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DISTRIBUTION AND ULTRASTRUCTURAL LOCALIZATION OF GEC1 IN THE RAT CNS

Y. WANG,[a] S. L. DUN,[a] P. HUANG,[a] C. CHEN,[a] Y. CHEN,[a] E. M. UNTERWALD,[a] N. J. DUN,[a] E. J. VAN BOCKSTAELE[b] AND L.-Y. LIU-CHEN[a]*

[a]*Department of Pharmacology, School of Medicine, Temple University, 3420 North Broad Street, Philadelphia, PA 19140, USA*

[b]*Department of Neurosurgery, Farber Institute for Neurosciences, Thomas Jefferson University, Philadelphia, PA 19107, USA*

Abstract—We have previously demonstrated that GEC1 interacts with the κ opioid receptor and GEC1 expression enhances cell surface expression of the receptor in Chinese hamster ovary cells. In this study, we generated an antiserum (PA629) directed against GEC1 in rabbits, characterized its specificity, and investigated distribution of GEC1 in tissues and in brain regions and spinal cord and its subcellular localization in hypothalamic neurons in the rat. Immunofluorescence staining demonstrated that PA629 recognized HA-GEC1 transfected into Chinese hamster ovary cells, but not HA-GABARAP or HA-GATE-16, although the three share high homology. Pre-incubation of PA629 with GST-GEC1, but not GST, abolished the staining. In immunoblotting, affinity-purified PA629 (PA629p) recognized GEC1, GABARAP and GATE-16. GEC1 migrated slower than GABARAP and GATE-16, with a M_r of 16 kDa for GEC1 and M_r of 14 kDa for GABARAP and GATE-16. Immunoblotting results showed that GEC1 level was higher in liver and brain than in lung and heart, and very low in kidney and skeletal muscle. GEC1 was present in all rat brain regions examined and spinal cord. Immunohistochemistry demonstrated that GEC1 immunoreactivity was distributed ubiquitously in the rat CNS with highly intense immunoreactivity in various brain nuclei and motor neurons of the spinal cord. Ultrastructural examination of neurons in the paraventricular nucleus of the hypothalamus showed that GEC1 was associated with endoplasmic reticulum and Golgi apparatus and distributed along plasma membranes and in cytosol. Coupled with our previous observation that GEC1 interacts with N-ethylmaleimide-sensitive factor, these findings strongly suggest that GEC1 functions in intracellular trafficking in the biosynthesis pathway and perhaps also the endocytic pathway. The widespread distribution of GEC1 suggests that GEC1 may be associated with many proteins, in addition to the κ opioid receptor.

*Corresponding author. Tel: +1-215-707-4188; fax: +1-215-707-7068.
E-mail address: lliuche@temple.edu (L.-Y. Liu-Chen).

Abbreviations: CHO, Chinese hamster ovary; DAB, 3,3′-diaminobenzidine; ER, endoplasmic reticulum; $GABA_A$ receptor, GABA type A receptor; GABARAP, $GABA_A$ receptor associated protein; GATE-16, Golgi-associated ATPase enhancer of 16 kDa; GEC1, glandular epithelial cell 1; GFAP, glial fibrillary acidic protein; GST, glutathione-*S*-transferase; IR, immunoreactivity; KOR, κ opioid receptor; NeuN, neuronal nuclei; PBS, phosphate-buffered saline; RT, room temperature; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TBS/T, Tris-buffered saline containing 0.1% Tween 20.

Key words: GABARAPL1, GABARAP, electron microscopy, kappa opioid receptor, NSF.

We have recently demonstrated that GEC1 (glandular epithelial cell 1) is associated with the κ opioid receptor (KOR) and expression of GEC1 enhances cell surface expression of the receptor (Chen et al., 2006). The cDNA encoding GEC1 was first isolated as an early estrogen-upregulated molecule from the cDNA library of the cultured guinea-pig endometrial glandular epithelial cells (Pellerin et al., 1993). Its level in these cells was increased by two-fold after 17β-estradiol treatment for 2 h in the presence of cycloheximide, which prolongs and superinduces early genes. The GEC1 gene was then identified by screening a guinea-pig genomic library (Vernier-Magnin et al., 2001). The human GEC1 cDNA was cloned from placenta and was named $GABA_A$ receptor-associated protein-like 1 (*GABARAPL1*) due to its high homology with the cDNA encoding $GABA_A$ receptor associated protein (*GABARAP*) (79% identity in nucleotide sequence) (Xin et al., 2001). The term GEC1 is used in this paper.

GEC1 is a small protein of 117 amino acids and has identical amino acid sequences across the species cloned to date, including frog (AAH72921), mouse (NP_065615), rat (XP_216288), guinea-pig (AAL32264), cattle (NP_001028788) and human (Q9H0R8), indicating that GEC1 is highly conserved in evolution. GEC1 belongs in a family of microtubule-associated proteins, which include the mammalian proteins GABARAP and Golgi-associated ATPase enhancer of 16 kDa (GATE-16) and the yeast protein Atg8. The amino acid sequence of GEC1 is similar to GABARAP (87% identity) and GATE-16 (61% identity). GABARAP interacts with the γ2 subunit of the GABA type A ($GABA_A$) receptor. The interaction promotes clustering of the receptor, alters its channel kinetics (Wang et al., 1999; Chen et al., 2000) and enhances its trafficking to the plasma membrane in neurons (Leil et al., 2004). GATE-16 plays an important role in intra-Golgi transport (Legesse-Miller et al., 1998; Sagiv et al., 2000). GEC1 was reported to interact with tubulin and promote its assembly *in vitro* (Mansuy et al., 2004). GEC1 also interacts with the γ2 subunit of $GABA_A$ receptor (Mansuy et al., 2004). In the yeast, Atg8 is involved in autophagy, a process that involves membrane engulfment of intracellular organelles followed by degradation during starvation (Kirisako et al., 1999).

GEC1 mRNA is distributed widely in tissues, as demonstrated by northern blot analysis (Xin et al., 2001; Vernier-Magnin et al., 2001) with the highest levels in the heart, brain, liver, skeletal muscle, kidney, placenta and

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peripheral blood leukocyte. The level of GEC1 mRNA was higher in human CNS than that of GABARAP mRNA (Nemos et al., 2003). However, to date, there is no report on the distribution of GEC1 protein, which may not correlate with that of mRNA in all tissues. In this study, we generated a rabbit antiserum against GST-GEC1 and found that it recognized GEC1 with high specificity in immunocytochemistry, but detected GEC1, GABARAP and GATE-16 equally in immunoblotting. We then investigated the distribution of GEC1 in the rat CNS. The ultrastructural localization of GEC1 was also examined using electron microscopy.

EXPERIMENTAL PROCEDURES

Animals and chemicals

Male adult Sprague–Dawley rats (200–250 g) were purchased from Ace Animals, Inc. (Boyertown, PA, USA). The animal experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals issued by the National Institutes of Health (USA) and were reviewed and approved by the Institutional Animal Care and Use Committees at Temple University and Thomas Jefferson University. Efforts were made to minimize animal suffering and the number of animals used. All the chemicals were purchased from Sigma-Aldrich, Corporation (St. Louis, MO, USA), unless indicated otherwise. The sources of other reagents are indicated in the methods in which they were used.

cDNA clones

The cDNA clones encoding the human GEC1 and GATE-16 were obtained by RT-PCR of RNA isolated from human embryonic kidney cells (HEK293). The human GABARAP cDNA was amplified from the cDNA clone kindly provided by Dr. Richard W. Olsen (University of California Los Angeles, Los Angeles, CA, USA). All three cDNA clones, with or without influenza hemagglutinin-HA-epitope added N-terminal to the N terminus, were cloned into *Eco*RI/*Xho*I sites of the vector pcDNA3.1/hygromycin (Invitrogen Corporation, Carlsbad, CA, USA). GEC1(38–117), the truncated form of GEC1 which encoded 38–117 of amino acids of GEC1, was also cloned into the same vector. cDNA clones of GEC1 and GABARAP were also cloned into pGEX-4T-1 glutathione *S*-transferase (GST) fusion vector (Amersham Biosciences, Piscataway, NJ, USA).

Protein expression and purification

GST, GST-GEC1 and GST-GABARAP cDNA clones in the vector pGEX-4T-1 were transformed into *E. coli* BL21-CodonPlus (DE3)-RP (Stratagene, La Jolla, CA, USA) and purified from *E. coli* using Glutathione Sepharose HP kit (Amersham Biosciences). GST-GEC1 and GST-GABARAP were digested with thrombin to cleave GST and GST was removed according to manufacturer's instructions (Amersham Biosciences). The cleavage by thrombin left two additional amino acids, Gly-Ser, N-terminal to the N-terminus of GEC1 and GABARAP sequences. Protein concentrations were determined by BCA assay (Pierce, Rockford, IL, USA) using bovine serum albumin for the standard curve.

Antiserum generation and purification

Antiserum against GEC1 was raised in New Zealand White rabbits by Covance Inc. (Princeton, NJ, USA) using GST-GEC1 as the antigen. PA629, antiserum from one of the rabbits, was used in the study. Pre-immune serum from the same rabbit and antiserum preincubated with GST-GEC1 (see below) were used as controls. Part of the antiserum was purified using Affi-Gel gel (Bio-Rad Laboratories, Inc., Hercules, CA, USA) conjugated with GEC1 protein according to manufacturer's instructions. The affinity-purified antibody was designated as PA629p.

Preabsorption of antiserum

PA629 for immunohistochemistry or PA629p for Western blot was incubated with about 5 μg/ml of GST or GST-GEC1 in antibody dilution buffer on a shaker overnight at 4 °C. The mixture was centrifuged at 15,000×*g* for 30 min at 4 °C and the supernatant was used in the indicated experiments.

Immunocytofluorescence

Chinese hamster ovary (CHO) cells were grown in Lab-Tek II eight-well slide chambers (Nalge Nunc International, Rochester, NY, USA). HA-tagged GEC1, GABARAP or GATE-16 was transfected into the cells with Lipofectamine 2000 (Invitrogen Corporation) according to the manufacturer's instructions. Twenty-four hours later, cells were fixed with 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS, pH 7.4) for 15 min at room temperature (RT), washed three times with 0.01 M PBS and incubated with blocking solution A (10% normal goat serum in PBS containing 0.1% Triton X-100) for 10 min. Cells were incubated with PA629 (1:3000) and monoclonal anti-HA antibody HA11 (1:1000) (Covance Inc.) for 2 h at RT. After thorough washing with PBS, cells were incubated with both goat anti-rabbit IgG conjugated with Texas Red and goat anti-mouse IgG conjugated with AlexaFluor488 (1:500) (Invitrogen Corporation) for 1 h at RT. Cells were rinsed several times and coverslipped with Citifluor (Ted Pella, Inc., Redding, CA, USA). Coverslips were sealed with nail polish. Cells were examined under an Eclipse TE300 fluorescence microscope (Nikon Inc., Japan) and images were captured with a CCD camera. Annotations and minimal adjustment of brightness and contrast were made with Photoshop Elements (Adobe Systems Inc., San Jose, CA, USA).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein staining

Five hundred nanograms of GEC1 and GABARAP were mixed with an equal volume of 2× Laemmli sample buffer (4% SDS, 0.1 M DTT, 20% glycerol, 62.5 mM Tris, pH 6.8) and subjected to 12% SDS-PAGE in Tricine–HCl buffer system (Schagger and von Jagow, 1987). The gels were washed with deionized water followed by incubation with GelCode Blue Stain Reagent (Pierce Co.) for 1 h. The gels were destained by washing with deionized water until good clarity was achieved.

Immunoblotting of GEC1 and GABARAP with PA629p

GEC1 or GABARAP was mixed with 2× Laemmli sample buffer and 1 ng protein was subjected to 12% SDS-PAGE in Tricine–HCl buffer system. The protein was then transferred onto Immobilon-P membranes (Millipore Corporation, Billerica, MA, USA) and membranes were incubated with the blocking solution B [5% non-fat dried milk in Tris-buffered saline (TBS, 150 mM NaCl, 25 mM Tris, pH 7.5) containing 0.1% Tween 20 (TBS/T)] for 30 min at RT to block nonspecific interaction. Membranes were then incubated with PA629p (1:10,000) in the blocking solution B at 4 °C overnight with agitation. Membranes were washed with TBS/T three times followed by incubation with goat anti-rabbit IgG conjugated with horseradish peroxidase (1:1000, Cell Signaling Technology, Inc., Beverly, MA, USA) for 2 h at RT. After washed with TBS/T, membranes were incubated with SuperSignal West chemiluminescent substrate (Pierce Co.) for 3–5 min and protein bands were visualized using Image Reader LAS-1000 plus image sys-