GGA1 Interacts with the Adaptor Protein AP-1 through a WNSF Sequence in Its Hinge Region*

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The Golgi-associated γ-adaptin-related ADP-ribosylation factor-binding proteins (GGAs) are critical components of the transport machinery that mediates the trafficking of the mannose 6-phosphate receptors and associated cargo from the trans-Golgi network to the endosomes. The GGAs colocalize in vivo with the clathrin adaptor protein AP-1 and bind to AP-1 in vitro, suggesting that the two proteins may cooperate in packaging the mannose 6-phosphate receptors into clathrin-coated vesicles at the trans-Golgi network. Here, we demonstrate that the sequence, WNSF, in the hinge region of GGA1 mediates its interaction with the AP-1 γ-ear. The Trp and Phe constitute critical amino acids in this interaction. The binding of Rabaptin5 to the AP-1 γ-ear, which occurs through a FXXF motif, is inhibited by a peptide encoding the GGA1 WNSF sequence. Moreover, mutations in the AP-1 γ-ear that abolish its interaction with Rabaptin5 also preclude its association with GGA1. These results suggest that the GGA1 WXXF-type and Rabaptin5 FXXF-type motifs bind to the same or highly overlapping sites in the AP-1 γ-ear. This binding is modulated by residues adjacent to the core motifs.

In eukaryotic cells, the budding and fusion of clathrin-coated vesicles (CCVs) mediate the transport of proteins and lipids from the trans-Golgi network (TGN) and the plasma membrane to the intracellular endosomal membrane system (1). In addition to clathrin, the Golgi- and plasma membrane-derived CCVs contain the adaptor protein (AP) complexes AP-1 and AP-2, respectively, as the principal coat proteins. The heterotetrameric AP complexes are comprised of two large adaptin subunits (γ and β1 for AP-1, α, and β2 for AP-2), which can be subdivided into an N-terminal trunk domain and a C-terminal appendage or ear domain linked by an extended flexible hinge, a medium adaptin subunit (μ1 or μ2), and a small adaptin subunit (σ1 or σ2). Electron microscopic images of purified AP-2 heterotetramers reveal two separate globular appendages, which correspond to the C-terminal portions of the large α and β2 chains, projecting from the central core of the complex. The AP-1 γ and β1-appendages are believed to have an analogous gross structure (2).

In addition to the AP complexes, the two subtypes of coated vesicles contain an array of distinct and, to a small degree, overlapping protein components that contribute to cargo selection and facilitate CCV formation by combining clathrin-binding and membrane association domains within an oligomeric protein complex. Among these are the accessory proteins eps15, epsin, amphiphysin, AP-180, auxilin, numb, and disabled-2, which function at the plasma membrane (3), and the GGAs (Golgi-associated γ-adaptin-related ADP-ribosylation factor-binding proteins), which are involved in vesicle budding from the TGN. The three mammalian GGAs are multidomain proteins that were identified on the basis of sequence similarity between the AP-1 γ-appendage domain and the C-terminal 150 amino acids of these proteins, also known as the γ-adaptin ear (GAE) domain (4–8). This domain is linked to the GAT domain by a flexile hinge region that interacts with clathrin (9, 10). The GAT domain binds ARF-GTP and mediates the membrane association of the GGAs with the TGN (9). The N-terminal region contains a VHS domain that has been shown to be important in the sorting of transmembrane proteins having acidic cluster-dileucine (AC-LL) signals, such as the mannose 6-phosphate receptors (MPRs) and sortilin, from the TGN to endosomes (10–13). In the case of the MPRs, interaction with GGAs is critical for the efficient delivery of acid hydrolases to lysosomes (14).

We have previously demonstrated that the GGAs and AP-1, along with MPRs, colocalize in clathrin-coated buds at the TGN of mouse L cells and human HeLa cells (15). We further showed a direct interaction between the hinge domains of the GGAs and the γ-ear domain of AP-1. In the case of GGA1, our in vivo binding data implicated residues 370–429 in the hinge segment as being important for its interaction with the AP-1 γ-ear. Moreover, a mutant MPR that was defective in binding the GGAs was poorly incorporated into AP-1 CCVs, suggesting that the GGAs and AP-1 cooperate to package MPRs into coated vesicles at the TGN. Thus, the in vitro interactions that we observed between the GGAs and AP-1 may be physiologically important in ensuring proper transfer of the MPRs from the GGAs to AP-1. In the current study, we sought to identify the sequence in the GGA1 hinge that is responsible for its association with AP-1. We show that the interaction between the AP-1 γ-ear and GGA1 is mediated by a WNSF sequence (residues 382–385) within the hinge region of GGA1 and that the 2 anchor aromatic amino acids constitute key residues. A functionally analogous WXXF/FW sequence found in the proteins NECAP1 and amphiphasin II also mediates their interactions with the AP-1 γ-ear.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—COS-7 cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s
medium (Invitrogen) containing 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum (HyCloneSM Laboratories, Inc., Logan, UT). S9 insect cells were maintained in the SF-900II SFM medium (Invitrogen) containing 25 units/ml penicillin, 25 μg/ml streptomycin. Glutathione-Sepharose 4B beads were purchased from Amersham Biosciences. The anti-c-myc (clone 9E10) monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), whereas the anti-hemagglutinin monoclonal was from Covance (Berkeley, CA). The anti-Rabaptin5 and anti-Xpress monoclonal antibodies were purchased from BD Transduction Laboratories and Invitrogen, respectively. The anti-AP-1–subunit-specific monoclonal antibody (100/3) was from Sigma. Frozen bovine adrenal glands were purchased from Pel-Freeze (Rogers, AR).

**Plasmid Construction and cDNA Cloning**—The plasmids encoding GST-AP-1 γ-ear (residues 703–822), GST-SIPWDLWTTS (the distal amphiphilic II clathrin-binding sequence), and myc-GGA1pCR3.1 have been described (10, 16–18). GST-GGA1 (376–391) was generated by annealing sense and antisense oligonucleotides and ligating the double-stranded product into EcoRI/Xhol-digested pGEX-5X-3 (Amersham Biosciences). His-Xpress-γ-ear (703–822) was constructed by PCR of the corresponding γ-ear coding sequence from GST-γ-ear (703–822)/pGEX-5X-3 (16) and ligation into the vector pTrchHisC (Invitrogen). Human NECAP1 was cloned from a brain cDNA library (Clontech) using primers corresponding to the 5′ and 3′ ends of the published hNECAP1 sequence (GenBankTM accession number AK074880) into the T7 promoter vector (Invitrogen). The cloned cDNA was sequenced in its entirety to ascertain the identity of the cloned product. GST-hNECAP1 was generated by PCR and ligation into EcoRI-digested pGEX-5X-3. The various GST-AP-1−γ-ear, myc-GGA1pCR3.1, GST-hNECAP1, and GST-SIPWDLWTTS mutant constructs were made using primers incorporating the desired mutations with the QuikChange system (Stratagene, La Jolla, CA). All constructs and mutations were confirmed to be correct by dideoxynucleotide sequencing.

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**Peptides**—The amino acid sequences derived from the GGA1 hinge region (376–391) corresponding to the peptides used in this study are as follows: SLDGCGWSFSGSSDADTDAT, termed GGA1A peptide; SLDQAFSFGSSDADAT, termed GGA1WnsF peptide; SLDGCGWSFSGSSDADAT, termed GGA1AnsA peptide; SLDQAFSFGSSDADAT, termed GGA1WnsF peptide; SLDQAFSFGSSDADAT, termed GGA1AnsA peptide; SLDQAFSFGSSDADAT, termed GGA1WnsF peptide; and SLDQAFSFGSSDADAT, termed GGA1AnsA peptide. These peptides were synthesized and purified at the Protein Chemistry Laboratory at Washington University in St. Louis, MO and purified by reverse phase high-performance liquid chromatography.

**Protein Expression and Purification**—myc-GGA1 (WT and mutants) were expressed in S9 insect cells, and cell lysates were prepared as described previously (18). COS-7 cells were transfected with plasmid DNA using LipofectAMINE Plus according to the manufacturer’s instructions (Invitrogen). Cells were harvested 48 h after transfection and lysed into cold assay buffer A (25 mM Hepes-KOH, pH 7.2, 125 mM potassium acetate, 2.5 mM magnesium acetate, 1 mM dithiothreitol, and 0.4% Triton X-100) by sonication. Following centrifugation at 20,000 × g, the supernatant containing the GGA protein was stored at 4 °C until there was no detectable protein being released and then repeated with wash buffer (binding buffer with 20 mM imidazole, pH 7.4). Elutions were repeated three times, and the fractions were pooled, concentrated using a Centricon-10 apparatus, and frozen in aliquots at ~80 °C for further use. The concentration of the purified protein was determined using the Bradford assay (39). GST pull-down assays were performed similarly except that reactions were carried out in a final volume of 300 μl containing the indicated concentrations of the various peptides.

**Electrophoresis and Immunoblotting**—Pellet and supernatant fractions (as indicated by P and S, respectively, in the figure legends) were resolved on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membrane. Blots were blocked with TBST (100 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100) containing 5% non-fat milk for 1 h at room temperature. Blots were then incubated with primary antibodies as indicated in the individual figure legends followed by horseradish peroxidase conjugated anti-mouse IgG. The immunoreactive bands were visualized on x-ray films using enhanced chemiluminescence (ECL) (Amersham Biosciences). Where necessary, the bands were quantified with the Kodak digital imaging system (Eastman Kodak Co.).

**RESULTS**

**GGA1 WXXF Motif Mediates Its Interaction with AP-1**—We have previously demonstrated that the hinge region of GGA1 between residues 370 and 429 mediates its interaction with the AP-1 γ-ear (15). To delineate the precise sequence requirement for AP-1 binding, a number of truncation mutants of myc-tagged GGA1 were expressed in COS-7 cells and tested for binding to AP-1–γ-ear protein (15–17). Bovine adrenal cytosol was prepared essentially as described (19) except that 7.4) until there was no detectable protein being released and then repeated with wash buffer (binding buffer with 20 mM imidazole, pH 7.4). Elutions were repeated three times, and the fractions were pooled, concentrated using a Centricon-10 apparatus, and frozen in aliquots at ~80 °C for further use. The concentration of the purified protein was determined using the Bradford assay (39). GST pull-down assays were performed similarly except that reactions were carried out in a final volume of 300 μl containing the indicated concentrations of the various peptides.

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peptide (Fig. 3C). This indicates that the WX XF motif of GGA1 is necessary and sufficient to mediate its interaction with the AP-1 γ-ear.

**WXXF- and FXXΦ-containing Proteins Bind to a Common or Overlapping Site on the AP-1 γ-Ear**—Recently, a number of accessory proteins that interact with the appendage domains of AP-1 and the GGA proteins were shown to contain sequences that fit a consensus motif, (D/E)FXXΦ (Φ represents leucine, phenylalanine, tryptophan, or methionine) (20–22). These include p56 and Rabaptin5 (Fig. 4A), as well as γ-synergin and EpsinR. The crystal structures of peptides with sequences conforming to this motif, in complex with the GAE domains of GGAs 1 and 3, have revealed the critical nature of the phenylalanine residue in the first position and the 3 residue for this interaction (20, 21). On the basis of mutagenesis data coupled with the structural homology between the AP-1 γ-ear and the GGA GAE domains, it appears likely that the accessory protein-binding site on both adaptors is very similar (20). To assess whether these proteins bind to the same site on the AP-1 γ-ear as does GGA1, we tested the effect of the GGA1 WXXF peptide on the binding of Rabaptin5 to GST-γ-ear. Rabaptin5 containing an FGFLV sequence (Fig. 4A) that interacts with the GGA GAE domain (23). As shown in Fig. 4B, Rabaptin5 binding was strongly inhibited by 0.2 mM WT GGA1 peptide but not by 0.5 mM AXXA mutant peptide. This suggests that the (D/E)FXXΦ and the WXXF motifs bind to a common or closely overlapping site on the AP-1 γ-ear.

We next tested whether the FXXΦ peptide (FGGF), derived from the p56 protein GAE-binding sequence, inhibits binding of myc-GGA1 to the AP-1 γ-ear. In competition assays (Fig. 5A), both the GGA and the p56 peptides displayed a similar inhibition profile except that the p56 peptide stimulated binding at the lowest concentration tested (30 μM). This was consistent with both peptides binding to a common or overlapping site on the AP-1 γ-ear. In contrast to this finding with the p56 peptide, the GGA1 mutant FXXF peptide is at least 8-fold less potent than the WT peptide in inhibiting binding of myc-GGA1 to GST-γ-ear (Fig. 5B). The most likely explanation for this difference is that residues in the proximity of the anchor aromatic amino acid influence the predilection for either a Phe or a Trp residue in the first position of the motif. These neighboring residues could possibly impact the specificity of the interactions of different accessory proteins with the GAE domains, as suggested previously (20). Curiously, the lowest concentration
of the GGA1 mutant FXXF peptide stimulated GGA1 binding to the AP-1 γ-ear, just as observed with the p56 peptide. An explanation for this anomaly is not clear at this point.

GGA1 WXXF Motif Engages the Same Key Residues in AP-1 γ-Ear as FXXΦ Motif—X-ray crystallography together with structure-based mutational analysis have revealed the binding site for accessory proteins within the AP-1 γ-ear (24, 25). The crystal structure showed the γ-ear domain to consist solely of an immunoglobulin-like fold with several basic and hydrophobic residues that are highly conserved between the γ-ear and GGA GAE domains forming a cluster on the surface of the β-sandwich structure. It has been shown that mutations K756Q, R793Q, R795Q, K797Q, E812K, and L762E in the γ-ear domain abolished its binding to the accessory proteins, γ-syn-ergin and Rabaptin5 (24, 25). Since the GGA1s also bind to the γ-ear, we wanted to determine whether the GGA-γ-ear interaction required the same structural determinants as did the other accessory proteins. To address this issue, the six mutations described above were introduced into GST-γ-ear, and the recombinant fusion were proteins tested for their ability to pull-down Rabaptin5 and GGA1. The results of the binding assays show that point mutations in 5 of the 6 residues within the γ-ear abolished its binding to GGA1 (Fig. 6). All of these mutants except for K797Q also impaired Rabaptin5 binding. The E812K mutation did not affect the ability of the γ-ear to interact with either GGA1 or Rabaptin5. Thus, with one exception, the same basic and hydrophobic residues on the γ-ear are required for interaction with both Rabaptin5 and GGA1.

NECAP and Amphiphysin Bind AP-1 in Vitro through WXX/F(W) Motifs—Two recent reports have identified a novel AP-2 ω-appendage-binding sequence, WXX/F(W)X/D/E, that is similar to the WXX motif described here (26, 27). In the protein NECAP (26), the WXXF sequence is positioned at the extreme C terminus of the protein. Hence, it was proposed that for the NECAP AP-2 ω-appendage-interacting motif, the terminal carboxyl group may replace the distal acidic side chain normally seen in the consensus sequence (27). Ritter et al. (26) also found that the AP-1 γ-ear interacted with NECAP proteins, but this interaction did not require the extreme C-terminal WXXF. Upon examining the NECAP amino acid sequence (Fig. 7A), we observed a second WXXF motif, 252WGDF255, positioned 16 residues proximal to the C-terminal ω-appendage-interacting WXXF motif. In view of our finding that the internal WXXF sequence of GGA1 interacted with the AP-1
we reasoned that the proximal WXXF sequence of NECAP1 may represent the γ-ear-interacting motif. To test this, various constructs of GST-NECAP1 were expressed and purified from bacteria for use in pull-down assays with bovine adrenal cytosol as a source of cytosolic AP-1. The results shown in Fig. 7, B and C, indicate that the proximal 252WGDF255 sequence of NECAP is the γ-ear-interacting motif and that it behaves in many respects similar to the GGA1 WXXF sequence. For instance, Trp-252 could not be substituted by either Phe or Ala at this position, but Phe-255 could be substituted by a Trp (Fig. 7C). Moreover, if the protein terminated with the WXXF sequence (Ser-256 stop), it was unable to bind to the γ-ear, similar to the observation with the GGA1 387 stop mutant. This is in contrast to the findings with the AP-2 a-appendage-interacting WXXF motif, which requires an Asp or Glu in the 5 position (where Trp is the 0 position) or the motif be positioned at the extreme C terminus of the protein (27).

In the course of characterizing the endocytic interactions between amphiphysin and clathrin, Drake and Traub (17) observed that a GST-SIPWDLWEPT peptide fusion harboring the distal amphiphysin II clathrin-binding sequence was able to associate with AP-1 from cytosol. Although these investigators did not ascertain whether the AP-1 binding to GST-SIPWDLWEPT was direct or the indirect consequence of its interaction with clathrin, it was shown that mutation of the proximal Trp to Ala, Phe, Tyr, or His resulted in the loss of AP-1 binding. Mutation of the distal Trp to Phe, however, had no effect on AP-1 binding. These results are reminiscent of our data with the GGA1 and NECAP γ-ear-interacting WXXF motifs. Hence, we sought to determine whether the GST-SIPWDLWEPT peptide fusion could associate directly with the
AP-1 γ-ear and whether this interaction was dependent on the 2 Trp residues. As shown in Fig. 7D, the Ni-NTA-purified AP-1 γ-ear domain was able to bind directly to the WT GST-SIP-WDLWEPT peptide fusion, but binding was severely compromised by mutating the proximal Trp to either an Ala or a Phe. Mutation of the distal Trp to Phe, however, had no detectable effect on the ability of the purified γ-ear to associate with the GST-ampiphysin II peptide fusion, but a Trp to Ala substitution at this position abolished binding. These results lend further support to the concept that the WXX(F/W) motif is a bona fide GAE-binding motif that has distinct characteristics from the (D/E)FXXΦ motif.

DISCUSSION

The data presented in this study establish that the 385WNSF385 sequence within the GGA1 hinge region is responsible for its interaction with the AP-1 γ-ear. Within this sequence, the 2 anchoring aromatic residues are critical determinants of the interaction. Recently, a peptide motif conforming to the consensus sequence, (D/E)FXXΦ (Φ represents leucine, phenylalanine, tryptophan or methionine), has been shown to be a GAE-binding motif (20–22). This motif is found in several accessory proteins that interact with both the AP-1 γ-ear and the GGA GAE domains, including γ-synexrin, Rabaptin 5, and EpsinR. Structural studies of peptides containing this motif in complex with the GAE domains of GGA1 and 3 show that the peptides bind in an extended conformation to two complementary hydrophobic pockets on the surface between strands β4 and β5 of the GAE domains (20, 21). The invariant anchoring phenylalanine residue (position 0), in particular, was observed to make specific hydrophobic contacts within the first pocket with 2 highly conserved arginine residues in the GAE domain. The flatness of both the aromatic ring of the phenylalanine and the guanidino group of the arginine made the resultant stacking interactions highly favorable. The crystal structures also revealed that the more general hydrophobic nature of the second pocket would permit accommodation of bulky hydrophobic residues in position +3. This was consistent with the fact that various bulky hydrophobic residues occupy this position in the GAE-binding motifs of the different accessory proteins. Since the GGA1 WNSF sequence has the aromatic amino acid, Trp, in its first position, we envisaged that Trp-382 of GGA1 participates in a very similar interaction with the AP-1 γ-ear. In agreement with this model, we found that the analogous arginine residues in the AP-1 γ-ear, Arg-793 and Arg-796, when mutated to glutamine, rendered the GST γ-ear domain unable to bind AP-1 in the presence of 1 mM of the GGA1 peptide (data not shown). Hence, we would predict the occurrence of analogous binding motifs (WXXF or FXXΦ) in the hinge regions of GGA2 and 3. An alignment of the three GGA hinge segments revealed no obvious candidate sequence in GGA2, but the 417WHLL sequence within the GGA3 hinge (long form) aligned with the 385WNSF sequence of GGA1 and could potentially be the γ-ear-binding motif. However, when we mutated both Trp-417 and Leu-420 to alanine, we observed no binding in the binding of the GGA3 mutants to GST-γ-ear (data not shown). Thus, the GGA2 and GGA3 AP-1-binding motifs may represent other novel sequences that fit into the same hydrophobic pockets of the AP-1 γ-ear domain.

While this manuscript was in preparation, Mattera et al. (28) reported that the NECAP1 GAE-binding motif residues in the sequence 252WGDF255, which is the same proximal WXXF motif that we have identified in the present study. In their study, the investigators performed a combinatorial analysis of GAE-binding motifs by phage display peptide library screening and found that the majority of the binding peptides had a tryptophan at position 0 and +3. Moreover, when they substituted a tryptophan for the phenylalanine in the Rabaptin5 FXXΦ GAE-interacting motif, they observed strong binding, suggesting that tryptophan may replace phenylalanine in FXXΦ-type motifs. Clearly, the reverse does not hold true, raising the possibility that the FXXΦ-type motif may have evolved from the WXX(F/W)-type motif with concomitant changes in the surrounding residues, thereby determining the predilection for either tryptophan or phenylalanine at position 0.

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