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Calnuc, an EF-Hand Ca^{2+}-Binding Protein, Is Stored and Processed in the Golgi and Secreted by the Constitutive-Like Pathway in AtT20 Cells

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Calnuc is an ubiquitous, EF-hand Ca^{2+} binding protein found in the cytoplasm where it binds to Gαi3, in the Golgi lumen where it constitutes a Ca^{2+} storage pool, and secreted outside the cell. Here we investigated the pathway of secretion of calnuc in AtT20 cells. We found by pulse-chase experiments that calnuc is synthesized in the endoplasmic reticulum, transported to the Golgi where it remains greater than 12 h and undergoes posttranslational modification (*O*-glycosylation and sulfation) followed by secretion into the culture medium. We examined if calnuc is secreted by the constitutive or regulated secretory pathway in AtT20 cells. By immunofluorescence and immunogold labeling, endogenous calnuc is found in immature secretion granules (ISG) but not mature regulated secretory granules (RSG), whereas overexpressed calnuc-green fluorescent protein (GFP) is found in both ISG and RSG, where it colocalizes with ACTH. Neither calnuc nor calnuc-GFP are released by the regulated secretory pathway, suggesting that endogenous calnuc and calnuc-GFP are progressively removed from ISG and RSG during granule maturation. We conclude that calnuc is secreted via the constitutive-like pathway and represents a useful endogenous marker for this pathway in AtT20 cells. Together, these observations indicate that calnuc has a unique itinerary as it is retained in the Golgi and is then constitutively secreted extracellularly where it may influence cell behavior via its Ca^{2+}-binding properties. (*Molecular Endocrinology* 16: 2462–2474, 2002)

CALNUC, ALSO KNOWN as nucleobindin, is an EF-hand calcium-binding protein that is widely expressed in cells and tissues (1–3) and is well conserved from flies to humans (3, 4). We have previously shown that calnuc is present both in the cytoplasm and in the Golgi lumen of AtT20 pituitary cells, PC12 cells, and many other cell types (3, 5). The cytoplasmic pool of calnuc interacts with Gαi3 at the cytoplasmic surface of Golgi membranes (6), affects the distribution of Gαi subunits (7), and modulates ACTH secretion in AtT20 cells. We have demonstrated that the Golgi pool of calnuc is located in the lumen of *cis*-Golgi cisternae and the *cis*-Golgi network, where it constitutes the major Ca^{2+}-binding protein in the Golgi (3) and a major Ca^{2+} storage pool (8). Calnuc is also released from the cell, as it has been found in bone extracellular matrix (1), in the sera of mice prone to the autoimmune disorder, systemic lupus erythematosis (9, 10), and in the culture supernatant of a B lymphocyte cell line established from these mice (11, 12). Nothing is known at present about the origin of the secreted pool of calnuc or its mode of secretion.

To investigate these questions, we have carried out detailed studies of the biosynthesis, intracellular transport, and secretion of calnuc in AtT20 cells and other cell lines. AtT20 cells have the advantage that they have well-characterized constitutive, constitutive-like, and regulated secretory pathways (13–18). The hormone ACTH destined for regulated secretion is packaged into immature secretory granules (ISG) that bud from the *trans*-Golgi network (TGN). These ISG mature to form dense core or regulated secretory granules (RSG), which fuse with the plasma membrane (PM) upon an appropriate stimulus and discharge ACTH by exocytosis. Other secretory proteins (*e.g.* proteoglycans and viral glycoproteins such as vesicular stomatitis virus-glycoprotein and gp70) are segregated at the TGN into constitutive secretory vesicles (19–21), which are rapidly and continuously discharged by exocytosis. In addition, a subset of proteins (constitutively secreted proteins, incompletely processed hormones, and SNAREs) are sorted from ISG during granule maturation via the constitutive-like secretory pathway (16–18, 22–24). We report here findings indicating that newly synthesized calnuc has a somewhat different fate after biosynthesis than other secreted proteins studied to date in AtT20 cells: it is synthesized in the endoplasmic reticulum (ER) traffics to and through the Golgi where it is posttranslationally modified and is then retained in the Golgi for a variable period of time ($>$12 h, depending on the cell type) until it is released from the cell via the constitutive and constitutive-like secretory pathways.

Abbreviations: ER, Endoplasmic reticulum; ERGIC, ER-Golgi intermediate compartment; GFP, green fluorescent protein; ISG, immature secretion granule; $NaClO_3$, sodium chlorate; NRK, normal rat kidney; PFA, paraformaldehyde; PM, plasma membrane; RSG, regulated secretion granule; TGN, *trans*-Golgi network.

RESULTS

Calnuc Remains in the Cell for a Variable Period and Is then Secreted

To clarify the relationship between the intracellular and extracellular pools of calnuc, we studied the kinetics of synthesis and secretion of calnuc in different cell types. We examined mouse pituitary AtT20 cells because calnuc has been well characterized in this cell line (3) and because this cell line has well-defined, regulated, and constitutive secretory pathways (13–17). Cells were pulse-labeled for 5 min with [^{35}S]-EasyTag and chased for different times (0–12 h), after which calnuc was immunoprecipitated from medium and cell extracts (Fig. 1). After 0 or 30 min chase, calnuc was detected as a 60-kDa band and was mostly found in cell extracts with very little or no calnuc detected in the culture medium. Beginning at 2 and especially at 5 h chase, a 63-kDa form of calnuc appeared, and calnuc was still mainly detected in cell extracts (93 ± 1% and 86 ± 2%, respectively). After a 12-h chase, 32 ± 2% of the calnuc was found in the culture medium where only the 63-kDa form was seen. We conclude that, in AtT20 cells, calnuc is retained inside the cell before being secreted and that it is posttranslationally modified before secretion.

To determine if secretion of calnuc takes place in other cell types expressing this protein, we next examined the kinetics of calnuc's synthesis and secretion in normal rat kidney (NRK) cells (Fig. 1), which are epithelioid cells that lack a regulated secretory pathway. Immediately post pulse, the 60-kDa form of calnuc was detected which was found exclusively in the cell extracts. After a 30-min chase, calnuc was still mainly (88 ± 7%) found in cell extracts, but it was already detected in the medium (12 ± 7%) even at this early time point. After a 2-h chase, the 63-kDa form of calnuc appeared in the cell extracts and some (33 ± 10%) of the calnuc was secreted. After 5 or 12 h, most (62 ± 9% and 77 ± 7%, respectively) of the newly synthesized calnuc was found in the medium. We conclude that newly synthesized calnuc is also synthesized, posttranslationally modified intracellularly and retained inside NRK cells, but it is secreted more rapidly than in AtT20 cells. In addition, we found that in NRK cells (but not AtT20 cells), the total label seen at 5 and 12 h was 83% and 69%, respectively, that at earlier time points, indicating approximately 17% and 31% of the labeled calnuc was lost during prolonged chase, most likely due to intracellular degradation.

We also examined HeLa and PC12 cells and found that, after a 3-h chase, 50% and 88%, respectively, of the calnuc remained inside the cell (data not shown). Thus in all cell types analyzed, calnuc is retained in the cell for a variable period (>12 h) and is then secreted into the medium. Interestingly, the time calnuc remains in the cell is shorter in NRK and HeLa cells that lack a regulated secretory pathway than in AtT20 and PC12 cells, two specialized secretory cells with both constitutive and regulated secretory pathways.

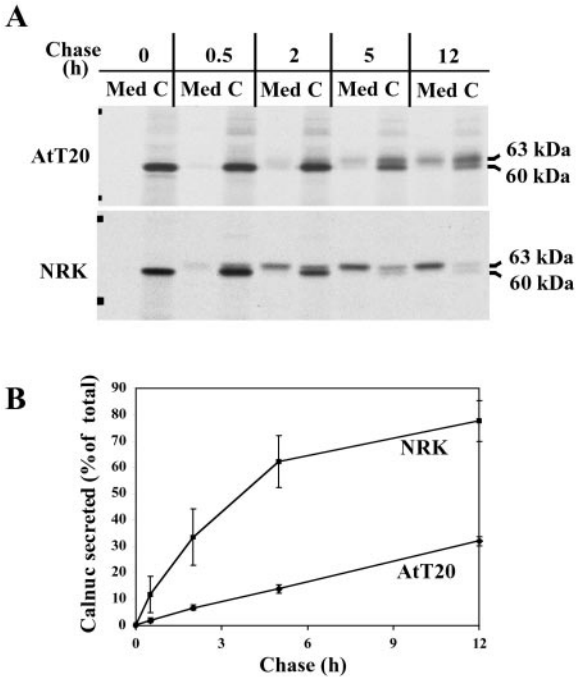

Fig. 1. Time Course of Calnuc Secretion in AtT20 and NRK Cells

In AtT20 cells, newly synthesized calnuc is detected only in cell lysates (C) and is not released into the culture medium (Med) immediately post pulse (0) or after a 30-min chase (0.5). After 2- and 5-h chases, calnuc is still found mainly in cell lysates, with only 7 ± 1% and 14 ± 2%, respectively, secreted into the medium. After a 12-h chase, 31 ± 4% of the calnuc is found in the medium. In NRK cells, 33 ± 10% of the calnuc is released at 2 h and 77 ± 7% after 12 h. A change in electrophoretic mobility of calnuc from 60 kDa to 63 kDa is observed during its biosynthesis. Only the 63-kDa form is secreted. A, Cells were pulse-labeled with [^{35}S]EasyTag for 5 min and chased for the indicated times. Radiolabeled calnuc was immunoprecipitated from the medium (Med) and cell extract (C) followed by separation on SDS-PAGE and detection by autoradiography. B, The amounts of calnuc present in the medium or cell extracts at different chase times were expressed as the percent of the total radiolabeled calnuc at each time point. SEM was calculated for at least three independent experiments.

Calnuc Is Posttranslationally Modified during its Transport to and through the Golgi

The changes in the electrophoretic mobility of calnuc suggest that it undergoes posttranslational modification(s) during its intracellular transport. To pinpoint the intracellular site where this occurs, we used 15-C and 20-C temperature blocks. When NRK cells were chased for 3 h at 15 C, which arrests protein transport in the ER-Golgi intermediate compartment (ERGIC) (25, 26), secretion of calnuc was completely blocked, and the 60-kDa form of calnuc predominated (Fig. 2).

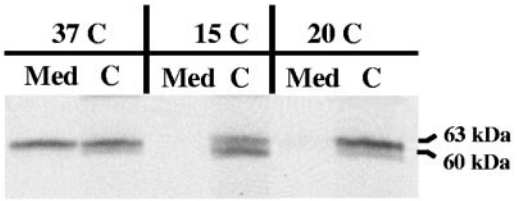

Fig. 2. Effects of Low Temperature on Calnuc Secretion and Processing

When NRK cells are pulse labeled and chased at 37 C for 3 h calnuc is fully processed to the 63-kDa form, and approximately 50% is released into the medium. In cells chased at 15 C (which arrests protein transport in ERGIC), secretion of calnuc into the medium (Med) is completely blocked, and the intracellular pool (C) of calnuc is partly processed to the 63-kDa form. When cells are incubated at 20 C (which arrests protein transport in the TGN), calnuc is fully processed to the 63-kDa form, but it is not released into the medium (Med). NRK cells were pulse-labeled with [^{35}S]EasyTag at 37 C for 5 min and chased for 3 h at 37, 15, or 20 C. Radiolabeled calnuc was immunoprecipitated from the medium (Med) and cell extract (C) and analyzed by SDS-PAGE and autoradiography.

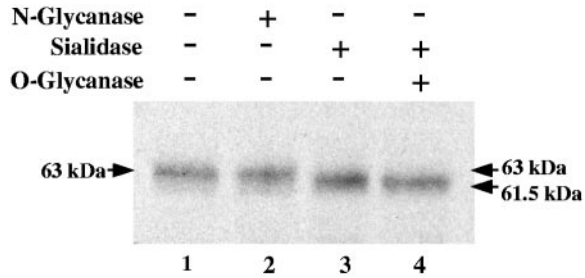

Fig. 3. Calnuc Contains *O*-Linked Oligosaccharides

Treatment of calnuc with sialidase type II induces a small shift (1 kDa) in the mobility of calnuc (lane 3) compared with the control (lane 1). When calnuc is digested with both *O*-glycanase and sialidase II, the mobility of calnuc is increased slightly (0.5 kDa) over sialidase alone (compare lanes 3 and 4). Digestion with *N*-glycanase has no detectable effect on calnuc (lane 2). Calnuc was immunoprecipitated from the medium of AtT20 cells labeled with [^{35}S]EasyTag for 1 h and chased for 7 h. Immunoprecipitates were incubated overnight at 37 C in the presence or absence of 2.5 mU *N*-glycanase (*F. meningosepticum*), 20 mU Sialidase II (*C. perfringens*), or 2 mU *O*-glycanase (*S. pneumonia*), and analyzed by SDS-PAGE and autoradiography.

The finding that the 60-kDa band is seen immediately post-pulse in cells incubated at 37 C (see Fig. 1), together with the fact that the 60-kDa band is the only band seen after incubation at 15 C, indicates that the 60-kDa band is the ER and ERGIC form of calnuc. This is in accord with the previous localization of calnuc in ERGIC and the *cis*-Golgi in AtT20 and NRK cells (3).

When cells were chased for 3 h at 20 C, which arrests protein transport in the TGN (27), secretion of calnuc into the medium was again completely blocked and the mature, 63-kDa form of calnuc predominated (Fig. 2). We conclude that the 60-kDa calnuc precursor is transported to the Golgi, where it is posttranslationally processed to the mature 63-kDa form.

Calnuc Is *O*-Glycosylated

To identify the nature of its posttranslational modification(s), we investigated if calnuc is glycosylated. Analysis of the calnuc sequence using the NetOGlyc 2.0 prediction server revealed two potential *O*-glycosylation sites but no putative *N*-glycosylation sites. Because sialic acids are the terminal sugars on many oligosaccharides, we initially determined if calnuc is sialylated in AtT20 cells. Cells were labeled for 1 h and chased for 7 h to allow posttranslational modification of radiolabeled proteins. Calnuc was then immunoprecipitated and digested with type II sialidase [removes sialic acids from both *N*- and *O*-linked oligosacharides (28)]. This treatment resulted in an approximately 1- to 2-kDa shift in the mobility of calnuc (Fig. 3, lane 3), indicating that calnuc is sialylated. When [^{35}S]EasyTag-labeled immunoprecipitates were digested with both sialidase and *O*-glycanase (which removes *O*-linked oligosacharides from glycoproteins), the electrophoretic mobility of calnuc was increased slightly (0.5 kDa) over sialidase alone (Fig. 3, compare lanes 3 and 4). When immunoprecipitates were incubated with *N*-glycanase (releases nearly all known *N*-linked oligosaccharides from glycoproteins), there was no detectable effect on the mobility of calnuc (Fig. 3, lane 2). This in keeping with calnuc's lack of putative *N*-glycosylation sites. As a positive control, digestion of fetuin (which has both *N*- and *O*-linked oligosaccharides) with *N*-glycanase or *O*-glycanase plus sialidase resulted in a shift of its mobility (data not shown). Calnuc immunoprecipitated from NRK cells also showed a shift in mobility after digestion with sialidase II. These results indicate that calnuc is *O*-glycosylated in AtT20 cells and NRK cells and provide further evidence that calnuc is transported through the exocytic or secretory pathway.

Calnuc Is Sulfated

Many secreted proteins are sulfated, and sulfation has been proposed to act as a signal for export from the cell (29, 30). To determine whether calnuc is sulfated, we labeled AtT20 cells with [^{35}S]sulfate or [^{35}S]-EasyTag (met/cys) and immunoprecipitated calnuc from media and cell lysates. After sulfate labeling, a band at approximately 63 kDa was seen in the medium and a band at approximately 62 kDa in the cell extract (Fig. 4A, lanes 1 and 2). The [^{35}S]sulfate-labeled, 62-kDa band is most likely due to incomplete processing. After a 3-h chase, far more (~50%) of the total [^{35}S]sulfate-labeled calnuc was recovered in the medium than [^{35}S]EasyTag-labeled calnuc (Fig. 4A, compare lanes 1 and 3). This is due to the fact that [^{35}S]-sulfate labels only the pool of calnuc that has reached the TGN of the Golgi apparatus where sulfation takes

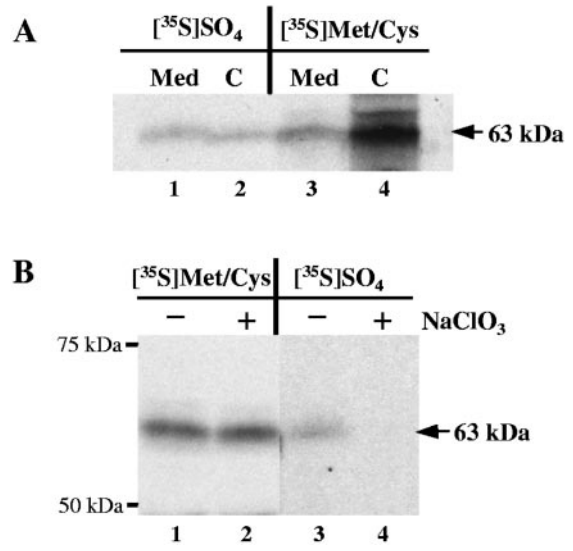

Fig. 4. Calnuc Is Sulfated, but Sulfation Is Not Required for its Secretion

A, [^{35}S]Sulfate labeling of AtT20 cells. A band at approximately 63 kDa (lanes 1 and 2), similar in mobility to [^{35}S]-EasyTag (Met/Cys)-labeled calnuc (lanes 3 and 4), is seen in both cell lysates (C) and medium (Med), indicating calnuc is sulfated. AtT20 cells were labeled with [^{35}S]sulfate for 30 min or with [^{35}S]met/cys for 5 min. Medium (Med) and cell lysate (C) were collected after a 3-h chase. Radiolabeled calnuc was immunoprecipitated and analyzed as in Fig. 2. B, Incubation in the presence (+) of sodium chlorate ($NaClO_3$) prevents sulfation (compare lanes 3 and 4), but does not prevent secretion as [^{35}S]EasyTag (Met/Cys)-labeled calnuc is released into the medium in both $NaClO_3$ (lane 2) and control (lane 1) cells. AtT20 cells were pretreated or not with 10 mM $NaClO_3$ for 1 h before labeling with [^{35}S]sulfate or [^{35}S]-EasyTag and chased for 5 h in the presence (+) or absence (−) of $NaClO_3$. Calnuc was immunoprecipitated from the medium and analyzed as described above.

place (18, 29, 31–34), whereas [^{35}S]EasyTag labels all newly synthesized calnuc. In contrast to most sulfated glycoproteins that are rapidly secreted after sulfation, sulfated calnuc remains in the cell for long periods (up to 6 h). We conclude that sulfated calnuc has a long intracellular half life and that calnuc secreted into the medium is more highly sulfated than cell-associated calnuc.

To further examine if sulfate is required for secretion of calnuc, cells were treated with sodium chlorate ($NaClO_3$), which prevents sulfation of both tyrosine and glycans (35, 36). $NaClO_3$ is an inhibitor of ATP-sulfurylase, the first enzyme in 3′-phosphoadenosine 5′-phosphosulfate biosynthesis (35, 36). When AtT20 cells were labeled with [^{35}S]sulfate or [^{35}S]EasyTag (met/cys) and calnuc was immunoprecipitated from the medium after a 5-h chase in the presence of $NaClO_3$, sulfation of calnuc was effectively inhibited (Fig. 4B, compare lanes 3 and 4), but there was no effect on secretion of [^{35}S]EasyTag-labeled calnuc (Fig. 4B, compare lanes 1 and 2). These results indicate that sulfation is not required for secretion of calnuc by AtT20 cells.

Calnuc Is Secreted by the Constitutive Pathway

According to our time-course data (see Fig. 1), mature calnuc remains in AtT20 cells for more than 12 h. This property is characteristic of proteins that are stored in dense core, RSG. AtT20 pituitary cells have both a regulated and a constitutive secretory pathway (13–17). ACTH is stored in RSG and is released into the culture medium after stimulation of the cells with the secretagogue 8-Br-cAMP. To determine the pathway by which calnuc is secreted, we tested whether secretion of calnuc is ongoing as is characteristic of constitutive secretion, or if it is induced by secretagogues as is characteristic of proteins such as ACTH that are released via the regulated secretory pathway. Cells were pulsed 1 h with [^{35}S]EasyTag and chased for 5 h, to allow calnuc to be posttranslationally processed (*O*-glycosylated and sulfated). They were then incubated for 2 h in the presence or absence of the secretagogue 8-Br-cAMP, and the presence of calnuc and ACTH in cell lysates and media was determined by immunoprecipitation. Secretion of calnuc was not significantly affected by 8-Br-cAMP treatment (Fig. 5A), whereas secretion of ACTH was stimulated more than 2-fold by the presence of this secretagogue (Fig. 5B). Even after a 12-h chase when a large amount of radiolabeled calnuc was still detected within the cell, stimulation with 8-Br-cAMP did not lead to increased release of calnuc (data not shown). These results indicate that little or no calnuc exits the AtT20 cell via the regulated pathway. From this together with the finding that calnuc is secreted by NRK and HeLa cells, which lack a regulated pathway, we conclude that calnuc undergoes constitutive secretion.

Endogenous Calnuc Does Not Reside in Regulated Secretory Granules

The inability of secretagogues to stimulate calnuc secretion suggested that this protein might be packaged into constitutive secretory vesicles rather than RSG. To clarify the nature of the vesicles with which calnuc is associated, we determined the distribution of calnuc in AtT20 cells. Calnuc has previously been shown by indirect immunofluorescence and immunoelectron microscopy (3) to be concentrated mainly in ERGIC and the *cis*-Golgi of AtT20 cells and cells of the rat pituitary. To gain further information on its localization, we used immunofluorescence and deconvolution microscopy which permits analysis of multiple sections (0.2 μm) of the cell at high resolution. We observed that endogenous calnuc is not only present in the Golgi, but it is also localized to vesicles found at the tips of cell processes (Fig. 6A). When the distribution of ACTH, the major secretory protein of RSG in AtT20 cells (37), was compared with that of calnuc by double labeling, both proteins were concentrated at the tips of

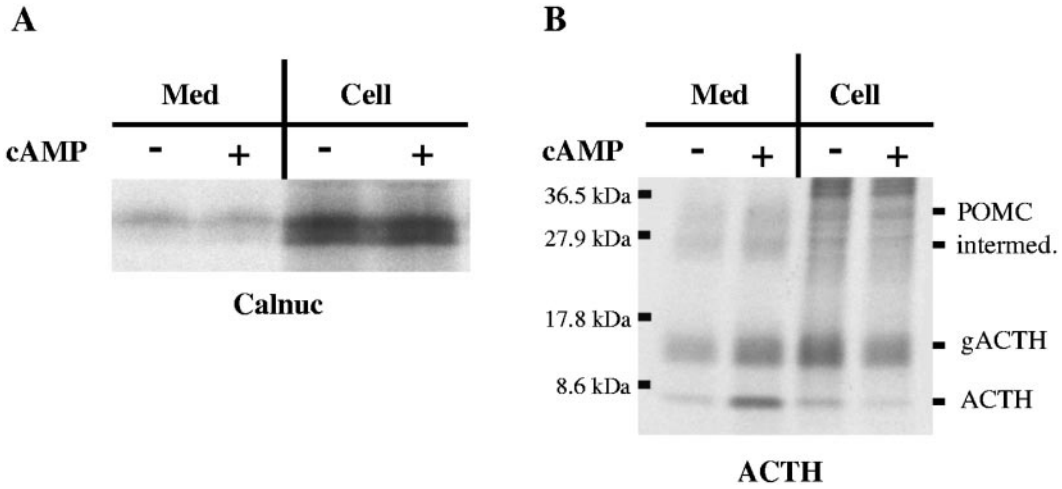

Fig. 5. Calnuc Is Secreted by the Constitutive Pathway in Parental AtT20 Cells

A, Secretion of calnuc into the medium (Med) is not significantly affected by incubation in the presence of 8-Br-cAMP (cAMP). B, Secretion of ACTH and glycosylated ACTH (gACTH) into the medium (Med) is induced more than 2-fold by this secretagogue. There is no difference in the amounts of POMC or intermediate product of POMC (intermed) secreted. AtT20 cells were labeled for 1 h with [^{35}S]EasyTag, chased for 5 h in the absence of secretagogue followed by incubation for 2 h in the presence (+) or absence (−) of 5 mM 8-Br-cAMP (cAMP). Radiolabeled calnuc and ACTH were immunoprecipitated from cells (C) and medium (Med) and analyzed as in Fig. 2.

the cell processes (Fig. 6, A and B), but their distribution did not overlap (Fig. 6C). These data indicate that calnuc is not present in ACTH-containing RSG in AtT20 cells.

To investigate the nature of the vesicles containing calnuc, we determined the distribution of calnuc and ACTH in AtT20 cells at the EM level by immunogold labeling of ultrathin cryosections. In cells double labeled for calnuc and ACTH, calnuc was mainly observed in *cis*-Golgi cisternae (Fig. 7A). Sometimes calnuc also colocalized with ACTH in ISG on the *trans*-side of the Golgi apparatus (Fig. 7A) and in RSG within cell processes (Fig. 7B). These results confirm that endogenous calnuc does not reside in RSG but is concentrated in constitutive vesicles for transport to the plasma membrane.

Calnuc-GFP Colocalizes with ACTH in Regulated Secretory Granules in AtT20 Cells Stably Expressing Calnuc-GFP

We also determined the distribution of calnuc by immunofluorescence in AtT20 cells stably overexpressing calnuc tagged with GFP at its C terminus (calnuc-GFP). Again, calnuc-GFP (Fig. 6D) and ACTH (Fig. 6E) were concentrated in vesicles located along cell processes and at their tips. The merged image (Fig. 6F) showed that, in contrast to endogenous calnuc, there was extensive overlap between calnuc and ACTH staining in these vesicles. By immunogold labeling at the EM level calnuc-GFP and ACTH codistributed in ISG on the *trans*-side of the Golgi apparatus (Fig. 8A) and in virtually every RSG within the cell processes (Fig. 8B). It was also found in the Golgi where it was concentrated mainly in *cis*-Golgi cisternae even in cells with high levels of expression (Fig. 8A). These results indicate that calnuc-GFP is associated with RSG.

Kinetics of Secretion of Calnuc-GFP

We next analyzed the kinetics of synthesis and secretion of calnuc-GFP in AtT20 cells stably expressing calnuc-GFP by immunoprecipitation of radiolabeled calnuc from the medium and cell extracts (Fig. 9) at various times of chase (up to 12 h). We found that calnuc-GFP was secreted far more rapidly than endogenous calnuc, as approximately 27% of the newly synthesized calnuc-GFP was secreted after 2 h, 51% after 5 h, and 55% after a 12-h chase. We conclude that calnuc-GFP is not retained inside the cell as effectively as endogenous calnuc.

Calnuc-GFP Is Secreted by the Constitutive or Constitutive-Like Secretory Pathway in AtT20 Cells

To investigate if the pathway by which calnuc-GFP is secreted is similar to that of endogenous calnuc, we evaluated if secretion of calnuc-GFP is responsive to secretagogues. AtT20 cells stably expressing calnuc-GFP were pulsed 20 min and chased in unlabeled medium for 1 or 5 h and stimulated with 8-Br-cAMP for 30 min. It has previously shown that early stimulation (1-h chase) tests entry of a protein into ISG, whereas later stimulation (5-h chase) tests its sustained presence in RSG (16). We found that secretion of ACTH

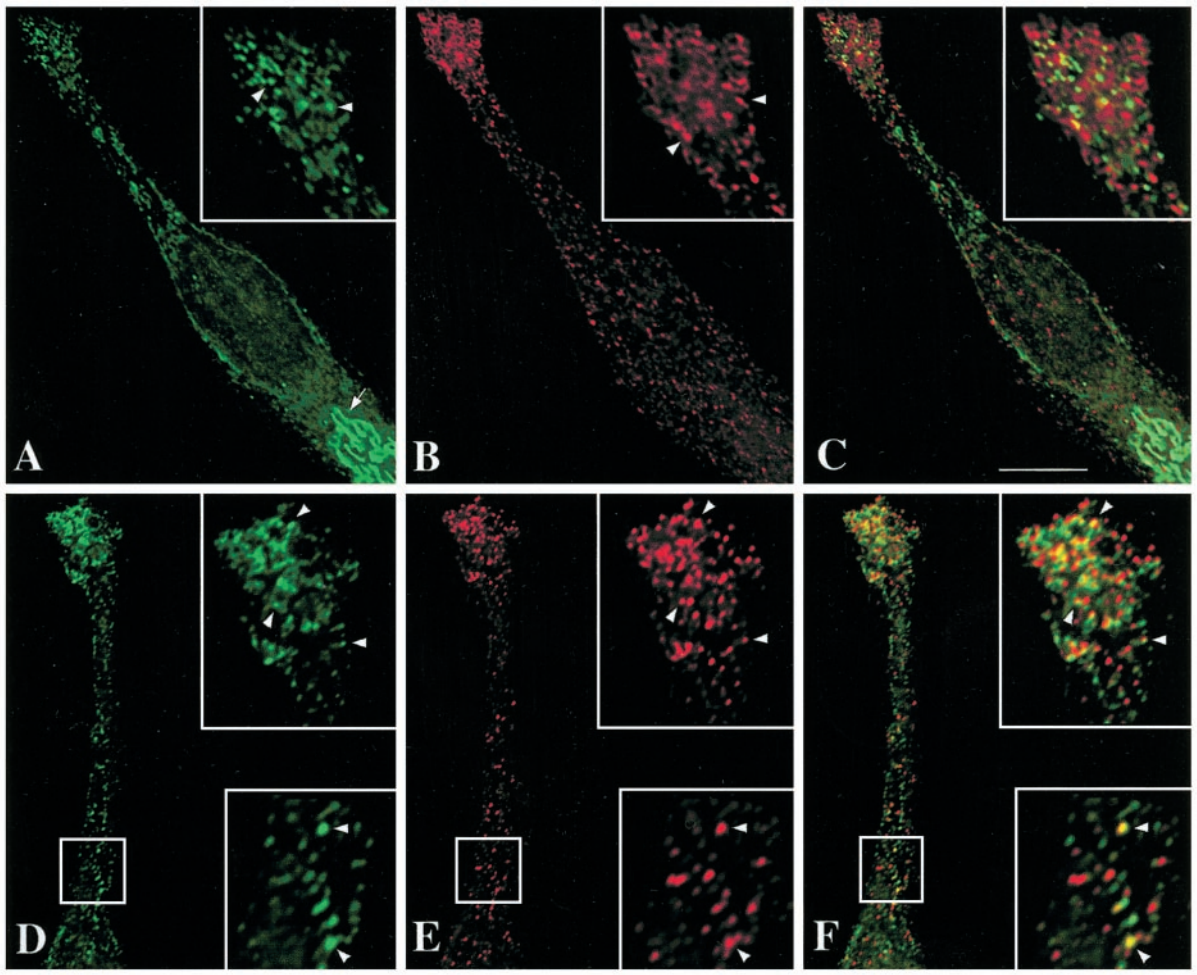

Fig. 6. Localization of Calnuc and ACTH by Immunofluorescence in Parental AtT20 Cells and Those Stably Expressing Calnuc-GFP

A, Endogenous calnuc is found mainly in the Golgi region (*arrow*) and in some vesicles concentrated (*arrowheads*) at the tip of the cell processes (enlarged in *inset*) of parental AtT20 cells. B, ACTH is stored in regulated secretory granules that are found throughout the cell but are most concentrated at the tips of the cell processes (*arrowheads*). C, Merged image showing little or no overlap in the distribution of calnuc and ACTH in parental cells. D and E, Distribution of calnuc-GFP (D) and ACTH (E) in AtT20 cells stably expressing calnuc-GFP. Increased numbers of vesicles stained for calnuc are observed at the tips of the cell processes (*arrowheads*, D). Extensive overlap (*yellow*) in the distribution of ACTH and calnuc (*arrowheads*) is seen along the cell processes and at their tips as shown in the *merged image* (F) enlarged in the *insets*. Parental AtT20 cells or AtT20 cells stably expressing calnuc-GFP were fixed in 2% PFA in phosphate buffer, permeabilized, and double labeled with rabbit affinity-purified anticalnuc IgG and mouse monoclonal anti-ACTH followed by highly cross-absorbed Alexa Fluor-488 goat antirabbit and Alexa Fluor-594 goat antimouse conjugates. *Bars*, 5 nm.

was stimulated after both 1 h (19 ± 6.7%) and 5 h (13 ± 2.5%) chases (Fig. 10), whereas secretion of calnuc-GFP (90 kDa) was slightly stimulated (5 ± 0.6%) after 1 h but not after 5 h (0.8 ± 1.4%) chase. These results suggest that, whereas radiolabeled ACTH is present in both ISG and RSG, radiolabeled calnuc-GFP enters ISG but is not released via RSG. From these results, together with the codistribution of calnuc-GFP and ACTH in ISG and RSG (Fig. 8), we conclude that most likely calnuc-GFP enters ISG and is progressively sorted out of these granules during their maturation to RSG before their discharge by exocytosis.

DISCUSSION

We have carried out a detailed analysis of the biosynthesis, intracellular processing and fate of the secreted pool of the EF-hand protein calnuc, in AtT20 pituitary cells. Our findings demonstrate that calnuc is synthesized as a 60-kDa protein in the rough ER and is retained in the Golgi for more than 12 h where it matures into a 63-kDa sialylated and sulfated form—some of which is released into the culture medium via the constitutive pathway. Even though calnuc has a long intracellular half-life in AtT20 cells, it is not stored in RSG and is not secreted by the regulated secretory

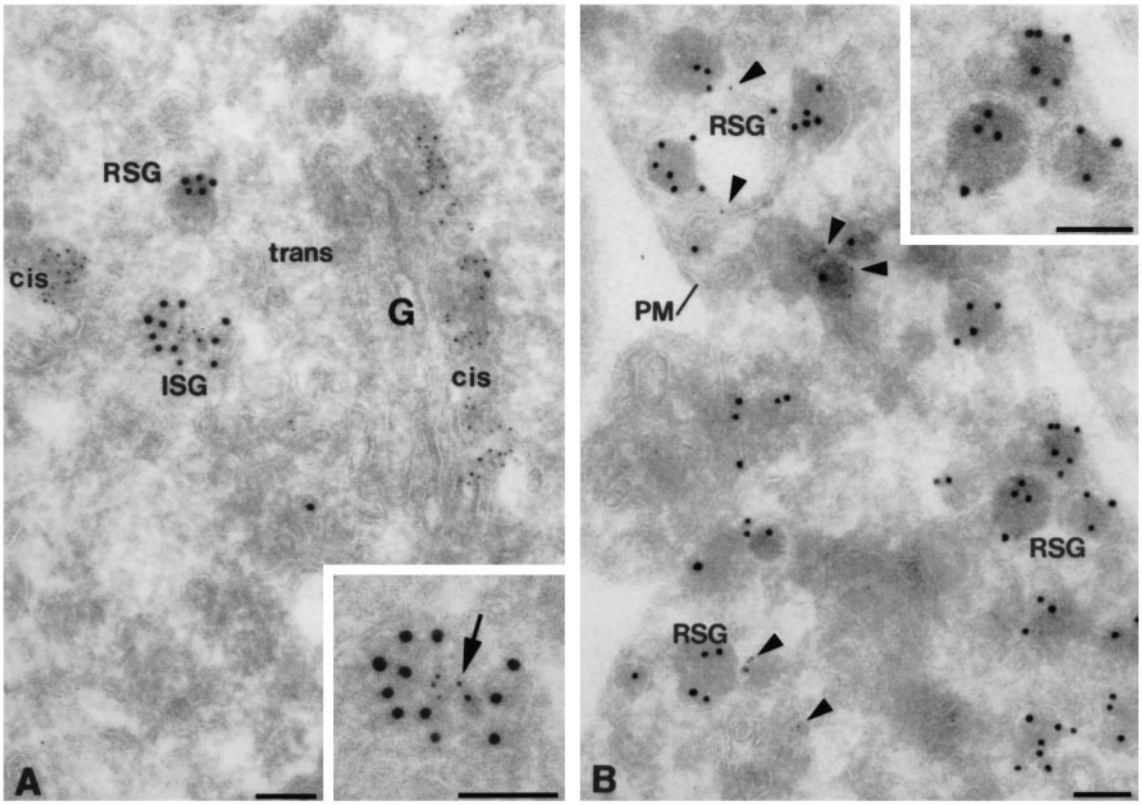

Fig. 7. Distribution of Endogenous Calnuc and ACTH by Immunoelectron Microscopy in Parental AtT20 Cells

A, Golgi region. Calnuc (small gold) is observed in *cis*-Golgi (cis) cisternae and an ISG located on the *trans*-side of the Golgi apparatus (trans). ACTH (large gold) is localized in this ISG as well as in an RSG. *Inset*, Enlargement of ISG showing colocalization of calnuc (*arrow*) with ACTH. G, Golgi apparatus. B, Tips of cell processes. ACTH (large gold) is found in numerous RSG, whereas relatively little calnuc (small gold, *arrowheads*) colocalizes with ACTH in these granules. *Inset*, Enlargement of RSG to the *right*. Cells were fixed with 4% PFA for 30 min followed by 8% PFA for 2 h. Ultrathin cryosections were prepared and incubated sequentially with primary antibodies followed by 5 or 10 nm gold, goat antirabbit, or goat antimouse IgG. Sections were postfixed and stained as described in *Materials and Methods*. *Bar*, 100 nm.

pathway. Instead, it is constitutively released into the culture medium. This is in accord with the reported presence of calnuc in blood, bone extracellular matrix, and culture media (1, 10–12, 38).

Calnuc is widely expressed in cells and tissues (1–3), and its secretion seems to be common to most if not all cells, as all cells examined (AtT20, PC12, NRK, and HeLa cells) secrete calnuc into the culture media. This raises a question concerning the functional significance of extracellular calnuc. Secretion of calnuc may be necessary for its turnover, or calnuc may have unknown extracellular functions. It is of interest that calnuc has been detected at high concentration in sera of mice prone to the autoimmune disorder, systemic lupus erythematosis, and has been implicated in the progression of this disease (10, 38), suggesting that extracellular calnuc is of clinical importance. Several other calcium binding proteins are also secreted, including calreticulin (39) and the EF-hand proteins calumenin (40) and BM-40 (SPARC) (41, 42). Of greatest interest is calreticulin, because there is significant homology between calnuc and calreticulin (3). Calreticulin is a resident protein of the ER, and, like calnuc, also exists in three pools—in the cytoplasm, within the ER and secreted (39). Extracellular calreticulin has been shown to bind to fibrinogen (43), laminin (44), and integrins (45, 46) and to influence cell adhesion and migration (39).

Calnuc is unusual as most proteins secreted via the constitutive pathway are rapidly secreted. Calnuc is secreted considerably more slowly (~2 h) from NRK cells than the time it takes vesicular stomatitis virus glycoprotein, a membrane glycoprotein, to appear at the cell surface ($t_{1/2} \approx 30$ min) (47). Similarly, in AtT20 cells, calnuc remains in the cell much longer (12 h) than the viral glycoprotein gp70 ($t_{1/2} \approx 40$ min) (19). Our studies suggest that in AtT20 cells calnuc remains in ERGIC and the *cis*-Golgi cisternae up to 5 h as indicated by the persistence of the 60-kDa precursor and reaches the *trans*-Golgi only after 5 h based on the appearance of the 63-kDa, fully processed form of calnuc. Furthermore, the sulfated form of calnuc stays in cells up to 6 h, which is six times longer than sulfated glycosaminoglycan chains, a marker for con-

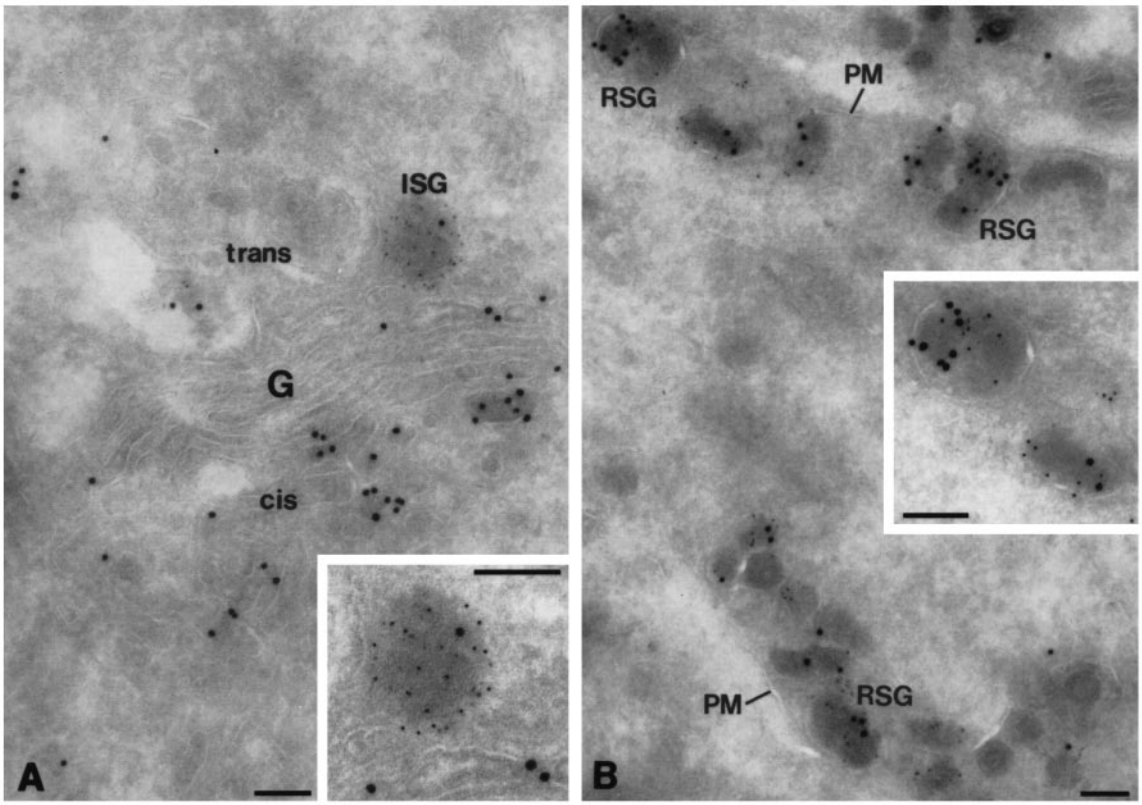

Fig. 8. Distribution of Calnuc and ACTH by Immunoelectron Microscopy in AtT20 Cells Stably Expressing Calnuc-GFP

A, Golgi region. Calnuc (large gold) is seen mainly in vesicles and cisternae on the *cis*-side (cis) of the Golgi and in the rims of some trans cisternae. Calnuc also codistributes with ACTH (small gold) in an ISG on the *trans*-side (trans) of the Golgi. *Inset*, Enlargement of ISG. G, Golgi apparatus. B, Tips of cell processes. Both calnuc (large gold) and ACTH (small gold) are found in virtually all the RSG. *Inset*, Enlargement of RSG on *upper left*. G, Golgi apparatus. Preparation and staining were the same as for Fig. 7. *Bar*, 100 nm.

stitutive secretion in AtT20 cells (7, 18). These results imply the existence of a mechanism that retards calnuc in the cell before its release into the medium, and our immunolocalization and biochemical results demonstrate this is not due to storage of calnuc in RSG. Calnuc's long intracellular half-life, its *cis* Golgi residence, together with its sulfation, a modification that takes place in the *trans*-Golgi or TGN (18, 29, 31–34) and the long intracellular half-life of [^{35}S]sulfate-labeled calnuc, suggest that calnuc may be retained by a combination of retention in ERGIC and the early Golgi, retrieval from the late Golgi, and recycling to the early Golgi.

Recently, it has become clear that at least some resident Golgi transmembrane proteins undergo recycling to the early part of the secretory pathway (48, 49), as many Golgi transmembrane proteins contain modifications (oligosaccharides, sulfate) that suggest passage beyond the point at which they normally reside (48, 50–55). However, there is little precedent for recycling of soluble proteins. Calnuc and Cab45, both calcium-binding proteins, are the only proteins identified to date that lack transmembrane domains and reside in the Golgi (3, 56). It is possible that calnuc may have a targeting domain in its sequence, or alternatively, may bind to a transmembrane protein which retains it in the *cis*-Golgi.

The mechanisms and sites of sorting of secretory proteins have been investigated for many years. Sorting of secretory proteins has been shown to occur in both the TGN and in ISG (57–61). It is widely believed that proteins destined for regulated secretion undergo aggregation, bind to a sorting receptor in the TGN, and are segregated into ISG that bud from the TGN (sorting for entry model), thereby excluding other proteins from entering the regulated secretory pathway. However, it has become evident that some constitutively secreted proteins also enter ISG and are progressively removed via vesicles that bud from maturing granules (sorting by retention model) (16, 24, 62). We found that endogenous calnuc enters ISG and is not present in RSG, and overexpressed canuc-GFP enters ISG and is found in RSG, but it is not released by the regulated pathway. Thus, our results indicate that calnuc and calnuc-GFP are actively removed from ISG during granule maturation. A number of constitutively secreted proteins when overexpressed have been shown to enter the regulated secretory pathway in

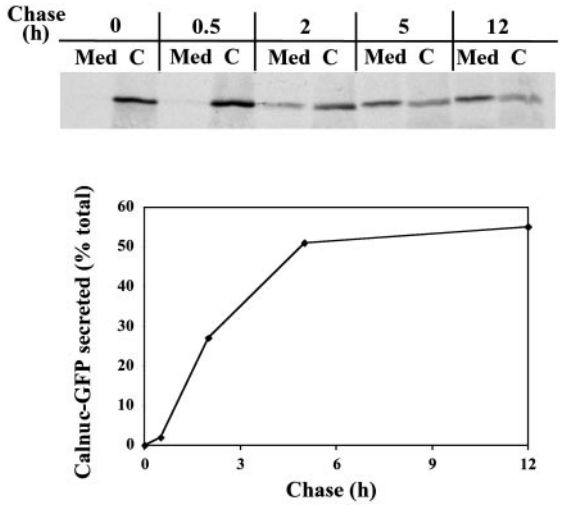

Fig. 9. Time Course of Secretion of Calnuc-GFP

In AtT20 cells stably expressing calnuc-GFP, 27% of the newly synthesized calnuc-GFP is released into the medium after 2 h chase, 51% after 5 h, and 55% after 12 h chase. Calnuc-GFP is secreted approximately 3 times more rapidly than endogenous calnuc (see Fig. 1). AtT20 cells stably expressing calnuc-GFP were pulsed-labeled for 5 min and analyzed as for Fig. 1.

AtT20 cells but are selectively removed from ISG and are released by the so-called constitutive-like pathway (16, 24, 57, 58, 63, 64). However, very few endogenous proteins have been shown to follow the constitutive-like pathway (57–61). Calnuc appears to represent a useful endogenous marker for this pathway in regulated secretory cells such as AtT20 cells and PC12 cells.

GFP tagging has proved to be very useful for studying the regulated secretory pathway in neuroendocrine cells (65–70), although the presence of GFP on some secreted proteins seems to affect their folding (71) or targeting (72). We have found that calnuc-GFP follows the same pathway as endogenous calnuc and is efficiently sorted and removed from ISG and RSG during their maturation. However, the kinetics of secretion of calnuc-GFP differs from that of endogenous calnuc in that it is secreted more rapidly than endogenous calnuc; more calnuc-GFP is present in ISG and it reaches RSG. These findings suggest that overexpressed calnuc-GFP may saturate the retention machinery that usually retains calnuc in the Golgi, but it does not saturate the sorting machinery in ISG and RSG.

In summary, calnuc is an unusual protein in many respects: it has multiple locations in the cell, multiple functions, and an unusual secretion itinerary. It is located in the cell in three pools. The cytosolic pool interacts with G protein α-subunits and is involved in regulated secretion (5), and the Golgi luminal pool functions in Ca^{2+} storage and has been implicated in Ca^{2+} homeostasis (3, 8). We have shown here that the secreted pool is derived from the Golgi pool, is discharged via constitutive secretory vesicles, and represents an endogenous marker for the constitutive-like pathway. In the future, it will be of interest to understand the mechanisms whereby calnuc is retained in the Golgi and to determine the physiological and pathological consequences of its secretion.

MATERIALS AND METHODS

Polyclonal anticalnuc (F-5059) against recombinant full-length calnuc was generated in rabbits and affinity purified as previously described (3). Rabbit anti-ACTH serum (UV16) used for immunoprecipitation was generously provided by Dr. J. D. Castle (University of Virginia, Charlottesville, VA), and mouse monoclonal anti-ACTH IgG used for immunofluorescence was from Novocastra Laboratories (Burlingame, CA). Highly cross-adsorbed Alexa Fluor 594 (red) goat antimouse $F(ab)_2$ (H + L) and Alexa Fluor 488 (green) goat antirabbit IgG (H + L) conjugates were from Molecular Probes, Inc. (Eugene, OR), and gold conjugates were from Amersham Pharmacia Biotech (Piscataway, NJ). Supersignal chemiluminescent reagent was purchased from Pierce Chemical Co. (Rockford, IL) and the enzymatic deglycosylation kit from Glyko (Novato, CA). [^{35}S]Sulfate was from ICN Biomedicals (Irvine, CA) and [^{35}S]EasyTag EXPRESS labeling mix (73% met/22% Cys) was from NEN Life Science Products (Boston, MA). Protein A-Sepharose CL-4B was from Amersham Pharmacia Biotech (Piscataway, NJ) and 10–20% Tris-Tricine gels from Bio-Rad Laboratories, Inc. (Hercules, CA). All chemicals were obtained from Sigma (St. Louis, MO) except as indicated.

Cell Culture

AtT-20 pituitary cells stably overexpressing GFP or calnuc-GFP were established as described previously (8). Cells were maintained in DMEM (high glucose) supplemented with 10% (vol/vol) horse serum, 2.5% (vol/vol) fetal calf serum (Life Technologies, Inc., Gaithersburg, MD), and 0.25 mg/ml G418. NRK cells (ATTC no. 52E) were cultured in DMEM (high glucose) supplemented with 5% (vol/vol) fetal calf serum and 2.5% (vol/vol) Nuserum (Becton Dickinson Labware, Bedford, MA). All media also contained 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate. Cells were used at 80% confluency.

Immunocytochemistry

For immunofluorescence, cells were processed as described previously (3). Briefly, they were fixed in 2% paraformaldehyde (PFA) in 100 mM phosphate buffer, pH 7.4, permeabilized with 0.1% Triton X-100 in PBS for 10 min, incubated with primary antibodies for 1 h in blocking buffer (1% BSA in PBS) followed by incubation with Alexa Fluor 488 (green) goat antirabbit and/or Alexa Fluor 594 (red) goat antimouse conjugates for 1 h in blocking buffer. Specimens were analyzed by deconvolution microscopy using the Applied Precision (Issaquah, WA) Delta Vision imaging system coupled to a Carl Zeiss (Thornwood, NY) S100 fluorescence microscope. For cross-sectional images of cells, stacks were obtained with 200 nm step width. Deconvolution was done on a Silicon Graphics workstation (Mountain View, CA) using Delta vision reconstitution software, and images were processed as Tiff files using PhotoShop 5.0 (Adobe Systems, Mountain View, CA).

For immunogold labeling, cells were fixed with 4% PFA in 0.1 M phosphate buffer for 30 min, followed by 8% PFA for 2 h. Samples were cryoprotected in 2.3 M sucrose at 4 C overnight and snap-frozen in liquid nitrogen. Ultrathin cryo-

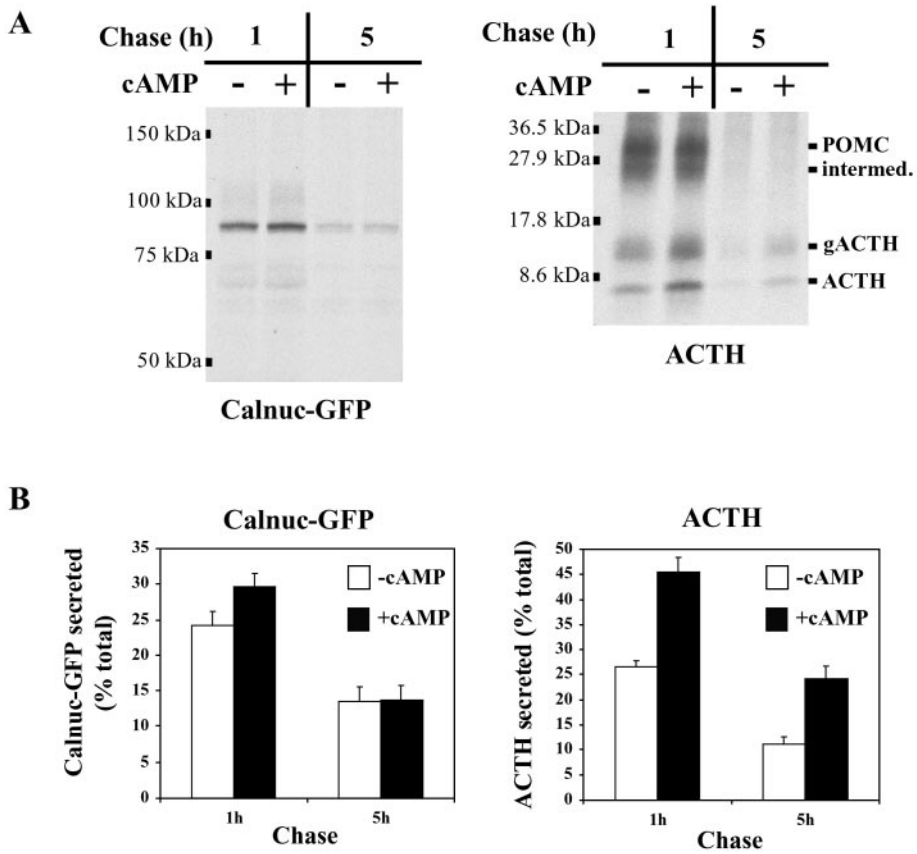

Fig. 10. Calnuc-GFP Is Secreted by the Constitutive Pathway in AtT20 Cells Stably Expressing Calnuc-GFP

A, The amount of calnuc-GFP released into the medium (*left panel*) is similar in the presence (+) and absence (−) of 8-Br-cAMP (cAMP). The amount of ACTH and glycosylated ACTH (gACTH) secreted (*right panel*) is increased in the presence of 8-Br-cAMP. AtT20 cells overexpressing calnuc-GFP were pulse-labeled for 20 min with [^{35}S]EasyTag and chased in unlabeled medium for 1 h or 5 h. Cells were then incubated for 30 min in the absence (−) or presence (+) of 5 mM 8-Br-cAMP (cAMP). Medium (Med) was harvested at the end of the incubations, and radiolabeled calnuc-GFP and ACTH were immunoprecipitated and analyzed as in Fig. 2. B, Quantitation of the stimulated secretion of calnuc-GFP and ACTH from AtT20 cells expressing calnuc-GFP. Calnuc-GFP values represent the percent of total labeled calnuc-GFP recovered in the medium. For ACTH, the values shown represent the percent of the total radiolabeled mature ACTH (gACTH + ACTH) recovered in the medium. *Error bars* show SEM of at least three independent experiments. The amounts of calnuc-GFP secreted after 30 min incubation in the presence or absence of secretagogue are not statistically different (Student's *t* test for 1-h or 5-h chase: $P > 0.05$), whereas ACTH secretion is significantly different (Student's *t* test for 1 h and 5 h: $P = 0.001$ and $P = 0.01$, respectively).

sections were cut at −100 C, picked up onto grids, and double-labeled with primary antibodies followed by 5 or 10 nm gold, goat antirabbit or goat antimouse IgG conjugates as previously described (3). Sections were stained with 0.4% uranyl acetate in 1.8% methyl cellulose and photographed on a JEOL 1200 EX II electron microscope.

Biosynthetic Labeling and Stimulation of Secretion

AtT20 cells expressing calnuc-GFP were seeded at a density of 1.5×10^5 cells/well in 24-well plates (Corning, Inc., Corning, NY). AtT20 cells expressing GFP and NRK cells were seeded at a density of 1.5×10^6 cells/100-mm dish and grown for 48 or 15 h, respectively. For protein labeling, cells were preincubated in Met-Cys-free DMEM for 30 min, followed by pulse-labeling with [^{35}S]EasyTag (300 μCi/ml) for 5–60 min at 37 C. For sulfate labeling, cells were preincubated in buffer A (110 mM NaCl; 5.4 mM KCl; 0.9 mM Na_2HPO_4; 10 mM $MgCl_2$; 2 mM $CaCl_2$; 1 g/liter glucose; 20 mM HEPES, pH 7.2) for 30 min, followed by labeling with [^{35}S]Sulfate (250 μCi/ml) in buffer A for 30 min at 37 C. Cells were then chased for 0–12 h in unlabeled DMEM plus 0.1% BSA. Where indicated, cells were subsequently incubated in fresh DMEM plus 0.1% BSA containing or not 5 mM 8-Br-cAMP. Medium samples were collected and cleared by centrifugation (1000 × *g* for 10 min). Cells were lysed in RIPA buffer (0.1% sodium dodecyl sulfate; 0.5% Triton X-100; 0.5% sodium deoxycholate; 20 mM Tris; 150 mM NaCl, pH 7.4) for immunoprecipitation of calnuc or buffer B (0.5% Triton X-100; 0.5% sodium deoxycholate; 25 mM EDTA; 15 mM Tris-HCl, pH 8.0) for immunoprecipitation of ACTH. Insoluble material was removed by centrifugation at 12,000 × *g* for 30 min, and the supernatant was used for immunoprecipitation.

Immunoprecipitation and SDS-PAGE

Calnuc and ACTH were immunoprecipitated as described earlier (5, 16). Briefly, medium samples or cell lysates (prepared has described above) were incubated with anticalnuc

or anti-ACTH serum overnight at 4 C. Fifty microliters of protein A-Sepharose CL-4B (50% slurry) were added and incubation continued for 2 h. The beads were washed four times in RIPA buffer (for calnuc) or buffer B (for ACTH) and once with PBS. The immune complexes were then separated by SDS-PAGE on 8% or 10% glycine gels (for calnuc) or 10–20% Tris-Tricine gels (for ACTH) and exposed to Kodak (Rochester, NY) Biomax MR film for autoradiography. Quantification of protein bands was measured by densitometry with ScanAnalysis Software (Biosoft, Cambridge, UK). To determine the kinetics of secretion, the percent of the total radiolabeled immunoreactive calnuc present in the medium or cell extracts was determined at each time point. SEM was calculated with at least three independent experiments.

Deglycosylation Experiments

Enzymatic deglycosylation was carried out according to the manufacturer's instructions. Briefly, [^{35}S]EasyTag-labeled immunoprecipitates were denatured in sodium dodecyl sulfate and β-mercaptoethanol and then incubated with *Flavobacterium meningosepticum* peptide:*N*-glycosidase F (*N*-glycanase) or *Clostridium perfringens* sialidase II in the presence or absence of *Streptococcus pneumonia O*-glycosidase DS (*O* glycanase) overnight at 37 C. The proteins were then separated on 8% glycine SDS-PAGE and exposed to Kodak Biomax MR film for autoradiography.

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REFERENCES

1. Wendel M, Sommarin Y, Bergman T, Heinegard D 1995 Isolation, characterization, and primary structure of a calcium-binding 63-kDa bone protein. J Biol Chem 270: 6125–6133
2. Miura K, Hirai M, Kanai Y, Kurosawa Y 1996 Organization of the human gene for nucleobindin (NUC) and its chromosomal assignment to 19q13.2-q13.4. Genomics 34: 181–186
3. Lin P, Le-Niculescu H, Hofmeister R, McCaffery JM, Jin M, Hennemann H, McQuistan T, De Vries L, Farquhar M 1998 The mammalian calcium-binding protein, nucleobindin (CALNUC), is a Golgi resident protein. J Cell Biol 141:1515–1527
4. Kawano J-I, Kotani T, Ogata Y, Ohtaki S, Takechi S, Nakayama T, Sawaguchi A, Nagaike R, Oinuma T, Suganuma T 2000 CALNUC (nucleobindin) is localized in the Golgi apparatus in insect cells. Eur J Cell Biol 79:208–217
5. Lin P, Fischer T, Weiss T, Farquhar MG 2000 Calnuc, an EF-hand Ca^{2+}binding protein, specifically interacts with C-terminal α5-helix of G αi3. Proc Natl Acad Sci USA 97:674–679
6. Weiss TS, Chamberlain CE, Takeda T, Lin P, Hahn KM, Farquhar MG 2001 Gαi3 binding to calnuc on Golgi membranes in living cells monitored by fluorescence resonance energy transfer of green fluorescent protein fusion proteins. Proc Natl Acad Sci USA 98:14961–14966
7. Lin P, Lavoie C, Fisher T, Farquhar M 2000 Calnuc regulates ACTH secretion in AtT20 cells. Mol Biol Cell 11: 500a (Abstract)
8. Lin P, Yao Y, Hofmeiser R, Tsien RY, Farquhar MG 1999 Overexpression of CALNUC (Nucleobindin) increases agonist and thapsigargin releasable Ca^{2+} storage in the Golgi. J Cell Biol 145:279–289
9. Kanai Y, Takeda O, Kanai Y, Miura K, Kurosawa Y 1993 Novel autoimmune phenomena induced *in vivo* by a new DNA binding protein Nuc: a study on MRL/n mice. Immunol Lett 39:83–89
10. Kubota T, Watanabe N, Kaneko T, Satake F, Miura K, Kurosawa Y, Miyasaka N, Kanai Y 2001 Activation of autoreactive T cells that help nucleobindin-injected mice produce anti-DNA antibodies. Immunol Lett 75:111–115
11. Kanai Y, Katagari T, Mori S, Kubota T 1986 An established MRL/Mp-lpr cell line with null cell properties produces a B cell differentiation factor(s) that promotes anti-single-stranded DNA antibody production in MRL spleen cell culture. Int Arch Allergy Appl Immun 81:92–94
12. Miura K, Titani Y, Kurosawa Y, Kanai Y 1992 Molecular cloning of nucleobindin, a novel DNA-binding protein that contains both a signal peptide and a leucine zipper structure. Biochem Biophys Res Commun 187:375–380
13. Moore HP, Walker M, Lee F, Kelly RB 1983 Expressing a human proinsulin cDNA in a mouse ACTH-secreting cell. Intracellular storage, proteolytic processing, and secretion on stimulation. Cell 35:531–538
14. Castle AM, Stahl LE, Castle JD 1992 A 13-amino acid N-terminal domain of a basic proline-rich protein is necessary for storage in secretory granules and facilitates exit from the endoplasmic reticulum. J Biol Chem 267: 13093–130100
15. Milgram SL, Eipper BA, Mains RE 1994 Differential trafficking of soluble and integral membrane secretory granule-associated proteins. J Cell Biol 124:33–41
16. Castle AM, Huang AY, Castle JD 1997 Passive sorting in maturing granules of AtT-20 cells: the entry and exit of salivary amylase and proline-rich protein. J Cell Biol 138: 45–54
17. Eaton BA, Haugwitz M, Lau D, Moore HP 2000 Biogenesis of regulated exocytotic carriers in neuroendocrine cells. J Neurosci 20:7334–7344
18. Fernandez CJ, Haugwitz M, Eaton B, Moore H 1997 Distinct molecular events during secretory granule biogenesis revealed by sensitivities to brefeldin A. Mol Biol Cell 8:2171–2185
19. Gumbiner B, Kelly RB 1982 Two distinct intracellular pathways transport secretory and membrane glycoproteins to the surface of pituitary tumor cells. Cell 28:51–59
20. Moore HH, Kelly RB 1986 Re-routing of a secretory protein by fusion with human growth hormone sequences. Nature 321:443–446
21. Miller SG, Moore HP 1992 Movement from trans-Golgi network to cell surface in semi-intact cells. Methods Enzymol 219:234–248
22. Grimes M, Kelly RB 1992 Intermediates in the constitutive and regulated secretory pathways released *in vitro* from semi-intact cells. J Cell Biol 117:539–549
23. Tooze SA 1998 Biogenesis of secretory granules in the trans-Golgi network of neuroendocrine and endocrine cells. Biochim Biophys Acta 1404:231–244
24. Castle AM, Huang AY, Castle JD 1998 Immunoglobulin-derived polypeptides enter the regulated secretory pathway in AtT-20 cells. FEBS Lett 439:341–345
25. Saraste J, Kuismanen E 1984 Pre- and post-Golgi vacuoles operate in the transport of Semliki Forest virus membrane glycoproteins to the cell surface. Cell 38:535–549

26. Schweizer A, Fransen JAM, Matter K, Kreis TE, Ginsel L 1990 Identification of an intermediate compartment involved in protein transport from endoplasmic reticulum to Golgi apparatus. Eur J Cell Biol 53:185–196
27. Matlin KS, Simons K 1983 Reduced temperature prevents transfer of a membrane glycoprotein to the cell surface but does not prevent terminal glycosylation. Cell 34:233–243
28. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K, Albright LM, Coen DM, Varki A 2000 Sialidases. Current protocols in molecular biology. New York: John Wiley & Sons, Inc.; 17.12.2–17.12.8
29. Huttner WB 1988 Tyrosine sulfation and the secretory pathway. Annu Rev Physiol 50:363–376
30. Hille A, Braulke T, von Figura K, Huttner W 1990 Occurrence of tyrosine sulfate in proteins—a balance sheet. 1. Secretory and lysosomal proteins. Eur J Biochem 188: 577–586
31. Baeuerle P, Huttner WB 1987 Tyrosine sulfation is a trans-Golgi-specific protein modification. J Cell Biol 105: 2655–2664
32. Rosa P, Mantovani S, Rosboch R, Huttner WB 1992 Monensin and brefeldin A differentially affect the phosphorylation and sulfation of secretory proteins. J Biol Chem 267:12227–12232
33. Spiro RC, Freeze HH, Sampath D, Garcia JA 1991 Uncoupling of chondroitin sulfate glycosaminoglycan synthesis by brefeldin A. J Cell Biol 115:1463–1473
34. Berstein HB, Compans RW 1992 Sulfation of the human immunodeficiency virus envelope glycoprotein. J Virol 66:6953–6959
35. Farley J, Nakawama G, Cryns D, Segel I 1978 Adenosine triphosphate sulfurylasefrom penicillium chrysogenum: equilibrium binding, substrate hydrolysis, and isotope exchange studies. Arch Biochem Biophys 185:376–390
36. Burnell JN, Roy AB 1978 Purification and properties of the ATP sulfurylase of rat liver. Biochem Biophys Acta 527:239–248
37. Gumbiner B, Kelly RB 1981 Secretory granules of an anterior pituitary cell line, AtT-20, contain only mature forms of corticotropin and β-lipotropin. Proc Natl Acad Sci USA 78:318–322
38. Kanai Y, Miura K, Uehara T, Amagai M, Takeda O, Tanuma S, Kurosawa Y 1993 Natural occurrence of NUC in the sera of autoimmune-prone MRL/LPR mice. Biochem Biophys Res Commun 196:729–736
39. Johnson S, Michalak M, Opas M, Eggleton P 2001 The ins and outs of calreticulin: from the ER lumen to the extracellular space. Trends Cell Biol 11:122–129
40. Vorum H, Hager H, Christensen BM, Nielsen S, Hionore B 1999 Human calumenin localize to the secretory pathway and is secreted to the medium. Exp Cell Res 248: 473–481
41. Hohenester E, Maurer P, Hohenadl C, Timpl R, Jansonius JN, Engel J 1996 Structure of a novel extracellular Ca^{2+}-binding module in BM-40. Nat Struct Biol 3:67–73
42. Hohenester E, Maurer P, Timpl R 1997 Crystal structure of a pair of follistatin-like and EF-hand calcium-binding domains in BM-40. EMBO J 16:3778–3786
43. Gray AJ 1995 The mitogen effects of the B β chain of fibrinogen are mediated through cell surface calreticulin. J Biol Chem 270:26602–26606
44. White TK 1995 cell surface calreticulin is a putative mannoside lectin which triggers mouse melanoma cell spreading. J Biol Chem 270:15926–15929
45. Zhu Q 1997 Calreticulin-integrin bidirectional signaling complex. Biochem Biophys Res Commun 232:354–358
46. Kwon MS 2000 Calreticulin couples calcium release and calcium influx in integrin-mediated calcium signaling. Mol Biol Cell 11:1433–1443
47. Babia T, Ayala I, Valderrama F, Mato E, Bosch M, Santaren JF, Renau-Piqueras J, Kok JW, Thomson TM, Egea G 1999 N-ras induces alterations in Golgi complex architecture and in constitutive protein transport. J Cell Sci 112:477–489
48. Hoe MH, Slusarewicz P, Misteli T, Watson R, Warren G 1995 Evidence for recycling of the resident medial/trans Golgi enzyme, *N*-acetylglucosamine transferase I, in ldlD cells. J Biol Chem 270:25057–25063
49. Cole NB, Ellenberg J, Song J, DiEuliis D, Lippincott-Schwartz J 1998 Retrograde transport of Golgi-localized proteins to the ER. J Cell Biol 140:1–15
50. Moremen KW, Touster O 1985 Biosynthesis and modification of Golgi mannosidase II in HeLa and 3T3 cells. J Biol Chem 260:6654–6662
51. Yuan L, Barriocanal JG, Bonifacino JS, Sandoval IV 1987 Two integral membrane proteins located in the cis-middle and trans-part of the Golgi system acquire sialylated N-linked carbohydrates and display different turnovers and sensitivity to cAMP-dependent phosphorylation. J Cell Biol 105:215–227
52. Gonatas JO, Mezitis SG, Stieber A, Fleischer B, Gonatas NK 1989 MG-160. A novel sialoglycoprotein of the medial cisternae of the Golgi apparatus. J Biol Chem 264: 646–653
53. Johnston PA, Stieber A, Gonatas NK 1994 A hypothesis on the traffic of MG160, a medial Golgi sialoglycoprotein, from the *trans*-Golgi network to the Golgi cisternae. J Cell Sci 107:529–537
54. Alcalde J, Egea G, Sandoval IV 1994 Gp74 a membrane glycoprotein of the cis-Golgi network that cycles through the endoplasmic reticulum and intermediate compartment. J Cell Biol 124:649–665
55. Opat AS, Houghton F, Gleeson PA 2001 Steady-state localization of a medial-Golgi glycosyltransferase involves transit through the *trans*-Golgi network. Biochem J 358:33–40
56. Scherer PE, Lederkremer GZ, Williams S, Fogliano M, Baldini G, Lodish HF 1996 Cab45, a novel Ca^{2+}-binding protein localized to the Golgi lumen. J Cell Biol 133: 257–268
57. Arvan P, Castle D 1998 Sorting and storage during secretory granule biogenesis: looking backward and looking forward. Biochem J 332:593–610
58. Gorr SU, Jain RK, Kuehn U, Joyce PB, Cowley DJ 2001 Comparative sorting of neuroendocrine secretory proteins: a search for common ground in a mosaic of sorting models and mechanisms. Mol Cell Endocrinol 172:1–6
59. Loh YP 1998 Protein secretion: puzzling receptors. Curr Biol 8:R41
60. Thiele C, Gerdes HH, Huttner WB 1997 Protein secretion: puzzling receptors. Curr Biol 7:R496–R500
61. Dannies PS 1999 Protein hormone storage in secretory granules: mechanisms for concentration and sorting. Endocr Rev 20:3–21
62. Kuliawat R, Arvan P 1992 Protein targeting via the "constitutive-like" secretory pathway in isolated pancreatic islets: passive sorting in the immature granule compartment. J Cell Biol 118:521–529
63. Varlamov O, Eng FJ, Nivikova EG, Fricker LD 1999 Localization of metallocarboxypeptidase D in AtT-20 cells. J Biol Chem 274:14759–14767
64. Stoller TJ, Shields D 1988 Retrovirus-mediated expression of preprosomatostatin: posttranslational processing, intracellular storage, and secretion in GH3 pituitary cells. J Cell Biol 107:2087–2095
65. Kaether C, Salm T, Glombik M, Almers W, Gerdes HH 1997 Targeting of green fluorescent protein to neuroendocrine secretory granules: a new tool for real time studies of regulated protein secretion. Eur J Cell Biol 74:133–142
66. Pouli AE, Emmanouilidou E, Zhao C, Wasmeier C, Hutton JC, Rutter GA 1998 Secretory-granule dynamics visualized *in vivo* with a phogrin-green fluorescent protein chimaera. Biochem J 333:193–199
67. El Meskini R, Jin L, Marx R, Bruzzaniti A, Lee J, Emeson R, Mains R 2001 A signal sequence is sufficient for green

fluorescent protein to be routed to regulated secretory granules. Endocrinology 142:864–873
68. Lochner JE, Kingma M, Kuhn S, Meliza CD, Cutler B, Scalettar BA 1998 Real-time imaging of the axonal transport of granules containing a tissue plasminogen activator/green fluorescent protein hybrid. Mol Biol Cell 9: 2463–2476
69. Haubensak W, Narz F, Heumann R, Lessmann V 1998 BDNF-GFP containing secretory granules are localized in the vicinity of synaptic junctions of cultured cortical neurons. J Cell Sci 111:1483–1493
70. Levitan ES 1998 Studying neuronal peptide release and secretory granule dynamics with green fluorescent protein. Methods 16:182–187
71. Pouli AE, Kennedy HJ, Schofield JG, Rutter GA 1998 Insulin targeting to the regulated secretory pathway after fusion with green fluorescent protein and firefly luciferase. Biochem J 331:669–675
72. Kunze II, Hensel G, Adler K, Bernard J, Neubohn B, Nilsson C, Stoltenburg R, Kohlwein SD, Kunze G 1999 The green fluorescent protein targets secretory proteins to the yeast vacuole. Biochim Biophys Acta 1410:287–298