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Rapid report

Involvement of transporter recruitment as well as gene expression in the substrate-induced adaptive regulation of amino acid transport system A

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Abstract

We investigated the molecular mechanism involved in the adaptive regulation of the amino acid transport system A, a process in which amino acid starvation induces the transport activity. These studies were done with rat C6 glioma cells. System A activity in these cells is mediated exclusively by the system A subtype, amino acid transporter A2 (ATA2). The other two known system A subtypes, ATA1 and ATA3, are not expressed in these cells. Exposure of these cells to an amino acid-free medium induces system A activity. This process consists of an acute phase and a chronic phase. Laser-scanning confocal microscopic immunolocalization of ATA2 reveals that the acute phase is associated with recruitment of preformed ATA2 from an intracellular pool to the plasma membrane. In contrast, the chronic phase is associated with an induction of *ata2* gene expression as evidenced from the increase in the steady-state levels of ATA2 mRNA, restoration of the intracellular pool of ATA2 protein, and blockade of the induction by cycloheximide and actinomycin D. The increase in system A activity induced by amino acid starvation is blocked specifically by system A substrates, including the non-metabolizable α-(methylamino)isobutyric acid.

Keywords: System A; Adaptive regulation; Amino acid starvation; Transporter recruitment; Gene expression; Glioma cell

System A is a Na^+-dependent active transport system for neutral amino acids expressed in most tissues [1]. A unique characteristic of this system is its ability to recognize *N*-alkylated amino acids as substrates [2]. α-(Methylamino)isobutyric acid (MeAIB) is routinely used as a model substrate for system A. Even though system A has been well recognized for its regulation [3–5], this phenomenon has so far been studied only at phenomenological level. The lack of molecular tools such as transporter-specific antibodies and cDNA probes precluded studies on the molecular mechanisms involved in the regulatory processes. Only recently, the molecular identity of the amino acid transport system A has been established [6–13]. Interestingly, these recent studies have identified three distinct transporter proteins that are capable of mediating the Na^+-dependent transport of the system A model substrate MeAIB. The three transporters are known as amino acid transporter A

Abbreviations: ATA, amino acid transporter A; MeAIB, α-(methylamino)isobutyric acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction

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(ATA)1, ATA2, and ATA3 [6–13]. ATA1 and ATA2 possess similar functional characteristics, but exhibit differential tissue expression pattern. ATA1 is expressed primarily in the placenta and brain whereas ATA2 is expressed ubiquitously in mammalian tissues. In contrast, ATA3 is functionally distinguishable from ATA1 and ATA2. Furthermore, the expression of ATA3 is almost strictly restricted to the liver. Based on these findings, only ATA2 represents the ubiquitously expressed system A transporter that is particularly known for its regulation.

The present study was undertaken to investigate the molecular mechanisms involved in the substrate-induced adaptive regulation of ATA2. Phenomenologically, the term 'adaptive regulation' refers to a process in which the activity of system A is up-regulated when cells are cultured in an amino acid-free medium and restoration of amino acid availability to the cells reverses the up-regulation [3–5]. The ability to regulate system A in this process is almost strictly restricted to the substrates of system A. To study the molecular events involved in this process, we selected the rat C6 glioma cells because these cells express only ATA2. Absence of ATA1 and ATA3 makes these cells ideal for studies involving the regulation of ATA2.

C6 glioma cells (American Type Culture Collection, Manassas, VA, USA) were cultured in minimum essential medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were seeded in 24-well culture plates and characterization of the transport of [^{14}C]MeAIB (American Radiolabeled Chemicals, St. Louis, MO, USA) was done with confluent cells. For studies involving adaptive regulation, confluent cells were exposed to either the regular culture medium (without the fetal bovine serum) or an amino acid-free salt solution for desired time periods, following which the transport of MeAIB was measured. The composition of the amino acid-free salt solution was 25 mM HEPES/Tris (pH 7.5) containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose. The same salt solution, after adjusting the pH to 8.5, was used as the uptake buffer for measurement of MeAIB transport. Transport was measured at 37°C with a 15 min incubation as described previously [8,9]. The transport of MeAIB in these cells was linear even up to 45 min.

The expression of different subtypes of system A in the rat brain and liver and in C6 glioma cells was analyzed by reverse transcription-polymerase chain reaction (RT-PCR). Poly(A)$^+$ RNA was isolated from C6 glioma cells, rat cerebral cortex, rat cerebellum, and rat liver. RT-PCR was done with primer pairs specific for rat ATA1, ATA2, and ATA3. A primer pair specific for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primer pairs used were as follows: 5′-ACGACTCTAATGACTTCACGG-3′ (sense) and 5′-ACTTACTGTTGAGTTCTGTTC-3′ (antisense) for ATA1; 5′-AACTACTCATACCCCACGAAG-3′ (sense) and 5′-AAAGGTGCCATTCACCGTTTC-3′ (antisense) for ATA2; 5′-AGCATTCAAAGCTGCTACACC-3′ (sense) and 5′-GAGTGTAGTCCACCATGAAGT-3′ (antisense) for ATA3; 5′-ACCACAGTCCATGCCATCAC-3′ (sense) and 5′-TCCACCACCCTGTTGCTGTA-3′ (antisense) for GAPDH.

Northern blot hybridization was carried out using poly(A)$^+$ RNA isolated from control and treated C6 glioma cells. The mRNA samples were size-fractionated on a denaturing formaldehyde-agarose gel and probed with [^{32}P]labeled rat ATA2 cDNA and rat GAPDH cDNA.

An anti-peptide antibody specific for rat ATA2, generated in rabbit, was used for immunolocalization of ATA2 in C6 glioma cells. The sequence of the antigenic peptide is DEDSSSYSSNGDFNYSYPTKQAA. This amino acid sequence is located in the *N*-terminal tail of rat ATA2 (amino acid positions 14–36) and is significantly different from the corresponding sequences in rat ATA1 and rat ATA3. In this region, only six amino acids are identical between ATA2 and ATA3 and only one amino acid is identical between ATA2 and ATA1. To assess the specificity of the antibody, we expressed rat ATA1, ATA2, and ATA3 heterologously in human retinal pigment epithelial cells using the vaccinia virus expression system as described previously [8] and used the membranes from these cells for Western analysis (data not shown). Vector-transfected cells served as the negative control. The antibody detected a 48 kDa protein in cells expressing rat ATA2. There was no immunoreactive protein detectable in vector-transfected cells and in cells expressing rat ATA1 and rat ATA3.

For laser-scanning confocal microscopic analysis of ATA2, C6 glioma cells were seeded on eight-well chamber slides and grown to confluency. Confluent cells were then exposed either to the regular culture medium or to the amino acid-free salt medium for 1 or 8 h. After treatment, the cells were prepared for laser-scanning confocal microscopic immunodetection of ATA2 as described previously [14]. The cells were fixed with ice-cold methanol and blocked with 10% normal goat serum. Incubation with the anti-ATA2 antibody was done for 3 h at room temperature. Incubation with 0.1% normal rabbit serum served as negative controls. After rinsing, cells were incubated overnight at 4°C with a fluorescein isothiocyanate-conjugated AffiniPure goat anti-rabbit IgG. Cells were then optically sectioned (z series) using a Nikon Diaphot 200 Laser-scanning Confocal Imaging System (Molecular Dynamics, Sunnyvale, CA, USA). Analyses of images were done using the Image Display 3.2 software package (Silicon Graphics, Mountain View, CA, USA).

To determine at the functional level which of the three subtypes of system A is/are expressed in the rat C6 glioma cells, the transport characteristics of MeAIB were first elucidated. The transport of MeAIB in these cells was Na^+-dependent. The system was specific for short-chain neutral amino acids as well as for glutamine and asparagine, but did not interact with anionic and cationic amino acids. The Na^+:MeAIB stoichiometry was 1:1. The Michaelis–

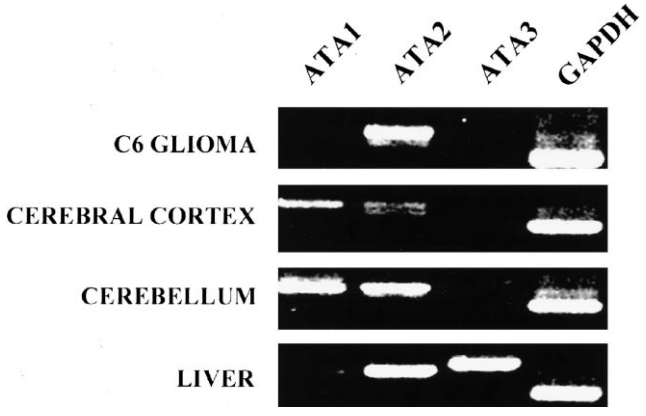

Fig. 1. Evidence for the expression of only ATA2 in C6 glioma cells. Poly(A)$^+$ RNA from C6 glioma cells, rat cerebral cortex, rat cerebellum, and rat liver was subjected to RT-PCR using primer pairs specific for rat ATA1, ATA2, ATA3 and GAPDH.

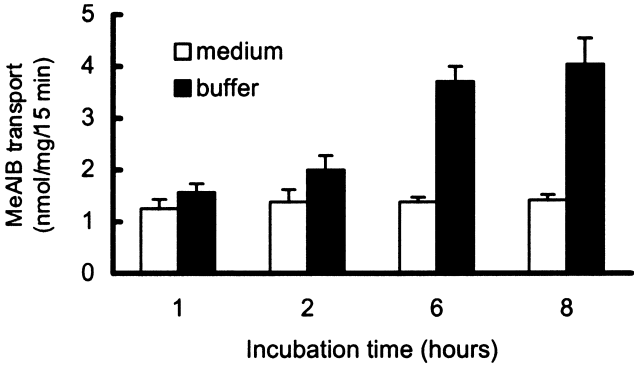

Fig. 2. Stimulation of system A activity induced by amino acid starvation. C6 glioma cells were exposed either to a complete medium (medium) or to an amino acid-free medium (buffer) for different time periods and then the transport of MeAIB (10 μM) was measured.

Menten constant (K_t) for MeAIB was 0.26 ± 0.02 mM. These functional characteristics of MeAIB transport in C6 glioma cells are similar to those described for ATA1 and ATA2 [6–11]. Both of these subtypes exclude anionic and cationic amino acids and exhibit a K_t value for MeAIB in the range of 0.2–0.3 mM. In contrast, ATA3 accepts cationic amino acids as substrates and has a K_t value for MeAIB in the range of 6–10 mM [12,13]. These data show that MeAIB transport in C6 glioma cells is mediated by ATA1 and/or ATA2, but not by ATA3.

Fig. 1 describes the results of RT-PCR for the molecular identity of the system A subtype that is expressed in C6 glioma cells. These cells express only ATA2 whereas cerebral cortex and cerebellum express both ATA1 and ATA2. The expression of ATA1 as well as ATA2 in rat brain corroborates the results from previous studies [6–11]. Rat liver was used as a positive control for ATA3 expression and, as expected, poly(A)$^+$ RNA from this tissue yielded the RT-PCR product for ATA3. RT-PCR was positive for GAPDH mRNA with all poly(A)$^+$ RNA samples. These data demonstrate that while the brain expresses ATA1 as well as ATA2, C6 glioma cells express only ATA2. ATA3 is not expressed in these cells nor in the brain.

The influence of amino acid starvation on the activity of system A was studied in C6 glioma cells by exposing the cells either to a complete medium or to an amino acid-free salt medium for varying periods of time and then assaying the transport of MeAIB. Exposure of the cells to the amino acid-free medium

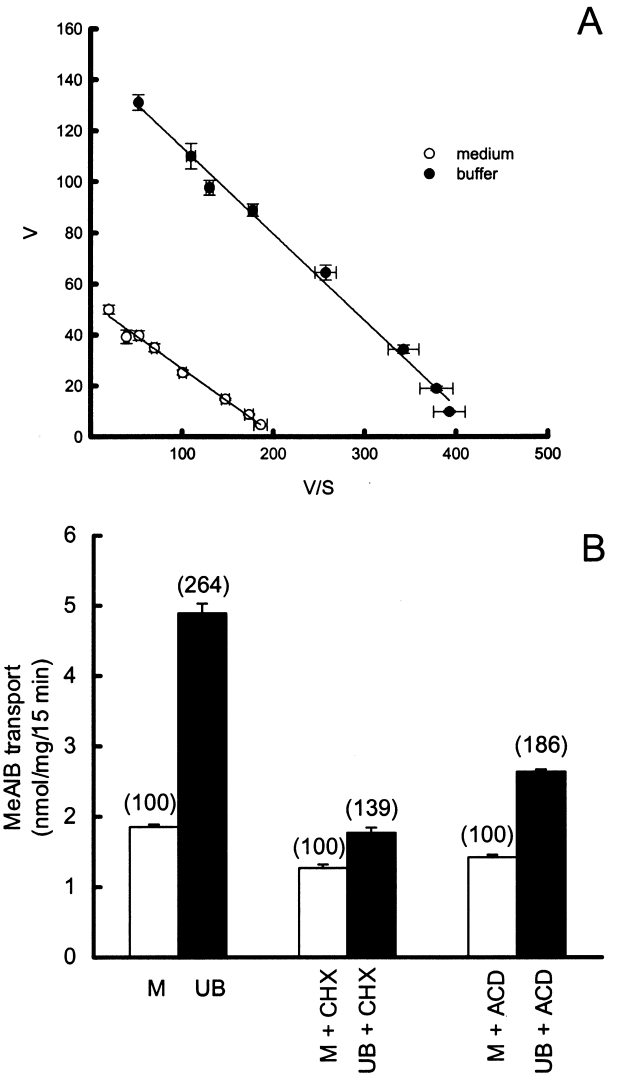

Fig. 3. (A) Saturation kinetics of MeAIB transport. C6 glioma cells were exposed either to a complete medium (medium) or to an amino acid-free medium (buffer) for 8 h and then the transport of MeAIB was measured over the concentration range of 0.025–2.5 mM. Results are given as Eadie–Hofstee plots. *V*, MeAIB transport in nmol/mg/15 min; *S*, MeAIB concentration in mM. (B) Influence of cycloheximide and actinomycin D. C6 glioma cells were first treated for 2 h with or without cycloheximide (CHX, 75 μg/ml) or actinomycin D (ACD, 7.5 μg/ml). Cells were then exposed either to a complete medium (M) or to an amino acid-free medium (UB) in the presence or absence of cycloheximide or actinomycin D for 8 h. The transport of MeAIB (10 μM) was then measured. Values in parentheses are percent of respective control transport measured in cells exposed to the complete medium.

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induced the activity of system A (Fig. 2). A significant induction (~25%) was seen even with 1 h exposure and the magnitude of induction increased as the exposure time increased. With 8 h exposure, the induction was 2.9-fold. There was no further change in the magnitude of induction when the exposure time was extended beyond 8 h (data not shown). The induction was specific for system A because the transport of glutamate and arginine was not affected under similar conditions (1 or 8 h exposure) (data not shown).

The saturation kinetics of MeAIB transport were then analyzed in control cells cultured in complete medium and in cells exposed to the amino acid-free salt medium for 8 h (Fig. 3A). The increase in the activity of system A caused by amino acid starvation was associated with an increase in the maximal velocity (V_{max}) of the transport system (control, 52.5 ± 1.3 nmol/mg of protein/15 min; amino acid starvation, 147.6 ± 2.8 nmol/mg of protein/15 min). The affinity of the transport system for MeAIB remained essentially unaltered. The values for K_t in control and in amino acid-starved cells were 0.26 ± 0.02 and 0.34 ± 0.01 mM, respectively. We then studied the possible role of RNA and protein synthesis in the adaptive regulation of system A. The influence of amino acid starvation on system A activity was attenuated significantly by cycloheximide (an inhibitor of protein synthesis) and actinomycin D (an inhibitor of RNA synthesis) (Fig. 3B). Taken collectively, these data show that chronic exposure (8 h) of the cells to an amino acid-free medium induces the system A activity most likely by enhancing de novo synthesis of the transporter protein and consequently increasing the density of the transporter protein in the plasma membrane.

We then investigated the ability of individual amino acids to reverse the influence of amino acid starvation on system A activity (Fig. 4A). As seen previously, exposure of the cells to an amino acid-free medium for 8 h induced system A activity by 2.5-fold. However, when the amino acid-free salt medium was supplemented with 1 mM MeAIB, glutamine, or asparagine, the induction was almost totally abolished. These three amino acids are substrates of system A and they were capable of reversing the influence of amino acid starvation on system A activity. In contrast, arginine was unable to reverse the

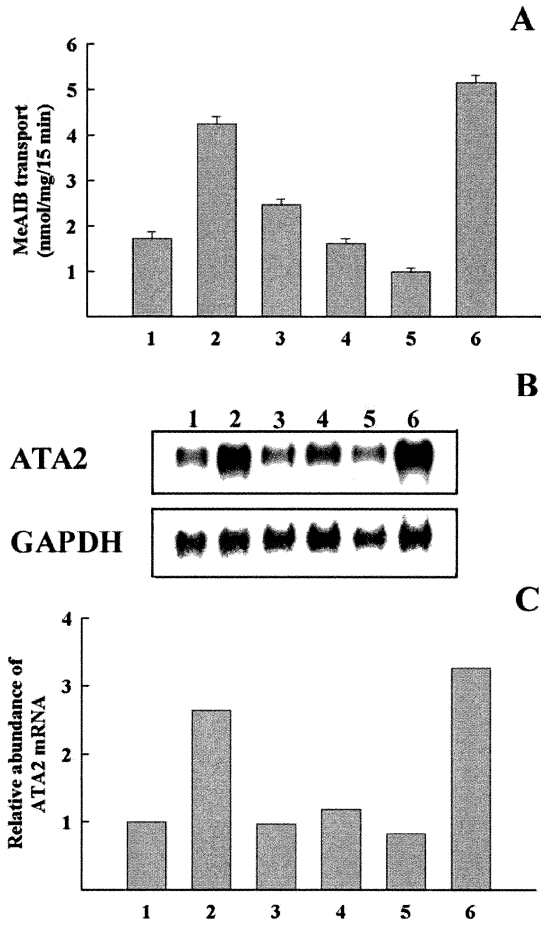

Fig. 4. Influence of system A substrates on amino acid starvation-induced changes in system A activity and in ATA2 mRNA levels. C6 glioma cells were exposed to a complete medium (1), an amino acid-free medium (2), or the amino acid-free medium supplemented with 1 mM MeAIB (3), glutamine (4), asparagine (5) or arginine (6) for 8 h. The transport of MeAIB (10 μM) was then measured. Poly(A)$^+$ RNA was isolated from these cells and subjected to Northern blot hybridization with [^{32}P]-labeled cDNA probes specific for ATA2 and GAPDH. (A) System A activity. (B) Hybridization signals for ATA2 mRNA and GAPDH mRNA. (C) Relative abundance of ATA2 mRNA after adjustment for variations in RNA loading by using GAPDH mRNA levels as the internal control.

influence of amino acid starvation. Arginine is not a substrate for ATA2. Northern blot analysis showed that amino acid starvation for 8 h increased the abundance of ATA2 mRNA by 2.7-fold. Addition of 1 mM MeAIB, glutamine, or asparagine to the amino acid-free salt medium abolished this increase in ATA2 mRNA abundance completely (Fig. 4B,C). Arginine did not have any effect on the increase in ATA2 mRNA abundance induced by amino acid starvation. These results show that chronic exposure of C6 glioma cells to an amino acid-free medium increases the steady-state levels of ATA2 mRNA and that the substrates of system A specifically reverse this effect.

We then analyzed the ATA2 protein expression in control cells cultured in the complete medium and in cells exposed to an amino acid-free salt medium (1 or 8 h). Laser-scanning confocal microscopic immunolocalization of the ATA2 protein showed that the increase in system A activity associated with acute exposure (1 h) of the cells to the amino acid-free medium is due to transporter recruitment from an intracellular pool to the plasma membrane (Fig. 5A,B). In cells cultured in the complete medium, ATA2 protein was detectable in the plasma membrane as well as in the cytoplasm (Fig. 5A, B). In contrast, the levels of ATA2 protein in the plasma membrane increased with a concomitant decrease in the cytoplasmic levels of the protein in cells exposed for 1 h to the amino acid-free medium (Fig. 5C, D). However, when the cells were exposed for 8 h to the amino acid-free medium, the cytoplasmic pool of the transporter protein was restored (Fig. 5G, H). The expression pattern of ATA2 in cells cultured for 8 h in the complete medium was not different from that in cells cultured for 1 h in the same complete medium (Fig. 5E, F). These results show that different mechanisms operate to increase system A activity in the acute and chronic phases of amino acid starvation. In the acute phase, amino acid starvation stimulates the recruitment of ATA2 from an intracellular pool to the plasma membrane, thus resulting in an increase in system A activity. The increase is small in magnitude, but the response is quick. This effect of amino acid starvation on the translocation of ATA2 is analogous to the influence of insulin on GLUT4 in adipocytes and skeletal muscle [15–17]. In the chronic phase, amino acid starvation activates *ata2* gene expression and enhances de novo synthesis of ATA2. This causes a much larger increase in system A activity compared to the acute phase response.

These studies describe for the first time the molecular mechanisms involved in the adaptive regulation of system A. The regulation consists of an acute phase involving the recruitment of preformed transporter protein from an intracellular pool to the plas-

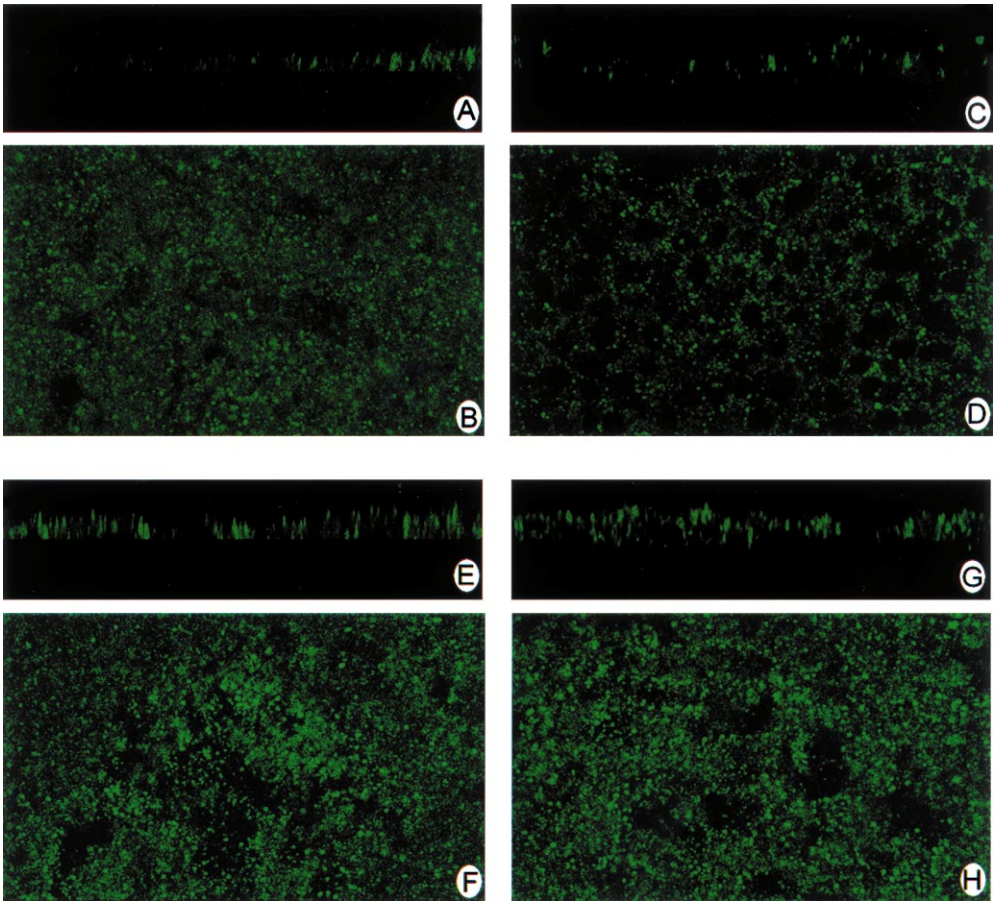

Fig. 5. Laser-scanning confocal microscopic immunolocalization of ATA2. C6 glioma cells were exposed either to a complete medium (A, B, E and F) or to an amino acid-free medium (C, D, G and H) for 1 h (A, B, C and D) or 8 h (E, F, G and H). Fluorescent immunolocalization was carried out with an antibody specific for rat ATA2. Optical sections were taken in a vertical plane (x and z) (A, C, E and G) as well as in a horizontal plane (x and y) (B, D, F and H).

ma membrane and a chronic phase involving activation of the transporter gene. A possible role of gene activation has been suggested previously as a mechanism of adaptive regulation of system A [3–5]. The present study provides unequivocal evidence for this mechanism. The involvement of protein recruitment in the adaptive regulation of system A is a novel finding in the present study. This may have relevance to the insulin-induced increase in system A activity in skeletal muscle [3–5]. Since insulin increases glucose transport in this tissue acutely by enhancing recruitment of preformed GLUT4, a similar mechanism may operate in the activation of system A in this tissue. Another interesting finding from the present study is the ability of MeAIB to reverse the activation of *ata2* gene expression induced by amino acid starvation. Previous studies have shown that MeAIB, though a non-metabolizable amino acid substrate for system A, is capable of reversing the increase in system A activity induced by amino acid starvation [5]. However, the underlying mechanism was thought to be *trans*-inhibition, a kinetic phenomenon that has no direct relevance to the process of adaptive regulation. The present study argues against this notion. MeAIB, though a non-metabolizable amino acid, is able to regulate the expression of the gene coding for ATA2. This raises an interesting question as to the mechanism involved in the process. It appears that the binding and translocation of the substrate via ATA2, irrespective of whether or not the substrate is metabolizable, is sufficient to provide the necessary signal to the cell to regulate the expression of the gene coding for the transporter. A possible mechanism for such a process may involve conformational

changes in the transporter protein induced by substrate binding and translocation which might influence the interaction of the protein with other signaling molecules inside the cell. Alternatively, there may be an amino acid sensor inside the cell that detects the intracellular concentrations of amino acids and transmits the signal to the nucleus to regulate *ata2* gene expression.

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