Interaction of the Cation-dependent Mannose 6-Phosphate Receptor with GGA Proteins*

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The GGAs (Golgi-localizing, γ-adaptin ear homology domain, ARF-binding) are a multidomain family of proteins implicated in protein trafficking between the Golgi and endosomes. Recent evidence has established that the cation-independent (CI) and cation-dependent (CD) mannose 6-phosphate receptors (MPRs) bind specifically to the VHS domains of the GGAs through acidic cluster-dileucine motifs at the carboxyl ends of their cytoplasmic tails. However, the CD-MPR binds the VHS domains more weakly than the CI-MPR. Alignment of the C-terminal residues of the two receptors revealed a number of non-conservative differences in the acidic cluster-dileucine motifs and the flanking residues. Mutation of these residues in the CD-MPR cytoplasmic tail to the corresponding residues in the CI-MPR conferred either full binding (H63D mutant), intermediate binding (R60S), or unchanged binding (E56F/S57H) to the GGAs as determined by in vitro glutathione S-transferase pull-down assays. Furthermore, the C-terminal methionine of the CD-MPR, but not the C-terminal valine of the CI-MPR, inhibited GGA binding. Addition of four alanines to the C-terminal valine of the CI-MPR bound to the VHS domains of the different GGAs with higher affinity than did the corresponding region of the CD-MPR (10, 13). There also was a significant difference in the relative affinities of the two MPRs for optimal GGA interaction. Mouse L cells stably expressing CD-MPRs with mutations that enhance GGA binding sorted cathepsin D more efficiently than wild-type CD-MPR. These studies provide an explanation for the observed differences in the relative affinities of the two MPRs for the GGA proteins. Furthermore, they indicate that the GGAs participate in lysosomal enzyme sorting mediated by the CD-MPR.

In mammalian cells, newly synthesized lysosomal enzymes are modified post-translationally to acquire the mannose 6-phosphate recognition marker (1). These enzymes bind to the luminal domains of sorting receptors through their mannose 6-phosphate recognition markers at the trans-Golgi network (TGN) and are targeted to acidified endosomes and lysosomes.

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† The abbreviations used are: TGN, trans-Golgi network; CD-MPR, cation-dependent mannose 6-phosphate receptor; CI-MPR, cation-independent mannose 6-phosphate receptor; GST, glutathione S-transferase; HA, hemagglutinin.
glutathione-Sepharose to concentrations of 3–6 mg/ml. The beads with bound proteins were pelleted by centrifugation at 750 × g for 1 min and the beads washed once with cold buffer B, and 300 μl of the SF9 cell lysate in buffer A at a final concentration of 2–5 mg/ml was added to the washed beads. To determine binding to AP-2, 300 μl of bovine adrenal cytosol (8 mg/ml) in buffer B was added to the washed beads. The binding reactions were allowed to proceed for 3 h at 4 °C with tumbling, following which the samples were subjected to centrifugation at 750 × g for 1 min. An aliquot of the supernatant was saved, and the pellets were washed four times each by resuspension in 1 ml of cold buffer B by centrifugation at 750 × g for 1 min and heated at 100 °C for 5 min. Unless indicated otherwise, 1% of each pellet and supernatant fraction was loaded on SDS gels.

ELECTROPHORESIS AND IMMUNOBLOTTING—Proteins were resolved on 10% SDS-polyacrylamide gels and transferred to nitrocellulose. Blots were blocked with TBST (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% non-fat milk for 1 h at room temperature. The blots were then probed 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 (Amersham Biosciences).

PULSING OF CD-MPR TRANSECTANTS—Stable cell lines expressing the various CD-MPR mutants were generated by transfection of the plBIINeo constructs into CI-MPR negative mouse L cells (D-9) and the subsequent selection of clones propagated in G418-containing media. Transgene protein expression was determined by Western blotting. The CD-MPR protein levels of the various cell lines were compared with the non-transfected D-9 cells by labeling equal quantities of total protein from cell lysates on SDS gels, transferring to nitrocellulose, and quantifying the immunoreactive bands by ECL using the Kodak Digital Imaging System (Eastman Kodak Co.).

RESULTS

Effect of Mutations in the CD-MPR Tail on GGA Binding—Although both the CD-MPR and the CI-MPR contain acidic cluster-dileucine sequences near the C terminus of their cytoplasmic tails, inspection of the amino acid sequences of the bovine species shows several differences in this region as well

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as in the flanking residues (Fig. 1). Setting the first leucine of the dileucine motif to 0, there are differences at positions 1, 4, 7, and 8 and at -5 and +3. To evaluate the importance of these differences in binding to the various GGAs, GST fusion peptides incorporating the C-terminal 18 amino acids of the receptors were generated with single or double substitutions of the CI-MPR residues into the equivalent position of the CD-MPR peptide backbone. The ability of these various mutant GST peptides to bind GGAs in pull-down assays was then compared with binding by the wild-type CI-MPR and CD-MPR peptides. In preliminary experiments, we found that the GST peptides with the wild-type MPR sequences bound GGAs to the same extent as the GST full-length cytoplasmic tails. Fig. 2A shows that GGA2 binds much better to the wild-type CI-MPR peptide than to the wild-type CD-MPR peptide, confirming the previous report (10). Thus, analysis of 1% of the GST CI-MPR peptide pellet gave a signal equivalent to that seen with 25% of the GST CD-MPR pellet. Mutation of the His residue at position 1 of the CD-MPR sequence to Asp resulted in a striking increase in GGA2 binding, equivalent to that seen with the CI-MPR peptide. Mutation of the Arg residue to a Ser at position 4 of the CD-MPR sequence greatly enhanced GGA2 binding as well, although not quite to the same extent as the His to Asp substitution. When the Glu and Ser flanking residues at positions 8 and 7 were changed to Phe and His, respectively, there was no significant alteration in GGA2 binding, indicating that these residues do not have a role in the interaction with GGA2. In contrast, the residues downstream of the dileucines strongly affect GGA2 binding. Thus substitution of the Pro and Met residues at positions -2 and 3 with alanines resulted in GGA2 binding equal to that observed with the CI-MPR peptide. This result is similar to that reported by Puertollano et al. (10) with GGA3 using the yeast two-hybrid assay. The same results were obtained when the binding of GGA1 and GGA3 to the various peptides was tested (Fig. 2, B–D). In both cases the CI-MPR peptide bound the GGAs much better than the CD-MPR peptide, and the His to Asp, Arg to Ser, and Pro to Ala/Met substitutions enhanced binding, whereas the Glu to Phe/Ser → His mutation had no detectable effect on binding.

The C-terminal Met Residue Inhibits GGA Binding—The finding that the Pro → Ala/Met → Ala substitution enhanced GGA binding indicated that one or both of these residues was inhibitory. To pursue this point, GST peptides were prepared in which the Pro and Met residues were individually mutated to alanines. When these were tested for binding to the GGA1 VHS domain, we found that the Pro → Ala substitution enhanced binding as much as the Pro → Met substitution. However, when both residues were substituted, binding was reduced to the level of the wild-type CI-MPR peptide, indicating that the C-terminal Met residue is inhibitory to GGA binding. To determine the role of this residue in binding, the Pro and Met residues were individually mutated to alanines. When these were tested for binding to the GGA1 VHS domain, we found that the Pro → Ala substitution enhanced binding as much as the Pro → Met substitution. However, when both residues were substituted, binding was reduced to the level of the wild-type CI-MPR peptide, indicating that the C-terminal Met residue is inhibitory to GGA binding. To determine the role of this residue in binding, the Pro and Met residues were individually mutated to alanines.
The peptide with the Met→Ala mutation exhibited a striking increase in GGA1 binding, whereas the peptide with the Pro→Ala substitution behaved similarly to the wild-type CD-MPR peptide (Fig. 2D). This demonstrates that the C-terminal Met completely accounts for the inhibitory effect. Because the C-terminal residue in the bovine CI-MPR is a valine, we asked whether substitution of this bulky hydrophobic residue with a methionine would influence GGA binding. As shown in Fig. 3, a GST CI-MPR peptide with the Val to Met substitution bound GGA1 and GGA2 poorly when compared with the wild-type peptide, supporting the interpretation that a Met at the +3 position inhibits binding to the GGAs.

Addition of Alanines to the C Termini of the MPRs Inhibits GGA Binding—The acidic cluster/dileucine motifs of both MPRs are located two residues from the C terminus of the cytoplasmic tails (Fig. 1). In sortilin, another membrane receptor known to interact with the GGAs, the acidic cluster/dileucine motif is one residue from the carboxyl end (12). This prompted us to ask whether the positioning of the acidic cluster/dileucine motif relative to the C terminus of the protein influences binding to the GGAs. To explore this issue we constructed GST CI-MPR and CD-MPR peptides with four alanine residues extending beyond the usual C-terminal residue. The CD-MPR peptide also contained the Pro→Ala/Met→Ala substitution so that GGA binding could be more easily detected. As shown in Fig. 4, the addition of the four alanines strikingly inhibited binding to all three GGAs. These findings indicate that the position of the acidic cluster/dileucine motif relative to the C terminus of the cytoplasmic tail has a major effect on GGA binding.

MPR Binding to the GGAs Correlates with Cathepsin D Sorting in Cells—In a previous study (17) published before the discovery of the GGAs, we reported that CI-MPR-negative mouse L cells expressing CD-MPR with the Pro→Ala/Met→Ala mutation sorted the acid hydrolase cathepsin D more efficiently than cells expressing greater amounts of wild-type CD-MPR. The current findings suggest that the improved sorting efficiency may have been the result of enhanced binding to the GGAs. To determine whether this correlation holds for the other mutations that increase GGA binding, CI-MPR-negative L cells (clone D-9) were stably transfected with plasmids encoding bovine CD-MPRs with either Arg→Ser, His→Asp, or Glu→Phe/Ser→His substitutions. The ability of these cells to sort cathepsin D in pulse/chase experiments was measured and compared with cells expressing wild-type CD-MPR or CD-MPR with the Pro→Ala/Met→Ala mutation. The results are summarized in Table I. The non-transfected D-9 cells sorted 44±3% of the cathepsin D due to the endogenous CD-MPR. The sorting efficiency increased to 69±4% in cells expressing 12-fold more wild-type CD-MPR but was essentially unchanged in a cell line expressing only 4-fold more wild-type CD-MPR.

### Table I

| Construct transfected | CD-MPR expression | % cathepsin D sorted | p value |
|-----------------------|-------------------|----------------------|---------|
| None (clone D-9)      | 1.0               | 44±3(30,60,n=10)     | <0.001  |
| Wild-type (clone ML4) - ESEERDDHLLPM | 12             | 69±4(50,83,n=7)      | >0.1    |
| Wild-type (clone ML5) | 4.0               | 45±5(28,63,n=6)      | >0.1    |
| Glu→Phe/Ser→His      | 4.0               | 53±4(49,69,n=4)      | >0.1    |
| His→Asp              | 2.0               | 70±4(44,85,n=9)      | >0.001  |
| Pro→Ala/Met→Ala      | 4.5               | 84±1(79,86,n=5)      | <0.001  |
| Arg→Ser              | 2.0               | 57±2(50,63,n=4)      | <0.05   |

* Levels of CD-MPR expression in the transfected cell lines are expressed relative to the non-transfected mouse D-9 cells.

* Cathepsin D sorting results are expressed as mean ± S.E.; the range of values is given in the parentheses, and n is the number of determinations.

* p values are obtained with statistical analysis comparing individually the whole sets of values of the mutations to the values of D-9.

### Fig. 5. CD-MPR mutants with enhanced GGA interaction do not show improved AP-1 binding.

To determine whether the increased sorting efficiency exhibited by the CD-MPR mutants was due to improved binding to AP-1, GST CI/CD-MPR tail FL (full-length cytoplasmic tail) and GST CI/CD-MPR pep (peptide) wild-type and mutants were tested for their ability to bind AP-1 in *in vitro* pull-down assays. 10% of pellet (P) and 3% of supernatant (S) were loaded on the SDS gel. As seen from the immunoblot, neither the wild-type GST CD-MPR peptide nor the mutants that showed markedly enhanced GGA interaction display any binding to AP-1.

Cells expressing CD-MPR with the Glu→Phe/Ser→His mutation sorted 53±2% of cathepsin D, which is not significantly different from the non-transfected D-9 cells. This mutation does not enhance GGA binding. In contrast, cells expressing CD-MPR with either the Arg→Ser or the His→Asp mutations exhibited a significant increase in cathepsin D sorting (57±2 and 70±4%, respectively) even though the receptor expression level was only two times the basal level. Both of these mutations increase GGA binding with the His→Asp substitution having a greater effect than the Arg→Ser substitution. The cells expressing receptor with the Pro→Ala/Met→Ala mutation sorted cathepsin D very efficiently (84±1%), confirming our previous findings. The expression level of this mutant receptor was 4.5-fold the basal level, which may explain why it is more effective than the His→Asp and Arg→Ser mutants. Taken as a whole, these data reveal a strong correlation between GGA binding and the efficiency of cathepsin D sorting in cells.

Another potential explanation for the improved ability of the mutant receptors to sort cathepsin D is that the amino acid substitutions enhance binding to the AP-1 adaptor protein complex that is also implicated in packaging the MPRs into transport vesicles in the TGN (19). The various GST fusion peptides were therefore tested for their ability to bind AP-1 in pull-down assays. As shown in Fig. 5, the full-length CD-MPR cytoplasmic tail bound only a trace amount of AP-1 in this assay compared with strong binding by the CI-MPR full-length cytoplasmic tail. None of the GST peptides having wild-type or mutant acidic cluster/dileucine sequences bound detectable levels of AP-1. This indicates that the AP-1 binding to the full-length CI-MPR cytoplasmic tail observed in this assay is not mediated by the acidic cluster-dileucine motif, in agreement with our previous study (11). Furthermore, it suggests that the improved efficiency of cathepsin D sorting exhibited by
three of the CD-MPR mutants is not a consequence of enhanced binding to AP-1.

DISCUSSION

The results presented in this study provide a number of new insights concerning the interaction of the GGAs with acidic cluster-dileucine motifs. First, our data show that the weak binding of the CD-MPR to the GGAs relative to that observed with the CI-MPR is due to two amino acid differences in the acidic clusters of these receptors along with a difference in the C-terminal residue located two residues downstream of the dileucines. Within the acidic clusters, we found that an Asp residue at the −1 position greatly enhanced GGA binding compared with a His at this position. This is consistent with our previous study (11) showing that multiple residues in the acidic cluster of the CI-MPR influence binding to GGA2. A Ser at position −4 also improved GGA binding relative to an Arg at that position. In both instances the favorable residue is present in the acidic cluster of the CI-MPR, whereas the unfavorable residue is found in the CD-MPR acidic cluster.

The other key observation is that a Met at the C-terminal position, as occurs in the CD-MPR, inhibits GGA binding, whereas a Val at that position, as found in the bovine CI-MPR, allows strong binding. This finding is significant because it indicates that the GGAs recognize the C-terminal residue as well as the acidic cluster-dileucine motif. Furthermore, because the addition of four alanines to the C-terminal residue strongly inhibited GGA binding, it appears that the location of the acidic cluster-dileucine motif two residues upstream from the C terminus is critical for optimal binding to the GGAs. The effects of these residues on binding was found with all three GGAs, demonstrating that the different GGAs recognize common elements in the acidic cluster-dileucine motifs.

While this manuscript was in preparation, two groups (20, 21) reported the crystal structure of the VHS domains of human GGA1 and GGA3 complexed with the acidic cluster-dileucine motifs of the MPRs. The VHS domain is a right-handed superhelix of eight helices. Helices 6 and 8 form a surface that makes extensive contacts with amino acid residues −5 to +3 of the CI-MPR cytoplasmic tail and residues −4 to +3 of the CD-MPR cytoplasmic tail. Among these residues, the dileucines and the aspartate at position −3 have the most extensive interactions with the VHS-binding site, whereas the other residues appear to contribute to a lesser extent to the binding. These authors also implicated the C-terminal flanking residues of the acidic cluster-dileucine motifs in the binding to the VHS domain. They noted that the C-terminal side-chain protrudes into a hydrophobic pocket. Furthermore, Misra et al. (20) found that MPR sequences with two residues distal to the dileucines gave optimal binding, whereas those with one or three bound more weakly, and those with zero or four did not bind. Peptides containing amidated C-terminal residues also bound very weakly. Thus binding to the VHS domains requires a precise spacing between the dileucines and the free C terminus. Finally, Misra et al. (20) reported that the CI-MPR peptide bound 4–6-fold better to the VHS domains of GGA1 and GGA3 than the CD-MPR peptide as determined by isothermal titration calorimetry. These findings are in good agreement with our results. Our data extend the work of Misra et al. (20) and Shibata et al. (21) by identifying the residues that account for the differences in CI-MPR and CD-MPR binding to the VHS domains.

The second important finding in our study is the strong correlation between the apparent binding affinities of the various acidic cluster-dileucine motifs to GGA1–3 in the in vitro pull-down assays and the efficiency of cathepsin D sorting in mouse L cells expressing CD-MPRs with the different mutations. These data provide evidence that GGAs play a key role in packaging CD-MPRs with bound acid hydrolases into transport vesicles that are delivered to the endosome/lysosome system. A similar correlation emerged from studies of CI-MPRs that have a variety of mutations in their acidic cluster-dileucine motifs (11, 18, 22–24). None of the mutations in the CD-MPR acidic cluster-dileucine motif that enhanced GGA binding resulted in AP-1 binding as measured by the pull-down assays. In fact, the full-length CD-MPR cytoplasmic tail bound AP-1 very poorly in this assay in contrast to the findings with the CI-MPR cytoplasmic tail. This probably reflects the insensitivity of the assay because Honing and co-workers (25) have reported that the cytoplasmic tail of the CD-MPR binds AP-1 with high affinity. These investigators used surface plasmon resonance to identify two independent sequences (amino acids 34–43 and 49–67) that bind AP-1 with Kd values of 14 nM. Whereas the 49–67 sequence includes the acidic cluster-dileucine motif, they showed that mutation of the dileucines abolishes GGA binding, and cells expressing CD-MPRs with this mutation exhibit extremely poor cathepsin D sorting (17, 26). Thus although AP-1 may have a role in packaging the CD-MPR into clathrin-coated vesicles at the TGN, either it handles only a small fraction of the cargo relative to the GGAs or it relies on the GGAs to present the MPRs for incorporation into AP-1 transport vesicles. Our data do not distinguish between these possibilities.

Finally, it is curious that the CD-MPR, in contrast to the CI-MPR, would evolve with an acidic cluster-dileucine motif that fails to provide optimal interaction with the GGAs. The CD-MPR does function to sort acid hydrolases in cells, so perhaps it works sufficiently well so that pressure has not been exerted to evolve into a more efficient receptor. Alternatively, the CD-MPR may be subject to some type of regulation that modulates GGA interaction. The cytoplasmic tail of the CI-MPR contains a casein kinase II phosphorylation site just upstream of the acidic cluster-dileucine motif (the Ser is at position −7, see Fig. 1). The importance of phosphorylation of Ser-57 is not clear at this time. Mauxion et al. (19) have suggested that phosphorylation of this serine facilitates AP-1 recruitment to the TGN. On the other hand, substitution of Ser-57 with an alanine or aspartate did not influence acid hydrolase sorting in mouse cells (17, 27). It may turn out that phosphorylation of Ser-57 regulates binding to the GGAs under some circumstances.

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