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Tissue specific variants of glutamate transporter GLT-1

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Abstract cDNA cloning of glutamate transporter GLT-1 from mouse brain and liver has revealed that 5′-ends of the messages are different between brain and liver. In addition, one of the GLT-1 cDNAs isolated from liver has been found to possess a 3′-end different from those of the others. Reverse transcription polymerase chain reaction (RT-PCR) amplification using primers specific for altered 5′-ends has confirmed that brain and liver messages possess their own specific 5′-ends. Both of the two 3′-ends have been demonstrated by RT-PCR to be present not only in liver but also in brain, indicating both brain and liver GLT-1 possess two types of 3′-ends. Although functional properties are not changed by the alteration of N-termini and C-termini when expressed in *Xenopus laevis* oocytes, co-expression of two liver type GLT-1 with different C-termini (mGLT-1A and mGLT-1B) has been found to result in the increase in V_{max} of transport without changing K_m. These results suggest the tissue specific alternative splicing at 5′-ends of GLT-1 messages and the interesting association of spliced variants with different C-termini.

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Key words: Glutamate transporter; Alternative splicing; Mouse brain; Mouse liver

1. Introduction

Uptake of glutamate into cells through plasma membrane is mediated via highly accumulative Na^+-coupled transporters [1–3]. By now four isoforms of glutamate transporters, GLAST, GLT-1, EAAC1 and EAAT4 have been identified [2,3]. In the central nervous system, glutamate transporters are expressed in both neurons (EAAC1 and EAAT4) and glial cells (GLT-1 and GLAST) [2,3]. The glutamate transporters in astrocytes in particular play critical roles to keep extracellular glutamate concentration below neurotoxic level to protect neurons from glutamate excitotoxicity. This idea has been confirmed recently by knockout of glutamate transporters [4,5]. Consistent with this, the regional loss of GLT-1, a major astrocyte glutamate transporter, has been associated with pathogenesis of a neurodegenerative disorder amyotrophic lateral sclerosis [6].

In peripheral tissues, glutamate transporters are supposed to play pivotal roles in epithelial transport of acidic amino acids and in cellular nutrition. Although EAAC1 is proposed to be an apical membrane transporter in the epithelia of intestine and kidney, other isoforms of glutamate transporters in peripheral tissues, however, have not been well characterized [7–10]. In the course of Northern blot analysis to determine the distribution of expression of glutamate transporters in peripheral tissues, we have found that GLT-1 is expressed in liver with different message size compared to brain. In this paper, we report the characterization of the liver GLT-1.

2. Materials and methods

A cDNA fragment of rat GLT-1 was obtained by reverse transcription polymerase chain reaction (RT-PCR) using synthetic oligonucleotide primers 5′-ATGGCATCAACCGAGGGTG-3′ and 5′-GCTGGATGCTAAAGCCAGC-3′ (corresponding to nucleotide 97–115 and 417–435 of rat GLT-1 cDNA [11], respectively). First strand cDNA was synthesized from brain poly(A)$^+$RNA obtained from adult Splague Daeley rat using oligo(dT)$_{12-18}$ (GibcoBRL, MD) as a primer and subjected to PCR amplification with Taq DNA polymerase described by the manufacturer (Perkin-Elmer). The PCR product (~0.3 kb) was labeled with [^{32}P]dCTP (^{T7}Quick prime, Pharmacia) and used as a probe for screening cDNA libraries [12]. Non-directional cDNA libraries were constructed from poly(A)$^+$RNA prepared from brain and liver of male Jcl:ICR mouse using Superscript Choice System (GibcoBRL). Random hexamer and oligo(dT)$_{12-18}$ were used as primers for first strand syntheses for brain and liver, respectively. cDNAs were ligated to λZipLox *Eco*RI arms (GibcoBRL). Screening cDNA libraries, isolation of positive plaques and in vivo excision were performed as described elsewhere [12]. cDNA inserts were subcloned into EcoRI site of pBluescript II SK$^-$ (Stratagene, CA) and sequenced in both direction as described [12].

For Northern analysis, Poly(A)$^+$RNA was prepared from male Jcl:ICR mouse tissues and blotted onto a nitrocellulose filter (3 μg/lane) [12]. The cDNA insert of clone GLB17 was used as a probe labeled with ^{32}P. Hybridization and high stringent washing were performed as described [12]. The final stringent washing was in 0.1×SSC/0.1% SDS at 65°C.

To determine the tissue distribution of spliced variants, RT-PCR was performed using primers unique to each spliced fragment: 5′-CTCCAGACCGTGCCCGGGAGAGG-3′ (sense primer *B*) designed in the 5′-untranslated region of clone GLB17 from brain (corresponding to nucleotide 623–645); 5′-CAGAACTTGGAAGCCAGTGCAC-3′ (sense primer *L*) designed in the 5′-untranslated region of clone GLL3 from liver (corresponding to nucleotide 1–22); 5′-CACGTTTCCAAGGTTCTTCCTC-3′ (antisense primer *a*) designed on C-terminus region of GLB17 (corresponding to nucleotide 2349–2370); 5′-TAAGGAGTCCAGCATGCACTAT-3′ (antisense primer *b*) designed in the 3′-untranslated region of GLL3 (corresponding to nucleotide 1716–1737). For the other sets of PCR, spliced fragment specific primers, *B*, *L*, *a* and *b*, were combined with primers common to all the spliced variants, antisense primer *X*: 5′-AGGCTAGACACCTCGTCG-3′ (corresponding to nucleotide 1136–1153 of GLB17) and sense primer *Y*: 5′-GTAAGCCTTACAGCCACC-3′ (corresponding to nucleotide 1938–1955 of GLB17).

Functional characteristics of proteins encoded by cDNAs were examined by *Xenopus laevis* oocytes expression as described [12]. cRNA was obtained by in vitro transcription using T7 RNA polymerase from cDNAs in pBluescript II SK$^-$ linearized with SpeI for GLB17 and BamHI for GLL1 and GLL3. Twenty-five ng of cRNA was injected to each oocyte. In the experiments in which cRNAs from GLL1 and GLL3 were co-injected, the total amount of cRNA injected was kept constant (25 ng). The uptake of [^{14}C]L-glutamate (New England Nuclear) was measured in the standard uptake solution (NaCl 100 mM, KCl 2 mM, $CaCl_2$ 1 mM, $MgCl_2$ 1 mM, HEPES 10 mM, Tris 5 mM, pH 7.4) containing 0.25 μCi/ml of [^{14}C]L-glutamate and non-labeled glutamate to make up the concentration indicated. For Na^+-free uptake solution, NaCl in the standard uptake solution was replaced by choline-Cl. The uptake was measured for 30 min and the values were expressed as pmol/oocyte/min. The values obtained were

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expressed as mean ± s.e.m. For each measurement, 7–8 oocytes were used. The statistical analysis between groups was determined by Student's unpaired *t*-test.

3. Results and discussion

Ten positive clones were isolated from a random-primed mouse brain cDNA library by screening 240 000 plaques. 5′- and 3′-end sequencing and the comparison of restriction enzyme cleavage patterns indicated that all 10 clones were identical except the extension of their 5′- and 3′-ends varied. The longest cDNA (GLB17) contained a 2996 bp cDNA insert with a single open reading frame from nucleotide 660 to 2375 encoding a 572-residue protein (designated mGLT-1) which exhibited 97% amino acid sequence identity to rat GLT-1 [11]. Amino acid sequence of mGLT-1 was identical to that of mouse GLT-1 sequence reported by Mukainaka et al. [9]. mGLT-1, however, exhibited some difference (98% identity at amino acid level) compared with mouse GLT-1 reported by Sutherland et al. (D (residue 26), G (62), A (112) and K (572) of mGLT-1 were replaced by E, R, V and EFD, respectively) [13].

Northern blot analysis using GLB17 cDNA as a probe revealed a long size of signal (11.3 kb) strongly expressed in mouse brain (Fig. 1), which is consistent with a rat Northern blot result [11]. GLT-1 was originally thought to be specific to brain, however, in this study, we found the expression of GLT-1 mRNA also in the peripheral tissue such as liver where a short band at 2.2 kb was more predominant than the 11.3 kb message (Fig. 1).

To understand the nature of the short message found in liver, an oligo(dT)-primed mouse liver cDNA library was

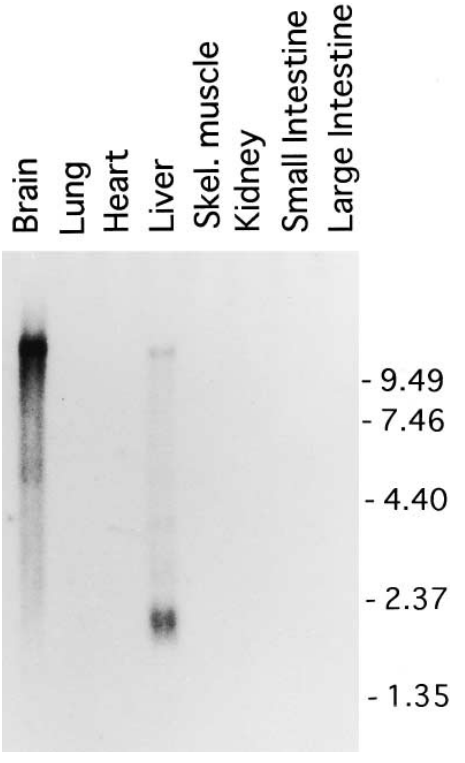

Fig. 1. High stringency Northern blot analysis of GLT-1 in mice. ^{32}P-labeled GLB17 cDNA was used as a probe. Skel. muscle: skeletal muscle.

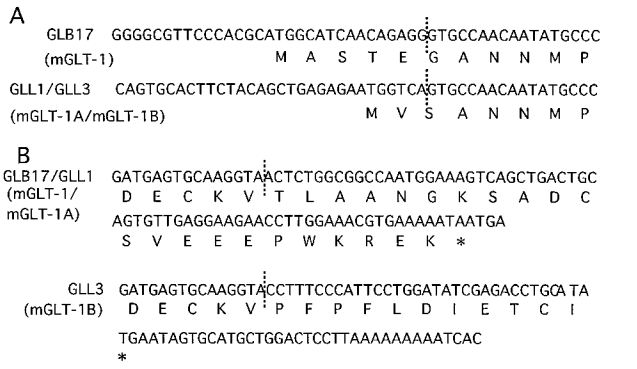

Fig. 2. Comparison of nucleotide sequences corresponding to N-termini (A) and C-termini (B) of mGLT-1, mGLT-1A and mGLT-1B which are encoded by cDNA clones GLB17, GLL1 and GLL3, respectively. Deduced amino acid sequences are attached to the nucleotide sequences. The broken lines indicate the predicted points of alternative splicing. *: Stop codon.

screened using the rat GLT-1 cDNA fragment as a probe. Five positive clones were isolated by screening 240 000 plaques. All the clones contained the identical 5′-ends except their extension varied. Surprisingly, the 5′-ends of liver GLT-1 cDNAs were, however, different from those of brain GLT-1 cDNAs, suggesting alternative splicing at the 5′-ends. Because this alteration at the 5′-ends involves the putative N-terminus of the protein, N-terminus 6 amino acids in brain GLT-1 are replaced by 3 amino acids in liver GLT-1 (Fig. 2A).

3′-Ends of 4 liver cDNAs out of 5 isolated clones were identical to each other and also to those of brain GLT-1 cDNAs except the variety in the sites of poly A tail addition. The poly A tail started at the poly adenylation site AATAAA at 28 base downstream from the stop codon in one of the liver cDNAs (GLL2) and at the poly adenylation signal-like sequence AAATAA 195 base downstream from the stop codon (GLL1, GLL4 and GLL5). The long GLT-1 message in brain is proposed to use the poly adenylation site at further downstream. Therefore, the liver mRNAs have shorter 3′-untranslated region compared to brain mRNA, which explains shorter messages in liver than those found in brain (Fig. 1). One of the liver cDNAs GLL3, a 2198 bp cDNA coding for the 558-residue protein, contained a unique 3′-end, which suggests alternative splicing occurring at the 3′-ends. Because of this alteration, C-terminus 22 amino acids encoded by GLB17 were replaced by 11 amino acids for GLL3 (Fig. 2B).

In order to determine the tissue specificity of spliced variants, RT-PCR was performed using primers specific for each spliced fragment. As is shown in Fig. 3A, PCR products were obtained for brain poly(A)$^+$RNA only when the primer *B* designed on 5′-untranslated region of brain GLT-1 GLB17 was used as a sense primer. Antisense primers *a* and *b*, designed on varied 3′-ends (see Fig. 4), both produced amplification products, indicating that brain expresses GLT-1 messages with two types of 3′-ends (Fig. 4). In brain, the PCR product produced by primer *b* was fainter than that by primer *a* (Fig. 3A and C). This is consistent with the fact that the 3′-end on which primer *b* was designed was not found in 10 cDNAs isolated from brain. In liver, only primer *L* produced amplification products regardless of 3′-end primers used (Fig. 3A), indicating that liver GLT-1 messages contain only liver type 5′-end and both of two types of 3′-ends (Fig. 4).

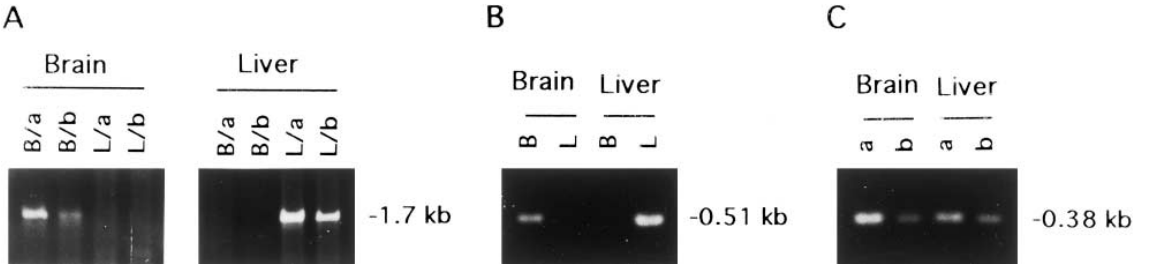

Fig. 3. RT-PCR using primers specific for GLT-1 variants. A: Sense primers *B* or *L* were combined with antisense primers *a* or *b*. B: Sense primers *B* or *L* were combined with an antisense primer *X*. C: A sense primer *Y* was combined with antisense primers *a* or *b* (see text).

The observations in Fig. 3A were further confirmed by additional RT-PCR in which the spliced fragment specific primers were combined with primers common to all the spliced variants (Fig. 3B and C). Fig. 3B indicates 'brain type 5′-end' amplified with primer *B* and 'liver type 5′-end' amplified with primer *L* were only found in brain and liver, respectively. Both of the 3′-ends identified with primer *a* (designated as 'type *a* 3′-end') and primer *b* (designated as 'type *b* 3′-end') were found in both brain and liver (Fig. 3C). We now name mouse liver GLT-1s encoded by GLL1 (liver type 5′-end/type *a* 3′-end) and GLL3 (liver type 5′-end/type *b* 3′-end) as mGLT-1A and mGLT-1B, respectively, while mouse brain GLT-1 encoded by GLB17 (brain type 5′-end/type *a* 3′-end) is mGLT-1.

In order to address the question whether the variations found among N-termini and C-termini of GLT-1 proteins affect transport functions, functional properties of mGLT-1, mGLT-1A and mGLT-1B were compared. When expressed in *Xenopus laevis* oocytes, mGLT-1, mGLT-1A and mGLT-1B all exhibited Na^+-dependent transports of [^{14}C]L-glutamate (data not shown). As shown in Fig. 5A, glutamate uptakes in the oocytes expressing mGLT-1, mGLT-1A and mGLT-1B exhibit similar concentration-dependent profiles with K_m values of 28.0±2.9, 32.3±1.9, 31.5+2.7 and 33.2±2.1 μM (mean±s.e.m. of 4 separate experiments), respectively, indicating no change in their affinity to glutamate. Glutamate uptakes via mGLT-1B and mGLT-1, furthermore, exhibited similar sensitivity to glutamate uptake inhibitors such as *threo*-β-hydroxyaspartate, dihydrokainate, L-*trans*-pyrrolidine-2,4-dicarboxylate and serine-*O*-sulfate (Fig. 5B) [8]. These results indicate that variations in N-terminus and C-terminus by the alternative splicing of GLT-1 do not affect the conformation of substrate binding sites. Therefore, it is suggested that both the N-terminus and C-terminus ends of GLT-1 which are predicted to be located intracellularly do not contribute to assemble the substrate binding sites.

Because spliced variants exhibited no detectable changes in their functions, significance of the 5′-end alternative splicing of GLT-1 would be in the tissue specific expression of the gene. The differences in 5′-ends of GLT-1 messages in brain and liver suggest the existence of tissue specific promoters upstream of the exons corresponding to tissue specific 5′-ends as was described in the other genes [14]. In brain GLT-1 gene expression is spatially and temporally regulated so as to suit the functional requirements [3]. For example, in the course of brain development, the dramatic upregulation of GLT-1 gene expression at around postnatal day 14 in cerebral cortex coincides with the postnatal development of glutamatergic transmission [15,16]. On the other hand, GLT-1 gene expression is down-regulated after regional deafferentiation and recovers on nerve fiber regeneration [17]. These facts suggest that astrocytes somehow sense glutamatergic transmission occurring in neighboring synapses and optimize GLT-1 gene expression so as to maintain extracellular glutamate concentration [3]. The brain specific promoter is probably responsible for the mechanism through which astrocytes respond to the surrounding neural activity. The comparison of brain specific *cis*-regulatory elements and liver specific ones would provide some clues to such a specialized function of astrocytes.

The significance of the alternative splicing at the 3′-ends is not clear because 3′-end spliced variants did exhibit neither differences in their functions (Fig. 5) nor differential tissue distribution (Fig. 3). Recently it was demonstrated that glutamate transporters exist as homomultimers [18]. Although different isoforms cannot form multimeric complex, it is still possible that spliced variants associate with each other. So, we tried co-expression of mGLT-1A and mGLT-1B which are both expressed in liver and possess different C-termini. As shown in Fig. 5A, oocytes co-expressed with mGLT-1A and mGLT-1B exhibited glutamate uptake with no change in K_m (K_m = 33.5±2.1 μM for mGLT-1A/mGLT-1B; mean±s.e.m. of 4 separate experiments, see above for comparison) and slight yet significant increase in V_{max} ($P<0.05$) compared with oocytes expressed solely with mGLT-1A and mGLT-1B (V_{max} = 2.40±0.17, 2.43±0.19, 3.41±0.2 pmol/oocyte/min for mGLT-1A, mGLT-1B and mGLT-1A/mGLT-1B, respectively; mean±s.e.m. of 4 separate experiments). When the different ratios of mGLT-1A and mGLT-1B cRNA (1:1, 1:2 and 2:1) were co-injected in the same batch of oocytes with the total amount of cRNA injected constant, the V_{max} value was the highest for mGLT-1A/mGLT-1B ratio 1:1 (V_{max} = 3.19, 2.72, 2.51, 2.09, 2.27 pmol/oocyte/min for mGLT-1A:mGLT-1B = 1:1, 1:2, 2:1, solely mGLT-1A, solely

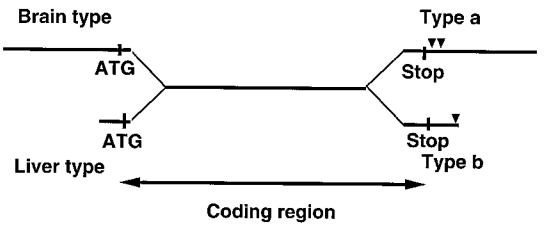

Fig. 4. Structures of GLT-1 mRNAs. ATG: start codon, Stop: stop codon, arrow head: poly adenylation site. Left: 5′-end, right: 3′-end. Brain type and liver type 5′-ends and type *a* and type *b* 3-ends are indicated. mGLT-1, mGLT-1A and mGLT-1B encoded by cDNA clones GLB17, GLL1 and GLL3 have brain type 5′-end/type *a* 3′-end, liver type 5′-end/type *a* 3′-end and liver type 5′-end/type *b* 3′-end, respectively.

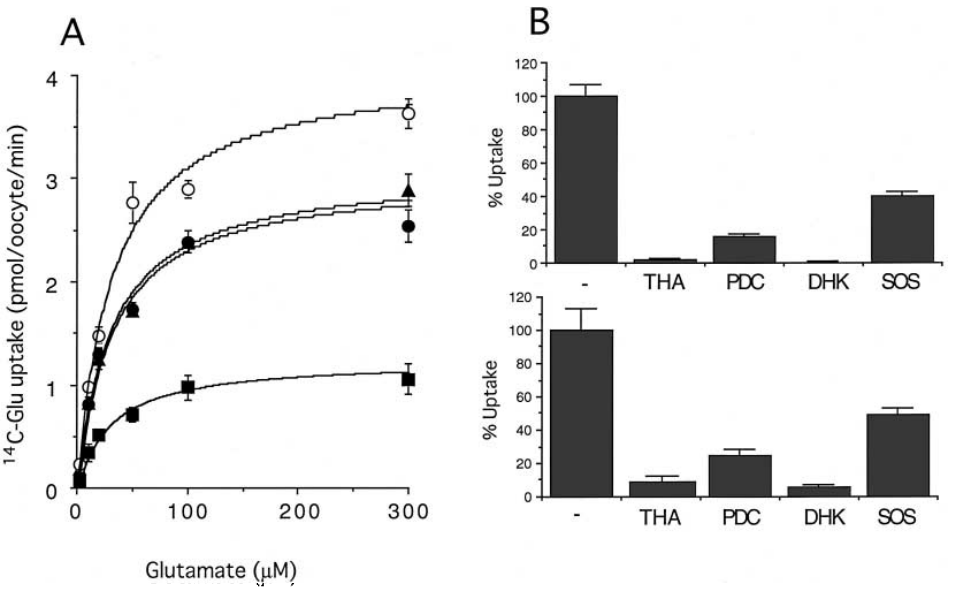

Fig. 5. Functional properties of brain and liver GLT-1. A: A representative result on concentration dependence of glutamate uptake via oocytes which express mGLT-1 (clone GLB17: filled square), mGLT-1A (clone GLL1: filled triangle), mGLT-1B (clone GLL3: filled circle) and both mGLT-1A and mGLT-1B (GLL1/GLL3 co-expression: open circle) measured in the same batch of oocytes. K_m (μM)/V_{max} (pmol/oocyte/min) are 23.2/1.14, 28.6/2.79, 26.5/2.74 and 29.2/3.89 for mGLT-1, mGLT-1A, mGLT-1B and mGLT-1A/mGLT-1B, respectively. Twenty-five ng of cRNA was injected into each oocyte for GLB17, GLL1 and GLL3 and 12.5 ng of GLL1 cRNA plus 12.5 ng of GLL3 cRNA for GLL1/GLL3 co-expression. B: Inhibition profiles of glutamate uptakes (10 μM) via mGLT-1B (upper) and mGLT-1 (lower) by glutamate uptake inhibitors (1 mM). –: water-injected control oocytes, THA: *threo*-β-hydroxyaspartate, PDC: L-*trans*-pyrrolidine-2,4-dicarboxylate, DHK: dihydrokainate and SOS: serine-*O*-sulfate. The glutamate uptakes were expressed as % of those of control oocytes. Each data point represents mean ± s.e.m. ($n = 7 \sim 8$).

mGLT-1B, respectively). Although the reason for this increase in V_{max} is not known, it is conceivable that C-terminus, which does not affect substrate binding (see above), somehow contributes to the translocation of substrates, which would be reflected by the increase in V_{max}. Another possibility is that the complex of mGLT-1A and mGLT-1B becomes more stabilized than homomultimers composed solely of mGLT-1A or mGLT-1B, so that they are able to stay longer in the plasma membrane. It is notable that the C-terminus encoded by type *a* 3′-end contains many charged residues whereas that encoded by type *b* 3′-end is rather hydrophobic. In liver, in particular, it is interesting to know whether the spliced variants with different C-termini co-exist on the plasma membrane of single hepatocytes.

In the present study, we have characterized glutamate transporter GLT-1 messages expressed in liver and indicated the alternative splicing at both 5′- and 3′-ends. For the next step of study, the characterization of the protein of each spliced variant with antibodies, in particular, specific for varied C-termini would be necessary to understand the mechanisms of seemingly co-operative properties and physiological and pathophysiological roles of GLT-1 spliced variants.

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