RESEARCH

Temporal and Spatial Regulation of the Expression of *BAD2*, a MAP Kinase Phosphatase, during Seizure, Kindling, and Long-term Potentiation

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Abstract

Recent studies indicate that stimulation of NMDA receptors in cultured hippocampal cells activates MAP kinase. Although the pathway whereby MAP kinase is activated has been characterized, little is known about the mechanisms that shut off MAP kinase. In the course of analyzing several immediate-early genes identified previously by differential screen as inducible by seizure activity, we found that one of them, *BAD2*, encodes dual purpose, threonine/tyrosine phosphatases with specific activity directed against MAP kinase (MKP-1). In situ hybridization of *BAD2* demonstrates that stimuli that produce seizure, kindling, and long-term potentiation cause a rapid increase in *BAD2* mRNA (within 0.5–1 hr after stimulation) that has, in each case, a distinctive pattern of expression in the brain. In these regions, the induction of a MAP kinase-specific phosphatase may provide a negative feedback control associated with long-term synaptic changes.

Introduction

Studies in invertebrates and vertebrates using inhibitors of protein and RNA synthesis (Davis and Squire 1984; Montarolo et al. 1986) provided the initial indications that changes in gene expression are involved in formation of long-term memory.

Genes induced by neuronal activity have been postulated to be important for initiating the stable structural modification that accompanies long-term neuronal plasticity during memory storage (Goelet et al. 1986; Greenberg et al. 1986; Kennedy 1989; Bailey and Kandel 1993; Alberini et al. 1994). To analyze the initial steps in activity-dependent gene expression in the mammalian brain, we have focused on the primary transcriptional events by attempting to clone new immediate-early genes that are rapidly induced by seizure activity in the absence of de novo protein synthesis (Qian et al. 1993). Using differential screening, we identified a set of immediate-early genes, the *BAD* genes, whose expression is brain-activity-dependent (Qian et al. 1993). In this paper we describe one of the genes, *BAD2*, and examine its induction and expression in response to seizure, kindling, and LTP. We find that *BAD2* is the rat homolog of CL100 and 3CH134 (Charles et al. 1992; Alessi et al. 1993). These genes encode for a dual purpose threonine and tyrosine protein phosphatase, MKP-1, which specifically dephosphorylates MAP kinase (Alessi et al. 1993; Charles et al. 1993; Sun et al. 1993).

MAP kinase is an important serine/threonine kinase activated by a variety of extracellular signals in both proliferating and nonproliferating cells (for review, see Crews et al. 1992; Davis 1993). MAP kinase is phosphorylated on threonine and tyrosine residues by MAP kinase kinase—a kinase that itself is regulated by Raf kinase. This dual phosphorylation of MAP kinase is required for its full activation (Anderson et al. 1990; Ahn et al. 1991; Boulton and Cobb 1991; Seger et al. 1991;

Robbins et al. 1993; for review, see Ahn et al. 1992). In the hippocampus, MAP kinase is phosphorylated by seizure activity (Baraban et al. 1993). Moreover, in hippocampal neurons in culture, the excitatory transmitter glutamate induces the phosphorylation of MAP kinase through activation of NMDA receptors (Bading and Greenberg 1991). In turn, MAP kinase is capable of phosphorylating several key transcription factors such as Jun and Myc (for review, see Davis 1993). Thus, MAP kinase seems to have an important role in the immediate-early response of a variety of cells.

However, the mechanism in the brain whereby the MAP kinase signaling is switched off is not known. The nucleotide sequence of *BAD2* reveals it to be the specific MAP kinase phosphatase, MKP-1. The rapid induction of *BAD2* as an immediate-early gene upon neuronal stimulation in the brain thus provides a negative feedback in the regulation of MAP kinase activity in a region-specific manner by seizure, kindling, and long-term potentiation (LTP). These data suggest that the *BAD2* MAP kinase phosphatase may play a critical role in attenuating or switching off gene expression in the brain triggered by MAP kinase activity.

Materials and Methods

DIFFERENTIAL SCREENING

Primary cDNA clones (30,000) from the seizure-induced rat hippocampal cDNA library were screened (Sambrook et al. 1989; Qian et al. 1993). Four replica filters were lifted from each plate. One set of the duplicates was hybridized to the ³²P-labeled cDNA probe (–) derived from poly(A)⁺ RNA isolated from the cortex of control rats. Another set of duplicates was hybridized to ³²P-labeled cDNA probe (–) derived from that of the animals that had undergone metrazole-induced seizure in the presence of cycloheximide. The filters were washed at high stringency. Clones that hybridized preferentially to the + probe were picked and subjected to further verification by slot–blot hybridization (Almendral et al. 1988).

SEIZURE INDUCTION AND NORTHERN BLOT ANALYSIS

Adult Sprague–Dawley male rats ~300 grams of body weight were injected intraperitoneally

with metrazole (50 mg/kg body weight) to produce convulsion. These rats were sacrificed for RNA isolation or in situ hybridization at various hours after onset of seizure. Total RNA from hippocampus of control animals and animals that had undergone metrazole-induced seizure either in the presence or absence of the protein synthesis inhibitor cyclohexamide were isolated at different time points using the cesium trifluoroacetate method (Pharmacia). Ten micrograms of RNA was loaded per lane. The blot was first hybridized to a random-primed ³²P-labeled probe specific for *BAD2* (Pharmacia) and subsequently rehybridized to a probe specific for actin to ensure the quality and quantity of mRNA used. Hybridization was performed in solution of 50% formamide, 0.5% SDS, 5× SSC, and 5× Denhardt's solution at 42°C overnight. The blot was washed in 1× SSC, 0.1% SDS, for 0.5 hr and then in 0.1× SSC, 0.1% SDS, for 1 hr at 65°C. Numbers above the lanes in Figure 2A indicate time in hours after the onset of seizure at which RNA was isolated. The positions of the 28S and 18S rRNAs are indicated at the left. The position of *BAD2* is indicated at the right.

CDNA ISOLATION AND SEQUENCING

Three independent full-length cDNA clones were isolated from our hippocampal cDNA library using homology screening at high stringency (Sambrook et al. 1990). Both strands of the three cDNAs in the pcDNAII vector (Invitogene) were sequenced by the chain termination sequencing Sanger method modified for Sequenase (v. 2.0, U.S. Biochemical).

IN SITU HYBRIDIZATION

Tissue sections (~18 μm) through the rostral hippocampus of control rats and rats seized after 30 min were prepared (Qian et al. 1993) as described previously. To detect *BAD2* protein tyrosine phosphatase mRNA, we synthesized a 45-mer oligonucleotide with sequence of CAGCT-TCACTCGGTTAGTCCTCATGAGGTAAGCAAG-GCAGATGGT complementary to amino acid sequence 267–281. The oligonucleotide was labeled with ³⁵S-labeled dATP to a sp. act. of 10⁹ cpm/μg (3'-Tailing kit, Boehringer Mannheim). Hybridization was performed overnight in hybridization so-

lution containing 50% formamide, 10% dextran sulfate, 0.7% Ficoll, 0.7% polyvinyl pyrrolidone, 350 mg/ml of bovine serum albumin, 0.15 mg/ml of yeast tRNA, 0.33 mg/ml of denatured herring sperm DNA, and 20 μ M dithiothreitol, using probe at 10^7 cpm/ml. The slides were then rinsed twice in $2\times$ SSC at room temperature and subsequently washed at 65°C in $2\times$ SSC, $1\times$ SSC, and $0.1\times$ SSC (20 min each). Air-dried slides were exposed to Amersham β max film for 2–3 weeks, developed, and counterstained with cresyl violet.

PREPARATION OF KINDLING AND LTP RATS

Male Long–Evan rats were anesthetized with pentobarbital, and chronic indwelling electrodes were implanted into the angular bundle of the perforant path and the hilus of the dentate gyrus, according to standard stereotaxic procedures (Gilbert and Mack 1990). Following a 2- to 3-week recovery period, three animals were given a series of biphasic wave stimulus pulses to initiate kindling (kindling stimulus, 500 μ A, 60 Hz, 1-msec/half-wave diphasic pulses for 1 sec). A mean of 34-sec afterdischarge was evoked and recorded as described. No behavioral seizures were seen. Three animals were stimulated to evoke LTP (LTP stimulus, 400 Hz for 50 msec using 0.1 msec pulses of 1000 μ A). This high-frequency stimulation evoked a rapid and long lasting increase in the field potential. In addition, three animals received the same number of stimulus pulses delivered at a low frequency (0.05 Hz) and two animals administered MK801 (2 mg/kg) 45 min prior to delivery of high-frequency stimulation. These five animals failed to evoke LTP. All animals mentioned above were sacrificed 1 hr after stimulation and processed for in situ hybridization.

Results

FULL-LENGTH cDNA CLONING AND SEQUENCE ANALYSIS

The full-length cDNA sequence of *BAD2* contains an open reading frame that codes for a protein of 367 amino acids with a calculated molecular weight of 39,613 and an estimated pI of 6.89 (Fig. 1A). The 5' end of the sequence contains

characteristic Kozak consensus sequence surrounding the initiator of methionine (ATG) (Kozak 1984). The 3' end of the sequence contains a typical polyadenylation site (Fig. 1A). Protein sequence MOTIF search and visual inspection revealed that the carboxy-terminal region of the protein contains a short stretch (Fig. 1A, boxed) that matches perfectly the consensus sequence of the catalytic box of known protein tyrosine phosphatases [I/V]HCXAGXXR(S/T) (Fig. 1B). A Kyte and Doolittle (1982) hydrophobicity plot shows that the polypeptide does not contain transmembrane-spanning hydrophobic stretches. This suggests that *BAD2* is likely a nonreceptor-type protein tyrosine phosphatase. Search of GenBank data bases revealed that *BAD2* polypeptide has ~96% identity to the previously identified proteins, human CL100 (from skin fibroblasts) and mouse 3CH134 (from 3T3 fibroblasts), and indicate that *BAD2*, CL100, and 3CH134 are the same gene in different species (Fig. 1B) (Charles et al. 1992; Keyes and Emslie 1992). Biochemical assay of the purified human CL100 recombinant protein has shown that it possesses a strong intrinsic phosphatase activity that can be abolished completely by addition of sodium vanadate and requires no divalent ions (Keyes and Emslie 1992). Recently, it has been shown that the human and mouse homologs of *BAD2* dephosphorylate MAP kinase specifically (Alessi et al. 1993; Charles et al. 1993; Sun et al. 1993). Therefore, *BAD2* is a MAP kinase-specific phosphatase that has now been named MKP-1 (for review, see Sun et al. 1993).

Visual inspection of the *BAD2* open reading frame reveals a potential nuclear localization sequence comprised of a short stretch of amino acids with a high proportion of positively charged arginine and lysine RRRAKG (Fig. 1B, residues 52–58) (Garcia-Bustos et al. 1991; Gerace 1992). In support of this observation, we have found that this nuclear localization sequence (RRRAR/KG) is also conserved in the PAC-1 phosphatase (residues 56–61), another inducible nuclear tyrosine phosphatase that has ~80% homology with *BAD2* phosphatase. Thus, other members of this subfamily of inducible nuclear protein tyrosine phosphatases may also translocate and dephosphorylate MAP kinase in the nucleus. Furthermore, we have observed that this nuclear localization sequence is flanked at the amino terminus by a phosphorylation site (RFSTIV) for both the cAMP protein kinase A (PKA) and for CAM kinase II. Phosphorylation sites have been shown to be important

Figure 1: (A) Nucleotide and deduced amino acid sequence of *BAD2* cDNA. The catalytic domain of tyrosine phosphatase is boxed. A polyadenylation consensus sequence is underlined. The sequence has been deposited in GenBank under the accession number U02553. (B) Sequence alignment of *BAD2* with mouse 3CH134, human CL100, and human PAC-1.

in the regulation of several nuclear targeting events (Garcia-Bustos et al. 1991; Gerace 1992).

NORTHERN BLOT ANALYSIS OF *BAD2* INDUCTION IN HIPPOCAMPUS AND TISSUE DISTRIBUTION

We have used metrazole (pentylene-tetrazol)-induced seizure as an experimental way to activate neurons in the brain. Metrazole is a GABA antagonist; it causes generalized seizure by suppressing inhibition normally produced by GABA-ergic inhibitory interneurons in brain (Ditcher and Ayala 1987). *BAD2* mRNA is induced by metrazole-induced seizures in rat brain cortex (Qian et al. 1993). We have now extended this analysis to the hippocampus and observed a similar induction pattern there (Fig. 2A). In the hippocampus as in the neocortex, expression of *BAD2* transcript reaches its peak within 30 min after onset of sei-

zure activity and then quickly returns to basal level by 2 hr. This increase in transcription of *BAD2* mRNA in response to activity occurs even in the presence of the protein synthesis inhibitor cycloheximide (data not shown), suggesting that *BAD2* is an immediate-early gene. Its transcriptional activation does not require new protein synthesis but, rather, depends on covalent modification of preexisting factors. A survey of *BAD2* expression in adult rat tissues shows that its mRNA is abundant in the heart and in brain (Fig. 2B), and is expressed at a lower level in lung and muscle, and at an even lower but still detectable level in liver and intestine. In contrast, PAC-1 is only expressed in spleen and thymus tissue; it is not detectable in brain, heart, lung, liver, muscle, and stomach. These different distribution patterns of *BAD2* and PAC-1 indicate tissue-specific functions for these two inducible nonreceptor protein phosphatases.

Figure 2: Northern blot analysis of *BAD2* in mRNA. (A) Induction kinetics of *BAD2* in rat hippocampus after seizure activity. (B) Tissue distribution of *BAD2* mRNA in control rat. Total cellular RNA from various tissue was isolated and fractionated using electrophoresis. Wash was done at high stringency (Qian et al. 1993). (C) Actin probe was used as loading control.

IN SITU HYBRIDIZATION REVEALS THAT *BAD2* IS ACTIVATED IN A RESTRICTED MANNER BY SEIZURE, KINDLING, AND LTP

We then were interested in studying the pattern of *BAD2* expression in response to three different patterns of neuronal activity: seizure, kindling, and LTP. Using in situ hybridization, we first studied the pattern of expression of *BAD2* in brain following convulsive seizure induced by injection of metrazole (Qian et al. 1993). Following seizure, there was a dramatic induction of *BAD2* in the thalamus, in the dentate gyrus of hippocampus, and in the neocortex. There was no significant increase in CA1–CA3 regions of the hippocampus, the caudate putamen, the hypothalamus, and the amygdala (Fig. 3). This contrasts with the expression of *Zif268*, a zinc finger transcription factor that is increased in expression following seizure in the CA1–CA3 regions as well as in the dentate gyrus of the hippocampus and cortex, but not in thalamus. The differential expression of *BAD2* and *Zif268* might reflect phenotypically different aspects of neuronal plasticity in different brain regions.

Kindling is a form of experimental epilepsy in which a stimulus, which initially induces only an afterdischarge limited to a specific brain area, becomes capable of evoking generalized seizure that when repeated daily for a number of days, spreads throughout much of the forebrain. We evoked an

Figure 3: Comparison of the induction of *Zif268* and *BAD2* phosphatase mRNA after metrazole-induced seizure activity. Brain sections (~18 μm) through the rostral hippocampus of control rats and seized rats after 30 min were prepared as described in Materials and Methods.

afterdischarge in the granule cells of the dentate gyrus, lasting, on average, 34 sec (from 29 to 40 sec) that was capable of initiating a generalized seizure were it to be given repeatedly. Following this afterdischarge, we detected an increase in expression of *BAD2* mRNA restricted to the dentate gyrus bilaterally in all these three animals (Fig. 4). Stimuli that were subthreshold for afterdischarge (100 μ A) produced no elevation of *BAD2* mRNA (Fig. 4). These data suggest that threshold for induction of kindling and *BAD2* phosphatase expression is comparable.

Finally, we studied the expression of *BAD2* gene in LTP, the most studied model of synaptic plasticity in the mammalian brain. LTP was evoked in the synaptic connections between the perforant path and the granule cells of the dentate gyrus in freely moving rats by delivering 10 high frequency trains (Qian et al. 1993). One hour after induction

Figure 4: Induction of *BAD2*-phosphatase transcript following kindling-inducing afterdischarge in hippocampus of freely moving rats. Five rats were prepared for kindling stimulation, and afterdischarge was evoked and recorded as described (Qian et al. 1993). No behavioral seizures were seen. Animals were sacrificed 1 hr after stimulation and processed for in situ hybridization as described in the legend to Fig. 2.

BAD2, low-frequency

BAD2, LTP

BAD, LTP-MK801

Figure 5: Induction of *BAD2* tyrosine phosphatase following the initiation of LTP in the synapses between the perforant path and the granule cells of the dentate gyrus of freely moving rats. The animals were sacrificed at 1 hr after stimulation and subsequently processed for in situ hybridization as in Fig. 3. (A) Low-frequency stimulation, which did not evoke LTP, failed to increase *BAD2* transcription. (B) High-frequency stimulation, which evoked LTP, increased *BAD2* expression in the granule cells ipsilateral dentate gyrus of the hippocampus. (C) Preinjection of MK801, an NMDA antagonist, prevented induction of LTP and also blocked the increase in *BAD2* expression.

there was a significant increase in the expression of *BAD2* in the ipsilateral granule cells of dentate gyrus (Fig. 5, *BAD2*, LTP). This unilateral increase in *BAD2* mRNA levels reflects activation of perforant path fibers that terminate mostly ipsilaterally. Animals in which no LTP was evoked after delivering the same number of stimulus pulses at a low frequency did not show any increase in the expression of *BAD2* [Fig. 5, (*low frequency*)]. Be-

cause the activation of NMDA receptors is necessary for the induction of LTP in the synapse made by the perforant pathway on the granule cells, we examined whether increase in *BAD2* transcripts also requires the activation of NMDA receptors. Preinjection of MK801 (2 mg/kg), a noncompetitive NMDA antagonist, which blocks LTP, completely prevented the increased expression of *BAD2* (Fig. 5, LTP-MK801). These results suggest that induction of *BAD2* expression is an NMDA receptor-mediated process.

Discussion

Previous studies on regulation of immediate-early genes by neuronal activity have focused mostly on transcription factors, such as *fos*, *jun*, and *Zif268*. Our data as well as that of others (Ernfors et al. 1991; Isackson et al. 1991; Merlio et al. 1993; Nediul et al. 1993) indicate that other types of genes are also induced. In our differential screen of activity-dependent immediate-early genes (Qian et al. 1993), we have found that *BAD5* is a tissue-type plasminogen activator (tPA), a secreted serine protease shown to be released during neuronal differentiation. We proposed that induction of tPA during LTP may play an important role in maintaining long-lasting LTP and the structural modification associated with this neuronal plasticity.

In this paper we have sequenced *BAD2* and find it to be 96% homologous to mouse 3CH134 and human CL100, suggesting that *BAD2* functions in brain as a specific phosphatase for MAP kinase. In contrast to PAC-1, another inducible nuclear tyrosine phosphatase that is not detected in brain and only expressed in spleen and thymus, *BAD2*, may be particularly important in brain, especially as it is activated in an activity-dependent manner.

Northern blot analysis shows that *BAD2* mRNA in hippocampus is elevated rapidly, within 30 min after neuronal stimulation, and returns to basal level within 2 hr. Because *BAD2* mRNA is induced in the presence of the protein synthesis inhibitor, cycloheximide (Qian et al. 1993), the induction of *BAD2* transcripts depends on covalent modification of preexisting transcription factors. Consistent with rapid turnover is the finding of several AUUUA repeats in the 3'-untranslated region indicating that the *BAD2* mRNA is unstable. These several observations indicate that *BAD2* is an immediate-early gene.

The mouse homolog (3CH134) of *BAD2* selectively dephosphorylates MAP kinase at both threonine and tyrosine residues and turns off the MAP kinase activity in vivo (Sun et al. 1993). Glutamate, acting through the NMDA receptor, activates MAP kinase in hippocampal neurons, perhaps as an early step in a growth process mediated by LTP. The increased expression of *BAD2* that we have found following neuronal stimulation may dephosphorylate MAP kinase and turn off its signaling.

BAD2 protein contains the nuclear localization sequence RRRAR/KG, which is conserved in another inducible tyrosine phosphatase, PAC-1. Identification of a dual-purpose threonine/tyrosine phosphatase with a nuclear localization signal raises another interesting possibility—that *BAD2* may also dephosphorylate MAP kinase in the nucleus. In several cell types, an active form of MAP kinase is localized in the nucleus following extracellular signals (Sanghera et al. 1992; Seth et al. 1992; Chen et al. 1992; Gonzales et al. 1993). In addition, *BAD2* nuclear localization signal is flanked by a consensus site for phosphorylation by CAM kinase II and PKA. Given the important role of these kinases in synaptic plasticity of the dentate granule cells, it is tempting to suggest that the ability of *BAD2* to dephosphorylate MAP kinase in the nucleus may be regulated by these kinases.

It will be extremely interesting to examine the role of *BAD2* MAP kinase phosphatase in LTP and long-term memory using gene knockout technique. Conversely, overexpression of *BAD2* in the brain could provide a specific means for inactivating MAP kinase and thus allow one to examine further the role of MAP kinase in LTP, learning, and memory.

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