Brain Glutamate Transporter Proteins Form Homomultimers*

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Removal of excitatory amino acids from the extracellular fluid is essential for synaptic transmission and for avoiding excitotoxicity. The removal is accomplished by glutamate transporters located in the plasma membranes of both glial cells and neurons (3). Several glutamate transporters have been cloned: GLAST1 (4), GLT-1 (5, 6), EAAC1 (7), and EAAT4 (8). The transporters are regulated (9–11) and highly differentially localized (12–15). Recent studies indicate that they modify glutamate receptor activation (16–20). Thus, the functions of these carriers may be more refined than simple removal of excitotoxic amino acids.

It is legitimate to ask whether the glutamate transporters might form oligomeric complexes. Several glutamate transporter subtypes exist (see above). These proteins have been reported to aggregate (21–23). GLAST and GLT have been observed in the same cells (15). Recent reports conclude that some other co-transporters may exist in vivo as oligomers (24–28). The glutamate transporters behave like a combination of carriers and chloride channels (8, 29). Several neurotransmitter receptors, including ionotropic glutamate receptors, operate as hetero-oligomeric complexes (30, 31).

Here we show by double labeling post-embedding electron microscopic immunocytochemistry that the two glial glutamate transporters GLT and GLAST are expressed in the same cell membranes. Furthermore, we demonstrate that GLT and GLAST, as well as the neuronal glutamate transporter EAAC, form oligomeric complexes but that GLT and GLAST do not complex with each other. Evidence suggests that oligomeric structure is required for transport activity.

EXPERIMENTAL PROCEDURES

Materials—Sodium dodecyl sulfate (SDS) of high purity (>99% C12 alkyl sulfate) and bis(sulfosuccinimidyl) suberate were from Pierce. Nitrocellulose sheets (0.22-μm pores, 100% nitrocellulose) and electrophoresis equipment were from Hoefer Scientific Instruments (San Francisco, CA). N,N,N′-Methylenediacylamide, acrylamide, ammonium persulfate, TEMED, and alkaline phosphatase substrates (nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate) were from Promega (Madison, WI). Alkaline phosphatase conjugated to mouse monoclonal anti-rabbit IgG (A-2556, clone RG-96) was obtained from Sigma (St. Louis, Missouri). [3H]Glutamic acid (50 Ci/mmol), molecular mass markers for SDS-polyacrylamide gel electrophoresis (PAGE), and cholate were from BDH (Gillingham, U.K.). All other reagents were either obtained from Sigma or from Fluka (Buchs, Switzerland).

The majority of excitatory signals in the mammalian central nervous system may be transmitted by glutamate (1). The extracellular glutamate concentration has to be kept low, both to secure a high signal-to-noise (background) ratio and because excessive glutamate receptor activation can lead to neuronal damage (2). This is achieved by the action of sodium-dependent glutamate transporters located in the plasma membranes of both glial cells and neurons (3). Several glutamate transporters have been cloned: GLAST1 (4), GLT-1 (5, 6), EAAC1 (7), and EAAT4 (8). The transporters are regulated (9–11) and highly differentially localized (12–15). Recent studies indicate that they modify glutamate receptor activation (16–20). Thus, the functions of these carriers may be more refined than simple removal of excitotoxic amino acids.

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¶ The abbreviations used are: GLAST, rat glutamate aspartate transporter (4); CHAPS, 3-(N,N,N,N-tetraethylammonium)propanesulfonate; DTNB, 5,5′-dithio-bis-2-nitrobenzoic acid; DTT, dithiothreitol; EAAC, an EAAC1-type transporter; EAAC1, rabbit excitatory amino acid carrier (7); GLT, a GLT-1-type transporter; GLT-1, rat glutamate transporter (5, 6); GLYT1, rat glycine transporter (33); NaP, sodium phosphate buffer with pH 7.4; FMPSF, phenylmethanesulfon fluoride; rEAAC1, rat excitatory amino acid carrier (36); PAGE, polyacrylamide gel electrophoresis; TEMED, N,N,N′,N′-tetramethylethylendiamine.
Production of Antibodies—Anti-peptide antibodies against three glutamate transporters (GLAST, GLT-1, and EAAC1) were prepared as described using synthetic peptides as antigens. The peptides representing parts of GLAST (rat EAAT1), GLT-1 (rat EAAT2), EAAC1 (rabbit EAAT3), and GLYT1 are referred to by capital letters A, B, C, and G, respectively, followed by numbers indicating the corresponding amino acid residues in the sequences (given in parentheses): A522–541 (PYQGLAQNDFEPRVQFDSET), B12–28 (KQVEVRMHD5S5L5SE5), B630–646 (GLATGVASLAKGQ3T5S-QF), and G623–638 (IVGNSGSSHLQD5RI) (4, 5, 7, 33). The corresponding anti-peptide antibodies are referred to as anti-A522 (rabbit 68488), anti-B12 (rabbit 68518), anti-B493 (rabbit 84946), anti-C510 (rabbit 96738), or anti-G623 (rabbit 80748). The anti-73-kDa antibodies (rabbit 85302) (21), which were raised and affinity purified against a purified glutamate transporter (34), were from the same purified batch as that previously described (14, 21, 22). The anti-A522 antibodies from rabbit 20604 were produced by immunizing with whole GLAST protein isolated from rat cerebellum and affinity purification of antibodies from the crude serum using the A522 peptide. The two anti-A522 antibodies from rabbit 68488 and 20604 gave identical immunoblot labeling patterns. Unless stated otherwise, anti-A522 refers to antibodies from rabbit 20604.

Electron Microscopic Immunocytochemistry—Tissue was perfusion-fixed with 2.5% glutaraldehyde, 1% formaldehyde, freeze-substituted with 0.5% uranyl acetate in methanol, embedded in Lowicryl HM20 at low temperature, and further processed as described before using the same antibodies and concentrations (14). Immunoblot experiments showed that the selectivity between the primary antibodies and GAR30 (30-nm gold particles) as reporter for the first primary antibody and GAR15 (15 nm) for the second. Omitting one of the primary antibodies leads to absence of label by the corresponding reporter particle type.

Expression of Glutamate Transporters in HeLa Cells—HeLa cells were maintained in monolayer culture and transfected with cDNAs encoding GLT (5) or rEAAC1 (36) using the vaccinia virus T7 expression system (36, 37–39). The virus was a generous gift from B. Moss (National Institutes of Health). The transfected HeLa cells were detached from the flasks and collected by centrifugation (1000 rpm, 5 min).

Tissue Preparation for Biochemistry—Wistar rats (180–250 g) of either sex (Mallegaard Hansen, Denmark) were killed by stunning and decapitation. The forebrain and cerebellum were dissected, homogenized immediately in 10–20 volumes of ice-cold hypotone solution (5 mM EDTA, 1 mM PMPSF, 1 mM PMPSF) using a Dounce glass-glass homogenizer. DTT (5 mM) was added when not stated otherwise. The homogenate was centrifuged (18,000 rpm, 30,000 × g, 4°C, 15 min) to sediment cell membranes (erythrocyte membranes were discarded). The supernatant was used in the same way. Below, the sedimented membranes are referred to as “the membrane pellet.” When stated, membranes were prepared as described (34) by homogenizing in 0.32 M mannitol, isolation of the crude synaptosomal fraction, and sedimentation of the membranes after osmotic shock.

Cross-linking of Membrane Proteins—For cross-linking of proteins in intact membranes, the membrane pellets (see under “Tissue Preparation” above) were resuspended in buffer (150 mM NaCl, 100 mM Na-HEPES, pH 7.5, 5 mM EDTA, 1 mM PMPSF, 5 mM DTT) to a final protein concentration of about 0.5 mg/ml and divided in aliquots. To the membrane suspensions, proteins, or liposomes a cross-linker was added (bis(sulfosuccinimidyl) suberate when not stated otherwise) to final concentrations of 0.3, 1, 3, 10, or 30 mM from a freshly prepared 100 mM stock solution in 20 mM HCl. After incubation (12 min, room temperature, end-over-end mixing), the reaction was terminated by adding 2× Tis-HCl, pH 9, to a final concentration of 200 mM. Then the membranes were solubilized in SDS-sample buffer (70 mM SDS, 62.5 mM Tris-HCl, pH 6.8, 0.3 M sucrose, 10 µg/ml bromphenol blue), gel-filtered (11) on Sephadex G-50 fine spin columns equilibrated with the above SDS-sample buffer (containing 5 mM DTT), and run on SDS-PAGE (see below).

Solubilization of Membranes for Cross-linking of Solubilized Proteins—The above membrane pellets were resuspended in equal volumes of buffer (150 mM NaCl, 100 mM Na-HEPES) and divided into aliquots. One volume of resuspended membranes was mixed with 0.35 volumes of the above saturated ammonium sulfate and 2 volumes of solubilization buffer consisting of buffer (5 mM EDTA, 1 mM PMPSF, 5 mM DTT, and 100 mM Na-HEPES, pH 7.5) with a detergent (33 mM CHAPS, 3.3 mM ml Triton X-100, or 50 mM cholate). After incubation (10 min, 0°C) and centrifugation (39,000 × g, 18,000 rpm, 4°C, 20 min), the supernatant was diluted 1 + 4 with buffer and detergent to give final detergent concentrations of 20 mM CHAPS, 1 µM Triton X-100, or 30 mM cholate. The salt-free supernatant (30%) and one volume (20%) was replaced with 5 mM NaCl for cross-linking experiments (see below) or omitted when the membranes were solubilized with 1% SDS.

Immunoabsorption of Glutamate Transporters—Aliquots of 100 µl of protein A-Sepharose Fast Flow was mixed with either anti-B12, anti-B493, anti-A522 or preimmune IgG, incubated (1 h, room temperature) with 20% newborn calf serum, and equilibrated with buffer (500 mM NaCl, 5 mM EDTA, 1 mM PMPSF, 10 mM NaF, pH 7.4) to a final protein A-Sepharose tube and incubated (4°C, end-over-end, 30 or 60 min). Then the supernatants were collected for reconstitution of transport activity (see below) and for SDS-PAGE. The precipitated protein was released from the Sepharose beads by boiling in SDS-sample buffer with 5% mercaptoethanol and subjected to SDS-PAGE. The proteins were immunoblotted with biotinylated (not shown) or regular non-biotinylated (Fig. 4) anti-GLT and anti-GLAST antibodies. The rabbit IgG that had been boiled in SDS-sample buffer containing mercaptoethanol and blotted, was poorly detected by the secondary antibody used.

Partial Purification of Glutamate Transporters—Glutamate transporters were partially purified from rat forebrain by lectin affinity chromatography (34) and eluted from the columns in buffer with a detergent (20 mM CHAPS, 50 mM β-ocetylglucoside, or 15 mM Zwittergent-314, 10 mM EDTA). Aliquots were incubated (15 min, 0°C) in the elution buffer, the proteins collected on DEAE-cellulose columns, and the detergent removed by washing with 20 mM CHAPS in 20 mM NaF. Then the proteins were released with high salt (500 mM NaCl, 20 mM CHAPS, and 50 mM NaF), reconstituted, and assayed for transport activity (see below).

Radiation Inactivation Analysis—Samples of rat cerebral cortex (0.2–0.35 g) from male Wistar rats (180 g) were kept at −20°C and exposed to high-energy electrons using the 10 MeV linear accelerator at Riso, Denmark. The dose of radiation was determined using calibrated thermosimeters (water). The samples were frozen (−10°C) during radiation which was delivered in runs of 0.5–2 Mrad. Between runs the samples were cooled to −15°C for at least 2 min to ensure that they remained completely frozen during the entire irradiation process. The total doses were from 0 to 20 Mrad. After radiation, the tissue was kept at −80°C until it was used. This irradiation procedure and the equipment have been calibrated by radiation inactivation of seven enzymes of known Michaelis constants (40 kDA) which give an agent constant of 730,000 Da × Mrad (40–42). Experience from the last 10 years shows that the interexperimental variation in the radiation effect is negligible and that extensive calibration is not required in every experiment. However, to verify the validity of the calibration, the binding of [3H]flunitrazepam and [3H]RO15-1788 to benzodiazepine receptors and the activity of pyruvate kinase were determined. The irradiated samples were homogenized in buffer (50 mM Tris acetate, pH 7.4, 5 mM EDTA, and 1 mM PMPSF) and centrifuged (39,000 × g, 18,000 rpm, 20 min, 4°C). The supernatants were assayed for pyruvate kinase activity using the Boehringer Mannheim test kit 126047. The pellets were either used for determination of benzodiazepine binding as described (41) or mixed with 12 volumes of solubilization buffer (32 mM cholate, 500 mM NaCl, 5 mM EDTA, 100 mM NaF, pH 7.4), incubated (10 min) on ice, and centrifuged as above. The supernatants were immediately frozen (−80°C) in portions of 300 µl and thawed immediately prior to reconstitution (see below).

Reconstitution of Glutamate Transporters in Liposomes—This was done as described (11, 34). Briefly, 100-µl sample with glutamate transporters and either 20 mM CHAPS, 30 mM cholate, or 1 µM Triton X-100 was mixed with 150 µl of a phospholipid/cholate/salt mixture, incubated on ice, and gel-filtered to remove detergent on spin columns equilibrated with the desired internal medium.

Determination of Glutamate Transport Activity—This was done exactly as described (34). Briefly, the uptake reaction was started by diluting 20 µl membrane liposomes in saline with trittiated amino acid and valinomycin. The reaction was terminated by dilution and filtration. The kinetic data were analyzed with the direct linear plot method (43).

Electrophoresis and Blotting—SDS-PAGE (15, 44) was done with separating gels consisting of 5% or 10% acrylamide. The electrophoresis trays had heat exchangers with cooling water kept at 0–5°C. After
RESULTS

Electron Microscopy—Here we studied the distribution of the two glutamate transporters GLT and GLAST by double label- ing post-embedding electron microscopic immunocytochem- istry. The same astroglial membranes were immunoreactive for both proteins, in hippocampus, as well as in cerebellum.

The particles, whether signaling GLT or GLAST, were not clustered together but were scattered randomly along the membranes. Most particles were farther apart than compatible with binding to the same macromolecular complex.

The resolution of the immunogold method (14) is such (Fig. 1) that although the antibodies are directed to epitopes on the inner side of the plasma membrane (15), some of the gold particles will be centered on the outside and may even overlay neighboring cells. The astroglial membranes were in contact with various other types of membrane, but since none of these showed appreciable labeling in the absence of astrocytic apposition, the labeling could be attributed to the astrocytes. Neuronal membranes, when not apposed to astrocytic processes, showed particle densities at background levels (<10% of those of astrocyte membranes).

Reducing Agents and Electrophoretic Mobility—When fresh rat brain tissue is solubilized with SDS and the proteins immediately separated on SDS-PAGE and immunoblotted, only bands corresponding to GLT and GLAST transporter monomers are seen even when reducing agents are omitted (Fig. 2, lanes 1, A and B, lane 1). Thus, in the fresh brain, the glutamate transporter proteins are not covalently bound to each other.

When intact membranes or detergent extracts are allowed to oxidize (Fig. 2), distinct higher molecular mass bands corresponding to oligomers appear on the immunoblots (Fig. 2A, lanes 2–6). The appearance of these bands is promoted by DTNB, a sulfhydryl oxidizer (compare lanes 5 and 6 in Fig. 2A), and prevented by sulfhydryl reducing agents, DTT (compare lanes 2 and 3 in Fig. 2A and B), and 2-mercaptoethanol (not shown). Once the monomers have been covalently connected to each other during the oxidation process, harsh treatment is needed to disrupt the oligomers. Even incubation with 2 mM DTNB for 2 h at room temperature had little effect on the SDS extracts (not shown). However, some diffuse increase in the immunoreactivity above the mon-
omer bands was noted (not shown) indicating nonspecific associations with other proteins of various molecular masses.

In the case of GLT (Fig. 2A), oxidation led to the formation of dimers, trimers, and higher molecular mass aggregates. GLAST behaved differently; in the same samples, trimer and higher molecular mass bands of GLAST were very weak compared to the monomer and dimer bands. Furthermore, oxidation of intact membranes, even with 2 mM DTNB for 2 h, did not give GLAST oligomer bands to any significant degree (Fig. 2B, lane 6).

Cross-linking of Membrane Proteins and Oligomer Size—It is clear from the above description that the glutamate transporter monomers have a tendency to associate. The transporters might therefore form oligomeric complexes in vivo. If present, these complexes would, however, dissociate during SDS-PAGE since they are not connected covalently. In order to prevent this dissociation, brain membranes were incubated with increasing concentrations of chemical cross-linkers (binary functional reagents) prior to solubilization, SDS-PAGE, and immunoblotting (see “Experimental Procedures”). Care was taken to avoid the oxidative cross-linking described above by using fresh brain tissue and by including DTNB in all buffers. Immunoblots (Fig. 3) of SDS-dissolved membrane proteins cross-linked with bis(sulfosuccinimidyl) suberate showed a striking pattern. In the absence of cross-linker, all the four transporter proteins studied (GLT, lane 1; GLAST, lane 5; EAAC, lane 9; and GLYT1, lane 12) ran exclusively as monomers on SDS-PAGE. Addition of increasing concentrations of cross-linker resulted in a shifting of the immunoreactivity of the glutamate transporters (GLT, lanes 2–4; GLAST, lanes 6–8; EAAC, lanes 10–11), but not that of the glycine transporter GLYT1 (lanes 13 and 14), toward bands corresponding to dimers and trimers. At the highest cross-linker concentrations, GLT was present predominantly as trimers (most clearly seen on blots with low amounts of GLT protein, e.g., lane B4), while GLAST was still present as an almost equal mixture of dimers and trimers (lane 8). Weak bands corresponding to higher molecular mass aggregates were sometimes visible (lanes 4 and 8) but were much weaker than those observed after oxidation (see above). At very high cross-linker concentrations, 10 and 30 mM, insoluble aggregates were observed after SDS solubilization of the cross-linked membrane proteins. In agreement, the overall immunoreactivity on the blots was reduced, and there was a diffuse staining with increasing intensity toward the top of the blots. In spite of this, the dimer bands did not disappear.

The immunoreactivity of EAAC was very weak as compared with GLT and GLAST in the brain but not when expressed in HeLa cells (Fig. 6) or after partial purification (Fig. 7) suggesting that the concentration of EAAC in the brain is low compared to those of GLT and GLAST. High cross-linker concentrations were required to demonstrate dimers and trimers in intact brain membranes (Fig. 3, lanes 10 and 11) but not in transfected HeLa cells (Fig. 6) or in partially purified extracts (Fig. 7).

Similar results were obtained for GLT and GLAST using suberic acid bis(N-hydroxysuccinimide ester), ethylene glycol-bis-(sulfosuccinimidylsuccinate), dimethyl pimelimidate, and 1-ethyl-3-(dimethylaminopropyl)-carbodiimide. With the latter reagent, which modifies carboxyl groups, the immunoreactivity was reduced in a concentration-dependent manner as expected because the antibody epitopes contain 3–5 carboxyl groups.

Immunoprecipitation of Glutamate Transport Activity—After having established that the proteins form separate oligomeric complexes, we asked if oligomeric structure is required for transport activity. Before attempting to answer this question, we wanted to find out how much GLAST, GLT, and EAAC contribute to the total reconstitutable glutamate transport activity in order to relate the immunoreactivity to the transport activity. This was done by using the glutamate transporter antibodies to immunoprecipitate transporter proteins from

**Fig. 3. Immunoblots of proteins cross-linked in intact membranes.** Membranes from a fresh rat forebrain (A) or rat cerebellum (B) were incubated with 5 mM DTT and increasing concentrations of cross-linker, bis(sulfosuccinimidyl) suberate, solubilized in SDS, electrophoresed, and immunoblotted with 200 ng/ml anti-B12 antibodies to GLT (lanes 1–4), 200 ng/ml anti-A522 antibodies to GLAST (lanes 5–8), 1000 ng/ml anti-C510 antibodies to EAAC (lanes 9–11), or 1000 ng/ml anti-G623 antibodies to GLYT1 (lanes 12–14). The bands representing GLAST monomers from the forebrain are narrow because they migrated close to the dye front. The cross-linker concentrations used were 0 (lanes 1, 5, 9, and 12), 0.3 (lanes 2 and 6), 1 (lanes 3, 7, and 13), 3 (lanes 4, 8, and 14), 10 (lane 10) or 30 mM (lane 11). The amounts of protein applied in each lane were 6 μg in lanes A1–8, 2 μg in lanes B1–8, 30 μg in lanes 9–11 and 25 μg in lanes B12–14.
The anti-GLT antibodies precipitated negligible amounts of GLT immunoreactivity (Fig. 4) from the supernatants (lanes 1–3) and pellets (lanes 4–6) of rat forebrain and then cross-linked and Immunoblotted. Again, bands corresponding to GLT, GLAST, and EAAC dimers and trimers were clearly present after cross-linking of the extracts obtained with CHAPS, cholate, Triton X-100. Membranes from rat forebrain were solubilized with CHAPS and reconstituted in liposomes. The liposomes were either incubated with bis(sulfosuccinimidyl) suberate (0, 1, and 3 mM) and 5 mM DTT, immediately electrophoresed, and blotted onto nitrocellulose (lanes 1–6) or incubated (48 h, on ice) without cross-linker or DTT (lanes 7 and 8). The blots were probed with 200 ng/ml anti-B12 antibodies (lanes 1–3 and 7) or 200 ng/ml anti-A522 (lanes 4–6 and 8). 3 μg of protein was applied in each lane. Some of the molecular mass markers (*) were affected by reducing agents.

Cross-linking and Transport Activity—From the above immunoprecipitation experiments, it was clear that GLT alone represents most of the transport activity detected with the reconstitution assay. Now we exposed several different glutamate transporter preparations to cross-linkers to see if the presence of GLT oligomers (as detected on immunoblots) was associated with glutamate transport activity.

GLT has previously been solubilized, purified, and reconstituted in active form (34). As is shown in Fig. 5, both GLT and GLAST multimers are present in the liposomes after reconstitution of CHAPS-solubilized transporters. In the liposomes trimers occurred regardless of whether the cross-linking was induced by bifunctional reagents (Fig. 5, lanes 3 and 6) or proceeded spontaneously by oxidation (lanes 7 and 8). Identical results were obtained after cross-linking of proteins after reconstitution from cholate or Triton X-100 extracts.

The glutamate transporters GLT-1 and rEAAC1 were expressed in HeLa cells using a recombinant vaccinia virus encoding the T7 RNA polymerase and transfected with cDNA encoding GLT-1 (5) or rEAAC1 (36). The cell membranes were incubated with DTT and 0 (lanes 1 and 4), 1 (lanes 2 and 5), or 3 mM (lanes 3 and 6) bis(sulfosuccinimidyl) suberate, electrophoresed, and immunoblotted with 200 ng/ml anti-B12 antibodies (lanes 1–3) or 1 μg/ml anti-C510 (lanes 4–6).

Cross-linking of membranes from HeLa cells transfected with cDNAs encoding GLT-1 (lanes 1–3) and rEAAC1 (lanes 4–6). HeLa cells were infected with a recombinant vaccinia virus encoding the T7 RNA polymerase and transfected with cDNA encoding GLT-1 (5) or rEAAC1 (36). The cell membranes were incubated with DTT and 0 (lanes 1 and 4), 1 (lanes 2 and 5), or 3 mM (lanes 3 and 6) bis(sulfosuccinimidyl) suberate, electrophoresed, and immunoblotted with 200 ng/ml anti-B12 antibodies (lanes 1–3) or 1 μg/ml anti-C510 (lanes 4–6).

7), and then cross-linked and immunoblotted. Again, bands corresponding to GLT, GLAST, and EAAC dimers and trimers were observed. Thus, oligomeric structure is preserved during solubilization and partial purification as well as during reconstitution.

During the purification of a glutamate transporter (34), it was shown that transport activity could be reconstituted after solubilization in CHAPS, cholate and Triton X-100. It was noted that the transporters rapidly inactivated and that this inactivation was much slower in cholate and CHAPS than in Triton X-100. No transport activity was observed when the proteins had been exposed to Zwittergent 3-12, β-octylglucoside, or SDS. We questioned whether this inactivation was due to dissociation of the oligomeric complexes. Membranes were, in the presence of DTT, solubilized with the different detergents and incubated with cross-linkers (see “Experimental Procedures” for details). The presence of bands corresponding to dimers and trimers were clearly present after cross-linking of the extracts obtained with CHAPS, cholate, Triton X-100, and Zwittergent 3-12 but to a lesser degree in the β-octylglucoside extracts and not at all in the SDS extracts (data not shown). Thus, the transport activities in the Triton and Zwittergent extracts are lost in spite of preserved oligo-
Fig. 7. Cross-linking of partially purified glutamate transporter proteins in solution. Membranes from rat forebrain were solubilized with CHAPS and the glycoproteins isolated by (wheat germ) lectin affinity chromatography. The glycoprotein fraction was divided in 4 aliquots to which was added 0 (lane 1), 0.1 (lane 2), 0.3 (lane 3), or 1 mM (lane 4) bis(sulfosuccinimidyl) suberate. After electrophoresis, the proteins were immunoblotted for GLT with 200 ng/ml anti-B12 antibodies (A), for GLAST with 200 ng/ml anti-A522 antibodies (B), or for EAAC with 1 µg/ml anti-C510 (C). 0.5, 0.5, and 5 µg of protein was applied in each lane in A, B, and C, respectively.

The possibility existed that Zwittergent 3-12 and β-octylglucoside interfered with reconstitution efficiency and the intactness of the liposomes rather than inactivating the glutamate transporter proteins. To exclude this possibility, a DEAE-cellulose column (34) was used to collect glutamate transporters solubilized with the mentioned detergents and replace these detergents with CHAPS. This detergent shift was not sufficient to regain transport activity (data not shown) suggesting irreversible conformational changes or detergent-induced loss of unknown co-factors.

**Radiation Inactivation Analysis**—The cross-linking and reconstitution experiments did not give any indication that the monomers are active since oligomers were present in all tested preparations exhibiting glutamate transport activity. To get more information, radiation inactivation analysis was performed. This technique determines the functional size of proteins. Therefore, the degree of polymerization required for transport activity is tested. Radiation inactivation analysis was done using rat cerebral cortex, exposed to 0–20 Mrad of electron radiation, followed by solubilization and reconstitution of the proteins (see “Experimental Procedures”). A molecular target size of 160 kDa was arrived at by multiplying the slope of the line (Fig. 8A) with the inactivation constant 730,000 Da × Mrad (41). Radiation inactivation analysis assumes that loss of functionality is due to a reduction in the number of active transporter molecular complexes. Therefore, \( K_m \) and \( V_{max} \) were determined at 0 and 5 Mrad. \( K_m \) was found to be 7.4 ± 1.3 and 8.1 ± 1.8 µM at 0 and 5 Mrad, respectively. These values, which represent mean ± S.E., are similar to previously reported values using the same reconstitution assay method (11).

**DISCUSSION**

**Co-localization of GLT and GLAST**—We show by double labeling post-embedding immunocytochemistry that the two excitatory amino acid transporters GLT and GLAST occur side by side in the same astroglial plasma membranes. This result demonstrates directly what was suggested by previous studies. Thus, light microscopic studies of neighboring sections showed the two immunoreactivities to be localized in the same astrocytic cells (15), whereas electron microscopic single labeling pre- and post-embedding immunocytochemistry showed that the two proteins reside in the same types of astrocytes but at different relative densities in different regions (14, 15).

The use of an antigen-friendly method of tissue preparation (freeze substitution) and embedding (acrylic resin at low temperature) made it possible to combine the preservation of immunoreactivity and ultrastructure (14, 15). We show by double labeling double immunocytochemistry that the two proteins reside in the same types of astrocytes but at different relative densities in different regions (14, 15).
Glutamate Transporter Oligomers

The glutamate transporters form oligomers—The cross-linking of intact membranes, detergent extracts, liposomes containing active reconstituted glutamate transporters, and transfected HeLa cells clearly shows the presence of GLAST, GLT, and EAAC multimers. Trimers are predominant in the case of GLT, whereas GLAST, in particular, may also exist as dimers. This conclusion is based on the resistance of the GLT dimer bands to high cross-linker concentrations and on the difference in the trimer-dimer relationships between GLT and GLAST.

The question remains if GLT also forms higher molecular mass complexes. The cross-linking experiments on intact membranes (Fig. 3) and the cross-linking and the oxidation experiments on the proteins reconstituted in liposomes (Fig. 5) suggest that trimers are the largest complexes formed by GLT as well as by the other two transporters. However, bands corresponding to higher molecular mass aggregates of GLT were observed after cross-linking of proteins in solution (Fig. 7) and after oxidation of membranes and detergent extracts (Fig. 2). To find out what this means will be the topic of further studies.

Oxidation changes the electrophoretic mobility of the transporters—The fact that only monomers are observed when fresh brain tissue is solubilized in SDS and quickly immunoblotted (Fig. 2, A and B, lane 1) shows that the oligomers in the living brain are held together by noncovalent interactions.

The appearance of oligomer bands in samples that have been stored prior to electrophoresis has previously been interpreted as aggregation (21, 34, 47, 48) which seemed to be sensitive to 2-mercaptoethanol (22). The formation of clearly defined oligomer bands indicates that the process is specific and that transporter proteins preferentially connect to each other rather than to other proteins. The specificity of the phenomenon is further illustrated by the fact that the GLT bands and the GLAST bands have different molecular masses. Our interpretation is that the appearance of oligomers shown in Fig. 2 is due to oxidation-induced formation of covalent bonds between the subunits of existing oligomer complexes so that they do not dissociate during SDS-PAGE. In other words, they form unnatural bonds with their natural partners. In agreement with this, immediate dissociation of the oligomer complexes with SDS inhibits the oxidative formation of covalent bonds between the monomers (Fig. 2, A and B). Since the oxidative cross-linking is inhibited by reducing agents and enhanced by DTNB, it is likely that oxidation of SH groups in neighboring polypeptides plays the predominant role. GLT-1 has 9 cysteine residues in its sequence, whereas GLAST has only 3. This may explain why oxidation (Fig. 2) and cross-linking via amino groups (Fig. 3) give somewhat different results. The presence of DTT during reconstitution of solubilized transporters strongly increases the transport activity of the ensuing proteoliposomes (data not shown) indicating that the oxidized complexes are less active. This is in line with previous reports on the inhibition of glutamate transport activity by free radicals and oxidizing agents (49, 50). The rapid oxidation of GLT may in part explain the loss of transport activity upon reconstitution as reported previously (34).

As shown in Fig. 2, oxidation of GLT, but not GLAST, in brain membranes leads to irreversible crosslinking. If this should happen in living cells, internalization of the damaged proteins would be expected. This could explain the observed (55) selective reduction in GLT in amyotrophic lateral sclerosis, a condition characterized by, among other things, oxidative damage.

GLT and GLAST do not complex with each other—The immunoblots of the cross-linked or oxidized membrane proteins directly demonstrate that GLT and GLAST do not associate with each other, in spite of being located in the same membranes in vivo.

The pilot experiment initiating this study was an immunoprecipitation experiment from a CHAPS extract of rat cerebellum resulting in co-precipitation of GLAST and GLT (not shown). From this we initially concluded that GLT and GLAST formed hetero-oligomers. However, significant co-precipitation from Triton X-100 and cholate extracts was not observed. The possibility existed that the hetero-oligomers were stable in CHAPS but not in Triton X-100 and cholate. This illustrates the difficulties. To study the noncovalent interactions between these hydrophobic membrane proteins, detergents have to be used to keep them solubilized and may interfere with the interaction under study. The cross-linking in intact membranes (Fig. 3) circumvents these problems and clearly demonstrates that the two proteins are not associated. The only way to avoid this conclusion is either to assume that the cross-linkers selectively connect GLT to GLT and GLAST to GLAST, but not GLT to GLAST, or to assume that only a minor fraction of the complexes are hetero-oligomers. The first assumption seems very unlikely since several different cross-linkers give the same result. The latter question has been addressed by immunoprecipitating cross-linked complexes. The immunoprecipitated GLAST complexes display no GLT immunoreactivity. We therefore conclude that the co-precipitation observed in the CHAPS extracts from cerebellum is due to poor solubilization or other unspecific interactions and not due to the presence of hetero-oligomers.

Might the glutamate transporters associate with other unidentified proteins?—As explained above, the three glutamate transporters form separate oligomeric complexes in the brain. However, it might be asked if these complexes consist of identical proteins, e.g., homotrimers or homodimers, or if they consist of additional proteins together with the glutamate transporters. If such extra proteins exist, they would have to have the same size as the glutamate transporter protein they complex with (see Fig. 3). This implies that there would have to be three different proteins. Such proteins would also have to be produced in the HeLa cells upon transfection of these cells with the glutamate transporter cDNAs (Fig. 6). If oligomeric structure is required for transport activity, it also implies that similar proteins are expressed upon transfection of Xenopus oocytes and COS cells. We therefore conclude that the most likely interpretation of the available data is that the complexes represent homo-oligomers.

The possibility that there might exist variants of each transporter subtype must be kept in mind. If this is the case, there could, for instance, be trimers consisting of three different GLT-variants.

GLT is the most abundant glutamate transporter in the brain—GLT was the glutamate transporter purified in an active form from rat brain using reconstitution of transport as an assay (34), and antibodies raised against this purified protein immunoprecipitated almost all of the reconstitutable transport activity (21) in spite of appearing to be specific for GLT (3, 15, 22). Here we show that GLT antibodies with defined specificities (antibodies to synthetic peptides) give the same result (Fig. 4). This demonstrates that most of the reconstitutable transport activity is due to GLT. Therefore it is legitimate to corre-

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K. Ullensvang, unpublished observations.
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late the GLT immunoblot labeling pattern with the reconstituted glutamate transport activity.

Oligomeric Structure May Be Required for Transport Activity—In the model systems investigated (brain membranes, detergent extracts, purified transporter, reconstituted transporters, and transfected HeLa cells), oligomeric structure is always seen together with transport activity. The subunits stick together strongly enough to maintain oligomeric structure during the mild conditions used for solubilization, reconstitution, and even purification in active form. SDS and β-mercaptoethanol, which effectively inactivate the transporters, also dissolve the oligomer complexes. The absence of activity with Triton X-100, which is effective inactivating the transporters, also shows that oligomeric structure is not the only requirement for transport activity. It might be legitimate to speculate if these detergents introduce conformational changes or cause dissociation of unknown cofactors.

Radiation inactivation studies further support the idea that oligomeric structure is required for transport activity. The reconstitutable transport activity from irradiated rat cerebral cortex declines exponentially corresponding to an estimated molecular target size of 160 kDa. The molecular mass of the polypeptide encoded by the GLT-1 clone (5) is 64 kDa. The native protein is glycosylated (21) and runs with an apparent molecular mass of 73 kDa on SDS-PAGE, but the sugar mass is not believed to contribute to the target size (51, 52) unless a possible reassociation for transport activity (53) which seems unlikely as the N-glycosylation has no impact on the transport activity of GLAST (47). Therefore, 160 kDa corresponds to a mass that is about 2.5 times larger than the individual polypeptides. The present data do not allow firm conclusions as to whether both the trimers and the dimer or one of them is active. If the trimer is the active species, a target size lower than 190 kDa would be expected if a partial reassociation with exclusion of damaged subunits takes place during solubilization and reconstitution.

After the completion of this study, we became aware of a paper by Béliveau and collaborators (54) who analyzed amino acid transport in membrane vesicles from intestinal epithelium by radiation inactivation. They found a target size of 250 kDa for glutamate transport which is compatible with trimers or tetramers. Taking into account that the latter results were obtained in a different tissue expressing different glutamate transporter(s), presumably EAAC (7), and with different procedures, they are in good agreement with ours. The uptake assays of the quoted study (54) were performed in nonsolubilized membranes, where reassociation of the complexes will be prevented, but in which radiation inactivation of other proteins governing the forces driving glutamate uptake (e.g. ion channels) may contribute to the estimated large target size.

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