# CHAPTER III: GAT (GGA and Tom1) Domain Responsible for Ubiquitin Binding and Ubiquitination

# ABSTRACT

GGAs are a family of monomeric adaptor proteins involved in membrane trafficking from the *trans*-Golgi network to endosomes. The GAT domains of GGAs have previously been shown to interact with GTP-bound ARF and to be crucial for membrane recruitment of GGAs. Here I show that the C-terminal subdomain (C-GAT) of the GAT domain, which is distinct from the N-GAT subdomain responsible for ARF binding, can bind ubiquitin. The binding is mediated by interactions between residues on one side of the α3 helix of the GAT domain and those on the so-called Ile44 surface patch of ubiquitin. The binding of the GAT domain to ubiquitin can be enhanced by the presence of a GTP-bound form of ARF. Furthermore, GGA itself is ubiquitinated in a manner dependent on the GAT-ubiquitin interaction. These results delineate the molecular basis for the interaction between ubiquitin and GAT, and suggest that GGA-mediated trafficking is regulated by the ubiquitin system as endosomal trafficking mediated by other ubiquitin-binding proteins.

# INTRODUCTION

GGAs are a family of monomeric adaptor proteins involved in membrane trafficking from the TGN to endosomes. There are three GGAs in humans, and they regulate clathrin-mediated trafficking of cargo proteins from the TGN to endosomes.

The GAT domain is conserved in GGA and Tom1 (target of Myb 1) (see Fig. 4 in GENERAL INTRODUCTION). Tom1 was originally identified as a protein whose expression was induced by v-Myb (97). Although Tom1 also contains the VHS domain, which also conserved in GGAs, its function is currently unknown; neither does its VHS domain binds to the ACLL motif nor does its GAT domain to ARF. In the course of searching for binding partners of Tom1, it has been found that the GAT domain of Tom1 interacts with ubiquitin. In CHAPTER III, I showed the GAT domain of GGAs also bind ubiquitin.

Ubiquitin is a highly conserved protein of 76 amino acids in all eukaryotic cells that is covalently conjugated to other proteins through an isopeptide bond between its C-terminal glycine and the ε-amino group of lysine residues in substrate proteins (Fig. III-1). Ubiquitin itself can function as an acceptor, through several of its seven lysine residues, to form a polyubiquitin chain. The canonical view is that ubiquitin modification (ubiquitination) of cytosolic proteins, when it is present as a polyubiquitin chain of four or more ubiquitins that are linked through lysine 48 functions as a general device for the targeting of proteins for proteolysis by the 26S proteasome. However, proteins can also be monoubiquitinated (98-101). Monoubiquitination of many cell-surface nutrient and ion transporters and signal-transducing receptors appears not to play a role in proteasomal breakdown; rather ubiquitination of these proteins serve as a signal for their internalization and sorting into inward-budding vesicles in the late endosome compartments, which give rise to multivesicular bodies (MVBs), and subsequent proteolysis in the lysosome. In mammalian cells, ubiquitin-dependent transport to the MVBs/lysosomes has so far been

demonstrated only along the endocytic pathway, such as that of the EGF receptor. However, in yeast, ubiquitinated transmembrane proteins are sorted into lumenal vesicles of the MVB and/or vacuole (equivalent to mammalian lysosomes) along both the endocytic and biosynthetic pathways.

A prerequisite for the sorting is the existence of components that specifically recognize the ubiquitinated proteins. To date, at least five classes of ubiquitin binding modules have been identified. Cells transmit the ubiquitination signals through proteins containing the conserved ubiquitin-binding modules, including the UBA (ubiquitin-associated domain), CUE (coupling of ubiquitin to endoplasmic reticulum degradation domain), UIM (ubiquitin interacting motif), UEV (ubiquitin E2 variant domain), and NZF (Npl4 zinc finger domain) (Ref. 101, and references therein). One or combinations of these modules are often found in proteins implicated in endocytic processes, such as the MVB pathway.

Proteins containing these modules bind and sort ubiquitinated proteins. An intriguing feature is that the ubiquitin-binding proteins often undergo monoubiquitination. For example, it has been shown that the presence of functional UIMs is a prerequisite for monoubiquitination of UIM-containing proteins. Importantly, because the UIMs themselves do not contain any lysine residues that serve as acceptors of ubiquitin, its requirement for monoubiquitination must associate with another aspect of the process, most likely the recognition of ubiquitin ligases.

Despite the studies in yeast, there has been no evidence for the existence of the ubiquitin system that regulates sorting at the TGN in mammalian cells. Therefore, I investigated the interaction between GGAs and ubiquitin to shed light on the new ubiquitin system that functions at the TGN.

# MATERIALS AND METHODS

*Plasmid Construction* — Vectors for domains of human GGA1 (VHS+GAT (residues 1-327); VHS (1-147); GAT (141-327); C-terminal region (306-639), GGA2 (GAT(157-342)), and GGA3L (VHS+GAT (1-320); VHS (1-146); GAT (140-320); C-terminal region (304-723)) were constructed as described previously (10,14). Deletions of the GGA3-GAT domain (C-GAT (209-320); α2+α3 (236-320); Δα3 (139-275)) were constructed by a PCR-based strategy. For expression in mammalian cells, the entire coding region or a domain fragment of the GGA cDNA was subcloned into pcDNA3 with an N-terminal HA- or $His_6$+FLAG-tag sequence (61).

*Two-hybrid Screening* — A human brain cDNA library (~1.1 X $10^6$ clones) was screened using the Tom1 VHS+GAT domain (residues 1-316) as bait in a manner described previously (37, 43). To identify GAT mutants defective in ubiquitin binding, a cDNA fragment for the GGA3 C-GAT subdomain was subjected to error-prone PCR, then to a reverse two-hybrid screening using ubiquitin as prey in a manner described in CHAPTER II.

*Pull-down Assays* — GST-fusion proteins (50 pmole) of GGA domains purified from *E. coli* BL21(DE3) cells were incubated with Ub- (15 μl) or protein A-agarose (30 μl) beads (Sigma) for 1 h at room temperature in buffer A (25 mM Hepes, pH 7.4, 125 mM KOAc, 2.5 mM MgOAc, 5 mM EGTA, 1 mM DTT) containing 0.1% BSA and 0.1% Triton X-100. The beads were then pelleted and washed three times with buffer A. Proteins associated with the beads were subjected to immunoblotting with anti-GST antibody (Sigma). Lysates from *E. coli* cells expressing $His_6$+T7-tagged GGA3-GAT was pulled down with GST-Ub as above and detected by anti-T7-tag antibody (Novagen, Madison, WI). To examine GAT interaction with ubiquitinated proteins, rat liver cytosol (containing 2 mg protein) was incubated with GST fusion proteins (~20 μg) of the GGA domain pre-bound to glutathione-Sepharose beads for 2 h at 4°C in buffer A containing a protease inhibitor mixture

(Complete™ EDTA-free). Proteins associated with the beads were subjected to immunoblotting with mouse monoclonal antibody to ubiquitin-conjugated proteins, FK2 (Affiniti Research Products, Ltd., Devon, UK). To examine ubiquitin interaction with endogenous GGA3, a cytosol or membrane fraction of HeLa cells (containing 350 μg protein) was incubated with GST-Ub pre-bound to glutathione-Sepharose at 4°C for 2 h in buffer A containing Complete™ EDTA-free. The proteins associated with the beads were subjected to immunoblotting with anti-GGA3 antibody (BD Biosciences, San Diego, CA).

The effect of ARF on the GAT-ubiquitin interaction was examined as follows. A mixture of multi-ubiquitin chain (2 μg; Affiniti Research Products) and varying amounts of purified recombinant ARF1ΔN17 (43) was incubated at room temperature for 30 min in 200 μl of buffer B (25 mM Hepes, pH 7.0, 100 mM NaCl, 1 mM $MgCl_2$, 2 mM EDTA, 0.1% Nonidet P-40) containing 25 μg/ml BSA and 200 μM GDP or GTPγS, and $MgCl_2$ was added to the incubation mixture to a final concentration of 7 mM to stop nucleotide exchange. After addition of 1 μg of recombinant GST-GGA3-VHS+GAT, the mixture was incubated at room temperature for 30 min and further incubated for 30 min after addition of 20 μl glutathione-Sepharose beads. The beads were then pelleted and washed three times with buffer B containing 7 mM $MgCl_2$ and 10 μM GDP or GTPγS. Proteins associated with the beads were subjected to immunoblotting with monoclonal anti-ubiquitin antibody, P4D1 (Santa Cruz Biotechnology).

*Immunofluorescence Analyses* — HeLa cells grown in 8-well Lab Tek-II chamber slides were transfected with an HA-GGA vector using FuGENE6 transfection reagent and incubated for 15-20 h. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, double-stained with monoclonal rat anti-HA antibody 3F10 and the FK2 antibody as described previously (42, 43), and observed with a confocal microscope (LSM 510, Carl

Zeiss, Oberkochen, Germany).

*In Vivo Ubiquitination Analyses* — HEK-293 cells grown on a 10-cm dish were transfected with expression vectors for $His_6$+FLAG-tagged GGA3 and HA-tagged ubiquitin (a kind gift from Dirk Bohmann, EMBL) (102) and incubated for 15-20 h. $His_6$+FLAG-GGA3 was purified by the method of Treler *et al.* (102) with a small modification. Briefly, denatured cell lysates (1 ml) were incubated with 50 μl of $Ni^{2+}$-NTA-agarose (Qiagen, Hilden, Germany) for 3 h at room temperature, washed and eluted with 100 μl of buffer containing 300 mM imidazole. The eluate was subjected to 7.5-20% or 7.5% SDS-PAGE, electroblotted onto Immobilon-P membrane (Millipore), and detected with the anti-HA 3F10 antibody (42, 43). The blot was reprobed with monoclonal mouse anti-FLAG M2 antibody (Sigma).

# RESULTS

*GAT Domains Interact with Ubiquitin* — A two-hybrid screening of a human brain cDNA library using the VHS+GAT domain of human Tom1 as bait yielded 44 positive clones. Among them, six encoded ubiquitin precursors containing one to six tandem ubiquitin units. Subsequent two-hybrid and pull-down analyses revealed that the GAT domain is responsible for the interaction with ubiquitin (data not shown).

To examine whether the ubiquitin binding is a general feature of the GAT domains, I performed a similar experiment with GGAs. As shown in Fig. III-2A, GST fusion proteins containing, but not lacking, the GAT domain of GGA1 was bound to Ub-agarose beads. As shown in Fig. III-2B, the GAT domains of GGA1 and GGA3, but not that of GGA2, were bound to Ub-agarose beads. These data demonstrate that the GAT domains of GGA1 and GGA3 directly interact with ubiquitin. Because the GGA3-GAT binding to ubiquitin was relatively efficient among the GGA-GAT domains examined, I thereafter focused mainly on the GGA3-GAT domain.

I then examined the binding ability of the GAT domains to ubiquitinated proteins. Rat liver cytosol was pulled down with the GST-GAT domains pre-bound to glutathione-Sepharose beads and subjected to immunoblotting using the monoclonal antibody FK2, which recognizes mono- and poly-ubiquitinated proteins but not free ubiquitin (103, 104). As shown in Fig. III-2C, the GAT domains of GGA1 (lane 9) and GGA3 (lane 5) but not that of GGA2 (lane 10) could bind ubiquitinated proteins, being in line with the data in Fig. III-2B. The VHS domain (lane 4) or the C-terminal region (lane 3) did not bind ubiquitinated proteins. It is notable that, compared with the band pattern of ubiquitinated proteins in the original cytosol (lane 11), the GAT domains appeared to bind selected ubiquitin-conjugated proteins (lanes 5 and 9). In a reciprocal experiment, GST-Ub could pull down endogenous GGA3 from both cytosolic and membrane-bound fractions (Fig. III-2D). In addition, expression

of GGA3 at moderate to high levels in cells caused accumulation of ubiquitinated proteins detected with the FK2 antibody in the Golgi region (Fig. III-2E, a and a'), indicating that GGA3 binds ubiquitinated proteins in the cell. The accumulation of ubiquitinated proteins was specific for GGA3, because such accumulation was not observed in cells expressing GGA2 at high levels (data not shown). The GAT-ubiquitin interaction contributes significantly to the GGA3-dependent accumulation of ubiquitinated proteins, because 60% of GGA3-overexpressing cells (n = 50) accumulated ubiquitinated proteins, while 34% of cells (n = 50) overexpressing a GGA3 mutant (see below) with a GAT domain defective in ubiquitin binding accumulated (b and b').

*ARF and Ubiquitin Bind Distinct GAT Subdomains* — Next, I attempted to delineate the region of the GGA3-GAT domain required for ubiquitin binding. Recent X-ray crystallographic studies of the GGA1-GAT domain from several laboratories have revealed that it possesses an α-helical fold comprising two subdomains (106-109) (see Fig. III-3, A and D). The N-GAT or hook subdomain is a helix-loop-helix structure composed of the first short helix α0 (the helix numbering is according to Ref. 108) and the N-terminal half of the second long helix α1 and is responsible for ARF-binding (105, 107, 108). The C-GAT subdomain constitutes a helix bundle composed of the C-terminal half of α1, and α2 and α3 (105-108). Surprisingly, deletion of the N-GAT subdomain significantly enhanced the binding efficiency to ubiquitinated proteins (compare lane 5 for GAT and lane 7 for C-GAT in Fig. III-2C). Deletion of the entire α1-helix abolished the binding to ubiquitinated proteins (lane 8), which presumably makes the three-helix bundle unstable. In addition, deletion of the entire α3-helix abolished the binding (lane 6). These data indicate that the C-GAT subdomain comprising a three-helix bundle is responsible for the ubiquitin binding, and more importantly that ARF and ubiquitin bind distinct subdomains, N-GAT and C-GAT, respectively (Fig. III-3D).

To further define the interface of the GAT domain with ubiquitin, I subjected the C-GAT subdomain to a reverse two-hybrid screening with ubiquitin to identify C-GAT mutants defective in ubiquitin binding. The screening identified six missense mutants (L247P, L262S, L276S, L280R, D284G and Y293H) (Fig. III-3A). Among these mutations, L247P, L262S and Y293H seemed to disrupt the packing of the helix bundle deduced from the structure of the GGA1-GAT domain (data not shown). On the other hand, the side chains of Leu276, Leu280, Asp284 appeared exposed on one side of α3 and could be responsible for protein-protein interactions (see Fig. III-3B). The two-hybrid data were confirmed by a pull-down experiment; neither the L276S, L280R nor D284G mutant was pulled down with Ub-agarose (Fig. III-4A). Moreover, unlike wild type full-length GGA3, its L280R mutant overexpressed in the cell did not cause accumulation of ubiquitinated proteins in the Golgi region (Fig. III-2E, b and b').

*Ubiquitin Binds GAT through its Ile44 Patch* — On ubiquitin, two surface patches have been shown to participate in binding to the proteasome, UIM, UBA and CUE domains and in intracellular trafficking; one patch including Ile44 and the other including Phe4 (98, 101, 109-112). To examine which patch is responsible for the GAT binding, $His_6$+T7-tagged GGA3-GAT was incubated with wild type Ub, Ub(F4A) or Ub(I44A) fused to GST, pulled down with glutathione-Sepharose, and subjected to immunoblotting with anti-T7 tag antibody. The experiment revealed that the GAT-ubiquitin interaction requires Ile44 but not Phe4 on the ubiquitin surface (Fig. III-3B).

*Mode of GAT-Ubiquitin Interaction* — On the basis of the above mutational data and three-dimensional structures of GGA1-GAT and ubiquitin, I constructed the most plausible model for the interaction between GGA3-GAT and ubiquitin by collaborating with Wakatsuki and colleagues (Fig. III-2, B and C); in this model, Asp284 interacts with $Arg42^U$ (the superscript U denotes a residue of ubiquitin), Leu276 with both $Leu8^U$ and $Val70^U$, and Leu280 with

both Ile44$^{U}$ and Val70$^{U}$. To address this model, I constructed additional ubiquitin mutants and examined their interactions with GGA3-GAT. As expected, L8A, R42A and V70A mutations of ubiquitin abolished the GAT binding (Fig. III-4B). These data make it most likely that the GAT domain interacts with ubiquitin in a manner presented in this model (Fig. III-3, B and C).

*ARF Binding to GAT Affects Ubiquitin Binding* — The data presented here along with the previous data (38, 43) show that the GAT domain can interact with ARF and ubiquitin through distinct subdomains, N-GAT and C-GAT, respectively. The interface between GAT and ARF and that between GAT and ubiquitin are sterically separated (Fig. III-3D). Furthermore, GGA3-GAT mutants defective in ubiquitin binding can bind ARF-GTPγS, and a GGA1-GAT mutant defective in ARF binding can bind ubiquitin (data not shown). However, the enhancement of ubiquitin binding by deleting the N-GAT subdomain (compare lanes 5 and 7 in Fig. III-2C) suggests a possibility that some conformational change in the N-GAT subdomain may affect ubiquitin binding to the C-GAT subdomain. To address this possibility, multi-ubiquitin chains were pulled down with the GGA3 VHS+GAT domain in the presence of increasing concentrations of purified ARF1-GDP or -GTPγS (in this experiment, I used VHS+GAT in place of GAT because the former bound ARF more strongly than the latter), and subjected to immunoblotting using the monoclonal anti-ubiquitin antibody P4D1. As shown in Fig. III-5, more ubiquitin chain was pulled down with the GGA3-VHS+GAT domain as increasing the concentration of ARF in the presence of GTPγS. In contrast, the ARF-dependent increase in the ubiquitin binding was marginal in the presence of GDP. The results suggest that the binding of ARF to the N-GAT subdomain might allosterically affect the ubiquitin binding to the C-GAT subdomain.

*GGA Ubiquitination Depends on GAT-Ubiquitin Interaction* — A number of, albeit not all, proteins that bind ubiquitin have been reported to undergo

monoubiquitination (113-117), although the regulatory mechanism underlying the coupling of ubiquitin binding and ubiquitination is currently uncertain. To examine whether this was also the case with GGA, lysates from cells transfected with expression vectors for various $His_6$+FLAG-tagged GGA3 constructs together with that for HA-ubiquitin were precipitated with $Ni^{2+}$-NTA-agarose beads under denaturing conditions and subjected to immunoblotting with anti-HA antibody to detect ubiquitinated GGA3 or with anti-FLAG antibody to confirm the efficiencies of protein expression and precipitation. As shown in Fig. III-6A, full-length GGA3 and constructs containing the GAT domain were efficiently ubiquitinated in the cells. The results indicate that the ubiquitination occurs within the GAT domain. In addition, the difference between the bands detected with anti-FLAG and anti-HA antibodies in size (~8 kDa) indicates monoubiquitination at least in the cases of the GAT and VHS+GAT constructs, although faint bands suggesting di- or tri-ubiquitination are also detectable. The VHS domain was slightly ubiquitinated, although I did not address its significance further. The C-terminal construct that includes the hinge region and the GAE domain was not ubiquitinated at all.

I then examined whether the GAT-ubiquitin interaction is prerequisite for ubiquitination. Unlike wild type, full length GGA3, ubiquitination of the L280R and D284G mutants was extremely reduced in the transfected cells (Fig. III-6, B and C), demonstrating that prior ubiquitin binding to the GAT domain make a major contribution to the GGA ubiquitination.

# DISCUSSION

The GAT domain has attracted attention by its role in ARF binding and concomitant recruitment onto TGN membranes of GGAs. In the present study, I have found that the GAT domains can interact with ubiquitin through the C-GAT subdomain, which is distinct from the N-GAT subdomain responsible for ARF binding. However, the GAT-ubiquitin interaction can be enhanced by ARF-GTP. Furthermore, ubiquitination of GGA3 occurs in a manner dependent on the GAT-ubiquitin interaction.

The N-GAT subdomain is a helix-loop-helix structure (105, 107, 108), whereas C-GAT is a three-helix bundle that resembles the N-terminal domains of syntaxin-1a and its relatives (105-108) and is implicated in interaction with Rabaptin-5 (108, 118). By analogy with SNARE-motif binding sites of the syntaxin N-terminal domains, a hydrophobic patch formed by residues of helices α2 and α3 of GAT was proposed to constitute a protein-protein interaction site (106). I have shown that, together with Asp284, the hydrophobic patch of GGA3-GAT indeed participates in ubiquitin binding.

On the other hand, I have also delineated the GAT-binding interface of ubiquitin by mutational experiments, although our attempts to make a co-crystal of GAT and ubiquitin have been unsuccessful so far. Like interactions with other ubiquitin-binding modules, the Ile44 surface patch of ubiquitin including Leu8, Ile44, Arg42 and Val70 mediates the GAT interaction. While this study was in progress, NMR and X-ray analyses revealed structural bases for ubiquitin interactions with CUE, NZF and UIM (119-122). All the studies pointed to the importance of the Ile44 hydrophobic patch in these interactions. The engagement of the overlapping ubiquitin surface by multiple ubiquitin-binding modules, including CUE, UIM, NZF and GAT, has important implications in membrane trafficking. For example, these ubiquitin-binding proteins might sequentially interact with the same ubiquitinated cargo protein along the transport pathway. Ubiquitination of these ubiquitin-binding proteins

themselves might contribute to the sequential interactions.

In contrast to the GAT domains of GGA1, GGA3 and Tom1, I failed to show ubiquitin binding of GGA2-GAT. In the case of other ubiquitin-binding modules, all the family members cannot interact with ubiquitin (114). However, the residues that I have shown to be essential for ubiquitin binding are identical in all human GGAs. One possible explanation for the apparent discrepancy is that residues other than those I have determined in GGA3-GAT are also essential for ubiquitin interaction but are different from corresponding residues of GGA2-GAT. Alternatively, the GAT domain of GGA2 might be somewhat different from those of GGA3 and GGA1 in the overall structure and be unable to accept ubiquitin. Structural determination of GGA2-GAT and a complex between GGA3-GAT and ubiquitin will help to discriminate between these possibilities.

Another key finding in the present study is that ubiquitinated proteins accumulate in the Golgi region in GGA3-overexpressing cells and the enhancement of binding between GAT and ubiquitin in the presence of the active ARF is observed, suggesting that the ubiquitin system functions in budding process at the TGN. In mammalian cells, there has been no evidence for the existence of biosynthetic pathway regulated by the ubiquitin system in contrast to the endocytic pathway, whereas in yeast, some biosynthetic cargos are sorted into MVB lumenal vesicles in a ubiquitin-dependent manner (123). The vacuolar enzymes, Cps1p and Phm5p, are ubiquitinated and sorted into MVB lumenal vesicles (124, 125). In addition, ubiquitination has been shown to regulate sorting of at least two plasma membrane transporters Gap1p and Tat2p at the level of the TGN or endosomes in response to the quality of the nitrogen source in the growth medium (126-129). In cells growing on poor nitrogen sources, Gap1p, a general amino acid transporter, is expressed at the plasma membrane in an active form, whereas in cells growing in high nitrogen sources, it is directly targeted from the TGN to the vacuole for degradation

without ever being delivered to the plasma membrane (126, 127). It has been reported that Gap1p in the TGN can be regulated by polyubiquitination (126); polyubiquitinated Gap1p is routed from the TGN to the vacuole, whereas the monoubiquitinated form is delivered to the plasma membrane. Such ubiquitination mechanism is also applied to the transport of Tat2p, a tryptophan permease (128, 129). Interestingly, mammalian glucose transporter GLUT4 and GLUT1, are appeared to be regulated by ubiquitin-like modifier protein SUMO-1 (130-132). It is possible that ubiquitin or a ubiquitin-like protein modulates the regulated protein translocation to the cell surface at the level of the TGN or endosomes. Clearly more work is needed to assess this speculation.

It is interesting to know what ubiquitinated proteins the GAT domain bind. The first possibility is that GAT domain might bind ubiquitinated transmembrane cargo proteins on TGN membranes and might regulate their transport processes. In line with this possibility, the GAT-ubiquitin interaction is enhanced by ARF-GTP, which recruits GGAs onto membranes. Secondly, the GAT domain might interact with another adaptor protein with ubiquitin modification to cooperate in trafficking processes. A third possibility is that GAT domain might recruit a ubiquitin-E2 or ubiquitin-E3 intermediate. Like other ubiquitin-binding proteins (113-117), GGA itself is ubiquitinated in a manner dependent on the GAT-ubiquitin interaction (Fig. III-6). Therefore, the temporal interaction between GAT and ubiquitin-E2 or -E3 ligase intermediate could contribute to the GGA3 ubiquitination. A fourth possibility is that the GAT domain recognizes monoubiquitin appended to GGA3 itself. It has been shown that the UBA domain containing protein, Rad23, blocks ubiquitin chain elongation by binding short, substrate anchored ubiquitin chains through its UBA domains (133, 134). Therefore, it is possible that the GAT domain also recognizes monoubiquitin appended to GGA3 itself, and this binding inhibits elongation of the ubiquitin chain on GGA3. The result in Fig. III-6A showing

the existence of monoubiquitinated GGA3 supported this idea. However, if GAT-ubiquitin interaction inhibits elongation of ubiquitin chain on GGA3 itself, polyubiquitination of the GGA3 mutant defective in ubiquitin binding must be observed. As shown in Fig. III-6., B and C, I failed to observe polyubiquitination of the GGA3 mutant even in the presence of proteasome inhibitors and to observe faster degradation of the mutant triggered by polyubiquitination than the wild type GGA3 (Fig. III-6. and data not shown). Therefore, it seems unlikely that the GAT domain intramolecularly interacts with the ubiquitin moiety appended to GGA3. I rather favor the possibility that the GGA3 monoubiquitination could be mediated by GAT and the ubiquitin-E3 ligase intermediate. Future studies are required to show the interaction between the GAT domain and its E3 ligase, and to address why GGA3 polyubiquitination does not occur.

What is the function of monoubiquitination in membrane trafficking? One possibility is that the balance between mono- and poly-ubiquitination regulates the cellular level of proteins involved in trafficking. It has been reported that a yeast coat protein Sec23p, a component of the COPII complex, that is essential for the transport from the ER to Golgi is monoubiquitinated (135). Accumulation of monoubiquitinated Sec23p facilitates its subsequent polyubiquitination and rapid degradation by the proteasome. Its rescue from degradation contributes to adapt a Sec23p expression level that is compatible with an efficient transport. Another report has shown that a COPI subunit β'-COP is also ubiquitinated and the Golgi to ER transport are regulated by the ubiquitination (136). Therefore, it is possible that monoubiquitinated GGA3 facilitates its polyubiquitination and the GGA3 degradation rate determines the TGN to endosome transport. I have also observed that the degradation rate of the GGA3 mutant defective in ubiquitin binding is delayed (data not shown). This result suggested that monoubiquitinated GGA3 could facilitate GGA3 polyubiquination. However, the difference between wild type and mutant

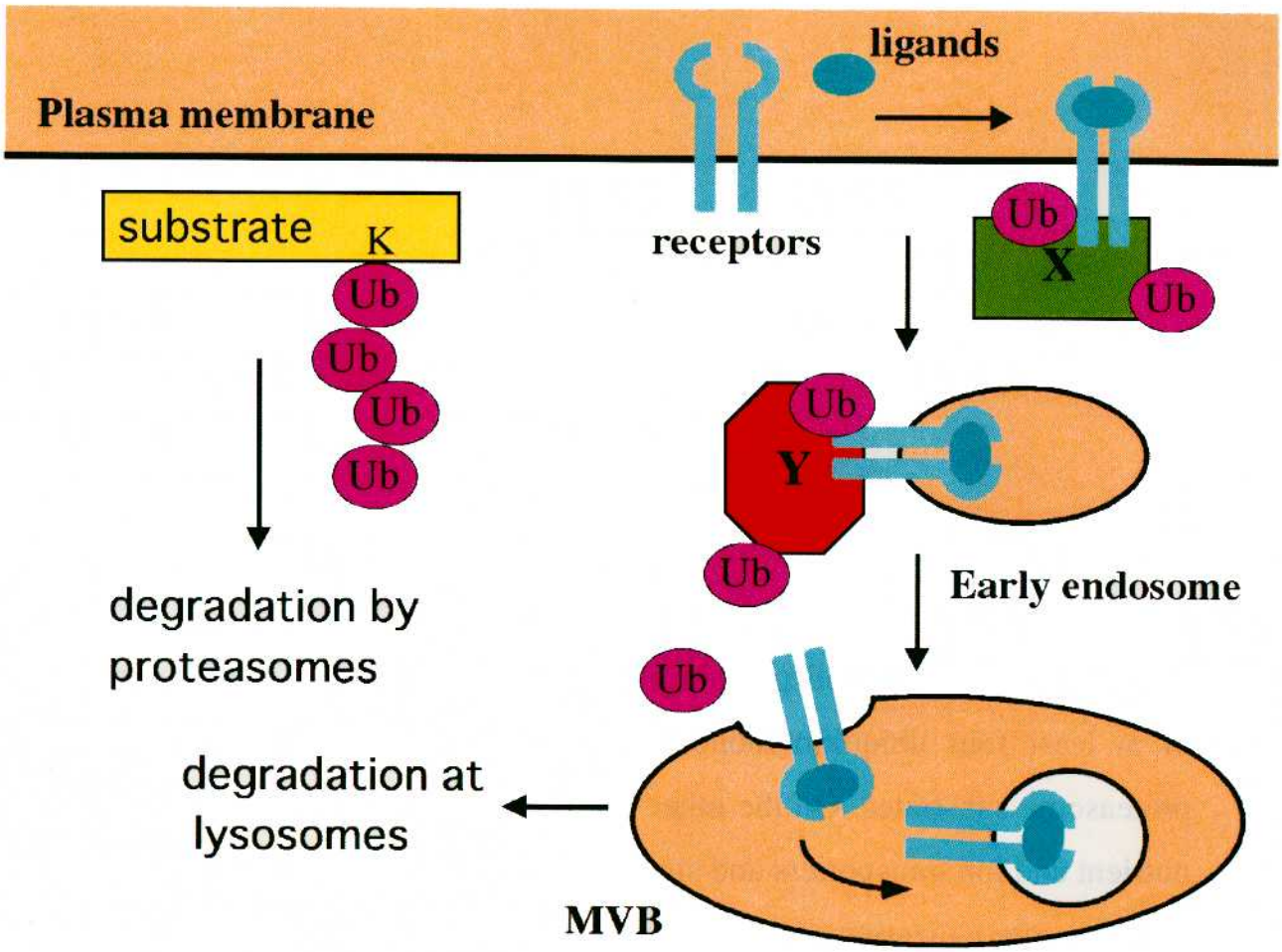
GGA3 emerged after 12 hr. The time course of the transport between the TGN and endosomes is several minutes to hours. It is therefore likely that even if the GGA3 degradation rate influences the transport, it could be an indirect effect.

The other possibility is that monoubiquitination modulates the activity of molecules. Ubiquitinated Sec23p can be recruited onto ER membrane but dissociates poorly (135). The inability of ubiquitinated Sec23p to interact with Sec24p, another COPII subunit, probably inhibits subsequent assembly of other components of COPII coats, preventing its dissociation from the ER membrane. Another example is β-arrestin, which binds AP-2 and clathrin, and its ubiquitination is required for $\beta_2$-adrenergic receptor ($\beta_2$AR) internalization (137). β-Arrestin directs $\beta_2$AR to clathrin-coated pits but does not internalize with it, subsequently $\beta_2$AR recycles to the plasma membrane rapidly. However, a β-arrestin-ubiquitin chimera, which cannot be deubiquitinated by cellular deubiquitinating enzymes and mimics a permanently ubiquitinated form of β-arrestin, does not dissociate from $\beta_2$AR, but is rather internalized with it into endosomes, resulting in enhancement of $\beta_2$AR internalization and degradation (138). Thus, ubiquitination status of β-arrestin determines the stability of the receptor-β-arrestin complex. Therefore, it is possible that GGA3 monoubiquitination alters its activity. It would be interesting to examine whether permanently ubiquitinated GGA3 is able to dissociate from the membrane.

My attempts to explore the physiological relevance of the GAT-ubiquitin interaction have been unsuccessful so far. For example, overexpression of GGA3 mutants defective in ubiquitin binding did not affect internalization or degradation of EGF receptor, which is internalized and degraded in lysosomes in a ubiquitin-dependent manner (98, 100). The GGA3 mutants neither affected localization of cation-independent MPR, which is known to interact with the VHS domain of GGAs through its ACLL motif (39, 41, 44). I am now searching for ubiquitinated proteins that interact with the GGA3-GAT domain

through their appended ubiquitin moieties.

# FIGURES


Plasma membrane
ligands
receptors
substrate
K
Ub
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Ub
X
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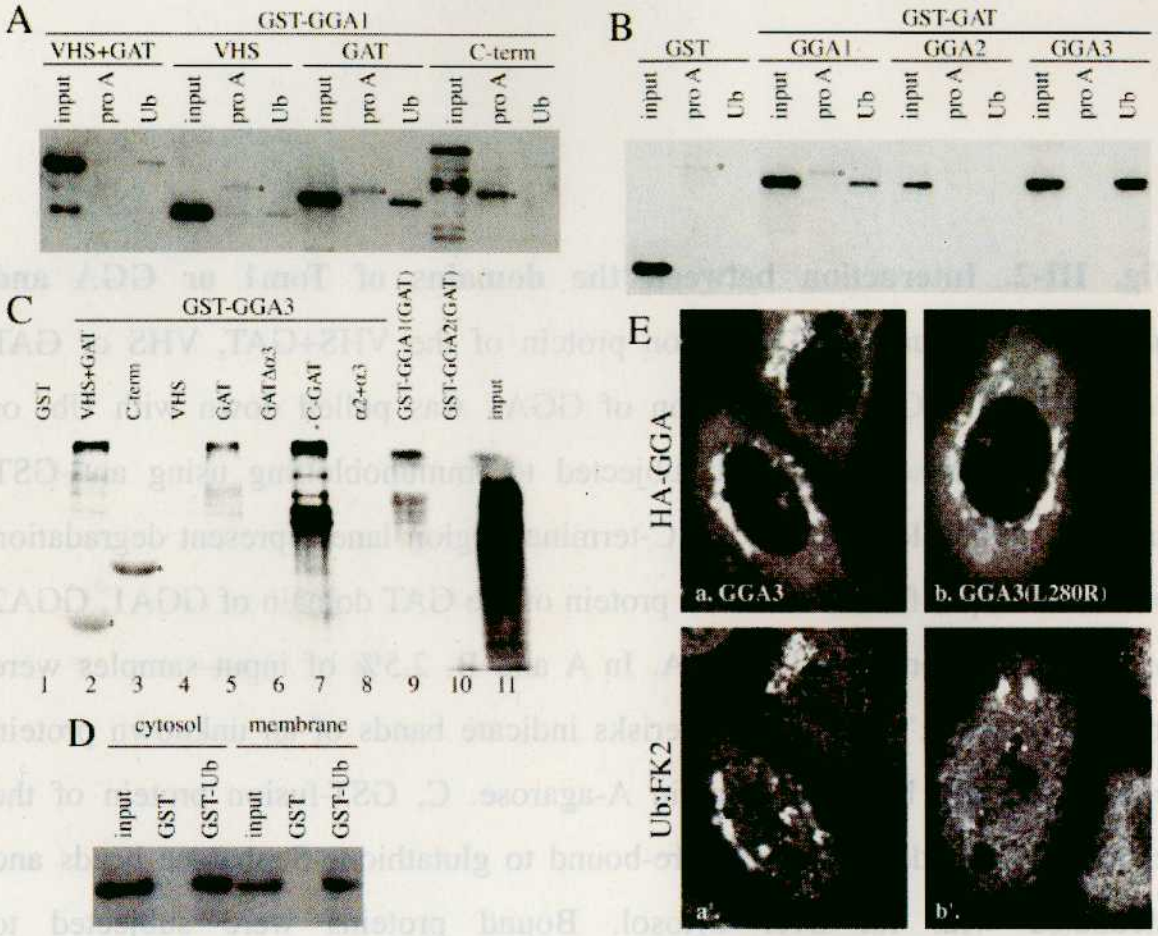
Ub
Y
Ub
Early endosome
degradation by
proteasomes
Ub
degradation at
lysosomes
MVB

**Fig. III-1. Ubiquitin and protein trafficking**. Polyubiquitin chains composed of at least four ubiquitin monomer units are found attached to most 26S proteasome substrates. On the other hand, ubiquitination of many cell-surface nutrient and ion transporters and signal-transducing receptors serves as a signal for their internalization via endocytic pathway and subsequent proteolysis in the lysosome/vacuole. Activated plasma membrane receptors are ubiquitinated and endocytosed to early endosomes. Ubiquitination also serves as a signal that transports ubiquitinated proteins into the inner lumenal vesicles of MVBs. Fusion of the MVBs and lysosome/vacuole results in the delivery of the lumenal vesicles to the hydrolytic environment. Proteins depicted as X and Y are proteins containing ubiquitin-binding domains. These proteins are thought to function in the transport of ubiquitinated transmembrane proteins.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input
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Ub
B
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GGA1
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GGA3
C
GST-GGA3
GST
VHS+GAT
C-term
VHS
GAT
GATΔα3
C-GAT
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cytosol
membrane
GST
GST-Ub
E
HA-GGA
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a. GGA3
b. GGA3(L280R)
a'.
b'.

**Fig. III-2. Interaction between the domains of Tom1 or GGA and ubiquitin.** A, purified GST-fusion protein of the VHS+GAT, VHS or GAT domain, or the C-terminal region of GGA1 was pulled down with Ub- or protein A-agarose beads and subjected to immunoblotting using anti-GST antibody. Multiple bands in the C-terminal region lane represent degradation products. B, purified GST-fusion protein of the GAT domain of GGA1, GGA2 or GGA3 was processed as in A. In A and B, 2.5% of input samples were loaded on 'input' lanes, and asterisks indicate bands of an unknown protein non-specifically bound to protein A-agarose. C, GST-fusion protein of the indicated GGA domains were pre-bound to glutathione-Sepharose beads and incubated with rat liver cytosol. Bound proteins were subjected to immunoblotting using the FK2 antibody. On 'input' lanes, 2% of input samples were electrophoresed. Asterisks indicate bands resulted from cross-reaction of the FK2 antibody with excess GST-fusion proteins. D, GST or GST-Ub pre-bound to glutathione-Sepharose was incubated with a cytosolic or membrane fraction of HeLa cells and subjected to immunoblotting using anti-GGA3 antibody.

On 'input' lanes, 8% of input samples were loaded. E, HeLa cells transfected with an expression vector for HA-GGA3 (a) or its L280R mutant (b) were double-stained with anti-HA antibody (a and b) and the FK2 antibody (a' and b').

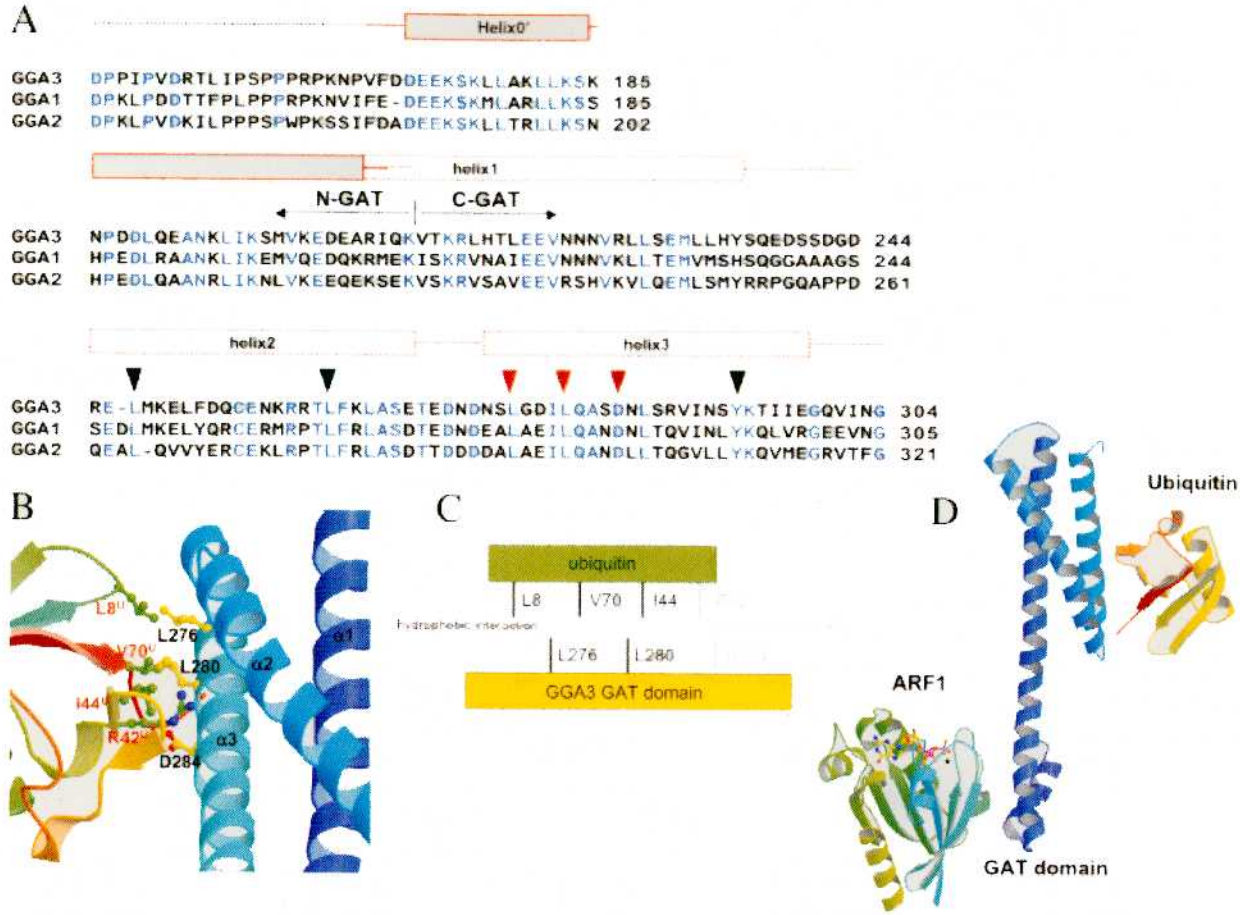


**Fig. III-3. Models for interaction between the GAT domain and ubiquitin.** A, alignment of amino acid sequences of the GAT domains from human GGAs. Residues conserved in all GGAs are shown in blue. Residues involved in packing of the helix bundle (Leu247, Leu262 and Tyr293) and those involved in interaction with ubiquitin (Leu276, Leu280 and Asp284) are indicated by black and red arrowheads, respectively. Boxes above the sequences depict α-helical regions. The helix numbering is according to Ref. 109. B, ribbon diagrams of a model for interaction mode between GGA3-GAT and ubiquitin deduced from experimental data. C, schematic representation of the GAT-ubiquitin interaction shown in B. D, a model of the interactions of the GAT domain with ARF and ubiquitin.

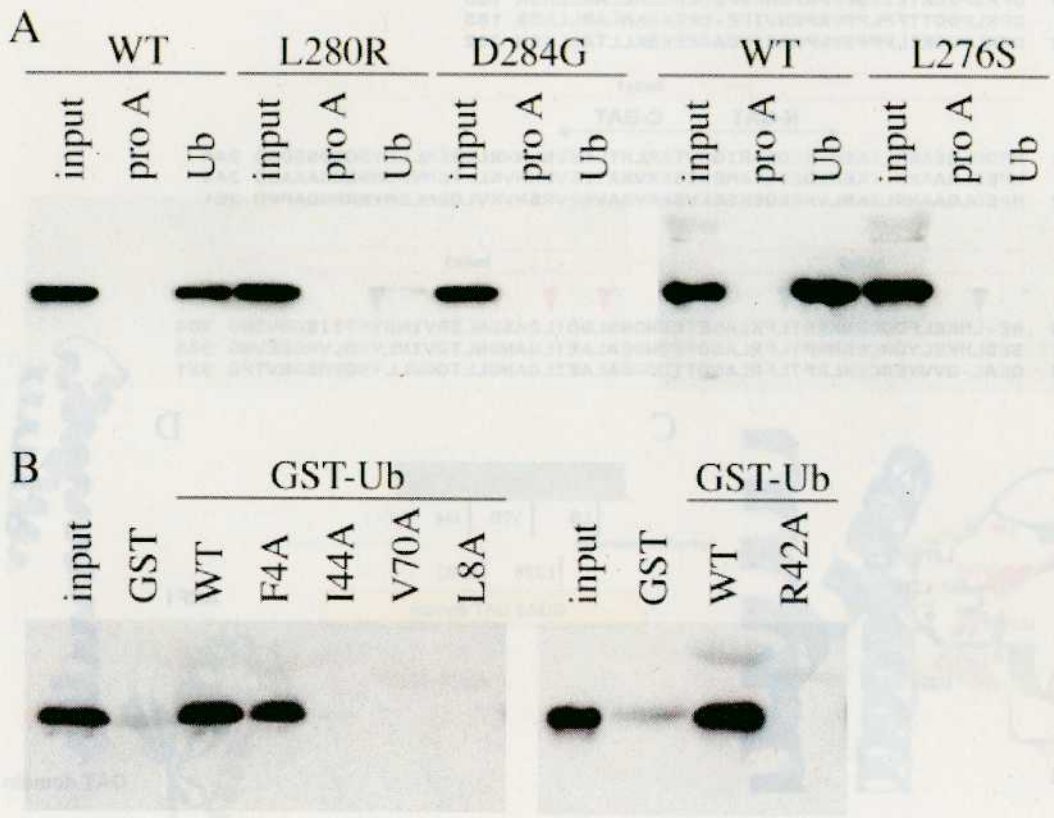


**Fig. III-4. Effects of mutations of residues in the GGA3-GAT domain and ubiquitin on their interaction.** A, wild type GGA3-GAT domain or its mutant fused to GST was pulled down with Ub- or protein A-agarose and subjected to immunoblotting using anti-GST antibody. On 'input' lanes, 2.5% of input samples were loaded. B, $His_6$+T7-tagged wild type GGA3-GAT or its mutant was incubated with GST-Ub pre-bound to glutathione-Sepharose and subjected to immunoblotting using anti-T7-tag antibody. On 'input' lanes, 2.5% of input samples were loaded.

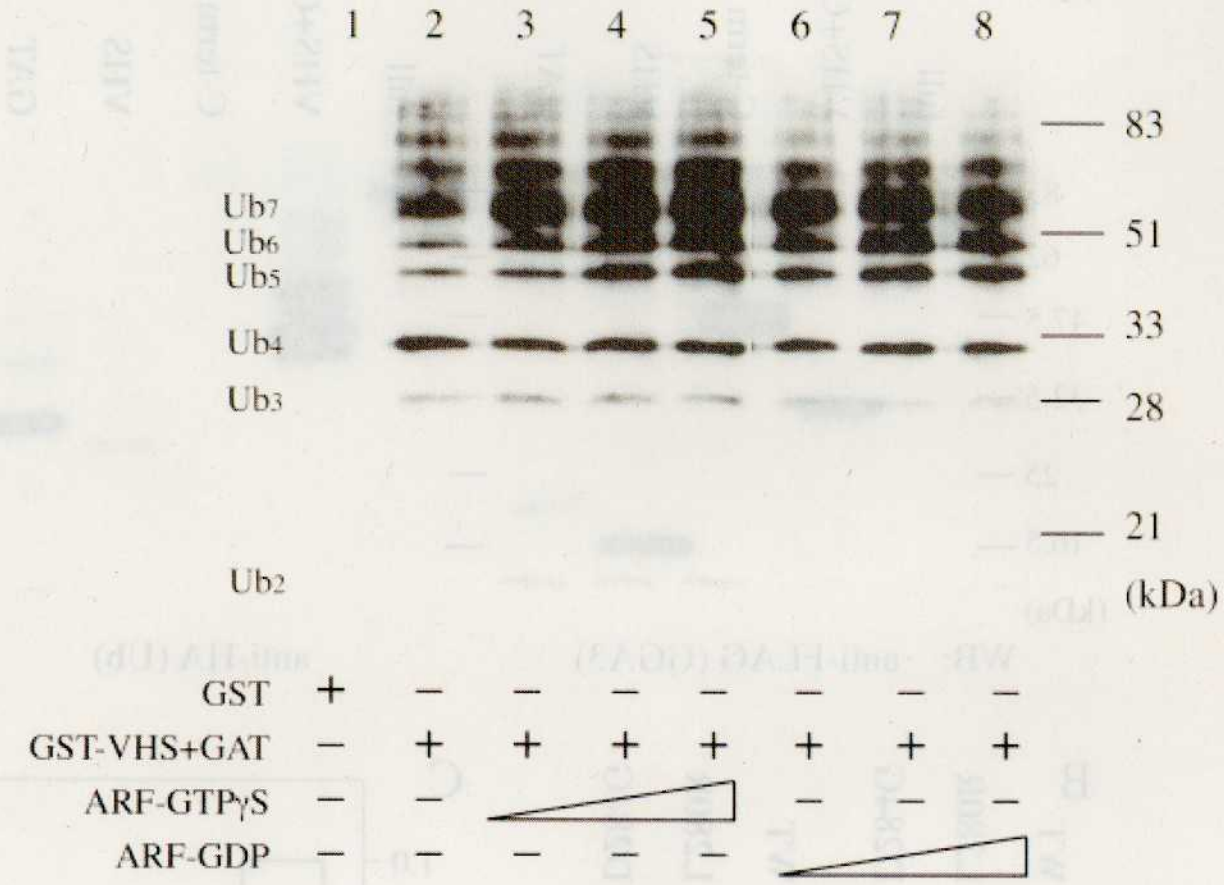


**Fig. III-5. Effect of ARF on the GAT-ubiquitin interaction.** A mixture of multi-ubiquitin chain (2 μg) and 0 (lanes 1 and 2), 1.1 (lanes 3 and 6), 2.2 (lanes 4 and 7) or 5.5 (lanes 5 and 8) μg of purified recombinant ARF1ΔN17 was incubated at room temperature for 30 min in the presence of GTPγS (lanes 2-5) or GDP (lanes 6-8) or in its absence (lane 1) and further incubated for 1 h after addition of GST-VHS+GAT (1 μg) and glutathione-Sepharose beads (20 μl) as described under 'Materials and Methods.' Materials associated with the beads were subjected to immunoblotting with anti-ubiquitin antibody.

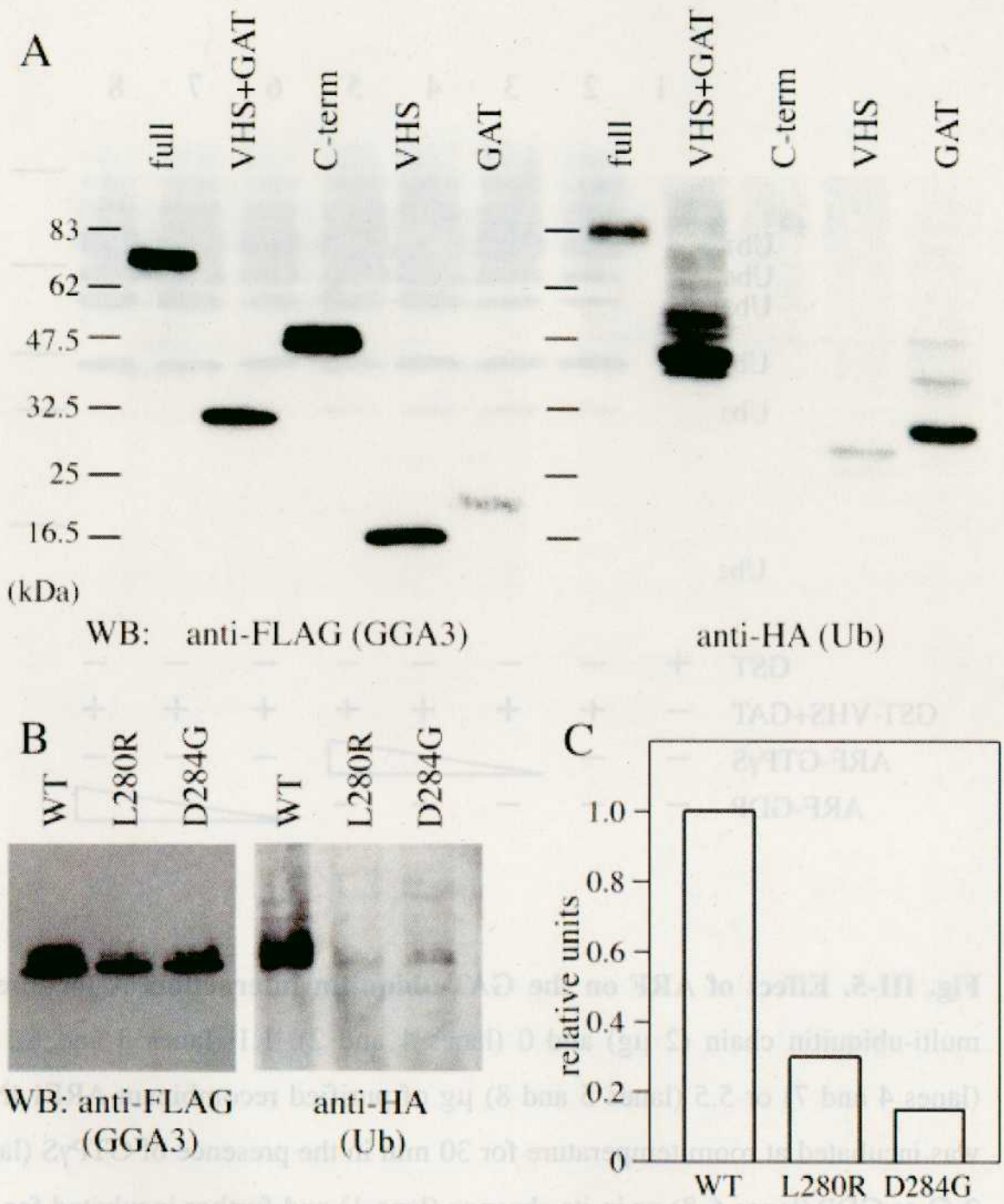
A
full
VHS+GAT
C-term
VHS
GAT
full
VHS+GAT
C-term
VHS
GAT
83
62
47.5
32.5
25
16.5
(kDa)
WB: anti-FLAG (GGA3)
anti-HA (Ub)
B
WT
L280R
D284G
WT
L280R
D284G
WB: anti-FLAG (GGA3)
anti-HA (Ub)
C
relative units
1.0
0.8
0.6
0.4
0.2
0
WT
L280R
D284G

**Fig. III-6. Ubiquitination of GGAs.** A, lysates from HEK-293 cells transfected with expression vectors for HA-ubiquitin and $His_6$+FLAG-tagged full-length GGA3 or its domain construct were precipitated with $Ni^{2+}$-NTA-agarose beads under denaturing conditions and subjected to immunoblotting with anti-FLAG (left panel) or anti-HA (right panel) antibody. B, lysates from HEK-293 cells transfected with expression vectors for HA-ubiquitin and $His_6$+FLAG-tagged full-length GGA3 or its mutant were processed as in A. C, relative densities the bands detected with anti-FLAG and anti-HA antibodies in B were quantitated by Image Gauge ver. 3.0 (Fuji Photo Film, Co.)