Golgi-localizing, γ-Adaptin Ear Homology Domain, ADP-ribosylation Factor-binding (GGA) Proteins Interact with Acidic Dileucine Sequences within the Cytoplasmic Domains of Sorting Receptors through Their Vps27p/Hrs/STAM (VHS) Domains*

Hiroyuki Takatsō, Yohei Katoh, Yoko Shiba, and Kazuhisa Nakayama§

From the Institute of Biological Sciences and Gene Experiment Center, University of Tsukuba, Tsukuba Science City, Ibaraki 305-8572, Japan

GGA (Golgi-localizing, γ-adaptin ear homology domain, ARF-binding) proteins are potential effectors of ADP-ribosylation factors, are associated with the trans-Golgi network (TGN), and are involved in protein transport from this compartment. By yeast two-hybrid screening and subsequent two-hybrid and pull-down analyses, we have shown that GGA proteins, through their VHS (Vps27p/Hrs/STAM) domains, interact with acidic dileucine sequences found in the cytoplasmic domains of TGN-localized sorting receptors such as sortilin and mannose 6-phosphate receptor. A mutational analysis has revealed that a leucine pair and a cluster of acidic residues adjacent to the pair are mainly responsible for the interaction. A chimeric receptor with the sortilin cytoplasmic domain localizes to the TGN, whereas the chimeric receptor with a mutation at the leucine pair or the acidic cluster is mislocalized to punctate structures reminiscent of early endosomes. These results indicate that GGA proteins regulate the localization to or exit from the TGN of the sorting receptors.

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§ To whom correspondence should be addressed. Tel.: 81-298-53-7725; Fax: 81-298-53-7725; E-mail: kazunaka@sakura.cc.tsukuba.ac.jp.

† The abbreviations used are: ARF, ADP-ribosylation factor; GGA protein, Golgi-localizing, γ-adaptin ear homology domain, ARF-binding protein; GGA1 and the VHS domains of human STAM1 (aa 1–143) and TOM1 (aa 1–152) and the GGA VHS domains.
Interaction between GGA VHS Domain and Sorting Receptors

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Antibodies—Monoclonal mouse anti-Tac antibody (7G7.B6) was obtained from a hybridoma cell line (Clonetech Laboratories, Palo Alto, CA). Antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Yeast Two-hybrid Screening and Assay—Yeast two-hybrid screening was performed as described previously (18). Briefly, yeast Y190 cells harboring the pGBT9 vector for the VHS+GGAH domain of GGA1 were transformed with a pACT2-based library of human brain cDNAs (Clontech), grown for 5–7 days on synthetic medium containing 25 mM 3-aminotriazole and lacking tryptophan, leucine, and histidine. The cells grown on the latter medium were then washed five times with phosphate-buffered saline.

Pull-down Assay—The VHS domain fused to TRX-His6 and the wild-type (WT) and mutated cytoplasmic domains fused to GST were expressed in E. coli. C6/36 cells and transfected with the reporter yeast cells harboring the pACT2-based library of human brain cDNAs. The yeast colonies were obtained by replica plating the reporter yeast cells on synthetic medium lacking tryptophan, leucine, and histidine. The colonies were then washed five times with phosphate-buffered saline and incubated sequentially with monoclonal mouse anti-Tac antibody and mouse anti-HA or polyclonal goat anti-EEA1 antibody and Alexa488-conjugated and Cy3-conjugated secondary antibodies. The stained cells were observed using a confocal microscope (TCS-SP2, Leica Mikrosysteme Vertrieb GmbH, Bensheim, Germany).

RESULTS

To identify proteins that interact with GGA proteins, we performed a yeast two-hybrid screening with the use of the VHS+GGAH domain of human GGA1 as bait. By screening a human brain cdna library, four positive cDNA clones were obtained. Among them, one clone covered a portion of the transmembrane domain and an entire cytoplasmic domain of sortilin (aa 774–831) and another covered a portion of the cytoplasmic domain of LRP3 (aa 748–770). As shown in Fig. 1A and summarized in Table I, reporter yeast cells harboring the VHS+GGAH domain of GGA1 and the cytoplasmic domain of either sortilin or LRP3 were able to grow on a histidine-deficient plate and exhibited β-galactosidase activity. When the VHS and GGAH domains were examined separately, only the former showed interactions with the sortilin and LRP3 cytoplasmic domains. We then examined whether the VHS domains of the other two GGAs could also interact with the cytoplasmic domain. The GGA2 VHS domain interacted with the sortilin cytoplasmic domain (Fig. 1A and Table I) and LRP3 (data not shown). By contrast, the VHS domain of GGA3 short (GGA3S), which lacks a 33-amino acid region within the VHS domain as compared with those of GGA1 and GGA2 (5–8) (see Fig. 1B), did not show a significant interaction. The sequence of GGA3-long (GGA3L), which contains the 33-amino acid region, has been also reported (5, 7) (see Fig. 1B). However, we could not examine whether the GGA3L VHS domain also interact with the cytoplasmic domains because our attempts to isolate its cDNA fragment by PCR of human brain, liver, and kidney libraries were unsuccessful.

The VHS domain is found in various proteins that function in phagocytic pathway and in signal transduction (11) (see Fig. 1B). Therefore, a reasonable speculation was that the VHS domains of other proteins could also interact with the sortilin and LRP3 cytoplasmic domain. However, this was not the case; the VHS domains examined, those of STAM1 and TOM1, failed to interact with the cytoplasmic domains of sortilin (Fig. 1A and Table I) and LRP3 (data not shown). In the VHS domain, GGA1 shares 64 and 73% of its amino acids with GGA2 and GGA3L, respectively, whereas 29 and 33% are shared with.
The interactions between the GGA VHS domains and the acidic dileucine sequences were also tested biochemically. To this end, GST-fusion proteins of the cytoplasmic domains and TRX-His<sub>6</sub> fusion proteins of the VHS domains expressed in *E. coli* were purified. Using these fusion proteins, we first examined the specificity of the sortilin cytoplasmic domain to the VHS domains. As shown in Fig. 2A, the VHS domains of GGA1 and GGA2 were pulled down efficiently with the GST-sortilin cytoplasmic domain, whereas the STAM1 VHS domain was not. Essentially the same results were obtained with the CI-MPR cytoplasmic domain fused to GST (Fig. 2A). These data are in good agreement with those obtained by two-hybrid assays. Apparently conflicting with the two-hybrid data was the result that the VHS domain of GGA3S was efficiently pulled down. The discrepancy appeared to be due to a nonspecific interaction of the GGA3S VHS domain with the GST portion of the GGA3S VHS domain fusion proteins because the GGA3S VHS domain fused to TRX-His<sub>6</sub> was efficiently pulled down with GST (Fig. 2A).

We then examined the sequence requirements of the sortilin cytoplasmic domain for interaction with the GGA1 VHS domain by the pull-down assay. As shown in Fig. 2B, the Ser → Asp mutant pulled down the GGA1 VHS domain as efficiently as the WT sortilin cytoplasmic domain, and the Ser → Ala and Asp → Asn mutants pulled down the VHS domain less efficiently. By contrast, the Asp → Asn/Glu mutated at the Leu/Ala mutations almost completely abrogated the interaction with the VHS domain. These data are consistent with the two-hybrid data.

MPRs are known to cycle between the TGN and late endosomes to deliver lysosomal hydrolases through the latter compartment to lysosomes (reviewed in Refs. 24–26). On the other hand, a previous study has shown that transiently expressed sortilin localizes in the TGN region, and a chimeric protein comprising the luminal and transmembrane domains of the Tac antigen and the cytoplasmic domain of sortilin is colocalized with CI-MPR (27). These data lead to the possibility that the acidic dileucine sequence of sortilin common to MPRs determines the subcellular localization of sortilin. To address this possibility, we examined the localization of the Tac chimera with the WT or mutated sortilin cytoplasmic domain by immunofluorescence microscopy. As shown in Fig. 3, the Tac chimera with the WT sortilin tail localized exclusively in the perinuclear region (panel B) as reported previously (27).
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, HeLa cells transfected with a vector for a Tac chimera contain-
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) antibodies. A merged image is shown in
anti-HA
(E
) and
tail and for HA-tagged GGA1 and double-stained with anti-Tac
with expression vectors for a Tac chimera containing the WT sortilin
tail
B

FIG. 2. Pull-down assays. A, the cytoplasmic domain of sortilin or
CI-MPR fused to GST or GST alone was prebound to glutathione-
Sepharose beads and incubated with the VHS domain of GGA1, GGA2,
GGA3S, or STAM1 fused to TRX-His6. The materials bound to the beads
were subjected to Western blot analysis using anti-TRX antibody as
described under “Experimental Procedures.” B, the WT or mutated
tail cytoplasmic domain fused to GST was prebound to glutathione-
Sepharose beads and incubated with the GGA1 VHS domain fused to
TRX-His6. The materials bound to the beads were subjected to Western
blot analysis using anti-TRX antibody as described under “Experimen-
tal Procedures.”

FIG. 3. Immunofluorescence analysis. A–D, HeLa cells were tran-
siently transfected with an expression vector for Tac (A), a Tac chimera
containing the WT sortilin tail (B), or the tail with an Leu → Ala/Leu →
Ala (C) or Asp → Asn/Glu → Gln/Asp → Asn (D) mutation and processed
for indirect immunofluorescence microscopy as described under “Exper-
imental Procedures.” E–E’, HeLa cells were transiently cotransfected
with expression vectors for a Tac chimera containing the WT sortilin
tail and for HA-tagged GGA1 and double-stained with anti-Tac (E)
and anti-HA (E’) antibodies. A merged image is shown in E’. F–F’, and
G–G’, HeLa cells transfected with a vector for a Tac chimera contain-
ing the sortilin tail with an Leu → Ala/Leu → Ala (F) for Asp → Asn/
Glu → Gln/Asp → Asn (G) mutation were double-stained with anti-Tac
(F and G) and anti-EEA1 (F’ and G’) antibodies. Merged images are
shown in F’ and G’.

whereas Tac itself was found largely on the cell surface (panel A).
Furthermore, the staining for the Tac chimera was superimposed on that for coexpressed GGA1 (panels E–E’), indicat-
ing that the chimeric protein localized to the TGN. By contrast,
the localization of the Tac-sortilin chimera with an Leu →
Ala/Leu → Ala mutation was different; it was found on punctu-
tate structures throughout the cytoplasm and in the cell pe-
riphy as well as in the perinuclear region (panel C). A similar
staining pattern was observed for the Tac-sortilin chimera with
an Asp → Asn/Glu → Gln/Asp → Asn mutation (panel D). The
Ser → Ala, Ser → Asp, or Asp → Asn/Asp → Asn mutation had
no obvious effect on the distribution of the Tac chimera (data
not shown). Thus, the ability of the sortilin cytoplasmic domain
to bind to the GGA VHS domain correlated well with its ability
to localize the Tac chimera to the TGN. The punctate staining
for the chimera with the Leu → Ala/Leu → Ala or Asp →
Asn/Glu → Gln/Asp → Asn mutation overlapped significantly
with that for EEA1 (panels F–F’ and G–G’), respectively, an
early endosome marker, but not with that for Lamp-1 (data not
shown), a late endosome/lysosome marker, indicating that
these mutations cause mislocalization of the chimera to early
endosomes.

DISCUSSION

GGAs are a family of TGN-associated peripheral membrane
proteins that have been proposed to function to facilitate pro-
tein trafficking between the TGN and lysosomes/vacuole.
The data presented here show that GGAs can interact with
the cytoplasmic domains of TGN-localized transmembrane pro-
teins including sortilin, CI-MPR, and LR3P. The interactions
occur between the VHS domains of GGAs and the conserved
acidic dileucine sequences at the COOH termini of the cyto-
plasmic domains. A mutation of the leucine pair or the cluster
of acidic residues proximal to the pair abrogates the interaction
and causes mislocalization of a chimeric receptor with the
sortilin cytoplasmic tail from the TGN to endosome-like struc-
tures. MPRs are known to cycle between the TGN and late
endosomes and to function as sorting receptors for lysosomal
hydrolases (24–26). Sortilin has, in its luminal domain, a cys-
teine-rich region homologous to two corresponding segments of
yeast Vps10p (27), which is a sorting receptor for vacuolar
proteins (reviewed in Ref. 28), leading to a possibility that
sortilin could also function as a sorting receptor for some lys-
sosomal proteins. GGAs are recruited onto TGN mem-
branes by virtue of ARFs and bind to clathrin in mammalian
cells (5, 7, 12), facilitating the transport of vacuolar proteins in
yeast (6, 7, 9, 10). Taken together with the data presented here,
it is likely that GGAs regulate the trafficking of lysosomal
proteins by interacting with the sorting receptors.

A Tac chimera containing the sortilin cytoplasmic domain
localizes to the TGN. By contrast, a chimera containing the
mutated sortilin tail (Leu → Ala/Leu → Ala or Asp →
Asn/Glu → Gln/Asp → Asn), which cannot interact with GGAs,
is mislocalized from the TGN to endosome-like structures. A pre-
vious study using systematic alanine-scanning mutagenesis
determined that the COOH-terminal acidic dileucine sequence
of CI-MPR is the major determinant of sorting of lysosomal
enzymes (29). Although that study did not see the localizations
of the CI-MPR mutants, their data are in keeping with the idea
that the interaction between GGAs and the receptor cytoplas-
mic domain is crucial for lysosomal protein sorting.

The acidic dileucine sequences are predicted to be able to
interact with adaptor molecules other than GGAs. For exam-
ple, the acidic clusters with serine residues phosphorylated by
the CK-II of MPRs were shown to interact with the AP-1
clathrin adaptor complex and proposed to be required for MPR
exit from the TGN (reviewed in Ref. 26). The interaction is
thought to be mediated by another adaptor protein, PACS-1,
which was originally identified as a protein that binds to the
phosphorylated acidic cluster of the TGN-localized endoprote-
ase furin (Ref. 30; reviewed in Ref. 31). However, the PACS-1
study suggested that the interaction between PACS-1 and the
phosphorylated acidic cluster mediates retrieval of furin from
post-TGN compartments rather than its anterograde transport.
from the TGN (23, 30, 31). Furthermore, the above CI-MPR mutational study suggested that a hydrophilic nature, but not phosphorylation/dephosphorylation cycles, at the serine position is important for lysosomal protein sorting (29), which is compatible with the data presented here. Taking into account the data presented thus far on GGAs, it is tempting to speculate that GGAs regulate the exit of sorting receptors from the TGN by recognizing the acidic dileucine sequences, whereas PACS-1 regulates their retrieval from post-Golgi compartments by recognizing the phosphorylated acidic clusters.

During the review process of this paper, a similar study was reported by Nielsen et al. (32). They identified GGA2 as a protein interacting with the sortilin cytoplasmic tail by two-hybrid screening and found that a deletion of the leucine pair abolished the ability of the tail to interact with GGA2 and led a Tac-sortilin chimera to a mild defect in its internalization. These data support our present data. However, apparently in conflict with our data was their finding that a deletion of the leucine pair abolished the ability of the tail to interact with GGA2 and led to a Tac-sortilin chimera to a mild defect in its internalization. This discrepancy might be because of the difference between our and their experimental systems; we observed the steady-state localization of the Tac-sortilin chimera, whereas they observed its internalization from the cell surface.

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