The Endoplasmic Reticulum Exit of Glutamate Transporter Is Regulated by the Inducible Mammalian Yip6b/GTRAP3-18 Protein

Alicia M. Ruggiero, Yiting Liu, Svetlana Vidensky, Susanne Maier, Elizabeth Jung, Hesso Farhan, Michael B. Robinson, Harald H. Sitte, and Jeffrey D. Rothstein

From the Departments of *Neuroscience and †Neurology, Johns Hopkins University, Baltimore, Maryland 21287, the ‡Institute of Pharmacology, Center for Biomolecular Medicine and Pharmacology, Medical University of Vienna, Waehringerstrasse 13a, A-1090 Vienna and the §Departments of Pharmacology and Pediatrics, Children’s Hospital of Philadelphia, University of Pennsylvania, Philadelphia, Pennsylvania 19104

GTRAP3-18 interacts with and reduces the activity of the neuronal specific Na+ /K+ glutamate transporter, EAAC1 both in vitro and in vivo. GTRAP3-18 and the related isoform, JM4, are distant relatives of the Rab GTPase-interacting factor PRA1 and share a topology of four transmembrane domains and cytosolic termini. GTRAP3-18 and JM4 are resident endoplasmic reticulum (ER) proteins. The physiological role of GTRAP3-18 is poorly understood. We demonstrate for the first time that GTRAP3-18 is a regulator of ER protein trafficking. Expression of GTRAP3-18 delays the ER exit of EAAC1, as well as other members of the excitatory amino acid transporter family. GTRAP3-18 uses hydrophobic domain interactions in the ER membrane to self-associate and cytoplasmic interactions at the C terminus to regulate trafficking. The features of GTRAP3-18 activity are consistent with recent phylogenetic sequence analyses suggesting GTRAP3-18 and JM4 be reclassified as mammalian isoforms of the yeast protein family Yip, Yip6b, and Yip6a, respectively.

We identified GTRAP3-18 (glutamate transporter-associated protein of EAAT3), a 22-kDa protein that is dynamically induced by retinoic acid both in vitro and in vivo and inhibits the activity of EAAC1 in a dose-dependent manner (GeneID 66028). EAAC1 (human nomenclature EAAT3) is an isoform of the plasma membrane glutamate transporter family localized to glutamatergic and GABAergic neuronal populations and is an important physiological component of normal and abnormal synaptic transmission (2–5). Antisense reduction in GTRAP3-18 or removal of the C-terminal interaction site eliminates GTRAP3-18-mediated alterations of EAAC1 activity (1). The mechanism of this activity was unknown but speculated to be related to interaction-dependent alterations to EAAC1 protein conformation or transport activity before cell surface delivery.

The expression of GTRAP3-18 has been found to be induced by diverse stimuli including oncogenic transformation and ER Ca2+ depletion. Chronic morphine administration leads to a 300–400% increase in GTRAP3-18 mRNA (6). Differentiation, heat shock, or oxidative stress increase the expression of the human isoform of GTRAP3-18, JWA (7, 8). These findings have been confirmed by microarray studies demonstrating increased expression of GTRAP3-18 mRNA under conditions of cell stress, inflammation, and cancer (GeoID 5194496).

Molecular modeling and reported data concur that GTRAP3-18 is an integral ER membrane protein (9) with four transmembrane domains and cytosolic N and C termini (10). GTRAP3-18 and JM4, its closest relative (39% identity 61% consensus; GeneID 11230), are structurally homologous to the Ras superfamily in their secondary structure and low molecular weight, but lack a GTP-binding consensus motif. They are conserved in human, rat, and mouse and have a homologue in Caenorhabditis elegans (D2096.2). Phylogenetic tree analysis of these conserved gene products was used to suggest reclassification as members of the expanded mammalian Yip family: GTRAP3-18/JWA as Yip6b and JM4 as Yip6a, despite an undetermined cellular function (10). The Yip family is loosely defined as integral membrane Ypt-interacting proteins, which often self-associate (11). Ypt proteins are yeast Rab GTPases (10, 12). One of the many functions assigned to Rab proteins is to regulate membrane trafficking spatially and temporally (13). Here we demonstrate that GTRAP3-18 and JM4 have a dominant effect to delay ER to Golgi trafficking.

**EXPERIMENTAL PROCEDURES**

Antibodies—Rabbit and chicken polyclonal anti-GTRAP3-18 antibodies were raised against peptide sequences from the N and C termini of the protein sequence: N1-GTRAP3-18: KFF/H11545/K, Yiting Liu/H20648, Harald H. Sitte/H20648, Michael B. Robinson/H, Susanne Maier/H, Elizabeth Jung/H, Hesso Farhan/H, Michael B. Robinson/H, Harald H. Sitte/H, Jeffrey D. Rothstein/H, and Jeffrey D. Rothstein/H.

Received for publication, February 2, 2007, and in revised form, December 28, 2007. Published, JBC Papers in Press, December 31, 2007, DOI 10.1074/jbc.M701006200

© 2008 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
Yip6b/GTRAP3-18 Regulates ER Exit

and N2-GTRAP3-18: KMDVNLAPRAWDDF; polyclonal EAAC1 antibody described previously (14, 15). Commercial antibodies included: anti-HA monoclonal and polyclonal, Gantit polyclonal, GFP and His6 mouse monoclonal: BAbCo/ Covance, anti c-Myc mouse monoclonal: Roche Applied Science, Calnexin polyclonal: Stressgen. Secondary Cy dye series (Cy2, Cy3, Cy5): Jackson Laboratories.

Plasmid Constructs—The eukaryotic expression vectors pcDNA3 and PRK5 were used for cDNA expression in the mammalian cell lines HEK 293T, COS-7 or in primary cortical neurons. GTRAP3-18 was cloned in-frame with the epitope tag HA into PRK5 using PCR-engineered Sal/Not sites. GTRAP3-18 was detectable at 23 kDa with a mouse monoclonal HA antibody (BAbCo) by Western blot analysis and corresponded to our rabbit and chicken anti-peptide polyclonal antibodies. YFP and CFP GTRAP3-18 were expressed in pEYFP-C1 and pECFP-C1 (Clontech) using PCR-engineered EcoRI/KpnI sites. EAAC1 cDNA was subcloned in myc-PRK5 to create EAAC1-myc fusion and in EcoRI/KpnI frame of pEYFP-C1 and pECFP-C1 (Clontech) using PCR-engineered restriction sites. Myc-N206S EAAT2 and full-length EAAT4 were provided by Mandy Jackson (University of Edinburgh) (16, 17). EAAT4-GFP in pEGFP was provided by Dan Gincel (Johns Hopkins). Full-length myc-GLT-1 in pCMV and hEAAT isoforms were cloned in pGBKT7 were provided by Mitsunori Watanabe (Johns Hopkins). Myc-GLT-1 was tagged with the FLAG epitope was provided by Julia Fuchs (Goettingen University) following modifications (18). The GTRAP3-18 homologue, JM4 was the gift of Marc Schweneker (University of Zurich).

Yeast Two-hybrid Screen—GTRAP3-18 was cloned into pACT2 and pPC86, and the HA and GTRAP3-18 isoforms and EAAC1 truncations were cloned in pGBK7 and pPC97, via PCR-engineered restriction sites EcoRI and SalI/NotI, respectively. The sequences were: E1 (462–542), E2 (462–574), E3 (429–525), E4 (486–565). The EAAC1 C terminus was further truncated into three regions: an extracellular loop (L, amino acids 431–443) that comprises part of both the glutamate binding and ER trafficking domains (19, 20), the final transmembrane domain (T, amino acids 444–469), and the cytosolic tail (C, amino acids 470–523) (21, 22). Dual transformed yeast was selected for growth on histidine drop-out media and for β-galactosidase expression in the auxotrophic yeast strains MaV203 and AH109. Detailed results of these assays may be obtained by contacting the authors.

Mammalian Cell Transfection—HEK293 and COS-7 cell lines were maintained according to standard protocols (ATCC), split 1 day before transfection and used at 50% confluency. All mammalian cells were transfected using FuGENE 6 (Roche Applied Science) according to the manufacturer’s directions. Cells were harvested at 16–72 h growth according to the experimental design.

Immunohistochemistry—Cells were fixed in 4% paraformaldehyde, rinsed, solubilized in 0.4% Triton X-100 for 30 min at 4 °C, and rinsed in TBS (50 mM Tris, pH 7.4, 150 mM NaCl). Primary (Roche Applied Science) antibodies were added at final concentrations ranging from 1:200 to 1:2000. Secondary antibodies Cy2 and Cy5 anti-mouse or anti-rabbit and Cy3 anti-chicken IgY (Jackson Antibodies) were chosen by experiment. Confocal microscopy of transfected cells in brain sections, hippocampal slice, dissociated cultures, and mammalian cells was performed with a Zeiss LSM 510 laser scanning microscope.

Na+-dependent Glutamate Uptake Assay—The activity and kinetics of expressed EAAT was assayed as described (1). Statistical analysis performed using Student's t test and two-way ANOVA.

Membrane Impermeant Biotinylation—Biotinylation was performed as described with some modifications (1, 17, 23). Visualized bands were analyzed and quantitated using electronic imaging and software (Versa Doc, Bio-Rad).

Enzymatic Deglycosylation—Endo H and PNGase were purchased from NEB. Cell lysates following biotinylation were digested at 37 °C for 12 h with gentle shaking with 2 units/μl of each enzyme in lysis buffer with the addition of 1% Nonidet P-40 detergent. Lysis buffer consisted of 100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, and protease inhibitor mixture (Roche Applied Science). Control samples were diluted and incubated without the addition of enzyme. The biotinylation assay was then completed as described above, or the samples were diluted in 2× loading buffer and analyzed by Western blotting.

Site-directed Mutagenesis—Site-directed mutagenesis of rEAAT3 at Asn-85, -128, -178, and -197 to Gln was performed using the QuikChange Site-directed Mutagenesis kit (Stratagene) as directed by the manufacturer. Mutagenesis of Pro-118 to Gly was performed in the same manner. Clones were verified by sequence analysis.

Metabolic Labeling—Metabolic labeling of HEK293T cells was performed as described (24). The cells were transfected with Myc-rEAAC1 and HA-GTRAP3-18 at a 1:2 or a 1:1 ratio and pulsed with Trans-Label (ICN) at 3.5 h post-transfection for 10 min followed by media chase for up to 16 h. Harvested cells were lysed and immunoprecipitated with c-Myc as described. Bound proteins were eluted and analyzed by SDS-PAGE and 35S emission on film.

FRET Analysis—Two methods were used to detect FRET: donor recovery after acceptor photobleaching (DRAP) (27); and the three-filter method according to Youvan (19, 28). The equipment used consisted of an epi-fluorescence microscope (Carl Zeiss TM210, Germany) COS7 cells (3 × 105/well); images were taken using a ×63 oil objective and a LUDL filter wheel that allows for rapid exchange of filters (less than 100 ms). The system was equipped with the following fluorescence filters: CFP filter (λ ex: 436 nm, λ dich: 455 nm, λ em: 480 nm), YFP filter (λ ex: 500 nm, λ dich: 515 nm, λ em: 535 nm), and FRET filter (λ ex: 436 nm, λ dich: mirror 455 nm, em: 535 nm). The acquisition of the images was performed with MetaMorph software (Meta Imaging, Universal Imaging Corporation, V. 4.6).

HEK293 cells were seeded on to poly-d-lysine-coated glass coverslips (24-mm diameter). The next day, cells were transiently transfected using the calcium phosphate precipitation method: 1–3 μg of cDNA was mixed with CaCl2 and HBS buffer (280 mM NaCl/10 mM KCl/1.5 mM Na2HPO4/12 mM dextrose/50 mM HEPES); after 6–10 min, the calcium phosphate-
DNA precipitate was added to the cells. After 4–5 h, the cells were washed twice with phosphate-buffered saline and briefly treated with glycerol, followed by the addition of fetal calf serum-containing medium. Media were replaced by Krebs-HBS buffer (10 mM HEPES, 120 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂). For DRAP, a CFP image was acquired before (lb) and after (la) photo-bleaching using the YFP filter settings for 90 s. DRAP in a specific region of interest (intracellular parts that mostly show red to white color in the representative image) was quantified by FRET efficiency (E) as described in (24) according to Equation 1.

\[
E = \frac{(la - lb)/la}{ICFP} \quad (Eq. 1)
\]

Images for the three-filter method were taken using a double dichroic mirror for all measurements. Various combinations of excitation and emission filters were used to produce the CFP, YFP, and FRET images (ICFP, IYFP, and IFRET, respectively). Background fluorescence was subtracted from all images; net FRET (nF) was calculated using Equation 2,

\[
nF = IFRET - (\alpha \times IYFP) - (\beta \times ICFP) \quad (Eq. 2)
\]

where \(\alpha\) and \(\beta\) represent the bleed-through values for YFP (0.319 ± 0.001; \(n = 15\)) and for CFP (0.656 ± 0.006; \(n = 14\)). To gauge the system both with positive and negative controls for FRET imaging, we used a fusion protein of CFP and YFP (termed CYFP; Ref. 25) and the CFP-tagged deletion mutant of the rat GABA transporter 1, CFP-rGAT1Δ37, lacking the last 37 amino acids, respectively.

**Statistical Analysis**—Statistical analysis was performed using Student’s t test and two-way ANOVA for all \(n\). All experiments were performed with a minimum \(n = 3\) replicates.

**RESULTS**

**GTRAP3-18 Prevents Complex Oligosaccharide Formation on EAAC1 in a Dose-dependent Manner**—EAAC1 and all members of the EAAT/ASCT gene family are \(N\)-linked (asparagine) glycosylated (26). The \(N\)-linked oligosaccharides are processed to the complex state by resident Golgi enzymes preceding cell surface delivery (27). Therefore, the state of the oligosaccharide may be used as an indicator of the extent of ER-Golgi trafficking and plasma membrane localization (27). EAAC1 expressed in
Yip6b/GTRAP3-18 Regulates ER Exit

mixed cortical cultures (Fig. 1A, lane 1) was separated into intracellular (lane 2) and plasma membrane (lane 3) pools using membrane-impermeant biotinylation (n = 3). EAAC1 predominantly resides in intracellular compartments (lane 2) (28). The cell surface population of EAAC1 (lane 3) is enriched for complex oligosaccharide EAAC1 (70 kDa) (27). EAAC1 protein forms a homotrimeric complex that is not completely disrupted by SDS-PAGE analysis (29). The visualization of these dimeric and trimeric protein bands in Western analysis are typical of all molecular subtypes of plasma membrane glutamate transporters (14).

We verified the assigned glycosylation state of EAAC1 protein using endoglycosidase treatment. The cortical culture cell lysates were exposed to the following treatments: no endoglycosidase (Fig. 1B, lane 1), Endo H (lane 2), or PNGase F (lane 3) (n = 3). Endo H selectively digests high mannose N-linked oligosaccharides. High mannose oligosaccharides are processed by enzymes resident in the Golgi; therefore, the presence of a high mannose oligosaccharide indicates that the protein has not left the ER membrane following translation. The endoglycosidase PNGase F, cleaves all N-linked oligosaccharides. The processed state of the oligosaccharide is noted by arrows; complex state (70 kDa) EAAC1 is Endo H-insensitive (black arrow), but the high mannose EAAC1 (60 kDa) is reduced by Endo H (lane 2, gray arrow). All oligosaccharides are cleaved by PNGase F, resulting in immunoreactive band collapse to 50 kDa (lane 3). We utilize endoglycosidase digest analysis in subsequent figures.

The expression of GTRAP3-18 is inducible and is maintained at very low levels in native tissue (1). In Fig. 1C, lysates of HEK 293T cells and adult mouse brain (20 μg per lane) are compared with that of HEK-293T cells transfected with GTRAP3-18 cDNA. The expression level of transfected GTRAP3-18 is comparable to that found in brain tissue lysate (Fig. 1C, lanes 2–3). The endogenous level of GTRAP3-18 is difficult to detect in mammalian cell lines (Fig. 1C, lane 1). We are unable to detect endogenous expression of GTRAP3-18 in multiple cell lines by Western blot analysis using 60 μg of cell lysate (data not shown). Mouse GTRAP3-18 is 97.9% identical to rat GTRAP3-18. The peptide sequence used to generate the antibody correspond to a fully conserved region. However at the low molecular weight this subtle difference creates a band shift on Western analysis (see supplemental Fig. S1). GTRAP3-18 protein has four transmembrane domains and cytosolic termini. The symmetrical GTRAP3-18 truncations, N-2TMD (transmembrane domains) (amino acids 1–93), and C-2TMD (amino acids 94–188), each have one cytosolic tail and two α-helical transmembrane domains. Following co-transfection (Fig. 2A), these truncated proteins are unable to alter the extent of plasma membrane expression of EAAC1 (lanes 6 and 8) or the ratio of high mannose to complex EAAC1 oligosaccharide (see arrows) (n = 5). They are also unable to reduce the Na^+ dependent glutamate uptake activity of EAAC1 (data not shown). However, these truncated GTRAP3-18 proteins retain ER localization (data not shown). The trafficking activity of the N-2TMD and C-2TMD is reconstituted by co-transfection of both fragments (lanes 9–10). This reconstitution of GTRAP3-18 activity in the ER suggests that the α-helical transmembrane domains of GTRAP3-18 self-oli-
Yip6b/GTRAP3-18 Regulates ER Exit

MARCH 7, 2008• VOLUME 283 • NUMBER 10
JOURNAL OF BIOLOGICAL CHEMISTRY

6179

The α-helical domains of GTRAP3-18 self-associate and mediate activity. A, GTRAP3-18 protein has four ER transmembrane domains with cytosolic termini (see diagram in supplemental Fig. S1). A truncation of GTRAP3-18 at its center point produces the proteins, N-2TMD (amino acids 1–93) and C-2TMD (amino acids 94–188). EAAC1 (lanes 1–2) and the co-transfection of full-length GTRAP3-18 with EAAC1 (lanes 3 and 4) demonstrates the GTRAP3-18 induced reduction in complex oligosaccharide (see arrows) and cell surface expression (lane 4). The co-transfection of either N-2TMD (lanes 4–6), or C-2TMD (lanes 7–8) GTRAP3-18 with EAAC1 does not alter oligosaccharide maturation or cell surface expression of the transporter. Co-transfection of both N-2TMD and C-2TMD reconstitutes GTRAP3-18 protein activity in the ER membrane as shown by a loss of complex oligosaccharide EAAC1 (see arrows) and cell surface expression (lane 10). B, GTRAP3-18 α-helical domain oligomerization in the ER membrane is measured by FRET analysis. C, summary of six experimental days with 6–10 values each is represented (shown as means ± S.E.). DRAP-FRET efficiency (E) was calculated as described under “Experimental Procedures.” When we co-transfected the CFP- and YFP-tagged forms of EAAC1, the value was indicative of homo-oligomerization between these subunits, in agreement with the observations by Gouaux’s group (29). Similar results have been obtained upon application of the three filter method (data not shown).

Are α-helical domains of GTRAP3-18 solely mediating activity? What is the relative contribution of the cytosolic N- and C-terminal sequence of GTRAP3-18? Deletion of either or both of the GTRAP3-18 cytosolic tails created the following truncated proteins: N- and TMD (amino acids 1–142), C- and TMD (amino acids 36–188), and TMD (amino acids 36–142) GTRAP3-18 (supplemental Fig. S1). The co-transfection of EAAC1 with N and TMD GTRAP3-18 (lanes 3 and 4) is unable to reduce the cell surface expression of EAAC1 (lane 4). However, the co-transfection of C and TMD (lanes 5 and 6) or TMD GTRAP3-18 (lanes 7 and 8) is able to reduce the level of complex oligosaccharide EAAC1 (see arrows) and the extent of cell surface expression. The membrane expression of EAAC1 is shown for comparison (lane 2).
These observations are in agreement with the biochemical protein involved in regulating ER to Golgi trafficking (32). alteration is often observed following the expression of a general ER exit block or induction of ER response. This noted that overexpression of GTRAP3-18 resulted in a significant change in cell morphology, probably resulting from a general ER exit block or induction of ER response. This alteration is often observed following the expression of a protein involved in regulating ER to Golgi trafficking (32). These observations are in agreement with the biochemical data (Figs. 1 and 2). Additional analysis found GTRAP3-18 is not localized to proteosmes or lysosomes and GTRAP3-18 expression reduced the trafficking and distribution of EAAC1 to these two organelles (data not shown). The morphology of the lysosome and proteasome organelles was not altered following GTRAP3-18 co-transfection (data not shown). These data suggest that GTRAP3-18 expression may control the ER exit of EAAC1.

GTRAP3-18 Expression Delays the High Mannose to Complex Oligosaccharide Transition of EAAC1—GTRAP3-18 co-transfection with EAAC1 inhibits ER to Golgi trafficking; do these trafficking changes alter EAAC1 protein stability? Metabolic labeling studies of EAAC1 have concluded that within 45 min of translation in mammalian cell culture, the N-linked oligosaccharide is processed to the complex state (27). We investigated the effects of GTRAP3-18 co-transfection on the oligosaccharide maturation of EAAC1 (Fig. 4, A and B (n = 4)). EAAC1 is in both high mannos and complex oligosaccharide states 30 min following 35S incorporation (Fig. 4A, see arrows and boxed lanes). The ratio of complex oligosaccharide EAAC1 increases with time and predominates within the hours following the metabolic labeling pulse (Fig. 4A). Co-transfection with
GTRAP3-18 delays the rapid trafficking of EAAC1 from the ER to the Golgi (Fig. 4B, see arrows and boxed lanes) thereby preventing the increase in the ratio of complex oligosaccharide EAAC1 on the same time scale. However, the EAAC1 protein delayed by GTRAP3-18 is able to reach the Golgi on a longer time scale of 8–12 h (data not shown). These data suggest GTRAP3-18 expression is able to alter intracellular trafficking events and thereby delay oligosaccharide processing.

GTRAP3-18 Restricts the ER Exit of Other Members of the Excitatory Amino Acid Transporter Family—We previously reported that the inhibitory effect of GTRAP3-18 on the neuronal glutamate transporter EAAC1 (1). Here, we tested GTRAP3-18 activity with other members of the excitatory amino acid transporter family. As seen in Fig. 5, a significant reduction in Na⁺/H⁺-dependent glutamate uptake activity following GTRAP3-18 co-transfection was found for, EAAT4 (5A), EAAT2 (5B), and EAAT1 (5C) (n = 3). Kinetic analysis of the effect of GTRAP3-18 is measured predominately as a change in Vmax (Table 1 analysis of EAAC1 and GLT-1). The activity of GTRAP3-18 on the EAAT family was found to be through a similar mechanism as for EAAC1 (data not shown). The glutamate transporter EAAT4 is illustrative of immunocytochemical findings (Fig. 5D, n = 4). EAAT4 is preferentially expressed on the cell surface and in recycling endosomes (panel 2, green). A small percentage of EAAT4 co-localizes with GTRAP3-18 (blue, panel 4) leading to a redistribution of EAAT4 to the ER (panels 4–7). This result is representative of the EAAT family.

---

**FIGURE 4.** GTRAP3-18 delays oligosaccharide maturation of EAAC1 but does not contribute to protein degradation. GTRAP3-18 delays the high mannose to complex oligosaccharide maturation of EAAC1. A and B, metabolic labeling of EAAC1 by [35S]incorporation demonstrates both high mannose and complex oligosaccharide states form within 0.5–1 h (A, see arrows and boxed lanes). Co-transfection with GTRAP3-18 delays the rapid trafficking of EAAC1 to the Golgi thereby preventing the high mannose to complex oligosaccharide transition (B, see arrows and boxed lanes).

**FIGURE 5.** GTRAP3-18 reduces the Na⁺/H⁺-dependent glutamate uptake activity of all EAAT isoforms through the delayed acquisition of the complex oligosaccharide state. A–C, activity of the other isoforms in the EAAT family is significantly decreased by equal molar co-transfection with GTRAP3-18, with expression levels of GTRAP3-18 that are low relative to expression of the transporters (p < 0.01-0.001). The transporters have some variation in the percent reduction in activity caused by GTRAP3-18; which is related to how well an isoform is trafficked to the surface. The majority of transfected EAAT2 and EAAT4 is localized to the plasma membrane (SD) and the reduction caused by GTRAP3-18 is relatively greater than for the isoforms EAAT1 and EAAT3 whose cellular distribution is biased toward intracellular vesicles. D, majority of EAAT4 (green, panel 1) does not localize to the ER (red, panels 2 and 3). Co-expression with GTRAP3-18 (blue, panel 4) leads to a redistribution of EAAT4 to the ER (panels 4–7). This result is representative of the EAAT family.

---

**GTRAP3-18 restricts the ER Exit of Other Members of the Excitatory Amino Acid Transporter Family**—We previously reported that the inhibitory effect of GTRAP3-18 on the neuronal glutamate transporter EAAC1 (1). Here, we tested GTRAP3-18 activity with other members of the excitatory amino acid transporter family. As seen in Fig. 5, a significant reduction in Na⁺/H⁺-dependent glutamate uptake activity following GTRAP3-18 co-transfection was found for, EAAT4 (5A), EAAT2 (5B), and EAAT1 (5C) (n = 3). Kinetic analysis of the effect of GTRAP3-18 is measured predominately as a change in Vmax (Table 1 analysis of EAAC1 and GLT-1). The activity of GTRAP3-18 on the EAAT family was found to be through a similar mechanism as for EAAC1 (data not shown). The glutamate transporter EAAT4 is illustrative of immunocytochemical findings (Fig. 5D, n = 4). EAAT4 is preferentially expressed on the cell surface and in recycling endosomes (panel 2, green). A small percentage of EAAT4 co-localizes with GTRAP3-18 (blue, panel 4) leading to a redistribution of EAAT4 to the ER (panels 4–7). This result is representative of the EAAT family.
Yip6b/GTRAP3-18 Regulates ER Exit

TABLE 1
Saturation kinetics analysis of EAAC1 and Glt-1/rEAAT2 following GTRAP3-18 co-transfection in HEK 293 cells

| Transfected cDNA | Mean V_max (μM) | Mean K_m (mM) | p value |
|------------------|----------------|--------------|---------|
| EAAC1            | 11.41          | 152.4        | <0.05   |
| EAAC1 and GTRAP3-18 | 5.393         | 75.21        |         |
| Glt-1/rEAAT2     | 17.43          | 211.7        | <0.006  |
| Glt-1/rEAAT2 and GTRAP3-18 | 5.554         | 82.24        |         |

with the ER marker calnexin (panels 1 and 3, red); GTRAP3-18 co-transfection (panel 4, blue) drastically alters EAAT4 subcellular distribution to the ER (panel 6) and leads to extensive co-localization with calnexin (panel 7, overlay).

DISCUSSION

Our group identified GTRAP3-18, a protein that is dynamically induced by retinoic acid both in vitro and in vivo and inhibits the activity of EAAC1 in a dose dependent manner (1). The mechanism by which GTRAP3-18 regulated EAAC1 activity and its basic cellular properties were not initially clear. In this report, we demonstrate that GTRAP3-18 is an integral ER membrane protein, and when it is co-expressed in heterologous cells, it is able to delay the ER to Golgi trafficking of EAAC1 and other members of the excitatory amino acid transporter family. Considering the localization of GTRAP3-18 in the ER, at the early stage of the protein trafficking, it is possible that the ER exit of other cell surface transporters or receptors may be regulated by GTRAP3-18. Besides the EAAT family members, our preliminary data show that GTRAP3-18 has the ability to regulate the trafficking of selected additional neuron-transmitter transporters and receptors. For example, the Na+ /Cl− family of neurotransmitter transporters are not well conserved with the EAAT family at the sequence level, but do share aspects of channel and transport function and a conserved oligomerized quaternary structure. They are predicted to have 12 transmembrane domains and form homo-oligomers of undetermined stoichiometry (33). Examples include the dopamine transporter (DAT) and the GABA transporter (GAT-1). GTRAP3-18 is able to delay the ER exit of these two proteins. The family of GPCR (G-protein-coupled receptors) is not structurally or functionally similar to transporter proteins. They have seven transmembrane domains and are heavily glycosylated (34). We examined trafficking of the β2-AR (adrenergic receptor), α1β receptor, and the D2R (dopamine receptor) of this family with GTRAP3-18 co-expression. As observed for transporter family members, GTRAP3-18 increases the population of high mannose oligosaccharide state of these proteins and restricts their subcellular localization to the ER. The common feature of these cell surface transporters and G-protein-coupled receptors is that they form oligomeric complex before exit out of the ER. In contrast, GTRAP3-18 was unable to clearly alter the cell surface expression of the plasma membrane proteins that do not oligomerize in the ER include the EGF receptor (EGFR) and SNAP-25.4

GTRAP3-18 utilizes hydrophobic α-helical interactions and weak oligomerization forces to function in the ER (35). The oligomerization of the four transmembrane domains of GTRAP3-18 is necessary for its activity (Fig. 2A). GTRAP3-18 molecules also self-associate, most likely via the hydrophobic α-helical domains. Their intramolecular oligomerization is measurable by FRET analysis (Fig. 2, B and C). The homo-oligomerization of GTRAP3-18 molecules may enhance its activity and specificity to certain oligomeric protein complexes (30, 36). The hydrophobic surface interactions between GTRAP3-18 and the oligomeric protein complexes may prevent these cargo proteins exiting out of the ER.

GTRAP3-18 and its homolog, JM4, arose as part of the expansion of Yip (yeast Rab GTPases Ypt-interacting proteins) family in mammals. This protein family emerged as strong candidates for GDFs (GDI-displacement factors), because at least one Yip protein Yip3/PRA1 can quantitatively dissociated prenylated-Rab9-GDI complexes and recruit Rab9 onto specific membrane locations. In mammals, a protein that is related to Yip3/PRA1 has been named Yip6/PRA2. Using this nomenclature, GTRAP3-18 would be classified as Yip6b and JM4 as Yip6a (12).

The expression of GTRAP3-18 is low but has been found to be induced by stressful physiologic stimuli such as calcium depletion, oncogenic transformation, nitric-oxide synthase induction and drug withdrawal (6–8, 32, 38). What is the physiological consequence of this injury induced overexpression of GTRAP3-18 and how does it alter ER exit of EAAC1 (and other proteins)? One possible mechanism underlying the inhibitory effect of overexpressed GTRAP3-18 on the ER exit of amino acid transporters could be that increased amount of GTRAP3-18 on the ER membrane locks Rab on the membrane and in turn disrupts its distribution between cytosolic pools (GDI-bound) and membrane pools. Eventually, this blocks protein trafficking out of the ER and results in the delayed ER exit of multiple proteins as we observed. Another possibility could be that increased level of GTRAP3-18 triggers ER stress responses, such as UPR (unfolded protein response), which limits ER to Golgi trafficking (39, 40). However, the inhibitory effect of overexpressed GTRAP3-18 is not universal and appears to be restricted to certain proteins that form oligomeric complexes in the ER, suggesting a specific cargo selection and packaging mechanism for the oligomeric protein complexes.

Acknowledgments—We thank Mandy Jackson, Margaret Dykes-Hoberg, Mitsunori Watanabe, Julia Fuchs, and Qiao Han for helpful guidance and suggestions. Randy Blakely, Haley Melikian, Gonzalo Torres, Marc Caron, and Avto Kalandadze provided reagents and critical review.

REFERENCES

1. Lin, C. I., Orlov, I., Ruggiero, A. M., Dykes-Hoberg, M., Lee, A., Jackson, M., and Rothstein, J. D. (2001) Nature 410, 84–88

4 J. D. Rothstein, unpublished observations.
2. Diamond, J. S. (2001) *J. Neurosci.* 21, 8328–8338
3. Danbolt, N. C. 65, 1–105, 2001
4. Sepkuty, J. P., Cohen, A. S., Eccles, C., Rafiq, A., Behar, K., Ganel, R., Coulter, D. A., and Rothstein, J. D. (2002) *J. Neurosci.* 22, 6372–6379
5. Mathews, G. C., and Diamond, J. S. (2003) *J. Neurosci.* 23, 2040–2048
6. Ikemoto, M. J., Inoue, K., Akiduki, S., Osugi, T., Imamura, T., Ishida, N., and Ohtomi, M. (2002) *Neuroreport.* 13, 2079–2084
7. Mao, W. G., Li, A. P., Ye, J., Huang, S., Li, A. Q., and Zhou, J. W. (2004) *Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi* 22, 60–63
8. Wang, N. P., Zhou J. W., Li, A. P., Cao, H. X., and Wang, X. R. (2003) *Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi* 21, 2040–2048
9. Abdul-Ghani, M., Gougeon, P.-Y., Prosser, D. C., Da-Silva, L. F., and Ng-see, J. K. (2001) *J. Biol. Chem.* 276, 6225–6233
10. Pfeffer, S., and Aivazian, D. (2004) *Nat. Rev. Mol. Cell Biol.* 5, 886–896
11. Calero, M., and Collins, R. N. (2002) *Biochem. Biophys. Res. Commun.* 290, 676–681
12. Pfeffer, S. R. (2005) *J. Biol. Chem.* 280, 15485–15488
13. Collins, R. N. (2003) *Mol. Membr. Biol.* 20, 105–115
14. Rothstein, J. D., Martin, L., Levey, A. J., Dykes-Hoberg, M., Jin, L., Wu, D., Nash, N., and Kudlacek, O. (1996) *Nature* 383, 266–276
15. Arriza, J. L., Fairman, W. A., Wadiche, J. I., Hirschberg, K., and Lippincott-Schwartz, J. (1991) *J. Cell Biol.* 165, 557–570
16. Teasdale, R. D., and Jackson, M. R. (2000) *Annu. Rev. Cell Dev. Biol.* 16, 27–54
17. Pfeffer, S. (2005) *Biochem. Soc. Trans.* 33, 627–630
18. Lee, S. P., O’Dowd, B. F., and George, S. R. (2003) *Life Sci.* 74, 173–180
19. Rutkowski, D. T., and Kaufman, R. J. (2004) *Trends Cell Biol.* 14, 20–28

**Proc. Natl. Acad. Sci. U. S. A. 98,** 15324–15329
21. Cheng, C., Glover, G., Banker, G., and Amara, S. G. (2002) *J. Neurosci.* 22, 10643–10652
23. Davis, K. E., Straff, D. J., Weinstein, E. A., Bannerman, P. G., Correale, D. A., Rothstein, J. D., and Robinson, M. B. (1998) *J. Neurosci.* 18, 2475–2485
25. Melikian, H. E., Ramamoorthy, S., Tate, C. G., and Blakely, R. D. (1996) *Mol. Pharmacol.* 50, 266–276
27. Yang, W., and Kilberg, M. S. (2002) *J. Biol. Chem.* 277, 31
29. Yernool, D., Boudker, O., Jin, Y., and Gouaux, E. (2004) *Nature* 431, 811–818
31. Trombetta, E. S. (2003) *Glycobiology* 13, 77R–91R
33. Sitte, H. H., Farhan, H., and Javitch, J. A. (2004) *Mol. Interventions* 4, 38–47
35. Teasdale, R. D., and Jackson, M. R. (1996) *Annu. Rev. Cell Dev. Biol.* 12, 27–54
37. Rutkowski, D. T., and Kaufman, R. J. (2004) *Trends Cell Biol.* 14, 20–28
