

Capturing protein interactions in the secretory pathway of living cells

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The secretory pathway is composed of membrane compartments specialized in protein folding, modification, transport, and sorting. Numerous transient protein–protein interactions guide the transport-competent proteins through the secretory pathway. Here we have adapted the yellow fluorescent protein (YFP)-based protein fragment complementation assay (PCA) to detect protein–protein interactions in the secretory pathway of living cells. Fragments of YFP were fused to the homooligomeric cargo-receptor lectin endoplasmic reticulum Golgi intermediate compartment (ERGIC)-53, to the ERGIC-53-interacting multicoagulation factor deficiency protein MCFD2, and to ERGIC-53's cargo glycoprotein cathepsin Z. YFP PCA analysis revealed the oligomerization of ERGIC-53 and its interaction with MCFD2, as well as its lectin-mediated interaction with cathepsin Z. Mutation of the lectin domain of ERGIC-53 selectively decreased YFP complementation with cathepsin Z. Using YFP PCA, we discovered a carbohydrate-mediated interaction between ERGIC-53 and cathepsin C. We conclude that YFP PCA can detect weak and transient protein interactions in the secretory pathway and hence is a powerful approach to study luminal processes involved in protein secretion. The study extends the application of PCA to carbohydrate-mediated protein–protein interactions of low affinity.

ERGIC-53 | lectin cargo receptor | protein fragment complementation assay | protein–protein interaction

Eukaryotic cells have evolved a secretory pathway that is composed of characteristic membrane compartments, including the endoplasmic reticulum (ER), the ER-Golgi intermediate compartment (ERGIC), and the Golgi apparatus. Approximately one-third of all cellular proteins are translocated into the lumen of the ER, where modification, folding, and oligomerization occur, before proteins are further transported along the secretory pathway. The folding and modification processes involve numerous ER resident proteins that are believed to operate as a quality control machinery that surveys correct folding in the ER (1, 2). After acquisition of transport competence, the secretory proteins exit the ER by a receptor-mediated mechanism (3, 4) or by bulk flow. The interactions between proteins of the ER quality control machinery and their substrates, as well as between cargo receptors and their cargo, are often of a weak and transient nature and therefore difficult to study. Traditional techniques for studying protein–protein interactions, such as yeast two-hybrid assays, may not be adequate to reveal interactions among these proteins, given that the yeast two-hybrid approach identifies interactions in a reducing (cytoplasm and nucleus) rather than oxidizing (ER) environment. We therefore explored the possibility of adapting the protein fragment complementation assay (PCA) (5) to studying protein interactions in the secretory pathway.

The basic concept of PCA relies on engineering reporter protein fragments that exhibit no functional activity by themselves and do not spontaneously fold. The fragments are fused to two interacting proteins. The interaction of the hybrid proteins brings the two reporter fragments into proximity, where they fold into the active 3D structure of the complete reporter

protein. PCA has been described by using a variety of proteins, including β -lactamase, dihydrofolate reductase, *Renilla* and firefly luciferases, and GFP and yellow fluorescent protein (YFP) as reporters (6–13). The GFP and YFP PCAs have proven particularly simple for detection and library screening of cytosolic, membrane, and nuclear protein–protein and protein–RNA interactions (13–16).

In the present study, we have explored the suitability of the YFP-based PCA technique for visualizing protein–protein interactions in the lumen of the early secretory pathway by using ERGIC-53 as model protein. ERGIC-53 is a homooligomeric nonglycosylated type I transmembrane protein cycling in the early secretory pathway (17, 18). It contains a functional lectin domain (19) and acts as a cargo receptor for a subset of glycoproteins, including blood coagulation factors V and VIII (20), cathepsin Z (catZ) (3), and presumably cathepsin C (catC) (21). Chemical crosslinking revealed a direct and carbohydrate-dependent interaction of the lysosomal protein catZ with ERGIC-53 (3). catC is also a lysosomal protein, related to catZ. The secretion of catC is reduced if a dominant-negative ER-locked form of ERGIC-53 is expressed, but all attempts to show a direct interaction between catC and ERGIC-53 have failed (21). Recently, MCFD2 was identified as an ERGIC-53 interacting protein (22). MCFD2 is a nonglycosylated luminal protein, which contains two EF-hands and binds ERGIC-53 in a calcium-dependent manner, but its precise role in cargo transport is unknown.

In the current study, we have adopted the YFP PCA to the secretory pathway and visualized the oligomerization of ERGIC-53 as well as its interaction with catZ and MCFD2. The power of YFP PCA is demonstrated by the detection of the lectin-mediated interaction between ERGIC-53 and catC. We anticipate that YFP PCA will be a valuable approach for studying the different processes involved in protein secretion and the basis for the first robust screens of protein–protein interactions in the secretory pathway.

Methods

Antibodies. The following antibodies were used: mouse mAb G1/93 against human ERGIC-53 (17), mouse mAb A1/182 against BAP31 (18), rabbit pAb against the KDEL receptor (23) (kind gift of H.-D. Söling, Max-Planck-Institut für Biophysikalische Chemie, Göttingen, Germany), mouse mAb G1/133 against giantin (24), and mouse mAb against GFP (Roche Applied Science). Note that anti-GFP recognizes YFP fragment 2 but not fragment 1.

DNA Constructs. The construction of pcDNA3 vectors (Invitrogen) containing the sequences of YFP fragments 1 (YFP1; amino acids 1–158) and 2 (YFP2; amino acids 159–239) was

Freely available online through the PNAS open access option.

Abbreviations: catC, cathepsin C; catZ, cathepsin Z; ER, endoplasmic reticulum; ERGIC, ER-Golgi intermediate compartment; PCA, protein fragment complementation assay; YFP, yellow fluorescent protein.

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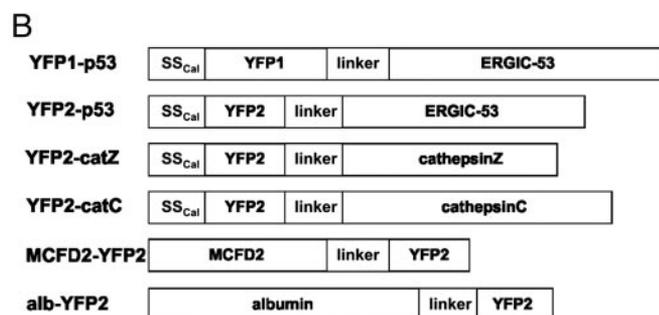
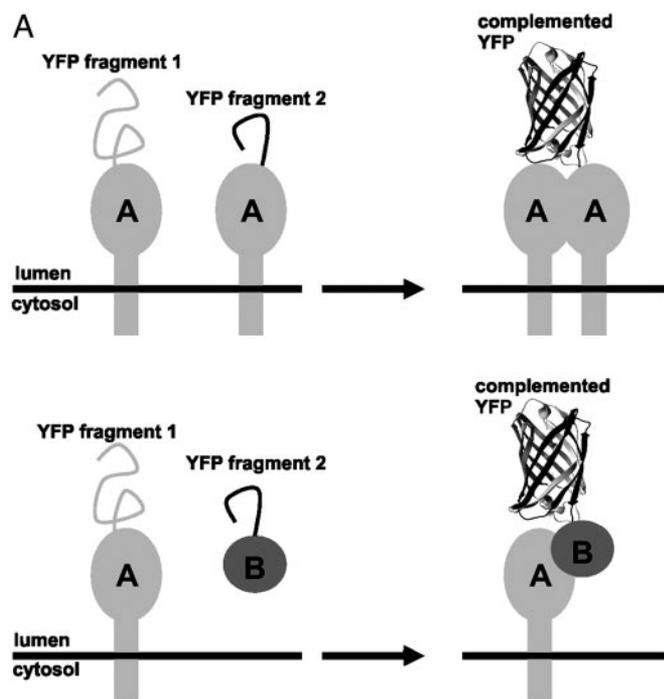


Fig. 1. YFP fragment complementation in the lumen of the secretory pathway. (A) Principle of the YFP PCA used in the current study. YFP fragments 1 (amino acids 1–158) and 2 (amino acids 159–239) are fused to the luminal part of a given transmembrane protein A or to a soluble luminal protein B. Homooligomerization of the membrane protein A or its interaction with B brings the two fragments of YFP into close proximity and leads to complementation into functional fluorescent YFP by folding into an active 3D structure. (B) Modular composition of the fusion proteins used in this study. ERGIC-53 (p53) served as an oligomeric transmembrane cargo receptor. catZ, catC, MCFD2, and albumin were used as soluble luminal proteins. For the N-terminal fusion of the YFP fragments, the endogenous signal sequence of the proteins was replaced by the signal sequence of calreticulin (SS_{cal}).

described previously (13). The sequence coding for the 10-aa (GGGS)₂ linker was introduced in 5' or 3' of the YFP fragments (13). cDNAs encoding MCFD2 and albumin were amplified by PCR and subcloned 5' of the linker-YFP2 sequence (resulting in fusion proteins with YFP fragments at the C terminus). ERGIC-53 cDNA had to be subcloned 3' of linker-YFP fragments, because its C terminus is cytosolic. For 3' subcloning, the DNA sequence coding for the signal sequence of calreticulin (SS_{cal}) was introduced into pcDNA3 by ligation of annealed phosphorylated oligonucleotides. The efficient signal sequence of calreticulin was used, because the endogenous signal sequence of ERGIC-53 was inefficient in ER translocation of recombinant ERGIC-53 fusion proteins. DNA sequences of YFP1- and YFP2-linker were amplified by PCR and subcloned 3' of SS_{cal}. cDNAs encoding ERGIC-53, catZ, and catC lacking the signal sequence were amplified by PCR and subcloned 3' of

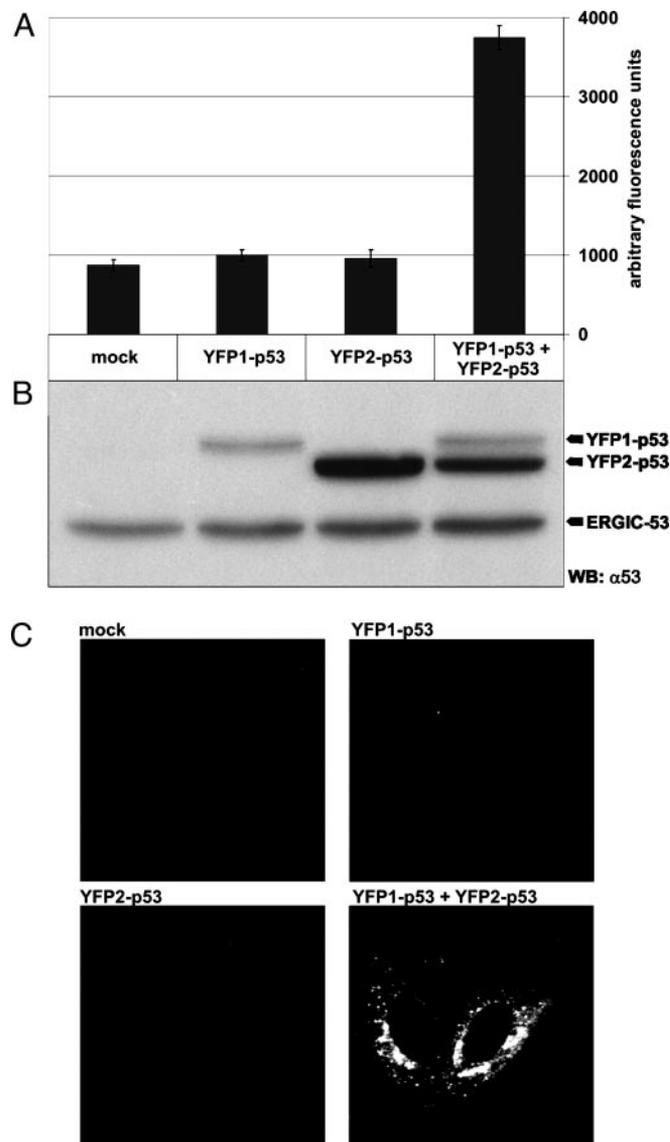


Fig. 2. Visualization of ERGIC-53 oligomerization by YFP PCA. (A) YFP fragment complementation was detected by fluorometric analysis by using microtiter plates with cell suspensions of HeLa cells expressing the indicated fusion proteins. Coexpression of YFP1-p53 and YFP2-p53 resulted in YFP fluorescence, whereas the expression of the single fusion proteins led to no detectable YFP fluorescence. (B) Immunoblot analysis using anti-ERGIC-53 verifies expression of the fusion proteins YFP1-p53 and YFP2-p53. Equal protein amounts are present in all lanes, as revealed by similar levels of endogenous ERGIC-53. (C) Visualization of the oligomerization of YFP1-p53 and YFP2-p53 in the early secretory pathway of HeLa cells by fluorescence microscopy of live cells. Note that the YFP fragments show no fluorescence signal when transfected individually.

the SS_{cal}-YFP fragment linker. The composition of all recombinant fusion proteins used in this study is shown in Fig. 1B. YFP1 containing the Q69M mutation (25), as well as ERGIC-53 containing the N156A mutation, was generated by introducing point mutations with the QuikChange site-directed mutagenesis kit (Stratagene).

Cell Culture and DNA Transfection. HeLa cells were grown in DMEM, supplemented with 10% FBS, 1× nonessential amino acids and antibiotics. For fluorometric analysis and metabolic labeling, HeLa cells were grown in six-well plates. For fluorescence microscopy, HeLa cells were grown on poly(L-lysine)-

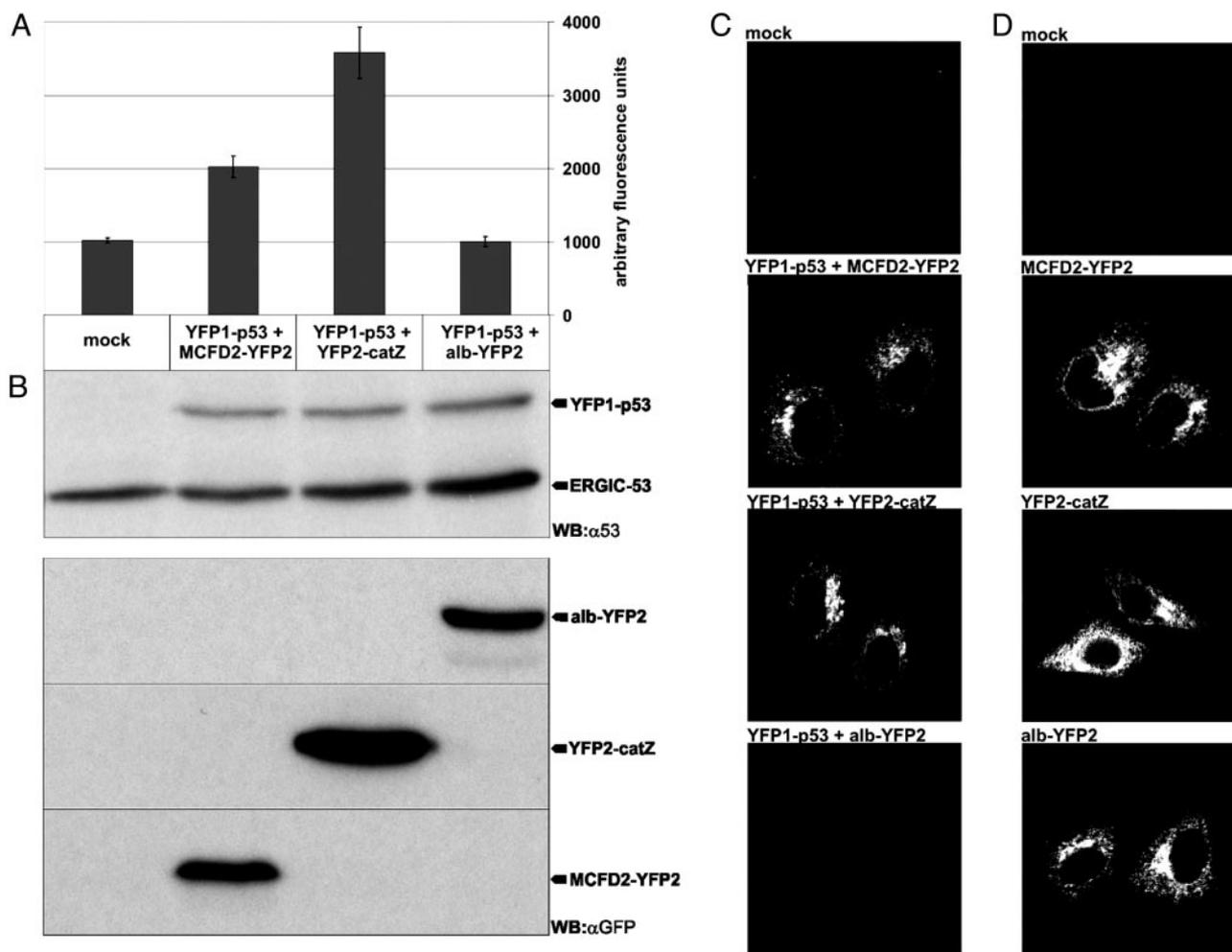


Fig. 3. YFP PCA can specifically detect interactions of ERGIC-53 with MCFD2 and catZ. (A) YFP fragment complementation indicates interactions of ERGIC-53 with MCFD2 and catZ but not with albumin. Fluorescence was measured by fluorometry of cell suspensions in microtiter plates. (B) Expression of the fusion proteins was visualized by immunoblotting with anti-ERGIC-53 and anti-GFP. (C) Fluorescence microscopy of live cells expressing the indicated fusion proteins. (D) Immunofluorescence using anti-GFP confirmed the expression of MCFD2-YFP2, YFP2-catZ, and alb-YFP2 in the early secretory pathway.

coated glass slides. Cells were transfected at $\approx 60\%$ confluence by using FuGENE6 (Roche Applied Science) according to the manufacturer's instructions.

YFP Fluorometric Analysis. Twenty-four hours after transfection, cells were washed with PBS, harvested by trypsinization, and resuspended in 1 ml of PBS. Cells were then pelleted by centrifugation, resuspended in 200 μ l of PBS, transferred to black 96-well microtiter plates (Nunc), and subjected to fluorometric analysis by using a Victor2 fluorometer (PerkinElmer). Excitation and emission wavelengths of 485 and 535 nm, respectively, were used. Data from three independent experiments were averaged, and error bars indicate standard deviations. For fluorescence microscopy, cells were washed twice with PBS and mounted under a glass coverslip. Live cells were analyzed by laser-scanning confocal microscopy (TCS NT, Leica, Deerfield, IL). Representative images of single optical sections are shown.

Immunofluorescence. Twenty-four hours after transfection, HeLa cells were fixed in 3% paraformaldehyde, permeabilized in PBS containing 0.1% saponin, and incubated with the primary antibody. For anti-GFP immunofluorescence, cells were stained with Alexa Fluor 488-conjugated goat-anti-mouse IgG (Molecular Probes). For colocalization studies, cells were stained with Alexa Fluor

568-conjugated goat-anti mouse IgG or goat-anti rabbit IgG (Molecular Probes) and analyzed by using confocal microscopy.

Immunoblot Analysis. Protein samples were prepared from cells used for fluorometric analysis in microtiter plates. Protein samples were separated by SDS/PAGE, transferred to nitrocellulose membranes, immunoblotted with anti-ERGIC-53 and anti-GFP, and visualized by enhanced chemiluminescence (Amersham Pharmacia Biosciences).

Metabolic Labeling. Twenty-four hours after transfection, cells were pulsed for 15 min with 100 μ Ci (1 Ci = 37 GBq) of [35 S]methionine (PerkinElmer) and chased for the indicated times in HeLa culture medium containing 10 mM L-methionine. Cells were lysed in 1% Nonidet P-40/50 mM Tris-HCl, pH 7.5/150 mM NaCl/PMSF, and the lysate was cleared by centrifugation at $100,000 \times g$ for 1 h. The chase medium was cleared from cell debris by centrifugation at $20,000 \times g$ for 10 min. Cleared samples were immunoprecipitated with anti-ERGIC-53 and -GFP. Immunoprecipitates were separated by SDS/PAGE and proteins visualized by fluorography.

Results

The principle of YFP PCA used in the current study is illustrated in Fig. 1A. To optimize YFP as a reporter for studying protein-

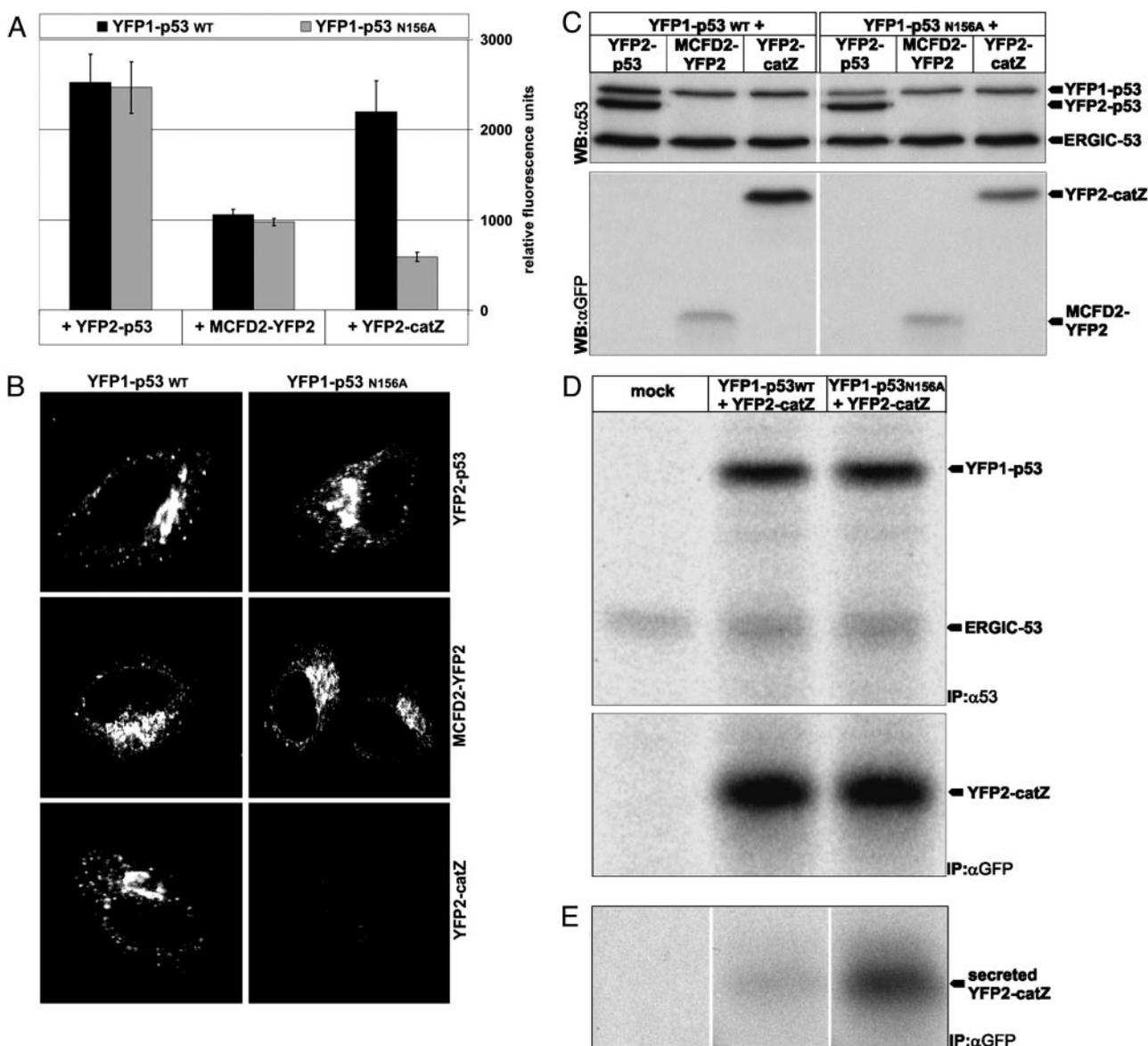


Fig. 4. Inactivation of the lectin activity of ERGIC-53 selectively impairs lectin-mediated interaction with catZ. (A) Fluorometric analysis of cell suspensions in microtiter plates shows that inactivation of the carbohydrate recognition domain of ERGIC-53 by the N156A mutation selectively impaired the lectin-mediated interaction with catZ. ERGIC-53 oligomerization and its interaction with MCFD2 are not affected. Relative fluorescence values are shown in which the background fluorescence of mock transfected cells was subtracted. (B) Fluorescence microscopy of living HeLa cells expressing the indicated fusion proteins. (C) Expression of all fusion proteins was analyzed by immunoblotting with anti-ERGIC-53 and anti-GFP. (D) A 15-min [35 S]methionine pulse followed by immunoprecipitation with anti-ERGIC-53 and anti-GFP shows equal rates of synthesis of YFP2-catZ cotransfected with either YFP1-p53_{WT} or YFP1-p53_{N156A} (fluorogram). (E) Transfected HeLa cells were pulsed for 15 min with [35 S]methionine and chased for 1 h. Anti-GFP immunoprecipitation of the chase medium reveals reduced secretion of YFP2-catZ cotransfected with YFP1-p53_{WT} as compared with YFP1-p53_{N156A} (fluorogram).

protein interactions in the secretory pathway, its glutamine residue at position 69 was replaced by a methionine. YFP Q69M, known as citrine, shows improved photostability and expression in different cellular compartments, including the secretory pathway (25). To test whether the YFP PCA reveals protein-protein interactions in the lumen of the secretory pathway, we first studied the homooligomerization of the type I transmembrane protein ERGIC-53. YFP fragments 1 and 2 were fused to the luminal N terminus of ERGIC-53, resulting in YFP1-p53 and YFP2-p53 (Fig. 1B). Individual expression of either YFP1-p53 or YFP2-p53 alone gave no detectable YFP fluorescence in living cells, indicating that the YFP fragments *per se* have no intrinsic fluorescence if expressed in the secretory pathway. Strong YFP fluorescence was observed, however, when YFP1-p53 and

YFP2-p53 were coexpressed, which allowed for YFP fragment complementation triggered by ERGIC-53 oligomerization (Fig. 2A). The fluorescence pattern (Fig. 2C) observed for complemented YFP in live cells is typical for the early secretory pathway (26). Double-labeling experiments showed overlap with the ER marker BAP31, the ERGIC- and cis-Golgi-localized KDEL-receptor, and the cis-medial Golgi marker giantin (Fig. 6, which is published as supporting information on the PNAS web site). We conclude that YFP PCA can detect homooligomerization of membrane proteins in the secretory pathway.

We next determined whether YFP PCA can detect the protein-protein-mediated interaction between ERGIC-53 and MCFD2 as well as the protein-carbohydrate-mediated interaction between ERGIC-53 and its glycoprotein cargo catZ. As a

negative control, we included the nonglycosylated protein albumin, the secretion of which does not depend on ERGIC-53. YFP fragment 2 was fused to the C terminus of MCFD2 and albumin as well as to the N terminus of catZ (Fig. 1B). C-terminal tagging of catZ was not possible, because it interferes with ERGIC-53 binding (C. Appenzeller and H.-P.H., unpublished data). Fluorometric analysis detected YFP fragment complementation if YFP1-p53 was coexpressed with MCFD2-YFP2 or YFP2-catZ (Fig. 3A). In both cases, the YFP fluorescence was localized to the early secretory pathway (Fig. 3C). Notably, albumin-YFP2 did not induce YFP fragment complementation with YFP1-p53, although it was correctly expressed at similar levels as MCFD2-YFP2 (Fig. 3B and D). The results demonstrate the selectivity and specificity of YFP PCA to detect luminal protein–protein interactions in the secretory pathway. Moreover, coexpression of YFP1-p53 with MCFD2-YFP2 showed similar fluorescence as coexpression of YFP2-p53 with MCFD2-YFP1 (data not shown), indicating exchangeability of the YFP fragments.

To further investigate the sensitivity and specificity of YFP PCA, we studied the effect of inactivation of the lectin domain of ERGIC-53 by the N156A mutation. This point mutation abolishes binding of ERGIC-53 to mannose beads (19) as well as to catZ (3). Fluorometric analysis revealed that the N156A mutation specifically decreased the lectin-dependent interaction of ERGIC-53 with catZ, whereas ERGIC-53 oligomerization, as well as its interaction with MCFD2, was not affected (Fig. 4A). The fluorometric results were confirmed by fluorescence microscopy of live cells (Fig. 4B). Immunoblot analysis showed higher steady-state levels of YFP2-catZ when coexpressed with YFP1-p53_{WT} than with YFP1-p53_{N156A} (Fig. 4C). A possible explanation for this difference may be decreased dissociation of YFP2-catZ from YFP1-p53_{WT} after complementation of the YFP fragments, which would result in intracellular accumulation of YFP2-catZ. Stabilization of the interaction between fusion proteins by YFP fragment complementation has indeed been noticed (12). To test this possibility, we studied synthesis and secretion of YFP2-catZ in pulse–chase experiments using [³⁵S]methionine. Fig. 4D shows that YFP2-catZ was synthesized at equal rates, irrespective of whether it was coexpressed with WT or N156A ERGIC-53. In contrast, YFP2-catZ secretion was less efficient when coexpressed with YFP1-p53_{WT} than with YFP1-p53_{N156A} (Fig. 4E). These findings explain the differences in the steady-state protein amount of YFP2-catZ (Fig. 4C) and support the notion that catZ is retained intracellularly by WT ERGIC-53 because of decreased dissociation after YFP fragment complementation.

So far, we have provided the proof of concept of YFP PCA for detecting luminal protein interactions in the secretory pathway that had previously been established by alternative techniques. To search for a novel interaction, we applied YFP PCA to catC. The secretion of catC was shown to be delayed in cells overexpressing dominant-negative ER-retained ERGIC-53 (21), but all previous attempts to demonstrate a direct interaction by pull-down or crosslinking experiments have failed. In contrast, YFP PCA can detect a direct interaction between ERGIC-53 and catC and shows that this interaction depends on a functional lectin domain of ERGIC-53 (Fig. 5). This result conclusively establishes that catC is a cargo glycoprotein of ERGIC-53 and demonstrates the power of YFP PCA to visualize novel protein–protein interactions of weak, transient, and glycan-mediated nature in the secretory pathway that escape detection by coimmunoprecipitation and chemical crosslinking.

Discussion

The control of secretion requires a plethora of protein interactions on both the cytoplasmic and luminal sides of the secretory pathway. A particular difficulty has been to detect luminal interactions, many of which are of low affinity and occur in an

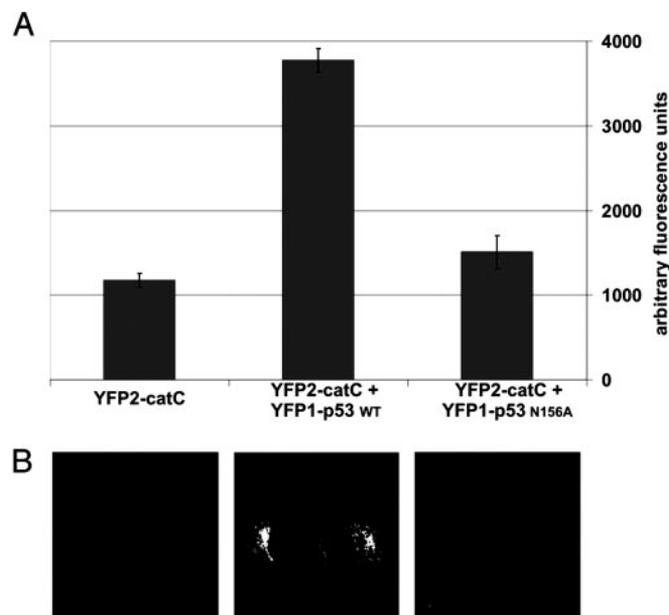


Fig. 5. ERGIC-53 interacts with catC. (A) Fluorometric analysis of YFP fragment complementation of ERGIC-53 and catC. The interaction of ERGIC-53 with catC depends on a functional lectin domain of ERGIC-53. (B) Fluorescence microscopy of live cells expressing the indicated fusion proteins.

oxidizing environment and hence are often not amenable to traditional approaches. The YFP PCA described here can overcome these limitations. It allows the visualization, in live cells, of luminal protein–protein interactions of different natures: (i) interactions among transmembrane proteins; (ii) protein–protein-based interactions between a transmembrane and a luminal protein; and (iii) glycan-mediated interactions between a membrane lectin and its cargo glycoprotein. Although not tested here, we anticipate that specific interactions between two soluble proteins in the lumen of the secretory pathway will also be detectable by YFP PCA. Further improvements to the citrine YFP PCA, such as increased solubility of fragments, could be achieved through protein engineering strategies (27), although solubility of YFP was not a problem in our experiments.

Most notably, YFP PCA can detect protein–protein interactions that are transient and of low affinity, as illustrated for the lectin interaction of ERGIC-53 with catZ. The detection of such low-affinity interactions may be facilitated by the stabilization of the complex by the reconstituted YFP. This also means that caution should be taken in applying the YFP PCA to quantitative kinetic analysis of protein complex dissociation in limited cases, for which dissociation is rapid compared with other processes like protein degradation. YFP PCA exhibits several major advantages over other methods for the investigation of protein–protein interactions, including: (i) its simplicity and reliability, (ii) its high sensitivity using YFP citrine that enables analysis of interactions among proteins expressed at levels comparable to many endogenous proteins, and (iii) direct visualization of protein–protein interactions in their normal compartmental environment of living cells.

Fluorometric analysis of complemented YFP results in a quantitative read-out. YFP complementation monitored for the interaction between ERGIC-53 and MCFD2 shows about half the fluorescence intensity compared with ERGIC-53 oligomerization and its interaction with catZ or catC. N-terminal tagging of MCFD2 did not significantly increase YFP fragment complementation for the interaction between ERGIC-53 and MCFD2 (data not shown). One possible reason for lower YFP fragment

complementation may be a lower expression level of MCFD2-YFP2 compared with YFP2-catZ (Fig. 3B). ERGIC-53 binding of catZ and catC is lectin-dependent, whereas binding of MCFD2 is lectin-independent (Figs. 4A and 5A). The position of this lectin-independent binding site on ERGIC-53 could also account for lower YFP fragment complementation by imposing spatial constraints.

In this study, we not only provide the proof of concept of the YFP PCA approach but also apply it to detect a direct protein interaction of low affinity between ERGIC-53 and catC that has been surmised but was experimentally unproven. The catC finding is important for several reasons. It validates a previous proposal based on catZ that catC carries an ER-export signal (28). Moreover, it paves the way for the identification of additional ligands of ERGIC-53. Such ligands have been proposed to exist on the basis of secretion assays performed in HeLa cells expressing dominant-negative ERGIC-53 (21). In more

general terms, the ability of the YFP PCA to detect transient protein interactions of low affinity will greatly facilitate the analysis of protein complexes involved in virtually all luminal functions of the secretory pathway. Of particular interest is that YFP PCA can detect carbohydrate-mediated interactions between a lectin and its ligands. Numerous lectins in the secretory pathway are involved in protein folding (2), degradation (29–31), transport (32, 33), and sorting (34). For some of these lectins, the ligands are unknown or insufficiently characterized and hence the YFP PCA may aid in the elucidation of lectin functions. Last, the feasibility of screening cDNA-fusion libraries for interacting partners identified by high-throughput YFP PCA analysis (35) should prove a useful approach for revealing new components involved in luminal processes controlling secretion.

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