Biosynthesis, Intracellular Targeting, and Degradation of the EAAC1 Glutamate/Aspartate Transporter in C6 Glioma Cells

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Rat C6 glioma cells were used as a model system to study the biosynthesis, intracellular targeting, and degradation of the EAAC1 transporter, a sodium-dependent glutamate/aspartate transport protein that encodes System Xₐₐₐₐ activity. At steady state, nearly 70% of the EAAC1 transporter was localized at the cell surface. The newly synthesized EAAC1 protein was co-translationally N-glycosylated with high mannosyl oligosaccharide chains that were processed into complex-type sugar chains as the protein matured. The final maturation steps for EAAC1 protein coincided with its plasma membrane arrival, which was first detected at about 45 min after the initial synthesis. The newly synthesized EAAC1 protein was protected from degradation during the maturation and targeting process, as well as during the first 5 h after plasma membrane arrival. After this initial lag period, both the newly synthesized transporter and the total cellular EAAC1 pool were degraded by first order kinetics with a half-life of 6 h. These results represent the first analysis of the synthesis and degradation of the EAAC1 amino acid transporter.

A family of Na⁺-dependent high affinity glutamate/aspartate transport systems, previously referred to collectively as System Xₐₐₐₐ, is essential for the glutamatergic transmission in the central nervous system (1–3), as well as for nutrition of many other cells. The human cDNAs encoding glutamate/aspartate transporter activity are designated as excitatory amino acid transporters, EAAT1–5 (4–6), but the rodent counterparts for EAAT1–3 were originally named as GLAST (7), GLT1 (8), and EAAC1 (excitatory amino acid carrier 1) (9), respectively. Rat EAAC1 cDNA encodes an integral membrane protein of 523 amino acids with a predicted nonglycosylated core molecular mass of 56.8 kDa (10), but it is N-glycosylated under normal conditions (11). It has been reported that GLAST, GLT1, and EAAC1 form homo-multimers, even when detected using immunoblotting under reducing conditions (12). On analysis with denaturing gels, the mature monomers of these transporters each runs as a broad band between 66 and 74 kDa, which is likely due to the microheterogeneity of their N-glycosylation sites and possibly other post-translational modifications such as phosphorylation (11).

Among the members of the glutamate/aspartate transporter family, the EAAC1 transporter appears to be the most ubiquitously expressed. Although quite abundant in brain, a significant level of expression of this transporter can also be detected outside the nervous system in small intestine, kidney, heart, skeletal muscle, lung, liver (9, 10), and placenta (13). It is postulated that the EAAC1 transporter may play a role in keeping the neuronal intracellular glutamate at high levels for use as a precursor for γ-aminobutyric acid synthesis or for other metabolic reactions in the brain (14). In addition, consistent with its ubiquitous expression among different tissues, EAAC1 transporter functions as a primary mechanism to provide glutamate and aspartate for general metabolism and other intracellular functions.

Rat C6 glioma cells exhibit several biochemical features of normal glial cells, such as expressing glial fibrillary acidic protein (15). These cells express a high level of System Xₐₐₐₐ transport activity (16). Although there are reports that GLT1 is expressed in C6 cells (17), most evidence indicates that C6 glioma cells express EAAC1 but not GLAST, GLT1, or EAAT4 (11, 18).

Although the distribution, regulation, and mechanism of anionic amino acid transporters has been extensively studied, much less is known about their biosynthesis and intracellular trafficking. It has been shown that the surface expression of EAAC1 can be rapidly up-regulated by both protein kinase C and phosphatidylinositol 3-kinase pathways (19, 20), suggesting the redistribution of EAAC1 from an intracellular compartment. Furthermore, Lin et al. (21) have made the interesting observation that EAAC1 activity can be modulated through protein-protein interactions with GTRAP3–18. For the studies described here, C6 glioma cells were used as a model system to study the biosynthesis, intracellular targeting, and degradation of EAAC1. The results reveal that the EAAC1 protein is N-glycosylated in a co-translational manner. Synthesis and trafficking to the plasma membrane required a minimum of 45 min, and there was a lag of about 5 h prior to degradation of transporters on the cell surface. With regard to turnover, the plasma membrane resident EAAC1 was endocytosed and then degraded with a half-life of about 6 h. Interestingly, the newly synthesized plasma membrane-associated transporter population was degraded at the same rate as the total pool of EAAC1, suggesting that both surface and intracellular EAAC1 proteins have similar half-life values. The results obtained provide the basis for studying the role of EAAC1 transporter synthesis and degradation during disease states.

MATERIALS AND METHODS

Cell Culture—C6 glioma cells were obtained from the American Type Culture Collection (CCL107) and maintained in supplemented Eagle's...
medium (MEM) containing 10% FBS as a monolayer culture under a humidified atmosphere of 5% CO₂, 95% air (37 °C) for a maximum of eight passages. The cultured cells were transferred to 24-well cluster dishes for whole cell transport assays or to 100–150-mm culture dishes for metabolic labeling, cell surface biotinylation, and total cellular protein or membrane protein collection.

**Transport Assay**—Amino acid uptake by C6 glioma cells was measured using the cluster tray method (22). One hundred thousand C6 cells were placed into each well of a 24-well tray and cultured for 24 h. To partially deplete the intracellular pool of amino acids and thus minimize trans-effects on transport and remove extracellular Na⁺, the cells were incubated at 37 °C twice for 15 min each in sodium-free Krebs-Ringers phosphate buffer (choline-KRP). To initiate transport, [3H]lysine (50 μCi/ml of either NaKRP or sodium-containing Krebs-Ringers phosphate buffer) or choline-KRP (37 °C) was added simultaneously to each of the 24 wells in the cluster tray for 1 min. The transport measurement was terminated by discarding the radioactivity and rapidly washing the cells five times with 2 ml of ice-cold choline-KRP. The Na⁺-dependent transport is taken as the difference between uptake in NaKRP and choline-KRP. The data are expressed as pmoles/mg⁻¹ protein/min⁻¹ and are presented as the averages of four assays on at least two different batches of cultured cells.

**Gel Electrophoresis and Immunoblotting**—Gel electrophoresis was performed in 7.5% polyacrylamide gels following the protocol originally described (23). The protein band of interest was cut out of the gel and placed into each well of a 24-well tray and cultured for 24 h. To partially deplete the intracellular pool of amino acids and thus minimize trans-effects on transport and remove extracellular Na⁺, the cells were incubated at 37 °C twice for 15 min each in sodium-free Krebs-Ringers phosphate buffer (choline-KRP). To initiate transport, [3H]lysine (50 μCi/ml of either NaKRP or sodium-containing Krebs-Ringers phosphate buffer) or choline-KRP (37 °C) was added simultaneously to each of the 24 wells in the cluster tray for 1 min. The transport measurement was terminated by discarding the radioactivity and rapidly washing the cells five times with 2 ml of ice-cold choline-KRP. The Na⁺-dependent transport is taken as the difference between uptake in NaKRP and choline-KRP. The data are expressed as pmoles/mg⁻¹ protein/min⁻¹ and are presented as the averages of four assays on at least two different batches of cultured cells.

**Pulse-Chase Metabolic Labeling of C6 Glioma Cells**

1. **Pulse-Chase Metabolic Labeling of C6 Glioma Cells**—To study the de novo biosynthesis and the intracellular targeting of the EAAC1 transporter protein in C6 cells, pulse-chase labeling with [3H]methionine-cysteine (ProMix; Amersham Biosciences) was used. After placing 9 × 10⁶ cells onto each 100-mm culture dish or 2.3 × 10⁵ cells onto each 150-mm dish, the cell monolayers were cultured for 24 h to permit growth to near confluence. The cells were washed once with sterile 37 °C phosphate-buffered saline, pH 7.4, and incubated with 15 ml/58-cm² surface area of methionine- and cysteine-free Dulbecco’s modified Eagle’s medium (Invitrogen) for 2 × 15 min at 37 °C to deplete the intracellular pool of free methionine and cysteine. The depletion medium then was aspirated, and the cells were incubated with 200 μCi/ml of [3H]Met-Cys in methionine- and cysteine-free Dulbecco’s modified Eagle’s medium at 37 °C for 15–30 min (see each figure legend). The cells were washed twice at 37 °C with MEM containing 5 mM each of nonradioactive methionine and cysteine (chasing medium) and then transferred to fresh chashing medium and incubated for 0–60 h (see each figure legend) followed by immunoprecipitation of the EAAC1 protein. For experiments in which the chase period was longer than 24 h, 1% FBS was added to the medium.

2. **Immunoprecipitation and Fluorography**—Immunoprecipitation of the EAAC1 transporter protein was performed following the procedure outlined by Harlow and Lane (24) with modifications. After pulse-chase labeling with [3H]Met-Cys, the cells were washed twice with ice-cold phosphate-buffered saline and once with SEB buffer (250 mM sucrose, 2 mM EDTA, 2 mM EGTA, and 10 mM HEPES, pH 7.5) and then frozen in 2.5 ml of SEB buffer containing proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride and 1 μg/ml each of leupeptin, aprotonin, pepstatin, N-tosyl-L-phenylalanine chloromethyl ketone, and N-p-tosyl-L-lysine chloromethyl ketone) at −80 °C. For analysis, the cells were thawed on ice, and another 2.5 ml of ice-cold hypotonic EB buffer (2 mM EDTA, 2 mM EGTA, and 10 mM HEPES, pH 7.5) was added. The cells were scraped from the plates and homogenized on ice with 15 passes through a prechilled steel block cell homogenizer with a clearance of 0.0025 inches (Auburn Tool & Dye, Warwick, RI). The cell homogenate was centrifuged at 400 × g for 10 min to remove unbroken cells and nuclei, and the supernatant was centrifuged at 280,000 × g for 1 h at 4 °C to collect a total membrane pellet, which was then extracted in PBS buffer (2% C₁₀₋₁₂E₅, 0.1% SDS, 1 mM EDTA in phosphate-buffered saline, pH 7.4) for 1 h on ice with constant stirring. After centrifugation at 40,000 × g for 1 h, the supernatant and the protein precipitation were transferred electrophoretically onto nitrocellulose membrane in 4 °C transfer buffer (25 mM Tris-base, 190 mM glycine, 20% methanol) at 299 mA and constant current for 20 h. After the transfer, the blot was stained briefly in Fast Green stain (0.1% Fast Green partate in 250 μl of either NaKRP (sodium-containing Krebs-Ringers phosphate buffer) or choline-KRP (37 °C) was added simultaneously to each of the 24 wells in the cluster tray for 1 min. The transport measurement was terminated by discarding the radioactivity and rapidly washing the cells five times with 2 ml of ice-cold choline-KRP. The Na⁺-dependent transport is taken as the difference between uptake in NaKRP and choline-KRP. The data are expressed as pmoles/mg⁻¹ protein/min⁻¹ and are presented as the averages of four assays on at least two different batches of cultured cells.

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incubated at 37 °C for 1 h and then mixed with an equal volume of 2× SDS buffer before the samples were loaded onto SDS-PAGE gel for separation and autoradiographic detection.

To determine the N-linked glycosylation of both the newly synthesized forms of the EAAC1 transporter protein, protein endoglycosidase F (PNGase F) digestion was performed. C6 glioma cell membrane proteins were collected from the cells, with or without pulse-chase labeling and then solubilized with PES buffer, and EAAC1 protein was immunoprecipitated. The precipitates were eluted with 10 μl of 5× denaturing solution (2.5% SDS, 5% β-mercaptoethanol) for 30 min at 37 °C and then diluted to 1× denaturing solution with water, as described above for the Endo H digestions. For each PNGase F digestion, 1/10 volume of 10% Nonidet P-40 and a buffer consisting of 10% Nonidet P-40, 500 μM sodium citrate, pH 7.5, as well as 500–1000 units of PNGase F (Bio Labs Inc.) were added. For the controls, the enzyme was replaced with equal volume of enzyme storage buffer. All of the samples were incubated at 37 °C for 60 min and then mixed with an equal volume of 2× SDS buffer before they were loaded onto a SDS-PAGE gel for separation and autoradiographic detection.

Cell Surface Protein Biotinylation—To determine the half-life of the cell surface EAAC1 protein, C6 glioma cells were washed twice with NaKR buffer (119 mM NaCl, 5.9 mM KCl, 1.2 mM KHCO3, 5.6 mM glucose, 25 mM Na2HPO4, 0.5 mM CaCl2, 1.2 mM MgSO4, pH 7.5) and incubated with 0.5–1 mg/ml of sulfo-NHS-LC-biotin (Pierce) in NaKR for 30 min. The specific conditions for each experiment are given in the figure legends. The biotin-containing buffer was aspirated, and the cells were rinsed twice with fresh MEM containing 50 mM glycine and then incubated in fresh MEM and 1% FBS at 37 °C for specific chase times ranging from 0 to 24 h at 37 °C. At the end of each chase period, the cells were washed once with ice-cold NaKR buffer, washed once with ice-cold SEB containing protease inhibitors (as above), and then frozen in 2 ml of SEB containing protease inhibitors at −80 °C. The percentage of the biotinylation for EAAC1 was 69%, compared with a value of less than 2% for the intracellular proteins, asparaginase synthetase (cytoplasmic) and GRP78 (endoplasmic reticulum) (data not shown).

To determine the transit time of newly synthesized EAAC1 transporter proteins to the plasma membrane, C6 cells were metabolically labeled with [35S]Met-Cys for 15–30 min and chased in medium containing 5 mM each of nonradiolabeled methionine and cysteine for 0–36 h. At the end of each chase time, these metabolically labeled C6 cells were washed twice with NaKR and then cell surface-biotinylated with 0.5–1 mg/ml of sulfo-NHS-LC-biotin in NaKR at 15 °C for 30–60 min. After aspirating the biotinylation solution, the cells were rinsed once in NaKR and then incubated with NaKR containing 50 mM glycine for 2 × 15 min at 15 °C to quench the remaining free biotin. The cells were washed once with ice-cold SEB containing protease inhibitors and frozen at −80 °C until all of the samples were collected. The surface-biotinylated C6 cells were thawed on ice and homogenized with a steel block homogenizer, and the total cellular membrane proteins were collected and solubilized with PES buffer, as described above. Equal amounts of proteins were incubated twice as described above. These results are consistent with the data by Dowd et al. (11) and show that glycosylation of the EAAC1 monomer does not reverse its oligomerization.

De Novo Biosynthesis Rate for EAAC1 Transporter—To determine the biosynthesis rate of EAAC1 protein in C6 glioma cells, the cells were metabolically labeled with 200 μCi/ml of [35S]Met-Cys for 15–120 min and then chased for 3 h. As shown in Fig. 2A, even a 15-min pulse labeling time is sufficient to detect newly synthesized EAAC1, and all three forms (monomer, dimer, and oligomer) of EAAC1 appear to be presented. These results suggest that the oligomerization is not a late step in the maturation process for the EAAC1 protein. Interestingly, the biosynthesis rate of EAAC1 was decreased when a higher number of cells were plated, indicating that EAAC1 biosynthesis is down-regulated by increased cell density (Fig. 2B). This observation is consistent with preliminary experiments showing that the total EAAC1 protein content was reduced as cells became more confluent (data not shown). Therefore, in all remaining experiments a fixed number of cells were plated (900,000/100-mm dish) and then cultured for an exact period of time (24 h) prior to labeling.

Maturation and Targeting of EAAC1 Transporter—To study the maturation process of EAAC1, C6 glioma cells were pulse-
The maturation of EAAC1 protein started after 45 min of chase and continued until 190 min. The data in Fig. 3 show that the 57-kDa form of EAAC1 matured into the 73-kDa protein. As the chase time increased, the 73-kDa protein became the predominant form of EAAC1.

At the address time required for the newly synthesized EAAC1 protein to be targeted to the plasma membrane, the cells were metabolically labeled with 200 μCi/ml of [35S]Met-Cys for 15 min and then chased for 0–120 min. When chased for less than 30 min, only the low molecular mass (57 kDa), immature form of EAAC1 was detected primarily (Fig. 3), but after longer chase periods, the 73-kDa form of EAAC1 matured into the 73-kDa protein. The maturation of EAAC1 protein started after 45 min of chase time and finished after 190 min. The data in Fig. 3 show that oligomeric forms were detected not only for the mature EAAC1 protein but also for the immature EAAC1 form (e.g., chase time = 0).

To address the time required for the newly synthesized EAAC1 protein to be targeted to the plasma membrane, the cells were metabolically labeled with 200 μCi/ml of [35S]Met-Cys for 15 min and chased in medium containing 5 mM each of nonradioabeled methionine and cysteine, a total cellular membrane fraction was solubilized in PES buffer, as described under Materials and Methods. An equal amount of starting protein was subjected to immunoprecipitation with anti-EAAC1 and then analyzed with SDS-PAGE and fluorography (A). B illustrates the densitometry data obtained from the data in A.
plateaus or increases only slightly thereafter. The densitometry analysis of the data shows that the targeting of the newly synthesized EAAC1 protein to the plasma membrane coincided with its maturation from the 57-kDa form to the mature 73-kDa monomer (Fig. 4B). Analysis of several experiments not included here and the data of Fig. 8 suggests that the apparent difference in the ratios at 2 h versus 6 h is due to experimental variability.

N-Glycosylation of EAAC1 during Maturation—To study the intracellular targeting of the newly synthesized EAAC1 protein, endoglycosidase digestions were employed. Resistance to Endo H digestion can be used as a hallmark to demonstrate that an N-glycoprotein has proceeded beyond the medial Golgi compartment (27). C6 glioma cells were pulse-labeled with 200 μCi/ml of [35S]Met-Cys for 15 min and then chased in medium containing 5 mM of nonradioactive Met and Cys, as described under “Materials and Methods.” At the end of each chase period, EAAC1 protein was immunoprecipitated from a PES-solubilized membrane fraction and then analyzed by SDS-PAGE and fluorography, as described under “Materials and Methods.” The data shown are representative of four independent experiments.

Degradation of the Cell Surface EAAC1 Protein—C6 plasma membrane proteins were labeled with sulfo-NHS-LC-biotin at 15 °C for 1 h and then chased in medium containing an excess of nonradiolabeled Met and Cys for 0–60 h. As shown in Fig. 6, the amount of radiolabeled EAAC1 protein diminished as the chase time was prolonged. From Fig. 6A, it is clear that the amount of all three forms of EAAC1 are proportional to each other throughout the chase period. Therefore, the densitometry readings for the monomer were used for quantification (Fig. 6B). When the data were plotted using the common logarithm of the remaining EAAC1 protein, it is evident that the decay process is a first order reaction (Fig. 6C). This lag period was observed consistently in all pulse-chase degradation experiments. Given that it takes about 3 h for all of the pulse-labeled EAAC1 protein to arrive at the plasma membrane, these new polypeptides must be protected from decay, not only during the de novo biosynthesis and targeting process as would be expected but also during the initial 5 h after the protein reaches the plasma membrane. However, once the decay process starts, it follows first order kinetics that yield a first order rate constant for degradation (k d) of 0.12 h−1 or a half-life of about 6 h (Fig. 6C).
ti-EAAC1 antibody (Fig. 7A). The half-life of the cell surface biotinylated EAAC1 protein was 6 h (Fig. 7B), a result consistent with the pulse-chase labeling described above. In contrast to the pulse-chase studies, which determine how long it takes for the newly synthesized EAAC1 protein molecules to be degraded, cell surface biotinylation determines the turnover of the cell surface EAAC1 protein as a whole without discriminating with regard to the age of the protein. The results obtained by both approaches show that the EAAC1 protein was degraded with a half-life of about 6 h under normal conditions in C6 glioma cells.

Residence Time of the EAAC1 Transporter at the Cell Surface—The length of time that the EAAC1 protein resides at the plasma membrane and whether or not intact EAAC1 protein could be detected following removal from the plasma membrane were assessed using pulse-chase labeling followed by cell surface biotinylation. Fig. 8A shows the rate of disappearance of the radiolabeled and biotinylated EAAC1 protein in C6 cells. Consistent with the pulse labeling experiments shown above, a lag period of about 8 h was observed prior to the initial loss of the EAAC1 from the cell surface. The loss of the radiolabeled, biotinylated EAAC1 protein represents the disappearance of the EAAC1 from the cell surface, which can result from either protein turnover at the membrane or protein internalization. The plasma membrane-resident EAAC1 protein disappeared from the cell surface at a rate of about 11.5%/h (half-life = 6 h), which is consistent with the total cellular EAAC1 protein decay rate defined by the pulse-chase studies (Figs. 6C and 8B). To confirm this observation and to compare these values within the same experiment, the disappearance rate of the newly synthesized (radiolabeled), plasma membrane-resident (biotinylated) EAAC1 protein was compared with the degradation rate for the total pool of newly synthesized EAAC1 protein (Fig. 9). The rate of EAAC1 removal from the plasma membrane was nearly identical to the rate of EAAC1 degradation.

DISCUSSION
The results represent a detailed study of the biosynthesis and turnover of the EAAC1 glutamate/aspartate transporter. Collectively, the data obtained indicate that the EAAC1 transporter is co-translationally modified by N-glycosylation and that the transition of the EAAC1 protein from its immature form to mature form coincides with the alteration of the attached oligosaccharide chains from the high mannose type to the complex type. These results also document that once the mature form is fully processed, it rapidly passes through the
Therefore, in pulse-chase experiments, when the chase time is
ified from high mannose- to complex-type carbohydrate chains.
and Golgi compartments, the oligosaccharide chains are mod-
detected. Further analysis of the oligomerization state of the
without additional processing, only the EAAC1 monomer was

total membrane fraction from C6 glioma cells was prepared
prolonged. These results suggest that oligomerization happens
oligomerization was observed as the pulse or chase time was

FIG. 8. Plasma membrane residence time of the EAAC1 trans-
porter. C6 glioma cells were pulse-chase labeled with 200 μCi/ml
[35S]Met-Cys in Met- and Cys-free medium for 30 min and then chas-
ed in MEM medium containing 1% FBS and 5 mM each of nonradiolabeled Met and Cys for 0–22 h. At the end of each chase period, the cells were surface-biotinylated with 0.5 mg/ml sulfo-NHS-LC-biotin in NaKRP for 1 h at 15 °C, and then the free biotin was quenched with 50 mM glycine in NaKRP for 2 × 15 min at 15 °C. After the PES-solubilized total membrane proteins were prepared, the total EAAC1 protein was immu
precipitated with anti-EAAC1 antibody, and then monomeric av-
idin-Sepharose beads were used to precipitate only the biotinylated
EAAC1 protein (A). The nonbiotinylated EAAC1 protein was immuno-
precipitated from the supernatant of the avidin precipitation through a
second incubation with anti-EAAC1 antibody. The logarithm of the
densitometry reading of the remaining radiolabeled EAAC1 monomer
was plotted against the chase time (B). The data are representative of
several individual experiments.

rest of the Golgi compartment and arrives at the plasma
membrane.

Haugeto et al. (12) reported that GLAST1, GLT1, and
EAAC1 transporters all formed homomultimers in rat brain
and transfected HeLa cells. Furthermore, based on results
obtained from radiation inactivation analysis, they postulated
that the glutamate transporters operate as homomultimeric
complexes in vivo. Consistent with that interpretation, our
anti-EAAC1 antibody detected three EAAC1 bands in C6 cell
extracts with estimated sizes of 73, 145, and >200 kDa, which
may represent the EAAC1 monomer, dimer, and trimer, re-
spectively. In addition, when C6 glioma cells were pulse-chase
labeled, the oligomer forms for the newly synthesized imma-
ture EAAC1 protein were also detected, and no change in
oligomerization was observed as the pulse or chase time was
prolonged. These results suggest that oligomerization happens
before EAAC1 synthesis is finished. However, that the
oligomerization is an artifact of transporter isolation or manipu-
lation cannot be ruled out. In several experiments when the

total membrane fraction from C6 glioma cells was prepared
without additional processing, only the EAAC1 monomer was
detected. Further analysis of the oligomerization state of the
transporter and the impact on function is needed.

As glycoproteins proceed through the endoplasmic reticulum
and Golgi compartments, the oligosaccharide chains are mod-
ified from high mannose- to complex-type carbohydrate chains.
Therefore, in pulse-chase experiments, when the chase time is
short, only the immature form of the protein will be detected
and, if it has not passed beyond the medial Golgi, this form will
be sensitive to endoglycosidase H digestion. For EAAC1, only
the immature EAAC1 protein species were detected when the
chase period was 45 min or less. These results indicate that the
core protein synthesis and the initial stages of glycosylation
occur within this time frame. Interestingly, the completion of
carbohydrate processing and the trafficking of the mature
EAAC1 protein to the plasma membrane were considerably
faster. Pulse-chase labeling followed by cell surface biotiny-
lation showed that the arrival of EAAC1 transporter to the cell
surface coincided with the shift of EAAC1 from its immature
forms to its mature form. These results indicate that there is no
significant lag time between these two processes. Furthermore,
the difference between the apparent molecular mass of the
mature EAAC1 monomer and its immature form is ~16 kDa,
and no intermediates were observed during its maturation.
These results suggest that during the maturation process the
trimming and readaptation steps for the N-glycosylation modi-
fication of each EAAC1 protein molecule occur relatively quickly.
The data support a model of rapid transit of the mature EAAC1
protein from the medial Golgi to the plasma membrane.

To establish the residence time of newly synthesized EAAC1
protein at the plasma membrane, cell surface biotinylation was
employed immediately after the pulse-chase labeling, followed
by anti-EAAC1 immunoprecipitation. When the rate at which
the EAAC1 protein disappeared from the surface was com-
pared with that of its degradation, the majority of the EAAC1
protein appeared to be degraded reasonably soon after it left
the cell surface. The disappearance rate of EAAC1 proteins from the cell surface was almost identical with the degradation rate of the total cellular EAAC1 protein pool. Our results indicate that 70% of EAAC1 resides at the plasma membrane, and for the 30% of transporters present in the intracellular pool, the synthesis and degradation rates are similar to those on the cell surface.

The present data indicate that the half-life for decay of radiolabeled EAAC1 protein is about 6 h. The decay process for the EAAC1 protein did not start as soon as it matured, even though biotinylation suggested that it was exposed at the extracellular surface of the plasma membrane. Instead, there was a lag time of about 8 h between EAAC1 synthesis and the beginning of its degradation, and at least 5 of those 8 h occurred after the protein had arrived at the plasma membrane. The newly synthesized EAAC1 protein then merges with the general EAAC1 protein pool to be degraded by a process exhibiting the expected first order kinetics. What makes these newly synthesized “protected” EAAC1 proteins distinguishable from the rest is unclear, but several possibilities exist. These newly synthesized EAAC1 protein molecules may: 1) be sequestered within specific membrane domains that are inaccessible to endocytosis; 2) lack a structural alteration or post-translational modification that is required for the recognition by an adaptor protein for endocytosis; or 3) lack the proper interaction with another regulatory protein (21) for the recognition by the degradation machinery. Collectively, these results represent the foundation on which to further investigate these possibilities as well as to consider regulation of the biosynthetic and degradative pathways as potential mechanisms for modulating EAAC1 activity under normal and disease states.

Interestingly, data from Davis et al. (19) and Sims et al. (20) indicate that when cells are treated with either protein kinase C or phosphatidylinositol 3-kinase activators, EAAC1 transporters can be recruited from an intracellular pool not accessible to an impermeant biotinylation reagent. The data in this report do not address the issue of EAAC1 recruitment or recycling. Recycling kinetics have been investigated for a number of receptors, but only a few solute transporters. Of the latter, the most thoroughly investigated is the GLUT4 glucose transporter that resides in one or more intracellular compartments and recycles between them and the plasma membrane in response to insulin (28). The current data outlining the synthesis and degradation of EAAC1 as well as documenting an intracellular pool of the transporter will permit more detailed studies on recruitment and recycling in the future.

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