Amyloid β Protein Precursor Is Phosphorylated by JNK-1 Independent of, yet Facilitated by, JNK-Interacting Protein (JIP)-1*

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Alzheimer’s disease (AD) is genetically linked to the processing of amyloid β protein precursor (AβPP). Aside from being the precursor of the amyloid β (Aβ) found in plaques in the brains of patients with AD, little is known regarding the functional role of AβPP. We have recently reported biochemical evidence linking AβPP to the JNK signaling cascade by finding that JNK-interacting protein-1 (JIP-1) binds AβPP. In order to study the functional implications of this interaction we assayed the carboxyl-terminal of AβPP for phosphorylation. We found that the threonine 668 within the AβPP intracellular domain (AID or elsewhere AICD) is indeed phosphorylated by JNK1. We surprisingly found that although JNK-1 can facilitate this phosphorylation, it is not required for this process. We also found that JNK-1 only facilitated phosphorylation of AβPP but not of the two other family members APLP1 (amyloid precursor-like protein 1) and APLP2. Understanding the connection between AβPP phosphorylation and the JNK signaling pathway, which mediates cell response to stress may have important implications in understanding the pathogenesis of Alzheimer’s disease.

Alzheimer’s disease (AD)1 is the most common neurodegenerative disease constituting approximately two thirds of all cases of dementia (1). AD is genetically linked to a few molecules, one being AβPP. AβPP is a type I transmembrane protein, which undergoes processing by the secretases to produce various fragments. Following processing by the β- and γ-secretases, the Aβ fragment (from the β to γ sites) and AID (from the γ site to the carboxyl-terminal) are produced (2, 3). Recently, another γ-secretase-dependent cleavage has been described to occur at the “epsilon” site, which lies within AID (4–7). This would cause shorter AID fragments of either 49 or 50 amino acids. The pathologic cascade, which leads to clinical manifestations of AD, has not been identified fully; however, the “Amyloid Hypothesis” has been used to explain certain aspects of AD pathology. According to this hypothesis, the accumulation of Aβ is the primary event that leads to all subsequent events in the pathology of AD (8).

However, considering that production of both Aβ and AID are dependent on the γ-secretase, we and others have attempted to understand the cellular effects of AID production. Indeed it has been found that AID is able to trigger apoptosis or lower the cell’s threshold to other apoptotic stimuli (9). Furthermore, many proteins have been found to interact with AID such as X11, Fe65, mDab, Sbe, Numb, and Numb-like (10–14). Use of “guilty by association” has been used to speculate possible roles for AID in the cell. One interacting protein, which has attracted much interest recently, is JNK-interacting protein (JIP)-1 (15). JIP-1 is a cytoplasmic protein that binds members of the JNK signaling cascade and scaffolds these proteins to allow for efficient activation of the JNK pathway (16). We and others (17–19) have shown that AID binds JIP-1. This biochemical connection between AβPP (and therefore AD), and JIP-1 (and therefore the JNK signaling cascade) provided an intriguing connection that could be important in understanding the pathology of AD. Pathological hallmarks of AD include amyloid plaques, neurofibrillary tangles, and neurodegeneration, with the last seeming to be most directly related to the clinical manifestations of AD (20). The JNK signaling cascade, which is responsible for responding to cell stress and mediating apoptosis (or survival) in different contexts, would seem likely to be involved in the neurodegeneration seen in AD (21). In fact, there have already been studies implicating the JNK signaling pathway in various aspects of AD pathology including both amyloid plaques and neurofibrillary tangles (22–26). While investigating the functional manifestations of this AβPP-JIP-1 interaction we have found that AID in combination with JIP-1 is able to cause transcriptional activation of a reporter gene (27).

In this study we present data supporting the possibility that there are two pathways for JNK1-dependent phosphorylation of threonine 668 on the cytoplasmic tail of AβPP. In one pathway, JIP-1 is able to scaffold the components necessary for JNK1 activation and AβPP phosphorylation, while in the second AβPP is phosphorylated independently of JIP-1. We further show that in the JIP-1-dependent pathway JNK1 is not capable of phosphorylating the cytoplasmic tails of APLP1 and APLP2, indicating that the functional role of JIP-1 is asymmetric with respect to action on AβPP and APLPs.

MATERIALS AND METHODS

DNA Constructs—The full-length JIP-1 construct used was the human JIP-1e described previously and is referred to as JIP-1 throughout this study. JIP-1JBD is also described elsewhere. Expression constructs for MLK3, JNK1, and MKK7 were obtained from Dr. C. W. Chow (Albert Einstein College of Medicine). Constructs for expression of GST fusion proteins were obtained from Dr. S. Matuda (GST-AID, GST-AID-Δ, GST-ALID1 (AβPP: Like Intracellular Domain), GST-ALID2 (Albert Einstein College of Medicine), or were constructed using the Transformer Site-directed Mutagenesis Kit (Clonetech, GST-AID-Y682G and GST-AID-T668A). mJIP1a was obtained from Dr. R. J.
Davis (Howard Hughes Medical Institute, University of Massachusetts Medical School, Worcester, MA) and the cloning of mJIP1b is described elsewhere.

Cell Culture—HEK293T cells were grown in RPMI 1640 media with 10% heat-inactivated fetal calf serum. HEK293 cells overexpressing A/H9252PP were grown in the same medium supplemented with 5 μg/ml of puromycin to maintain stable expression.

Immunoprecipitations, JNK Activation, Kinase Assays, and Western Blotting—Immunoprecipitations were performed as described elsewhere with the addition of the phosphatase inhibitors sodium fluoride (50 mM) and sodium vanadate (0.2 mM) to all buffers. To immunoprecipitate A/H9252PP, antibody P2–1 (BIOSOURCE) was used. HEK293 cells were treated with 60 mJ/cm² of UVB using a Bio-Rad cross-linker to activate JNK. SP600125 (Biomol) was added at the indicated concentrations 10 min before UVB treatment. Kinase assays were performed using lysis buffer and kinase buffer from Cell Signaling Technology as follows. For experiments in Figs. 3, 4, and 5, cells were lysed by sonication for 10 s. After spinning down, the supernatant was added to GST fusion protein and incubated overnight at 4 °C. The beads were washed twice with lysis buffer and twice with kinase buffer followed by the addition of 50 μl of kinase buffer supplemented with 100 μM ATP. Following incubation at 30 °C for 30 min, the reaction was halted by boiling in loading buffer. For experiments in Figs. 2 and 5b, proteins were purified by immunoprecipitation for 4.5 h at 4 °C and washed twice with lysis buffer and twice with kinase buffer. Appropriate purified proteins were then added to GST-AID. The kinase assay was then performed as described above. Proteins were separated by SDS-PAGE and Western blotting was carried out with the following antibodies: AβPP, (22C11, Chemicon or APP-C-terminal, Zymed Laboratories Inc.); FLAG, (Sigma); MLK3, JNK1, Phospho-JNK1, Jun, phospho-Jun, pan-phospho-threonine (Cell Signaling Technology), monoclonal AβPP-Phospho-T668 (P. Davies).

RESULTS

JNK1 Activation Results in a JIP-1-independent AβPP Phosphorylation in Vivo—We and others (17, 18) have previously identified an interaction between AβPP and JIP-1. Considering that JIP-1 is a scaffold protein which is able to bind components of the JNK signaling cascade and AβPP contains a consensus sequence for JNK phosphorylation (Thr-Pro or TP) in the intracellular domain, we questioned whether AβPP is phosphorylated by JNK1 in a JIP-1-dependent manner. HEK293 cells expressing AβPP but not JIP-1, and MLK3 or pcDNA3 were transfected into HEK 293 cells, and immunoprecipitation was performed to isolate AβPP. AβPP was only phosphorylated in the presence of JIP-1, JNK1, and MLK3. b, combinations of JIP-1, JNK1, and MLK3 were transfected and analyzed as in a. Only samples containing active JNK had phosphorylated AβPP. c, combinations of JIP-1, JNK1, and MKK7 were transfected and analyzed as in a. Once again, only samples containing active JNK had phosphorylated AβPP. d, AβPP phosphorylation induced by UVB is blocked by the JNK inhibitor SP600125.
1/JNK1/MLK3 but not in the control cells. In order to determine which components of the signaling cascade are required for this phosphorylation, we transfected the various combinations of JIP-1, JNK1, and MLK3. Surprisingly, we found (Fig. 1b) that the combination of JNK1 and MLK3 was sufficient to cause phosphorylation, in fact to an even greater extent than in combination with JIP-1. A similar experiment was carried out using the MAP kinase kinase M KK7 instead of MLK3 to activate JNK1. Once again we found that phosphorylation was stronger in the absence of JIP-1 (Fig. 1c). Although these data could be explained by stating that JIP-1 acts as a JNK inhibitor and therefore there is less phospho-JNK1 in the sample, Fig. 1, b and c indicate that there is equal if not more phospho-JNK1 in the JIP-1-supplemented samples. A more likely explanation would be that JIP-1 could play a direct role in facilitating AβPP phosphorylation but due to JIP-1 overexpression there is quenching due to distribution of AβPP and active kinases to different JIP-1 complexes. Nevertheless, these data indicate that active JNK1, even not in the context of JIP-1, is able to cause AβPP phosphorylation.

To determine whether activation of endogenous JNK results in AβPP phosphorylation, we transfected HEK293 cells with UVB to activate JNK. Thirty minutes after UVB exposure JNK was activated (data not shown), and AβPP was phosphorylated on threonine 668 (Fig. 1d). Moreover, the specific JNK inhibitor SP600125 blocked AβPP phosphorylation on threonine 668 in a dose-response manner (Fig. 1d). Thus, AβPP is a JNK substrate.

**Active JNK1 Directly Phosphorylates AID in Vitro Independent of JIP-1**—The above results suggest that active JNK1 can phosphorylate AβPP at threonine 668 in vivo. However, it is also possible that AβPP could be phosphorylated by other endogenous kinases activated by JNK1. To determine whether JNK1 is directly responsible for this phosphorylation, we used an *in vitro* system. Cells were transfected individually with JIP-1 or JNK1, with JNK1 either being activated with anisomycin or not. Cells were lysed and proteins were purified by immuno-precipitation (Fig. 2a). Different combinations of these proteins were incubated with GST-AID in the presence of ATP and phosphorylation was analyzed by Western blotting. Fig. 2b shows that active JNK1 is sufficient to phosphorylate AID, even in the absence of JIP-1. These data indicate that JNK1 can directly phosphorylate AβPP.

**AβPP Is Phosphorylated by JNK1 in Vitro in a JIP-1-dependent Manner**—Although the above data indicated that JIP-1 was not necessary for AβPP phosphorylation, we speculated that JIP-1 might function under certain circumstances to bring JNK1 and AβPP together to allow efficient phosphorylation. In order to test for this, we overexpressed combinations of JIP-1, JNK1, and MLK3 (Fig. 3a) and analyzed whether GST-AID could pull-down JIP-1 already loaded with active JNK1 that could phosphorylate AID (Fig. 3b). Fig. 3c shows using a pan-phospho-threonine antibody that JIP-1 is able to serve as the link between GST-AID and active JNK1 to facilitate AID phosphorylation *in vitro*. Using an antibody specific for AβPP Thr-668 phosphorylation we determined that threonine 668 was being phosphorylated (Fig. 3d). In order to determine whether this was the only location being phosphorylated, we incubated the JIP-1, JNK1, and MLK3 combination along with GST-AID-T668A and compared it to GST-AID. Fig. 3d shows that although binding of the JIP-1/JNK1/MLK3 complex is not decreased by this mutation, no threonine phosphorylation is detected using a pan-phospho-threonine antibody or an antibody recognizing phosphorylation at Thr-668. Importantly, use of both a GST-AID-YENPTY motif mutant (GST-AID-Δ) and a GST-AID-Y682G point mutant, (both of which do not bind JIP-1), was used to confirm that binding of JIP-1 to AID is required for *in vitro* association and phosphorylation in this system (Fig. 3, c and d).
There are two forms of JIP-1, which have been identified in both mouse and rat (31), JIP1b contains a complete PTB domain that interacts with AβPP while JIP1a is missing 47 amino acids at the beginning of the PTB domain and is therefore unable to bind AβPP. In order to determine whether JIP1a and JIP1b may mediate AβPP phosphorylation in vitro, we performed a kinase assay as above however this time using either JIP1a or JIP1b. Fig. 4a shows that only JIP1b is able to bind GST-AID and mediate phosphorylation of AID in vitro. These data suggest that there may be two pools of JIP-1, one that is involved in AβPP function and one that functions independently of AβPP pathways. We next wanted to clarify the role of JNK1 along this JIP-1-mediated pathway. A JIP-1 mutant lacking the JNK binding domain (JIP-1ΔJBD) was used to perform the kinase assay such that JNK1 would not be recruited to AβPP. Fig. 4b shows that although both the wild-type JIP-1 and the JIP-1ΔJBD mutant associate with AID, JNK1 (and therefore phospho-JNK1) is not recruited by JIP-1ΔJBD and AID is not phosphorylated. These data indicate that JNK1 recruitment plays an essential role in the JIP-1-dependent AβPP Thr-668 phosphorylation in vitro.

**JIP-1 Facilitates Phosphorylation of AβPP but Not the APLPs—**AβPP is part of a larger gene family that is comprised of AβPP, APLP1, and APLP2; however, most research on the family has been focused on AβPP because only it has been directly implicated in AD (2, 33). Work using mice with either single or compound knockouts of the different family members indicate that although there are unique roles for each of the different AβPP family members during development, there is also functional redundancy among them that allows for compensation between the family members (34, 35). APLP1 and APLP2 are also able to bind JIP-1 although they bind JIP-1 more weakly than does AβPP (27). Sequence alignments between the three AβPP family members show that all three family members contain the YENPTY motif, which is essential for JIP-1 binding (Fig. 5a). Furthermore, both AβPP and APLP2 contain the TP motif, which may be phosphorylated by JNK1, whereas APLP1 does not contain this motif (Fig. 5a).

We first wanted to determine whether active JNK can phosphorylate APLP1 and APLP2. Fig. 5b shows that as predicted from the absence or presence of the TP motif APLP1 cannot be phosphorylated by JNK while APLP2 can. This also would indicate that JIP-1 is not essential for APLP2 phosphorylation. We therefore wanted to determine whether JIP1 can facilitate phosphorylation of APLP2 similar to AβPP. To do this, we transfected the JIP-1/JNK1/MLK3 complex into 293 cells and performed a kinase assay such that JNK1 would not be recruited by JIP1. GST fusion proteins were used to pull-down JIP1 and JIP lacking the JNK binding domain cannot facilitate AID phosphorylation. a, JIP1a or JIP1b along with JNK1 and MLK3 were transfected into HEK 293 cells. Protein complexes were pulled-down with GST-AID. Only JIP1b is able to facilitate Thr-668 phosphorylation. b, JIP-1 or JIP-1 lacking the JNK binding domain (JIP-1ΔJBD) along with JNK1 and MLK3 were transfected into HEK 293 cells. Although JIP-1 is pulled down in both samples, only wild-type JIP-1, which has the JBD can bring active JNK1 to phosphorylate GST-AID.

**Fig. 5. JIP-1 facilitates phosphorylation of APP but not the APLPs.** a, alignment of AβPP, APLP1, and APLP2. The conserved YENPTY motif responsible for AβPP to JIP-1 binding, as well as the JNK consensus phosphorylation motif (TP) is indicated. Note that APLP1 does not contain the TP motif. b, inactive (JNK) or active JNK (*JNK) was added to GST fusion proteins (AID, AID T668A, ALID1, ALID2) and incubated to allow for phosphorylation. Only AID and ALID2 were phosphorylated by active JNK. c, GST fusion proteins were used to pull-down JIP1 and other components that might be bound. Kinase assays were performed, and Western blotting was carried out. Both pan-phospho-threonine antibody and phospho-T668 antibody reveal that only AID is phosphorylated.
Finally, we have also found that that in vitro only AID is phosphorylated in a JIP-1-dependent manner, while the ALIDs are not. We have also previously found that only AID and not the ALIDs are capable of combining with JIP-1 to form a transcriptionally active complex. AD is genetically linked to AβPP but not the APLPs, presumably because only AβPP yields Aβ fragment following processing by the secretases. Our data now suggest a new JIP-1-related difference between AβPP and the APLPs. Phosphorylated AID fragments may mediate the toxicity found in AD while ALID fragments, which are not phosphorylated in a JIP-1-facilitated manner, cannot mediate these functions. Further work will be necessary to identify the functional consequences of JNK1-mediated phosphorylation at Thr-668 particularly if it is involved in the pathogenesis of Alzheimer’s Disease.

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REFERENCES

1. Nussbaum, R. L., and Ellis, C. E. (2003) N Engl. J. Med. 348, 1356–1364
2. Sisodia, S. S., and St. George-Hyslop, P. H. (2002) Nat. Rev. Neurosci. 3, 281–290
3. Koo, E. H. (2002) Traffic, 3, 763–770
4. Sastre, M., Steiner, H., Fuchs, K., Capell, A., Multiaup, G., Condon, M. M., Teplow, D. B., and Haass, C. (2001) EMBO Reports 2, 835–841
5. Yu, C., Kim, S. H., Besuchet, T. X., H. Gasparini, L., Wang, R., and Sisodia, S. S. (2001) J. Biol. Chem. 276, 43756–43769
6. Weidemann, A., Eggett, S., Reinhard, F. B., Vogel, M., Paliga, K., Bailer, G., Masters, C. L., Beyreuther, K., and Erv, G. (2002) Biochemistry 41, 2925–2935
7. Sato, T., Dohmae, N., Qi, Y., Kukuda, N., Misonou, H., Matsuromi, R., Ma- rayama, H., Koo, E. H., Haass, C., Takio, K., Morishima-Kawashima, M., Ishiura, S., and Ihara Y. (2003) J. Biol. Chem. 278, 24294–24301
8. Hardy, J., and Selkoe, D. J. (2002) Science 297, 353–356
9. Passer, B., Pellegrini, L., Russo, C., Siegel, R. M., Lenardo, M. J., Sceltzini, G., Buckingham, M., Taktion, M., and D’Adamio, L. (2000) J. Alzheimers Dis. 2, 289–301
10. Borg, J. P., Ogi, J., Levy, E., and Margolis, B. (1996) Mol. Cell. Biol. 16, 6229–6241
11. Fiore, F., Zambrano, N., Minopoli, G., Donini, V., Dullio, A., and Russo, T., (1995) J. Biol. Chem. 270, 36883–36886
12. Trommsdorff, M., Borg, J. P., Margolis, B., and Herz, J. (1998) J. Biol. Chem. 273, 33556–33560
13. Tarr, P. E., Roncarati, R., Pellico, G., Pellico, P. G., and D’Adamio, L. (2002) J. Biol. Chem. 277, 16798–16804
14. Roncarati, R., Sestan, N., Scheinfeld, M. H., Berechid, B. E., Lopez, P. A., McGlade, J. C., Meucci, O., Rakic, P., and D’Adamio, L. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7102–7107
15. Dickens, M., Rogers, J. S., Cavanagh, J., Raitano, A., Xia, Z., Halpern, J. R., Greenberg, M. E., Sawyers, C. L., and Davis, R. J. (1997) Science 277, 693–696
16. Whitmarsh, A. J., Cavanagh, J., Tarrant, A., Xia, Z., Halpern, J. R., Greenberg, M. E., Sawyers, C. L., and Davis, R. J. (1997) Science 281, 1677–1681
17. Matsuda, S., Yasukawa, T., Homma, Y., Ita, Y., Niikura, T., Hiraki, T., Hirai, S., Ono, S., Kita, Y., Kawasaki, M., Ueyama, K., Yamamoto, T., Kyriakis, J. M., and Nishimoto, I. (2001) J. Neurosci. 21, 6657–6667
18. Scheinfeld, M. H., Roncarati, R., Vito, P., Lopez, P. A., Abdallah, M., and D’Adamio, L. (2002) J. Biol. Chem. 277, 3767–3775
19. Inomata, H., Nakamura, Y., Hayakawa, A., Takata, H., Suzuki, T., Miyazawa, K., and Kitamura, N. (2003) J. Biol. Chem. 278, 22346–22355
20. Niikura, T., Hashimoto, Y., Tajima, H., and Nishimoto, I. (2002) J. Neurosci. 22, 380–391
21. Lin, A. (2003) Bioessays. 25, 17–24
22. Zhu, X., Raina, A. K., Rottkamp, C. A., Aliev, G., Perry, G., Boux, H., and Smith, M. A. (2001) J. Neurochem. 76, 435–441
23. Shojo, M., Iwakami, N., Takeuchi, S., Waragai, M., Suzuki, M., Kasazawa, I., Lippari, C. F., Ono, S., and Okazawa, H. (2002) Brain Res. Mol. Brain Res. 103, 211–233
24. Reynolds, C. H., Utton, M. A., Gibb, G. M., Yates, A., and Andison, B. H. (1997) J. Neurochem. 68, 1736–1744
25. Goedert, M., Hasegawa, M., Jakes, R., Lawler, S., Cundella, A., and Cohen, P. (1997) FEBS Lett. 409, 57–62
26. Zhu, X., Castellani, R. J., Takahashi, A., Nonomura, A., Atwood, C. S., Perry, G., and Smith, M. A. (2001) Mech. Ageing Dev. 123, 39–46
27. Scheinfeld, M. H., Matsuda, S., and D’Adamio, L. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 1729–1734
28. Aplin, E. A., Gibb, G. M., Jacobsen, J. S., Gallo, J., and Andison, B. H. (1996) J. Neurochem. 67, 659–707
29. Suzuki, T., Oishi, M., Marushak, D. R., Carrikin, A. J., Nairn, A. C., and Greenfield, P. (1994) EMBO J. 13, 1114–1122
30. Standen, C. L., Brownlee, J., Grierson, A. J., Krishnayapan, S., Lau, K. F., McLoughlin, D. M., and Miller, C. C. (2001) J. Neurochem. 76, 316–320
31. Coffey, E. T., Hongisto, V., Dickens, M., Davis, R. J., and Courtney, M. J. (2000) *J. Neurosci.* 20, 7602–7613
32. Ando, K., Iijima, K. I., Elliott, J. I., Kirino, Y., and Suzuki, T. (2001) *J. Biol. Chem.* 276, 40353–40361
33. Haass, C. and De Strooper, B. (1999) *Science* 286, 916–919
34. von Koch, C. S., Zheng, H., Chen, H., Trumbauer, M., Thinakaran, G., van der Ploeg, L. H., Price, D. L., and Sisodia, S. S. (1997) *Neurobiol. Aging.* 18, 661–669
35. Heber, S., Herrus, J., Gajic, V., Hainfellner, J., Aguzzi, A., Bulicke, T., von Kretzschmar, H., von Koch, C., Sisodia, S. S., Tremml, P., Lipp, H. P., Wolfer, D. P., and Muller, U. (2000) *J. Neurosci.* 20, 7851–7963
36. Ramelot, T. A., and Nicholson, L. K. (2001) *J. Mol. Biol.* 307, 871–884
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