with commercially available antibodies.

cretory granule-rich regions of anterior pituitary endocrine cells (Fig. 8, C and D). Staining for GFP and GH was coincident in GH cells (Fig. 8, E and F). The transfected signal-GFP also showed substantial accumulation in regions of anterior pituitary endocrine cells rich in secretory granules (Fig. 8H).

Expression of pre-NPY-GFP and signal-GFP in PC-12 and anterior pituitary cells: biochemistry

We sought a biochemical method to confirm the results of the immunofluorescence studies. Stimulation studies showed that both the pre-NPY-GFP and signal-GFP constructs yielded proteins that were stored in regulated secretory granules in PC-12 cells and in primary anterior pituitary cells (Fig. 9). For all of the cell types tested (AtT-20, PC-12, primary anterior pituitary), the relative stimulation of GFP secretion from pre-NPY-GFP cells (Fig. 9, A and B) was substantially greater than from signal-GFP cells (Fig. 9, C and D). Nevertheless, stimulation secretion of GFP produced from signal-GFP was clearly seen for every cell type, indicating that a significant amount of GFP is stored in LDCVs.

To analyze the intracellular localization of the protein stored in pre-NPY-GFP and signal-GFP cells in more detail, subcellular fractionation was performed on PC-12 cultures expressing the transfected constructs (Fig. 10A). The majority of the GFP in pre-NPY-GFP and signal-GFP cells was found in the P2 pellet, as expected for a protein stored in regulated secretory granules. Further fractionation using sucrose gradients (Fig. 10B) showed that the GFP products continued to comigrate with secretory granule markers such as synaptotagmin, and to separate from other markers such as γ-adaptin (marker for AP1-containing clathrin-coated vesicles) (40).

Discussion

GFP-tagged molecules have been used to dissect the pathways of secretion for peptides in endocrine and neuroendocrine cell lines, and in primary pituitary cultures. Using immunocytochemistry and subcellular fractionation to localize the GFP chimeras, and secretagogues to stimulate secretion, we confirmed that NPY-GFP was expressed in secretory granules in all the cell types tested. NPY-GFP was not transported to granules as efficiently as ACTH or NPY, as evidenced by its accumulation in the TGN area. However, once the chimera was transported beyond the TGN area, it was stored in mature secretory granules in a similar fashion to ACTH and NPY. In addition, NPY-GFP expression overlapped that of another secretory granule constituent, PC1. These findings were observed in multiple cell lines, and importantly were reproduced in primary endocrine cells. In an independent study, the longer preproNPY-GFP fusion protein (97 residues plus GFP) directed GFP to LDCVs, and secretion was stimulated in a potassium and calcium-dependent manner (8). In this work, we first established that the 66 residues of prepro-NPY (signal sequence plus 38 amino acids of NPY) were sufficient to convert GFP from a cytosolic protein to a resident of the secretory granules, and then demonstrated that the signal sequence alone was sufficient to yield GFP residing in LDCVs. Using the signal and prosequences of preprosomatostatin, globin can be delivered

into the lumen of the ER in GH3 somatomammotrope cells (41). However, without the 82 residues of the prosomatostatin sequence, the globin was rapidly destroyed in the ER (41).

Native NPY precursor (proNPY) is known to be very efficiently cleaved in AtT-20 cells (28, 30). In fact, a great many precursors are cleaved in the secretory granules of endocrine and neuronal cell lines (42–47). Thus, the potential of many neuroendocrine cells to cleave fusion molecules involving full-sized peptide precursors raises serious questions about the interpretation of experiments relying solely on the localization and apparent secretion of the GFP moiety of those chimeras. Indeed, AtT-20 cells are quite adept at cleaving NPY from the GFP region of the preproNPY-GFP construct (Fig. 1) (28, 30). In this study, we established that the NPY-GFP fusion protein derived from pre-NPY-GFP remained intact in primary anterior pituitary cells, AtT-20 cell lines and PC-12 cells, in cell extracts and also in the medium following secretagogue-induced secretion.

The autocatalytic formation of its fluorophore, and the lack of known specific targeting information in the GFP molecule, have led to the increasing popularity of GFP as a tag for studying the movement of proteins within cellular compartments (2, 48). However, there are a few reports that GFP can reroute tagged proteins in some cell types or have targeting information of its own. For example, whereas native GFP is cytosolic in AtT-20 cells, unmodified GFP in COS-1 cells exhibits a nuclear localization (49, 50). Moreover, GFP fused to proinsulin was misfolded and retained in the ER of insulinoma INS-1 β-cells (10), and appending GFP retargeted some proteins to the vacuole in yeast (50). These findings make it necessary to examine the routing of GFP-tagged molecules within different cell types thoroughly, and to ensure that GFP tagging does not disrupt the normal targeting of the fusion partner (49). The results presented in this study were reproduced in several cell systems, including an endocrine cell line (AtT-20), a pheochromocytoma-derived cell line that possesses neuroendocrine features (PC-12), and in primary pituitary cells. Thus, these important findings are not limited to a single cell line, nor are the results a peculiarity of immortalized cell lines maintained in tissue culture for long periods of time.

Several models of regulated secretion have been proposed. One model proposes that proteins destined for regulated granules are selectively aggregated in the presence of Ca^{2+} and the acidic environment of the immature secretory granules (13, 20, 51). Another model of sorting to regulated granules posits the presence of specific receptors that recognize specific structural motifs within the sorted proteins (24, 52). Such sorting motifs reportedly include disulfide bridges and associated hairpin loop structures (24). A third model argues that the regulated granules are the default pathway, from which inappropriate proteins are progressively removed (13, 16, 17, 53). Another model suggests that a protease cleavage site is sufficient to direct sorting and retention in the regulated secretory pathway (46, 47). Although there are a great many routing determinants identified in the cytoplasmic domains of transmembrane proteins (54–57), no such signals have been identified in soluble proteins destined for the LDCV.

In this work, we demonstrated that the signal peptide of NPY alone was sufficient to target GFP into the lumen of the regulated secretory pathway in several cell types; as predicted from past work on signal peptides and proteins with transmembrane domains, signal-GFP should certainly enter the lumen of the ER (58–61). In cells expressing signal-GFP, GFP was readily demonstrated within secretory granules, and its secretion was stimulated above basal levels by treatment with secretagogue. Thus, as signal-GFP enters the lumen of the endoplasmic reticulum, cleavage of the NPY signal peptide occurs. GFP, normally a cytosolic protein, enters the regulated secretory pathway with varying efficiency in AtT-20, PC-12 and in primary pituitary cells. It is worth noting that the efficiency with which GFP enters the regulated secretory pathway is not substantially different from the efficiency with which various forms of PC1 enter the regulated secretory pathway.

The signal peptide cleavage must be correct within about 5 amino acid residues, because the sizes of the signal-GFP and cytosolic GFP products are indistinguishable on high resolution peptide gels. In addition, the signal cleavage site is predicted to occur with 99.8% certainty immediately after the NPY signal in the signal-GFP construct (60, 61). In the usual terminology, this would argue that GFP entered regulated secretory granules by default, as no known targeting or sorting motifs for the mammalian regulated pathway have been described for native *Aequorea victoria* GFP; native jellyfish GFP is cytosolic in AtT-20 cells (Fig. 2). It must be recalled that this result is not true for every protein that enters the lumen of the ER, however, because amylase is sorted to exocrine granules but constitutively released in endocrine cells (21).

It has been proposed that segregation of proteins between constitutive and regulated pathways occurs within the Golgi region (13, 46, 62). Evidence from our own studies indicate that PAM and ACTH are present together in some *trans* cisternae of the Golgi, and both are absent from other stacks, implying sorting before reaching the *trans* Golgi stack, perhaps within the lumen of *cis* or *medial* Golgi stacks (63). There is little evidence for sorting as early as the ER. Signal sequences are cleaved cotranslationally, and proteins in the lumen of the ER move rather freely for tens of minutes (2, 58, 60, 64–67), making it implausible that vesicle targeting information could be retained from the chosen signal sequence.

In this study, we established that the signal sequence of NPY was sufficient to reroute GFP into large dense core vesicles and support regulated secretion of GFP. Furthermore, we have demonstrated that biologically inactive GFP fusion proteins can be used as suitable markers of dense core granules. In addition, signal GFP may be useful as an easily visualized but biologically inactive replacement for precursors to active peptides in the generation of peptide knockout animals.

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