Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 2586–2591, March 1998
Neurobiology

A splicing variant of a death domain protein that is regulated by a mitogen-activated kinase is a substrate for c-Jun N-terminal kinase in the human central nervous system

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Communicated by Seymour Benzer, California Institute of Technology, Pasadena, CA, December 29, 1997 (received for review October 3, 1997)

ABSTRACT **The mitogen-activated kinase activating death domain protein (MADD) that is differentially expressed in neoplastic vs. normal cells (DENN) was identified as a substrate for c-Jun N-terminal kinase 3, the first demonstration of such an activity for this stress-activated kinase that is predominantly expressed in the brain. A splice isoform was identified that is a variant of MADD. A protein identical to MADD has been reported to be expressed differentially in neoplastic vs. normal cells and is termed "DENN." We demonstrated differential effects on DENN/MADD in a stressed vs. basal environment. Using *in situ* hybridization, we localized both the substrate and the kinase to large pyramidal neurons in the human hippocampus. It was interesting that, in four of four patients with neuropathologically confirmed acute hypoxic changes, we detected a unique translocation of DENN/MADD to the nucleolus. These changes were apparent only in neurons sensitive to hypoxia. Moreover, in those cells, translocation of the substrate was accompanied by nuclear translocation of JNK3. These findings place DENN/MADD and JNK in important hypoxia insult-induced intracellular signaling pathways. Our conclusions are important for future studies for understanding these stress-activated mechanisms.**

The c-Jun N-terminal kinases (JNKs), or stress-activated kinases, belong to the mitogen-activated kinase family sharing sequence homology with other members, including the extracellular signal-regulated kinases (ERKs). Three JNKs have been identified: JNK1, 2, and 3 (for reviews, see refs. 1 and 2). Each JNK can be spliced differentially, yielding two to four isoforms depending on the tissues and species, and with varying C termini and internal substitutions of the putative c-Jun recognition and binding region (3, 4). Unlike JNK1 and 2, which are expressed ubiquitously in a variety of human tissues, JNK3 is found predominantly in the brain within neurons (5). Developmentally, it is expressed in postmitotic neurons undergoing differentiation (6). As with the ERKs, activation of JNK requires dual phosphorylation of both a threonine and a tyrosine residue located in a consensus tripeptide sequence.

Several substrates for JNK have been identified, such as c-Jun, ATF-2, Elk1, and p53 (7–10). Phosphorylation of these substrates results in elevated transcriptional activity (7, 9, 11, 12). Each JNK isoform binds the various substrates with different affinities. It is not known whether individual JNKs respond to distinct extracellular signals or lead to different downstream effects by preferentially phosphorylating specific substrates.

JNK activity can be elevated by a variety of stimuli, including environmental stress (13–16), apoptotic agents (17), or neurotoxic insults (18). A kinase cascade immediately upstream of JNK leads to its induction (19–25), reminiscent of ERK activation, which involves a parallel mechanism. It is possible that mitogenic and stress signals are transduced by ERK and JNK, respectively, through their selective phosphorylation of different transcription factors.

Evidence for the direct involvement of JNK in apoptosis comes from several studies in PC12 cells; overexpression of a constitutively activated JNK kinase potentiates apoptosis induced by nerve growth factor (NGF) deprivation (26). Conversely, microinjection of a c-Jun-dominant negative mutant into rat sympathetic neurons protects the cells from apoptosis (27). In addition, increased c-Jun activity alone in NIH 3T3 fibroblasts is sufficient to trigger cell death (28).

Based on the expression pattern of JNK3 restricted to neurons and the established role for JNK in cell death pathways under stress, we explored the possibilities of neuron-specific functions of JNK3 beyond the general effects of c-Jun phosphorylation. We used the yeast two-hybrid system to search for novel proteins that interact with JNK3. One such protein that proved to be a splice variant of the mitogen-activated kinase activating death domain protein (MADD) that is differentially expressed in neoplastic vs. normal cells (DENN) revealed a relationship between JNK3 activation and the neuronal stresses of hypoxia/ischemia and the inflammatory response in the human central nervous system (CNS).

MATERIALS AND METHODS

Yeast Two-Hybrid Library Screening. The MATCHMAKER two-hybrid system 2 from CLONTECH was used. The bait construct was generated as follows: two PCR primers were designed (primers were made by the Molecular Core Facility at the University of Southern California), the 5′ primer GTT ACC CGG GG*A* TGA GCC TCC ATT TCT TAT AC and the 3′ primer TAT GGT CGA CCA CTC TCA CTG CTG CTG TTC ACT G. The underlined sequences from the two primers were complementary to the nucleotides 231–251 and nucleotides 1494–1513 of JNK3 cDNA, respectively. The amplified JNK3 cDNA was inserted in-frame with the Gal4 DNA binding domain. Successful construction of the fusion protein was verified on Western blots by using both anti-JNK antibody (Santa Cruz Biotechnology) and anti-Gal4-binding domain antibody (CLONTECH). The construct was then cotransformed into yeast cells with a human brain cDNA library fused to the Gal4 activation domain (Gal4-AD), and the cells were screened for colonies that activated the reporter genes LacZ

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Abbreviations: JNK, c-Jun N-terminal kinase; TNF, tumor necrosis factor; TNFR, TNF receptor; ERK, extracellular signal regulated kinase; DENN, differentially expressed in neoplastic vs. normal cells; MADD, mitogen-activated kinase-regulated death domain protein; GST, glutathione *S*-transferase; CNS, central nervous system.

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and His3. Plasmids were purified from yeast as described by Ward *et al.* (29) and transformed into Max efficiency-competent DH5α (GIBCO/BRL). Automated plasmid sequencing was performed by the Molecular Core Facility at the University of Southern California School of Medicine.

Construction of Glutathione *S*-Transferase (GST) 1.1 and Hemagglutinin (HA)–DENN/MADD. Clone 1.1 was subcloned into the *Xho*I and *Eco*RI sites of pGEX-4T-2 (Pharmacia). Four reverse transcription-PCR fragments were obtained, representing fragments of the full length DENN (using the numbering system by Chow and Lee, GenBank accession no. U44953): from nucleotide 165 to 1543, from nucleotide 1149 to the alternative splice junction in clone 1.1, from the splice junction to nucleotide 4054, and nucleotides 3920 to 4947. The most 5′ fragment was PCR-amplified again, adding an HA epitope (YPYDVPDYA) following the start codon. These fragments and clone 1.1 were ligated and subcloned into pcDNA3 (Invitrogen, Carlsbad, CA).

Generation of Anti-MADD Antibodies. A synthetic peptide corresponding to the C terminus of DENN/MADD (CSSEEDLRTPPRPVSS) was used for rabbit polyclonal antisera generation (Bio-Synthesis, Lewisville, TX).

Cell Cultures. Mouse neuroblastoma Neuro-2A cells were obtained from American Type Culture Collection, routinely cultured in MEM (Sigma) and transfected by using lipofectAmine (GIBCO/BRL). Cells were harvested 2 days post-transfection, and the postnuclear supernatant was analyzed by Western blotting or immunoprecipitation by using antibodies including anti-HA (Boehringer Mannheim) and anti-DENN/MADD peptide antibodies and detected with horseradish peroxidase-conjugated secondary antibody (Transduction Laboratories, Lexington, KY) and the enhanced chemiluminescence method (Amersham).

***In Vitro* Kinase Assay.** Bacterially expressed GST-1.1 was purified on glutathione Sepharose 4B beads (Pharmacia) and incubated with 0.5 μg of recombinant rat JNK3 (Stratagene) in a kinase buffer (Stratagene) in the presence of 5 μCi of γ-^{33}P-ATP (Amersham). In some experiments, alternative kinases were used: p34^{cdc2}/cyclin B (25 units) and Erk2 (50 units) (New England Biolabs). The reaction mixture was then analyzed by SDS/PAGE.

***In Situ* Hybridization.** Sense or antisense riboprobes were prepared by *in vitro* run-off transcription in the presence of α-^{35}S-UTP (Amersham). Unincorporated radionucleotides were removed by passing the reaction mixture through a G50 Sephadex quick spin column (Boehringer Mannheim). *In situ* hybridization was performed according to the standard protocol (30).

Immunohistochemistry. Human CNS tissues were obtained postmortem from five neurologically and histologically normal control patients with no infarction or agonal hypoxia and from four patients with known premortem hypoxia and with histological confirmation of hypoxic nerve cell changes. Based on the hematoxylin and eosin stain, these include a spectrum of changes starting with condensed, eosinophilic cytoplasm followed by nuclear pyknosis and neutrophil and macrophage infiltration. Tissues (1 cm^3) were snap-frozen in liquid nitrogen-chilled isopentane and stored at −90°C. The postmortem interval of the tissues from both sets of patients ranged from 2 to 8 h. Cryostat sections (10 μm) of hippocampus or cerebellum, the most hypoxia-sensitive regions, or Neuro2A cells cultured on Biocoat chamber slides (Becton Dickinson) were used. Slides were air dried and fixed in ice-cold acetone. After blocking with 5% goat serum, primary antibodies including rabbit anti-DENN/MADD C-terminal antibody or rabbit anti-JNK3 antibody (Upstate Biotechnology, Lake Placid, NY) were each diluted 1:100 and allowed to incubate at room temperature for 1 h. After washing with PBS, a biotinylated goat anti-rabbit IgG secondary antibody (Vector Laboratories) was added, and the avidin–biotin complex (Vector Laboratories) and AEC (Zymed) methods were used for visualization. Sections were counterstained with Mayer's hematoxylin solution (Sigma).

RESULTS

Yeast Two-Hybrid Screening and Expression of DENN/MADD in the CNS. Using the yeast two-hybrid system (CLONTECH), $\approx 1.5 \times 10^6$ colonies were screened in a human brain cDNA library, and 41 positive clones were obtained that activated transcription of the reporter genes. These clones did not interact with the Gal4-AD itself nor with Gal4-AD fused to either p53 or lamin. Sequence analysis revealed that one clone, clone 1.1, represented a partial human cDNA of DENN (submitted to GenBank by Chow and Lee, accession no. g1399092). As illustrated in Fig. 1*A*, the 370 amino acids encoded by clone 1.1 were identical to those of DENN, with the important exception that amino acids 762–804 were absent from the yeast clone, suggesting the possibility of a splice variant.

To determine whether clone 1.1 was contiguous with the remainder of the DENN sequence, primers were designed to encompass the junction of the putative splice site and were hybridized only with the spliced form consistent with clone 1.1. Reverse transcription followed by PCR amplification of human brain mRNA was carried out (Fig. 1*A*, reactions 1 and 2). Sequencing of both PCR products confirmed that clone 1.1 was a spliced form of DENN in the human brain. In addition, our data revealed an additional G at nucleotide 4022, upstream from the original stop codon assigned by Chow and Lee at nucleotide 4041. This frameshift results in utilization of the stop codon at nucleotide 4940 and translation of a polypeptide 300 residues longer. Recently, cloning of the mitogen-activated kinase-activating death domain protein (MADD) that interacts with the tumor necrosis factor receptor (TNFR) was reported (31, GenBank accession no. g2102697). The MADD sequence is virtually identical to that of DENN. Consistent with our results, MADD includes the C-terminal 300-amino acid extension. We will therefore refer to the clone used as DENN/MADD.

Examination of the primary sequence of human DENN/MADD reveals two leucine zippers, a putative nuclear localization signal (PRGKRRAK) and a potential proline-rich SH3 binding domain (KPLPSVPP) (32) (Fig. 1*A*). The SH3 binding domain is also present in clone 1.1, adjacent to the variably spliced site. Homologs of human DENN/MADD have been isolated from rat and *Caenorhabditis elegans* as important mediators of regulated neurotransmitter release. The rat DENN/MADD shares >90% identity with its human counterpart, whereas the *C. elegans* homolog (AEX-3) exhibits various degrees of similarity in three domains, with extensive variability in the rest of the sequence (Fig. 1*A*). In addition, the putative nuclear localization signal and the SH3 binding domain, as well as one of the leucine zippers, are missing from AEX-3.

To determine the expression pattern of DENN/MADD in human tissues, Northern analysis was performed. A transcript of $\approx$7.2 kb was evident in heart, brain, placenta, muscle, and pancreas but was absent in lung, liver, and kidney (Fig. 1*B*). The mRNA species detected in the brain appeared to be homogeneous. To determine whether one or both of the two splice variants were represented, primers flanking the putative splice site were used in reverse transcription-PCR reactions. As shown in Fig. 1*C*, PCR II, two bands of 355 and 226 bp were amplified, consistent with the predicted sizes of the fragments derived from DENN/MADD and clone 1.1, respectively. Sequencing of the two bands confirmed that the 226-bp band corresponded to clone 1.1, whereas the broader, 355-bp band was a mixture of the original DENN/MADD and another sequence (not shown). Of interest, a similar reaction, using

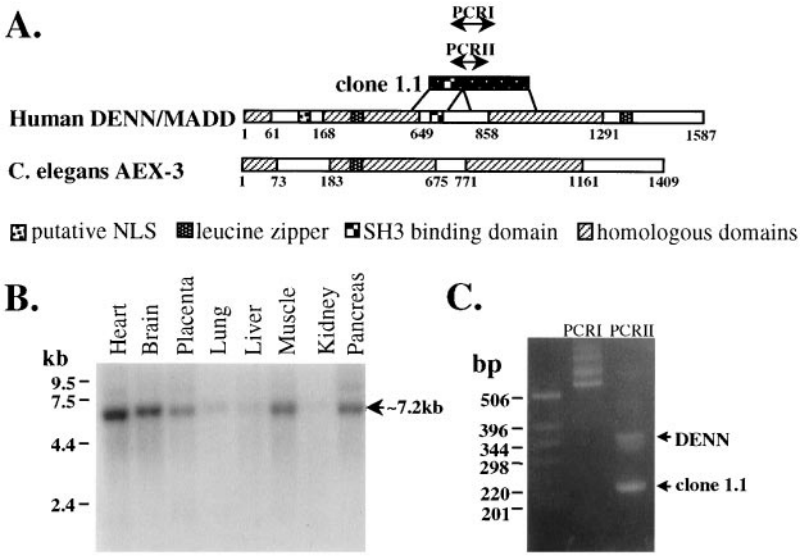

FIG. 1. Cloning of DENN/MADD and its splice variant. (*A*) Graphic representation of clone 1.1 and human DENN/MADD. Sequence comparison with *C. elegans* homolog AEX-3 also is illustrated. Shaded boxes are regions conserved between the two proteins. Three motifs in human are shown, one of which also occurs in AEX-3. (*B*) Northern analysis. A human tissue mRNA blot (CLONTECH) was used with clone 1.1 as the probe. Hybridization of the same blot with an actin cDNA probe indicated that similar amounts of RNA were present in each lane (data not shown). (*C*) Reverse transcription-PCR of DENN/MADD. mRNA (1 μg) from human brain was reverse-transcribed and amplified by using the primers illustrated in *A*: PCRI, nucleotides 2334–2689; PCRII, nucleotides 2334–3024. In both PCR reactions, multiple bands are seen. Clone 1.1 corresponds to the lower band in PCRII.

primers outside of those used in PCRII (Fig. 1*C*, PCRI), yielded multiple bands, indicating the existence of additional splice isoforms. Thus, at least three splice variants are present in the adult human brain, although their functional differences remain to be elucidated. It is likely that the 129-bp difference in size between DENN/MADD and clone 1.1 was too small to be distinguishable on the Northern blot.

***In Vitro* Phosphorylation of Clone 1.1 by JNK.** A purified recombinant GST-1.1 fusion protein was used for the *in vitro* kinase assay. As shown in Fig. 2*A*, incubation of GST-1.1 with increasing amounts of JNK3 resulted in increased phosphorylation (Fig. 2*A*, lanes 2, 3, and 4). The band slightly below GST-1.1 probably represented partially proteolyzed substrate. GST–c-Jun also was included as a positive control (Fig. 2*A*, lane 1).

The specificity of clone 1.1 phosphorylation by JNK was compared with two other proline-directed serine-threonine kinases, $p34^{cdc2}$/cyclinB and ERK. Similar to c-Jun (Fig. 2*B*, lanes 7–9), clone 1.1 (Fig. 2*B*, lanes 1–3) was phosphorylated preferentially by JNK but much less so by $p34^{cdc2}$/cyclinB or ERK2. In contrast, myelin basic protein (Fig. 2*B*, lanes 4–6) was phosphorylated by both $p34^{cdc2}$/cyclinB and ERK2, whereas JNK failed to phosphorylate myelin basic protein.

Expression of Full Length DENN/MADD in Neuro2A Cells. HA-tagged, full length DENN/MADD was constructed with the HA epitope added at the N terminus of the fusion protein. Expression of the HA–DENN/MADD in Neuro2A cells was confirmed by Western blot by using a rabbit polyclonal antibody generated against a C-terminal peptide of DENN/MADD. As shown in Fig. 3*A*, a band of ≈200 kDa corresponding to the protein expressed by the full length cDNA was recognized by both anti-HA and anti-peptide antibody only in the transfected samples (Fig. 3*A*, lanes 1 and 3). To demonstrate the specificity of the anti-DENN/MADD antibody for immunostaining, mock- (no DENN/MADD cDNA transcript)

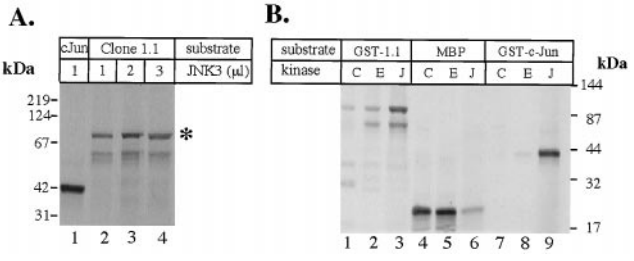

FIG. 2. Phosphorylation of GST-1.1. (*A*) GST-1.1 expressed in *Escherichia coli* was purified and incubated with 1, 2, or 3 μl of JNK3 in the presence of γ-^{33}P-ATP at 30°C for 20 min. Reactions were stopped by the addition of SDS sample buffer and resolved on the gel (lanes 2, 3, and 4). The asterisk (*) indicates the phosphorylated GST-1.1. GST–c-Jun (1 μg) is shown in lane 1 as a positive control. (*B*) Comparison of phosphorylation of GST-1.1, myelin basic protein, and GST–c-Jun by three different kinases: C ($p34^{cdc2}$/cyclinB), E (ERK2), or J (JNK3). Note that GST-1.1 is phosphorylated preferentially by JNK3 (lane 3).

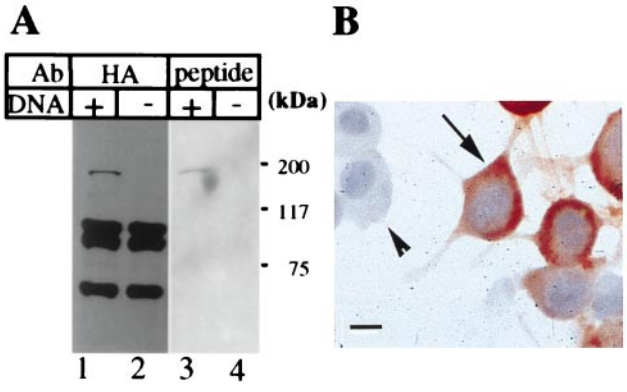

FIG. 3. Expression of full length DENN/MADD in Neuro2A cells. (*A*) Transfected with either pcDNA3 (lanes 2 and 4) or HA-tagged DENN/MADD cDNA in pcDNA3. Western blots are shown that included either anti-HA antibody (lanes 1 and 2) or anti-DENN/MADD peptide antibody (lanes 3 and 4). DENN/MADD appears as a band of ≈200 kDa in lanes 1 and 3. (*B*) Cells transfected with DENN/MADD cDNA were analyzed for DENN/MADD expression by immunostaining by using the anti-peptide antibody. Arrowhead points to a nontransfected cell; arrow indicates a transfected cell that shows diffuse cytoplasmic staining. (Bar = 10 μm.)

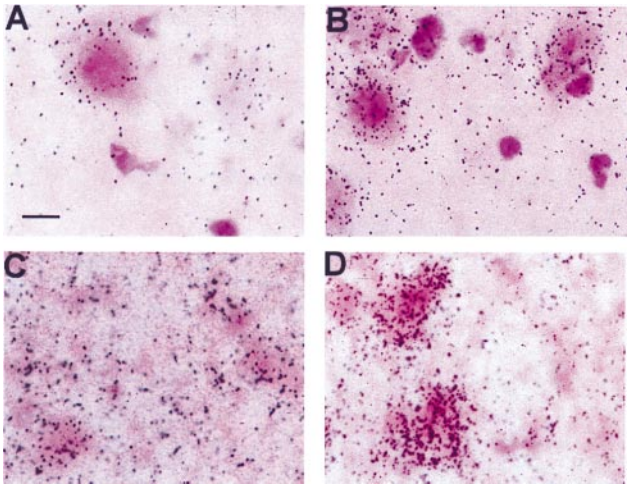

FIG. 4. *In situ* hybridization in normal hippocampus CA4 region. The probes are: sense JNK3 (*A*); antisense JNK3 (*B*); sense clone 1.1 (*C*); and antisense clone 1.1 (*D*). Labeling over neuronal cytoplasm with antisense probes exceeds the nonspecific background with the sense markers in both cases. All figures are at the same magnification. (Bar in *A* = 10 μm.)

or human DENN/MADD cDNA-transfected Neuro2A cells grown on chamber slides were examined with the antibody. Brightly stained cells were only apparent in the cDNA-transfected cells (Fig. 3*B*) but not in the negative control (not shown).

Co-Localization of JNK3 and DENN/MADD by *in Situ* Hybridization. One prerequisite for JNK3 to phosphorylate DENN/MADD *in vivo* is that they be expressed in the same cell type. *In situ* hybridization was used to detect the mRNA distribution of the kinase and its substrate in the human hippocampus. Fig. 4 shows abundant silver grains with both antisense probes compared with the sense probes, indicating the presence of both JNK3 and DENN/MADD mRNA (Fig. 4*D*) in large neurons in the hippocampal CA4 region. Similar results also were observed in pyramidal neurons in the CA1 region and subiculum (data not shown).

Localization of DENN/MADD by Immunohistochemistry in the Normal and Hypoxic Nervous System. Immunohistochemical studies were performed on cryostat sections obtained from brain tissues obtained postmortem from four normal patients. As shown in Fig. 5*A*, JNK3 immunostaining was sparse and diffusely distributed in the cytoplasm of pyramidal neurons in the hippocampus. These neurons also expressed scant to moderate amounts of DENN/MADD in their somas (Fig. 5*B*). Preimmune sera gave none of the signals (data not shown). In the cerebellum, DENN/MADD was found in the Purkinje cell cytoplasm (Fig. 5*D*) and in the strongly stained processes and terminals extending transversely across the molecular layer nearly to the pial surface (Fig. 5*C*). In contrast, in the hippocampi of four patients with typical histological evidence of acute hypoxia including condensed, eosinophilic cytoplasm and with neuronophagia (macrophage invasion), (Fig. 5*G*), JNK3 was shown to be more strongly expressed, especially in the nucleus (Fig. 5*E*). Concomitantly, anti-DENN/MADD staining also was modified by striking labeling of the nucleolus (Fig. 5*F*). This nuclear and nucleolar staining of DENN/MADD only was observed in the CA1 region and subiculum, regions known to be selectively sensitive to hypoxia (Fig. 5*H*). In two of two other patients with cerebellar hypoxic changes, some Purkinje cells also showed nucleolar staining of DENN/MADD (Fig. 5*H*). This was not observed in any of the five normal control patients whose brain lacked signs of hypoxia/ischemia.

DISCUSSION

We report the identification and sequencing of a new splice variant, GST-1.1, of human DENN/MADD. Recombinant GST-1.1 was phosphorylated preferentially by JNK *in vitro* but not by $p34^{cdc2}$/cyclinB or ERK, suggesting that DENN/MADD is a favored substrate for JNK. DENN/MADD is heavily phosphorylated, and JNK is the first kinase accounting for this function (31). There are five Ser-Pro and one Thr-Pro sequences in clone 1.1 that potentially serve as acceptor sites for JNK. The actual residues used have not been identified yet.

Although c-Jun and ATF-2 both bind JNK with considerable affinity *in vitro* (9, 11), DENN/MADD failed to do so with JNK, either in the coimmunoprecipitation or the *in vitro* pull-down assay (data not shown), despite the fact that the DENN/MADD variant was discovered in the yeast screen. Gupta *et al.* (2) hypothesized that binding and phosphorylation are separate attributes of JNK. At least two other established substrates, Elk-1 and JunD, do not exhibit detectable *in vitro* binding to JNK. We generated two truncated JNK3 cDNA constructs that encompassed either the N terminus (residues 1–245) or the C terminus (residues 147–381). Both fragments contained the corresponding region in JNK2 previously re-

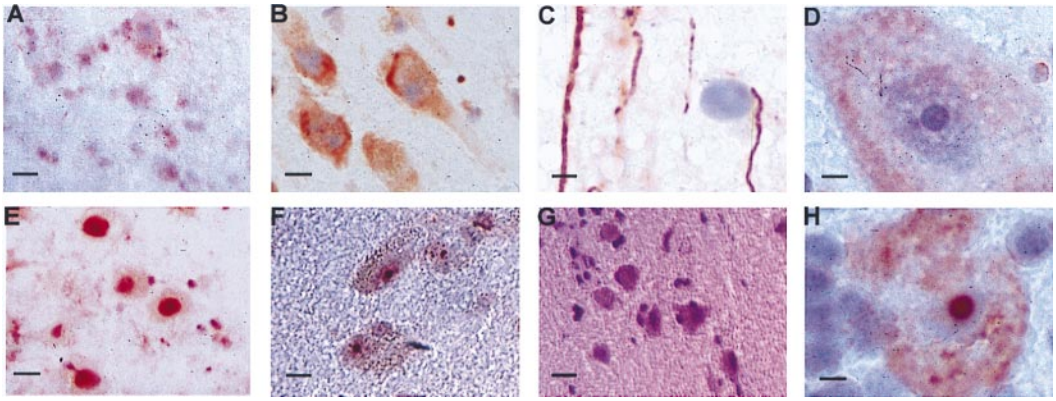

FIG. 5. Immunohistochemical localization of JNK3 and DENN/MADD. Cryostat sections of normal (*A–D*) or hypoxic/ischemic brain (*E–H*). (*A*) In normal hippocampus (CA-1) stained with anti-JNK3, there is minimal, diffuse cytoplasmic staining of pyramidal neurons. (*E*) In contrast, hippocampus with hypoxic change shows strong nuclear immunoreactivity. Anti-DENN/MADD antibody shows weak to moderate cytoplasmic staining of normal hippocampal pyramidal neurons in *B*. (*G*) The hypoxic/ischemic hippocampus shows typical eosinophilic pyramidal neurons. Note the perineuronal macrophages (hematoxylin and eosin). (*F*) Anti-DENN/MADD immunostain of a comparably affected hippocampus shows intense nucleolar stain. In the normal cerebellum in *D*, Purkinje cells show diffuse cytoplasmic staining. (*C*) There is also intense staining of neurites in the molecular layer of the normal cerebellum. In the hypoxic/ischemic cerebellum, there is strong nucleolar staining of the Purkinje cells in *H*. (Bar = 25 μm in *A*, *E*, and *G*; bar = 10 μm in *B* and *F*; and bar = 2.5 μm in *C*, *D*, and *H*.

ported to be essential for efficient c-Jun binding (3). However, neither of these truncated JNK3 forms interacted with clone 1.1 in the yeast system (data not shown), suggesting that the entire JNK sequence may be required for measurable binding to occur, consistent with results obtained by Gupta *et al.* (2) for other substrates. Future mutagenesis studies may determine precisely which residues in clone 1.1, including the SH3 binding domain, are important for JNK recognition.

Our data indicated that JNK phosphorylated DENN/MADD with significantly higher activity than did ERK. In addition to JNK, the C terminus of DENN/MADD (not overlapping with clone 1.1) also interacted with TNFR1 as part of the TNFR signaling complex (31). Schievella *et al.* (31) demonstrated that, in COS cells, overexpression of a partial cDNA starting at F1269, but not the full length DENN/MADD, strongly induces JNK activity independently of TNF. In contrast, ERK activity is augmented by full length DENN/MADD and TNF has an additive effect. Concomitant activation of ERK and JNK has been documented to occur with TNFα acting through either TNFR1 or TNFR2 (31, 33), although ERK and JNK appear to have opposing effects on cellular growth and death. For example, ERK activation counteracts Fas- or nerve growth factor deprivation-induced/JNK-mediated apoptosis (26, 34). In this scenario, ERK is the principal effector of the TNF–TNFR1–DENN/MADD pathway. Subsequently, TNF stimulates JNK, probably by other pathways (33), which, in turn, phosphorylates DENN/MADD. How, then, does phosphorylation of DENN/MADD impact on its activation of the mitogen-activated kinases? We speculate that phosphorylated DENN/MADD may suppress the ERK pathway when the JNK pathway is active. TNFα also induces a mitogen-activated protein kinase kinase kinase, ASK1, with apoptosis as the consequence (35). It remains to be examined whether DENN/MADD is involved in the action of ASK1 as well as the physiological consequences of TNFα induction of JNK.

DENN/MADD homologs have been cloned from rat and *C. elegans* as GDP/GTP exchange proteins specific for the Rab3 subfamily members to regulate exocytosis of neurotransmitters (36, 37). Consistent with such a function in vesicle release, we observed expression of DENN/MADD in the neuronal processes in the cerebellum and hippocampus (Fig. 5). In the latter site, Rab3 is essential for long term potentiation (38). It is yet unknown whether phosphorylation alters the GTP/GDP exchange activity of DENN/MADD.

Immunohistochemical localization of DENN/MADD in normal human CNS tissues revealed focal cytoplasmic immunoreactivity of neuronal subpopulations, including their proximal and distal neurites. In all four patients with either acute hypoxic cell change, including with neuronophagia, the cytoplasmic DENN/MADD immunostaining showed both enhanced expression and an altered subcellular distribution. Striking nucleolar staining was apparent in neurons of the hypoxia-sensitive regions of the hippocampus, including CA1 and the subiculum, as well as in Purkinje cells in the cerebellum.

These changes reflect the nonuniform susceptibility of neurons to hypoxia/ischemia in the CNS. There is a well established hierarchy of selective sensitivity among neurons throughout the nervous system. Most susceptible are pyramidal neurons of the subiculum, CA1 regions of the hippocampus, and Purkinje cells of the cerebellum. Microscopically, a fairly constant time course evolves after the hypoxic/ischemic insult. The earliest and characteristic hallmark, visible after 4 h, is the eosinophilic or "red neurons" followed by nuclear pyknosis and disappearance of the nucleoli. By 15–24 h, neutrophils infiltrate the tissues, and by 2–3 days, there is an influx of macrophages. Thus, the association of DENN/MADD with the nucleolus is a change that happens very early, perhaps when the damage is still reversible. As a stress-activated kinase, JNK3 presumably would be activated under the hypoxic conditions in these neurons. Of interest, expression of JNK3 in the hippocampus also was found to be increased, in particular in the nucleus (Fig. 5*F*), placing the kinase and substrate in the same intracellular compartment, consistent with our hypothesis for their interaction. These observations support the results of Dragunow *et al.* (39), who report that hypoxia/ischemia increased c-Jun expression in CA1 of the rat hippocampus mediated by *N*-methyl-D-aspartate. In our experiments, when Neuro2A cells were transfected with DENN/MADD cDNA, the expressed protein was concentrated in the cytoplasm, suggesting that exogenous signals may be necessary for translocation to the nucleolus to occur. Because our antibody recognizes the C terminus of DENN/MADD that is shared among various splice isoforms, it is unclear presently whether one or all isoforms are translocated into the nucleus.

A growing list of nuclear proteins have been found to be recruited into the nucleolus. This diverse array includes the tumor suppressor Rb (40), transcription factor YY1 (41), heat-shock protein 70 (42), histone deacylase (43), TATA-binding protein (44), some viral proteins such as HIV Rev (45), and herpesvirus MEQ (46). Although a consensus sequence has yet to be defined, the nucleolar localization signal generally involves a stretch of positively charged residues. Functionally, these proteins may influence DNA replication or transcriptional activity. The role of DENN/MADD in nucleolar function remains unclear, but the restricted pattern concentrated in the hypoxia-sensitive regions suggests that it may be part of a neuronal injury response. As a stress-activated kinase, JNK3 potentially is induced under these conditions, and activated JNK has been shown to be targeted to the nucleus (refs. 47 and 48; Fig. 5). It is tempting to speculate that, after translocation of both the substrate and kinase transducing the stress signal in the hypoxia-sensitive neurons, JNK3 then phosphorylates DENN/MADD.

We propose that DENN/MADD has dual activities: Normally, it modulates neurotransmitter release of a subset of neurons functioning as a "housekeeping" gene evolutionarily conserved from *C. elegans* to human. This function is likely to be independent of the phosphorylation state because the domain important for JNK recognition (clone 1.1) is localized in the divergent region between *C. elegans* and human (Fig. 1*A*). In addition, JNK may be bound tightly to a cytoplasmic anchoring inhibitor (49) and therefore be unavailable for DENN/MADD phosphorylation. Under stress conditions, such as hypoxia or macrophage invasion, DENN/MADD may translocate into the nucleolus. Elucidation of the complex pathway of DENN/MADD and JNK could help to understand how neurons respond to stress and could provide insight into nucleolar regulatory mechanisms.

We greatly appreciate the allocation of autopsied brain tissues by the Alzheimer's Disease Research Center at University of Southern California. We also thank Celia Williams, Ning Sun, Zhiqun Tan, and Steven Schreiber for technical advice on *in situ* hybridization and immunohistochemistry and Bernard Freidin for his computer expertise. This work was supported in part by the National Institute of Mental Health (5R37MH39145), the National Institute on Aging (5P50AG05142), the Sankyo Corporation (Sankyo) to C.A.M., and National Institutes of Health training grant (5K12AG00521) to Y.Z.

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