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Increased GTP-Binding to Dynamin II Does Not Stimulate Receptor-Mediated Endocytosis

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Regarding the molecular mechanism of dynamin in receptor-mediated endocytosis, GTPase activity of dynamin has been thought to have a critical role in endocytic vesicle internalization. However, a recent report suggested that GTP-binding to dynamin itself activates the dynamin to recruit molecular machinery necessary for endocytosis. In this study, to investigate the role of GTP binding to dynamin II, we generated two mutant dynamin II constructs: G38V and K44E. G38V, its GTP binding site might be mainly occupied by GTP caused by reduced GTPase activity, and K44E mutant, its GTP binding site might be vacant, caused by its decreased affinity for GTP and GDP. From the analysis of the ratio of GTP vs GDP bound to dynamin, we confirmed these properties. To test the effect of these mutant dynamins on endocytosis, we performed flow cytometry and confocal immunofluorescence analysis and found that these two mutants have inhibitory effect on transferrin-induced endocytosis. Whereas fluorescent transferrin was completely internalized in wild-type (WT) dynamin II expressing cells, no intracellular accumulation of fluorescent transferrin was found in the cells overexpressing K44E and G38V mutant. Interestingly, the amount of GTP bound to K44E was increased when endocytosis was induced than that bound to WT. The present results suggested that the GTPase activity of dynamin II is required for formation of endocytic vesicle and GTP-binding to dynamin II per se is not sufficient for stimulating endocytosis.

Key Words: **GTP-binding; dynamin II; receptor-mediated endocytosis.**

Receptor-mediated endocytosis occurs via the formation of clathrin-coated vesicles and involves coat assembly, receptor recruitment, coated pit invagination, and finally vesicle budding (1, 2). Dynamin is a GTPase protein that is believed to play a direct role in detaching clathrin-coated vesicles from the plasma membrane. Until now, it is known that dynamin tetramers in the GDP-bound states are recruited to the neck, which acts as a template for formation of the dynamin spiral. Bound GDP is exchanged for GTP, and on completion of a spiral formation around the neck of a bud, when dynamin tetramers at one end of the spiral stack on tetramers from the other, GTP hydrolysis is accelerated. The energy released from the hydrolysis sends the conformational signal to proceed with scission (3, 4). Many experimental data have supported this model. For example, when GTP hydrolysis was blocked (5) or dynamin's ability to self-assemble on membranes was inhibited (6, 7), endocytosis no longer occurred. But a recent report proposed its role as a switch to regulate the endocytic step (8). Sever and her colleagues (8) hypothesized that dynamin is not a force-generating molecule but a regulatory molecule like classical ras proteins to transmit signals from upstream molecule to downstream effectors. According to their hypothesis, dynamin is active only in GTP-bound states and, after the bound GTP is hydrolyzed, dynamin becomes inactive form. GTP hydrolysis converts an active dynamin to an inactive GDP-bound form and triggers disassembly of the stacked dynamin tetramers, releasing dynamin and the fission machinery for further rounds of vesicle formation.

Despite of many efforts of some reviewers, above two models seem to be conflicting and hardly reconciled. To define the role of GTPase activity or GTP-bound form of dynamin during receptor-mediated endocytosis, we constructed two mutants of dynamin II, K44E and G38V, based on the similarity of the GTPase domain between ras and dynamin, and characterized them by flow cytometric and confocal laser microscopic analysis. G38V mutant is largely in GTP-occupied form caused

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by its reduced GTPase activity, but surprisingly overexpression of this mutant dynamin II resulted in significant inhibition of receptor-mediated endocytosis as efficiently as K44E mutant. It has generally been thought that K44E mutant might be mainly GTP-free form caused by its decreased affinity for GTP and GDP. From these results we suggest that GTP-bound state of dynamin II is not an active form and basal GTPase activity is essential for effective endocytosis.

EXPERIMENTAL PROCEDURES

Expression constructs and site-directed mutagenesis. The point mutants, K44E and G38V, were generated by standard site-directed mutagenesis techniques. PCR was performed using PCR system (Perkin Elmer, GeneAmp PCR System 9600). PCR products were subcloned into pBluescript (Invitrogen, San Diego, CA) and sequenced on an automated DNA sequencer (Perkin Elmer). Wild-type, K44E, and G38V dynamin II constructs were then cloned into pCMVTag-1 mammalian expression vector (Stratagene) with *Bgl*II (5′ end) and *Hin*dIII (3′ end) (Boehringer Mannheim) sites in frame with pCMVTag, placing the Flag at the N-terminal end of the construct.

Cell culture and transient transfection. BOSC23 (derived from the Ad5-transformed human embryonic kidney 293 cell line) and NIH3T3 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY), penicillin G (100 U/ml), streptomycin sulfate (100 μg/ml), amphotericin B (0.25 μg/ml) and 2-mercaptoethanol (50 μM) at 37°C in a 5% CO_2 humidified incubator. All transient transfections were done in six-well plates where 2.5×10^6 cells were plated in 2.5 ml of growth medium 24 h prior to transfection. Where indicated, 25 μM of chloroquine was added to serum-free medium 5 min prior to adding DNA. Either 250 or 500 μl of 2× HBS (pH 7.05) was added to an equal volume of DNA/$CaCl_2$ solution while bubbling, and the resulting solution was immediately added onto each well. After 24 h, the medium was changed with DMEM containing 10% FBS. Cells were harvested 48–72 h after transfection.

Analysis of GTP bound to dynamin II. For metabolic labeling, NIH3T3 cells were washed once with PBS and incubated with phosphate-free Dulbecco's MEM (5% FBS) for 15 min at 37°C. Cells were labeled for 3 h in phosphate-free medium supplemented with 0.25 mCi/ml [^{32}P] H_3PO_4, and then harvested. Cells were washed twice with PBS, scraped off the plate in PBS, and were lysed in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.5, 0.15M NaCl, 1% Triton X-100, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin). After 60 min incubation on ice, the lysate was cleared by centrifugation and the protein concentration was determined with the BioRad assay kit. Transfected cell lysates were incubated with affinity gel (anti-FLAG M2) for 2 h at 4°C. Immunoprecipitates were resuspended in 30 μl of 1M KH_2PO_4 (pH 3.4) and incubated at 85°C for 3 min. Samples were centrifuged at 12,000 rpm for 5 min, 15 μl of supernatant was decanted to a new tube, and the next day 10 μl were decanted for spotting, 1 μl at a time, onto 20 × 20 cm Silica gel 60 thin layer chromatography plates (Merck). Plates were developed in 1M KH_2PO_4 (pH 3.4) for 2 h. The percent of GTP bound to dynamin was calculated as cpm in GTP/(GTP + GDP) by Phosphoimager (Kodak). These cpm values normalized for moles of phosphate.

Immunoprecipitation and Western blot analysis. Cells were washed twice in ice-cold phosphate-buffered saline (PBS) and were lysed in ice-cold lysis buffer. After 60 min incubation on ice, the lysate was cleared by centrifugation (13,000 rpm, Eppendorf centrifuge, 20 min) and the protein concentration was determined with the BioRad assay Kit. Cell lysates in lysis buffer were incubated with affinity gel (anti-FLAG M2) for 2 h at 4°C. Equal amounts of immunoprecipitates were run out on SDS–polyacrylamide gels and blotted onto nitrocellulose filters (Amersham). The filters were blocked with 5% skim milk in PBST. Primary antibody was anti-FLAG antibody (anti-FLAG M2 monoclonal Ab). After incubation with secondary HRP-conjugated antibody (Upstate Biotechnology Incorporated), signals were visualized using the Amersham ECL kit.

Endocytosis assay. Endocytosis of transferrin was assessed as follows: Each dynamin construct was introduced into coverslip-plated BOSC23 cells by using DNA/calcium phosphate coprecipitation method. At 48 h after transfection, cells were starved for 1 h in serum-free media at 37°C and were labeled for 40 min with 20 μg/ml of transferrin (Sigma) or FITC- or tetramethylrhodamin-conjugated transferrin (Molecular Probes). Endocytosis was stopped by cooling on ice and washing with the same medium at 0°C. Cells were washed further with PBS, fixed with 4% paraformaldehyde (20 min, 4°C) and processed for immunofluorescence.

Flow cytometric analysis. Cells were detached from the tissue culture dishes by 1× EDTA (in DPBS, GibcoBRL), centrifuged and resuspended in 200 μl DMEM, containing FITC-conjugated transferrin. As above, transferrin was internalized for 40 min (37°C) and endocytosis was stopped by cooling on ice. The cells were subsequently washed with the same medium and PBS at 0°C. Analysis of the fluorescence intensity was performed with a Becton Dickinson FACSCalibur instrument. At least 10,000 cells were analyzed for each sample.

Confocal laser scanning microscopy. For immunofluorescence assay, BOSC23 cells were grown on glass coverslips for 48 h to ~60% confluence. The cover-slips bearing transfected cells were rinsed briefly in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) and fixed for 20 min by immersion in 4% paraformaldehyde fixative (4°C), followed by permeabilization with 0.1% Triton X-100. The cells were blocked with 1.0% BSA in PBS, and incubated for 1 h at 37°C with anti-Flag Ab (anti-FLAG M2 monoclonal) as primary antibody diluted in PBS containing 1.0% BSA. After three washes in PBS, the coverslips were incubated with affinity isolated secondary antibody (fluorescein-conjugated goat anti-mouse IgG) (Biosource, Camarillo, CA). The coverslips were washed three times with PBS and mounted in Fluorescent Mounting Medium (DAKO). Cells were observed for epifluorescence using a confocal laser scanning microscope (TCS400, LEICA, Inc.). Acquired images were manipulated with SCANware 5.0 (LEICA, Inc.) and digitized using Adobe Photoshop software (Adobe Photosystems, Inc., Mountain View, CA).

RESULTS

Transient expression of mutants in cultured BOSC 23 cells. To define the cellular function of dynamin II in mammalian cells, we constructed two mutant dynamins (the lysine-to-glutamic acid mutation [K44E] and the glycine-to-valine mutation [G38V]) based on the similarity of GTPase domain between ras and dynamin) (Fig. 1A). K44E mutation in dynamin II was based on K16N mutation in H-ras (9). Deduced from the fact that the ras mutation had an inhibitory phenotype due to its decreased affinity for both GTP and GDP (ensuring that it was of empty state in GTP binding site (10)), K44E was thought to be also in empty state. Actually, like H-ras K16N, K44E has been known to have low GTPase activity as assessed in previous study (11). G38V mutation in dynamin II was equivalent to G12V mutation in conventional H-ras protein. Mutations in amino acids 12, 59, and 61 of

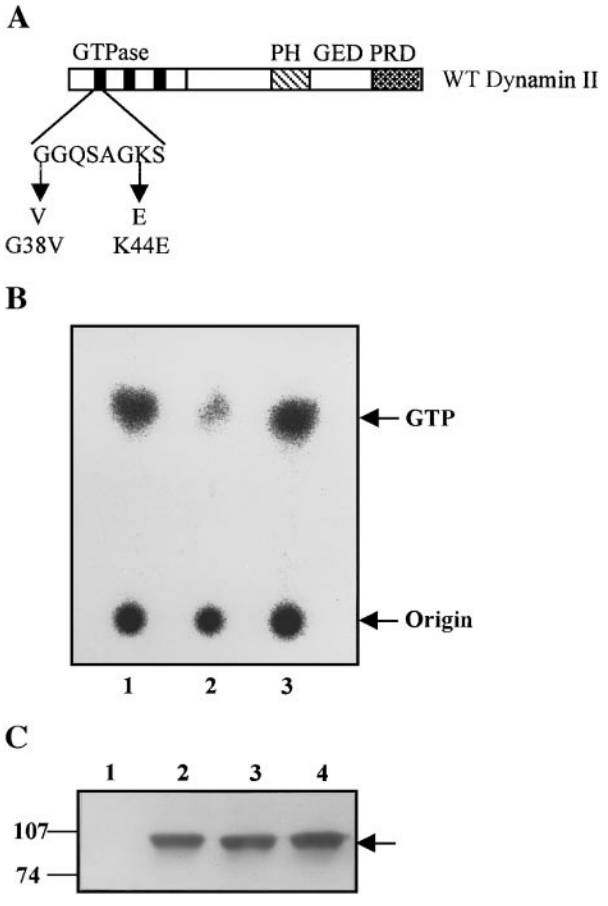

FIG. 1. Characterization of expressed dynamin mutants. (A) Schematic representation of full-length dynamin II showing the N-terminal GTPase domain, the PH (pleckstrin-homology), the GED (GTPase effector domain), and the C-terminal PRD (proline rich domain). Tripartite of GTPase domain (black boxes, left) is present at the amino terminus. (B) Autoradiographs after chromatographic separation of GTP and GDP extracted from dynamin II in NIH3T3 Cells. Transfected cells with WT (lane 1), K44E (lane 2), and G38V (lane 3) construct. (C) Transient expression of WT and mutant dynamin constructs in BOSC 23 cells. No transfected cells (lane 1), cells transfected with WT (lane 2), K44E (lane3), and G38V (lane 4). Arrow indicates band of dynamin II. In all of these experiments, 1–1.5 mg of cell lysate proteins was used. Numbers on the left are molecular masses, in kilodaltons. (B) and (C), representative results of an experiment performed on three separated cell preparation.

H-ras have their major effects on GTP hydrolysis rather than GTP binding (12). Therefore, G38V mutant was expected to have low GTPase activity, which might leave the mutant in GTP-bound state.

To demonstrate this more directly, we analyzed the relative levels of GTP bound to WT, K44E, and G38V. After transfection of WT and these two mutant constructs, transfected cells were labeled with [^{32}P] orthophosphate for 3 hr, lysed, and dynamin II was collected by immunoprecipitation with affinity gel (anti-FLAG M2). Bound nucleotides were extracted and separated by thin layer chromatography. We observed that a high amount of GTP is bound to G38V (34 ± 5%, lane 3), compared with those of WT (23 ± 3%, lane 1) and K44E (17 ± 2%, lane 2) (Fig. 1B), and found that total amount of GTP and GDP bound to K44E (31% compared with that of WT) was lower than that to others. From these results we confirmed that GTP binding site of G38V mutant is preferentially occupied by GTP and K44E has lower affinity for both GTP and GDP.

To determine whether the constructed proteins were expressed from introduced plasmids, we examined the expression level of WT, K44E, and G38V dynamin II protein in transfected BOSC23 cells. Figure 1C demonstrated that WT (lane 2), K44E (lane 3), and G38V (lane 4) proteins were expressed in transfected cells while Flag-tagged dynamin was not detectable in untransfected- or vector transfected-cells as expected (lane 1 and data not shown). And when the same blot was incubated with anti-dynamin Ab (Hudy2) to ensure that Flag-tagged dynamin was quantitatively overexpressed compared with endogenous dynamin, Flag-tagged dynamin was found to be expressed six times as much as the endogenous dynamin (data not shown).

K44E and G38V inhibit receptor-mediated endocytosis of transferring. Next we tested if K44E and G38V-expressing cells could normally internalize fluorescence-labeled transferrin, a marker for receptor-mediated endocytosis, by flow cytometric analysis. BOSC 23 cells transfected with WT, K44E, and G38V were challenged to internalize FITC-conjugated transferrin for 40 min. Flow cytometric analysis revealed that the extent of transferrin uptake in K44E and G38V expressing cells was significantly reduced in comparison with untransfected cells and WT transfected cells (Fig. 2A). The inhibitory effect of K44E on transferrin uptake was consistent with previous studies reporting impaired endocytosis in COS-7 cells expressing this mutant dynamin (10). It was notable that the G38V did inhibit the receptor-mediated endocytosis as efficiently as K44E (Fig. 2B). To compare the efficiency of transferrin uptake, mean values of fluorescence intensity in BOSC 23 cells were expressed as a percentage of that in no transfected control cells (37°C, 100%). This is a representative experiment of three independent experiments with relatively similar percentage and about 40% of the cells were transfected. Percent of transfected cells were calculated by cotransfection of control GFP vector with dynamin expression construct.

To definitely verify that the G38V inhibited the process of the receptor-mediated endocytosis and to investigate how G38V worked to produce the inhibitory effects, we performed confocal microscopic analysis. As shown in Fig. 3, strong fluorescence intensity of FITC-labeled dynamin was found near the plasma membrane of both WT (panel A1) and mutant expressing

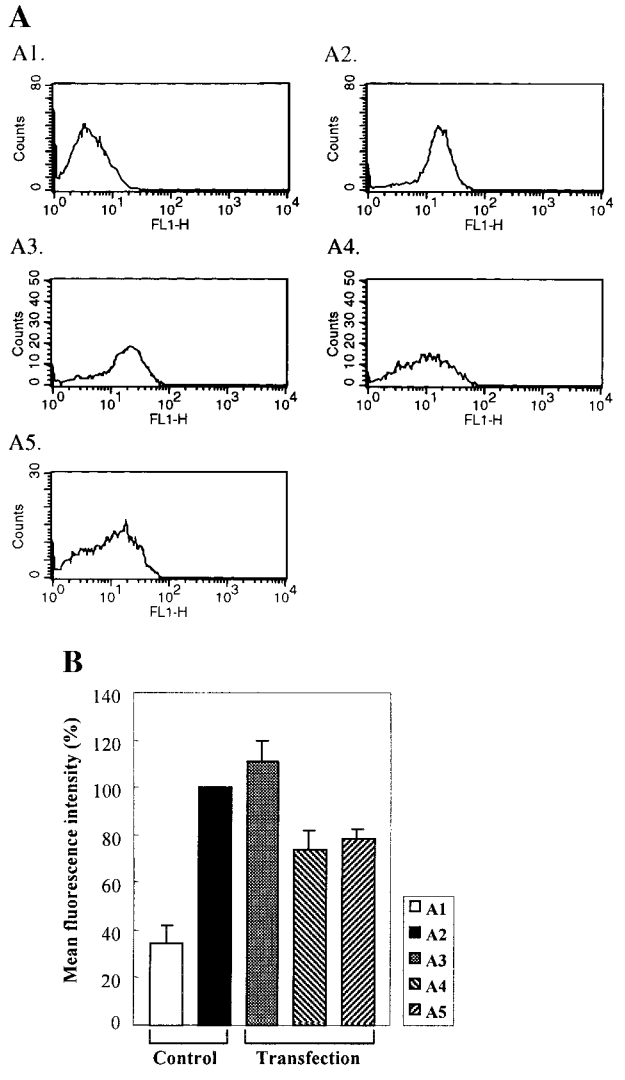

FIG. 2. Receptor-mediated endocytosis of FITC-transferrin is inhibited by the mutant dynamin expression. To analyze the effect of mutant dynamin expression on the transferrin internalization, we performed FACS analysis. (A1) Negative control (4°C); (A2) no transfection; (A3) WT; (A4) K44E mutant; (A5) G38V mutant. For comparison, the mean values of transferrin uptake in BOSC 23 cells in A are shown in B. The efficiencies of endocytosis in all transfected cells were expressed as a % of mean fluorescence intensity in no transfected control cells. The results, expressed as mean ± SD, represent data obtained in three independent experiments.

cells (panels B1 and C1). Complete internalization of labeled transferrin into vesicular elements, which was indicated by the strong red fluorescence intensity in cytoplasmic region near the nucleus, was observed in cells overexpressing WT (Fig. 3, panel A2). As shown in the overlapped image (panel A3), intense red fluorescence was well correlated with intense bright fluorescence indicating high expression of transfected WT in the same cells. By contrast, the cells overexpressing K44E (panel B2) and G38V mutant (panel C2) showed little intracellular accumulation of fluorescent transferrin. Instead, fluorescent transferrin was confined to plasma membrane. Nearly identical localization (panels B3 and C3) of mutant dynamin II (bright fluorescence) and transferrin (red fluorescence) at the plasma membrane represented that overexpressed mutant dynamin II associated with invaginated membrane, while internalization of transferrin was observed in cells not expressing Flag-tagged dynamin (data not shown).

GTP bound state of dynamin II in receptor mediated endocytosis. To identify the GTP bound state of dynamin II more precisely, we analyzed the relative levels of GTP bound to WT, K44E, and G38V during receptor-mediated endocytosis. After transfection of WT, K44E and G38V constructs, transfected cells were labeled with [^{32}P] orthophosphate for 3 h, and then challenged to internalize transferrin for 40 min. The cells lysed and dynamin II was collected by immunoprecipitation with affinity gel (anti-FLAG M2). Bound nucleotides were extracted and separated by thin layer chromatography (Fig. 4B). As shown in Fig. 4A, a high amount of GTP was bound to K44E (50%, lane 2) and G38V (54% GTP, lane 3) than WT (37%, lane 1). From these results we confirmed that GTP preferentially occupies GTP binding site of G38V mutant. Together, we also observed that K44E mutant during receptor-mediated endocytosis is occupied by GTP in spite of its lower affinity for both GTP and GDP (11).

DISCUSSION

Dynamin has been known to have an important role in receptor-mediated endocytosis (13), but precise function and action mechanisms were largely unknown. Dynamin has four discrete domains—a GTPase domain which exhibits high intrinsic rate of GTP hydrolysis, an assembly domain through which the protein can oligomerize into rings and spirals (14), a pleckstrin-homology (PH) domain which binds phosphoinositide (15), and proline-rich domain (PRD) to enable the protein to bind several proteins those have SH3 domain (16).

It was recently found that assembly domain has an additional function, assembly- dependent GTPase activating function. For this reason, the assembly domain of dynamin is also called GTPase effector domain (GED) (17). By over-expressing two kinds of mutant dynamin (K694A and R725A) with mutations in GED, Sever and her colleagues (8) suggested that dynamin might be a classical regulatory GTPase, similar to ras proteins. Since the mutants with impaired GED showed low GTPase activating protein (GAP) activity (R725A) or defect in self-assembly (K694A), GTPase activity of both mutants became much lower than that of wild-type dynamin. According to prevalent hypothesis in which GTPase activity of dynamin provides energy to drive membrane fission in endocytosis (18,

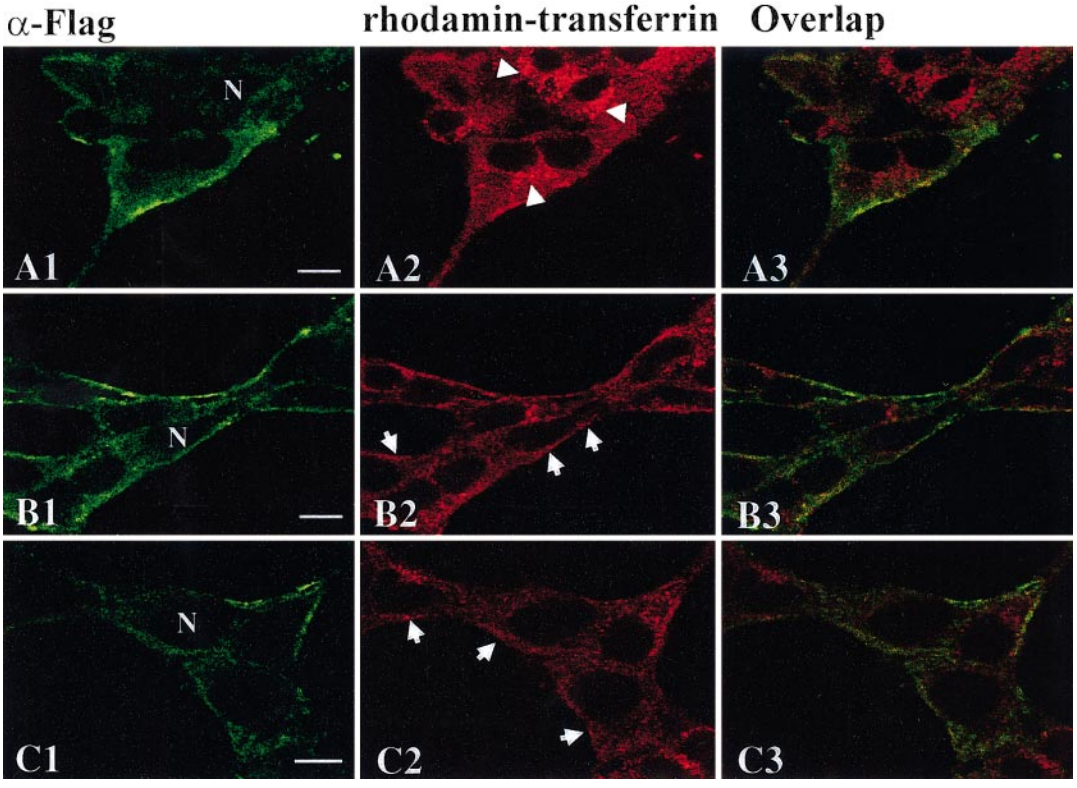

FIG. 3. Confocal images of transferrin uptake in WT, K44E, and G38V dynamin II transfected BOSC 23 cells. (A1, B1, C1) Dynamin expression with anti-Flag antibody. (A2, B2, C2) Fluorescence signals from the cells, which have internalized tetramethylrhodamin-transferrin. (A3, B3, C3) Overlapping images. (A) Wild-type; (B) K44E; (C) G38V dynamin II. Transferrin remained associated with the plasma membrane in cells expressing K44E and G38V mutant dynamin II, while pronounced internalization of transferrin was observed in cells expressing WT dynamin II. Arrowheads point to the internalized transferrin in A2 and arrows in B2 and C2 indicate transferrin located in plasma membrane. N, nucleus. Bars indicate 10 μm.

19), the mutants with lower GTPase activity were expected to inhibit endocytosis (5). However, these mutants led to accelerated endocytosis. Sever and her colleague explained their unexpected results by proposing that dynamin is active only in GTP-bound state; the function of GTP hydrolysis is not to generate force

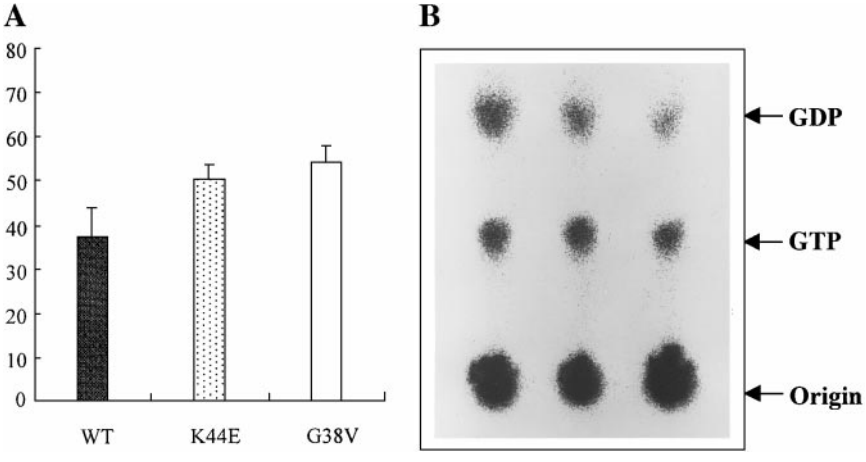

FIG. 4. Autoradiographs of GTP and GDP bound to dynamin II in receptor-mediated endocytosis. To verify the GTP bound state of dynamin II, we measured the relative levels of GTP bound to WT, K44E, and G38V in receptor-mediated endocytosis. Lane 1, WT; lane 2, K44E; lane 3, G38V. A high amount of GTP is bound to K44E (50%) and G38V (54%) compared with that of WT (37%) (A). GTP/GDP was extracted and separated by thin layer chromatography (B).

but to regulate activation state of dynamin. Their hypothesis predicts that over-expression of GTP-bound dynamin, which recruits molecular effectors necessary for progress of endocytosis, stimulate the endocytosis.

To investigate the role of GTP binding of dynamin, we constructed a mutant dynamin (G38V) with abnormally high occupancy by GTP. On the basis of the similarity with H-ras mutant (20), G38V was expected to have reduced GTPase activity, maintaining GTP-bound state. Our attempt to measure the GTPase activity of G38V, was not successful due to extraordinary high Km value of dynamin for GTP (21). Instead, we directly analyzed the ratio of GTP/GDP bound to dynamin. G38V was found to be preferentially occupied by GTP as expected.

In the process of receptor mediated endocytosis, the formation of membrane invagination and the proper stacking of dynamin around the neck are prerequisite to membrane fission (4). In cells expressing K694A and R725A mutants, the length of the neck was thought to be longer due to decreased GTPase activity, which impedes well-timed GTP hydrolysis. Assuming that the GTPase activity of K694A (when assembled, same as wild-type) and R725A (when assembled, one tenth of wild-type) were sufficient for driving membrane fission, the elongated neck might not only overcome the decreases of GTPase activity and but also even enhance endocytosis. Our result clearly showed that relatively high amount of GTP was bound to G38V and K44E than WT in receptor-mediated endocytosis (Fig. 4). It was expected that K44E and G38V also induced the elongation of the neck of invaginated membrane due to their low GTPase activity. However, in these mutants, GTPase activity could not reach to basal intrinsic GTPase activity due to the impairment of GTPase itself, which might result in the inhibition of endocytosis. It is known that all the GTPase mutant dynamins such as K44A and S45N had low intrinsic GTPase activity and inhibited receptor-mediated endocytosis (5, 11). The inhibition of endocytosis by these mutants can be explained in the same basis with K44E.

We expected that the amount of GTP bound to K44E was lower than WT when endocytosis occurs, but the result was opposite. Damke *et al.* (11) showed that K44E mutant has lower GTPase activity, but they did not show GTP bound state of this mutant. Recently, Yang and Cerione (22) have described that although K44E mutant show to block receptor-mediated endocytosis, it is not clear that this mutant binds to GTP with wild-type affinity. Therefore, from our analysis, we proposed that K44E mutant has lower affinity for GTP in normal condition (Fig. 1B), but this mutant has some affinity for GTP when endocytosis occur (Fig. 4).

Thus we hypothesize that GTPase activity of dynamin provides force to drive membrane fission, and the relatively high intrinsic GTPase activity, even when not fully activated by GED, can generate the force. Ras protein (a typical regulatory GTPase) has relatively high affinity for GTP and low catalytic activity (23). Therefore, low affinity of dynamin for GTP and high catalytic activity of its GTPase also imply that dynamin is not a regulatory GTPase like ras protein. The data in the present study clearly showed that GTP-binding to dynamin was not sufficient to progress endocytosis if the dynamin had little GTPase activity. The molecules known to bind dynamin such as Grb2 (24, 25) and amphyphysin (26) seem not to contribute to membrane fission. It remains to be solved whether there are any other molecules necessary for scissoring membrane. However, Stowell *et al.* (27) showed that the assembly of dynamin spirals on PIP_2-containing lipid tubes enhanced the rate of GTP hydrolysis. Moreover Sweitzer and Hinshaw (28) demonstrated that the dynamin tubes undergo dramatic conformational changes upon the addition of GTP, which results in the constriction and vesiculation of the lipid tubes. These results strongly suggested that dynamin itself might act as a structural, mechanochemical enzyme.

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