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GLAST stability and activity are enhanced by interaction with the PDZ scaffold NHERF-2

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ABSTRACT

The astrocytic glutamate transporter GLAST (also known as EAAT1) is a key regulator of extracellular glutamate levels in many regions of vertebrate brains. To identify novel interacting partners that might regulate the localization and function of GLAST in astrocytes, we screened the transporter's C-terminus (GLAST-CT) against a proteomic array of 96 different PDZ domains. The GLAST-CT robustly and specifically interacted with PDZ domains from two related scaffolding proteins, the Na^+/H^+ exchanger regulatory factors 1 and 2 (NHERF-1 and NHERF-2). Studies on cultured rat cortical astrocytes revealed that these cells are highly enriched in NHERF-2 relative to NHERF-1. Endogenous GLAST and NHERF-2 from cultured astrocytes were found to robustly co-immunoprecipitate, and further co-immunoprecipitation studies on mutant versions of GLAST expressed in transfected cells revealed the GLAST/NHERF-2 interaction to be dependent on the last amino acid of the GLAST-CT. Knockdown of endogenous NHERF-2 in astrocytes via siRNA treatment resulted in a significant reduction in GLAST activity, which corresponded to significantly reduced total expression of GLAST protein and reduced half-life of GLAST, as assessed in pulse-chase metabolic labeling studies. These findings reveal that NHERF-2 can interact with GLAST in astrocytes to enhance GLAST stability and activity.

Glutamate is the most abundant neurotransmitter in the mammalian central nervous system and the mediator of excitatory neurotransmission at the majority of synapses in the brain. The extracellular concentration of glutamate must be tightly regulated, however, as excessive glutamate signaling can lead to excitotoxic cellular death [4]. Five transporters have been identified as the principal regulators of extracellular glutamate levels and therefore have been named excitatory amino acid transporters EAAT-1 (rat homologue known as GLAST), EAAT-2 (rat homologue known as GLT-1), EAAT-3, EAAT-4, and EAAT-5. Genetic studies have shed light on the functions of the individual transporter sub-types, revealing a dominant role for GLAST and GLT-1 in controlling synaptic glutamate levels [16,21]. Interestingly, GLAST and GLT-1 are exclusively expressed in astrocytes [3,12], thereby speaking to the importance of astrocytes in clearing synaptic glutamate. Moreover, mutations in GLAST are linked with a variety of disease states, including epilepsy, stroke, Alzheimer's Disease, Parkinson's Disease, and schizophrenia [2], further highlighting the importance of understanding GLAST function.

We set out to identify novel interacting partners that might regulate the selective localization and function of GLAST in astrocytes. Since the C-terminus (CT) of GLAST possesses a consensus motif for association with PDZ domains, a family of protein-protein interaction domains named after the first three proteins in which they were identified (post-synaptic density protein of 95 kDa, discs-large, and zona-occludens 1), we screened the GLAST-CT against a proteomic array of PDZ domains in order to identify novel GLAST interacting partners.

Overlays of the PDZ domain array were performed as previously described [6,8]. Briefly, 1 μg of His- and S-tagged PDZ domain fusion proteins were spotted onto nitrocellulose, dried overnight, and overlaid with GST-alone (control) or GST-GLAST-CT. Membranes were washed and incubated with an HRP-coupled anti-GST monoclonal antibody (Amersham Pharmacia Biotech) and binding was visualized using enhanced chemiluminescence.

HEK-293 (ATCC) cells were cultured in Dulbecco's modified Eagle medium containing GlutaMAX™, 10% fetal bovine serum (Atlanta Biologicals) and 1% penicillin/streptomycin, and were maintained at 37 °C in an atmosphere of 95% air/5% CO_2. Transfections were performed with Lipofectamine 2000, as previously

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described [10]. DNA constructs used were rat GLAST in pcDNA3 (kindly provided by Jeff Rothstein, Johns Hopkins), human FLAG-GLAST (kindly provided by Susan Amara, Univ. Pittsburgh), and rabbit HA-NHERF-2 in pBK-CMV.

Purified primary astrocyte cultures were prepared by the method of McCarthy and de Vellis [14]. All animal work was performed under the guidance of the National Institute of Health Guide for the Care and Use of Laboratory Animals. Briefly, neocortices were dissected from E18-19 Sprague Dawley rat embryos and dissociated in medium by trituration. The cells were re-suspended in GlutaMAX™ DMEM (10% FBS and 1% Pen/Strep) on poly-D-lysine coated tissue culture flasks. The medium was changed the next day and then every 48 h. After 6–8 days, cells were shaken overnight (280–310 rpm) to remove microglia and oligodendrocytes and passaged 48 h later. Immunostaining with the astrocytic marker GFAP revealed that this culturing method results in at least 90% purified GFAP-positive astrocyte cultures (data not shown).

Overlay assays, immunoprecipitation experiments, and Western blotting were performed as previously described [10]. For overlay experiments, 1 μg of purified protein was separated by SDS-PAGE gel electrophoresis, transferred onto nitrocellulose, and overlaid with purified His/S-tagged NHERF-2-PDZ domain 2 fusion protein (prepared using a pET30A construct and corresponding to amino acids 149-337 of human NHERF-2) in increasing concentrations. Binding of His/S-tagged NHERF-2 to GLAST was visualized with S-protein HRP and quantified via densitometry. Numbers were expressed as percentage of maximum binding to generate binding curves for affinity estimates.

Immunoprecipitation experiments were performed using anti-FLAG M2 affinity gel (Sigma), or NHERF-2 antibody bound to Protein A/G agarose beads (Pierce). Astrocyte lysates were also incubated with purified NHERF-2 or GST-alone bound to beads for pull-downs. For all experiments, cells were lysed with a buffer containing 50 mM NaCl, 20 mM Hepes, 5 mM EDTA, 1 protease inhibitor cocktail tablet (Roche Diagnostics), 1% Triton X-100, pH 7.4. To detect endogenous and/or recombinant NHERF-1, NHERF-2, and GLAST, the reagents used were S-protein HRP conjugate (1:4,000, Novagen), polyclonal guinea pig anti-GLAST (1:10,000, Chemicon), rabbit polyclonal anti-GLAST (H-50, Santa Cruz), rabbit anti-NHERF-1 (1:7000) and rabbit anti-NHERF-2 (1:5000) [24].

Mutagenesis of the last amino acid of the GLAST (Amara construct) from a methionine to an alanine (M542A) was performed using the QuikChange Site-directed mutagenesis kit (Stratagene). Primers used for PCR were 5′-GACAGCGAAACCAAGGCGTAG-3′ and its reverse complement. Sequencing was performed to confirm mutation of the target bases. Knockdown of NHERF-2 was accomplished using TransIT-siQuest transfection reagent (Mirus). NHERF-2 siRNA was Silencer pre-designed siRNA (ID# 55838; Ambion). Control siRNA was Silencer Negative Control #1 siRNA (Ambion).

For the amino acid uptake experiments, astrocytes were incubated at 37 °C in assay buffer containing 1 mM of the GLT-1-selective inhibitor dihydrokainate (DHK) (Tocris), followed by increasing concentrations of [^{3}H]aspartate for 6 min in a 37 °C water bath. Uptake was stopped with three washes of ice-cold buffer containing choline chloride in replacement of sodium chloride. Cells were lysed with 0.5 M NaOH and samples were counted in 30% Scintisafe (Fisher).

Pulse-chase assays were performed as previously described [1]. Briefly, primary astrocytes that had been treated with either control siRNA or NHERF-2 siRNA were incubated in methionine-free DMEM for 30 min, then 60 μCi of Redivue L-[^{35}S]-methionine (Amersham Biosciences) was added to each plate to incubate at 37 °C for an additional 30 min. Cells were washed and immediately chased with 3 mM cold L-methionine for various time points (0, 2, 4, 8, 12, 24, 48, and 72 h). Samples were adjusted to normalize for protein concentration, GLAST was immunoprecipitated using guinea pig anti-GLAST antibody, and the amount of ^{35}S incorporated into the GLAST protein was determined.

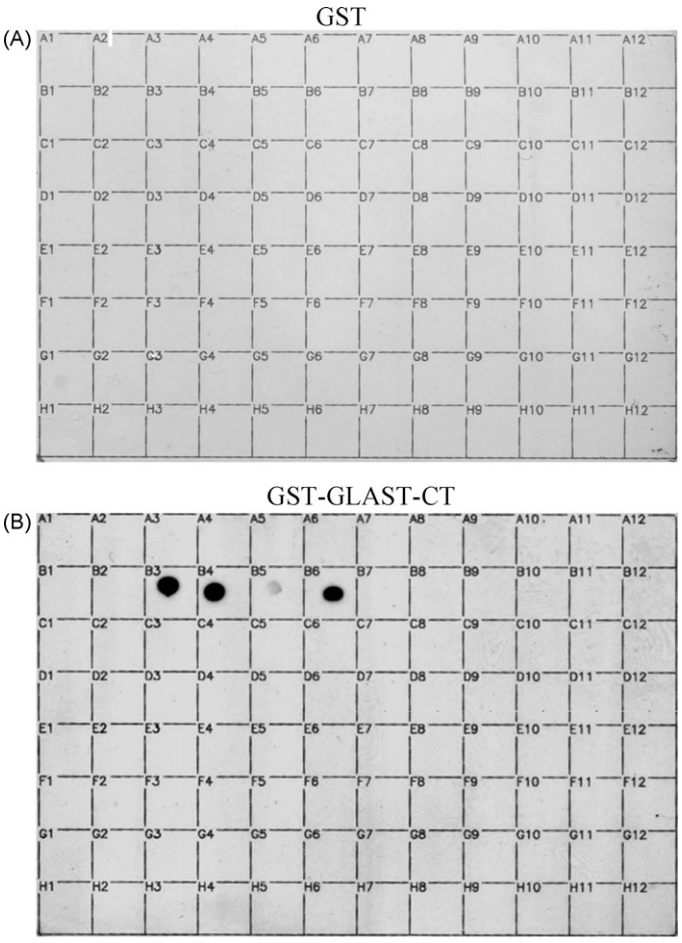

Fig. 1. The C-terminus of GLAST binds selectively to NHERF PDZ domains. Control GST (A) or a GST-fusion protein comprising the last 25 amino acids of GLAST (B) were overlaid at 100 nM onto a proteomic array containing 96 distinct PDZ domains ($n = 3$). A complete list of the PDZ proteins on this array has been described previously by He et al. [8].

In order to gain a panoramic view of potential PDZ interactions for GLAST, we screened a GST-fusion protein of the GLAST-CT against a previously described PDZ proteomic array containing 96 distinct PDZ domains (Fig. 1). The GLAST-CT did not detectably bind to the vast majority of PDZ domains on the array. However, strong binding of the GLAST-CT was observed to both PDZ domains of the Na^+/H^+ exchanger regulatory factor (NHERF-1), as well as to PDZ domain 2 of the related protein NHERF-2 (Fig. 1). Weaker binding was also observed to the first PDZ domain of NHERF-2. The interaction of GLAST with NHERF-1 has been reported previously [11], but the association of GLAST with NHERF-2 is novel. Thus, we characterized the GLAST/NHERF-2 interaction in further detail.

Saturation binding assays overlaying His-tagged NHERF-2-PDZ2 onto purified GLAST-CT-GST revealed the K_D of the GLAST/NHERF-2-PDZ2 interaction to be 196 nM (Fig. 2A). This affinity is in the range of other PDZ domain-mediated associations that are known to be physiologically relevant [18]. Furthermore, full-length GLAST from astrocyte lysates was robustly pulled down by GST-NHERF-2 (Fig. 2B). NHERF-2 seemed to preferentially interact with oligomers of GLAST in these experiments, but it should be noted that it is unclear whether oligomers of GLAST observed in SDS-PAGE gels correspond to oligomers of GLAST in living cells.