Ubiquitination and Endocytosis of Cell Adhesion Molecule DM-GRASP Regulate Its Cell Surface Presence and Affect Its Role for Axon Navigation*

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DM-GRASP, cell adhesion molecule of the immunoglobulin superfamily, has been shown to promote growth and navigation of axons. We here demonstrate that clustering of DM-GRASP in the plasma membrane induces its rapid internalization via dynamin- and clathrin-dependent endocytosis, which is controlled by phosphatidylinositol 3-kinase and mitogen-activated protein kinase ERK. The clustering of DM-GRASP activates ERK; the intensity and duration of ERK activation by DM-GRASP do not depend on rapid clathrin-mediated internalization of DM-GRASP. Moreover, the preference of retinal ganglion cell axons for DM-GRASP-coated micro-lanes requires clathrin-mediated endocytosis for the appropriate axonal turning reactions at substrate borders. Because the intracellular domain of DM-GRASP does not contain motifs for direct interactions with the endocytosis machinery, we performed a yeast two-hybrid screen to identify intracellular proteins mediating the uptake of DM-GRASP and isolated ubiquitin. Immunoprecipitation of DM-GRASP coexpressed with ubiquitin revealed that one or two ubiquitin(s) are attached to the intracellular domain of cell surface-resident DM-GRASP. Furthermore, elevated ubiquitination levels result in a decrease of cell surface-resident DM-GRASP as well as in the amount of total DM-GRASP. The endocytosis rate is not affected, but the delivery to multivesicular bodies is increased, indicating that DM-GRASP ubiquitination enhances its sorting into the degradation pathway. Together, our data show that ubiquitination and endocytosis of DM-GRASP in concert regulate its cell surface concentration, which is crucial for its function in axon navigation.

During the development of the nervous system, cell adhesion molecules of the immunoglobulin superfamily (IgSF-CAMs)3 play critical roles in neuronal migration, axon growth, and axon navigation (1). The IgSF-CAM DM-GRASP (2) (also termed SC1 (3), BEN (4), and JC7 (5)) has been shown to play a role in cell adhesion (2, 6), axon growth (7, 8), axon navigation (9, 10) as well as in neuronal migration (11), differentiation (12), and synapse formation (13, 14). Highly conserved mammalian orthologs of DM-GRASP have been found in human (ALCAM/CD166 (15)), rodents (ALCAM/CD166 (16, 17)), and bovine (CD166 (18)). DM-GRASP interacts homophilically with itself and heterophilically with the IgSF-CAM L1/NgCAM (5, 6, 19, 20). DM-GRASP also interacts with CD6, which is, however, not present in the visual system (18, 21). DM-GRASP is an integral membrane protein of 100 kDa with a very short cytoplasmic domain (2), which makes it, together with two Ig domains of the rare V-type, an unusual member among the IgSF-CAMs.

IgSF-CAMs display spatially and temporally dynamic, discrete expression patterns during development and mediate (homophilic and heterophilic) cell-cell and cell-substrate interactions. For the formation of a functional neuronal network, the motile, sensory tip of the elongating axons, the growth cone, rapidly reacts to environmental cues. To regulate adhesion and intracellular signaling pathways triggered by surface-resident IgSF-CAMs, the regulation of their density and presence on the growth cone is thought to be crucial. This could be achieved by a balanced integration of synthesized/recycled IgSF-CAMs into the plasma membrane and their endocytosis. Clathrin-dependent endocytosis and recycling has been found for L1/NgCAM or NCAM (22, 23).

For the formation of clathrin-coated pits, clathrin coassembles with adaptor proteins that form a link between the clathrin lattice and the plasma membrane proteins (24) by recognizing specific consensus sequences or dileucine motifs (25). To pinch off the membrane, dynamin self-assembles into rings around the neck of the clathrin-coated pits, and coated vesicles are formed (26, 27). For transmembrane proteins lacking any adaptor-binding site, mono-ubiquitination, the addition of a single ubiquitin to their cytoplasmic domain (2), which makes it, together with two Ig domains of the rare V-type, an unusual member among the IgSF-CAMs.

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3 The abbreviations used are: IgSF-CAM, cell adhesion molecule of the immunoglobulin superfamily; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; RGC, retinal ganglion cell; wt, wild type; dn, dominant-negative; MEK, MAPK/ERK kinase; MDC, monodansyl-cadaverine.

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35-amino acid cytoplasmic domain. In the present study, we investigated how the presence of DM-GRASP in the plasma membrane is regulated by internalization; DM-GRASP is internalized by a dynamin- and clathrin-dependent endocytosis in growth cones and critically depends on the activity of mitogen-activated protein kinase (MAPK) ERK and phosphatidylinositol 3-kinase (PI3K). We show that regulated internalization of DM-GRASP is pivotal for the orientation of axons along DM-GRASP lanes. In a screen for binding partners of the intracellular domain of DM-GRASP, we found, among others, ubiquitin. We also demonstrate that DM-GRASP is indeed ubiquitinated and that this affects intracellular trafficking. Ubiquitin-regulated endocytosis of DM-GRASP might thus be involved in the dynamic regulation of DM-GRASP functions in navigating growth cones.

EXPERIMENTAL PROCEDURES

Animals, Antibodies, Plasmids, and Reagents—Fertilized white leghorn chicken eggs were obtained from a local provider. Antibodies used for this report were rabbit antibodies against DM-GRASP (9) and ERK1/2 (Calbiochem), goat antibody against ALCAM (R & D Systems), mouse monoclonal antibody against NgCAM (1E12 (34)), active ERK (MAPK-YT; Sigma), and FLAG (M2; Sigma); secondary antibodies were purchased from Jackson Laboratories and Molecular Probes. The following cDNAs were used for expression in mouse N2a neuroblastoma cells or human embryonic kidney 293 (HEK293) cells: pMIW-DM-GRASP (Hideaki Tanaka, Kumamoto University, Honjo, Japan), pCMV-SPORT-ALCAM (Deutsches Resourcenzentrum für Genomforschung), pcDNA3-dynamin and pcDNA3-dynamin(K44A) (Sandra Schmid, The Scripps Institutes), and pcDNA3-FLAG-ubiquitin (Ivan Dikic, Goethe University, Frankfurt, Germany). All of the inhibitors were purchased from Calbiochem.

Retinal Single Cell Cultures—Single cell cultures of embryonic day 6 retina were prepared as described (9, 35). The cultures were fixed in 4% paraformaldehyde for 30 min and stained by indirect immunofluorescence as described (36).

Immunofluorescence Detection of DM-GRASP Endocytosis—For differential labeling of cell surface DM-GRASP and endocytosed DM-GRASP in living cells, a modification of the method of Needham et al. (37) was used; 24 h after transfection (Lipofectamine; Invitrogen), N2a cells were plated on glass coverslips coated with poly-L-lysine (50,000 cells/coverslip), and nontransfected retinal ganglion cells (RGCs) were seeded on laminin-coated coverslips (30,000 cells/coverslip). 24 h after seeding, cells were incubated with 20 µg/ml immunoaffinity-purified polyclonal DM-GRASP antibody (9) for 5–30 min at 37 °C to allow for endocytosis of the complexes formed by cell surface DM-GRASP and DM-GRASP antibodies; for controls (0 min), the cells were not treated with DM-GRASP antibodies. To stop endocytosis, the cells were fixed for 20 min with 4% paraformaldehyde. To visualize all of the DM-GRASP proteins (clustered and nonclustered) present on the cell surface, the cells were labeled with the same polyclonal DM-GRASP antibody as used for triggering endocytosis. DM-GRASP-antibody complexes present at the cell surface were then labeled by incubation with fluorescein isothiocyanate- or Alexa 488-conjugated goat anti-rabbit IgG antibodies. Any remaining unlabeled cell surface DM-GRASP-antibody complexes, which had not been saturated by secondary antibodies, were blocked by 200 µg/ml goat anti-mouse IgGs for 1 h. The cells were then treated with 4% paraformaldehyde for 10 min to fix the cell surface-labeled DM-GRASP-antibody complexes and were then permeabilized by 0.1% Triton X-100 for 1 h. The cells were blocked by 10% goat serum for 30 min, and the internalized DM-GRASP-antibody complexes were labeled by Texas Red- or Alexa 546-conjugated goat anti-rabbit IgG antibodies. After additional washing steps, the coverslips were mounted with Mowiol and were viewed in an inverted microscope (Axiovert 200M; Zeiss) equipped with a digital camera (AxioCam; Zeiss). All of the micrographs and quantification of N2a cells shown were taken from the equatorial plane as determined in phase contrast with a focal depth of 1 µm (38).

Quantification of Fluorescence Intensity—Using phase contrast optics to avoid biased selection, 18 or more growth cones/sample group were randomly selected. Then fluorescent images were captured, with exposure time being kept constant and taking care to avoid pixel saturation. For quantification of fluorescence intensity, the growth cone outlines were traced on the phase contrast images by ImageJ and superimposed on the fluorescent images, and the fluorescent intensity within the growth cone was determined, giving a measurement of pixel intensity/unit area. The background fluorescence intensities were measured in an adjacent area clear of cellular material and subtracted from the growth cone reading, yielding the background-corrected intensity. Statistical analyses were performed using the t test.

DM-GRASP Internalization and Cell Surface Biotinylation Assay—To quantify DM-GRASP endocytosis, the proteins remaining on the cell surface after exposure to the DM-GRASP antibody were labeled by a membrane-impermeant biotinylation reagent. For this, cells in 35-mm tissue culture dishes were incubated for 1 h in presence of the indicated inhibitor before the onset of the experiment. The inhibitors used were PI3K inhibitor LY294002 (10 µM), MEK inhibitor U0126 (10 µM), protein kinase C inhibitor bis-indolylmaleimide (1 µM), and phospholipase D inhibitor ethanol (0.5%). The cells were kept for 30 min at 4 °C before the addition of the polyclonal DM-GRASP antibody and further incubation at 4 °C for 30 min. The cells were then washed once with medium and incubated at 37 °C for the indicated times. The cells were removed, immediately washed with ice-cold phosphate-buffered saline, and kept for the rest of the experiment at 4 °C. The cells were treated with sulfo-NHS-SS-biotin (1 mg/ml; Pierce) in phosphate-buffered saline at 4 °C for 30 min followed by washing with 100 mM glycine. The cells were solubilized with radioimmune precipitation assay buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton-X-100, 1% sodium deoxycholate, 0.1% SDS) and then centrifuged at 17,000 × g for 15 min at 4 °C. Biotinylated proteins were separated from nonbiotinylated proteins by incubation with avidin-agarose (Calbiochem) for 1 h at room temperature. The beads were washed five times with 1 ml of radioimmunoprecipitation assay buffer, and the adsorbed proteins were eluted with SDS sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 6% β-mercaptoethanol, and 0.001% bromphenol blue). The proteins were separated by SDS-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose sheet. Western blot
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analysis with a polyclonal DM-GRASP antibody was performed as described before (9).

Immunoprecipitation and Immunoblotting—HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum in a humidified atmosphere containing 5% CO2 at 37 °C. HEK293 cells were transfected with plasmids using Lipofectamine as described (37). 24 h after transfection, the cell surface DM-GRASP was biotinylated and clustered by incubation with DM-GRASP antibody for 15 min. The cells were washed with phosphate-buffered saline and extracted in radioimmune precipitation assay lysis buffer. The cell lysates were clarified by centrifugation at 16,000 × g for 15 min at 4 °C and incubated with DM-GRASP-specific antibodies and protein A-Sepharose for 1–2 h before onset of the experiments, the medium was replaced with serum-free Dulbecco’s modified Eagle’s medium/F-12 (Invitrogen) supplemented with 1% fetal bovine serum. 1–2 h before onset of AXON terminals, the cells were seeded at 106 cells/well (coated with 10 µg/ml laminin) and cultured for 24 h in Dulbecco’s modified Eagle’s medium/F-12 (Invitrogen) supplemented with 1% fetal bovine serum. 1–2 h before onset of the experiments, the medium was replaced with serum-free Dulbecco’s modified Eagle’s medium/F-12. The cells were pre-incubated with DMSO or monodansyl-cadaverine (MDC) for 60 min prior to addition of DM-GRASP clustering antibody. The cells were lysed in buffer containing 1% Nonidet P-40, 0.25% sodium deoxycholate, 50 mM HEPES (pH 7.4), 137 mM NaCl, 1 mM Na-EDTA, 10 mM phenylmethylsulfonyl fluoride, 10 mM α-glycerophosphate, 2 mM calcyclin A, and protease inhibitors. The lysates were clarified by centrifugation, and equal quantities of protein were separated by SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and detected with the anti-phospho-ERK antibody. The membranes were then stripped and reprobed to confirm equal loading using the antibody against ERK1/2. The ratio of phospho-ERK to total ERK was determined by densitometry.

Axon Preference Assay—Preference assays were performed as described (9), except that recombinant, soluble DM-GRASP (3 µg/ml) was employed (39). 24 h after start of the explant culture, MDC was added in a final concentration of 100 µM for an additional 24 h before fixation with 4% paraformaldehyde. A substrate lane was considered to be showing preference if its RGC axons (with the exception of a very few). A retinal explant strip was considered to be showing preference if its RGC axons respected the borders of at least 50% of the lanes of a given substrate. Images were taken and assembled with a Nikon Eclipse 90i equipped with a Nikon DS-1QM camera (Nikon Imaging Center, University of Heidelberg) using the scan large image function.

Yeast Two-hybrid Screen—For the yeast two-hybrid screen, an embryonic day 9.5/10.5 CD1 mouse library (a kind gift of Stefan Offermanns, University of Heidelberg, Heidelberg, Germany), generated by random-primed cDNA synthesis, was used. The library pVP16 (carrying the LEU2 gene) cDNA library contains ~5 × 106 clones with insert sizes ranging from 350 to 700 nucleotides (40). As a bait, the intracellular domain of DM-GRASP fused to the LexA sequence of the pBTM116 containing the TRP1 gene was employed. The yeast two-hybrid results were confirmed by retransformation of bait (pBTM1 or pBTMIII) and prey (interacting clones) vectors.

RESULTS

DM-GRASP Is Endocytosed in the Central Domain of Growth Cones—The turnover of DM-GRASP in growth cones of extending RGC axons was studied in retinal single cell cultures (Fig. 1). Normally, DM-GRASP is distributed in a punctate pattern over the entire cell surface of the growth cone (Fig. 1A). To trigger DM-GRASP endocytosis, it was clustered by the addition of polyclonal DM-GRASP antibody, and the internalization was analyzed by differential immunofluorescence labeling of internalized and cell surface DM-GRASP. After 10 min, cell surface DM-GRASP is enriched at neck and central domain of the growth cone, and DM-GRASP-containing vesicles are present in the central domain (Fig. 1B). After 30 min, DM-GRASP is predominantly located in vesicles, which are almost exclusively restricted to the central growth cone domain (Fig. 1C). Quantification of the internalized and cell surface DM-GRASP after clustering (Fig. 1D) reveals a swift onset of endocytosis because internalized DM-GRASP is detected already 5 min after clustering. The amount of internalized DM-GRASP increases 3-fold in the next 5 min; after 15 min the internalized DM-GRASP reaches a maximum that declines to lower levels after 30 min. Together these data indicate that clustering of DM-GRASP induces its rapid translocation into the central growth cone where it is endocytosed and removed by retrograde transport or degradation.

Endocytosis of DM-GRASP Depends on Dynamin and Clathrin—To analyze the mechanisms underlying DM-GRASP endocytosis, we tested whether the disruption of dynamin function affects DM-GRASP internalization (Fig. 2). For this, N2a cells (devoid of endogenous DM-GRASP) were cotransfected with DM-GRASP and wild type (wt)-dynamin or DM-GRASP and dominant-negative (dn)-dynamin. The expressed DM-GRASP is equally distributed all over the cell surface in its typical punctate pattern, independent of coexpression of wt-dynamin or dn-dynamin.

When DM-GRASP clustering is induced by antibody addition in N2a cells expressing DM-GRASP and wt-dynamin, internalized DM-GRASP is found in vesicles located beyond the cell surface after 5 min (Fig. 2A). After 10 min, almost all DM-GRASP-containing vesicles are located in the cell center (Fig. 2B); the same distribution is observed at 15 min (Fig. 2C) as well as at 20 and 25 min (not shown). In contrast, in cells cotransfected with dn-dynamin, almost no DM-GRASP is internalized at 5 min (Fig. 2D), and the first DM-GRASP-containing vesicles appear in the vicinity of the plasma membrane only after 10 min (Fig. 2E). Within the next 5 min, more DM-GRASP is internalized, and the vesicles translocate to the central cell region (Fig. 2F) now displaying the same distribution as
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![Image](https://example.com/image.png)

**FIGURE 1. Endocytosis of DM-GRASP in growth cones.** Endocytosis of DM-GRASP (induced by antibodies against DM-GRASP) into retinal ganglion cell growth cones was visualized by double immunofluorescence labeling of DM-GRASP on the cell surface (left column), internalized DM-GRASP (middle column), and merged images (right column). A, in nontreated RGCs, DM-GRASP is equally distributed over the growth cone surface including lamellipodia and filopodia, and no internalized DM-GRASP is visible; speckled DM-GRASP staining indicates spontaneous aggregation of DM-GRASP in the plasma membrane. B, 10 min after antibody application, DM-GRASP aggregates at the cell surface, and DM-GRASP-positive vesicles can be detected. C, At 30 min, DM-GRASP is hardly visible on the cell surface but in vesicle clusters containing internalized DM-GRASP in the central growth cone domain. D, quantification of cell surface DM-GRASP (green) and internalized DM-GRASP (red) after clustering of DM-GRASP shows the time course of its endocytosis and removal from the growth cone. The error bars represent S.E. **, p < 0.01; ***, p < 0.001; bar, 10 μm. 

observed for wt-dynamin expressing N2a cells; this pattern remains stable (20 and 25 min; not shown).

To quantify the time course of DM-GRASP endocytosis, the number of DM-GRASP expressing N2a cells displaying DM-GRASP-containing vesicles was determined over time (Fig. 2G). After 5 min, the fraction of dn-dynamin cotransfected cells internalizing DM-GRASP is almost five times lower than the one of wt-dynamin cells (7 ± 4 and 33 ± 13%, respectively; p < 0.05). Within the next 5 min, however, this difference decreases to only about two times because of an overly proportionate increase in the fraction of dn-dynamin cells internalizing DM-GRASP (27 ± 3 and 62 ± 3%; p < 0.0006). In the following 10 min, the number of DM-GRASP internalizing cells reaches the same level (75 ± 1 and 75 ± 12%), which does not significantly change in the following (78 ± 3 and 78 ± 10%).

We next analyzed whether clathrin is involved in the internalization of DM-GRASP. For this, MDC, a specific inhibitor of clathrin-dependent endocytosis, was applied to DM-GRASP-transfected N2a cells, which were then induced to internalize DM-GRASP by antibody addition. To quantify DM-GRASP endocytosis, cell surface proteins were labeled by biotinylation and purified, and the presence of DM-GRASP was determined by densitometric evaluation of Western blots (Fig. 2H). If clathrin is not inhibited, the amount of surface DM-GRASP after 15 min is reduced to about one-third (37 ± 5%, p < 3.4 × 10⁻³) compared with cells not treated by antibody application and decreases to about one-fifth after 30 min (22 ± 9%, p < 0.007). Under inhibition of clathrin, surface DM-GRASP is hardly decreased (89 ± 8%, p > 0.05) 15 min after antibody application. Only after 30 min, is a significant reduction in surface DM-GRASP detected; the amount of DM-GRASP remaining on the cell surface (60 ± 8%, p < 0.003) is yet three times higher compared with the one of the same time point under control conditions (p < 0.02).

Together, the results show that dynamin and clathrin are crucial for DM-GRASP internalization. The inhibition of dynamin as well as clathrin is almost completely counterbalanced by a prolonged internalization, indicating that predominantly the rapid DM-GRASP internalization depends on these two proteins.

**DM-GRASP Internalization Is Regulated by ERK**—We next investigated the regulatory mechanisms underlying DM-GRASP endocytosis (Fig. 3). For this, N2a cells were treated with various inhibitors before the cell surface DM-GRASP was clustered, biotinylated, purified, and immunoblotted as described above. We tested protein kinase C because it enhances the association of DM-GRASP to the actin cytoskeleton (41), and actin association had been shown to lower the endocytosis of other CAMs. Application of protein kinase C inhibitor bis-indolylmaleimide does not affect the internalization of DM-GRASP significantly (Fig. 3A); only a quarter of DM-GRASP (25 ± 8%) remains on the cell surface compared with controls (DM-GRASP signal on the surface of bis-indolylmaleimide-pretreated N2a cells without antibody clustering), which corresponds to the normal reduction (Fig. 2H). Also inhibition of phospholipase D, an enzyme crucial for the clathrin-dependent internalization of the epidermal growth factor receptor (42), by ethanol does not significantly
affect the normal level of DM-GRASP left in the plasma membrane (10 ± 9%). In contrast, inhibition of PI3K by LY294002 prevents any significant changes of DM-GRASP levels on the cell surface (149 ± 20%). Preincubation with U0126, an inhibitor of the ERK-activating kinase MEK, causes an almost 5-fold increase of cell surface DM-GRASP (472 ± 52%), demonstrating that MAPK ERK is crucial for an efficient removal of DM-GRASP from the cell surface.

We selectively immunofluorescently labeled cell surface DM-GRASP and internalized DM-GRASP to further investigate whether the ERK inhibition affects the internalization of DM-GRASP. Inhibitor U0126 significantly reduces the number of N2a cells displaying DM-GRASP-containing vesicles upon DM-GRASP clustering (for 10 min) by one-third compared with controls (42 ± 5% of U0126-treated cells and 62 ± 4% of control cells, respectively; p < 0.002), indicating that a decreased endocytosis underlies the observed accumulation of DM-GRASP in the cell surface upon ERK inhibition.

To investigate whether ERK also regulates the levels of DM-GRASP in the cell surface of growth cones, retinal single cells were kept in sparse culture for 24 h to allow for the formation of RGC axons and growth cones. The cultures were then treated with inhibitor U0126. DM-GRASP was clustered for 20 min and labeled, and the levels of DM-GRASP in the plasma membrane were analyzed by quantitative immunocytochemistry (Fig. 3B). Without inhibitor, the levels of cell surface DM-GRASP decrease upon DM-GRASP clustering to almost half (58 ± 6%; p < 0.001) compared with those in controls (DM-GRASP signal on the surface of RGC growth cones without antibody clustering). Under ERK inhibition, in contrast, significantly higher levels of DM-GRASP (82 ± 9%) stay on the cell surface; the effect is considerably milder, however, than the one observed for N2a cells (Fig. 3A). Taken together, the results show that the rapid internalization, i.e. clathrin-dependent endocytosis of DM-GRASP depends, at soma and growth cone, on PI3K and ERK.

DM-GRASP Endocytosis Does Not Affect ERK Activation—Because clustering and endocytosis of another IgSF-CAM, L1, had been shown to activate ERK (43, 44), we investigated whether the clathrin-mediated internalization of DM-GRASP has an impact on ERK activation. For this, we induced DM-GRASP clustering in retinal cell cultures in the presence of MDC and determined the amount and time course of activated ERK by quantitative Western blots employing a phospho-ERK-specific antibody (independent of the antibody used, avian ERK appears as a single band (66, 67)) (Fig. 3C). Already 5 min after onset of clustering, the level of activated ERK in these cells increases almost 5-fold (4.7 ± 0.5%; p < 0.002) compared with the one in cells not stimulated by DM-GRASP clustering. The stimulation of ERK activity persists for the next 5 min (3.0 ± 0.1%; p < 0.001) and declines to original levels only after 15 min (2.4 ± 0.6%; not significant). The dynamics of ERK activation do not differ significantly at any time point from the one determined in absence of MDC, demonstrating that the clathrin-mediated endocytosis does not play a role for the stimulation of ERK by DM-GRASP and that clustering of DM-GRASP in the cell surface is sufficient to activate ERK in this signaling pathway.

Preference of Axons for DM-GRASP Depends on Clathrin-mediated Endocytosis—To address the question whether clathrin-mediated internalization of DM-GRASP plays a role for axon navigation that depends on DM-GRASP-DM-GRASP
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To investigate whether ubiquitin affects the turnover of DM-GRASP (Fig. 6), the amount of ubiquitin expressed in DM-GRASP transfected cells was varied. Western blots of DM-GRASP immunoprecipitates (from homogenates) show that DM-GRASP levels decrease with increasing ubiquitin expression (Fig. 6A). To selectively monitor levels of cell surface DM-GRASP, all of the cell surface proteins were biotinylated and isolated (Fig. 6B). The data show that the cytoplasmic domain of plasma membrane-resident DM-GRASP can be mono-/di-ubiquitinated.

Ubiquitination Affects DM-GRASP Trafficking—To investigate whether ubiquitin affects the turnover of DM-GRASP (Fig. 6), the amount of ubiquitin expressed in DM-GRASP transfected cells was varied. Western blots of DM-GRASP immunoprecipitates (from homogenates) show that DM-GRASP levels decrease with increasing ubiquitin expression (Fig. 6A). To selectively monitor levels of cell surface DM-GRASP, all of the cell surface proteins were biotinylated and isolated (Fig. 6B). Quantitative Western blot analysis reveals that ubiquitination of DM-GRASP clearly reduces its presence in the plasma membrane, increasing amounts of transfected ubiquitin cDNA, causing decreasing levels of cell surface DM-GRASP.

trans-interaction, we employed a substrate preference assay (Fig. 4) offering alternating lanes of DM-GRASP plus laminin versus laminin only as axonal growth substrate (9, 35). This assay reflects the situation of RGC axons navigating in the developing retina where they selectively extend in the optic fiber layer, a structure composed of RGC axons and the only layer providing DM-GRASP; laminin is ubiquitously present. Also in vitro, RGC axons emerging from embryonic day 6 retina explants show a clear preference for the DM-GRASP-containing substrate (Fig. 4A). The RGC axons respect the borders of almost all of the DM-GRASP lanes (83 ± 13%), and they perform turning reactions to stay on the preferred lanes and only rarely cross onto the laminin lanes (Fig. 4B). Under inhibition of the clathrin-mediated endocytosis by MDC, in contrast, RGC axons display a random growth pattern (Fig. 4C). The axons leave the DM-GRASP lanes crossing onto the laminin lanes without any turning reactions at the borders (Fig. 4D) and only occasionally respect the borders of DM-GRASP lanes (15 ± 9%; p < 0.001). The data thus show that the rapid clathrin-mediated internalization of DM-GRASP is crucial for the fast, appropriate reactions of the highly dynamic growth cone to DM-GRASP cues in its environment.

The Intracellular Domain of Membrane-integrated DM-GRASP Is Ubiquitinated—Because the intracellular domain of DM-GRASP does not contain motifs for binding partners, e.g., the clathrin machinery, we aimed at the identification of such interaction partners. For this we performed a yeast two-hybrid screen employing an embryonic day 9.5/10.5 mouse library and the intracellular domain of DM-GRASP as a bait (Fig. 5). One of the cDNAs isolated from a pool of 5 × 10⁶ independent clones (Fig. 5A) was identified as the ubiquitin cDNA by sequence analysis (Fig. 5B). To test whether DM-GRASP is indeed ubiquitinated, it was coexpressed with FLAG-tagged ubiquitin in HEK293 cells followed by purification of biotinylated cell surface-resident DM-GRASP. The isolate shows a diffuse DM-GRASP double band with a molecular mass of 116 and 100 kDa, respectively; the upper band is ubiquitinated as revealed by employing the FLAG-specific antibody (Fig. 5C). Removal of the N-linked oligosaccharides of DM-GRASP by N-Glycosidase F treatment results in a single band of 90 kDa, and FLAG detection visualizes two bands of 97 and 104 kDa that correspond to DM-GRASP carrying one or two ubiquitin molecules, respectively (Fig. 5C). To prove that the cytoplasmic domain of DM-GRASP is ubiquitinated, the full-length protein or a mutant lacking the entire cytoplasmic domain was expressed together with FLAG-ubiquitin. Western blots of the DM-GRASP isolates show that ubiquitin is only bound to DM-GRASP containing the cytoplasmic domain (Fig. 5D). Together, the data show that the cytoplasmic domain of plasma membrane-resident DM-GRASP can be mono-/di-ubiquitinated.

FIGURE 3. P13K- and ERK-regulated DM-GRASP endocytosis. A, N2a cells expressing DM-GRASP were treated with bis-indolylmaleimide (protein kinase C (PKC) inhibitor), ethanol (phospholipase D (PLD) inhibitor), LY294002 (P13K inhibitor), or U0126 (ERK inhibitor), and after endocytosis (induced for 15 min with DM-GRASP antibodies) the cells were surface-biotinylated and lysed; the biotinylated proteins were precipitated and immunoblotted to detect cell surface DM-GRASP. DM-GRASP bands of three or four independent experiments were densitometrically analyzed. B, retinal single cell cultures were treated with Me₂SO (control) or U0126 (ERK inhibitor) and after endocytosis (induced by DM-GRASP antibodies for 20 min), surface DM-GRASP on RGC growth cones was visualized by immunofluorescence staining to quantify the fluorescence intensity of DM-GRASP present at the cell surface of growth cones; fluorescence intensities are plotted as percentages of those before induction of endocytosis. C, impact of clathrin-mediated DM-GRASP endocytosis on ERK activation. Retinal cultures were treated with or without MDC (clathrin inhibitor), and after endocytosis (induced by DM-GRASP antibodies for the indicated time), the cells were lysed; the lysates were subjected to SDS-PAGE and immunoblotting with a phospho-ERK-specific antibody (phospho-ERK, upper panel); the same blots were reprobed with an antibody recognizing ERK independent of its phosphorylation status (total ERK, lower panel; the single band of 43 kDa is typical for avian ERK (66, 67)). ERK activity in nontreated and in MDC-treated retinal cultures was densitometrically quantified. Already without induction of DM-GRASP endocytosis by antibody addition (0 min), a basal, endogenous ERK activity is detected (most likely because of stimulation by cell-cell contact). The ERK activity means of three to five independent experiments are plotted (ERK activity of untreated cells set to 1). The error bars represent S.E. **, p < 0.01; ***, p < 0.001.
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To test whether ubiquitination of DM-GRASP affects its endocytosis, N2a cells were transfected with DM-GRASP and cotransfected with ubiquitin or not, and endocytosis assays were performed. The degree of DM-GRASP endocytosis does not differ under both conditions (Fig. 6, C and D); after 10 min, for example, about half of the cells display DM-GRASP-containing endocytic vesicles (Fig. 6E). This suggests that ubiquitination of DM-GRASP does not affect the level of its endocytosis itself but rather regulates the sorting of DM-GRASP into recycling or degradation pathway.

Quantitative analysis of the endocytic vesicles reveals that ubiquitin transfection increases the percentage of large vesicles containing internalized DM-GRASP; the total number of DM-GRASP positive vesicles/cells remains unchanged (Fig. 6F). In controls, approximately half of the vesicle population has a diameter smaller than 300 nm; the other vesicles display larger diameters (up to 600 nm). Upon ubiquitin transfection, about two-thirds of the DM-GRASP-containing vesicles possess diameters larger than 300 nm (p < 0.05), a hallmark for vesicles bound to the lysosomal pathway. These data indicate that ubiquitination of DM-GRASP enhances channeling of this protein (also) into the degradation pathway, thus regulating the levels of plasma membrane DM-GRASP.

DISCUSSION

We here show for the first time that a cell adhesion molecule of the immunoglobulin superfamily, DM-GRASP, is ubiquitinated. The ubiquitination affects membrane-resident DM-GRASP, enhancing its degradation and thus decreasing its cellular concentration in neuronal cells. This is based on a rapid endocytosis of clustered DM-GRASP, which depends on dynamin and clathrin and is regulated by ERK and PI3K. The endocytosis of DM-GRASP in the growth cone is crucial for the preference of axons for DM-GRASP as a growth substrate.

Endocytosis of DM-GRASP—Rapid, clathrin-dependent endocytosis is an internalization mechanism also used by the IgSF-CAMS apCAM, L1, and NCAM (23, 45, 46) as well as many other integral membrane proteins, regulating their cell surface presence. The control of the plasma membrane concentration of DM-GRASP might be important for navigating axons by adjusting the adhesion of the growth cone. Too strong adhesion is known to impede motility (47); in sensory neurons, it has been found that a highly adhesive substrate (poly-L-lysine) causes a decrease in neurite extension (48). Moreover, the cell surface concentration of integrins in sensory neurons negatively correlates with the density of extracellular matrix molecules offered as substrate, resulting in delicately tuned adhesive properties required for neurite growth (49).

Besides regulating the adhesive properties of cell membranes, DM-GRASP internalization conceivably could also affect intracellular signaling. Our results show that endocytosis of DM-GRASP has no effect on activation or termination of ERK signaling in retinal cells. Independent of endocytosis, ERK activity raises already upon clustering of DM-GRASP in the plasma membrane. Endocytosis of DM-GRASP might be required, however, for mediating precise spatiotemporal control of the DM-GRASP-activated intracellular signaling complexes comprising among others ERK (50). Growth cones, where DM-GRASP clusters and signaling complexes con-
growth factor receptor, nerve growth factor receptor) is crucial to prevent excessive stimulation of cells and to allow their regeneration. The mechanism(s) controlling the duration of DM-GRASP-induced ERK activation remain to be elucidated.

Clathrin-mediated endocytosis of transmembrane proteins has been shown to be tightly regulated by phosphorylation or dephosphorylation (52, 53). Activity of both PI3K and ERK reduce the concentration of DM-GRASP in the plasma membrane; we could show that inhibition of ERK decreases the internalization of DM-GRASP in RGC growth cones and increases its plasma membrane concentration in N2a cells (the stronger effect of the inhibition in N2a cells might be due to the overexpression). PI3K has been shown to be necessary for the internalization of several growth factor receptors as it controls initial stages of the coated pit assembly (54–58). Several studies suggest that ERK is not directly involved in the regulation of the clathrin-dependent endocytosis machinery; for the Na,K-ATPase, however, it has been shown that ERK-mediated phosphorylation of the cytoplasmic domain is required for its endocytosis (59).

For L1 it has been demonstrated that dephosphorylation of a tyrosine residue in the cytoplasmic domain is crucial for its clathrin-mediated endocytosis and signaling (60). Conceivably, ERK-mediated phosphorylation of the cytoplasmic domain of DM-GRASP reduces the proposed interaction with members of the ezrin-radixin-moesin family of actin linkers (61) and thereby facilitates DM-GRASP endocytosis. ERK-mediated phosphorylation of cytoskeletal linker proteins could also weaken the binding of DM-GRASP to the cortical cytoskeleton and thus promote its internalization.

**Ubiquitination of DM-GRASP**—Binding of one or several single ubiquitin molecules to transmembrane proteins has been shown to act as an internalization signal. The short cytoplasmic domain of DM-GRASP (32 amino acids) contains seven lysine residues that can serve as acceptors for ubiquitination. Our results show that only one or two ubiquitin molecules are bound to plasma membrane-resident DM-GRASP, potentially because of steric restrictions. Ubiquitinated transmembrane proteins are recruited into clathrin-coated pits/vesicles by linker proteins containing ubiquitin interacting motifs, e.g. Eps15 and epsin (28, 62). Our observation that DM-GRASP endocytosis is not affected by the overexpression of ubiquitin does not exclude that ubiquitination of the cytoplasmic domain is crucial for DM-GRASP internalization. It has been shown that an increase in ubiquitination of (overexpressed) transmembrane proteins does not affect their internalization rate, probably because of sufficient endogenous ubiquitin levels (30); it is hence likely that the endogenous ubiquitin amount is also effectual for an efficient DM-GRASP internalization.

The DM-GRASP-ubiquitin stoichiometry might affect the fate of internalized DM-GRASP. The twice mono-ubiquitinated DM-GRASP can be assumed to be subjected to a higher rate of intracellular retention and degradation than the once ubiquitinated DM-GRASP because of the increased probability of the twice mono-ubiquitinated DM-GRASP to be bound by ubiquitin-sorting receptors. The latter bind ubiquitin with low affinity (10$^{-4}$ to 10$^{-3}$ M) (63, 64) and target the ubiquitinated proteins into the degradation pathway. It is noteworthy in this
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context, however, that the relative low number of ubiquitins (one or two) probably allows for de-ubiquitination of DM-GRASP during or shortly after its delivery to the early endosomes, resulting in recycling instead of degradation. We indeed find that upon overexpression of ubiquitin, DM-GRASP accumulates in large vesicles (probably multivesicular bodies of the degradation pathway (65)), and the cellular concentration of DM-GRASP decreases.

Preference for DM-GRASP—During development of the visual system, RGC axons extend along earlier formed RGC axons, the only area in the environment of the growth cone providing DM-GRASP, thus serving as a substrate pathway. The interactions of DM-GRASP present on the substrate axons with DM-GRASP on the advancing growth cones are crucial for proper axonal orientation in the retina (9). The data presented here now show that the endocytosis of DM-GRASP is crucial for the preferential growth of RGC axons on DM-GRASP-containing micro-lanes that mimic the earlier formed RGC axons. The control of the DM-GRASP concentration in the plasma membrane of RGC growth cones by endocytosis can be assumed to be an important prerequisite for their optimal adhesion and ultimately axon navigation.

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REFERENCES

1. Manes, P. F., and Schachner, M. (2007) Nat. Neurosci. 10, 19–26
2. Burns, F. R., von Kannen, S., Guy, L., Raper, J. A., Kambholz, J., and Chang, S. (1991) Neuron 7, 209–220
3. Tanaka, H., and Obata, K. (1984) Dev. Biol. 106, 26–37
4. Pourquié, O., Collety, M., Thomas, J. L., and Le Douarin, N. M. (1990) Development 109, 743–752
5. el-Deeb, S., Thompson, S. C., and Covault, J. (1992) Dev. Biol. 149, 213–227
6. Tanaka, H., Matsu, T., Agata, A., Tomura, M., Kubota, I., McFarland, K. C., Koh, B., Lee, A., Phillips, H. S., and Shelton, D. L. (1991) Neuron 7, 535–545
7. DeBernardo, A. P., and Chang, S. (1995) Dev. Biol. 169, 65–75
8. Pollerberg, G. E., and Mack, T. G. (1994) Dev. Biol. 165, 670–687
9. Avci, H. X., Zelina, P., Thelen, K., and Pollerberg, G. E. (2004) Dev. Biol. 271, 291–305
10. Weiner, J. A., Koo, S. J., Nicolas, S., Fraboulet, S., Pfaff, S. L., Pourquié, O., and Sanes, J. R. (2004) Mol. Cell Neurosci. 27, 59–69
11. Heffron, D. S., and Golden, J. A. (2000) J. Neurosci. 20, 2287–2294
12. Stephan, J. P., Bald, L., Roberts, P. E., Lee, J., Gu, Q., and Mather, J. P. (1999) Dev. Biol. 212, 264–277
13. Chedotal, A., Pourquié, O., Ezan, F., San Clemente, H., and Sotelo, C. (1996) J. Neurosci. 16, 3296–3310
14. Yamagata, M., Herman, J. P., and Sanes, J. R. (1995) J. Neurosci. 15, 4556–4571
15. Bowen, M. A., Patel, D. D., Li, X., Modrell, B., Malacock, A. R., Wang, W. C., Marquardt, H., Neubauer, M., Pesando, J. M., Francke, U., et al. (1995) J. Exp. Med. 181, 2213–2220
16. Kanki, J. P., Chang, S., and Kuwada, J. Y. (1994) J. Neurobiol. 25, 831–845
17. Sekine-Aizawa, Y., Omori, A., and Fujita, S. C. (1998) Eur. J. Neurosci. 10, 2810–2821
18. Konno, A., Ahn, J. S., Kitamura, H., Hamilton, M. J., Gebe, J. A., Aruffo, A., and Davis, W. C. (2001) J. Leukocyte Biol. 69, 944–950
19. DeBernardo, A. P., and Chang, S. (1996) J. Cell Biol. 133, 657–666
20. van den Kemp, L. C., Nelissen, J. M., Degen, W. G., Torensma, R., Weidle, U. H., Bloomers, H. P., Figdor, C. G., and Swart, G. W. (2001) J. Biol. Chem. 276, 25783–25790
21. Yang, P., Chen, L., Zwart, R., and Kijlstra, A. (2002) Invest. Ophthalmol. Vis. Sci. 43, 1488–1492
22. Kamiguchi, H., and Lemmon, V. (1998) J. Neurosci. 18, 3749–3756
23. Minana, R., Duran, J. M., Tomas, M., Renau-Piqueras, J., and Guerri, C. (2001) Eur. J. Neurosci. 13, 749–756
24. Chang, M. P., Mallet, W. G., Mostov, K. E., and Brodsky, F. M. (1993) EMBO J. 12, 2169–2180
25. Mellman, I. (1996) Annu. Rev. Cell Dev. Biol. 12, 575–625
26. Hinshaw, J. J., and Schmid, S. L. (1995) Nature 374, 190–192
27. Takeda, K., McPherson, P. S., Schmid, S. L., and De Camilli, P. (1995) Nature 374, 186–190
28. Hicke, L., and Dunn, R. (2003) Annu. Rev. Cell Dev. Biol. 19, 141–172
29. Fujita, Y., Krause, G., Scheffner, M., Zeenher, D., Laddy, E. H., Behrens, J., Sommer, W. T., and Birchmeier, W. (2002) Nat. Cell Biol. 4, 222–231
30. Haglund, K., Sigismund, S., Polo, S., Szymkiewicz, I., Di Fiore, P. P., and Dikic, I. (2003) Nat. Cell Biol. 5, 461–466
31. Kaabeche, K., Guenou, H., Bouvard, D., Didelot, N., Listrat, A., and Marie, P. J. (2005) J. Cell Sci. 118, 1223–1232
32. Buttner, C., Sadtler, S., Leyendecker, A., Laube, B., Grifon, N., Betz, H., and Schmalzing, G. (2001) J. Biol. Chem. 276, 42978–42985
33. Burma, M., Dreier, L., Dittman, J. S., Grunwald, M. E., and Kaplan, J. M. (2002) Neuron 35, 107–120
34. Kayem, J. F., Roman, J. M., Von Boxberg, Y., Schwarz, U., and Dreyer, W. J. (1992) Eur. J. Biochem. 208, 1–8
35. Zelina, P., Avci, H. X., Thelen, K., and Pollerberg, G. E. (2005) Development 132, 3609–3618
36. Pollerberg, G. E., Sadoul, R., Goridis, C., and Schachner, M. (1985) J. Cell Biol. 101, 1921–1929
37. Needham, L. K., Thelen, K., and Maness, P. F. (2001) J. Neurosci. 21, 1490–1500
38. Holton, K. L., Loder, M. K., and Melian, H. E. (2005) Nat. Neurosci. 8, 881–888
39. Thelen, K., Wolfram, T., Mairer, B., Jähring, J., Tinazli, A., Piehler, J., Spatz, J. P., and Pollerberg, G. E. (2007) Soft Matter 3, 1486–1491
40. Vojet, A. B., Cooper, J. A., and Honesbellen, S. M. (1997) in The Yeast Two-hybrid System (Bartel, P. M., and Fields, S., eds), pp. 29–42, Oxford University Press, Oxford
41. Zimmerman, A. W., Nelissen, J. M., van Emst-de Vries, S. E., Willems, P. H., de Lange, F., Collard, J. G., van Leeuwen, F. N., and Figdor, C. G. (2004) J. Cell Biol. 8, 16, 271, 264–277
42. Lee, C. S., Kim, I. S., Park, J. B., Lee, M. N., Lee, H. Y., Suh, P. G., and Ryu, S. H. (2006) Nat. Cell Biol. 8, 477–484
43. Schaefer, A. W., Kamiguchi, H., Wong, E. V., Beach, C. M., Landreth, G., and Lemmon, V. (1999) J. Biol. Chem. 274, 37965–37973
44. Schmid, R. S., Pruitt, W. M., and Maness, P. F. (2000) J. Neurosci. 20, 7448–7458
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4177–4188
45. Bailey, C. H., Kaang, B. K., Chen, M., Martin, K. C., Lim, C. S., Casadio, A., and Kandel, E. R. (1997) Neuron 18, 913–924
46. Long, K. E., Asou, H., Snider, M. D., and Lemmon, V. (2001) J. Biol. Chem. 276, 1285–1290
47. Holly, S. P., Larson, M. K., and Parise, L. V. (2000) Exp. Cell Res. 261, 69–74
48. Kuhn, T. B., Brown, M. D., and Bamburg, J. R. (1998) J. Neurobiol. 37, 524–540
49. Condic, M. L., and Letourneau, P. C. (1997) Nature 389, 852–856
50. von Zastrow, M., and Sorkin, A. (2007) Curr. Opin. Cell Biol. 19, 436–445
51. Piper, M., Salih, S., Weinl, C., Holt, C. E., and Harris, W. A. (2005) Nat. Neurosci. 8, 179–186
52. Holbrook, M. R., O’Donnell, J. B., Jr., Slakey, L. L., and Gross, D. I. (1999) Biochemistry 38, 9348–9356
53. Lamaze, C., and Schmid, S. L. (1995) J. Cell Biol. 129, 47–54
54. Gommerman, J. L., Rottapel, R., and Berger, S. A. (1997) J. Biol. Chem. 272, 30519–30525
55. Joly, M., Kazlauskas, A., and Corvera, S. (1995) J. Biol. Chem. 270, 13225–13230
56. Joly, M., Kazlauskas, A., Fay, F. S., and Corvera, S. (1994) Science 263, 684–687
57. York, R. D., Molliver, D. C., Grewal, S. S., Stenberg, P. E., McCleskey, E. W., and Stork, P. J. (2000) Mol. Cell. Biol. 20, 8069–8083
58. Caruso-Neves, C., Kwon, S. H., and Guggino, W. B. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 17513–17518
59. Khundmiri, S. J., Bertorello, A. M., Delamere, N. A., and Lederer, E. D. (2004) J. Biol. Chem. 279, 17418–17427
60. Schaefer, A. W., Kamei, Y., Kamiguchi, H., Wong, E. V., Rapoport, I., Kirchhausen, T., Beach, C. M., Landreth, G., Lemmon, S. K., and Lemmon, V. (2002) J. Cell Biol. 157, 1223–1232
61. Yonemura, S., Hirao, M., Doi, Y., Takahashi, N., Kondo, T., Tsukita, S., and Tsukita, S. (1998) J. Cell Biol. 140, 885–895
62. Aguilar, R. C., Watson, H. A., and Wendland, B. (2003) J. Biol. Chem. 278, 10737–10743
63. Piper, R. C., and Luzio, J. P. (2007) Curr. Opin. Cell Biol. 19, 459–465
64. Raiborg, C., Bache, K. G., Gillooly, D. J., Madshus, I. H., Stang, E., and Stenmark, H. (2002) Nat. Cell Biol. 4, 394–398
65. Gu, F., and Gruenberg, J. (1999) FEBS Lett. 452, 61–66
66. Perron, J. C., and Bazb, J. L. (1999) Mol. Cell Neurosci. 13, 362–378
67. Sanada, K., Hayashi, Y., Harada, Y., Okano, T., and Fukada, Y. (2000) J. Neurosci. 20, 986–991