AD_____

Award Number: DAMD17-01-1-0150

TITLE: The Non-Classical Secretion of Thioredoxin from Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Steven L. Chuck, M.D.

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center
Boston, Massachusetts 02215

REPORT DATE: June 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2002	Annual (1 Jun 01 - 31 May 02)

4. TITLE AND SUBTITLE
The Non-Classical Secretion of Thioredoxin from Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-01-1-0150

6. AUTHOR(S)
Steven L. Chuck, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Beth Israel Deaconess Medical Center
Boston, Massachusetts 02215

E-Mail: schuck@caregroup.harvard.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Thioredoxin is a small protein with a catalytic site for redox reactions that is involved in several physiologic and pathophysiologic processes in humans. A ubiquitous cytosolic protein, thioredoxin also is secreted by some types of cells including cells derived from leukemia, lung, colon, and breast cancers. Extracellular thioredoxin acts as an autocrine growth factor and can potentiate the action of other cytokines on these tumor cell lines. The novel pathway by which thioredoxin is secreted has not been elucidated. Our specific aims are to: identify the amino acid sequence within thioredoxin that directs its secretion from cells, and to identify the cellular gene products that secrete thioredoxin from breast cancer cells. We aim to develop a molecular view of the non-classical process by which thioredoxin is secreted. Armed with a thorough understanding of this export process, it may be possible to slow the growth of breast cancers in humans in an entirely new way by inhibiting the release of thioredoxin.

14. SUBJECT TERMS	15. NUMBER OF PAGES
breast cancer, non-classical protein secretion, thioredoxin	38
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Thioredoxin is a small protein with a catalytic site for redox reactions (1) that is involved in several physiologic (2-5) and pathophysiologic (6-9) processes in humans. A ubiquitous cytosolic protein, thioredoxin also is secreted by some types of cells including cells derived from leukemia (6), lung, colon, and breast cancers (10). Extracellular thioredoxin acts as an autocrine growth factor and can potentiate the action of other cytokines on these tumor cell lines. In MCF-7 breast cancer cells, extracellular thioredoxin stimulates more rapid growth of the cells in culture and greater colony formation in soft agar (9). Thioredoxin is not exported by the classical secretory pathway involving the endoplasmic reticulum, the Golgi apparatus, and vesicular transport, nor is the protein exported via multi-drug resistance channel proteins (10). The novel pathway by which thioredoxin is secreted has not been elucidated. Furthermore, it is not known what the structural features of thioredoxin are that direct it but not other cytosolic proteins to a non-classical secretory pathway. Proteins secreted through the classical pathway usually contain amino-terminal signal sequences of roughly 20-30 amino acids that target the nascent chain to the membrane of the endoplasmic reticulum (ER). These sequences open the protein-conducting channel in the membrane. Similarly, nuclear, mitochondrial, and chloroplast proteins are all directed by signals encoded within the preproteins. We hypothesize that distinct amino acids in thioredoxin direct its export through a non-classical secretory pathway comprised of specific cellular proteins. Our specific aims are to:

1. Identify the amino acid sequence within thioredoxin that directs its secretion from cells.
2. Identify and clone the cellular gene products that secrete thioredoxin from breast cancer cells.

We aim to develop a molecular view of the non-classical process by which thioredoxin is secreted. Armed with a thorough understanding of this export process, it may be possible to slow the growth of breast cancers in humans in an entirely new way by inhibiting the release of thioredoxin.

Body of Report

Aim 1. Identify the amino acid sequence within thioredoxin that directs its secretion from cells

Our plan was first to assay the secretion of truncated forms of thioredoxin to localize the region that directs secretion of the protein. Second, we planned to construct fusion proteins that could be secreted non-classically in which thioredoxin is fused in-frame with a normally cytosolic protein. We planned to fuse short lengths of thioredoxin that are candidates for a targeting signal to this passenger and then assay for secretion.

Truncations

We found that truncations of thioredoxin are often unstable and become degraded. Human thioredoxin contains 104 amino acids and migrates as a 12 kDa protein by SDS-PAGE. Using PCR, we engineered coding regions for truncated thioredoxin (Trx) proteins corresponding to Trx91, Trx85, Trx70, and Trx55 (the number denotes the number of amino-terminal amino acids in the construct—i.e., these are all carboxy-terminal truncations). We expressed these cDNAs in Chinese hamster ovary (CHO) cells via transient transfection using Lipofectamine 2000. The next day, the cells were starved in methionine-free medium for 30 minutes then labeled for 30 minutes with ^{35}S-methionine. The cells were then chased in complete medium for 0 or 6 hours. The media was collected and the cells were lysed in buffer containing 1% Triton X-100. The samples were subjected to immunoprecipitation using antibody against thioredoxin. We found that these C-terminal truncations were synthesized but over the 6-hour chase period, they were degraded intracellularly. Figure 1 shows the results for one truncation, Trx91, which is representative of our findings for the other truncations. In preliminary experiments, we have treated cells with 50 µg/ml ALLN, a protease and proteasome inhibitor, to try to prevent degradation but this chemical did not appear to prevent the disappearance of the thioredoxin truncations (data not shown).

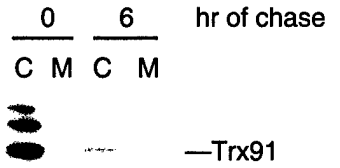

Figure 1. Trx91 is degraded and not secreted from CHO cells. CHO cells transiently transfected with Trx91 via Lipofectamine 2000 were pulse-labeled with ^{35}S-methionine for 30 minutes and chased in complete medium for 0 or 6 hours. Cell lysates (C) and media (M) were immunoprecipitated with antibody against thioredoxin and samples were displayed by SDS-PAGE and fluorography. See text for details.

Our plans are to repeat the labeling of these truncated thioredoxin proteins in the presence of ALLN or lactacystin (a more specific inhibitor of proteasomes), and to study these truncations in fusion proteins (as described below) which may stabilize them.

Fusion proteins

We have engineered and studied the secretion of several fusion proteins consisting of thioredoxin and a passenger domain. The passenger domain has ranged from as short as an epitope tag (e.g., FLAG or myc) to whole proteins. The ideal passenger domain will be long enough to permit detection by SDS-PAGE when fused to short sequences of thioredoxin. In addition, the passenger domain must be inert for non-classical secretion; that is, it must not be secreted non-classically on its own yet must be permissive for export when targeted to this pathway.

We added various epitope tags to the amino- or carboxy-terminal ends of thioredoxin and assayed secretion of the resulting proteins. The myc epitope appears to inhibit secretion of thioredoxin. A construct encoding thioredoxin tagged at its carboxy terminus with the myc epitope is poorly secreted (Figure 2). We engineered two FLAG-tagged thioredoxin proteins with the epitope at either the amino- or carboxy-terminus. The secretion of these two FLAG-tagged proteins varied. When the FLAG epitope is at the carboxy-terminus, the tagged thioredoxin is secreted in a proportion

similar to wild-type thioredoxin (Figure 3). However, when the FLAG epitope is at the amino-terminus, secretion is reduced. Therefore, we decided to use the FLAG epitope for tagging the carboxy-terminus of fusion proteins.

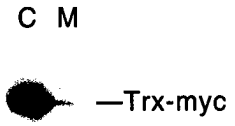

Figure 2. Myc-tagged thioredoxin is secreted poorly. The secretion of thioredoxin-myc was assessed as in Figure 1. Cell lysate (C) and medium (M) were immunoprecipitated with antibody against myc after a 6 hour chase period.

Figure 3. Secretion of FLAG-tagged thioredoxin. The secretion of thioredoxin-FLAG (Trx-FLAG) and FLAG-thioredoxin (FLAG-Trx) was assessed as in Figure 1. Cell lysate (C) and medium (M) were immunoprecipitated with antibody against FLAG after a 6 hour chase period.

The first fusion protein that we created consisted of thioredoxin and green fluorescent protein (GFP). This fusion protein (Trx-GFP) is secreted well from transiently transfected CHO cells. As a control, we expressed GFP and assayed its secretion from CHO cells. To our surprise, GFP is also secreted quite well from CHO cells. Over 50% of newly-synthesized GFP is secreted via a non-classical secretory pathway that is insensitive to brefeldin A. We characterized the non-classical secretion of GFP in CHO and other cells. Furthermore, we discovered that the secreted GFP is not fluorescent, indicating that it is not properly folded. Our data suggests that non-classical secretion can be a route for the secretion of improperly-folded proteins. These data have been submitted for publication (see Appendix).

We have examined several other proteins that could serve as a suitable passenger. We engineered thioredoxin upstream of glutathione S-transferase from *Schistosoma japonicum* and found that the fusion protein is secreted but analysis of secretion is complicated by an unexplained doublet (Figure 4). We used a domain from beta lactamase as a passenger downstream of thioredoxin but the resulting fusion protein is not secreted (Figure 5). We subcloned thioredoxin into an expression vector encoding a modified region from the FK506-binding protein (FKBP) that we obtained from Ariad. This vector, a part of the Argent homodimerization system, results in the expression of fusion proteins with a carboxy-terminal hemagglutinin (HA) epitope that can dimerize in the presence of a small chemical molecule that binds the FKBP domain. When we used antibody against thioredoxin to immunoprecipitate this protein, we found that thioredoxin-FKBP-HA is secreted well from transfected CHO cells (Figure 6). In contrast, this fusion protein is not immunoprecipitated from the medium when antibody against the HA epitope is used (Figure 6). The epitope is recognized on the fusion protein in the cells, and the migration of the protein in the cells and medium is identical when antibody against thioredoxin is used for detection. Thus, the fusion protein is altered without changing its apparent migration on SDS-PAGE so that the carboxy-terminal HA epitope is not recognized in the medium. One explanation is that the fusion protein is improperly folded so that the HA epitope is masked. This scenario is similar to the secretion of non-fluorescent GFP. In any case, it will be difficult to detect fusion

proteins based on this modified FKBP (Argent) domain since the HA epitope is not recognized in the medium. We also tried using the antibody against the FKBP domain but did not get very good detection of the protein (data not shown). Moreover, some molecules containing the Argent domain appear to be modified as evidenced by a slight change in mobility by SDS-PAGE and the appearance of a doublet. Therefore, although the Argent domain is secreted well when fused to thioredoxin, it is not a suitable passenger domain.

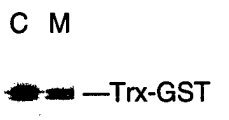

Figure 4. Thioredoxin-GST is secreted as a doublet. The secretion of thioredoxin-GST was assessed as in Figure 1. Cell lysate (C) and medium (M) were immunoprecipitated with antibody against thioredoxin after a 6 hour chase period.

Figure 5. Thioredoxin-beta-lactamase is not secreted from CHO cells. The secretion of thioredoxin-beta-lactamase was assessed as in Figure 1. Cell lysate (C) and medium (M) were immunoprecipitated with antibody against thioredoxin after a 6 hour chase period.

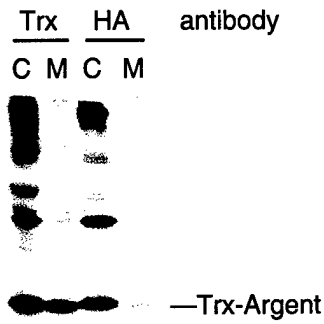

Figure 6. A thioredoxin-FKBP domain fusion protein is secreted well but the C-terminus is not recognized. The secretion of thioredoxin fused to the modified FKBP domain (Argent) of the Argent homodimerization kit (Ariad) was assessed as in Figure 1. Cell lysate (C) and medium (M) were immunoprecipitated with antibody against thioredoxin (Trx) or the HA epitope (HA) after a 6 hour chase period.

We are investigating two other fusion proteins currently. We have engineered a fusion protein consisting of thioredoxin followed in-frame by murine dihydrofolate reductase and a FLAG epitope (Trx-DHFR-FLAG). This construct was to be used in our second aim as described below. This fusion protein is secreted from transfected CHO cells (Figure 7). Similar to thioredoxin-Argent, it appears that Trx-DHFR-FLAG is only faintly detected in the medium when antibody against FLAG is used for immunoprecipitation (data not shown). Unlike Trx-Argent, however, Trx-DHFR-FLAG does not have a doublet. We are now determining whether the FLAG epitope can be recognized by western blotting. We are also assaying whether the control protein, DHFR-FLAG, is secreted. The second protein that we are investigating now consists of thioredoxin followed by a chitin binding domain (CBD) from *B. circulans* and the FLAG epitope (Trx-CBD-FLAG). The CBD is derived from the IMPACT intein-mediated

protein expression system (New England BioLabs). This small (~6 kDa) domain has the added benefit of binding chitin beads. Hence, proper folding of this domain is assayable by binding to chitin beads. We find that Trx-CBD-FLAG is secreted from CHO cells (Figure 8) but, similar to other non-classically secreted thioredoxin fusion proteins, it is not properly folded in the media. This conclusion is based on the binding of this fusion protein to chitin beads in cell lysates but not media (Figure 8). We are also determining whether this fusion protein can be detected in the media using antibody against FLAG by western blotting. If either of these proteins proves to be a suitable passenger domain, we will use it for further studies to localize a targeting sequence.

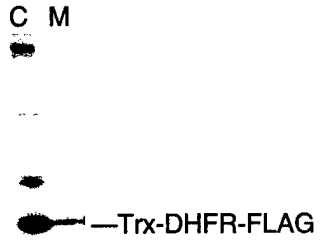

Figure 7. Thioredoxin-dihydrofolate reductase (DHFR)-FLAG is secreted from CHO cells. A fusion protein encoding thioredoxin (Trx) followed in-frame by murine DHFR was engineered and expressed in CHO cells. Secretion was assessed as in Figure 1. Cell lysate (C) and medium (M) were immunoprecipitated with antibody against thioredoxin after a 6 hour chase period.

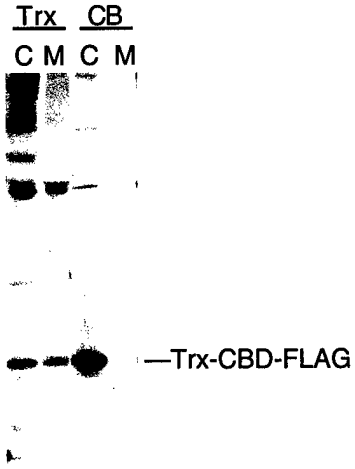

Figure 8. Thioredoxin-chitin-binding-domain-FLAG (Trx-CBD-FLAG) is secreted from CHO cells but not properly folded in the medium. The fusion protein Trx-CBD-FLAG was created via PCR and expressed in transiently-transfected CHO cells. Secretion was assessed as in Figure 1. Cell lysate (C) and medium (M) were immunoprecipitated with antibody against thioredoxin (Trx) or chitin beads (CB) after a 6 hour chase period.

We plan to finish evaluating DHFR and CBD as passenger domains and to examine the secretion of fusion proteins that encode truncated thioredoxin and a passenger domain.

Aim 2. Identify and clone the cellular gene products that secrete thioredoxin from breast cancer cells

Our plan was to crosslink a bulky fusion protein that is targeted for non-classical secretion but stuck in its passage. This approach has been used successfully to crosslink components of translocation channels (11-13). We are still investigating this approach. We tried using Trx-DHFR-FLAG since this construct is targeted for secretion. We treated cells expressing Trx-DHFR-FLAG with methotrexate which has been shown to bind to DHFR and induce a globular conformation. However, we found that this treatment did not impair secretion of Trx-DHFR-FLAG. Perhaps methotrexate is unable to bind to improperly-folded Trx-DHFR-FLAG molecules that are then secreted. We are looking at the crosslinking pattern using Trx-beta-lactamase or another fusion protein that is not secreted. It is possible that such a protein is targeted to but unable to pass through a translocation channel.

We also have begun using yeast for studies of non-classical secretion. The sec18 strain has a temperature-sensitive mutation that blocks classical secretion at the non-permissive temperature. We are determining whether this strain secretes human thioredoxin at the non-permissive temperature. If so, we will perform mutagenesis and screen for loss of secretion of thioredoxin or a thioredoxin fusion protein.

Key Research Accomplishments

* Tested several thioredoxin fusion proteins for targeting and secretion

* Discovered and characterized the non-classical export of improperly-folded green fluorescent protein

* Determined that improperly-folded thioredoxin fusion proteins can be substrates for non-classical secretion

Reportable Outcomes

Tanudji M, Hevi S, and Chuck SL. Improperly-folded green fluorescent protein is secreted via a non-classical pathway. *Submitted*

Conclusions

The non-classical secretion of thioredoxin from breast cancer cells has been associated with enhanced tumorigenesis. We have studied the non-classical secretion of several thioredoxin fusion proteins with the aim of defining the targeting signal and export machinery for this pathway. Although many such fusion proteins are secreted, several of the secreted forms appear to be improperly folded at their carboxy-terminus as indicated by lack of recognition by antibodies or substrate-coated beads for the C-

terminal domains. However, in every case, the thioredoxin domains are properly folded and recognized by specific antibody. At present, our knowledge of this pathway cannot be developed as a medical product. We are continuing our studies, however, with the goal of completing our aims.

References

1. Holmgren A. Redox regulation by thioredoxin and thioredoxin reductase. *Biofactors* 11: 63-4, 2000.
2. Hirota K., M. Murata, Y. Sachi, H. Nakamura, J. Takeuchi, K. Mori and J. Yodoi. Distinct roles of thioredoxin in the cytoplasm and in the nucleus. A two-step mechanism of redox regulation of transcription factor NF- kappaB. *J Biol Chem* 274: 27891-7, 1999.
3. Saitoh M., H. Nishitoh, M. Fujii, K. Takeda, K. Tobiume, Y. Sawada, M. Kawabata, K. Miyazono and H. Ichijo. Mammalian thioredoxin is a direct inhibitor of apoptosis signal- regulating kinase (ASK) 1. *Embo J* 17: 2596-606, 1998.
4. Balcewicz-Sablinska M. K., E. E. Wollman, R. Gorti and D. S. Silberstein. Human eosinophil cytotoxicity-enhancing factor. II. Multiple forms synthesized by U937 cells and their relationship to thioredoxin/adult T cell leukemia-derived factor. *J Immunol* 147: 2170-4, 1991.
5. Di Trapani G., C. Orosco, A. Perkins and F. Clarke. Isolation from human placental extracts of a preparation possessing 'early pregnancy factor' activity and identification of the polypeptide components. *Hum Reprod* 6: 450-7, 1991.
6. Tagaya Y., M. Okada, K. Sugie, T. Kasahara, N. Kondo, J. Hamuro, K. Matsushima, C. A. Dinarello and J. Yodoi. IL-2 receptor(p55)/Tac-inducing factor. Purification and characterization of adult T cell leukemia-derived factor. *J Immunol* 140: 2614-20, 1988.
7. Wakasugi N., Y. Tagaya, H. Wakasugi, A. Mitsui, M. Maeda, J. Yodoi and T. Tursz. Adult T-cell leukemia-derived factor/thioredoxin, produced by both human T-lymphotropic virus type I- and Epstein-Barr virus-transformed lymphocytes, acts as an autocrine growth factor and synergizes with interleukin 1 and interleukin 2. *Proc Natl Acad Sci U S A* 87: 8282-6, 1990.
8. Wang J., M. Kobayashi, K. Sakurada, M. Imamura, T. Moriuchi and M. Hosokawa. Possible roles of an adult T-cell leukemia (ATL)-derived factor/thioredoxin in the drug resistance of ATL to adriamycin. *Blood* 89: 2480-7, 1997.
9. Gallegos A., J. R. Gasdaska, C. W. Taylor, G. D. Painemurrieta, D. Goodman, P. Y. Gasdaska, M. Berggren, M. M. Briehl and G. Powis. Transfection With Human Thioredoxin Increases Cell Proliferation and a Dominant-Negative Mutant Thioredoxin Reverses the Transformed Phenotype of Human Breast Cancer Cells. *Cancer Research* 56: 5765-5770, 1996.
10. Rubartelli A., A. Bajetto, G. Allavena, E. Wollman and R. Sitia. Secretion of thioredoxin by normal and neoplastic cells through a leaderless secretory pathway. *J Biol Chem* 267: 24161-4, 1992.
11. Mothes W., S. Prehn and T. A. Rapoport. Systematic probing of the environment of a translocating secretory protein during translocation through the ER membrane. *Embo J* 13: 3973-82, 1994.

12. Kouranov A. and D. J. Schnell. Analysis of the interactions of preproteins with the import machinery over the course of protein import into chloroplasts. *J Cell Biol* 139: 1677-85, 1997.
13. Eilers M. and G. Schatz. Binding of a specific ligand inhibits import of a purified precursor protein into mitochondria. *Nature* 322: 228-232, 1986.

Appendix

1. Tanudji M, Hevi S, and Chuck SL. Improperly-folded green fluorescent protein is secreted via a non-classical pathway. *Submitted*

Improperly-folded green fluorescent protein is secreted via a non-classical pathway

Marcel Tanudji, Sarah Hevi and Steven L. Chuck†

From the Molecular Medicine Unit, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215

†To whom correspondence should be addressed: Steven Chuck, Molecular Medicine Unit, Beth Israel Deaconess Medical Center, RW663, 330 Brookline Avenue, Boston, MA 02215

Office: 617-667-1625
Fax: 617-667-8040
Email: schuck@caregroup.harvard.edu

Short title: Non-classical secretion of GFP

Keywords: Green fluorescent protein, Brefeldin A, non-classical protein secretion, protein trafficking.

Summary
The green fluorescent protein is a cytosolic protein frequently used as a molecular tag to study protein localization in intact cells. We discovered that this protein is secreted into the medium by several but not all cell lines through a non-classical secretory pathway that is insensitive to brefeldin A. Green fluorescent protein is secreted efficiently by Chinese hamster ovary cells with 60% of synthesized proteins secreted over 8 hours. This pathway is sensitive to changes in temperature but not to factors in serum or chemicals known to affect other non-classical protein secretion pathways. Fluorescence is observed in cells expressing green fluorescent protein indicating that some of the protein must be fully folded in the cytosol. However, secreted green fluorescent protein is not fluorescent and therefore not folded properly. Furthermore, cellular fluorescence does not change over 6 hours while a significant proportion of green fluorescent protein is secreted. Thus, nascent green fluorescent protein either is folded correctly or incorrectly, and the improperly-folded molecules can be exported. Non-classical secretion might be a route by which cells remove an excess of improperly-folded, cytosolic proteins.

Footnotes
[1] Abbreviations used: GFP, green fluorescent protein; ER, endoplasmic reticulum; BFA, brefeldin A; FGF, fibroblast growth factor; IL-1, interleukin-1; CHO, Chinese hamster ovary; TXSWB, Triton X-100 salt wash buffer; PMSF, phenylmethylsulfonyl fluoride; LDH, lactate dehydrogenase.
[2] MT, SH, SLC, unpublished data.

Introduction

Green fluorescent protein (GFP)[1] is the molecule that provides bioluminescence in coelenterates such as the jelly fish *Aequorea victoria*. GFP folds into a barrel-shaped structure made up of beta-strands with the chromophore located in the center (Tsien, 1998). Fully-folded GFP requires no cofactors or substrate to be functional, and excitation of the chromophore at a specific wavelength results in the emission of fluorescent light (Chalfie, 1995). Furthermore, the fluorescence is stable, species-independent, and can be observed non-invasively in any cell expressing GFP (Kain et al., 1995). These convenient properties of GFP have been exploited as a tool to study various cellular processes (Cinelli et al., 2000).

Due to its intrinsic fluorescence, GFP is commonly used as a molecular tag to study intracellular protein trafficking. GFP appears to be an inert and stable molecule localized in the cytosol. Furthermore, it is small enough (29 kDa) to be used as a passenger protein for fusion constructs. In these fusion proteins, it is assumed that GFP does not contain intrinsic targeting information. To date, various proteins have been tagged with the GFP molecule to study localization and sorting in compartments such as the mitochondria, nucleus, chloroplasts, endoplasmic reticulum (ER), Golgi apparatus, and plasma membrane (Chatterjee and Stochaj, 1996; Choy et al., 1999; Lee et al., 2001; Niwa et al., 1999). GFP-tagged proteins can be observed and their movement tracked in intact cells simply by looking for fluorescence at the targeted location.

Protein secretion in mammalian cells generally occurs via the classical secretory pathway that traverses the ER and Golgi apparatus. Secreted proteins contain a signal sequence with all the necessary information that is required to target them for secretion. A protein that is not normally secreted can be targeted for secretion by attaching a signal sequence (Simon et al., 1987). The classical secretory pathway is completely inhibited by brefeldin A (BFA) which causes reversible resorption of the Golgi apparatus back into the ER (Doms et al., 1989).

Over the past decade, it has been shown that several proteins are secreted independently of the ER-Golgi pathway. For example, basic fibroblast growth factor (FGF), interleukin (IL)-1β, HIV-tat, galectin-3, and thioredoxin are secreted in a non-classical manner (Chang et al., 1997; Mehul and Hughes, 1997; Mignatti et al., 1992; Rubartelli et al., 1992). These proteins do not display any signal sequence or protein motif known to act as a signal for export (for a review see (Muesch et al., 1990; Rubartelli and Sitia, 1997). Furthermore, their secretion is not inhibited by the addition of BFA. The pathways used for the export of these proteins are still poorly understood and multiple pathways for non-classical protein secretion may exist in cells.

In our laboratory, we wished to use GFP as a passenger in a fusion construct to study non-classical secretion by Chinese hamster ovary (CHO) cells. As a control, we expressed GFP alone. To our surprise, most of the GFP expressed in CHO cells is secreted into the medium in a non-classical manner. Many but not all types of cells examined also secrete GFP. We characterized the kinetics, sensitivity to temperature and factors in serum, and effects of known specific chemical inhibitors on the export of GFP from transfected CHO cells. In contrast to cytosolic GFP, secreted GFP does not fluoresce indicating that it is not properly folded.

Materials and Methods

Reagents

Trans ^{35}S-label (>70% L-methionine; >1000 Ci/mmol) was purchased from ICN Biomedicals. Mouse monoclonal antibodies specific for GFP used for immunoprecipitation and western blotting were purchased from QBiogene. Monoclonal antibodies against the myc epitope were purified by protein G affinity chromatography from media of MYC 1-9E10.2 cells (Evan et al., 1984). Polyclonal antibodies against the FLAG epitope (DYKDDDDK) were purchased from ProSci. Protein A-agarose, Lipofectamine 2000, OptiMEM, fetal calf serum, and Glutamax I were purchased from Gibco/Life Technologies. The pQBI25-fN3 plasmid, encoding modified GFP with a single emission (474 nm) and excitation peak (509 nm) is from QBiogene. BFA and cycloheximide was purchased from Sigma.

Plasmid constructions

The GFP construct used in this study was created as follows. Using the pQBI25-fN3 plasmid as a template, the forward (encoding BamHI site, translation initiation consensus sequence and inititation methionine: CGGGATCCGCCACCATGGCTAGCAAAGGAGAAGAACTCTTC) and reverse (encoding a stop codon and XbaI site: CGTCTAGATAGTCAATCGATGTTGTAGAG) primers were used to amplify the GFP coding region with Platinum pfx DNA polymerase (Gibco/ Life Technologies). The PCR product was digested, isolated, and subcloned into pcDNA3 at the BamHI and XbaI site of the multiple cloning site. A similar method was used to create the preprolactin-myc construct using the forward (CGGGATCCGCCACCATGG ACAGCAAAGGTTCGTCGC) and reverse (ATAGTTTAGCGGCCGCGCAGTTGT TGTTGTAGATGATTCTGC) primers to amplify the bovine preprolactin gene from the plasmid pT7-Bprl. The PCR product was isolated and cloned between the BamHI and NotI sites of pcDNA3 with a pre-existing myc epitope coding sequence following the Not I site. The murine dihydrofolate reductase (DHFR)-FLAG mammalian expression construct was created as follows. Using pDS5/3 plasmid ((Rassow et al., 1989); a kind gift of Dr Elzbieta Glaser) as a template, the DHFR gene was amplified using forward (CGGGATCCGCCACCATGGAATTCATGGTTCGACCATTG) and reverse (CCTCTAGATTACTTGTCGTCATCGTCCTTGTAGTCGTCTTTCTTCTCGTAGACTTCAAAC) primers. The PCR product was digested with BamHI and NotI and subcloned into pcDNA3 with a pre-exisiting FLAG epitope coding sequence following the NotI site. The same approach was used to create the *Schistosoma japonicum* glutathione S-transferase mammalian expression construct except that pGEX-3 plasmid (Pharmacia) was used as a template and the different forward (GGAATTCTATGTCCCCTATACTAGGTTATTGG) and reverse primers (CCTCTAGATCACGATAAATTCCGGGGATCCC) were used for amplification. All constructs were verified by DNA sequencing.

Cell culture and transient transfection

CHO, A375, COS, NIH 3T3, HEK-293, MCF-7 and HT-29 cells were maintained at 37°C/5% CO2 in culture medium (DMEM , 10% fetal bovine serum, 1% penicillin/streptomycin and 1% non-essential amino acids). One day prior to transfection, the cells were trypsinized and counted. One million cells were used to seed 60-mm culture dishes and left overnight to form >90% confluent monolayers. Confluent layers of cells were transiently transfected with Lipofectamine 2000 according to the manufacturer's instructions. Briefly, 3 μg of plasmid DNA and 15 μl of Lipofectamine 2000 was used for each transfection. Each of the components were resuspended in

450 µl of OPTI-MEM, incubated for 5 min at room temperature, mixed and incubated for a further 23 min before the addition of 4 ml of OPTI-MEM. The DNA-Lipofectamine 2000 suspension was then added to the cells (prewashed twice in PBS to remove residual serum proteins) and incubated at 37°C overnight. The next day, transfected cells were washed twice in PBS to remove residual DNA-Lipofectamine 2000 complexes prior to metabolic labeling.

Metabolic labeling with ^{35}S-methionine

The cells were incubated in 1 ml starving medium (DMEM minus methionine and cysteine, 5% fetal bovine serum, 1% Glutamax I, 1% non-essential amino acids, 1X penicillin and streptomycin) for 30 min at 37°C in 5% CO2. Metabolic labeling was carried out by the addition of 100 µCi of ^{35}S-methionine and incubating at 37°C for 30 min. At the end of the labeling period, the cells were washed in 1 ml of chase medium (DMEM, 1X penicillin and streptomycin, 1% non-essential amino acids) and chased in 800 µl of chase medium for the indicated amount of time. Some cells were treated with 1 µg/ml BFA during the starvation, labeling, and chase periods.

Immunoprecipitation, SDS-PAGE and phosphor-imaging

For each pulse-chase assay, the medium was collected and cells on the dish were lysed by the addition of 1 ml of 1X Triton X-100 salt wash buffer (TXSWB; 1% Triton X-100, 100 mM Tris-HCl pH 8, 100 mM NaCl, 10 mM EDTA) in the presence of 2 mM PMSF. Both cell lysate and medium were centrifuged at 16,000 x *g* for 15 min to remove cell debris and the supernatant was transferred to a fresh 1.5 ml tube. At this point, cleared cell lysate and medium were used for spectrofluorometric measurements or LDH assays (see below). For immunoprecipitation, 200 µl of 5X TXSWB (5% Triton X-100, 500 mM Tris-HCl pH 8, 500 mM NaCl, 50 mM EDTA) and 2mM PMSF were added to the cleared medium. Either 1 µl of anti-GFP antibodies or 6 µl of anti-myc antibodies bound to protein G beads was added to each 1 ml of the cell lysate or medium. The samples were mixed by inversion and incubated at 4°C for 1 hr before addition of 10 µl of a suspension of protein G-agarose beads. The samples were rotated at 4°C overnight, washed twice in 1X TXSWB and twice in wash buffer (100 mM Tris-HCl pH8, 100 mM NaCl), and resuspended in 1X SDS-PAGE buffer with 500 mM DTT. The samples were incubated at 37°C for 30 min prior to boiling and loaded on a 15% polyacrylamide gel. At the completion of electrophoresis, the gels were destained for 30 min, soaked in 1 M sodium salicylate for 30 min, and dried. The gels were exposed to x-ray film for viewing or a phosphor-imaging screen (Molecular Dynamics) for quantitation.

Lactate dehydrogenase assay

Assays for lactate dehydrogenase (LDH) were carried out on 5 µl samples of cell lysate and medium after the chase period to assess cell lysis. The TOX7 LDH assay kit (Sigma) was used according to the manufacturer's instructions.

Spectrofluorometric measurements

Two hundred microliters of cleared cell lysate and medium were aliquoted into 96-well plates with blank 1X TXSWB and fresh chase medium as the respective controls. The plates were scanned on a Cytofluorescent 2350™ Fluorescence Measurement System (Millipore) with filter sets covering the GFP excitation and emission wavelengths (Ex: 485±20 nm; Em: 530±25 nm).

Results

Transiently-transfected CHO cells secrete GFP via a non-classical mechanism

We wished to use GFP as a passenger protein to assay for non-classical secretion. As a control, we expressed GFP to investigate its cellular location in CHO cells. ^{35}S-methionine-labelled GFP was detected as an immunoreactive 29 kDa protein in cell lysate (Fig. 1, lane 5) which is absent in the lysate or medium of untransfected cells (Fig.1A, lanes 1 and 2). To our surprise, however, we saw GFP in the medium after 6 hr of chase (Fig.1A, lane 6). We investigated whether GFP was secreted via the classical secretory pathway. CHO cells were transiently transfected with a plasmid encoding GFP then pulse labeled and chased in the presence of 1 μg/ml BFA, an effective inhibitor of the classical secretory pathway. GFP was detected in similar amounts in both the cell lysate and medium in the absence or presence of BFA (Fig. 1A, Lanes 7 and 8 vs Lanes 5 and 6). To verify the effectiveness of BFA on the classical ER-Golgi pathway, CHO cells were transfected with bovine preprolactin. This protein was synthesized and secreted (Fig. 1, lanes 9 and 10), and its export was inhibited by 1 μg/ml of BFA (Fig. 1A, lanes 11 and 12). Thus, this concentration of BFA was sufficient to inhibit ER-Golgi dependent protein secretion. We confirmed that the GFP detected in the medium was secreted and not released by cell lysis by carrying out an assay for LDH. In each sample, less than 5% of total cellular LDH was detected after 6 hr chase period (data not shown). To investigate whether other cytosolic proteins of similar size are non-specifically exported after transient transfection, we transfected CHO cells with genes coding for murine dihydrofolate reductase (mDHFR) and *Schistosoma japonicum* glutathione S-transferase. However, over-expression of these proteins in CHO cells did not result in their secretion into the medium (Fig. 1B). Recent reports have shown that the expression of GFP in mammalian cells can be toxic (Liu et al., 1999). It is possible that GFP expression in CHO cells causes these cells to be more susceptible to lysis. Our assay for LDH does not discriminate lysis from transfected or untransfected cells. Therefore, we co-transfected CHO cells with the plasmid constructs coding for GFP and mDHFR proteins. If GFP expression is toxic and results in cell lysis, then GFP and mDHFR proteins would be expected to be present in the medium in equivalent proportions after 6 hrs of chase. When both proteins are co-expressed in CHO cells (Fig. 1C, lane 1), however, only GFP is detected in the medium after 6 hr of chase (Fig. 1C, lane 2). This result indicates that GFP is specifically secreted by CHO cells and is not present in the medium because of lysis of the transfected cells.

We also assessed the ability of other commonly used cell lines to secrete GFP. NIH 3T3 and HEK293 cells transiently transfected with a plasmid encoding GFP secrete the protein non-classically over 6 hr in the presence of BFA (Fig. 2, lanes 1 to 4). In contrast, COS cells secrete GFP poorly over 6 hr (Fig. 2, lane 5 vs 6). Certain cancer cells export thioredoxin via a non-classical pathway (Berggren et al., 1996). Therefore, we investigated if thioredoxin-secreting cancer cells, such as A375, MCF-7 and HT-29, can also efficiently secrete GFP. We observed that GFP is generally secreted by these cancer cells in the presence of BFA (Fig. 2, lanes 7-10). One exception, however, are HT-29 cells which do not secrete GFP (Fig. 2A, lanes 11 and 12) yet export thioredoxin well (Berggren et al., 1996). For each cell line tested, no more than 5% of total cellular LDH was detected in the medium indicating that very little cell lysis had occured. Overall, compared to CHO cells, less GFP is secreted from the cell lines studied in Fig. 2. Taken together, these results indicate that GFP can be secreted by some cells via a brefeldin A-resistant pathway that is particularly active in CHO cells.

Kinetics of GFP secretion by transiently transfected CHO cells

We next investigated the kinetics of GFP secretion. CHO cells transfected with GFP were labeled with ^{35}S-methionine and chased at 37°C in medium lacking serum for 2, 4, 6 and 8 hr. After separation by SDS-PAGE, the protein bands were quantitated using a phosphorimager and the relative secretion for each time point was calculated (Fig. 3). The secretion of GFP is a slow but steady process with up to 60% being secreted into the medium after 8 hr of chase. The increase in secretion is not due to cell lysis since an assay for LDH showed that less than 5% of total cellular LDH was detected in the medium after the maximum chase time of 8 hr (data not shown). Since an adequate proportion of GFP is secreted into the medium after 6 hr of chase (Fig. 3, lanes 5 and 6), we chose to use this time point for subsequent assays of GFP secretion.

GFP secretion is altered by temperature

Facilitated protein secretion is generally a temperature-dependent process. For example, secretion of proteins via the classical ER-Golgi secretory pathway is affected by alterations in temperature (Saraste et al., 1986). On the other hand, passive diffusion through a pore is not affected by a change in temperature (Melchior and Gerace, 1995). We investigated the temperature sensitivity of GFP secretion to gain insight into whether it is a facilitated or passive process. CHO cells transfected with GFP were starved, labeled at 37°C, and then incubated in serum-free chase medium at 25°C, 37°C and 42°C for 6 hr. As expected, about 45% of GFP synthesized in the cells is secreted after 6 hr at 37°C (Fig. 4, lanes 3 and 4). Lowering the chase temperature down to 25°C drastically inhibits GFP secretion (Fig. 4, lanes 1 and 2). On the other hand, increasing the chase temperature to 42°C increased the secretion of GFP modestly by 10% (Fig. 4, lanes 5 and 6). However, assays of LDH indicated an increase of cell lysis at this temperature (15% of total cellular LDH detected in the medium) that can account entirely for the slight increase in GFP detected in the medium.

GFP secretion is not affected by the amount of serum in the media

Some non-classical secretory pathways are sensitive to factors in serum. For example, the secretion of HIV-tat and IL-1β is inversely proportional to the amount of serum in the medium (Chang et al., 1997; Rubartelli et al., 1990). The secretion of thioredoxin is also reduced with increasing amounts of serum in the medium[2]. We investigated whether factors present in fetal bovine serum also affect the secretion of GFP. CHO cells transfected with the GFP plasmid were starved, labeled with ^{35}S-methionine, and chased in medium containing 0%, 5%, 10% and 20% fetal bovine serum (Fig. 5). Quantitation using a phosphorimager revealed no significant differences in the proportion of secreted GFP despite increasing concentrations of serum.

Various chemicals do not affect GFP secretion

The results above suggest that GFP is secreted by a non-classical secretory pathway in CHO cells. To investigate whether the pathway for GFP secretion might be the same as that used by other non-classically secreted proteins, we tested the effect of various chemical inhibitors on the secretion of GFP (Table 1) (Hughes, 1999; Mignatti et al., 1992; Rubartelli et al., 1990). Although some of these compounds alter the non-classical secretion of other proteins, none appeared to have a significant effect on the secretion of GFP. For example, methylamine, which has been shown to inhibit the non-classical secretion of thioredoxin by blast cells (Rubartelli et al., 1992), has no effect in the secretion of GFP. Compounds such as monensin and A23187, which have been shown to enhance the release of thioredoxin, IL-1β and galectin-3 (Mehul and

Hughes, 1997; Rubartelli and Sitia, 1991), also have no effect on GFP secretion. These data suggest that GFP may use a different non-classical secretory pathway.

Secreted GFP is not associated with externalized membrane vesicles

At least one non-classically secreted protein, galectin-3, is secreted via plasma membrane blebbing (Mehul and Hughes, 1997). We studied whether GFP likewise is secreted in externalized membrane vesicles. Such a mechanism could enable the post-translational export of fully-folded cytosolic proteins from the cell. CHO cells transfected with the GFP plasmid were subjected to a 6 hr pulse-chase assay. At the end of the chase, the medium was clarified by low-speed centrifugation followed by high-speed centrifugation to pellet membrane vesicles in the medium (Mehul and Hughes, 1997). However, after immunoprecipitation, no GFP protein was detected in the pellet recovered after the high-speed centrifugation (Fig. 6, lane 4). Indeed, all of the GFP still remained in the supernatant fraction (Fig. 6, lane 3) and in similar amounts to that detected in the medium after low-speed centrifugation (Fig. 6, lane 2). This result suggests that GFP is not secreted into the medium via membrane blebbing.

Improperly-folded GFP is secreted

GFP is a stable molecule that is resistant to spontaneous unfolding or degradation in the cell (Bokman and Ward, 1981). By fluorescence microscopy, GFP-transfected CHO cells are brightly fluorescent (data not shown) indicating that some of the GFP is folded properly in these cells. However, a loosely-folded or unfolded conformation is generally required for protein translocation through channels (Schatz and Dobberstein, 1996). Since the structure of fluorescent GFP is a bulky beta barrel, it would seem likely that the protein must be at least partly unfolded for export through a channel. We investigated whether GFP is secreted as an unfolded molecule by assessing its fluorescence in the medium. First, we assessed the quantities of unlabeled GFP secreted into the media relative to the cell, by carrying out a western blot analysis. Untransfected and transfected CHO cells were placed in serum-free medium for 6 hr. At the end of the incubation, the medium and cell lysate were harvested, aliqouts were saved for spectrofluorometric assays, and the rest of the samples were subjected to immunoprecipitation, separated by SDS-PAGE, and immunoblotted with anti-GFP antibodies (Fig. 7A). As expected no GFP was detected in untransfected CHO cells and medium (Fig. 7A, lanes 1 and 2). GFP was detected in comparable amounts in the cells and medium of transfected cells by immunoblotting (Fig. 7A, lanes 3 and 4). We then carried out the spectrofluorometric assay on aliquots of the same samples used for the western blot. Very little auto-fluorescence was detected in the cell lysate or medium of untransfected CHO cells (Fig 7B, CHO). On the other hand, the cell lysate of GFP-transfected CHO cells showed a large increase in fluorescence in agreement with the fluorescence seen by microscopy (Fig. 7B, CHO+GFP). However, no change in fluorescence was seen in the medium despite significant secretion of GFP (Fig. 7A).

We ruled out two possible artifacts that might account for the lack of fluorescence in the media. First, to test whether the lack of GFP fluorescence was due to quenching by components in the medium, we carried out a 1:1 dilution of cell lysate with fresh medium or phosphate buffered saline (PBS). The relative fluorescence detected in the sample diluted with fresh medium was half that of the undiluted sample and similar in value to the fluorescence measured when PBS was used as a diluent indicating that the medium does not quench GFP fluorescence (Fig. 7B, CHO+GFP lysate diluted). Second, we examined whether the lack of fluorescence in the medium results from GFP becoming non-fluorescent after export into the medium. CHO cells

expressing GFP were lysed in fresh medium containing 1% Triton X-100. After clarification by centrifugation, the medium containing released cytosolic GFP was incubated at 37°C for 0, 3 and 6 hr and the fluorescence was measured at each time point. Fluorescence remained virtually constant over 6 hrs of incubation in medium (Fig. 7C). Thus, the lack of fluorescence in the medium is not due to inactivation or unfolding of functional GFP once it is exported. From these results, we conclude that secreted GFP is not folded properly and therefore not fluorescent.

Two different forms of GFP are present in CHO cells but only the improperly-folded form is secreted.

The secretion of unfolded, non-fluorescent GFP could result from either of two possible models. In the first model, all cytosolic GFP is fluorescent, and to be exported, it must first be unfolded prior to or during secretion. This implies that the reduction in intracellular GFP would be reflected by a similar reduction in fluorescence under conditions where no new GFP molecules are synthesized. In the second model, GFP is present in the cell in two different forms as either a properly folded, fluorescent molecule that is not secreted, or as an unfolded protein that can be secreted. In this model, the fluorescence from cellular GFP would remain constant despite a decrease in the total cytosolic GFP.

We investigated these two models of GFP secretion. CHO cells transiently transfected with GFP were placed in medium supplemented with cycloheximide to a final concentration of 100 μM which is sufficient to inhibit protein synthesis (data not shown). The cells were incubated at 37°C in this medium for 0 or 6 hr, and the media and cell lysates were harvested. Aliquots were saved for spectrofluorometric assays, and the rest of the samples were analyzed by western blotting (Fig. 8A). As expected, initially all of the GFP synthesized is present in the cell (Fig. 8A, lane 1) and not in the medium (Fig. 8A, lane 2). This data correlated with the spectrofluorometric result which showed that all of the fluorescence is associated in the cell lysate and none is detected in the medium (Fig. 8B, 0 hr chase, cell lysate vs medium). After 6 hr of chase, the immunoblot showed that approximately one third of the synthesized GFP is present in the medium (Fig. 8A, lanes 4) with a similar reduction of GFP in the cell lysate (Fig. 8A, lane 3). The relative amounts of GFP secreted into the medium is similar to the secretion observed in the absence of cycloheximide (Fig. 7A) indicating that the protein synthesis inhibitor did not affect the non-classical export of GFP. Assays for LDH confirmed that the GFP dectected in the medium is not due to lysis caused by cycloheximide (data not shown). However, the fluorescence in the cell lysate after 6 hr is virtually identical to that at the outset (Fig. 8B, 6 hr , cell lysate) while no significant fluorescence is detected in the medium despite the significant secretion of GFP protein (Fig. 8B, 6 hr, medium). This data strongly supports the second model. Thus, GFP exists in two different pools in transiently transfected CHO cells--the properly folded, fluorescent molecules, and unfolded, non-fluorescent proteins--and only the second form can be secreted (Fig. 9).

Discussion

We demonstrate that GFP is secreted by a variety of cells via a non-classical secretory pathway. Significant secretion was observed from transiently-transfected CHO cells with over half of the labeled GFP exported to the medium. The secretion of GFP is sensitive to low temperature but not factors in serum. Commonly used cell lines and several cancer cell lines also demonstrate the ability to secrete GFP albeit with less efficiency. GFP secreted from transiently-transfected CHO cells does not fluoresce indicating that the improperly-folded form is secreted via this non-classical pathway.

It is surprising that GFP is secreted from transiently transfected cells. GFP is a cytosolic protein with no known targeting signal. The intrinsic targeting of GFP for non-classical export might not have been observed previously because the molecule is often targeted to a specific location in cells. To our knowledge, this is the first report of GFP secretion by mammallian cells. In yeast, GFP can be localized to the vacuole presumably due to a cryptic targeting signal (Kunze et al., 1999). Previously assumed to be an inert molecule, GFP can have other unanticipated effects on cells. For example, transgenic mice that express GFP in the heart develop cardiomyopathy (Huang et al., 2000). Furthermore, prolonged overexpression of GFP triggers apoptosis in cells (Liu et al., 1999). Many visual applications, such as fluorescence microscopy, may not be sensitive to minor toxic effects that go unnoticed (Schmitz and Bereiter-Hahn, 2001). Taken together, the unanticipated effects and secretion of GFP raise doubts regarding the inertness of this molecule.

Our experiments indicate that the secretion of GFP is not simply due to cell lysis caused by either a cytotoxic effect of GFP or liposome-mediated transfection (Fig. 1C). GFP but not mDHFR is secreted from CHO cells that are co-transfected with cDNA encoding both proteins. If GFP or liposome-mediated transfection caused cell lysis, both expressed proteins would be found in the media. Furthermore, CHO cells, when transfected using calcium phosphate instead of a lipsosome formulation, also secrete GFP (data not shown). Finally, only non-fluorescent GFP is secreted (Fig. 7). If released by cell lysis, both fluorescent and non-fluorescent GFP would be expected in the media. Hence, the export process can discriminate between properly- and improperly-folded forms. This specificity indicates that GFP is not released due to cell lysis.

Although GFP can diffuse through the nuclear pore into the nucleus (Chatterjee and Stochaj, 1996; Chatterjee and Stochaj, 1998), we believe that the export of GFP is not due to non-selective diffusion for several reasons. First, lowering the chase temperature to 25°C significantly inhibits GFP secretion (Fig. 4). Low temperatures are known to inhibit a variety of facilitated translocation processes but not diffusion (Melchior and Gerace, 1995). Second, GFP is poorly secreted from certain cell lines such as COS and HT-29 (Fig. 2, lanes 6 and 12). This result suggests that certain cellular factors or machinery are required for GFP export and that export of GFP is not due to non-specific diffusion of a molten globule isoform through the plasma membrane as has been suggested for other proteins (Bychkova et al., 1988). Such translocation machinery has been characterized in the ER, mitochondria, and chloroplasts (Chen et al., 2000; Johnson and van Waes, 1999; Rehling et al., 2001). Finally, the pathway used by GFP for export appears to be selective since other heterologous, cytosolic proteins overexpressed in CHO cells are not secreted (Fig. 1B and C). This selectivity implies that a specific region of the GFP protein may behave as a cryptic targeting signal as has been demonstrated in yeast (Kunze et al., 1999). Thus, the non-classical secretion of GFP is selective and not universal among mammalian cells.

Very little is known about non-classical secretory pathways in eukaryotes. Several proteins such as basic FGF, IL-1β, HIV-Tat, thioredoxin, and galectin-3 are secreted in a non-classical manner (Cleves, 1997; Rubartelli and Sitia, 1997). However, only a few of these pathways have been studied and their exact mechanisms have not been identified. For example, the Na+/K+ ion channel has been implicated in the secretion of basic FGF (Florkiewicz et al., 1998), whereas an ATP-binding cassette (ABC) transporter appears to be involved in the export of IL-1β (Andrei et al., 1999). Galectin-3 is secreted in vesicles from membrane blebbing; this pathway appears to be capable of post-translational export of fully folded proteins (Hughes, 1999). We failed to detect any GFP associated with vesicles. Furthermore, factors in serum and various chemical inhibitors that affect the secretion of other non-classically secreted proteins have no apparent effect on GFP secretion. Our data suggest the existence of another non-classical export pathway in eukaryotic cells capable of secreting unfolded GFP.

Protein translocation across membranes generally requires proteins to be in a loosely folded or unfolded conformation. One exception is the prokaryotic twin arginine translocase (Tat) which is capable of exporting fully-folded proteins--including GFP--into the periplasm (Berks et al., 2000; Thomas et al., 2001). In our studies, the GFP secreted from CHO cells is not fluorescent and therefore not properly folded. Perhaps the GFP is loosely folded or unfolded as a condition for secretion. Once secreted, GFP does not fold into a fluorescent conformation because of the absence of chaperone proteins (Feilmeier et al., 2000; Sacchetti et al., 2001). In contrast, GFP secreted via the ER-Golgi pathway is fluorescent in the medium because proper folding is maintained during secretion (Laukkanen et al., 1996).

GFP is fluorescent in the cytoplasm of transfected cells indicating that some of the protein is properly folded. The GFP in the medium, however, is not fluorescent (Fig. 7B). Thus, to be secreted by CHO cells, GFP must be in an unfolded conformation. Two models could account for this unfolding. In the first model, fully folded GFP must be unfolded prior to or during its export. In the second model, not all of the GFP synthesized in the cytosol is folded to the fluorescent conformation. This pool of nascent, unfolded GFP might remain associated with chaperones in the cytosol to maintain a loosely folded or unfolded conformation. This scenario is much more likely for post-translationally translocated proteins (Schatz and Dobberstein, 1996). The prolonged but efficient secretion of GFP despite treatment with cycloheximide (Fig. 8) suggests that export occurs post-translationally. We discovered that the pool of intracellular, fluorescent GFP did not decrease over 6 hr while a substantial fraction was secreted into the medium (Fig. 8). This result indicates that the second model is correct: two separate pools of nascent GFP —one folded and fluorescent; the other unfolded, non-fluorescent, and able to be secreted—exist in cells (see model in Fig. 9). Being a protein of jellyfish origin, GFP does not always fold properly at 37°C (Ogawa et al., 1995; Patterson et al., 1997; Siemering et al., 1996; Tsien, 1998). Thus, the two pools of GFP in CHO cells likely arise from an inefficiency in proper folding even though we used an engineered form of GFP. Our data does not indicate the relative size of these two pools, although the high proportion that is secreted over several hours (Fig. 3) suggests that the pool of unfolded, non-fluorescent GFP consists of at least half the newly-synthesized GFP. The pool of unfolded GFP does not appear to localize in aggresomes formed by aggregates of misfolded proteins in the cytosol (Garcia-Mata et al., 1999; Garcia-Mata et al., 2002). We observed a diffuse pattern of GFP fluorescence throughout the cytosol as is typically observed (Garcia-Mata et al., 1999; Kain et al., 1995). By immunofluorescence microscopy, an identical pattern of GFP staining is seen in the cytosol of these cells (data not shown). Thus, in cells, two overlapping pools of GFP can be found throughout the cytosol.

Since GFP is not present endogenously in CHO cells, the physiological substrate for this non-classical pathway remains to be identified. To be active extracellularly, proteins secreted via this pathway must be able to attain the proper conformation outside the cell in the absence of chaperone proteins. Alternatively, this non-classical secretory pathway might be a means to dispose of improperly-folded proteins from the cytosol.

This work was supported by grant DAMD17-01-1-0150 from the United States Army.

References

Andrei, C., Dazzi, C., Lotti, L., Torrisi, M. R., Chimini, G. and Rubartelli, A. (1999). The secretory route of the leaderless protein interleukin 1beta involves exocytosis of endolysosome-related vesicles. *Mol. Biol. Cell* **10**, 1463-1475.

Berggren, M., Gallegos, A., Gasdaska, J. R., Gasdaska, P. Y., Warneke, J. and Powis, G. (1996). Thioredoxin and Thioredoxin Reductase Gene Expression in Human Tumors and Cell Lines, and the Effects of Serum Stimulation and Hypoxia. *Anticancer Research* **16**, 3459-3466.

Berks, B. C., Sargent, F. and Palmer, T. (2000). The Tat protein export pathway. *Mol. Microbiol.* **35**, 260-274.

Bokman, S. H. and Ward, W. W. (1981). Renaturation of Aequorea gree-fluorescent protein. *Biochem. Biophys. Res. Commun.* **101**, 1372-1380.

Bychkova, V. E., Pain, R. H. and Ptitsyn, O. B. (1988). The 'molten globule' state is involved in the translocation of proteins across membranes?. *FEBS Lett.* **238**, 231-234.

Chalfie, M. (1995). Green fluorescent protein. *Photochem. Photobiol.* **62**, 651-656.

Chang, H. C., Samaniego, F., Nair, B. C., Buonaguro, L. and Ensoli, B. (1997). HIV-1 Tat protein exits from cells via a leaderless secretory pathway and binds to extracellular matrix-associated heparan sulfate proteoglycans through its basic region. *Aids* **11**, 1421-1431.

Chatterjee, S. and Stochaj, U. (1996). Monitoring nuclear transport in HeLa cells using the green fluorescent protein. *Biotechniques* **21**, 62-63.

Chatterjee, S. and Stochaj, U. (1998). Diffusion of proteins across the nuclear envelope of HeLa cells. *Biotechniques* **24**, 668-674.

Chen, K., Chen, X. and Schnell, D. J. (2000). Mechanism of protein import across the chloroplast envelope. *Biochem. Soc. Trans.* **28**, 485-491.

Choy, E., Chiu, V. K., Silletti, J., Feoktistov, M., Morimoto, T., Michaelson, D., Ivanov, I. E. and Philips, M. R. (1999). Endomembrane trafficking of ras: the CAAX motif targets proteins to the ER and Golgi. *Cell* **98**, 69-80.

Cinelli, R. A., Ferrari, A., Pellegrini, V., Tyagi, M., Giacca, M. and Beltram, F. (2000). The enhanced green fluorescent protein as a tool for the analysis of protein dynamics and localization: local fluorescence study at the single-molecule level. *Photochem. Photobiol.* **71**, 771-776.

Cleves, A. E. (1997). Protein transports: the nonclassical ins and outs. *Curr. Biol.* **7**, R318-320.

Doms, R. W., Russ, G. and Yewdell, J. W. (1989). Brefeldin A redistributes resident and itinerant Golgi proteins to the endoplasmic reticulum. *J. Cell Biol.* **109**, 61-72.

Evan, G. I., Lewis, G. K. and Bishop, J. M. (1984). Isolation of monoclonal antibodies specific for products of avian oncogene myb. *Mol. Cell Biol.* **4**, 2843-2850.

Feilmeier, B. J., Iseminger, G., Schroeder, D., Webber, H. and Phillips, G. J. (2000). Green fluorescent protein functions as a reporter for protein localization in Escherichia coli. *J. Bacteriol.* **182**, 4068-4076.

Florkiewicz, R. Z., Anchin, J. and Baird, A. (1998). The inhibition of fibroblast growth factor-2 export by cardenolides implies a novel function for the catalytic subunit of Na+,K+-ATPase. *J. Biol. Chem.* **273**, 544-551.

Garcia-Mata, R., Bebok, Z., Sorscher, E. J. and Sztul, E. S. (1999). Characterization and dynamics of aggresome formation by a cytosolic GFP-chimera. *J Cell Biol* **146**, 1239-1254.

Garcia-Mata, R., Gao, Y. S. and Sztul, E. (2002). Hassles with Taking Out the Garbage: Aggravating Aggresomes. *Traffic* **3**, 388-396.

Huang, W. Y., Aramburu, J., Douglas, P. S. and Izumo, S. (2000). Transgenic expression of green fluorescence protein can cause dilated cardiomyopathy. *Nat. Med.* **6**, 482-483.
Hughes, R. C. (1999). Secretion of the galectin family of mammalian carbohydrate-binding proteins. *Biochim. Biophys. Acta.* **1473**, 172-185.
Johnson, A. E. and van Waes, M. A. (1999). The translocon: a dynamic gateway at the ER membrane. *Annu. Rev. Cell Dev. Biol.* **15**, 799-842.
Kain, S. R., Adams, M., Kondepudi, A., Yang, T. T., Ward, W. W. and Kitts, P. (1995). Green fluorescent protein as a reporter of gene expression and protein localization. *Biotechniques* **19**, 650-655.
Kunze, I. I., Hensel, G., Adler, K., Bernard, J., Neubohn, B., Nilsson, C., Stoltenburg, R., Kohlwein, S. D. and Kunze, G. (1999). The green fluorescent protein targets secretory proteins to the yeast vacuole. *Biochim. Biophys. Acta.* **1410**, 287-298.
Laukkanen, M. L., Oker-Blom, C. and Keinanen, K. (1996). Secretion of green fluorescent protein from recombinant baculovirus-infected insect cells. *Biochem. Biophys. Res. Commun.* **226**, 755-761.
Lee, Y. J., Kim, D. H., Kim, Y. W. and Hwang, I. (2001). Identification of a Signal That Distinguishes between the Chloroplast Outer Envelope Membrane and the Endomembrane System in Vivo. *Plant Cell* **13**, 2175-2190.
Liu, H. S., Jan, M. S., Chou, C. K., Chen, P. H. and Ke, N. J. (1999). Is green fluorescent protein toxic to the living cells?. *Biochem. Biophys. Res. Commun.* **260**, 712-717.
Mehul, B. and Hughes, R. C. (1997). Plasma membrane targetting, vesicular budding and release of galectin 3 from the cytoplasm of mammalian cells during secretion. *J. Cell Sci.* **110**, 1169-1178.
Melchior, F. and Gerace, L. (1995). Mechanisms of nuclear protein import. *Curr. Opin. Cell Biol.* **7**, 310-318.
Mignatti, P., Morimoto, T. and Rifkin, D. B. (1992). Basic fibroblast growth factor, a protein devoid of secretory signal sequence, is released by cells via a pathway independent of the endoplasmic reticulum-Golgi complex. *J. Cell Physiol.* **151**, 81-93.
Muesch, A., Hartmann, E., Rohde, K., Rubartelli, A., Sitia, R. and Rapoport, T. A. (1990). A novel pathway for secretory proteins?. *Trends Biochem. Sci.* **15**, 86-88.
Niwa, Y., Hirano, T., Yoshimoto, K., Shimizu, M. and Kobayashi, H. (1999). Non-invasive quantitative detection and applications of non-toxic, S65T-type green fluorescent protein in living plants. *Plant J.* **18**, 455-463.
Ogawa, H., Inouye, S., Tsuji, F. I., Yasuda, K. and Umesono, K. (1995). Localization, trafficking, and temperature-dependence of the Aequorea green fluorescent protein in cultured vertebrate cells. *Proc Natl Acad Sci U S A* **92**, 11899-11903.
Patterson, G. H., Knobel, S. M., Sharif, W. D., Kain, S. R. and Piston, D. W. (1997). Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy. *Biophys J* **73**, 2782-2790.
Rassow, J., Guiard, B., Wienhues, U., Herzog, V., Hartl, F. U. and Neupert, W. (1989). Translocation arrest by reversible folding of a precursor protein imported into mitochondria. A means to quantitate translocation contact sites. *J Cell Biol* **109**, 1421-1428.
Rehling, P., Wiedemann, N., Pfanner, N. and Truscott, K. N. (2001). The mitochondrial import machinery for preproteins. *Crit. Rev. Biochem. Mol. Biol.* **36**, 291-336.

Rubartelli, A., Bajetto, A., Allavena, G., Wollman, E. and Sitia, R. (1992). Secretion of thioredoxin by normal and neoplastic cells through a leaderless secretory pathway. *J. Biol. Chem.* **267**, 24161-24164.
Rubartelli, A., Cozzolino, F., Talio, M. and Sitia, R. (1990). A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence. *EMBO J.* **9**, 1503-1510.
Rubartelli, A. and Sitia, R. (1991). Interleukin 1 beta and thioredoxin are secreted through a novel pathway of secretion. *Biochem. Soc. Trans.* **19**, 255-259.
Rubartelli, A. and Sitia, R. (1997). Secretion of mammalian proteins that lack a signal sequence. In *Unusual Secretory Pathways: From Bacteria to Man.*, (ed. K. Kuchler, A. Rubartelli and B. Hollan), pp. 87-114. New York: R.G. Landes Company.
Sacchetti, A., Cappetti, V., Marra, P., Dell'Arciprete, R., El Sewedy, T., Crescenzi, C. and Alberti, S. (2001). Green Fluorescent Protein variants fold differentially in prokaryotic and eukaryotic cells. *J Cell Biochem* **81**, 117-128.
Saraste, J., Palade, G. E. and Farquhar, M. G. (1986). Temperature-sensitive steps in the transport of secretory proteins through the Golgi complex in exocrine pancreatic cells. *Proc. Natl. Acad. Sci. U S A.* **83**, 6425-6429.
Schatz, G. and Dobberstein, B. (1996). Common principles of protein translocation across membranes. *Science* **271**, 1519-1526.
Schmitz, H. D. and Bereiter-Hahn, J. (2001). GFP associates with microfilaments in fixed cells. *Histochem. Cell Biol.* **116**, 89-94.
Siemering, K. R., Golbik, R., Sever, R. and Haseloff, J. (1996). Mutations that suppress the thermosensitivity of green fluorescent protein. *Curr Biol* **6**, 1653-1663.
Simon, K., Perara, E. and Lingappa, V. R. (1987). Translocation of globin fusion proteins across the endoplasmic reticulum membrane in Xenopus laevis oocytes. *J. Cell Biol.* **104**, 1165-1172.
Thomas, J. D., Daniel, R. A., Errington, J. and Robinson, C. (2001). Export of active green fluorescent protein to the periplasm by the twin- arginine translocase (Tat) pathway in Escherichia coli. *Mol. Microbiol.* **39**, 47-53.
Tsien, R. Y. (1998). The green fluorescent protein. *Annu. Rev. Biochem.* **67**, 509-544.

Figure legends

Table 1. Many chemical compounds--including inhibitors of protein export--do not affect GFP secretion.
CHO cells transfected with a plasmid encoding GFP were starved, labeled with ^{35}S-methionine, and chased for 6 hr in the presence of various chemicals at the concentrations indicated in the table. See text for details.

Figure 1. GFP is secreted via a non-classical pathway in CHO cells. A, Untransfected CHO cells (Ctrl) and cells transiently transfected with either GFP (GFP) or preprolactin-myc (PPL) were labeled with ^{35}S-methionine and chased for 6 hr. The cell lysates (C) and media (M) were subjected to immunoprecipitation with either antibodies against GFP (lanes 1-8) or myc (lanes 9-12), and the washed immunoprecipitates were displayed by SDS-PAGE and fluorography. Some cells (+BFA) were treated with 1 μg/ml brefeldin A for one hour prior to labeling (lanes 3-4, 7-8, and 11-12). "% Sec." denotes the percentage of secretion of GFP into the media as determined by a phosphorimager. **B**, CHO cells transfected with plasmids encoding either mouse dihydrofolate reductase (mDHFR) or *Schistosoma japonicum* glutathione S-transferase (GST) both tagged with the FLAG epitope. The cells were labeled with ^{35}S-methionine and chased in serum-free medium. The cell lysate (C) and media (M) were immunoprecipitated with anti-FLAG antibodies and processed as above. **C**, Plasmids coding for mDHFR and GFP were used to co-transfect CHO cells. The cells were labeled, chased and processed as described in B.

Figure 2. GFP is secreted by various cells including cancer cells.
NIH 3T3, HEK-293, COS, MCF-7, A375 and HT-29 were transiently transfected with a plasmid encoding GFP. The day after transfection, the cells were labeled with ^{35}S-methionine and chased in the presence of BFA. After 6 hr, the cell lysates (C) and media (M) were harvested and immunoprecipitated with anti-GFP antibodies. The washed immunoprecipitates were resolved by SDS-PAGE followed by fluorography. "% Sec." denotes the percentage of GFP secreted by transfected cells.

Figure 3. Kinetics of GFP secretion from CHO cells.
CHO cells transiently expressing GFP were starved, metabolically labeled with ^{35}S-methionine, and chased for various lengths of time as indicated. At each time point, the cell lysate (C) and medium (M) were harvested and immunoprecipitated with anti-GFP antibodies and analyzed by SDS-PAGE followed by fluorography. The graph depicts the percentage of secretion at each time point.

Figure 4. GFP secretion is a temperature-dependent process.
CHO cells transiently expressing GFP were starved, labeled with ^{35}S-methionine at 37°C, and chased for 6 hr at various temperatures as indicated above each set of lanes. The cell lysates (C) and media (M) were harvested and immunoprecipitated with anti-GFP antibodies. "% Sec." denotes the percentage of secretion.

Figure 5. GFP secretion is independent of factors present in serum.
CHO cells transiently expressing GFP were starved, labeled with ^{35}S-methionine, and chased for 6 hr in the presence of various concentrations of serum as indicated. The cell lysates (C) and media (M) were harvested and immunoprecipitated with anti-GFP antibodies. "% Sec." denotes the percentage of secretion.

Figure 6. Externalized membrane vesicles are not involved in GFP secretion.
CHO cells transiently transfected with the plasmid encoding GFP were starved, labeled with ^{35}S-methionine, and chased for 6 hr. At the end of the chase period, the cell lysate (C) and medium (M) were collected, centrifuged at 2,000 x *g* for 30 min, and each divided into two equal aliquots. One aliquot was immunoprecipitated with anti-GFP antibodies. The second aliquot of the medium was subjected to further centrifugation at 90,000 x *g* for 2 hr to pellet vesicles. The supernatant (S) was collected and the pellet (P) was resuspended in 1X TXSWB buffer and immunoprecipitated with anti-GFP antibodies. The samples were separated by SDS-PAGE and analyzed by autoradiography.

Figure 7. Non-classically secreted GFP is not fluorescent.
Untransfected (CHO) and GFP-transfected CHO cells (CHO+GFP) were washed and incubated in fresh medium for 6 hr. **A**, The cell lysate (C) and media (M) were immunoprecipitated with anti-GFP antibodies, separated by SDS-PAGE, and immunoblotted with anti-GFP antibodies. **B**, The fluorescence was measured from 200 µl aliquots of the cell lysate and media harvested as above. The graph shows the relative fluorescence units (RFU) per ml for the samples. The fluorescence also was measured from lysate of GFP-expressing cells that was diluted 1:1 in fresh medium or PBS (CHO+GFP lysate diluted). Each bar on the graph represents the average value derived from at least 3 independent samples. **C**, CHO cells expressing GFP were lysed in medium supplemented with 1X Triton X-100. The fluorescence was measured from 200 µl aliquots of the medium after 0, 3 and 6 hr incubation at 37°C. The values are plotted as RFU per ml.

Figure 8. Two different forms of GFP are present in CHO cells but only the improperly-folded form is secreted.
CHO cells expressing GFP (CHO+GFP) were incubated at 37°C in the presence of 100 µM cycloheximide for 0 or 6 hr. **A**, The cell lysate (C) and media (M) were harvested at the end of the incubation, immunoprecipitated, and immunoblotted with anti-GFP antibodies. **B**, Spectrofluorometric measurements of 200 µl aliquots of the cell lysate and media harvested as above are plotted as RFU per ml. Each bar on the graph represents the average value derived from 5 independent samples.

Figure 9. Two-pool model of GFP secretion.
See text for details.

Compound	Molecular Target	Conc. tested
Brefeldin A	Golgi apparatus	2μg/ml
Methylamine	exocytosis	10mM
Monensin	Na^+ ionophore	100μm
Verapamil	Ca^{2+} ion channel	100μM
Phloridzin	glucose transporters	200μM
Staurosporine	protein kinase(broad)	100nM
H-89	protein kinase(broad)	10μM
A23187	Ca^{2+} ionophore	1μM
Thapsigargin	Ca^{2+} release blocker	1μM
EDTA	ion chelator	1mM

Table 1

Non-classical secretion of GFP

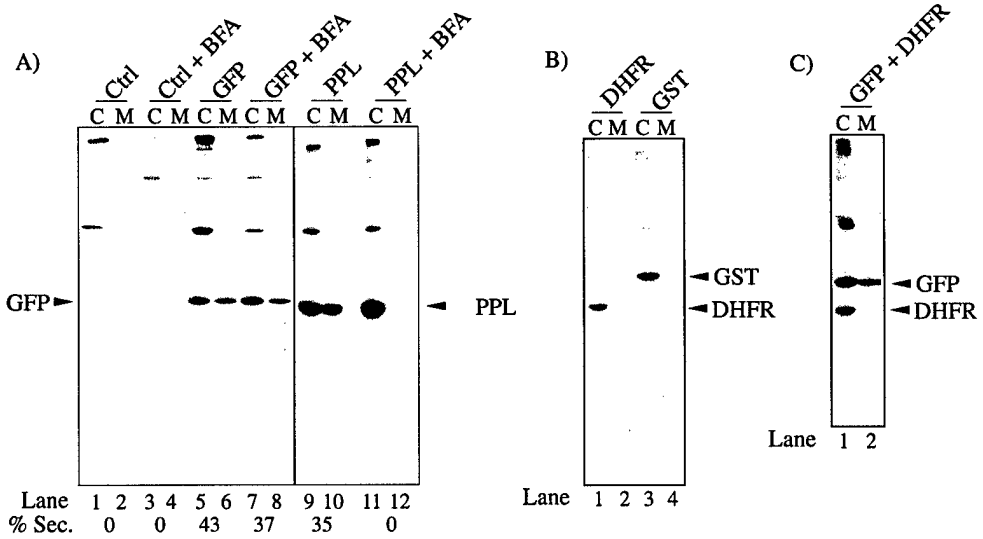

Fig. 1

Non-classical secretion of GFP

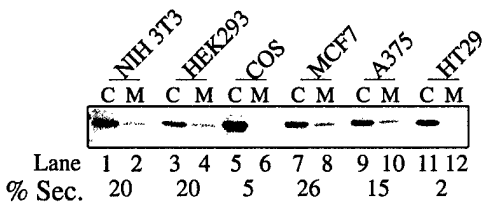

Fig. 2

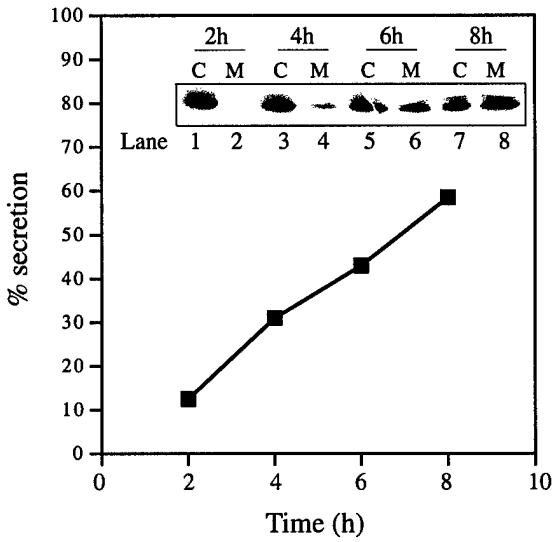
2h
4h
6h
8h
C M C M C M C M
Lane 1 2 3 4 5 6 7 8
% secretion
Time (h)
0
10
20
30
40
50
60
70
80
90
100
0
2
4
6
8
10

Fig. 3

Non-classical secretion of GFP

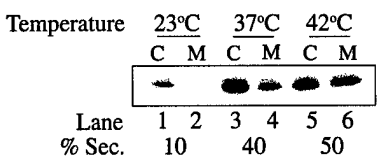

Fig. 4

Non-classical secretion of GFP

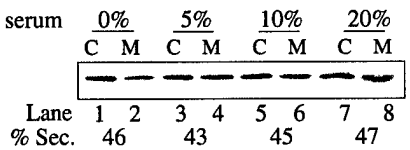

Fig. 5

Non-classical secretion of GFP

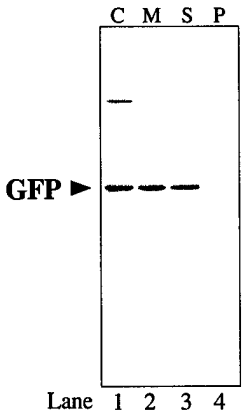

Fig. 6

Non-classical secretion of GFP

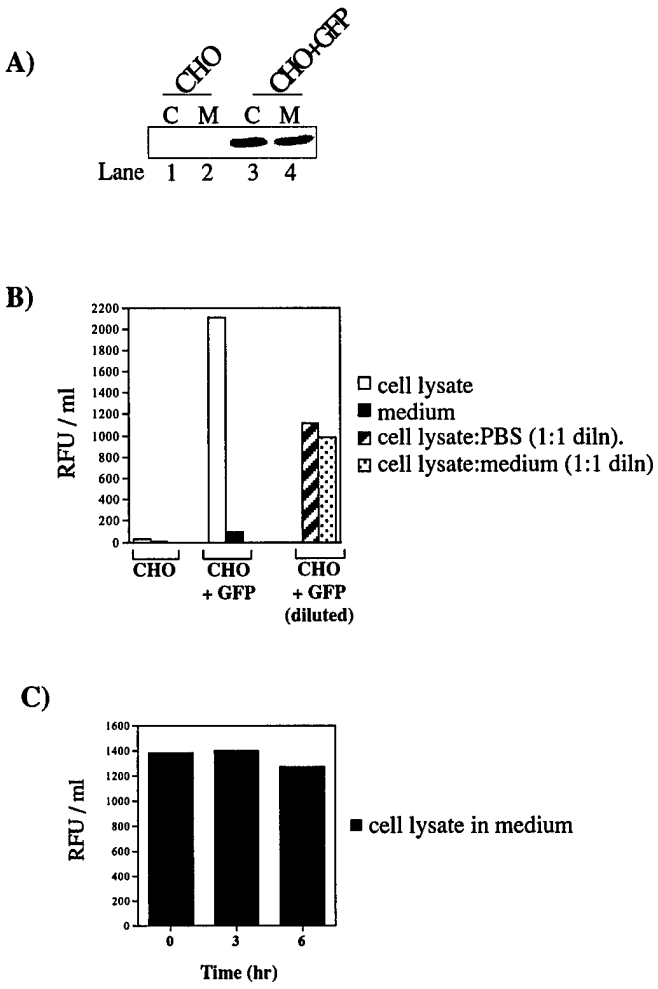

Fig. 7

Non-classical secretion of GFP

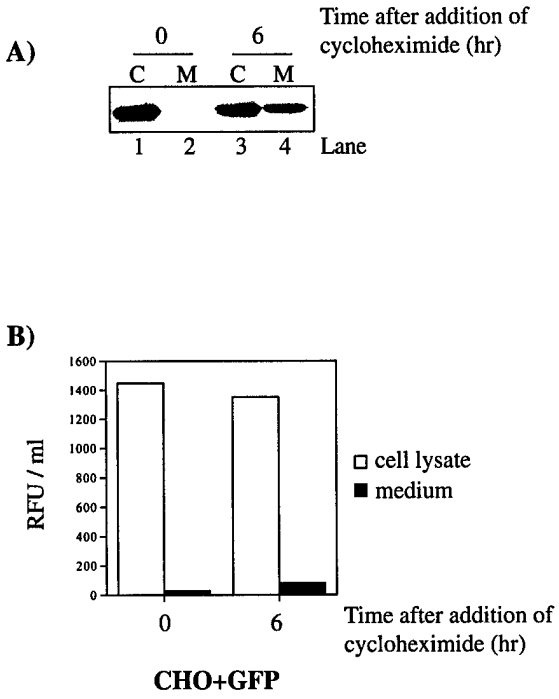

Fig. 8

Non-classical secretion of GFP

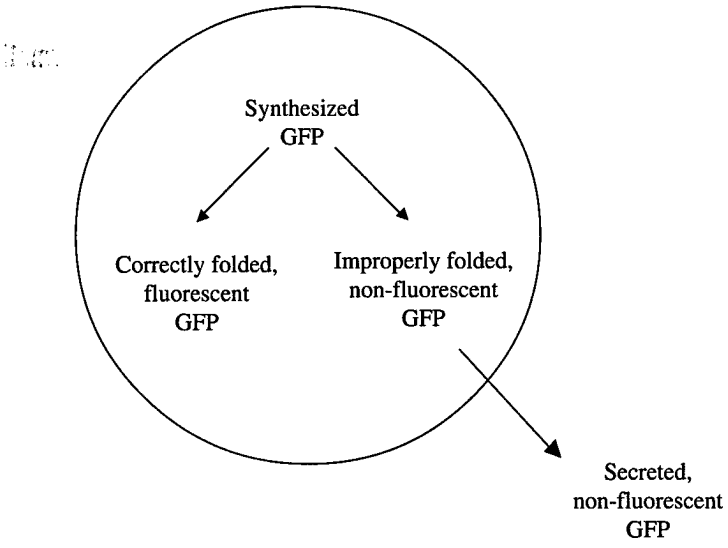

Fig. 9