Oligomerization of green fluorescent protein in the secretory pathway of endocrine cells

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Green fluorescent protein (GFP) is used extensively as a reporter protein to monitor cellular processes, including intracellular protein trafficking and secretion. In general, this approach depends on GFP acting as a passive reporter protein. However, it was recently noted that GFP oligomerizes in the secretory pathway of endocrine cells. To characterize this oligomerization and its potential role in GFP transport, cytosolic and secretory forms of enhanced GFP (EGFP) were expressed in GH4C1 and AtT-20 endocrine cells. Biochemical analysis showed that cytosolic EGFP existed as a 27 kDa monomer, whereas secretory forms of EGFP formed disulphide-linked oligomers. EGFP contains two cysteine residues (Cys⁴⁹ and Cys⁷¹), which could play a role in this oligomerization. Site-directed mutagenesis of Cys⁴⁹ and Cys⁷¹ showed that both cysteine residues were involved in disulphide interactions. Substitution of either cysteine residue

resulted in a reduction or loss of oligomers, although dimers of the secretory form of EGFP remained. Mutation of these residues did not adversely affect the fluorescence of EGFP. EGFP oligomers were stored in secretory granules and secreted by the regulated secretory pathway in endocrine AtT-20 cells. Similarly, the dimeric mutant forms of EGFP were still secreted via the regulated secretory pathway, indicating that the higher-order oligomers were not necessary for sorting in AtT-20 cells. These results suggest that the oligomerization of EGFP must be considered when the protein is used as a reporter molecule in the secretory pathway.

Key words: dimerization, disulphide, mutant, regulated pathway, secretion.

INTRODUCTION

Fluorescent proteins are finding increased use in cell-biological applications. In particular, the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has been used extensively in recent years to monitor cellular processes in real time [1]. Unlike other commonly used reporter proteins, including β -galactosidase, firefly luciferase, alkaline phosphatase, chloramphenicol acetyltransferase and β -glucuronidase, detection of GFP does not require an additional substrate, as the protein can be easily monitored using long-wave UV light sources. GFP has been reported to remain stable in the presence of many denaturants and proteases as well as over a broad range of pH and temperature [2]. The intrinsic fluorescence and relatively small size (27 kDa) of GFP make it a good reporter protein for use in fusion proteins (transfer chimaeras). Thus GFP has been used to label and locate fusion proteins, and to study intracellular protein traffic [3,4].

GFP appears to be an ideal reporter protein for the secretory pathway where proteins are difficult to access in individual organelles. In order to use GFP as a marker for secretory transport, the protein should not alter the transport route or localization of GFP fusion proteins. Although preliminary data suggested that GFP met this condition [5], it was recently reported [6,7] that GFP exhibits unusual targeting in the secretory pathway of endocrine cells. Rather than acting as a passive reporter molecule that is directly secreted from these cells [5], GFP is sorted to the regulated secretory pathway and stored in

secretory granules [6,7]. It was noted that GFP forms oligomers in the secretory pathway that could alter the intracellular transport of this protein [7]. In the present study we show that these oligomers are disulphide-linked and involve both cysteine residues of enhanced GFP (EGFP). Substitution of either cysteine residue results in the loss of higher-order oligomers of EGFP, while retaining the dimerization of EGFP in the secretory pathway. The dimeric protein is still sorted to the regulated secretory pathway in endocrine cells.

MATERIALS AND METHODS

Cell culture

Rat pituitary GH4C1 cells were maintained and transfected as previously described [8]. Mouse pituitary AtT-20 cells were cultured in Dulbecco's modified Eagle's medium containing equine serum (15 %, v/v), fetal bovine serum (5 %, v/v), penicillin (100 μ g/ml) and streptomycin (100 units/ml) at 37 °C in a humidified atmosphere with 5 % CO₂.

Transfection and mutagenesis

AtT-20 cells were detached with 0.25 % trypsin containing 0.03 % EDTA. The cells were then plated at 5×10^5 cells/well in 6-well culture plates and transfected as described for GH4C1 cells [8]. The cDNA for EGFP (Clontech, Heidelberg, Germany) was cloned into pcDNA3 (Invitrogen, Groningen, The Netherlands). Secreted forms of EGFP were produced as described [7] (see

Abbreviations used: GFP, green fluorescent protein; EGFP, enhanced GFP; FLAG, Asp-Tyr-Lys-Asp-Asp-Asp-Lys; Cf-EGFP, cytosolic FLAG epitope-containing EGFP; S-EGFP, secreted EGFP; Lf-EGFP, secreted long joining peptide-FLAG epitope-containing EGFP; KRH, Krebs-Ringer-Hepes; NEM, N-ethylmaleimide; IAA, iodoacetic acid; TCA, trichloroacetic acid.

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Figure 1 Schematic representation of cytosolic and secretory forms of EGFP and their mutants

The location of the cysteine residues (C), signal sequence (stippled), FLAG epitope (hatched), joining peptide (black) and EGFP (white) are shown. The mutations [Cys⁴⁹ → Ser (Cys49Ser), Cys⁷¹ → Ser (Cys71Ser) and Cys⁴⁹ → Ser/Cys⁷¹ → Ser (Cys49/71Ser)] are indicated.

Figure 1). The mutant forms of each protein were obtained by site-directed mutagenesis using the QuikChange™ mutagenesis kit (Stratagene, LaJolla, CA, U.S.A.). The oligonucleotides EGFP-CF (5'-CCCTGAAGTTCATCAGTACCACCGCAA-GC-3') and EGFP-CR (5'-GCTTGCCGGTGGTACTGAT-GAACTTCAGGG-3') were used to change Cys⁴⁹ → Ser (Cys49Ser), and C71SF (5'-CTGACCTACGGCGTGCAAAG-CTTCAGCCGCTACCCC-3') and C71SR (5'-GGGGTAGCG-GCTGAAGCTTTGCACGCCGTAGGTCAG-3') were used to change Cys⁷¹ → Ser (Cys71Ser). The double mutants (both cysteine residues changed to serine residues) were generated using cDNAs containing the Cys49Ser mutation as the template for a second round of mutagenesis to introduce the Cys71Ser mutation. All of the oligonucleotides used in mutagenesis were purchased from BioCorp (Montreal, Canada). The products of mutagenesis were verified by automated DNA sequencing.

Fluorescence studies

AtT-20 cells were transiently transfected to express EGFP and its mutants. After 2 days following transfection, live transfected cells were analysed with a Nikon photomicroscope (Fryer; Huntley, IL, U.S.A.) equipped for epifluorescence with FITC filters. Digital images were recorded with a Kodak MS digital camera (Rochester, NY, U.S.A.).

Preparation of cell extracts

Transiently transfected GH4C1 cells were treated with 5 mM sodium butyrate for 20 h prior to each experiment. The cells were incubated in Krebs–Ringer–Hepes (KRH) buffer [129 mM NaCl, 5 mM NaHCO₃, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1 mM CaCl₂, 1.2 mM MgCl₂, 2.8 mM glucose and 10 mM Hepes (pH 7.4)], scraped and lysed in 1 ml/well of lysis buffer (KRH buffer containing 0.3 % Tween-20, 5 mM EDTA and 1 mM PMSF). The cell extracts were precipitated with 10 % (v/v) trichloroacetic acid (TCA) to concentrate cellular proteins.

N-Ethylmaleimide (NEM) and iodoacetic acid (IAA) treatment

GH4C1 cells, transiently transfected with secreted forms of EGFP, were rinsed with KRH buffer and incubated for 2 h with KRH buffer containing 20 mM NEM or IAA. Medium was collected and the cells were scraped and lysed with lysis buffer containing 20 mM NEM or IAA. The medium samples and cell lysates were precipitated with 10 % (v/v) TCA.

Secretion experiments

Transiently transfected AtT-20 cells were washed twice with 1 ml of KRH buffer. For basal secretion, the cells were incubated for 1 h in 1 ml of KRH buffer at 37 °C. For stimulated secretion, the cells were incubated for 1 h with 1 ml of low-salt KRH buffer [79 mM NaCl, 5 mM NaHCO₃, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgCl₂, 2.8 mM glucose and 10 mM Hepes (pH 7.4)] containing 2.5 mM CaCl₂, 50 mM KCl and 10 μM forskolin. PMSF, EDTA and Tween-20 were added to basal- and stimulated-secretion media to final concentrations of 1 mM, 5 mM and 0.3 % respectively, and proteins in the media precipitated with 10 % (v/v) TCA.

Gel electrophoresis and immunoblotting

The acid-precipitated protein samples were resuspended in SDS/PAGE sample buffer in the absence (non-reducing) or presence (reducing) of 0.7 M 2-mercaptoethanol and heated for 10 min at 100 °C. The samples were analysed by SDS/PAGE on 10 % gels [9]. Immunoblotting was conducted essentially as described [8] using a polyclonal antiserum raised against GFP (1:10000 dilution; Clontech). The blots were visualized by chemiluminescent assay and exposed to Kodak Biomax or X-omat film.

RESULTS AND DISCUSSION

EGFP oligomerizes in the secretory pathway of endocrine cells [7]. To characterize this oligomerization, rat pituitary GH4C1 cells were transiently transfected with either cytosolic or secreted forms of EGFP. The cytosolic form of EGFP contained the Asp-Tyr-Lys-Asp-Asp-Asp-Lys (FLAG) epitope, which was in-

Figure 2 Ribbon model of EGFP

The structure of EGFP, as defined by 1.9 Å (where 1 Å = 0.1 nm) resolution X-ray diffraction analysis [11], was produced by RasMol [16] from data deposited at the Protein Data Bank [17]. The positions of Cys⁴⁹ and Cys⁷¹ are indicated (dark grey).

Figure 4 Alkylation does not affect oligomerization of S-EGFP

GH4C1 cells were transfected with S-EGFP. Cell lysates were prepared with 20 mM NEM or IAA and analysed by SDS/PAGE under non-reducing conditions, followed by immunoblotting with the GFP antiserum. Lane 1, S-EGFP in the absence of NEM or IAA (control); lane 2, S-EGFP in the presence of 20 mM NEM; lane 3, S-EGFP in the presence of 20 mM IAA. The molecular masses (in kDa) of monomeric (27), dimeric (60) and oligomeric (200) forms are shown. The blot shown is representative of two independent experiments performed in duplicate.

Figure 3 Immunoblot analysis of GFP in GH4C1-cell extracts under reducing and non-reducing conditions

GH4C1 cells were transfected with wild-type Cf-EGFP, S-EGFP and Lf-EGFP (**A**) or their Cys⁴⁹ → Ser (Cys49Ser) mutants (**B**). Cell lysates were prepared and samples were analysed by SDS/PAGE under non-reducing conditions followed by immunoblotting with GFP antiserum. (**A**) Lanes 1 and 4, Cf-EGFP; lanes 2 and 5, S-EGFP; lanes 3 and 6, Lf-EGFP. (**B**) Lanes 1 and 4, Cf-EGFP-Cys49Ser; lanes 2 and 5, S-EGFP-Cys49Ser; lanes 3 and 6, Lf-EGFP-Cys49Ser. Samples were run under non-reducing (lanes 1–3) and reducing (lanes 4–6) conditions. GFP indicates the position of monomeric GFP. The molecular masses (in kDa) of monomeric (27), dimeric (60) and oligomeric (200) forms are shown. The blots shown are representative of two independent experiments performed in duplicate.

tended to aid in its identification [7]; this protein was termed Cf-EGFP (cytosolic FLAG epitope-containing EGFP). Two secreted forms of EGFP were also tested. One of these, secreted EGFP (S-EGFP), contained the pre-proinsulin signal peptide and a short joining peptide (Asp-Pro-Pro-Val-Ala-Thr) fused in-frame to the N-terminus of EGFP. The other secreted form of EGFP, Lf-EGFP (secreted long joining peptide-FLAG epitope-containing EGFP), contained the same signal peptide as S-EGFP, the FLAG epitope used in Cf-EGFP and a longer form of the joining peptide (GTELGSTANGRQCAGILQSTVPRA-RDPPVAT; one-letter amino acid notation), followed by the complete sequence of EGFP (Figure 1). Note that the long joining peptide used in Lf-EGFP contains a cysteine residue, which is lacking from Cf-EGFP and S-EGFP. To study further the oligomerization and secretion of GFP in endocrine cells, we synthesized various mutant forms of Cf-EGFP and S-EGFP with one or both of the cysteine residues of native EGFP (Figure 2) mutated to serine residues. These mutants were designated Cys49Ser, Cys71Ser or Cys49/71Ser (Figure 1).

The cytosolic and secreted forms of wild-type EGFP were expressed in pituitary GH4C1 cells and analysed by SDS/PAGE under non-reducing and reducing conditions. Under non-reducing conditions, Cf-EGFP was present as a single 27 kDa band (Figure 3A, lane 1), whereas the secretory forms, S-EGFP and Lf-EGFP, exhibited multiple bands at 27, 60 and 200 kDa respectively (Figure 3A, lanes 2 and 3). These results indicate that EGFP undergoes oligomerization in the secretory pathway of GH4C1 cells, whereas it remains as a monomer in the cytosol. All three forms of EGFP exhibited a single 27 kDa band under reducing conditions (Figure 3A, lanes 4–6), suggesting that the oligomers of EGFP are due to disulphide interactions between EGFP molecules.

The monomeric form of S-EGFP only represented about 12 % of the total amount of EGFP detected by immunoblotting (Figure 4). A number of controls were considered to rule out the

Figure 5 Immunoblot analysis of GH4C1-cell extracts containing S-EGFP or its mutants

GH4C1 cells were transfected with S-EGFP or its mutants. Cell lysates were prepared and samples were analysed by SDS/PAGE under non-reducing conditions followed by immunoblotting with GFP antiserum. Lane 1, S-EGFP (S); lane 2, S-EGFP-Cys49Ser (S49); lane 3, S-EGFP-Cys71Ser (S71); lane 4, S-EGFP-Cys49/71Ser (S49&71); lane 5, cells transfected with vector alone (control). The molecular masses (in kDa) of monomeric (27), dimeric (60) and oligomeric (200) forms are shown. The blot shown is representative of two independent experiments performed in duplicate.

possibility that oligomerization was an artefact of the cell lysis procedure, acid precipitation or the presence of non-EGFP peptide sequences. To show that EGFP existed as an oligomer *in vivo* prior to cell lysis, the cells were treated with an alkylating agent (20 mM NEM or IAA) during the secretion experiment and preparation of cell lysates. This treatment did not alter the oligomerization of S-EGFP (Figure 4). Thus the oligomerization of S-EGFP was not an artefact of the sample preparation procedure. Furthermore, the highly charged FLAG peptide did not contribute to oligomerization. This peptide is present in Cf-EGFP and Lf-EGFP, but not in the S-EGFP. Thus oligomerization correlated with secretion, but not with the presence of the FLAG peptide. The Lf-EGFP band appeared on occasions as a doublet, but the molecular basis or significance of this doublet was not determined.

Since the oligomers of secreted forms of EGFP were due to disulphide interactions, the protein sequence of EGFP was analysed for the presence of cysteine residues, which could interact to form the disulphide linkages. EGFP consists of a single polypeptide chain of 238 amino acids arranged in an 11-stranded β -barrel wrapped around a single central helix (Figure 2) [10,11]. There are two cysteine residues, which are located at positions 49 and 71 of the EGFP molecule (Figure 2) [10,11]. Based on the crystal structure of EGFP, Cys⁴⁹ is predicted to be on the outside of the β -barrel, whereas Cys⁷¹ is located internally in the folded molecule (Figure 2). In Lf-EGFP, a third cysteine residue is present in the long joining peptide (Figure 1). Because Cys⁴⁹ is located on the exterior of the protein, where it would be available for disulphide interaction, we first substituted this residue for a serine residue in the secreted forms of EGFP to produce S-EGFP-Cys49Ser and Lf-EGFP-Cys49Ser. The amount of higher-molecular-mass oligomers formed by S-EGFP-Cys49Ser (Figure 3B, lane 2) was less than the oligomerization of non-mutated S-EGFP (Figure 3A, lane 2), demonstrating that Cys⁴⁹ was involved in oligomerization of S-EGFP. The finding that S-EGFP-Cys49Ser still formed disulphide-linked dimers

Figure 6 Fluorescence microscopy of Cf-EGFP and its mutants in AtT-20 cells

AtT-20 cells were transiently transfected with Cf-EGFP (C) and its mutants [Cf-EGFP-Cys49Ser (C49) and Cf-EGFP-Cys71Ser (C71)]. Control, cells transfected with vector alone. Live cells were observed by fluorescence microscopy. Results are representative of two independent experiments performed in duplicate.

(Figure 3B, lane 2) suggested that Cys⁷¹ was sufficient for dimerization of EGFP. Lf-EGFP-Cys49Ser, on the other hand, formed both dimers and higher-order oligomers (Figure 3B, lane 3). This finding suggested that both Cys⁷¹ and the cysteine residue present in the joining peptide of Lf-EGFP (Figure 1) were involved in oligomerization.

To test further the role of Cys⁷¹ in oligomerization, this residue was substituted with a serine residue alone (Cys71Ser) or in combination with Cys49Ser (Cys49/71Ser). The S-EGFP-Cys71Ser mutant formed mostly dimers (Figure 5, lane 3). Thus, in this mutant, Cys⁴⁹ is responsible for disulphide-bond formation. The absence of higher-molecular-mass oligomers confirmed that Cys⁷¹ must participate in disulphide oligomerization of S-EGFP. This was confirmed by the analysis of the double mutant S-EGFP-Cys49/71Ser, which existed as a monomer (Figure 5, lane 4). The finding that Cys⁷¹, a residue located internally in the folded EGFP molecule, participates in disulphide oligomerization suggests that oligomerization occurs prior to complete folding of EGFP.

In control experiments, Cys⁴⁹ and Cys⁷¹ of Cf-EGFP were mutated to produce Cf-EGFP-Cys49Ser and Cf-EGFP-Cys71Ser respectively. As expected, both proteins were monomeric, similar to the non-mutated Cf-EGFP (Figure 3B, lane 1). Moreover, mutations of Cys⁴⁹ and Cys⁷¹ did not affect the fluorescence of Cf-EGFP (Figure 6).

Neither wild-type nor mutated secreted forms of EGFP were reproducibly secreted from GH4C1 cells, although they were readily detected in the cell extracts. Since secretory forms of GFP are secreted from AtT-20 cells [6], we tested secretion of the EGFP constructs in this cell line. Both single cysteine residue mutants of S-EGFP (S-EGFP-Cys49 and S-EGFP-Cys71) were fluorescent and existed as dimers and monomers in AtT-20 cell extracts (results not shown). We have previously extensively characterized the sorting and localization of secretory forms of EGFP in secretory granules of endocrine cells [7]. To test whether

Figure 7 Stimulated secretion of S-EGFP mutants from AtT-20 cells

AtT-20 cells were transiently transfected with S-EGFP-Cys49Ser (S49) and S-EGFP-Cys71Ser (S71). The secretion media were analysed by SDS/PAGE under reducing conditions, followed by immunoblotting with the GFP antiserum. Lanes 1 and 3, basal secretion of S-EGFP-Cys49Ser and S-EGFP-Cys71Ser respectively; lanes 2 and 4, stimulated secretion of S-EGFP-Cys49Ser and S-EGFP-Cys71Ser respectively. The blot shown is representative of two independent experiments performed in duplicate.

the mutated forms of S-EGFP were also sorted to the secretory-granule pathway, the stimulated secretion (a measure of granule storage) was quantitated. Interestingly, both molecules (S-EGFP-Cys49 and S-EGFP-Cys71) exhibited stimulated secretion from AtT-20 cells (Figure 7), suggesting that they were sorted to the secretory granule pathway. Unfortunately, the double mutant of S-EGFP (S-EGFP-Cys49/71Ser) could not be detected in AtT-20 cells (results not shown). Hence, the role of dimerization on EGFP trafficking could not be determined. Nevertheless, although oligomerization of secretory proteins has been proposed to play a role in sorting to the regulated secretory pathway [12–14], the present results suggest that higher-order oligomerization is not necessary for sorting of EGFP to secretory granules in AtT-20 cells.

The data presented above show that the molecular forms of EGFP differ in the cytosol and secretory pathway of endocrine cells. The disulphide-linked oligomerization of EGFP reported in the present study presumably is different from the ionic-strength-dependent EGFP dimers formed in the crystal form [11]. The finding that EGFP forms disulphide-bonded oligomers in the secretory pathway suggests that this protein is not a passive reporter molecule in the secretory pathway of endocrine cells. Since oligomerization involves Cys⁷¹, which is located internally in the wild-type molecule (Figure 2), it is likely that EGFP undergoes oligomerization before complete folding in the secretory pathway of endocrine cells. It is not clear how oligomerization affects the fluorescence of EGFP. The wild-type and the mutated forms of cytosolic and secreted EGFP were fluorescent, although the secretory forms exhibit less fluorescence than the cytosolic forms (R. K. Jain and S.-U. Gorr, unpublished work). Thus, since multiple forms of EGFP (monomer, dimer and oligomer) exist in the secretory pathway, we cannot exclude that one or more of these forms are not fluorescent. Therefore the effect of oligomerization on fluorescent techniques, such as

fluorescence resonance energy transfer ('FRET'), in the secretory pathway must be assessed independently. Together with a recent report on aberrant trafficking of GFP in yeast [15], the present study raises concerns about the use of EGFP as a passive reporter molecule for studies on protein trafficking in the secretory pathway in general and, in particular, in endocrine cells.

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