Biochimica et Biophysica Acta 1788 (2009) 1861–1868

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

The ATRA-dependent overexpression of the glutamate transporter EAAC1 requires RARβ induction

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ARTICLE INFO

Article history:
Received 16 February 2009
Received in revised form 29 April 2009
Accepted 8 May 2009
Available online 18 May 2009

Keywords:
EAAT3
C6 cells
Glioma
Retinoids
RXR
Slc1a1
Transport

ABSTRACT

The mechanisms underlying trafficking and membrane targeting of EAAC1, the rodent counterpart of the human EAAT3 carrier for anionic amino acids, are well characterized. In contrast, much less is known on the regulation of Slc1a1, the gene that encodes for the transporter. We have recently found that all-*trans* retinoic acid (ATRA) stimulates EAAC1 expression and anionic amino acid transport in C6 rat glioma cells. We report here that the ATRA effect on EAAC1 activity was inhibited by the specific RAR antagonist LE540 and mimicked by Am80, a RAR agonist, but not by the RXR agonist HX630. Moreover, the ATRA-dependent induction of Slc1a1 mRNA required the synthesis of a protein intermediate and was not associated with changes in the messenger half-life. ATRA treatment induced the expression of both Rarb mRNA and RARβ protein several hours before the induction of Slc1a1, while the mRNA for RFX1, a transcription factor recently involved in Slc1a1 transcription, was unchanged. In addition, Rarb silencing markedly inhibited the ATRA-dependent increase of both Rarb and Slc1a1 mRNAs. We conclude that in C6 glioma cells the induction of Slc1a1 by ATRA requires the synthesis of RARβ, suggesting that the receptor is involved in the regulation of the transporter gene.

1. Introduction

The glutamate transporter EAAT3 and its rodent counterpart EAAC1, although present in several cell types, are mainly expressed in central nervous system (CNS) neurons [1]. As other members of the EAAT family, EAAC1, which is encoded by the *Slc1a1* gene, is involved in the regulation of the extracellular glutamate concentration and in synaptic plasticity [2]. Alterations in the activity and/or expression of the transporter have been implied in several neurological and psychiatric conditions [3]. For these reasons, the regulation of EAAC1/EAAT3 activity has been investigated in a number of studies (see [3] for a recent review). Most of these involve rapid mechanisms that modify transporter trafficking between the plasma membrane and intracellular compartments, where most of the carrier proteins are located [4].

Much less is known about EAAC1/EAAT3 regulation at the gene level. Ma et al. [5] have shown that the transfection of rat glioma C6 cells with the human transcription factor RFX1 induces EAAC1 expression. More recently, we have found that in the same cell model the chronic exposure to all-*trans* retinoic acid (ATRA) causes a marked induction of Slc1a1, causing the increased abundance of EAAC1 carrier proteins and the proportional stimulation of transport activity [6]. Under the same conditions, no induction of Slc1a2 (for the GLT-1 transporter) or Slc1a3 (for the GLAST transporter) is observed [6], suggesting that inducibility by ATRA is a peculiarity of Slc1a1, at least in C6 cells. In this cell model ATRA treatment also causes the increased expression of Plp, a marker of the oligodendrocytic differentiation pathway and a well known ATRA target gene [7]. The induction of Plp mRNA by ATRA in C6 cells is a slow phenomenon, requires a protein intermediate and has been attributed to an increase in the half-life of the messenger [8].

A variety of genes are direct or indirect targets of retinoids and their expression is modulated by ATRA at both transcriptional and post-transcriptional levels. In particular, the transcription of direct ATRA targets is promoted by a complex formed by ATRA, a heterodimer of RAR–RXR receptors and several other co-stimulator molecules. Indirect regulation of gene expression by ATRA comprises various mechanisms such as (a) the induction of an intermediary (usually a transcription factor) that is directly responsible for the effect, (b) the modulation of mRNA stability, and (c) the interaction

Abbreviations: ATRA, all-*trans* retinoic acid; CNS, Central Nervous System; DMEM, Dulbecco's modified Eagle Medium; EAAC1, Excitatory Amino Acid Carrier 1; EAAT, Excitatory Amino Acid Transporter; EBSS, Earle's Balanced Salt Solution; FBS, Fetal Bovine Serum; Rarb, retinoic acid receptor, beta, gene; RAR, Retinoic Acid Receptors; RXR, Retinoid X Receptors

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doi:10.1016/j.bbamem.2009.05.005

with nuclear receptors other than the classical RAR–RXR dimers [9]. Moreover, retinoids can affect transcription through non genomic effects, such as the modulation of MAPK cascade [10] or of Akt signaling pathway [11].

The essential step required for the direct transcriptional effects of ATRA is represented by the interaction of the retinoid with the heterodimer formed by a member of RAR receptor family and a RXR receptor. For each of the three RAR subtypes (α, β, and γ), several isoforms exist that differ for their N-terminal regions [12]. There are two major isoforms for RARα (α1 and α2) and for RARγ (γ1 and γ2) and four major isoforms for RARβ (β1 and β3, initiated at the P1 promoter, and β2 and β4, initiated at the P2 promoter). Two isoforms also exist for each of the three RXR receptors thus far characterized (α, β, and γ) [13]. Upon the interaction with the ligand, the RAR–RXR heterodimer exchanges co-repressor partners with co-activators and the transcription of the target gene can start. While RARα is expressed in most tissues [12], the expression of RARβ and RARγ is tissue specific and highly regulated [14]. Interestingly, two of the RARβ isoforms, RARβ2 and 4, are transcribed from a promoter that contains a DR5 sequence and, hence, are inducible by retinoids [15,16]. Consistently, ATRA treatment markedly affects the expression of RARβ receptors in many cell types. In particular, in C6 cells ATRA stimulates the expression of one of the "heavy", 55 kDa isoform of RARβ, while RARα is not affected by the retinoid [17].

In this report we have studied the mechanisms underlying the effect of the retinoid on EAAC1, obtaining evidence that involves the synthesis and the activation of a RARβ receptor as one of the steps in the regulatory process.

2. Methods and materials

2.1. Cells and treatments

The rat CNS-derived C6 glioma cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown at 37 °C and 5% CO_2 in Dulbecco's modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 4 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were used for less than 10 passages from thawing with no apparent change in morphology or sensitivity to treatments.

For ATRA treatment, unless otherwise specified, culture medium was substituted 12 h after the passage with fresh medium supplemented with 0.1% FBS in the absence or in the presence of ATRA. ATRA, obtained from Sigma, was used at the concentrations detailed for each experiment starting from a 10 mM stock solution in DMSO. The RAR and RXR agonists were synthesized by one of us (HK) according to the methods reported previously [18–20]. The identification and purity of the compounds were determined by melting point, NMR, and Mass Spectroscopy. The compounds are very stable to heat, light, acids and bases and do not decompose under the experimental conditions adopted. RAR and RXR agonists and inhibitors were used at the concentrations detailed in the experiments starting from stock solutions in DMSO (10 mM, 10 mM, 1 mM, and 1 mM for, respectively, Am80, HX630, LE540 and HX531). In the controls, the vehicle was used at the maximal concentration adopted in the single experiment.

2.2. Transport activity

The initial influx of D-[^{3}H]aspartate, a high-affinity substrate of EAATs [1], was measured in 96-well multidish plates (Falcon, Becton, Dickinson Biosciences, Franklin Lakes, NJ, USA), where C6 cells had been seeded at a density of $10 \cdot 10^3$ cells/well, or in Falcon 24-well multidish plates, where cells had been seeded at a density of $50 \cdot 10^3$ cells/well. For the experiments, cells were washed twice in a modified EBSS (Earle's Balanced Salt Solution, containing (in mM) 117 NaCl, 5 KCl, 1.8 $CaCl_2$, 1 NaH_2PO_4, 0.8 $MgSO_4$, 5.5 glucose, buffered with 20 mM HEPES-NaOH at pH 7.4) and incubated in the same saline solution supplemented with D-[^{3}H]aspartate (1 μM, 2 μCi/ml) for 1 min.

At the end of the assay, multiwell dishes were washed twice with ice-cold urea (300 mM) and cell monolayers were extracted with absolute ethanol. The extracts were added to scintillation fluid and counted with a Wallac Trilux^2 liquid scintillation spectrometer (Perkin-Elmer, Boston, MA, USA). Cell proteins were determined directly in the well with a modified Lowry procedure and measured with a Wallac Victor^2 Multilabel Counter (Perkin-Elmer). Amino acid influx is expressed as pmoles mg of protein^{-1}·min^{-1}.

2.3. RT-qPolymerase Chain Reaction

Total RNA was isolated with RNeasy Mini Kit® (Qiagen S.p.a., Milan, Italy). After reverse transcription, 25 ng of cDNA from each sample was amplified in a total volume of 25 μl with 2× SYBRGreen qPCR SuperMix-ROX (Rovalab, Taltow, Germany), along with the following forward and reverse primers (5 pmol each) for the genes: 5′ CGA CTT GCC GTA CCT GGA CT 3′ and 5′ GCC CAC GGG ACT CAA CAC GA 3′ for Slc1a1; 5′ ATA CCC CAG AGC AAG ACA CC 3′ and 5′ AGC AGA TGG CAC TGA GAA GA 3′ for Rarb; 5′ ACT GCC TCT TTC TTC TTC CT 3′ and 5′ ATT TTC CCA AAC AAT GAC AC 3′ for Plp; 5′ AAC CAG AGA GCC GAT TTT AGA G 3′ and 5′ AAC TGT TGC TAC CCA CCC TAC T 3′ for Rfx1; 5′ AGC CTC AAG ATC ATC AGC AAT G 3′ and 5′ CAC GAT ACC AAA GTT GTC ATGGA 3′ for Gapdh. All the primers were designed with the help of Primer 3 program [21] according to the known or predicted (in the case of Rfx1) rat sequences reported in GenBank. Quantitative PCR was performed in a 36 well Rotor-Gene 3000 (Corbett Research, Rotor-Gene™ 3000, version 5.0.60, Mortlake, Australia). For all the probands each cycle consisted of a denaturation step at 95 °C for 30 s, followed by separate annealing (30 s) and extension (30 s) steps at a temperature characteristic for each proband. Fluorescence was monitored at the end of each extension step. A no template, no-reverse transcriptase control was included in each experiment. At the end of the amplification cycles a melting curve analysis was added. The analysis of the data was made according to the Relative Standard Curve Method [22]. qRT-PCR data were expressed as the ratio between proband mRNA and Gapdh mRNA.

2.4. Western analysis

For RARβ expression, protein extraction and quantification were performed with the method of Farinha et al. [23] with minor modifications. C6 cells, grown to subconfluence on 10-cm tissue culture plates, were rinsed twice in PBS and lysed in 300 μl of Sample Buffer 1× (31.25 mM Tris–HCl pH 6.8, 3% SDS, 10% glycerol, 100 mM DTT, 0.02% bromophenol blue). After solubilization, cell lysates were collected in Eppendorf tubes and passed 10 times into 25 G and then 27 G needles. The quantification of proteins was performed with a modified Lowry assay [23]. Briefly, the protein sample was added with 1 ml of H_2O and 100 μl of sodium deoxycholate (0.15%) and the mixture incubated for 10 min. At the end of this period, 100 μl of 72% trichloroacetic acid was added to the sample that was vortexed and span at 14,000 rpm for 5 min at room temperature. The supernatant was removed and the pellet was resuspended in 400 μl of H_2O, supplemented with 400 μl of reagent A (1 volume of CTC; 1 volume of 10% sodium dodecyl sulphate; 1 volume of 0.8 M NaOH; 1 volume of H_2O, where CTC is a mixture of 25 ml of 0.4% $CuSO_4 \cdot 5H_2O$, 25 ml of 0.8% potassium tartrate, and 200 ml of 5% Na_2CO_3), incubated for 10 min, and added with 200 μl of Reagent B (1 volume of Folin–Ciocalteau reagent + 4 volumes of H_2O). Absorbance at 750 nm was read after 30 min and protein content was calculated from bovine serum albumin standards. Aliquots of 60 μg were loaded on a 10% gel for SDS-PAGE. After the electrophoresis, proteins were transferred to nitrocellulose membranes (Schleicher and Schuell Bioscience, Dassel,