The Acidic Cluster of the CK2 Site of the Cation-dependent Mannose 6-Phosphate Receptor (CD-MPR) but Not Its Phosphorylation Is Required for GGA1 and AP-1-Binding*  

Received for publication, December 10, 2003, and in revised form, March 24, 2004  
Published, JBC Papers in Press, March 24, 2004, DOI 10.1074/jbc.M313525200  

Jacqueline Stöckli, Stefan Hönigs, and Jack Rohrer‡†  

From the Institute of Physiology, University of Zurich, Zurich 8057, Switzerland and the Institute for Biochemistry II, University of Göttingen, 37073 Göttingen, Germany  

Lysosomal biogenesis depends on proper transport of lysosomal enzymes by the cation-dependent mannose 6-phosphate receptor (CD-MPR) from the trans-Golgi network (TGN) to endosomes. Trafficking of the CD-MPR is mediated by sorting signals in its cytoplasmic tail. GGA1 (Golgi-localizing, γ-ear-containing, ARF-binding protein-1) binds to CD-MPR in the TGN and targets the receptor to clathrin-coated pits for transport from the TGN to endosomes. The motif of the CD-MPR that interacts with GGA1 was shown to be 61DXLL. Reports on increased affinity of cargo, when phosphorylated by casein kinase 2 (CK2), to GGA1s focused our interest on the effect of the CD-MPR CK2 site on binding to GGA1. Here we demonstrate that Glu56 and Glu59 of the CK2 site are essential for high affinity GGA1 binding in vitro, whereas the phosphorylation of Ser57 of the CD-MPR has no influence on receptor binding to GGA1. Furthermore, the in vivo interaction between GGA1 and CD-MPR was abolished only when all residues involved in GGA1 binding were mutated, namely, Glu56, Glu59, Asp61, Leu64, and Leu65. In contrast, the binding of adaptor protein-1 (AP-1) to CD-MPR required all the glutamates surrounding the phosphorylation site, namely, Glu55, Glu56, Glu58, and Glu59, but like GGA1 binding, was independent of the phosphorylation of Ser57. The binding affinity of GGA1 to the CD-MPR was found to be 2.4-fold higher than that of AP-1. This could regulate the binding of the two proteins to the partly overlapping sorting signals, allowing AP-1 binding to the CD-MPR only when GGA1 is released upon autoinhibition by phosphorylation.

The cation-dependent mannose 6-phosphate receptor (CD-MPR) is a type I integral membrane protein that is involved in the transport of lysosomal hydrolases to the lysosomes (1, 2). Newly synthesized lysosomal enzymes acquire a mannose 6-phosphate tag on their N-linked oligosaccharides by sequential action of two enzymes in the Golgi and the trans-Golgi network (TGN) (3, 4). The mannose 6-phosphate tag acts as a lysosomal targeting signal and is recognized by the CD-MPR in the TGN. Upon binding lysosomal enzymes, CD-MPR is packaged into clathrin-coated vesicles and transported to the acidified endosomes, where the receptor dissociates from the ligand, which is subsequently packaged into lysosomes. However, the receptor is either transported to the plasma membrane, where it is rapidly internalized, or recycled back to the TGN to mediate another round of sorting. The trafficking of the CD-MPR is directed by signals located in its 67-amino-acid cytoplasmic tail.

The binding of the CD-MPR cytoplasmic tail to GGA1 (Golgi-localizing, γ-ear-containing, ARF-binding protein-1) mediates its transport out of the TGN (5). GGA1 is a monomeric, soluble adaptor with four domains, an N-terminal VHS (Vps37p/Hrs/STAM) domain, a GAT (GGA and TOM1) domain, a connecting hinge segment, and a C-terminal GAE (γ-adaptin ear) domain (6–8). Recruitment of GGA1 to the TGN is mediated by an interaction of the GAT domain with ADP-ribosylation factor (ARF) (9). The VHS domain of GGA1 binds cargo, which is subsequently targeted to clathrin-coated pits, mediated through an interaction between the GGA1 hinge domain with clathrin and adaptor protein-1 (AP-1) (5, 10). The key residues in cargo, such as CD-MPR, cation-independent MPR (CI-MPR), sortilin, memapsin 2, and low density lipoprotein receptor-related protein 3 (LRP3), for binding to the VHS domain of GGA1 were shown to be DXXLL (5, 11–13). For the CI-MPR, it was shown that the phosphorylation by casein kinase 2 (CK2) of the serine preceding the aspartate increases its affinity to GGA1 (11). Furthermore, the acidic cluster-dileucine motif in the hinge domain of GGA1 requires phosphorylation by CK2 of the serine three residues upstream of the aspartate to facilitate autoinhibition of the VHS domain, by intramolecular or intermolecular binding (14). The CD-MPR also contains a serine upstream of the 61DXXLL, which is phosphorylated by CK2, but its involvement in GGA1 binding has not been investigated so far (15, 16).

Reports on the functional importance of the CK2 phosphorylation site are controversial. Normal as well as impaired lysosomal enzyme delivery was reported for CD-MPR with mutant CK2 phosphorylation sites (17–19). Furthermore, the phosphorylation of Ser57 was suggested to be required for surface delivery of the CD-MPR either directly from TGN or by inhibiting transport from endosomes to TGN (17). On the other surface plasmatic resonance; WT, wild-type; PIC, pro tease inhibitor cocktail.
hand, phosphorylation of Ser-207 was shown to be essential for AP-1 binding to the TGN for proper sorting to endosomes (19, 20). However, Höning et al. (21) showed high affinity binding of AP-1 to non-phosphorylated CD-MPR peptides (residues 1–67 and 49–67). Altogether, it is uncertain whether the phosphorylated serine or the non-phosphorylated serine leads to plasma membrane delivery of the CD-MPR, resulting in mis-sortings of lysosomal enzymes, or whether it has an effect on lysosomal enzyme sorting at all.

Before the GGAs and their function of sorting in the TGN were identified, AP-1 was thought to mediate TGN sorting of cargo, such as CD-MPR (21–23). Since the discovery of the GGAs, the function of AP-1 became less clear, and thereafter, some models accounting for the function of AP-1 were suggested. Fibroblasts deficient in the μ1-subunit of AP-1 accumulated CD-MPR in endosomes and showed reduced retrograde transport in vitro, which led to the conclusion that AP-1 could be involved in retrograde transport from endosomes to the TGN (24, 25). However, the localization of AP-1 to the TGN and in clathrin-coated vesicles originating from the TGN, as well as its increased binding to CD-MPR upon phosphorylation of the receptor, led to a model in which GGA1 recruits cargo, and by phosphorylation of both GGA1 and cargo, GGA1 hands over the cargo to AP-1, which mediates vesicle budding from TGN (10, 20, 23, 26).

Our aim was to explore the involvement of the CK2 phosphorylation site in GGA1 and AP-1 binding to determine the importance of the CK2 phosphorylation of CD-MPR in TGN sorting. In this study, we show that two glutamates, Glu-55 and Glu-56, are involved in GGA1 binding in vitro in addition to the known DXXLL motif and that phosphorylation of Ser-207 of the CD-MPR had no effect on GGA1 binding. The importance of Glu-53 and Glu-59 of the CD-MPR for high affinity binding of GGA1 was further confirmed by kinetic studies. Analysis of the interaction of GGA1 with the CD-MPR in vivo demonstrates that the binding was abolished only when residues Glu-53, Glu-59, Asp-61, Leu-81, and Leu-99 were mutated simultaneously. A comparison of the binding properties of AP-1 and GGA1 to the CD-MPR revealed subtle but essential differences. Most importantly, the CD-MPR has a lower binding affinity to AP-1 than to GGA1. Furthermore, all four glutamates, Glu-55, Glu-56, importantly, the CD-MPR has a lower binding affinity to AP-1 than to GGA1. Furthermore, all four glutamates, Glu-55, Glu-56, Glu-58, and Glu-59, but not the phosphorylation of Ser-57, were involved in AP-1 binding.

**EXPERIMENTAL PROCEDURES**

**Materials**—Enzymes used in molecular cloning were obtained from Roche Diagnostics, New England Biolabs (Beverly, MA), or Promega (Madison, WI); general chemicals were from Fluka (Buchs, Switzerland); protease inhibitors were from Sigma; Dubecco’s modified Eagle’s medium (DMEM), fetal calf serum, G418, and LipofectAMINE Plus were from Invitrogen; polyethyleneimine, 25 kDa (catalog number 23966) was from Polysciences, Inc. (Warrington, PA); cell culture dishes were from Falcon (Franklin Lakes, NJ); nitrocellulose was from Schleicher & Schuell; enhanced chemiluminescence Western blotting reagents were from PerkinElmer Life Sciences; glutathione-Sepharose 4B and low molecular weight protein markers were from Amersham Biosciences; Prolong Antifade were from Molecular Probes (Eugene, OR). Oligonucleotides were synthesized either by the DNA synthesis facility of the Friedrich Miescher Institute (Basel, Switzerland) or by the DNA synthesis facility of the Microsynth GmbH (Balgach, Switzerland) after digestion with pEGFP-C1 vector (Clontech). The construct was confirmed by DNA sequencing.

**Antibodies**—Horseradish peroxidase-conjugated antibodies against mouse and rabbit were from Amersham Biosciences. Alexa 568-conjugated goat anti-mouse antibody and Alexa 488-conjugated goat anti-rabbit antibody were from Molecular Probes. The monoclonal antibody 22D4 specific for the bovine CD-MPR was generously provided by D. Messner (27). The polyclonal anti-CD-MPR antibody was a kind gift from S. Kornfeld (Washington University School of Medicine, St. Louis, MO). The anti-giantin monoclonal antibody was kindly provided by H. P. Hauri.

**Recombinant DNA**—All basic DNA procedures were as described (28). The PCR procedure of Ho et al. (29) was used to generate the MPR-FFWYLLA, MPR-C30A/C34A, MPR-S27A, MPR-S57T, MPR-C118S, MPR-Clus A, MPR-Clus D, MPR-E55Q, MPR-E56Q, MPR-E55/Q-E56Q, MPR-E56Q, MPR-E56Q, MPR-E56Q/E59Q, MPR-D61N, MPR-R61D/L64A/L65A, MPR-E56Q/E59Q/D61N, L64A/L65A constructs with pSFFV-MPR (30) serving as a template together with MPR-BglII-I-down (5-CGGAGAATTCCTCACCTAGGCGTGG-3) and pSFFV-neup (5-CTGGCTATGCACTCGCATTATT-3) as the downstream and upstream primers respectively. Appropriate partial complementary pairs of oligonucleotides in which the desired amino acid replacement had been incorporated were chosen as internal primers. The final PCR products were subcloned into pSFFVneo as described (30) and confirmed by sequencing.

**GST-GGA1-A240stop construct** was generated by PCR amplification, using Myc-GGA1pFB1 (a generous gift from S. Kornfeld) as a template and subsequent cloning into the BamHI-NotI sites of pGEX-6p3 (Amersham Biosciences). The GFF-GGA1-A240stop construct was generated in two steps as follows. The GGA1-A240stop insert was subcloned into the BamHI-NotI sites of pcdNA3.1+, and the resulting plasmid was then digested with Xhol, and the overhanging 5’ end was filled up with the Klenow fragment of DNA polymerase I and subsequently digested with KpnI. The fragment was ligated with KpnI-SmaI-digested eGPFG-C1 vector (Clontech). The construct was confirmed by sequencing.

**Cell Culture and Transfection**—A mammalian 6-Phinulin-like growth factor receptor-deficient mouse L cell line designated D9 (LRec−) was maintained in DMEM containing 10% fetal calf serum. The cells were transfected with XbaI-linearized DNA with LipofectAMINE Plus according to the manufacturer’s directions. Selection for resistance to neomycin (G418) was carried out using 500 μg/ml G418 as the final concentration. Resistant colonies were picked individually and screened for expression of bovine CD-MPR by immunoblotting. Clones expressing similar amounts of receptor as compared with ML4 cells, the reference cell line expressing WT bovine CD-MPR (31), were expanded for further study and maintained in selective medium.

Mouse L cells stably expressing wild-type or mutant CD-MPR were grown on coverslips in 6-well plates in DMEM to 50% confluency before transfection with GST-GGA1-A240stop or pSFFV-neup (5-mg/ml polyethyleneimine (25 kDa). 2.2 μg of DNA in 75 μl of DMEM was mixed with 8 μl of 1 mg/ml polyethyleneimine in 75 μl of DMEM, vortexed, and incubated for 15 min at room temperature following the addition of 1.05 ml of DMEM. The mix was added to the cells in the 6-well plate, and after 24 h, the cells were fixed and prepared for immunofluorescence.

**In Vitro GST Pull-down Experiment**—GST-GGA1-A240stop or GST-CD-MPR were expressed in Escherichia coli strain DH5α. A saturated overnight culture was diluted 1:10 in 25 ml of growth medium and incubated at 37 °C until A600 was 0.6–0.8 before induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h. The cells were harvested by centrifugation, washed with ice-cold phosphate-buffered saline (PBS), and lysed by sonication in 2 ml of pull-down buffer (50 mM Hepes, pH 7.4, 150 mM KCl, 1 mM MgCl2) containing a 1:500 dilution of a protease inhibitor cocktail (5 mg/ml benzamidine and 1 mg/ml each of pepstatin A, leupeptin, antipain, and chymostatin in 40% dimethyl sulfoxide, 60% ethanol) (PIC) and phenylmethylsulfonyl fluoride (PMSF) (40 μg/ml in ethanol). Insoluble material was removed by centrifugation at 12,500 rpm for 10 min at 4 °C in a Sorvall GSA centrifuge. The supernatant was incubated for 30 min at room temperature on a rotating shaker with 400 μl of glutathione-Sepharose 4B beads, which were prewashed three times with pull-down buffer containing 0.1% bovine serum albumin in a silanized Eppendorf tube. The beads with GST or GST-GGA1-A240stop were washed three times and then resuspended in 1 ml of pull-down buffer containing PIC and PMSF, the amount for 10 assays. Extracts from mouse L cells expressing wild-type or mutant CD-MPR constructs were prepared from cells grown on 15-cm Falcon tissue culture dishes in the following way. Confluent 15-cm dishes of cells were put on ice and washed once with 10 ml of ice-cold PBS and scraped into 5 ml of pull-down buffer containing PIC and PMSF, then pelleted at 1000 rpm for 5 min at 4 °C in a Heraeus centrifuge and resuspended in 1 ml of pull-down buffer containing PIC and PMSF. The cells were homogenized in a ball-bearing homogenizer of 16-μm clearance using 12 strokes on ice and centrifuged at 700 × g for 10 min at 4 °C. The protein concentration of the resulting post-nuclear supernatant was measured and a protein assay of appropriate amounts of PNS from each of the mutant cell lines and wild-type cell line yielding the same amount of CD-MPR were determined by Western blotting with anti-CD-MPR monoclonal antibody 22D4 followed by densitometric scanning and quantification using Image-
QuanNT 5.0 software (Amersham Biosciences). These PNS samples adjusted for expression levels were used for the assay. 100 μl of the resuspended beads bound with either GST or GST-GGA1-A240stop were incubated with 100 μg of PNS from the wild-type cell line or an equivalent amount of PNS from mutant cell lines adjusted for the expression level as described above in 300 μl of pull-down buffer containing PIC and PMSF and 0.1% Triton X-100 in a silanized Eppendorf tube for 2 h at 4 °C on a rotating shaker. Beads were spun at 2500 rpm for 2 min at room temperature in an Eppendorf tabletop centrifuge. The supernatant was collected and stored. The beads were washed three times with pull-down buffer containing 0.1% Triton X-100. 40 μl of non-reducing SDS-PAGE sample buffer (94 mM Tris-HCl (pH 6.8), 3% SDS, 15% glycerol, 0.001% bromphenol blue) was added to the beads, boiled and analyzed by SDS-PAGE and Western blotting containing PIC and PMSF for 30 min at room temperature. The beads were spun down at 700 × g for 2 min, the supernatant containing the GST-GGA1-A240 protein was dia lyzed against buffer A (50 mM Tris, pH 8.7, 250 mM NaCl, 1 mM dithiothreitol) containing PIC and PMSF. The GST-GGA1-A240 was recovered, and the concentration was measured using a protein assay.

Analysis of Protein-Protein Interaction by SPR—To monitor the binding of AP-1 and GGA1 to the CD-MPR C-terminal domain, a synthetic peptide corresponding to the tail residues 49–57 and mutants within this peptide were immobilized on a CM5 surface of a BIAcore 3000 biosensor. AP-1 was purified from pig brain using published methods (21), and GGA1 was purified as a GST fusion protein. Both proteins were immobilized on the sensor surface. The proteins were eluted from the sensor surface using 500 mM NaCl in buffer A (50 mM Tris, pH 8.7, 250 mM NaCl, 1 mM dithiothreitol) at a flow rate of 20 μl/min. Binding and dissociation were recorded for 2 min. A short pulse injection (5 s) of 50 mM NaOH was then used to remove bound material from the sensor surface. The rate constants were determined using the software supplied by the manufacturer assuming a single binding site for AP-1 and GGA1.

SDS-PAGE and Immunoblotting—The proteins were separated on a 10% SDS-polyacrylamide minigel by using the Laemml system (32). After electrophoresis, gels were transferred into nitrocellulose membranes according to the method of Towbin et al. (33). The membrane was blocked with 5% non-fat dry milk powder (Sano Lait, Coop, Switzerland) in PBS. The blot was subsequently incubated with monoclonal antibody 22D4 (diluted 1:500 in PBS-3% powdered milk) followed by a horseradish peroxidase-conjugated anti-mouse secondary antibody (di- antibody 22D4 (diluted 1:500 in PBS-3% powdered milk) followed by a horseradish peroxidase-conjugated anti-mouse secondary antibody (di- antibody 22D4 (diluted 1:500 in PBS-3% powdered milk) followed by a horseradish peroxidase-conjugated anti-mouse secondary antibody (di- antibody 22D4 (diluted 1:500 in PBS-3% powdered milk) followed by a horseradish peroxidase-conjugated anti-mouse secondary antibody (diluted 1:500 in PBS-3% powdered milk). Immunoreactive proteins were visualized using the enhanced chemiluminescence detection system according to the manufacturer’s instructions.

Confocal Immunofluorescence Microscopy—Cells were grown on coverslips and, if mentioned, transiently transfected with pEGFP-GGA1-A240stop. After 24 h, cells were washed with PBS and fixed in 3% paraformaldehyde, pH 8.3, for 20 min followed by four washes with 20 mM glycine in PBS. The cells were permeabilized in saponin buffer (0.1% saponin, 20 mM glycine in PBS) for 20 min. All the following steps were performed in saponin-containing buffer. Cells were incubated with anti-CD-MPR monoclonal antibody 22D4 (1:500) or anti-giantin monoclonal antibody (1:250) for 30 min and washed four times followed by the incubation with goat anti-mouse Alexa 568 antibody. The non-transfected cells were incubated with anti-CD-MPR polyclonal antibody followed by an incubation with goat anti-rabbit Alexa 488 antibody. The coverslips were washed four times and mounted on glass slides using the ProLong Antifade for viewing with a Leica SP2 AOBS UV confocal laser-scanning microscope. Serial sections in the z-axis through the entire cells were taken, and the resulting stacks of images were analyzed using the Imaris program (Bitplane AG, Zurich, Switzerland). The quantification of the fluorescence was performed using the Leica Confocal Analysis software. A region of interest was set around the Golgi/TGN area, and the amount of fluorescence was determined for the green (GGA1 or giantin) and the red (CD-MPR) channel by the program. A second region of interest was laid around the entire cells, and the fluorescence was measured again for both channels. Using these values, the percentage of fluorescence in the Golgi/TGN area was calculated relative to the total staining.

RESULTS

Acidic Cluster of the CK2 Site of CD-MPR but Not Phosphorylation Is Essential for GGA1 Binding—To analyze the involvement of the CK2 phosphorylation site of the CD-MPR in GGA1 binding, several mutants of the bovine CD-MPR were constructed and stably transfected into mouse L cells. We used full-length CD-MPR, expressed in mammalian cells to allow the formation of the correct posttranslational modification and the three-dimensional structure, which provided physiological conditions for protein-protein interactions. To differentiate between the phosphorylation and the acidic CK2 site, several point mutations of the CD-MPR sequence were created (Fig. 1A). The Ser57 was replaced either by an alanine to disable phosphorylation or by an aspartate to disable phosphorylation, but mimicking the negative charge of phosphorylation. In addition to the three variants at position 57, the cluster of four glutamates Glu55–Glu59 of the CK2 site was mutated to glutamines, generating the cluster-minus mutants (MPR-Clus−S, MPR-Clus−A, and MPR-Clus−D). Apart from the CK2 site mutants, two additional mutants were tested for GGA1 binding, the palmitoylation-deficient (MPR-C30A/C34A) mutant and the internalization-deficient (MPR-FFWYLL-A) mutant, lacking the dileucine motif, which is part of the known GGA1 binding motif (QXXXXL/D) (5, 11, 12). The GGA1 binding assay was performed with wild-type or mutant CD-MPR, in PNS from stably transfected cells and a truncated form of GGA1 fused to GST (GST-GGA1-A240stop), since the full-length GGA1 has been shown to be autoinhibitory for binding to the receptor (14). GST-GGA1-A240stop comprises the VHS domain for cargo binding and a larger part of the GAT domain for the interaction with ARF. In the GST pull-down assay, we could detect strong binding of the wild-type MPR to the GGA1 fragment and no binding of the FFWYLL-A mutant, as expected (Fig. 1B), with the PNS containing an equal amount of the CD-MPR protein (Fig. 1C). The specificity of the assay was confirmed by the fact that the CD-MPR did bind to the GST-GGA1-A240stop and not to GST alone (Fig. 1D). The palmitoylation-deficient CD-MPR mutant (MPR-C30A/C34A) bound to GGA1 comparable with wild-type (Fig. 1, B and C). This mutant is known to accumulate in lysosomes due to the lack of palmitoylation (34); however, the deficiency in palmitoylation did not influence the recognition of GGA1, suggesting that this posttranslational modification is not essential for TGN sorting. Interestingly, the binding of the Ser57 mutants (MPR-S57A, MPR-S57D) to GGA1 was not impaired, indicating that phosphorylation does not play a role in GGA1 binding (Fig. 1, B and C). However, the cluster-minus mutants, in which the negatively charged glutamates were changed to non-charged glutamines, did not bind to GGA1. This result was again independent of the amino acid substitution at position 57. Glu58 and Glu59 of the CK2 Site Are Essential for GGA1 Binding—To identify more precisely the residues of the CK2 site in the CD-MPR, which are responsible for the impaired binding to GGA1, we created mutant receptors with single and double exchanges of the glutamates Glu55–Glu59 (Fig. 2A). Additionally, the residues composing the motif already known to be essential for GGA1 binding were replaced to generate the mutants MPR-D61N and MPR-D61N,L64A/L65A (5, 11, 12). All mutant CD-MPR constructs were stably transfected into mouse L cells, and the PNS of these cells was used in the GGA1 binding assay. Changing the charged residues Glu55 and Glu56 of the cytoplastic tail to glutamines did not have an effect on GGA1 binding, neither as single mutants (MPR-E55Q, MPR-E56Q) nor combined as a double
mutant (MPR-E55Q/E56Q) (Fig. 2, B and C). However, the binding of MPR-E58Q to GGA1 was reduced, and the binding of MPR-E59Q was affected even more. The binding of MPR-E58Q/E59Q to GGA1 was not detectable, like the CD-MPR mutants known for impaired GGA1 binding, such as MPR-D61N and MPR-D61N,L64A/L65A (Fig. 2, B and C). Thus, the complete GGA1 binding motif of the CD-MPR includes Glu58 and to some extent Glu59: 58EEXXXDXXLL65.

Mutation of Glu58 and Glu59 to Alanines Decreased Binding Affinity—To analyze the kinetics of the GGA1-CD-MPR interaction and to verify the results obtained in the GST-GGA1-A240 pull-down experiments, surface plasmon resonance (SPR) technology was used. For this purpose, CD-MPR tail peptides from residues 49 to 67, with certain residues replaced by alanines (Fig. 3A) and containing either a normal serine or a phospho-serine, were coupled to the surface of a biosensor. GGA1 was purified as GST-GGA1-A240stop fusion protein and used at concentrations ranging from 25 to 500 nM. Binding and dissociation were recorded for 2 min, and the rate constant was calculated (Fig. 3B). The rate constant of the binding to the CD-MPR wild-type, non-phosphorylated peptide was set to one, and the binding affinities of the mutant peptides were displayed as relative values in Fig. 3C. The CD-MPR wild-type peptide and the MPR-E55A/E56A peptide both bound to GGA1 with a comparable binding affinity. For both peptides, phosphorylation had only a minor effect, increasing the binding by 0.17-fold (Fig. 3, B and C). All the other mutations in the peptides caused a decreased binding affinity, indicating their requirement for GGA1 binding (Fig. 3C). The binding of GGA1 to the D61A/D62A peptide was too low to be detected. The L64A/L65A peptide showed a 6-fold decrease in binding affinity as compared with the CD-MPR wild-type peptide, and its phosphorylation increased the binding of GGA1 only by 0.18-fold. The new residues found to be essential for GGA1-CD-MPR interaction in the GST pull-down experiment (Glu58 and Glu59) were also required for binding in the SPR experiment. As compared with MPR-WT peptide, the MPR-E58A/E59A peptide showed a 3.2-fold decreased binding affinity to GGA1. Furthermore, this peptide displayed a major difference between the non-phosphorylated and the phosphorylated form. The GGA1 had almost a normal binding affinity to the phosphorylated MPR-E58A/E59A peptide but not to the non-phosphorylated form; thus, a 2.6-fold difference in rate constant was measured. Hence, the binding of GGA1 to the MPR-E58A/E59A peptide was dramatically decreased.

**Fig. 1.** Interaction *in vitro* between wild-type and mutant CD-MPR constructs with GGA1. A, schematic illustration of the cytoplasmic tails of CD-MPR wild type and mutants. The 67 amino acids of the cytoplasmic tail are shown in single-letter codes. In the mutant constructs, the mutated amino acids are indicated by bold letters. TMD, trans-membrane domain. B, GST-GGA1-A240stop pull-down with CD-MPR wild-type and mutants. GST-GGA1-A240stop was purified from bacterial culture and bound to glutathione-Sepharose 4B beads, which was subsequently incubated with PNS from mouse L cells, stably transfected with CD-MPR wild-type or mutants. Following incubation at 4 °C for 2 h, the Sepharose was washed three times, boiled in sample buffer, and subjected to SDS-PAGE. Bound receptor was detected by immunoblotting with anti-CD-MPR antibody. C, to detect CD-MPR expression levels, 10% of the PNS used for the GST-GGA1-A240stop pull-down was subjected to SDS-PAGE and immunoblotting with anti-CD-MPR antibody. D, pull-down of GST and GST-GGA1-A240stop with PNS from mouse L cells stably transfected with wild-type CD-MPR. The protein standard shows the molecular mass in kDa.
could be restored by the phosphorylation of Ser57, whereas the phosphorylation in the other mutants displayed only a minor increase in the binding of GGA1. The inhibition of GGA1 binding was tested by incubating GGA1 with short inhibitory peptides prior to the binding assay. GGA1 was incubated with a 10-fold molar excess of soluble peptide A-(49–58) or B-(58–67) (Fig. 3A) for 15 min followed by recording of the binding to the immobilized MPR wild-type peptide on the biosensor. Only
peptide B was able to block binding to the MPR wild-type peptide, whereas peptide A had no effect (data not shown), confirming the data from the mutational analysis.

Acidic Cluster of the CK2 Site of CD-MPR but Not Phosphorylation Is Essential for AP-1 Binding—To explore the involvement of the CK2 phosphorylation site of the CD-MPR in AP-1 binding and its kinetics, SPR experiments were performed. The same peptides as for the GGA1 binding were used (Fig. 3A). Purified AP-1 was used at concentrations ranging from 25 to 500 nM. Binding and dissociation were recorded for 2 min, and the rate constant was calculated. The affinity of AP-1 to the wild-type CD-MPR peptide was 2.4-fold lower than that of the GGA1 to the same peptide (Fig. 3, B–D). This result explained the difficulties in detecting AP-1 in a co-immunoprecipitation of CD-MPR or GST-pull-down experiments (data not shown). For the binding of AP-1 to the peptides MPR-D61A/D62A and MPR-L64A/L65A, the binding affinities were in a similar range as for CD-MPR wild-type peptide (Fig. 3D). However, the mutation of both pairs of glutamates to alanines, MPR-E55A/E56A and MPR-E58A/E59A, decreased binding by a factor of 2.7 and 3.0, respectively (Fig. 3D). In all peptides, phosphorylation of the serine only showed a minor increase in binding to AP-1, 0.1-fold. Thus, the glutamates of the CK2 phosphorylation site, but not the phosphorylation itself, are essential for AP-1 binding.

TGN Redistribution of CD-MPR in Vivo by Dominant-negative GGA1 Is Impaired in the Mutant Containing E58Q

Fig. 4. Interaction of dominant-negative GGA1 with CD-MPR wild-type and mutant forms. A–E, mouse L cells stably transfected with CD-MPR wild-type (A and B) and mutant constructs (C–E) were grown on coverslips. Cells were transiently transfected with dominant-negative GFP-GGA1 (green), fixed, and permeabilized after 24 h. The cells were then incubated with anti-giantin monoclonal antibody (A) or anti-CD-MPR monoclonal antibody 22D4 (B–E) followed by incubation with goat anti-mouse Alexa 568 antibody (red). Serial sections in the z-axis through the entire cells were taken with a confocal laser-scanning microscope. Arrows indicate the redistribution of the CD-MPR. Scale bars are 10 μm. F, fluorescence in Golgi/TGN area was compared with the fluorescence of whole cells of double-transfected cells treated as described (B–E) (white bars) and as a control of non-transfected cells (gray bars) incubated with anti-giantin monoclonal antibody followed by incubation with goat anti-mouse Alexa 568 antibody and a subsequent incubation with anti-CD-MPR polyclonal antibody followed by incubation with goat anti-rabbit Alexa 488 antibody. The amount of giantin in the Golgi/TGN area was set to 100%. Several experiments have been performed analyzing individual double-transfected cells (white bars) or non-transfected cells (gray bars), and the quantification represents an average of four independent experiments. The values are shown as the mean ± S.E. from these experiments.
E59Q,D61N,L64A/L65A Mutations—To determine whether the in vitro results reflect the situation in living cells, interaction between GGA1 and CD-MPR was investigated in vivo. It was previously shown that a dominant-negative fragment of GGA1, containing the VHS domain for cargo binding and the GAT domain to be recruited to the TGN, could redistribute and accumulate CI-MPR and CD-MPR in the TGN (5, 35). GFP-GGA1-A240stop, although not containing the complete GAT domain, comprised the essential domains for interaction with ARF to be recruited to the TGN but lacked the hinge and GAE domains and is therefore unable to recruit clathrin and accessory proteins required for budding (9). Thus, GFP-GGA1-A240stop was acting as a dominant-negative GGA1 (5), when transiently transfected into mouse L cells, which were stably transfected with CD-MPR wild-type. GFP-GGA1-A240stop co-localized with the Golgi marker protein giantin, indicating that it is localized to the Golgi/TGN, and as a consequence, it did trap the wild-type CD-MPR in this compartment (Fig. 4, A and B). The amount of fluorescence of the CD-MPR in the Golgi/TGN area in cells transiently transfected with the dominant-negative GGA1 and in non-transfected cells was quantified, and the different mutants of the CD-MPR were compared. The amount of the fluorescence of giantin in the Golgi/TGN area as compared with the fluorescence of the whole cell was set to 100%. The cells transiently transfected with dominant-negative GGA1 showed a redistribution of the CD-MPR from 31 to 67% in the Golgi/TGN and consequently a depletion from the periphery (Fig. 4B, arrow, and F). As expected, the CD-MPR mutants, which did interact with GGA1 in vitro, were redistributed to the Golgi/TGN as well, when dominant-negative GGA1 was expressed (data not shown). Interestingly, the CD-MPR mutants MPR-D61N,L64A/L65A and MPR-E58Q/E59Q presented the same effect, indicating that their binding to GGA1 was not completely impaired, although they were not detectable in the GST-GGA1 pull-down, and the corresponding peptides had a very low affinity for the interaction with GGA1 (Fig. 4, C, D, and F). Therefore, another mutant construct was created in which all residues involved in GGA1 binding were changed simultaneously, the MPR-E58Q/E59Q,D61N,L64A/ L65A. The transient transfection of dominant-negative GGA1 did not redistribute the MPR-E58Q/E59Q,D61N,L64A/L65A to the Golgi/TGN, indicating that this mutant CD-MPR is not interacting with GGA1 (Fig. 4, E and F). The stably transfected wild-type and mutant CD-MPR show approximately a 40-fold overexpression relative to the endogenous level of mouse CD-MPR (18). The observed redistributions of some of the receptors are therefore not likely due to dimerization of transfected CD-MPR with endogenous wild-type receptor, and furthermore, the inability of the MPR-E58Q/E59Q,D61N,L64A/L65A to interact with GGA1 in vivo. The different affinities of CD-MPR to AP-1 and GGA1 might present a regulatory mechanism of the binding of these adaptors to the overlapping sorting signals in the cytoplasmic tail of the CD-MPR.

The results presented in this study demonstrate that the acidic cluster of the CK2 site upstream of the DXXXL motif of the CD-MPR but not its phosphorylation is required for GGA1 and AP-1 binding. Furthermore, we show that only the CD-MPR mutant with all residues of the GGA1 binding site changed (MPR-E58Q/E59Q,D61N,L64A/L65A) is unable to interact with GGA1 in vivo. The different affinities of CD-MPR to AP-1 and GGA1 might present a regulatory mechanism of the binding of these adaptors to the overlapping sorting signals in the cytoplasmic tail of the CD-MPR.

GGAs have been shown to be essential for the transport of CD-MPR and other cargo from the TGN to endosomes. The consensus binding motif of the VHS domains of GGAs and their cargo was revealed to be DXXXL (5, 12, 36). Given that the CK2 phosphorylation of a serine upstream of the DXXXL motif increased binding affinity of the CI-MPR peptide to the VHS domains of GGA1 and GGA3, additional residues might be involved in GGA binding (37). Furthermore, the autoinhibitory function of the acidic cluster-dileucine motif in the hinge domain of GGA1 and GGA3 by intramolecular or intermolecular binding of the VHS domain depends on the CK2 phosphorylation of the serine three residues upstream (14). The existence of a CK2 phosphorylation site upstream of the DXXXL motif in the CD-MPR suggested a similar influence on GGA1 binding. However, our results revealed that the phosphorylation of Ser57 had no effect on GGA1 binding. Mutants with the serine replaced by an alanine or an aspartate to mimic phosphorylation bound GGA1 similar to wild-type receptor. Conversely, the mutations of the glutamates surrounding the serine in the receptor led to an inhibition of GGA1 binding. Further characterization of the glutamates revealed the Glu30Glu40 residues to be essential for GGA1 binding. The kinetic studies of the interaction between CD-MPR and CD-MPR confirmed that indeed the MPR-E58A/E59A peptide showed a 3-fold decreased binding as compared with wild-type. The binding affinities of GGA1 to peptides containing mutations of the DXXXL motif were even lower, the binding to the MPR-L64A/L65A peptide being 6-fold decreased and the binding to the MPR-D61,L62A peptide being not measurable, reflecting the very low affinity of GGA1 to these residues. In vivo interaction of CD-MPR with dominant-negative GGA1 demonstrated that mutating the residues D61N,L64A/L65A and the E58Q/E59Q individually did not inhibit GGA1 binding. Both mutant constructs redistributed and accumulated in the TGN upon expression of the dominant-negative GGA1, showing a clearly different pattern as compared with non-transfected cells. Therefore, the CD-MPR containing all amino acids mutated, namely the new residues from this study, E58Q/E59Q, combined with the already known residues, D61N,L64A/L65A, was generated. Upon expression of dominant-negative GGA1, no redistribution and accumulation of the MPR-E58Q/E59Q,D61N,L64A/L65A were observed. This indicates that the interaction of MPR-E58Q/E59Q,D61N,L64A/L65A with GGA1 is inhibited. Altogether, these results led to the extension of the GGA1 interaction motif of the CD-MPR to 5EEXDXXXL.²⁹

The alignment of the GGA1 binding sites of cargo revealed that all the proteins have in the positions upstream from the Asp³¹ up to the Glu⁵⁰ in the CD-MPR (position −3 to −1 from the crucial aspartate 0, Fig. 5) at least one acidic residue or a serine phosphorylation site (Fig. 5, gray box, italic letters). This indicates that a negative charge, not specifically a glutamate, within this range of the position −1 to −3 might be crucial for GGA1 binding. This is confirmed by the requirement of the phosphorylation of the serine at position −3 in the GGA1 hinge.
domain for its autoinhibitory function and the increased affinity to GGA when the serine at position −1 in the CI-MPR is phosphorylated (Fig. 5) (14, 37). Furthermore, the rescue of high affinity GGA1 binding in the E58A/E59A peptide, when the serine was phosphorylated (Fig. 3B), led to the assumption that the required negative charges for GGA1 binding could be compensated by enough negative charges in the vicinity, which might range maximally between position −4 and −1 (Fig. 5). The data from the structural analysis with VHS domains of GGA and CI-MPR or CD-MPR peptides revealed that the residues −6 to −3 were disordered and the residues from −2 to 0 were well ordered with the aspartate 0 forming the most extensive interaction with the VHS domain (11, 38). Three positively charged amino acids in the GGA1 and GGA3 VHS domains interact with the cargo peptide. Lys31 of GGA1 (GGA3 amino acid numbers are decreased by one) mainly interacts with Asp at position 0, whereas Lys87 and Arg89 interact with phospho-serine at position −1 of the CI-MPR (37, 38). It might be possible that the latter two basic residues of the VHS domain of GGA1 could interact with the glutamates at position −3 and −2 (Glu59–Glu59) of the CD-MPR. The absence of positional restriction and irrelevance of the type of negative charge, which we suggested to range between −4 or −3 to −1, might explain the less ordered nature of the interaction upstream of the aspartate 0, which was found in the structural analysis. However, it should be considered that only short peptides were used for the structural analysis, not enabling the formation of a three-dimensional structure, which might result in a different kind of interaction of residues upstream of the aspartate.

The kinetic analysis of the interaction between AP-1 and CD-MPR revealed the involvement of the four glutamates, Glu57–Glu59 of the CK2 site, rather than the dileucine motif of the CD-MPR. This is in contrast to other proteins that rely on a dileucine motif for their interaction with AP-1 such as HIV-1 Nef and Limp-II (39). Therefore, the interaction of the CD-MPR with AP-1 might be a special case in which glutamates of a CK2 site are preferred binding partners over the dileucine motif. Mutation of each pair of glutamates to alanines led to a 3-fold increase of the rate constant of AP-1 binding. Comparable with the GGA1 binding, phosphorylation of the serine only had a negligible effect on CD-MPR binding to AP-1. Thus, AP-1 and GGA1 had overlapping binding sites, both comprising the Glu59–Glu59 and both independent of the Ser57 phosphorylation. Interestingly, the binding affinity of wild-type CD-MPR peptide to AP-1 was 2.4-fold lower than the affinity to GGA1. The overlapping binding sites combined with the different binding affinities for CD-MPR led to the conclusion that AP-1 is unable to bind to CD-MPR in the presence of GGA1, unless GGA1 is inactivated by phosphorylation. Thus, our results confirm the suggested model, in which GGA1 relays CD-MPR to AP-1, with slight modifications concerning the regulation (26). Our model suggests that GGA1 binds to CD-MPR with high affinity, thereby blocking the overlapping binding sites and disabling AP-1 binding. Subsequent phosphorylation of GGA1 by CK2, which is associated with AP-1, causes autoinhibition of GGA1 followed by the release of CD-MPR, rendering the overlapping binding site accessible for the weaker binding to AP-1. The localization of both adaptors, GGA1 and AP-1, to the TGN supports our model (6–8, 23). Thus, we suggest that both adaptors are involved in sorting in the TGN independent of cargo phosphorylation, regulated by different rate constants for the overlapping sorting signals.

Acknowledgments—We thank Dr. S. Kornfeld for generously providing the Myc-GGA1pFB1 construct and the anti-CD-MPR polyclonal construct. The hybridoma cell line producing monoclonal antibody 22D4 against CD-MPR was a kind gift of Dr. D. Messner. The anti-giantin monoclonal antibody was kindly provided by Dr. H. P. Hauri. P. Nair and B. Schaub are acknowledged for critical reading of the manuscript. We acknowledge Dr. E. Berger for continuous support and critical reading the manuscript.

REFERENCES
