GGAs: A Family of ADP Ribosylation Factor-binding Proteins Related to Adaptors and Associated with the Golgi Complex

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Abstract. Formation of intracellular transport intermediates and selection of cargo molecules are mediated by protein coats associated with the cytosolic face of membranes. Here, we describe a novel family of ubiquitous coat proteins termed GGAs, which includes three members in humans and two in yeast. GGAs have a modular structure consisting of a VHS domain, a region of homology termed GAT, a linker segment, and a region with homology to the ear domain of γ-adaptins. Immunofluorescence microscopy showed colocalization of GGAs with Golgi markers, whereas immunoelectron microscopy of GGA3 revealed its presence on coated vesicles and buds in the area of the TGN. Treatment with brefeldin A or overexpression of dominant-negative ADP ribosylation factor 1 (ARF1) caused dissociation of GGAs from membranes. The GAT region of GGA3 was found to: target a reporter protein to the Golgi complex; induce dissociation from membranes of ARF-regulated coats such as A P-1, A P-3, A P-4, and COPI upon overexpression; and interact with activated ARF1. Disruption of both GGA genes in yeast resulted in impaired trafficking of carboxypeptidase Y to the vacuole. These observations suggest that GGAs are components of ARF-regulated coats that mediate protein trafficking at the TGN.

Key words: trans-Golgi network • ADP ribosylation factor • coats • adaptins • VHS

Introduction

Protein trafficking at various stages of the endocytic and secretory pathways is mediated by vesicular intermediates that carry cargo molecules between different membrane-bound compartments. Both the formation of these intermediates and cargo selection depend upon the function of a set of protein coats that are recruited from the cytosol to membranes (reviewed by Mellman, 1996; Rothman and Wieland, 1996; Schekman and Orci, 1996; Kirchhausen et al., 1997). Some coats contain as their key constituents either one of four heterotetrameric adaptor protein (AP)1 complexes (subunit compositions indicated in parentheses): A P-1 (γ-β1-α1-μ1); A P-2 (α-β2-μ2-α2); A P-3 (δ-β3-μ3-α3); or A P-4 (ε-β4-μ4-α4); reviewed by Hirst and Robinson, 1998; Bonifacino and Dell’Angela, 1999; Kirchhausen, 1999). A P-2 has the shape of a “head” with two protruding “ears” (Heuser and Keen, 1988); the other three complexes are thought to have a similar structure. A more distantly related coat protein complex, known as coatomer or COPI (α-β-γ-δ-ε-ζ), has been shown to play a role in ER-Golgi and endocytic trafficking pathways (reviewed by Lippincott-Schwartz et al., 1998; Wieland and H arter, 1999).

Recruitment of A P-1, A P-3, A P-4, and COPI, but not A P-2, from the cytosol to membranes is regulated by members of the ADP-ribosylation factor (ARF) family of small GTP-binding proteins (Donaldson et al., 1992a,b; Robinson and Kreis, 1992; Palmer et al., 1993; Stamnes and Rothman, 1993; Traub et al., 1993; Faundez et al., 1998; Ooi et al., 1998; Dell’Angela et al., 1999a; Hirst et al., 1999). A R F1 is the most abundant member of the family and also the most active for recruitment of COPI and A P-3 to membranes (Liang and Kornfeld, 1997; Ooi et al., 1998); other A R Fs (A R F2-5) may also be able to regulate

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Abbreviations used in this paper: 3AT, 3-amino-1,2,4-triazole; AP, adaptor protein; ARF, ADP-ribosylation factor; BFA, brefeldin A; CPY, carboxypeptidase Y; GAE, γ-adaptin ear; GAL4ad, GAL4 transcription activation domain; GAL4bd, GAL4 binding domain; GAT, GGA and TOM1; GFP, green fluorescent protein; GST, glutathione-S-transferase; GTPγS, guanosine 5’-O-(3-thiotriphosphate); ORF, open reading frame; TOM1, target of myb1.
Materials and Methods

Nucleic Acid Manipulations

A cDNA segment comprising codon 5 to the stop codon of the long form of human GGA1 was PCR-amplified from a human heart cDNA library, using primers based on partial genomic DNA sequences from GenBank/EMBL/DBJ (accession number D63876). The 5′ primer included an additional sequence to place a MucI site adjacent to the codon for amino acid 5. The cDNA fragment was subcloned into the EcoRI–SalI sites of the pGEX-5X-1 vector. GFP-fusion constructs, comprising different regions of the GGA 3 long isoform, were prepared by PCR and subsequent cloning into the EcoRI–NotI sites of the PGFP-C2-MCS vector (Clontech), as follows: GFP-VHS (residues 1-146); GFP-VHS-GAT (residues 1-327); and GFP-GAT (residues 147-313). The insert of the two-hybrid construct GST-GGA3 was subcloned into the EcoRI– BamHI sites of the pgEFP-C2-MCS vector to generate the GFP-GAE construct. The two-hybrid ARF1/Q71L construct was engineered by PCR from the HA-ARF1-Q71L mammalian expression plasmid (Ooi et al., 1998) and cloned in-frame (without the HA sequence) into the EcoRI–Xhol sites of the pgEFP vector (Clontech). The corresponding two-hybrid ARF1/T31N plasmid and the GST-γ-2-adaptin plasmid were kindly provided by Chean Eng Ooi (National Institutes of Health, Bethesda, MD).

Antibodies

Polynuclear antibodies to human GGA2 and GGA3 were raised in rabbits using as immunogens purified GST-GGA2 and GST-GGA3, respectively. Both antibodies were affinity-purified on columns containing their corresponding immunogen. The antibodies were gifts of Dall’A neglica et al., 1999a,b).

Cell Culture and General Biochemical Procedures

The sources and culture conditions of the human cell lines HeLa, M1, H4, Jurkat, Caco-2, MEG-01, and MNT-1 have been described previously (Dell’A neglica et al., 2000). Metabolic labeling of cells in culture with [35S]methionine, preparation of Triton X-100 and detergent-free extracts, salt extraction of podomembrane membranes, ultracentrifugation on sucrose gradients, and immunoprecipitation-recapture were carried out as described previously (Dell’A neglica et al., 1997). Recombinant GST-fusion proteins were purified by using glutathione-Sepharose 4B beads (Pharmacia Biotech).

Transfection and Immunofluorescence

Transfection of HeLa cells was performed by using FuGENE-6 (Roche Molecular Biochemicals). Immunofluorescence microscopy analysis was carried out as described (Dell’A neglica et al., 1999a). Images were acquired on a Zeiss LSM 410 or LSM 510 confocal microscopes. The lack of antibody cross-reactivity or optical cross-over on two-color fluorescence experiments was corroborated using appropriate controls done in parallel.

Immunoelectron Microscopy

Immunoelectron microscopy of ultrathin frozen sections of HeLa cells transfected with a GGA3 construct was performed essentially as described (Slot and Geuze, 1983). In brief, cells were fixed in 4% formaldehyde, 0.1 M phosphate buffer (pH 7.4) for 2 h at room temperature, scraped, and embedded in 10% gelatin. Gelatin blocks were incubated for 16 h in 2 M sucrose at 4°C, and then frozen in liquid nitrogen. Ultrathin frozen sections were made with a cryoultramicrotome (Richert Ultratoc 5) and transferred to grids using sucrose-methy cellulose droplets. Sec-
tions were treated with 1% fish skin gelatin and 0.15 M glycine before incubation at room temperature with rabbit antibody to GGAs. Bound antibody was detected by incubation with protein A-conjugated to 10-nm (for double-labeling) or 10-nm (for single-labeling) gold particles (Utrecht University, The Netherlands). For double-labeling experiments, sections were subsequently incubated with sheep anti-human TGN46, rabbit anti-lam B, and GAE (C) regions of human GGAs and the corresponding mouse antibody to the M yc epitope and rabbit antibody to TGN46 followed by a lexa-488-conjugated donkey anti-mouse IgG (B and C, green channel) and Cy3-conjugated donkey anti-rabbit IgG (B and C, red channel); D–F, rabbit antibody to GGA2 and mouse antibody to the HA epitope followed by Cy3-conjugated donkey anti-rabbit IgG (D and F, red channel) and A lexa-488-conjugated donkey anti-mouse IgG (E and F, green channel); and G–I, mouse antibody to the M yc epitope and rabbit antibody to GGA3 followed by a lexa-488-conjugated donkey anti-mouse IgG (G and I, green channel) and Cy3-conjugated donkey anti-rabbit IgG (H and I, red channel). Stained cells were examined by confocal fluorescence microscopy. Bar, 10 μm.

Results

Identification and Domain Organization of GGAs Proteins

Homology searches of sequences derived from both genomic and EST sequencing projects revealed the existence of at least three novel human genes encoding proteins with similarity to the ear domain of the γ1-adaptin subunit of AP-1. The complete ORFs of these proteins, herein referred to as GGA1 (GenBank/EMBL/DDC accession number A F218584), GGA2 (GenBank/EMBL/DDC accession number A C002400) and GGA3 (GenBank/EMBL/DDC accession numbers A F219138 and A F219139 for long and short alternatively spliced forms, respectively), were isolated from human cDNA libraries using a combination of PCR and 5’ RACE. Complete or partial sequences of GGAs and GGA3 (short isoform) recently have been published by others as a result of large-scale DNA sequencing projects (Nagase et al., 1995; Kikuno et al., 1999; Loftus et al., 1999). The GGAs have also been independently identified by Boman et al. (2000) and Hirsh et al. (2000). Northern blot analyses have shown that the three human GGAs are expressed in all human tissues examined (our unpublished observations; see also Nagase et al., 1995; K Ikuno et al., 1999). Subsequent database searches revealed the occurrence of GGAs-related proteins in other eukaryotic organisms. In particular, two ORFs from S. cerevisiae, Y DR 358w and Y HR 108w, were deemed to encode the yeast counterparts of human GGAs; we refer to these proteins as yeast Gga1p and Gga2p, respectively.

The three human and two yeast GGAs were found to share a similar domain organization consisting of four distinct regions (Fig. 1; and Supplemental Figure 1, http://www.jcb.org/cgi/content/full/149/1/81/D C1). The first region, comprising ~140 residues proximal to the GGAs’ NH2 termini, displays significant homology to the VHS domain, a protein module previously found in the vertebrate proteins TOM1L1 (i.e., target of myb 1; not to be confused with mitochondrial outer membrane proteins), TOM1L1, Hrs, Hrs-2, and STAM1, as well as in yeast Vps27p (Lohi and Lehti, 1998, and references therein).

The second region, comprising the next ~160 residues, is highly homologous among the human GGAs and contains Bank/EMBL/DDC accession numbers are indicated in the legend to Fig. 1. The positions of the segments within the proteins’ primary structures are indicated by residue numbers next to the sequences. Highlighted letters represent residues conserved in at least four (A) or three (B and C) of the proteins.

Supplemental Figure 2. Intracellular localization of Myc-GGA1 and GGA2 analyzed by indirect immunofluorescence microscopy of transfected cells. Hela cells transfected with either Myc-GGA 1 (A–C, G–I) or both GGA 2 and HA-tagged TGN38 (D–F) were fixed, permeabilized, and stained as follows: A–C, mouse antibody to the M yc epitope and rabbit antibody to TGN46; D–F, rabbit antibody to GGA2 and mouse antibody to the HA epitope followed by Cy3-conjugated donkey anti-rabbit IgG (D and F, red channel) and A lexa-488-conjugated donkey anti-mouse IgG (E and F, green channel); and G–I, mouse antibody to the M yc epitope and rabbit antibody to GGA3 followed by a lexa-488-conjugated donkey anti-mouse IgG (G and I, green channel) and Cy3-conjugated donkey anti-rabbit IgG (H and I, red channel). Stained cells were examined by confocal fluorescence microscopy. Bar, 10 μm.

Online Supplemental Materials

The online version of this article includes figures that accompany the information presented here and is available at http://www.jcb.org/cgi/content/full/149/1/81/D C1.

Supplemental Figure 1. Multiple sequence alignments of VHS (A), GAT (B), and GAE (C) regions of human GGAs and the corresponding segments of related human proteins γ1, γ2-adaptin; γ2, γ2-adaptin. Gen-
an ~85 residue segment with low but significant homology to human TOM1 and TOM1L1. We refer to this region as “GAT” to denote the occurrence of homology among the GGA and TOM1 proteins. The GAT region has a high propensity to fold into coiled coil structures. The third region is much less conserved among the GGAs; it is of variable size, rich in prolines and serines, and predicted to adopt a random coil conformation. Finally, the ~125 residues proximal to the GGAs COOH termini display significant homology to the ear domains of the γ1-adaptin subunit of mammalian AP-1 and of the closely related γ2-adaptin protein (Robinson, 1990; Lewin et al., 1998; Takatsu et al., 1998). We refer to this region as GAE, for γ-adaptin ear homology domain.

Biochemical Characterization of Endogenous GGA2 and GGA3 in Human Cells

To study the biochemical properties of the human GGAs, we raised polyclonal antibodies to GST-fusion proteins bearing the corresponding GAE domains. Antibodies to GGA2 and GGA3 allowed us to identify the endogenous proteins in HeLa cells, by using an immunoprecipitation-recapture procedure (Fig. 2 A). The electrophoretic mobility of the protein recognized by the anti-GGA2 antibody corresponded to a polypeptide of ~70 kD, a molecular mass that was in close agreement with that predicted for human GGA2 (67.2 kD). The apparent molecular mass of the protein isolated with the antibody to GGA3 (~90 kD) was larger than that expected for the long isoform of GGA3 (78.3 kD). However, specificity controls convinced us that the ~90-kD protein corresponded to GGA3. First, the protein was isolated only when the anti-GGA3 antibody was used in both the first and second immunoprecipitation steps (Fig. 2 A). Second, recognition of this protein by the anti-GGA3 antibody was completely inhibited by excess GST-GGA3 GAE, but not by similar amounts of GST or GST-GGA2 GAE (data not shown). Finally, the anti-GGA3 antibody did not crossreact with endogenous GGA2, the γ1-adaptin subunit of AP-1, or overexpressed Mcy-GGA1 (data not shown). Both GGA2 and GGA3 were expressed in a wide variety of human cell lines (Fig. 2 B), in agreement with the results of Northern blot analyses.

Subcellular fractionation of metabolically labeled HeLa cells yielded most of endogenous GGA2 and GGA3 in a cytosolic fraction. A small (~10%) fraction, however, was associated with a postnuclear membrane pellet (Fig. 2 C). The membrane-associated forms of both GGA2 and GGA3 could be extracted with 0.2 M sodium carbonate, pH 11 (data not shown). These results suggested that GGA2 and GGA3 exist as both soluble and peripherally membrane-bound forms.

To estimate the sizes of the soluble forms of GGA2 and GGA3, a cytosolic extract was fractionated by ultracentrifugation on a 5–20% sucrose gradient, and the resulting fractions were analyzed by immunoprecipitation-recapture (Fig. 2 D). Both GGA2 and GGA3 were recovered from fractions corresponding to a sedimentation coefficient of ~3.5 S. This value was significantly smaller than those obtained for heterotetrameric AP complexes (e.g., 10 S for AP-4; Dell’Angelica et al., 1999a), thus suggesting that GGA2 and GGA3 are not components of AP-like complexes. Rather, the calculated sedimentation coefficients of GGA2 and GGA3 are more compatible with these proteins existing as monomers or dimers with an asymmetric shape. GGA2 did not coprecipitate with GGA3 (Fig. 2 A) or GGA1 (Hirst et al., 2000), suggesting that they do not form stable dimers with one another.

Localization of GGAs to the Golgi Complex and Peripheral Cytoplasmic Structures

To examine the intracellular localization of the human GGA proteins, we initially performed indirect immunofluorescence microscopy analysis on fixed-permeabilized HeLa cells using the rabbit polyclonal antibodies to the...
were labeled with \[35S\]methionine and subjected to immunoprecipitation, SDS-PAGE, and fluorography. B, The indicated human cell lines were metabolically labeled with \[35S\]methionine for 85 h and lysed in the presence of Triton X-100. The cleared extract was immunoprecipitated using antibodies to GGA2 or GGA3, and the resulting precipitates were subjected to a second immunoprecipitation. The final immunoprecipitates were analyzed by 4–20% SDS-PAGE and fluorography to ascertain whether GGA2 or GGA3 were detected in these conditions (Fig. 3). This antibody gave punctate staining of a juxtanuclear structure resembling the Golgi complex (Fig. 3, A, D, G, J, M, and P). Staining was judged to be specific, as it could be inhibited by addition of a GST-GGA3\_GAE fusion protein (Fig. 3 B) but not by GST-GGA2\_GAE (Fig. 3 C), GST-\gamma-2-adaptin\_GAE (data not shown) or GST (Fig. 3 A).

The juxtanuclear structure stained by the anti-GGA3 antibody displayed partial colocalization with the TGN markers, TGN38 (Fig. 3, D–F) and furin (Fig. 3, G–I), and, to a lesser extent, with the cis-medial Golgi marker, 58-K protein (Fig. 3, J–L). We also observed partial colocalization of GGA3 with AP-1 in the juxtanuclear area of the cell, although the patterns of staining were not coincident (Fig. 3, M–O). There was little colocalization of GGA3 with AP-3 in the area of the Golgi complex (Fig. 3, P–R, left inset). However, we could observe faint staining for GGA3 in peripheral cytoplasmic foci, some of which did colocalize with AP-3 (Fig. 3, P–R, right inset), as well as with AP-1 (data not shown). We did not detect any colocalization of GGA3 with the plasma membrane-associated AP-2 complex (data not shown). Taken together, these observations suggest that GGA3 is mainly associated with the TGN, with an additional population of molecules localized to a peripheral cytoplasmic compartment.

Myc-epitope-tagged GGA1 (Myc-GGA1) or untagged GGA2 expressed at moderate levels by transfection into HeLa cells also exhibited good colocalization with TGN markers (Supplemental Figure 2, http://www.jcb.org/cgi/content/full/149/1/81/DC1). Similar observations were made for endogenous GGA1 and epitope-tagged GGA2 by Hirst et al. (2000). These observations suggest that, like GGA3, GGA1 and GGA2 are predominantly localized to the TGN.

**Association of GGA3 with Coated Membranes in the Area of the TGN**

The intracellular localization of human GGA proteins was further investigated by immunoelectron microscopy on ultrathin HeLa cell sections. A labeling of endogenous GGA3 yielded a very low signal, we expressed higher levels of the untagged protein by transfection. Staining of cells expressing moderate levels of the transgene with anti-GGA3 antibody revealed specific labeling in an area of tubules and vesicles adjacent to the Golgi stack (Fig. 4 A). A more detailed inspection of structures stained for GGA3 revealed that the gold particles were associated with budding membrane profiles covered with an electron-dense coat (Fig. 4 B). The region labeled for GGA3 was identified as the TGN by double-labeling with anti-GGA3 and anti-TGN46 antibodies (Fig. 4 C). Similar analyses performed on HeLa cells transfected with Myc-GGA1 also showed concentration of label in the area of the TGN, although significant labeling of the Golgi stack was also observed (data not shown).

**Regulation of GGA Localization by ARFs**

Since ARF1 regulates recruitment of various protein coats to membranes, it was of interest to investigate whether this was also the case for GGA1. To this end, we first examined the effect of treating HeLa cells with brefeldin A (BFA), a pharmacological inhibitor of ARF1 guanine nucleotide exchange activity (Donaldson et al., 1992b). We observed that treatment with 2 \(\mu\)g/ml BFA for 5 min was sufficient to effect complete disappearance of endogenous GGA3 staining from both the juxtanuclear area and peripheral foci (Fig. 5, A and B). At this early time of treatment, the Golgi cisternae and TGN had not yet disrupted, suggesting that the disappearance of GGA3 staining was not due to redistribution of the Golgi complex to the ER or endo-

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**Figure 2.** Biochemical characterization of endogenous GGA2 and GGA3. A, HeLa cells were metabolically labeled with \[35S\]methionine and lysed in the presence of Triton X-100 (Dell’Angelica et al., 1997). The cleared extract was immunoprecipitated using antibodies to GGA2 or GGA3, and the resulting precipitates were subjected to a second immunoprecipitation step using antibodies to GGA2, GGA3, or two irrelevant proteins. The final immunoprecipitates were analyzed by 4–20% SDS-PAGE and fluorography. B, The indicated human cell lines were metabolically labeled with \[35S\]methionine and subjected to immunoprecipitation–recapture, using antibodies to GGA2 (top) or GGA3 (bottom). C, A detergent-free extract (Dell’Angelica et al., 1997) of metabolically labeled HeLa cells was centrifuged first at 600 g for 5 min to remove nuclei and then at 120,000 g for 90 min to obtain cytosolic (C) and post-nuclear membrane (M) fractions. Both fractions were subjected to immunoprecipitation–recapture with antibodies to GGA2 or GGA3. D, A cytosolic fraction obtained as in C was loaded onto a 4–20% sucrose gradient and centrifuged in an SW-41 Beckman rotor at 39,000 rpm for 17 h at 4°C. Collected fractions were analyzed for GGA2 and GGA3 by immunoprecipitation–recapture. The positions of protein standards and the AP-4 complex in the gradient are indicated by arrows and an arrowhead, respectively.

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**GAE domains of GGA2 and GGA3.** Only the antibody to GGA3 was found to detect the endogenous antigen under these conditions (Fig. 3). This antibody gave punctate staining of a juxtanuclear structure resembling the Golgi complex (Fig. 3, A, D, G, J, M, and P). Staining was judged to be specific, as it could be inhibited by addition of a GST-GGA2\_GAE fusion protein (Fig. 3 B) but not by GST-GGA2\_GAE (Fig. 3 C), GST-\gamma-2-adaptin\_GAE (data not shown) or GST (Fig. 3 A).

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somes, but rather to dissociation into the cytosol. Removal of BFA from the culture medium resulted in reassociation of GGA3 with both the juxtanuclear and peripheral structures (Fig. 5 C), indicating that the effects of the drug were reversible. Similar effects of BFA were observed for Myc-GGA1 and GGA2 expressed by transfection into HeLa cells (data not shown).

Another test for the involvement of ARF1 in recruitment of protein coats to membranes is the expression of dominant-negative ARF1 mutants. Overexpression of such dominant-negative mutants renders endogenous ARF1 inactive by sequestering guanine nucleotide exchange factors required for their activation (Peyroche et al., 1999); this in turn elicits dissociation of protein coats similar to that caused by BFA (Dascher and Balch, 1994; Peters et al., 1995; Ooi et al., 1998). We observed that overexpression of dominant-negative ARF1/ T31N in HeLa cells resulted in disappearance of endogenous GGA3 from its juxtanuclear location (Fig. 5, D and E). A dominant-negative ARF5/T31N mutant had a simi-
lar, albeit less dramatic, effect (data not shown), consistent with the reduced potency of this construct in the regulation of other protein coats (Ooi et al., 1998). Overexpression of a dominant-negative ARF6/T27N construct, on the other hand, had no effect on GGA3 association with the juxtanuclear structure (Fig. 5, F and G). Taken together, the effects of BFA and the ARF1 dominant-negative mutant suggest that association of GGA with membranes is regulated by ARF1.

Targeting to the TGN Mediated by the GAT Region

We next sought to identify the region of GGA3 responsible for targeting this protein to the TGN. Expression constructs were made by fusing the green fluorescent protein (GFP) to the VHS domain alone, the VHS domain plus GAT region, the GAT region alone, or the GAE domain, as shown in Fig. 6. The constructs were expressed by transfection into HeLa cells, and their intrinsic fluorescence used to compare their localization in moderately expressing cells relative to endogenous TGN46. We observed that the VHS (Fig. 6, A–C) or GAE (Fig. 6, J–L) domain constructs were cytosolic, whereas the VHS plus GAT (Fig. 6, D–F) or GAT region (Fig. 6, G–I) constructs colocalized with TGN46. This experiment thus demonstrated that the GAT region alone (~160 amino acids) is sufficient for targeting a reporter protein to the TGN. Treatment with BFA also caused rapid dissociation of GFP-GAT from the TGN (Fig. 6, M–O).

Dissociation of ARF-regulated Coats Induced by Overexpression of the GAT Region

In the course of the experiments described above, we noticed that a fraction of the transiently transfected cells expressing the highest levels of GFP-GAT or GFP-VHS-GAT (typically 5–10%) accumulated these proteins in the cytosol, probably due to saturation of binding sites at the Golgi complex (Fig. 7, A, C, E, and I). Strikingly, these cells exhibited dissociation of AP-1, AP-3, AP-4, and COPI, as well as endogenous GGA3, from their corresponding intracellular membranes (Fig. 7, A–F, and data not shown). In contrast, the localization of AP-2 was not affected by overexpression of any of these constructs (data not shown). Overexpression of GFP-VHS or GFP-GAE constructs did not affect the distribution of the protein coats mentioned above (see for example Fig. 7, G and H). Thus, the effects of overexpressing the GAT region (or proteins having this domain) are reminiscent of manipulations that inactivate endogenous ARF1, which suggests a possible functional relationship of the GAT region to ARF1.

We also observed that high expression levels of GFP-GAT (Fig. 7, I and J), GFP-VHS-GAT, but not GFP-VHS (data not shown), caused a redistribution of TGN46 to a more diffuse peripheral location. The nature of this location was not established.

Interaction of the GAT Region with Activated ARF1

The fact that overexpression of proteins having the GAT
region caused redistribution specifically of coats regulated by ARF1 prompted us to test whether the GAT region or other portions of the GGA proteins interact directly with ARF1. To this end, we used the yeast two-hybrid system, by cotransforming a suitable yeast strain with various combinations of ARF1 and GGA-domain plasmid constructs. Interactions were assayed by complementation of histidine auxotrophy in the absence or presence of 3-amino-1,2,4-triazole (3AT) and by expression of β-galactosidase activity (Fig. 8 A). We observed that GGA3-derived VHS-GAT and GAT constructs, but not VHS or GAE constructs, interacted with the constitutively activated ARF1 mutant, ARF1/Q71L (Fig. 8 A). Interactions were strong, as growth could still be observed in the presence of 10 mM 3AT (Fig. 8 A), and β-galactosidase activity developed after just 30 min. VHS-GAT constructs derived from GGA1 and GGA2 also interacted with ARF1/Q71L (data not shown). In contrast, the inactive ARF1 mutant, ARF1/T31N, failed to interact with VHS-GAT or GAT constructs from GGA3 (Fig. 8 A), GGA1, or GGA2 (data not shown) in both the growth and β-galactosidase assays. The yeast two-hybrid assays were corroborated using in vitro binding assays in which recombinant ARF1/Q71L, or native ARF from bovine brain cytosol, were tested for interaction with GST-fusion proteins comprising different regions of GGA3 (Fig. 8 B). The GST-fusion protein having the VHS-GAT segment, but not those having the VHS or GAE regions, bound both recombinant ARF1/Q71L and bovine brain ARF upon activation with GTPγS (a nonhydrolyzable GTP analogue). In contrast, we did not observe binding in samples that had been mock-activated in the absence of GTPγS (Fig. 8 B). Furthermore, neither of two other GTP-binding proteins, Rab4 and dynamin 1, interacted with the VHS-GAT segment (Fig. 8 B). Taken together, the above experiments demonstrated that the GAT region is capable of interacting with activated ARF1.
Disruption of Both Yeast GGA Genes Impairs CPY Sorting to the Vacuole

The predominant localization of the human GGA s to the TGN pointed to their possible role in sorting at this organelle. To test this hypothesis, the yeast GGA genes were disrupted by homologous recombination. Both single (gga1Δ and gga2Δ), as well as the double (gga1Δ/gga2Δ), mutant strains were viable and grew at rates indistinguishable from that of the wild-type parental strain at 23, 30, and 37°C (data not shown). To investigate a possible sorting defect in the mutant strains, we examined the trafficking of the vacuolar enzyme, CPY.

Immunoblot analysis revealed that the gga1Δ and gga2Δ strains contained mostly mature, 61-kD CPY, similar to the parental wild-type strain (Fig. 9A). The gga1Δ/gga2Δ strain, on the other hand, displayed a reduced amount of mature CPY and increased amounts of larger CPY species, particularly the Golgi precursor form (p2; Fig. 9A).
Pulse–chase and immunoprecipitation analyses showed that all of the CPY was processed from the p1 and p2 precursors to the mature form within 15 min of chase in the wild-type strain (Fig. 9 B). In contrast, only ~50% of the CPY precursors were processed to the mature form, even after 30 min of chase in the gga1Δ/gga2Δ strain, with the rest accumulating as the Golgi precursor form p2 (Fig. 9 B). These analyses thus demonstrated that mutation of both GGA genes results in delayed processing of p2 to mature CPY (Fig. 9 B). In addition, a colony blotting assay revealed significant secretion of immunoreactive CPY in the gga1Δ/gga2Δ strain (Fig. 9 C). Thus, the delayed processing of CPY to the mature, vacuolar form was likely due to impaired transport to the vacuole and partial mis-sorting to the periplasmic space.

Discussion

A assembly of protein coats involved in vesicle formation and cargo selection is a complex process requiring the concerted action of many structural and regulatory proteins. These proteins establish dynamic networks of low affinity interactions that cooperatively lead to the assembly of stable coats on membranes. Most proteins involved in coat assembly have a modular structure, which enables them to interact simultaneously with multiple other proteins and/or membrane lipids. The three human and two yeast members of the GGA family reported fit this description, as they are composed of at least four distinct regions referred to as VHS, GAT, linker and GAE. Despite the homology of this latter region to the ear domain of the γ1-adaptin subunit of AP-1, the GGAs represent a novel type of adaptor-related proteins that are not part of AP complexes.

The VHS domain has been previously identified in a group of proteins thought to play roles in protein trafficking and/or signal transduction (Lohi and Lehto, 1998, and references therein). Although some of these proteins have been localized to endocytic vesicles (Piper et al., 1995; Asao et al., 1997; Komada et al., 1997; Lohi et al., 1998), it is currently unclear whether the VHS domain can function as a localization determinant. In this regard, our experiments show that a GFP fusion protein containing the VHS domain of GGA3 is cytosolic, suggesting that this domain is insufficient to confer association with intracellular membranes.

Immediately adjacent to the COOH terminus of the VHS domain of the GGA proteins is a region that we call GAT. The three human GGA proteins are highly homologous in the GAT region, and also exhibit significant, albeit more limited, homology to analogous regions in the two yeast Ggaps and the human TOM1 and TOM1L1 proteins (Seroussi et al., 1999). We report here that, unlike the VHS domain of GGA3 is cytosolic, suggesting that this domain is insufficient to confer association with intracellular membranes.

Figure 7. Effects of overexpression of GFP-GAT or GFP-VHS on protein coats and TGN46 analyzed by fluorescence microscopy. HeLa cells were transfected with plasmids encoding GFP fused to either the GAT (A–F, I and J) or VHS (G and H) regions of GGA3. Cells were fixed, permeabilized, and incubated with the 100/3 antibody to the γ1-adaptin subunit of AP-1 (B and H), the β3C1 antibody to the β3A subunit of AP-3 (D), or rabbit antibodies to the β-COP subunit of COPI (F) or TGN46 (J). Bound antibodies were revealed by staining with either Cy3-conjugated donkey anti–mouse IgG (B and H) or Cy3-conjugated donkey anti–rabbit IgG (D, F, and J). A, C, E, and I, GFP-GAT; G, GFP-VHS. Arrows point to cells that overexpress the GFP constructs. Bar, 10 μm.
The intervening segment between the GAT region and GAE domain of both human GGA s and yeast Gga1p is rich in proline and serine residues and has a high probability of random coils. These features suggest that this region may act as a flexible hinge that links the VHS-GAT and GAE domains of the proteins. The linker region of human GGA2 contains the sequence LIDLE, and that of yeast Gga1p the sequences LIDFD and LLDFD, all of which conform to the motif L(D,E,N)(L,F,D,E) implicated in binding to the clathrin terminal domain (Dell’Angelica et al., 1998). Imperfect variants of this motif are actually capable of mediating interactions of GGA s with clathrin. Whether any of these motifs are actually capable of mediating interactions of GGA s with clathrin will have to be investigated experimentally.

By analogy with the ear domains of the α/γ/β/δ ε AP subunits, the GAE domain of GGA s could interact with regulatory molecules. Indeed, GST pulldown assays have shown that the GAE domains of GGA s and γ-adaptins bind a number of cytosolic proteins, some of which may be involved in coated vesicle formation (Hirst et al., 2000).

Our immunoelectron microscopy and subcellular fractionation analyses suggest that GGA s are components of protein coats that cycle between the cytosol and membranes. Moreover, the dissociation of GGA3 or GFP-GAT from membranes induced by treatment with BFA or expression of a dominant-negative ARF1, as well as the direct interaction of the GAT domain with activated ARF1, suggest that recruitment of GGA s to membranes is regulated by the ARF GTP/GDP cycle. Since the GGA s do not contain domains that are characteristic of guanine nucleotide exchange factors or GTPase activating proteins, they are most likely to function as ARF effectors. This places the GGA s in the same category as other ARF1-regulated protein coats, such as COPI, AP-1, AP-3, and AP-4. It remains to be established, however, whether GGA s are themselves structural components of novel coats, or regulators of the assembly of other coats.

Immunofluorescence and immunoelectron microscopy analyses suggest that GGA3 is mainly associated with the TGN and may therefore be involved in trafficking events at this organelle. We have also noticed faint punctate staining for GGA3 in the peripheral cytoplasm and partial colocalization of GGA3 with A P-1 and A P-3 on immunofluorescence microscopy; this suggests the existence of an additional pool of GGA3 associated with peripheral (perhaps endosomal) structures. The GFP-GAT chimera also shows good colocalization with TGN markers. However, its pattern of staining is continuous rather than punctate. A possible explanation for this observation is that GFP-GAT is properly targeted to the TGN, but lacks the ability to concentrate in coated vesicles and buds.

Overexpression of GFP-GAT, but not GFP fusion proteins having other domains of GGA3, causes dissociation of ARF1-regulated coats from membranes. This correlates well with the ability of the GAT region to interact with activated ARF1. This could mean that GGA s play roles in the ARF1-mediated deposition of protein coats onto membranes. A more likely explanation, however, is that overexpression of GFP-GAT either sequesters activated ARF1 or blocks a common effector binding site within the ARF1 molecule, thus preventing recruitment of other coats to membranes.

The existence of two GGA homologues in yeast has allowed us to assess the function of these proteins in vivo.

**Figure 8.** Binding of activated ARF1 to the GAT domain of GGA3. A, Two-hybrid assays. Yeast transformants expressing the combinations of constructs indicated in the figure were spotted onto plates lacking leucine and tryptophan, with or without histidine (+His and −His, respectively), and in the absence or presence of 10 mM 3AT. Filter β-galactosidase (β-gal) assays were performed on cells grown in the presence of histidine. Notice the growth in the absence of histidine and the positive β-galactosidase activity of transformants expressing the activated ARF1 mutant (Q71L) and the GGA3 constructs that include the GAT domain. B, In vitro binding assays. Bovine brain cytosol and recombinant ARF1/Q71L were either treated with 0.1 mM GTP•γS (+GTP•γS) or mock-treated (−GTP•γS), and subsequently incubated with glutathione-Sepharose beads containing GST or GST-fusion proteins comprising the VHS, VHS-GAT, or GAE domains of human GGA3. Bound proteins were analyzed by immunoblotting, using the 1D9 antibody to ARF or antibodies to the Rab4 and dynamin I GTP-binding proteins. Notice the GTP•γS-dependent binding of bovine brain ARF1, and of recombinant ARF1/Q71L, to the GST-fusion protein bearing the VHS-GAT segment, but not the VHS or GAE regions, of GGA3.
Double-mutants of genes encoding these proteins displayed delayed processing of CPY from a Golgi to a vacuolar form. In addition, we observed secretion of immunoreactive CPY from a Golgi to a vacuolar form. This secretion was compatible with impairment of transport to the vacuole, a phenotype characteristic of vps mutants (H orazovsky et al., 1995; Conibear and Stevens, 1998). M utation of either of the two yeast GGA genes individually had little or no effect on CPY processing and secretion, suggesting that both genes produce similar functions. Perhaps this is also the explanation for the observation that the TGN in an ARF1-dependent manner, and involved in protein trafficking at this organelle.

In conclusion, our studies of human and yeast GGA’s suggest that these proteins are components of coats associated mainly with the TGN in an ARF1-dependent manner, and involved in protein trafficking at this organelle.

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References

A sao, H. Y. Sasaki, T. Arita, N. Tanaka, K. Endo, H. Kasai, T. Tashihita, Y. Endo, T. Fujita, and K. Sugamura. 1997. Hrs is associated with STAM, a signal-transducing adaptor molecule. Its suppressive effect on cytokine-induced cell growth. J. Biol. Chem. 272:32706–32711.

Barr, F. A. 1989. A novel rab-interacting domain defines a family of Golgi-targeted coiled-coil proteins. Curr. Biol. 9:381–384.

Berberen, G., J. Dumont, V. Gilleit, P. A. Bolle, and F. Hilger. 1991. The Ydp plasmids: a uniform set of vectors bearing versatile gene disruption cassettes for Saccharomyces cerevisiae. Yeast. 7:475–477.

Boman, A. L., and R. A. Kahn. 1995. A r proteins: the membrane traffic police? Trends Biochem. Sci. 20:147–150.

Boman, A. L., C.-J. Zhang, X. Zhu, and R. A. Kahn. 2000. A family of A r effecors that can alter membrane transport through the trans-Golgi. Mol. Biol. Cell. In press.

Bonifacino, J. S., and E. C. Dell’A nigelica. 1999. Molecular bases for the recognition of tyrosine-based sorting signals. J. Cell Biol. 145:293–326.

Conibear, E., and T. H. Stevens. 1998. Multiple sorting pathways between the late Golgi and the vacuole in yeast. Biochim. Biophys. Acta. 1404:211–230.

Daucher, C., and W. E. Balch. 1994. Dominant inhibitory mutants of ARF1 block endoplasmic reticulum to Golgi transport and trigger disassembly of the Golgi apparatus. J. Cell Biol. 269:1437–1448.

Dell’A nigelica, E. C., H. O hno, C. E. Ooi, E. Rabinovich, K. W. Roche, and J. S. Bonifacino. 1997. A P-3: an adaptor-like protein complex with ubiquitous expression. EMBO J (Eur. Mol. Biol. Organ.) 15:917–928.

Dell’A nigelica, E. C., J. Klumperman, W. Stoorvogel, and J. S. Bonifacino. 1998. A association of the A P-3 adaptor complex with clathrin. Science. 280:431–434.

Dell’A nigelica, E. C., M. Mullins, and J. S. Bonifacino. 1999a. A P-4, a novel protein complex related to clathrin adaptors. J. Biol. Chem. 274:7279–7285.

Donaldson, J. G., D. Finazzi, and R. D. Klausner. 1992b. Brefeldin A inhibits coatomer protein -G to Golgi membranes. Proc. Natl. Acad. Sci. USA. 89:6406–6412.

Donaldson, J. G., D. Finazzi, and R. D. Klausner. 1992c. Bre feldin A inhibits Golgi membrane-catalysed exchange of guanine nucleotide onto A R F protein. Nature. 360:350–352.

Fauzade, V., J. T. Horng, and R. B. Kelly. 1998. A function for the A P3 coat complex in synaptic vesicle formation from endosomes. Cell. 93:423–432.

Hampton, R. Y., and J. Rine. 1994. Regular degradation of H MG-CoA reductase, an integral membrane protein of the endoplasmic reticulum, in yeast. J. Biol. Chem. 269:125–312.

Herman, P. K., and S. D. Emr. 1990. Characterization of V PS34, a gene required for vacuolar protein sorting and vacuole segregation in Saccharomyces cerevisiae. Mol. Cell. Biol. 10:6742–6754.

Heuser, J. E., and J. Krenn. 1988. Deep-etch visualization of proteins involved in clathrin assembly. J. Cell Biol. 107:877–880.

Hirst, J., and M. S. Robinson. 1998. Clathrin and adaptors. Biochim. Biophys. Acta. 1404:173–193.

Hirst, J., N. A. Bright, B. Rous, and M. S. Robinson. 1999. Characterization of a fourth adapt or-related coat complex. J. Biol. Chem. 274:2871–2877.

Hirst, J., W. Y. Lui, N. A. Bright, N. Totty, M. N. Seaman, and M. S. Robinson. 2000. A family of proteins with γ-adaptin and V HS domains that facilitate trafficking between the trans-Golgi network and the vacuole/lysosome. J. Cell Biol. 149:67–79.

Horazdovsky, B. F., D. B. Devy d, and M. S. Robinson. 1995. Protein transport to the vacuole. Curr. Opin. Cell Biol. 7:544–551.

Ikuhara, T., J. S. Bonifacino, and H. Riezman. 1997. Linking cargo to vesicle formation: receptor tail interactions with coat proteins. Curr. Opin. Cell Biol. 9:489–495.

Komada, M., R. Masaki, A. Yamamoto, and N. Ikamura. 1997. Hrs, a tyrosine kinase substrate with a conserved dual zinc finger domain, is localized to the cytoplasmic surface of early endosomes. J. Biol. Chem. 272:20538–20544.

Kunisada, Y., H. A. Brown, M. G. Waters, P. C. Sternweis, and M. G. Roth. 1996. Evidence that phospholipase D mediates ADP ribosylation factor-dependent formation of Golgi-coated vesicles. J. Cell Biol. 134:295–306.

Lewin, D. A., D. Sheff, C. E. Ooi, J. W. Whitney, E. Yamamoto, L. M. Chicheon, P. Webster, J. S. Bonifacino, and J. M. Elmamal. 1998. Cloning, exp onentiation, and localization of a novel γ-adaptin-like molecule. F E B S Laett. 435:263–268.

Li, l., D. Small, and S. Kornfeld. 1997. Comparative activity of ADP-ribosylation factor family members in the early steps of coated vesicle formation on rat liver Golgi membranes. J. Biol. Chem. 272:4141–4148.

Lippincott-Schwartz, J., N. B. Cole, and J. M. Dalsgard. 1998. Building a secretory apparatus: role of A R F and COPI in Golgi biogenesis and maintenance. Histochem. Cell Biol. 109:449–462.

Lottus, B. J., U. J. Kim, V. P. Sneddon, F. Kalus, R. Brandon, J. Fuhrmann, T.

Figure 9. Effects of disruption of yeast GGA genes on CPY processing and secretion. A , Immunoblot analysis of CPY in whole-cell lysates of wild-type, gga1Δ, gga2Δ, and gga1Δ/ gga2Δ strains. The positions of the p2 and m forms of CPY are indicated. The asterisk indicates an unidentified intermediate species. B, Wild-type and gga1Δ/ gga2Δ strains were pulse-labeled with [35S]methionine for 10 min at 30°C and then chased for 0, 15, or 30 min at 30°C. CPY was isolated by immunoprecipitation and analyzed by SDS-PAGE and fluorography. The positions of the p1, p2, and m forms of CPY are indicated. C, Colony blotting analysis of CPY secretion. Nitrocellulose replicas of streaks of the strains mentioned in A and of a vps1 mutant strain (positive control) were probed with anti-CPY antiserum to reveal secreted CPY.
Mason, M. L., Crosby, M., Barnstead, L., Cronin, et al. 1999. Genome duplications and other features in 12 Mb of DNA sequence from human chromosome 16p and 16q. Genomics. 60:295–308.

Lohi, O., and V.P. Lehto. 1998. VHS domain marks a group of proteins involved in endocytosis and vesicular trafficking. FEBS Lett. 440:255–257.

Lohi, O., A. Poussu, J. Merilainen, S. Kellokumpu, V. M. Wasenius, and V. P. Lehto. 1998. EA ST, an epidermal growth factor receptor- and Epst51-associated protein with Src homology 3 and tyrosine-based activation motif domains. J. Biol. Chem. 273:21408–21415.

Meliman, I. 1996. Endocytosis and molecular sorting. Annual Rev. Cell Dev. Biol. 12:575-625.

Moss, J., and M. Vaughan. 1998. Molecules in the ARF orbit. J. Biol. Chem. 273:21431–21434.

Mullins, C., Y. Lu, A. Campbell, H. Fang, and N. Green. 1995. A mutation affecting signal peptidase inhibits degradation of an abnormal membrane protein in Saccharomyces cerevisiae. J. Biol. Chem. 270:17139–17147.

Nagase, T., N. Seki, A. Tanaka, K.-I. Ishikawa, and N. Nomura. 1995. Prediction of the coding sequences of unidentified human genes. IV. The coding sequences of 40 new genes (KIAA0011-KIAA0050) deduced by analysis of cDNA clones from human cell line KG-1. DNA Res. 2:167–174.

Ooi, C.E., E.C. Dell’Angelica, and J. S. Bonifacino. 1998. ADP-ribosylation factor 1 (ARF1) regulates recruitment of the AP-3 adaptor complex to membranes. J. Cell Biol. 142:391–402.

Palmer, D. J., J. B. Helms, C. J. Beckers, L. Orci, and J. E. Rothman. 1993. Binding of coatomer to Golgi membranes requires ADP-ribosylation factor. J. Biol. Chem. 268:12083–12089.

Peters, P. J., V. W. Hsu, C. E. Ooi, D. Finazzi, S. B. Teal, V. Oorschot, J. G. Donaldson, and R. D. Klausner. 1995. Overexpression of wild-type and mutant ARF1 and ARF6: distinct perturbations of nonoverlapping membrane compartments. J. Cell Biol. 129:1003–1017.

Peyroche, A., B. Atonny, S. R. Ribeiro, J. Acker, J. Cherfils, and C. L. J. Jackson. 1999. Brefeldin A acts to stabilize an abortive ARF-GDP-Sec7 domain protein complex: involvement of specific residues of the Sec7 domain. Mol. Cell. 3:275–285.

Piper, R.C., A.A. Cooper, H. Yang, and T.H. Stevens. 1995. VPS27 controls vacuolar and endocytic traffic through a prevacuolar compartment in Saccharomyces cerevisiae. J. Cell Biol. 131:603–617.

Radakrishna, H., R. D. Klausner, and J. G. Donaldson. 1996. A luminal fluoride stimulates surface protrusions in cells overexpressing the ARF6 GTPase. J. Cell Biol. 134:935–947.

Roberts, C.J., C.K. Rayment, C.T. Yamashiro, and T.H. Stevens. 1991. Methods for studying the yeast vacuole. Methods Enzymol. 194:644–661.

Robinson, M.S. 1990. Cloning and expression of γ-adaptin, a component of clathrin-coated vesicles associated with the Golgi apparatus. J. Cell Biol. 111:2139–2156.

Robinson, M.S., and T. E. Kreis. 1992. Recruitment of coat proteins onto Golgi membranes in intact and permeabilized cells: effects of brefeldin A and G protein activators. Cell. 69:129–138.

Rothman, J. E., and F. T. Wieland. 1996. Protein sorting by transport vesicles. Science. 272:227–234.

Schekman, R., and L. Orci. 1996. Coat proteins and vesicle budding. Science. 271:1526–1533.

Sereoussi, E., D. Kedra, M. K. A limova, A. C. S. Nordqvist, I. Fransson, J. F. Jacobs, Y. Fu, H. Q. Pan, B. A. R. Roe, S. Imreh, and J. P. Dumanski. 1999. TOM1 genes map to human chromosome 22q13.1 and mouse chromosome 8C1 and encode proteins similar to the endosomal proteins HGS and STAM. Genomics. 57:380–388.

Simonsen, A., R. Lippe, S. Christoforidis, J. M. Gauthier, A. Brech, J. Callaghan, B. H. Toh, C. M. Murphy, M. Zerial, and H. Stenmark. 1998. EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. Nature. 394:494–498.

Slot, J. W., and H. J. Geuze. 1983. The use of protein A–conjugated gold (PAG) complexes as immunolabels in ultrathin frozen sections. In Immunocytochemistry. A. C. Cuello, editor. J. Wiley & Sons, Chichester. 323–346.

Stamnes, M. A., and J. E. Rothman. 1993. The binding of AP-1 clathrin adaptor particles to Golgi membranes requires ADP-ribosylation factor, a small GTP-binding protein. Cell. 73:999–1005.

Takatsu, H., S. Michinari, H. Y. Shin, K. Murakami, and K. Nakayama. 1998. Identification and characterization of novel clathrin adaptor-related proteins. J. Biol. Chem. 273:24693–24700.

Traub, L. M. J., A. Ostrom, and S. Kornfeld. 1993. Biochemical dissection of AP-1 clathrin adaptor complexes as immunolabels in ultrathin frozen sections. In Immunocytochemistry. A. C. Cuello, editor. J. Wiley & Sons, Chichester. 323–346.

Zhao, L., J. B. Helms, B. Brugger, C. Harter, B. Martoglio, R. Graf, J. Brunner, J. W. Slot, and H. J. Geuze. 1983. The use of protein A–colloidal gold (PAG) complexes as immunolabels in ultrathin frozen sections. In Immunocytochemistry. A. C. Cuello, editor. J. Wiley & Sons, Chichester. 323–346.