Visualization of protein transport along the secretory pathway using green fluorescent protein

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Abstract **We have expressed green fluorescent protein (GFP) from *A. victoria* in the secretory pathway of HeLa cells by fusing it to the C-terminus of a secretory protein, chromogranin B. Under normal culture conditions at 37°C maturation of GFP to the fluorescent form was not detectable. However, fluorescent GFP was observed when biosynthetic protein transport was arrested at the intermediate compartment or the *trans*-Golgi network by temperature blocks (15°C and 20°C, respectively). Reversal of the temperature blocks allowed the visualization of secretion of fluorescent GFP and offers the possibility to analyse transport in the secretory pathway in living cells.**

Key words: Green fluorescent protein; GFP; Chromogranin B; Secretory pathway; Intermediate compartment; *trans*-Golgi network

1. Introduction

The secretory pathway with its various compartments is one of the most complex transport routes in eukaryotic cells. Proteins are delivered to various destinations including lysosomes, plasma membrane and the extracellular space [1]. During the past few years substantial progress has been made in the identification and characterization of the cellular compartments involved and the vesicular transport events mediating the vectorial transport throughout the pathway [2,3]. At the same time, however, no experimental approach has allowed the analysis of such vesicular traffic under in vivo conditions in real time. Such studies would require a reporter protein which can be visualized in vivo.

With this in mind, the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* [4] attracted our interest. In the jellyfish, GFP is synthesized as a cytosolic protein [4]. Maturation to its fluorescent form occurs post-translationally within a few hours and involves cyclization and oxidation of the residues serine-tyrosine-glycine of the polypeptide [4,5]. Heterologous expression of GFP generates striking green fluorescence in many different organisms without additional substrates or cofactors, thus making it an excellent reporter for in vivo use [6–8]. Moreover, these studies show that no species-specific factors are needed for GFP to fluoresce and suggest that maturation occurs by autocatalysis or through an ubiquitous cellular component [6].

So far GFP has been used to monitor the transfection of cells, as a reporter for promoter activity or to localize fusion proteins in the cytosol [6,8]. In the present study we address the question whether (i) GFP becomes fluorescent when targeted to the secretory pathway and if so, whether (ii) it would allow the visualization of compartments and vesicular traffic in this pathway.

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Abbreviations: GFP, green fluorescent protein; hCgB, human chromogranin B; TGN, *trans*-Golgi network; ER, endoplasmic reticulum; IC, intermediate compartment.

2. Materials and methods

2.1. Plasmid construction

Standard molecular cloning techniques were used throughout [9]. The hCgB-GFP construct was obtained by fusing the coding sequence of GFP without the start codon to the 3′ end of hCgB cDNA (clone hSgI/19, [10]) lacking the stop codon (see Fig. 1). Fusion of the two sequences led to the introduction of two codons, Val and Pro. hCgB in the pGEM4 vector (Promega) was first amplified by PCR using as forward primer a T7-primer and as reverse primer oligonucleotide 5′-CCGATCGATCATGGGTACCCCCCTTTGGCTGAATTTC, the latter being complementary to nucleotides 2125–2140 of the hCgB sequence (GenBank No Y00064). The introduced *Kpn*I site is underlined. The amplified hCgB-fragment was digested with *Hin*dIII and *Kpn*I and cloned into vector pSP73 (Promega) which was digested with *Hin*dIII and *Kpn*I, yielding pSP73/hCgB. The cDNA of GFP was amplified by PCR from the pGFP10.1 plasmid ([4], kindly provided by M. Chalfie, New York). The forward primer 5′-GCGCGGTACCCAGTAAAGGAGAAGAACTTTTC (GFP-A) included nucleotides 29–49 from the GFP-sequence (GenBank No M62653) and introduced a *Kpn*I site (underlined). The reverse primer 5′-GCGCGAATTCATACAATTTTAATGAATC (GFP-B) is complementary to nucleotides 842–863 and introduced an *Eco*RI site (underlined). The amplified GFP fragment was digested with *Kpn*I and *Eco*RI and cloned into vector pSP73/hCgB (see above) digested with *Kpn*I and *Eco*RI yielding pSP73/hCgB/GFP.

A mutated form of GFP (Ser65 to Thr65, [11]) was constructed using the mutagenic oligonucleotide 5′-GCGGCCATGGCCAACACTTGTCACTACTTTCACTTATG (GFP-C) including the unique *Nco*I site of GFP (underlined). PCR was performed with GFP-C and GFP-B using pSP73/hCgB/GFP as a template. The wild type GFP *Nco*I/*Eco*RI fragment of pSP73/hCgB/GFP was exchanged by the corresponding mutated fragment and subcloned as a *Hin*dIII/*Eco*RI fragment into the mammalian expression vector pCDM8 (Invitrogen). The mutation was verified by sequencing.

2.2. Cell culture and transfection

HeLa-cells (ATCC CCL185) were cultured at 37°C and 5% CO_2 in EMEM medium containing 10% fetal calf serum. Cells were grown on coverslips to 50% confluency overnight. Transient DNA transfections were performed using calcium phosphate precipitation [9] and led to 20 to 50% positive transformants. Control cells were transfected without hCgB-GFP DNA. Analysis was performed 2 days after start of transfection. For [^{35}S]sulfate labeling cells were pretreated for 17 h with 7 mM sodium butyrate.

2.3. Sulfate labeling and protein analysis

Pulse-chase labeling of HeLa cells (6 cm dishes) with [^{35}S]sulfate was carried out as previously described for PC12 cells [12,13] except that the pulse and chase media were serum-free. Cells were harvested in a buffer containing 10 mM Hepes pH 7.4, 1% Triton X-100, 250 mM sucrose, 1 mM EDTA, 1.25 mM PMSF, 1 μg/ml leupeptin. Cell lysates and chase media were centrifuged at 1200 rpm for 10 min (Eppendorf

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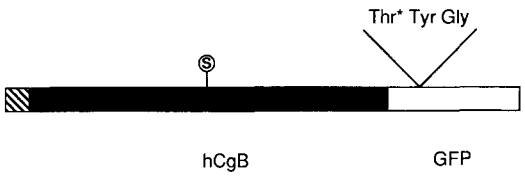

Fig. 1. Diagram of the structure of hCgB-GFP. GFP (white box) was fused to the C-terminus of hCgB (black box). The N-terminal signal sequence of hCgB is shown (hatched box). The insertion shows the modified GFP chromophore with the serine to threonine mutation (asterisk), the circled S indicates the tyrosine sulfation site identified in hCgB [10].

5415C), acetone precipitated with 50 μg haemoglobin as carrier and analysed by SDS-PAGE. For western blotting, cell lysates and chase media were transferred to nitrocellulose and probed with the monoclonal antibody 67-C7-2 against hCgB [14]. The blot was processed by ECL (Amersham).

2.4. Temperature blocks and analysis of cells

Two days after transfection temperature blocks were performed using a waterbath [15]. During the temperature blocks the medium was buffered with 10 mM HEPES. After the indicated times cells were either placed immediately on ice or incubated for indicated times at 37°C and 5% CO_2. Release of the block was started by replacing HEPES-buffered culture medium by prewarmed culture medium without Hepes. For the microscopic analysis cells were fixed with 3% paraformaldehyde on ice for 20 min. Alternatively living cells on coverslips were placed on a glass slide and analysed immediately. A Zeiss axiophot with FITC filter sets was used.

3. Results

3.1. Construction of secretory hCgB-GFP

To obtain a secreted form of GFP we fused it to the C-terminus of hCgB (Fig. 1). Although hCgB is a protein of the regulated secretory pathway of neuroendocrine cells [10,16], it was shown to be constitutively secreted in transfected HeLa cells (a cell line without a regulated pathway used in the present study) with a $t_{1/2}$ of 26 min (A. Krömer and H.-H. Gerdes, unpublished). We used a mutated form of GFP (Ser65 to Thr65; see section 2). Besides a stronger fluorescent signal this mutant is converted to its fluorescent form about four times more rapidly than the wildtype GFP [11]. By choosing this mutant we expected that the generation of its fluorescent form would occur faster than its secretion from the cell, thus resulting in the visualization of its transport along the secretory pathway.

3.2. Expression of hCgB-GFP in the secretory pathway

HeLa cells were transiently transfected with hCgB-GFP. To confirm the expression of full length hCgB-GFP cell lysates were analysed by a Western blot using a monoclonal antibody recognizing the hCgB-moiety of the chimeric protein. As shown in Fig. 2a hCgB-GFP with an M_r of about 155 kDa was detected in hCgB-GFP transfected cells (lane 1) but not in control cells (lane 2). In addition also a minor band of lower molecular weight was visible in lane 1 (asterisk). As a proteolytic product of similar difference in mobility was described for hCgB alone with the same antibody [14] we favour the idea that this lower molecular weight band reflects an N-terminally processed fusion protein, although we cannot rule out C-terminal processing of hCgB-GFP.

We next investigated whether hCgB-GFP was targeted to the secretory pathway and subsequently secreted into the medium. For this we used tyrosine sulfation, a TGN-specific post-translational modification ([17], Fig. 1). After a 5 min [^{35}S]sulfate pulse a band of the same mobility (and a minor band of lower molecular weight, asterisk) as recognized by the monoclonal antibody

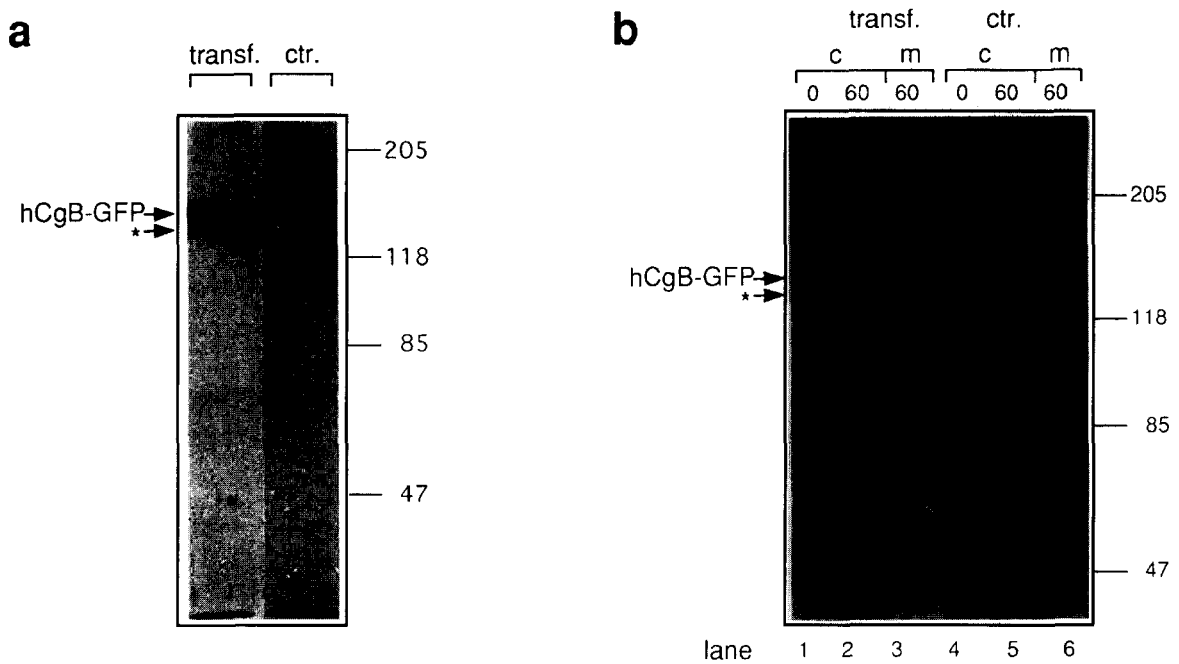

Fig. 2. hCgB-GFP is targeted to the secretory pathway and secreted into the medium. HeLa cells were transfected with (transf.) or without DNA encoding hCgB-GFP (ctr.). (a) A Western blot of cell lysates was probed with monoclonal antibody 67-C7-2 against hCgB and processed by ECL. (b) Cells were pulse-labeled for 5 min with [^{35}S]sulfate followed by a chase for indicated times (min). Equal aliquots of cell lysates (c) and chase media (m) were analysed by SDS-PAGE and fluorography. The positions of hCgB-GFP, the putative degradation product of hCgB-GFP (asterisk) and molecular weight standards are indicated.

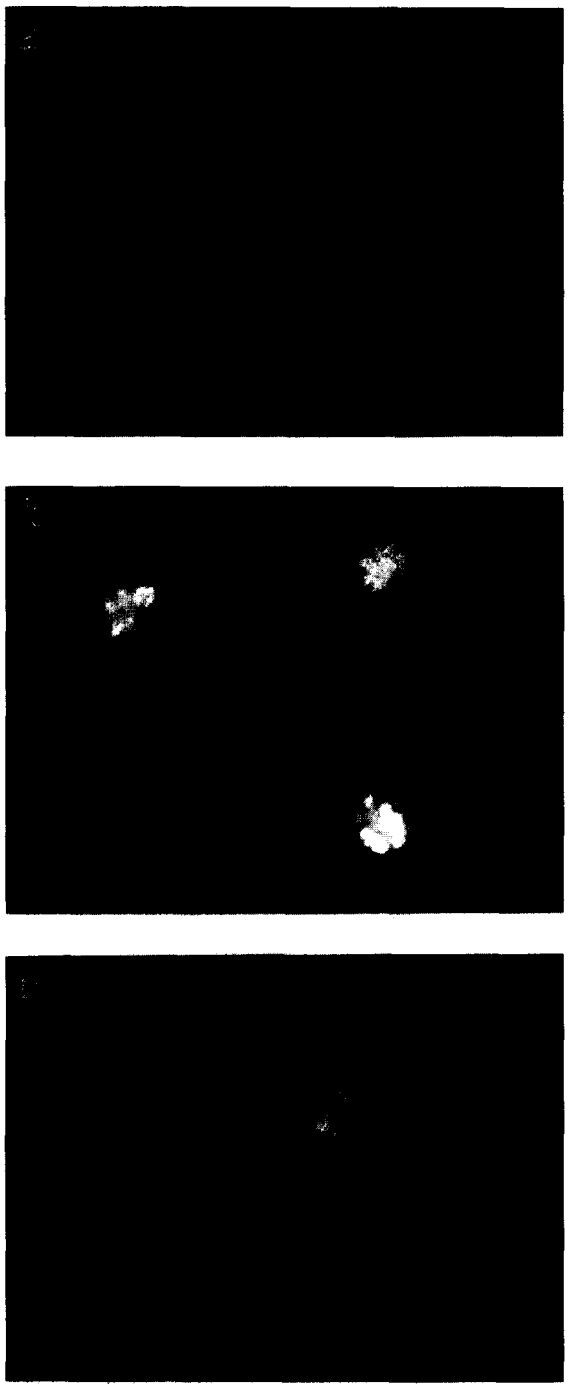

Fig. 3. Block of secretion results in formation of fluorescent GFP. HeLa cells were transfected with hCgB-GFP and analysed without (a) or with (b,c) application of a 20°C block for 2 h. Fluorescence analysis with FITC filter sets was performed after fixation (a,b) or directly on living cells (c). The same exposure time was used in a–c.

(Fig. 2a) was detectable in cell lysates of transfected cells (Fig. 2b, lane 1) but not in control cells (Fig. 2b, lane 4). To demonstrate the secretion of hCgB-GFP [^{35}S]sulfate pulse-labeled cells were chased for 1 hr and analysed together with the media. As shown in Fig. 2b the full length hCgB-GFP of 155 kDa was detected in the medium of transfected (lane 3) but not of control cells (lane 6). Consistent with the secretion the cellular amount of labeled hCgB-GFP was significantly reduced (compare lanes 1 and 2 in Fig. 2a) after 1 h of chase. Taken together we conclude that hCgB-GFP was targeted to the lumenal side of the secretory pathway in HeLa cells and secreted into the medium.

3.3. *Detection of fluorescent hCgB-GFP*

When transiently transfected HeLa cells were cultured under normal conditions, fixed and analysed, they showed very little, if any, fluorescence (Fig. 3a). In contrast, when vesicular transport was arrested at the *trans*-Golgi network (TGN) by a 20°C temperature block for 2 h [15], a strong perinuclear fluorescence was visible in fixed cells indicating the presence of active GFP in a compartment likely to be the TGN (Fig. 3b). To test whether fluorescence of GFP was also observed in living cells, cells grown on a coverslip were not fixed but analysed directly after culturing at 37°C and after a 2 h 20°C block. As with the fixed cells a strong perinuclear staining was observed only after a 20°C temperature block (Fig. 3c) but not under normal culture conditions (not shown), demonstrating the usefulness of GFP as an in vivo reporter for the secretory pathway.

3.4. *Visualization of different compartments in the secretory pathway*

A block of protein transport at the intermediate/*cis*-Golgi network compartment can be obtained by incubation at 15°C [18]. To test whether fluorescent GFP could be visualized under such conditions, we incubated transfected HeLa cells at 15°C. Already after 15 min of incubation at 15°C green fluorescence was visible as a reticular and punctate staining throughout the cytoplasm (Fig. 4a) resembling a pattern obtained for the endoplasmic reticulum (ER) and intermediate compartment (IC) [19]. After 60 min of incubation at 15°C the fluorescent GFP displayed a strong perinuclear staining (Fig. 4b) which remained unchanged at least for 1 h of further incubation at 15°C (data not shown). Due to the strong signal in Fig. 4b, an underlying punctate pattern could not be excluded. Irrespective of the presence of such a pattern these data suggest that the fluorescent GFP visible as a punctate pattern after 15 min of incubation moved more distal in the secretory pathway during longer incubation times. Whether the perinuclear pattern obtained after 1 h reflects a subdomain of the IC proximal to the Golgi stack or a compartment distal to the Golgi stack can not be concluded from our data and will be subject of future studies.

We next investigated whether a shorter incubation at 20°C would also lead to the visualization of GFP in transfected HeLa cells. The signal for fluorescent GFP was much weaker after 15 min incubation at 20°C (Fig. 4c) than at 15°C (Fig. 4a). Fluorescence could only be seen on the film after a longer exposure time (Fig. 4c′). Incubation at 20°C for 30 min yielded a clearly stronger signal (Fig. 4d) reaching a plateau after 1 h (not shown). The detection of fluorescent GFP after 15 min of incubation at 15°C but not at 20°C indicates that higher local concentrations of fluorescent GFP are reached earlier at 15°C than at 20°C.

3.5. *Release of the 15°C block results in the secretion of hCgB-GFP*

Next we wanted to test if the movement of recombinant GFP along the secretory pathway could be monitored upon release of the temperature-dependent secretion block. Therefore cells

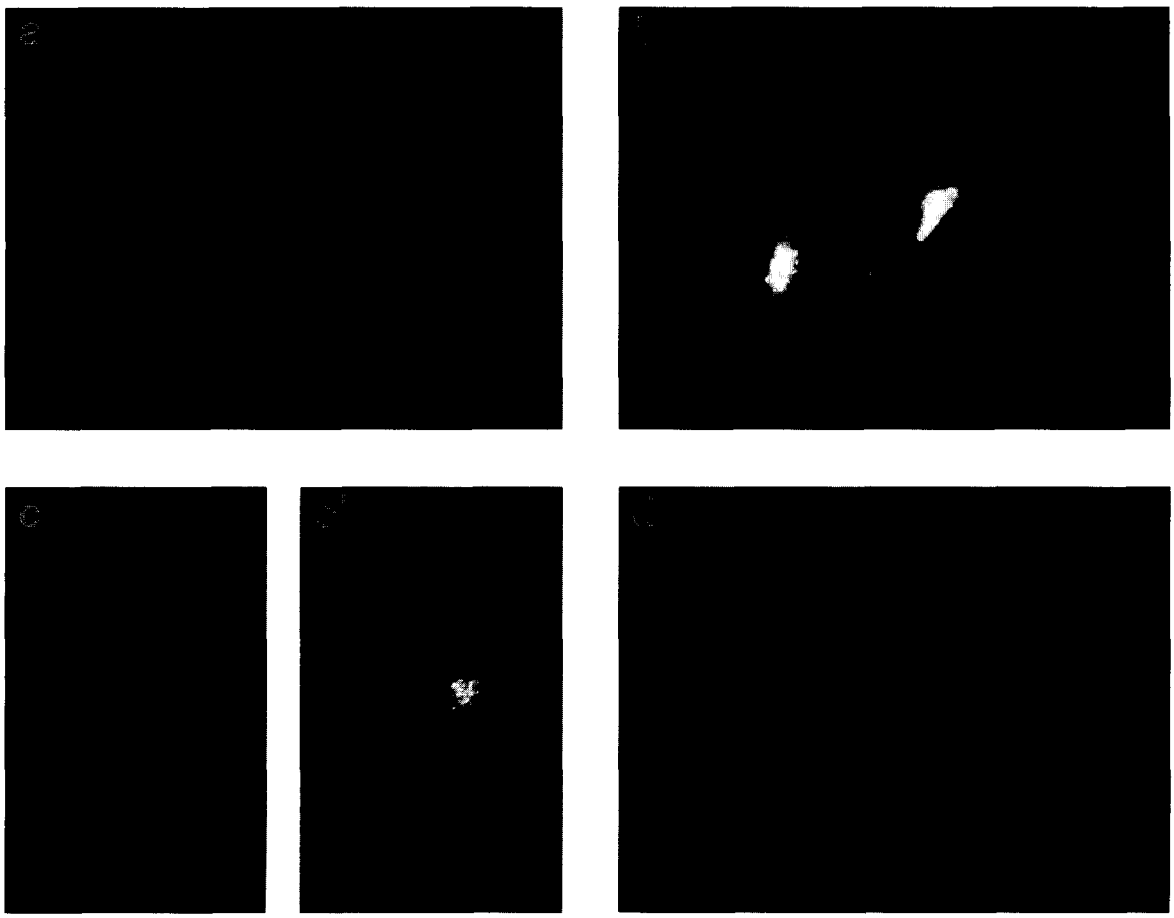

Fig. 4. Different compartments can be visualized by distinct temperature blocks. Transfected HeLa cells were incubated at 15°C for 15 min (a) or 60 min (b) or at 20°C for 15 min (c,c') or 30 min (d). Cells were fixed and analysed for green fluorescence. Micrographs show the same exposure time except panel c' for which the exposure time was 3 times longer. Different cell sizes reflect the heterogeneity of the HeLa cell population.

were incubated at 15°C for 2 h and chased for various times at 37°C, fixed and analysed. After short chase times of 5 min at 37°C (Fig. 5a) the perinuclear fluorescence pattern was unchanged. Incubation for 15 min, however, resulted in a punctated presumptive vesicular pattern (Fig. 5b). This vesicular staining reached a maximum after 30 min (Fig. 5c) and disappeared entirely after 1 h of incubation (Fig. 5d) suggesting the complete secretion of fluorescent hCgB-GFP.

4. Discussion

We have expressed a secretory form of GFP in HeLa cells. Fluorescent GFP was only obtained after arrest of transport of hCgB-GFP mediated by a temperature block at 15°C or at 20°C. Short blocking times at 15°C revealed a reticular and punctate pattern throughout the cell consistent with localization of CgB-GFP in the ER and IC. Prolonged transport blocks at 15°C or a block at 20°C showed a perinuclear staining consistent with Golgi and TGN localization. Reversal of both transport blocks [18,20] led to a complete disappearance of fluorescent GFP from the cells (Fig. 5, only data for 15°C are shown). The observed loss of fluorescence was most likely due to secretion of fluorescent hCgB-GFP rather than degradation since full-length hCgB-GFP was detected in the medium (Fig. 2b, lane 3).

In addition to our studies on the traffic of GFP along the secretory pathway we gained new insights in the maturation of GFP. One unsolved question concerns the mechanism of chromophore formation. From several heterologous expression studies leading to fluorescent GFP it was concluded that either chromophore formation is dependent on ubiquitous protein factors or occurs autocatalytically [6]. Our study shows that chromophore formation can occur independent of cytosolic factors. Since insertion of secretory proteins into the endoplasmic reticulum occurs cotranslationally, maturation of hCgB-GFP must take place in the lumen of the secretory pathway. Furthermore our data suggest that chromophore formation or concentration of fluorescent GFP occurs faster in the ER than in the Golgi/TGN (Fig. 4). Incubation at 15°C has been shown to result in a delay of exit from the ER [18]. Therefore the longer retention of GFP in the ER could lead to faster maturation due to the high concentration of chaperones or could result in higher local concentrations seen at 15°C and not at 20°C.

In conclusion our system opens the unique possibility to visualize a secreted form of GFP in a pulse-chase like manner in living cells. This is possible because we have shown that the fast secretion of hCgB-GFP under normal conditions does not lead to fluorescence within the cell. Only block of secretion results in fluorescent GFP in a pulse-like manner. Upon block release this distinct population of fluorescent GFP can be fol-

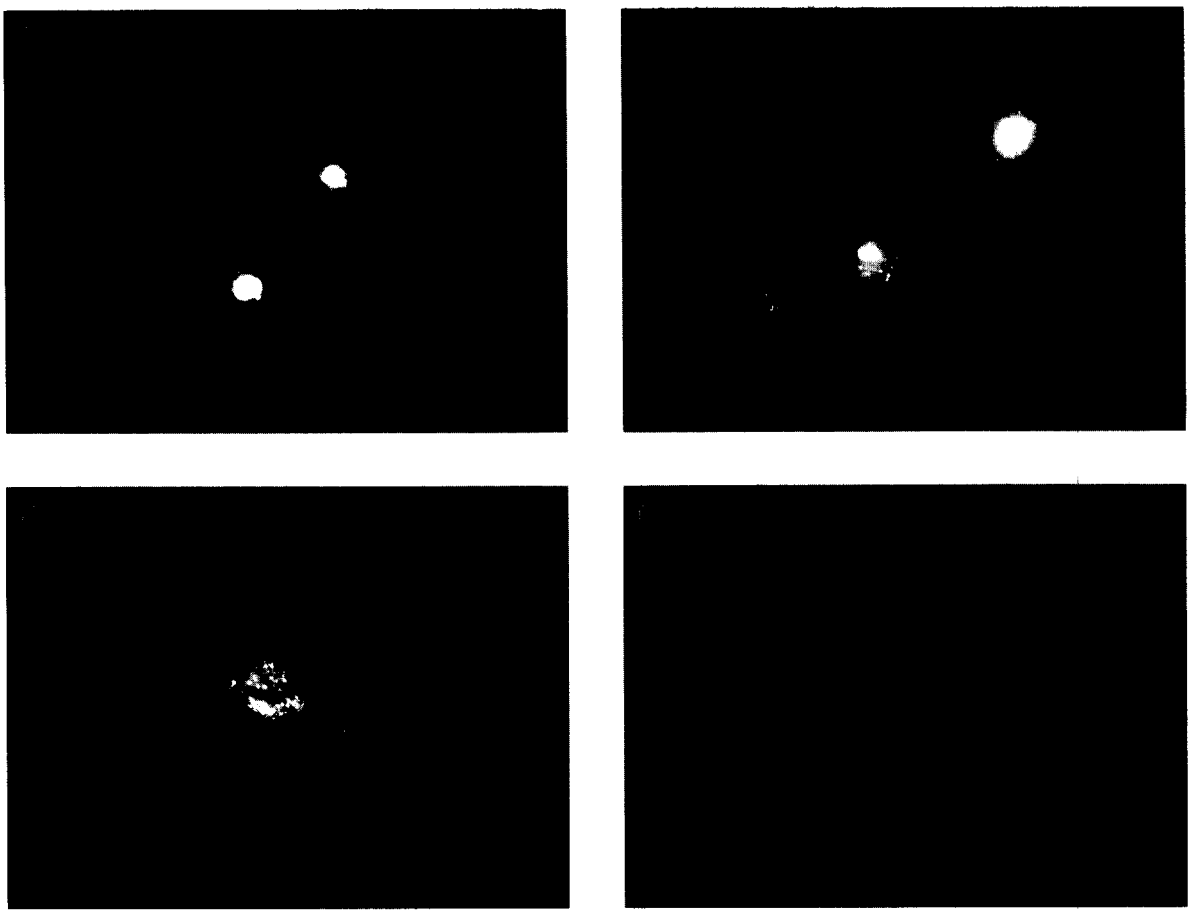

Fig. 5. Release of the 15°C block results in vesicular transport of hCgB-GFP. hCgB-GFP-transfected HeLa cells were subjected to a 15°C block for 2 h. Thereafter the cells were fixed after an additional incubation at 37°C for 5 min (a), 15 min (b), 30 min (c) or 60 min (d) followed by the analysis of FITC-fluorescence.

lowed in a chase-like manner, since hCgB-GFP distal to this population has left the cell, and hCgB-GFP proximal to this population does not fluoresce. This system will allow us to study in real time the dynamics of vesicular traffic along the secretory pathway. In particular budding and fusion steps, the movement of secretory vesicles and the application of drugs affecting secretion can be analysed in living cells and will contribute to our understanding of the dynamics of transport processes.

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