22. MDCK or COS-7 cells grown on cover slips were transfected with FuGENE-6 (Roche Molecular Biochemicals). At 15 to 20 hours after transfection, cells were fixed in methanol/acetone at −20°C for 10 min. Immunofluorescent staining and confocal microscopy were done as described (*10*).

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Binding of GGA2 to the Lysosomal Enzyme Sorting Motif of the Mannose 6-Phosphate Receptor

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The GGAs are a multidomain protein family implicated in protein trafficking between the Golgi and endosomes. Here, the VHS domain of GGA2 was shown to bind to the acidic cluster–dileucine motif in the cytoplasmic tail of the cation-independent mannose 6-phosphate receptor (CI-MPR). Receptors with mutations in this motif were defective in lysosomal enzyme sorting. The hinge domain of GGA2 bound clathrin, suggesting that GGA2 could be a link between cargo molecules and clathrin-coated vesicle assembly. Thus, GGA2 binding to the CI-MPR is important for lysosomal enzyme targeting.

The CI-MPR serves a key role in the biogenesis of lysosomes (*1*). This receptor binds newly synthesized lysosomal enzymes through their mannose 6-phosphate recognition marker in the trans-Golgi network (TGN). The ligand-receptor complex is then packaged into transport vesicles that dock on endosomal compartments where the enzymes are released and subsequently transferred to lysosomes. Efficient lysosomal enzyme sorting by this intracellular pathway is dependent on an acidic cluster–dileucine motif near the COOH-terminus of the cytoplasmic tail of the receptor, as shown by the finding that mutations in this motif result in hypersecretion of the enzymes (*2–5*). In spite of the importance of this sorting signal, the binding partner of the acidic cluster–dileucine motif has not been identified.

The GGAs are a newly described protein family named for being Golgi-localized, γ-ear–containing, ARF-binding proteins (*6–10*). Each of the five members (two in yeast and three in mammalian cells) contains four domains. The COOH-terminal domain is homologous to the ear domain of the γ-adaptin subunit of AP-1. This domain is linked to the GAT domain by a hinge region. The GAT domain binds ARF–guanosine triphosphate and mediates membrane association with the TGN. The NH_2-terminal region contains a VHS domain to which no function has been assigned.

Deletion of the yeast GGA genes impairs sorting of carboxypeptidaseY from the Golgi to the vacuole and delivery of Pep12p from the Golgi to late endosomes (*7*, *8*, *11*, *12*), suggesting that the GGAs may be required for the assembly of transport vesicles (*12*). These molecules have been localized to coated buds in the TGN in mammalian cells but have not been detected in clathrin-coated vesicles (CCVs) (*7*, *8*), and no specific function for Golgi-associated GGAs has been defined.

We hypothesized that these proteins might interact with receptors such as the CI-MPR to facilitate their packaging into transport vesicles. To test whether GGA2 binds the CI-MPR, we solubilized rat liver Golgi membranes with detergent and used them as a source of receptor for binding to glutathione *S*-transferase (GST) or GST-GGA2 coupled to glutathione beads. The CI-MPR bound specifically to GST-GGA2 (Fig. 1A). By contrast, the polymeric immunoglobulin A receptor (Fig. 1A), Lamp1, Lamp2, LimpII, and TGN38 did not bind (*13*). Furthermore, purified full-length CI-MPR bound directly to GST-GGA2, whereas soluble CI-MPR lacking the cytoplasmic tail failed to bind (Fig. 1A). We next determined which domain of GGA2 mediates the interaction with the CI-MPR. GST-GGA2 COOH-end (residues 170 to 613) with the VHS domain deleted failed to bind the receptor, implicating the VHS domain in this interaction (Fig. 1A). However the GST-GGA2 VHS domain by itself (residues 29 to 165) also failed to bind the CI-MPR. Extension of the VHS domain by 23 amino acids into the GAT domain (from residues 165 to 188) restored receptor binding (Fig. 1B). Addition of more residues did not increase receptor binding.

To identify the determinant in the CI-MPR cytoplasmic tail that binds the VHS domain, we used a library of CI-MPR negative mouse L cell fibroblast lines that had been stably transfected with cDNAs encoding either wild-type bovine CI-MPR or mutants with various deletions or amino acid substitutions in the cytoplasmic tail (Fig. 1C) (*2*, *4*, *5*). Cell pellets were solubilized with detergent and tested for binding to GST-GGA2 in pull-down binding assays. Mutant bovine CI-MPRs with COOH-terminal deletions of 40 or more amino acids of the 163–amino acid cytoplasmic tail were greatly impaired in binding to GST-GGA2 (Fig. 1D). We next tested the AC series of mutants, each having four consecutive residues changed to alanines, starting at the COOH-terminal LLHI (*14*) sequence of the murine CI-MPR (*5*). Mutants AC1 to 3 were severely impaired in binding GST-GGA2 (<5% of wild type), whereas mutants AC4 and AC5 exhibited only a small decrease in binding (80% of wild type), and AC8 was somewhat more impaired (35% of wild type) (Fig. 1, E and F). Receptors with point mutations in this distal region (D157A, D158A, D160A, E161A, and D162A) (*14*) also exhibited poor binding to GST-GGA2 (12 to 20% of wild type) (Fig. 1G). The cytoplasmic tail of CI-MPR contains a second acidic motif that lacks the dileucine sequence (Fig. 1C). Mutations in this motif (AC17 and AC18) did not substantially affect GGA2 binding, indicating that this upstream motif is not necessary for GGA2 binding (Fig. 1F). Finally, a mutant receptor with the COOH-terminal LLHI sequence deleted (Δ4) failed to bind GST-GGA2 (Fig. 1F). These results demonstrate that the acidic cluster–dileucine sequence ^{157}DDSDEDLLHI^{166} (*14*) mediates the interaction with the VHS domain of GGA2 and establish a very strong correlation between reduced GGA2 binding and defective lysosomal enzyme sorting. The failure of rat LimpII with a DEXXXLL (*14*) motif in its cytoplasmic tail to bind GGA2 illustrates the specificity of this interaction.

The CI-MPR has been localized to AP-1–containing CCVs on the TGN (*15*). According-

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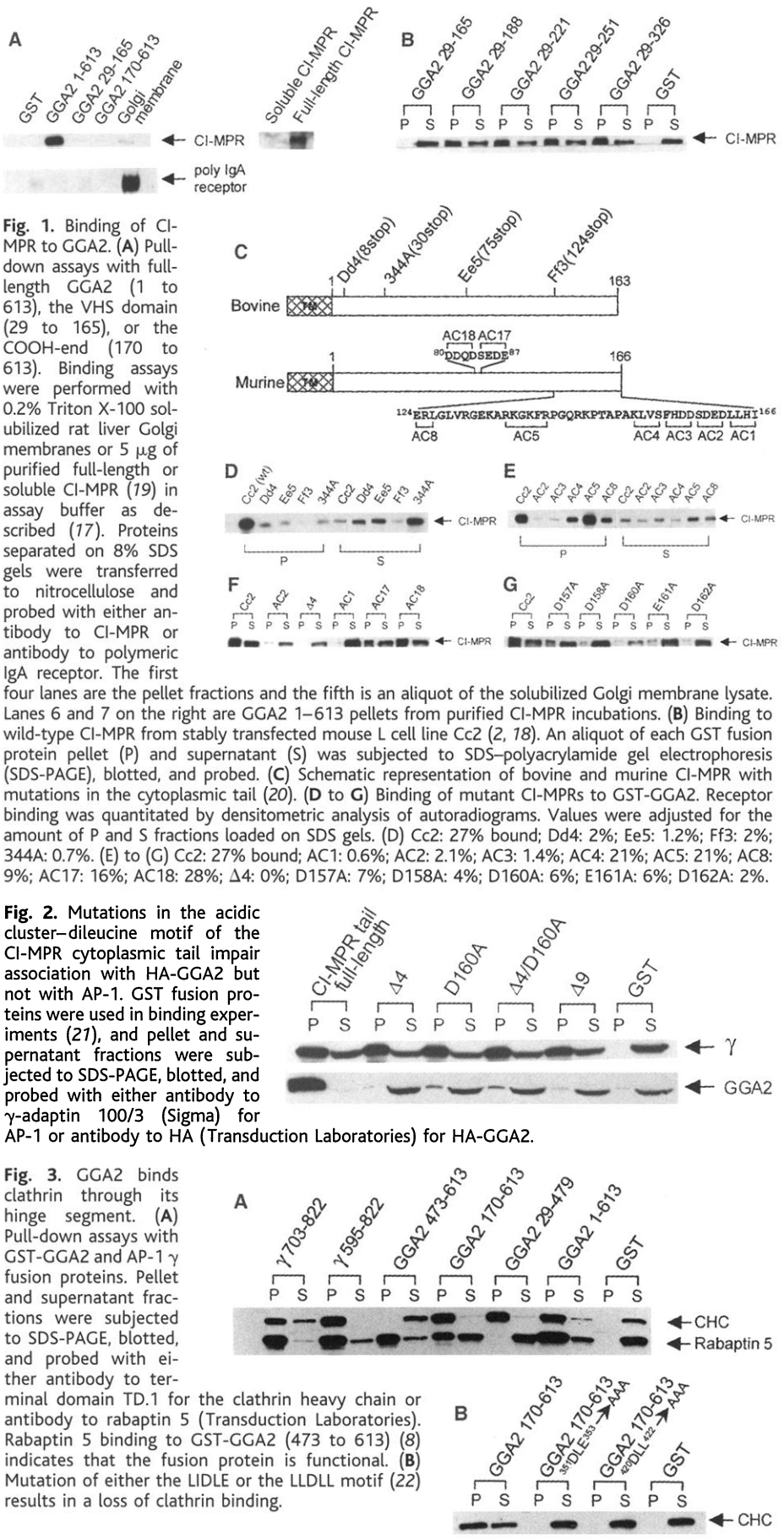

Fig. 1. Binding of CI-MPR to GGA2. (**A**) Pull-down assays with full-length GGA2 (1 to 613), the VHS domain (29 to 165), or the COOH-end (170 to 613). Binding assays were performed with 0.2% Triton X-100 solubilized rat liver Golgi membranes or 5 μg of purified full-length or soluble CI-MPR (*19*) in assay buffer as described (*17*). Proteins separated on 8% SDS gels were transferred to nitrocellulose and probed with either antibody to CI-MPR or antibody to polymeric IgA receptor. The first four lanes are the pellet fractions and the fifth is an aliquot of the solubilized Golgi membrane lysate. Lanes 6 and 7 on the right are GGA2 1–613 pellets from purified CI-MPR incubations. (**B**) Binding to wild-type CI-MPR from stably transfected mouse L cell line Cc2 (*2*, *18*). An aliquot of each GST fusion protein pellet (P) and supernatant (S) was subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE), blotted, and probed. (**C**) Schematic representation of bovine and murine CI-MPR with mutations in the cytoplasmic tail (*20*). (**D** to **G**) Binding of mutant CI-MPRs to GST-GGA2. Receptor binding was quantitated by densitometric analysis of autoradiograms. Values were adjusted for the amount of P and S fractions loaded on SDS gels. (D) Cc2: 27% bound; Dd4: 2%; Ee5: 1.2%; Ff3: 2%; 344A: 0.7%. (E) to (G) Cc2: 27% bound; AC1: 0.6%; AC2: 2.1%; AC3: 1.4%; AC4: 21%; AC5: 21%; AC8: 9%; AC17: 16%; AC18: 28%; Δ4: 0%; D157A: 7%; D158A: 4%; D160A: 6%; E161A: 6%; D162A: 2%.

Fig. 2. Mutations in the acidic cluster–dileucine motif of the CI-MPR cytoplasmic tail impair association with HA-GGA2 but not with AP-1. GST fusion proteins were used in binding experiments (*21*), and pellet and supernatant fractions were subjected to SDS-PAGE, blotted, and probed with either antibody to γ-adaptin 100/3 (Sigma) for AP-1 or antibody to HA (Transduction Laboratories) for HA-GGA2.

Fig. 3. GGA2 binds clathrin through its hinge segment. (**A**) Pull-down assays with GST-GGA2 and AP-1 γ fusion proteins. Pellet and supernatant fractions were subjected to SDS-PAGE, blotted, and probed with either antibody to terminal domain TD.1 for the clathrin heavy chain or antibody to rabaptin 5 (Transduction Laboratories). Rabaptin 5 binding to GST-GGA2 (473 to 613) (*8*) indicates that the fusion protein is functional. (**B**) Mutation of either the LIDLE or the LLDLL motif (*22*) results in a loss of clathrin binding.

ly, we tested whether the acidic cluster–dileucine motif also mediates AP-1 binding. AP-1 bound equally well to GST fusions containing either wild-type or mutant CI-MPR cytoplasmic tails, whereas the mutants displayed only background levels of hemagglutinin (HA)-GGA2 binding (Fig. 2). Furthermore, a GST fusion protein containing only the acidic cluster–dileucine motif bound HA-GGA2, but not AP-1 (*13*). Thus, impaired AP-1 binding was not the basis for the sorting defect in cells expressing receptors with mutations in the acidic cluster–dileucine motif.

The hinge region of GGA2 contains two consensus clathrin-binding motifs, LIDLE (*7*) and LLDLL (*14*, *16*). GST fusion proteins containing full-length GGA2 (residues 1 to 613), the NH_2-terminal region (residues 29 to 479), and the COOH-terminal region (residues 170 to 613) all bound clathrin from rat liver cytosol in a pull-down experiment (Fig. 3A). These three constructs share the GAT domain and the hinge region. In contrast, the γ-adaptin earlike domain (residues 473 to 613) failed to bind clathrin, whereas the AP-1 γ ear domain (703 to 822) did. When either of the putative clathrin-binding motifs in the hinge was mutated to alanines in the COOH-terminal construct, clathrin binding was markedly reduced, establishing that the hinge region is necessary for clathrin binding by GGA2 (Fig. 3B).

The fact that mutations in the CI-MPR acidic cluster–dileucine motif impair lysosomal enzyme sorting and decrease binding to GGA2, but not AP-1, indicates that GGA2 has a major role in this sorting process. This could occur by two mechanisms. First, GGA2 could bind the CI-MPR in the TGN and facilitate its entry into forming AP-1 CCVs. The ability of GGA2 to bind clathrin could mediate or enhance its association with AP-1 CCVs. Alternatively, GGA2 could bind the CI-MPR and function as a coat protein, nucleating its own coated vesicles. In this case, GGA2 and AP-1 would be functioning in parallel to package CI-MPR into different vesicular carriers at the TGN, similar to what has been proposed in yeast (*12*). GGA2 may also perform both of these functions. These findings reveal an unexpected level of complexity in the sorting of lysosomal enzymes.

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14. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; and X, any amino acid.
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18. Mouse L cells (Cc2) expressing bovine CI-MPR were grown to confluency on 15-cm tissue culture dishes in α-MEM (Mediatech) and 10% fetal bovine serum. The cells were washed twice with phosphate-buffered saline, scraped into assay buffer containing 0.2% Triton X-100, and sonicated. After centrifugation at 15,000*g*, the supernatant was used immediately in binding assays (*17*). GST-GGA2 fusion constructs were made by polymerase chain reaction (PCR) with GST-GGA2(1-613)pGEX4T (*6*) as template DNA and ligation into the vector pGEX6P (Amersham Pharmacia). Fusion proteins were expressed and purified as described (*17*).
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20. The construction of the deletion mutants (Dd4, Ee5, Ff3, and 344A) and the AC mutants and their effect on sorting are described elsewhere (*2*, *3*, *5*).
21. GST fusion protein constructs encoding the wild-type bovine 163–amino acid cytoplasmic tail and the deletion or point mutants were made by PCR as described (*17*). HA-GGA2(1–613)pRK5 was transfected into COS cells (*6*), and the cell lysate was prepared as with mouse L cells (*18*). GST pull-down assays were performed with either bovine brain cytosol (AP-1) or the COS cell lysate (HA-GGA2).
22. Site-directed mutagenesis was carried out with primers incorporating the desired mutations with the QuickChange system (Stratagene). GST pull-down assays were performed with rat liver cytosol as the source of soluble clathrin. The TD.1 monoclonal antibody was a gift from F. Brodsky
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G Protein Signaling from Activated Rat Frizzled-1 to the β-Catenin–Lef-Tcf Pathway

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The *frizzled* receptors, which mediate development and display seven hydrophobic, membrane-spanning segments, are cell membrane–localized. We constructed a chimeric receptor with the ligand-binding and transmembrane segments from the β_2-adrenergic receptor (β_2AR) and the cytoplasmic domains from rat Frizzled-1 (Rfz1). Stimulation of mouse F9 clones expressing the chimera (β_2AR-Rfz1) with the β-adrenergic agonist isoproterenol stimulated stabilization of β-catenin, activation of a β-catenin–sensitive promoter, and formation of primitive endoderm. The response was blocked by inactivation of pertussis toxin–sensitive, heterotrimeric guanine nucleotide–binding proteins (G proteins) and by depletion of Gαq and Gαo. Thus, G proteins are elements of Wnt/Frizzled-1 signaling to the β-catenin–lymphoid-enhancer factor (LEF)-T cell factor (Tcf) pathway.

Wnts constitute a family of vertebrate genes encoding ligands essential to signaling in early development, signaling that includes control of cell proliferation, cell fate, and embryonic patterning (*1*). These secreted glycoproteins act via members of the *frizzled* gene family (*2–4*). Signaling downstream of some Frizzled homologs in response to Wnt-1 or Wnt-8 leads to activation of the phosphoprotein Dishevelled (Dsh/Dvl), which then represses the function of glycogen synthase kinase-3β (GSK-3) activity (*5*, *6*). In the absence of Wnt, GSK-3 phosphorylates β-catenin, reducing its stability and abundance. Wnt signaling represses GSK-3 activity, thereby increasing the stability and intracellular accumulation of β-catenin, which then accumulates in the nucleus where it binds to members of the Lef-Tcf classes of architectural high-mobility group box transcription factors to activate genes involved in early development. Analysis of this pathway has been hindered by the lack of easy experimental methods for tightly controlling the receptor activation state or inhibition of the receptor.

To allow the rapid activation and inhibition of Frizzled coupled to the β-catenin pathway, we created a chimeric receptor consisting of the extracellular and transmembrane segments of the hamster β_2AR and the cytoplasmic domains of the Rfz1 (Fig. 1A). The sequence of the Rfz1 cytoplasmic domains diverge from those of the β_2AR (*7*, *8*). This chimeric receptor has the potential to be activated by soluble drugs of well-known pharmacology. Mouse F9 teratocarcinoma cells were stably transfected with an expression vector harboring the β_2AR-Rfz1 chimera (*9*). Clones expressing mRNA encoding the Rfz-1 chimeric receptor in large amounts were identified by reverse transcription–polymerase chain reaction (RT-PCR) and propagated (Fig. 1B). Expression of the chimeric receptor was quantified readily using labeled iodocyanopindolol (ICYP), a high-affinity β-adrenergic antagonist ligand that binds specifically to the transmembrane domain of the β_2AR. ICYP-binding studies of Chinese hamster ovary (CHO) clones (which do not express endogenous β_2AR) stably transfected with pβ_2AR-Rfz1 vector demonstrate a K_d of ~80 pM and a maximal binding capacity (B_{max}) of 2 to 4 pmol ICYP binding per milligram of protein (*10*, *11*). Immunoblots of cell membranes from F9 clones expressing the β_2AR-Rfz1 chimera stained with antibodies to an extracellular epitope of the β_2AR identified the endogenous 65-kD β_2AR and a 55-kD molecular species with the predicted size of the chimera (Fig. 1C). ICYP binding to the β_2AR-Rfz1 chimera displays a rightward shift of the affinity of the chimera for the β-adrenergic agonist isoproterenol (ISO) in the presence of a GTP analog (GTP-γ-S, Fig. 1D). All heptihelical receptors known to operate via heterotrimeric G proteins display this characteristic GTP-dependent shift in agonist affinity (*12*).

Before testing the signaling activity of β_2AR-Rfz1, we needed an assay for signaling by Rfz1. F9 cells stably transfected to express wild-type Rfz1 form primitive endoderm (PE) when treated with conditioned medium containing Xwnt-8, as monitored by positive staining for the PE markers cytokeratin endo-A (i.e., antigen for the TROMA monoclonal antibody) and tissue plasminogen activator (*13*, *14*). Clones expressing the β_2AR-Rfz1 chimera were therefore treated with the β-adrenergic agonist ISO or the β-adrenergic antagonist propranolol, or both. Isoproterenol stimulated formation of PE in stably transfected F9 stem cells and propranolol blocked this response (Fig. 2A). Taken together these data demonstrate the ability of the Rfz1 chi-

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