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SPECIFICITY OF ANTIBODIES: UNEXPECTED CROSS-REACTIVITY OF ANTIBODIES DIRECTED AGAINST THE EXCITATORY AMINO ACID TRANSPORTER 3 (EAAT3)

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Abstract—Specific antibodies are essential tools for identifying individual proteins in biological samples. While generation of antibodies is often straightforward, determination of the antibody specificity is not. Here we illustrate this by describing the production and characterization of antibodies to excitatory amino acid transporter 3 (EAAT3). We synthesized 13 peptides corresponding to parts of the EAAT3 sequence and immunized 6 sheep and 30 rabbits. All sera were affinity purified against the relevant immobilized peptide. Antibodies to the peptides were obtained in almost all cases. Immunoblotting with tissue extracts from wild type and EAAT3 knockout animals revealed that most of the antibodies did not recognize the native EAAT3 protein, and that some recognized other proteins. Several immunization protocols were tried, but strong reactions with EAAT3 were only seen with antibodies to the C-terminal peptides. In contrast, good antibodies were obtained to several parts of EAAT2. EAAT3 was only detected in neurons. However, rabbits immunized with an EAAT3-peptide corresponding to residues 479–498 produced antibodies that labeled axoplasm and microtubules therein particularly strongly. On blots, these antibodies recognized both EAAT3 and a slightly smaller, but far more abundant protein that turned out to be tubulin. The antibodies were fractionated on columns with immobilized tubulin. One fraction contained antibodies apparently specific for EAAT3 while another fraction contained antibodies recognizing both EAAT3 and tubulin despite the lack of primary sequence identity between the two proteins. Addition of free peptide to the incubation solution blocked immunostaining of both EAAT3 and tubulin. Conclusions: Not all antibodies to synthetic peptides recognize the native protein. The peptide sequence is more important than immunization protocol. The specificity of an antibody is hard to predict because cross-reactivity can be specific and to unrelated molecules. The antigen preabsorption test is of little value in testing the specificity of affinity purified antibodies.

Key words: glutamate uptake, immunocytochemistry, polyreactive, antibodies, tubulin, specificity testing, oligodendrocyte.

The amino acid glutamate is the major excitatory neurotransmitter in the mammalian CNS. The only significant mechanism for inactivation of extracellular glutamate appears to be cellular uptake mediated by a family of five glutamate (excitatory amino acid) transporter proteins (EAAT1–5; for review see: Danbolt, 2001). EAAT3 is expressed in neurons (Kanai and Hediger, 1992; Rothstein et al., 1994; Shashidharan et al., 1997; He et al., 2001), including GABAergic ones, in most parts of the nervous system. EAAT3 is concentrated in the neuronal cell bodies (somata) and dendrites apparently avoiding the nerve terminals. Later studies (Conti et al., 1998; Kugler and Schmitt, 1999) have confirmed these findings, but have reported that astrocytes of the cerebral cortex and white matter also express EAAT3 (Conti et al., 1998). Kugler and Schmitt (1999) detected the protein in oligodendrocytes and noted co-localization with tubulin using an antibody directed to a synthetic peptide corresponding to residues 480–499 of rat EAAT3.

We have previously produced antibodies to EAAT1, EAAT2 and EAAT4, and used them to identify the transporter proteins in tissue sections and protein extracts (e.g. Danbolt et al., 1992; Levy et al., 1993; Lehre et al., 1995; Dehnes et al., 1998; Lehre and Danbolt, 1998). In parallel with this work, we have also generated antibodies to EAAT3 by immunizing animals with synthetic peptides corresponding to different parts of the EAAT3 protein sequence. Here we describe the production and testing of the latter antibodies in order to demonstrate some of the difficulties in determining the specificity of an antibody. We show that rabbits immunized with a peptide corresponding to residues 479–498 of rat EAAT3 gave rise to antibodies recognizing both EAAT3 and tubulin. Using antibodies specific to EAAT3, no EAAT3 immunoreactivity was detected in oligodendrocytes in contrast to the previous report based on antibodies to EAAT3 residues 480–499 (Kugler and Schmitt, 1999).

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Abbreviations: BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate; EAAC1, rabbit glutamate transporter (Kanai and Hediger, 1992); EAAT, excitatory amino acid transporter (=glutamate transporter); EDTA, sodium ethylenediamine tetraacetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HSA, human serum albumin; KLH, keyhole limpet hemocyanin; Map, multiple antigenic peptide; MBP, myelin basic protein; MBS, *m*-maleimido benzoyl-*N*-hydroxysuccinimide ester; NaPi, sodium phosphate buffer with pH 7.4; NSC, newborn calf serum; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBST, Tris-buffered saline with 0.1% Triton X-100.

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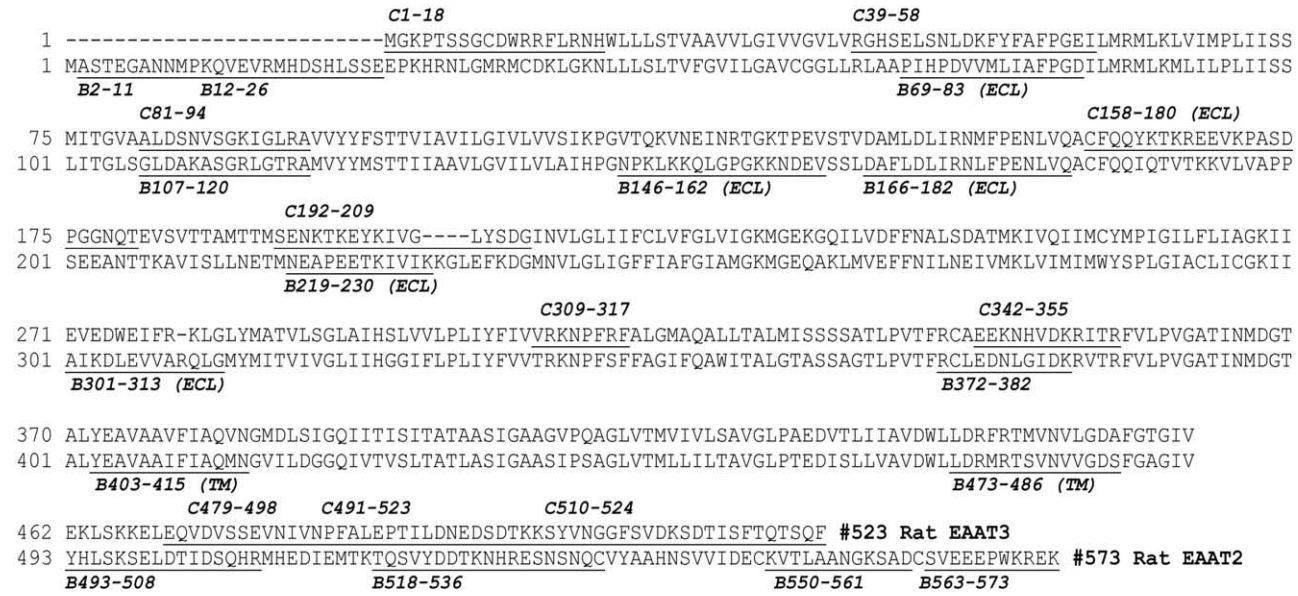

Fig. 1. Sequence alignment of rat EAAT2 and rat EAAT3. The amino acid sequences used for peptide synthesis are underlined and the peptide names given either above (EAAT3) or below (EAAT2) the sequences. Some peptides represent parts of putative extracellular (ECL) or transmembrane (TM) domains as indicated. All the other peptides are selected from putative intracellular domains. The peptides C468–482 and C486–499 correspond to parts of rabbit EAAT3 differing from rat EAAT3 and are therefore not shown in this figure. Peptide C510–524 is also from rabbit, but this sequence is identical to rat, corresponding to rat amino acids 509–523. To avoid confusion, we have kept this peptide's original (rabbit) numbering, as it is used throughout this paper. Peptide C1–13 is not shown because of the overlap with C1–18.

EXPERIMENTAL PROCEDURES

Materials

Sodium dodecyl sulfate (SDS) of high purity (>99% C12 alkyl sulfate) and bis(sulfosuccinimidyl) suberate were obtained from Pierce (Rockford, IL, USA). *N*,*N*′-methylene-bisacrylamide, acrylamide, ammonium persulfate, TEMED and alkaline phosphatase substrates (nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate) were from Promega (Madison, WI, USA). Biotinylated anti-rabbit, anti-sheep and anti-mouse immunoglubulins, streptavidin-biotinylated horseradish peroxidase complex, and colloidal gold-labeled anti-rabbit and anti-mouse immunoglubulins, electrophoresis equipment, molecular mass markers for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), nitrocellulose sheets (0.22 m pores, 100% nitrocellulose), Protein A-Sepharose Fast Flow and Sephadex G-50 fine were from Amersham Biosciences (Buckinghamshire, UK). Alexa fluor goat anti-rabbit 555 and goat anti-mouse 488 were from Molecular Probes (Eugene, OR, USA). Paraformaldehyde and glutaraldehyde EM grade were from TAAB (Reading, UK). Fluoromount G and Lowicryl HM20 were from Electron Microscopy Sciences (Fort Washington, PA, USA). Alkaline phosphatase-conjugated monoclonal antibodies to rabbit and sheep IgG, anti-beta-tubulin, bovine serum albumin (BSA), 3-[(3-cholamido-propyl)dimethylammonio]-1-propanesulphonate (CHAPS), dithiotreitol (DTT), EDTA, guanosine-5′-triphosphate (GTP), HEPES, human serum albumin (HSA), keyhole limpet hemocyanin (KLH), *m*-maleimido benzoyl-*N*-hydroxysuccinimide ester (MBS), phenylmethanesulfonyl fluoride (PMSF), rabbit serum albumin, thyroglobulin, Trizma base, Trisma–HCl and tubulin were obtained from Sigma (St. Louis, MO, USA). Other reagents were obtained from Fluka (Buchs, Switzerland). Anti-myelin basic protein (MBP) and anti-CNPase were from Sternberger Monoclonals (Lutherville, MD, USA).

Peptides

Peptides representing parts of EAAT2 (Pines et al., 1992; 573 amino acid residues) and EAAT3 are referred to by capital letters "B" and "C," respectively, followed by numbers indicating the corresponding amino acid residues in the sequences (given in parentheses). The first EAAT3-peptides were made based on the rabbit sequence which is 524 amino acid residues long (Kanai and Hediger, 1992). The rat sequence was used when it became available (Bjørås et al., 1996) and is 523 residues long (lacking residue 191 in the rabbit sequence). The peptide sequences are shown in Fig. 1. Note that the C510–524 peptide is numbered according to the rabbit sequence although identical to the rat 509–523. The following two rabbit peptides are not shown in Fig. 1 because the sequences are different: C468–482 (KELEQMDVSSEVNIV-*amide*) and C486–499 (ALESATLDNEDSDT-*amide*).

Only the peptides representing the C-termini of the native proteins were synthesized as free C-terminal acids (B563–573, C491–523 and C510–524). The remaining peptides shown were synthesized as C-terminal amides. B301–313 and C1–13 were also synthesized as multiple antigenic peptides (map). Map-peptides were used for immunization without coupling to carrier protein while the other peptides were coupled to either KLH, rabbit serum albumin or thyroglobulin with either glutaraldehyde (with or without reduction with sodium borohydride) or MBS as described previously (Danbolt et al., 1998). The production of gold particles (Frens, 1973) and the conjugation of gold to immunogens (Pow and Crook, 1993) were performed as described.

Antigenicity profiles (Fig. 2) were calculated for rat EAAT2 and EAAT3 according to Jameson and Wolf (1988) using the Protean program (DNASTAR, Inc., Madison, WI, USA).

Animals, immunizations and collection of tissue

All animal experimentation was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 and