Phosphorylation by cAMP-dependent Protein Kinase Inhibits the Degradation of Tau by Calpain*

Joel M. Litersky and Gail V. W. Johnson‡

From the Departments of Psychiatry and Cell Biology, University of Alabama at Birmingham, Birmingham, Alabama 35294

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The effects of cAMP-dependent protein kinase (cAMP-PK) phosphorylation on the degradation of the microtubule-associated protein tau by calpain were studied. Purified bovine brain tau that had been phosphorylated by cAMP-PK had a slower migration pattern on sodium dodecyl sulfate-polyacrylamide gels and a more acidic, less heterogeneous pattern on two-dimensional, nonequilibrium pH gradient electrophoresis (NEPHGE) gels compared with untreated tau. Phosphorylation of tau by cAMP-PK significantly inhibited its proteolysis by calpain compared with untreated tau. To our knowledge this is the first demonstration that phosphorylation of tau by a specific kinase results in increased resistance to hydrolysis by calpain. Tau dephosphorylated by alkaline phosphatase migrated more rapidly on sodium dodecyl sulfate-polyacrylamide gels and also showed an altered two-dimensional NEPHGE pattern. Dephosphorylation of tau had no effect on its susceptibility to calpain proteolysis, indicating that regulation of the susceptibility to calpain hydrolysis is due to the phosphorylation of a specific site(s). These results suggest a role for phosphorylation in regulating the degradation of tau. Abnormal phosphorylation could result in a protease-resistant tau population which may contribute to the formation of paired helical filaments in Alzheimer’s disease.

Tau is a microtubule-associated protein consisting of a group of isoforms with apparent molecular masses ranging from approximately 45 to 62 kDa (Cleveland et al., 1977). The major isoforms of tau have been reported to be the products of alternative RNA splicing of a single gene (Goedert et al., 1989; Himmler, 1989). Two-dimensional analysis of purified tau, however, has revealed greater than 30 discrete isolectric variants, many of which are the result of posttranslational modifications such as phosphorylation (Butler and Sleimans, 1986).

Tau is a major antigenic component of paired helical filaments (PHFs) present in the neurofibrillary tangles (NFTs) found in abundance in the brains of patients with Alzheimer’s disease (Delacourte and Defossez, 1986; Grundke-Iqbal et al., 1986a; Goedert et al., 1988). Abnormally phosphorylated species of tau are also present in Alzheimer’s disease brain (Grundke-Iqbal et al., 1986b, Wood et al., 1986; Flament and Delacourte, 1989; Flament et al., 1990). There is also evidence that a modified form of tau in Alzheimer’s disease brain (A68) is more acidic and less heterogeneous than tau from normal brain (Ksiezak-Reding et al., 1990), which could also be the result of an altered phosphorylation state (Lee et al., 1991). The accumulation of abnormally phosphorylated tau in susceptible neurons has been suggested to precede the formation of NFTs (Bancher et al., 1989). In vitro, phosphorylation of tau by calcium/calmodulin-dependent protein kinase slows the migration of tau isoforms on sodium dodecyl sulfate (SDS)-polyacrylamide gels, and a resemblance has been noted between this altered band pattern and that obtained with tau from Alzheimer’s disease brain (Baudier and Cole, 1987). The phosphorylation state of a protein may affect its susceptibility to hydrolysis by proteases. For example, phosphorylation of actin-binding protein by cAMP-dependent protein kinase (CAMP-PK) increases its resistance to proteolysis by the calcium-dependent, thiol protease, calpain (Zhang et al., 1988; Chen and Stracher, 1989). Therefore, the phosphorylation of tau might inhibit proteolytic degradation by calpain (Bancher et al., 1989; Johnson et al., 1989b). Calpain is present in high concentrations in the brain, is localized within virtually all neurons (Perlmutter et al., 1990), and has been postulated to play a role in the processing and degrading of cytoskeletal proteins (Vitto and Nixon, 1986; Siman et al., 1989; Johnson et al., 1989b).

In this report, modification of the calpain-induced hydrolysis of tau by altering its phosphorylation state is demonstrated. Treatment of tau with either the catalytic subunit of cAMP-PK or alkaline phosphatase significantly altered the electrophoretic mobilities and patterns as determined by SDS-polyacrylamide gel electrophoresis (PAGE) and two-dimensional nonequilibrium pH gradient electrophoresis (NEPHGE). In addition, we have demonstrated for the first time that the phosphorylation of tau by cAMP-PK significantly reduces its susceptibility to proteolysis by calpain.

EXPERIMENTAL PROCEDURES

Proteins—Tau was purified from twice-cycled microtubules prepared from fresh bovine brain as previously described (Johnson et al., 1989b), and the concentration was determined by the method of Lowry et al. (1951) after acid precipitation of the protein. The catalytic subunit of cAMP-PK was purified to homogeneity from beef heart using the method of Reimann and Beham (1983). Molecular biology grade calf intestinal alkaline phosphatase was from Boehringer Mannheim, calpain was from Sigma, and [γ-32P]ATP was from ICN.

*P-Labeled Phosphorylation of Tau—cAMP-PK phosphorylation of tau was conducted with [γ-32P]ATP to determine the rate and extent
of phosphorylation of untreated tau and tau previously dephosphorylated with alkaline phosphatase (see below). 32P-Labeled phosphorylation of tau was measured by incubation of tau (0.2 mg/ml) with CAMP-PK (5 µg/ml) in 20 mM Tris·Cl, pH 7.2, 10 mM MgCl2, 1 mM dithiothreitol (DTT), 50 µM [γ-32P]ATP (0.1 µCi/µmol) at 30 °C. Aliquots (2.5 µg of tau) were removed at the indicated times, and the reaction was stopped by the addition of 100 µl of cold 25% trichloroacetic acid and 10 µl of bovine serum albumin (5 mg/ml). The samples were mixed thoroughly, incubated in an ice bath for 20 min, and centrifuged for 4 min at 14,000 rpm in an Eppendorf centrifuge. The supernatant was removed, and the pellet was washed twice with acetone and the supernatant was removed, and the pellet was washed once with acetic acid and 10 µl of bovine serum albumin were added to each aliquot and treated as described above.

To dephosphorylate tau, 20 µg of tau was incubated at 37 °C with 1 µg of the catalytic subunit of CAMP-PK in 25 mM Tris·Cl, pH 7.2, 10 mM MgCl2, 10 µM ZnCl2, 1 mM DTT, and 100 µM ATP in a volume of 50 µl. The reaction was stopped after 240 min by incubating the mixture in a boiling water bath. Control samples were treated identically except that heat-killed, instead of active CAMP-PK, was used. The samples were diluted with buffer to produce final concentrations of 0.1 mg/ml tau, 55 mM Tris·Cl, pH 7.4, 100 mM NaCl, 2.5 mM MgCl2, 2.5 mM ZnCl2, 2 mM DTT, and 1 mM EDTA prior to phosphorylation.

To dephosphorylate tau, 20 µg of tau was incubated at 37 °C with 2 units of alkaline phosphatase in 25 mM Tris·Cl, pH 8.6, 10 mM MgCl2, 10 µM ZnCl2, and 1 mM DTT in a final volume of 50 µl for 240 min. The reaction was stopped and the samples were analyzed as described above.

To determine the effects of CAMP-PK phosphorylation on the calpain proteolysis of tau were reversible, some samples of phosphorylated tau were treated with alkaline phosphatase, as described above, prior to calpain-induced degradation.

The proteolysis of tau by calpain (1:20 enzyme-to-substrate ratio, w/w) was carried out exactly as previously described (Johnson et al., 1989b, 1991).

Electrophoresis—Aliquots were run on 6–12.5% gradient SDS-polyacrylamide gels or with or without 6 M urea and Coomassie-stained or transferred to nitrocellulose (Towbin et al., 1979) and immunoblotted with the phosphate-independent monoclonal antibody Tau-2 as described previously (Papasozomenos and Binder, 1987; Johnson et al., 1989b). Immunoblots from the proteolysis experiments were quantitated using a Bio-Rad video densitometer, and the data were evaluated using analysis of variance. Values were considered significantly different when p < 0.05.

Two-dimensional NEPHGE was carried out as described by O'Farrell et al. (1977). Samples were lyophilized and reconstituted in two-dimensional buffer (9.5 M urea, 2% Nonidet P-40 (Calbiochem), 5% β-mercaptoethanol, and 2% ampholytes (1.5%, pH 6–8; 0.5%, pH 3–10)). Carbonic anhydrase was used as a marker in the first dimension.

RESULTS

Quantitation of Phosphate Incorporation—Both untreated and dephosphorylated tau were readily phosphorylated by the catalytic subunit of CAMP-PK. Although there was a trend toward more phosphate being incorporated into tau dephosphorylated by alkaline phosphatase compared with untreated tau, the difference was not significant. Maximal 32P-phosphorylation of untreated and dephosphorylated tau was 1.4 ± 0.1 mol of 32P/mol of tau and 1.7 ± 0.2 mol of 32P/mol of tau, respectively. Maximal 32P-phosphorylation of tau was achieved between 120 and 180 min of incubation (Fig. 1). The insets of Fig. 1 show the electrophoretic mobility shifts that occur when untreated and dephosphorylated tau are phosphorylated by CAMP-PK.

Dephosphorylation of 32P·Tau—Tau that has been phosphorylated by CAMP-PK can be dephosphorylated readily by alkaline phosphatase. At 10 min more than 50% of the 32P was removed from tau by the alkaline phosphatase. However, the remaining 32P on tau was released from tau at a slower rate. 11% of the total 32P was still associated with tau at 180 min (Fig. 2). Incubation of tau in a boiling water bath prior to alkaline phosphatase treatment did not alter the rate or extent of removal of 32P from tau. At 180 min only 11% of the 32P remained associated with tau. Values indicate mean ± S.E., n = 4. The insets are immunoblots of control (C) and dephosphorylated (D) tau demonstrating the electrophoretic mobility shifts induced by CAMP-PK phosphorylation.

Fig. 1. Time course of the phosphorylation of tau by CAMP-PK. Control (●) or dephosphorylated (○) tau (0.2 mg/ml) was incubated in the presence of the catalytic subunit of CAMP-PK (5 µg/ml) and 50 µM [γ-32P]ATP. 32P incorporation into tau was determined at the times indicated. Maximal incorporation of 32P into both control and dephosphorylated tau was achieved by 120 min. Values indicate mean ± S.E., n = 4. The insets are immunoblots of control (C) and dephosphorylated (D) tau demonstrating the electrophoretic mobility shifts induced by CAMP-PK phosphorylation.

Fig. 2. Autoradiographic (A) and quantitative analysis (B) of the dephosphorylation of 32P·tau by alkaline phosphatase. Tau was 32P-phosphorylated with CAMP-PK prior to incubation with alkaline phosphatase (0.1 unit/µg of tau) and determination of the removal of 32P from tau. At 180 min only 11% of the 32P remained associated with tau. Values indicate mean ± S.E., n = 4.
extent of dephosphorylation (data not shown).

Phosphorylation and Dephosphorylation of Tau—In initial experiments, tau was phosphorylated by incubation with the catalytic subunit of cAMP-PK for 15, 30, 60, 180, or 240 min. A representative immunoblot of the time course of the phosphorylation of tau by cAMP-PK is shown in Fig. 3A. Phosphorylation reduced the number of bands from five to four and also slowed the migration of the protein bands.

Dephosphorylation of tau by alkaline phosphatase altered the electrophoretic profile as demonstrated by the typical immunoblot shown in Fig. 3B. Again there was a reduction in band number from five to four, but dephosphorylation resulted in increased mobility of the tau proteins.

The shifts in the tau band pattern due to phosphorylation by cAMP-PK or dephosphorylation by alkaline phosphatase are compared on the immunoblot shown in Fig. 4A. In this figure tau was treated for 240 min with either cAMP-PK (lane 1) or alkaline phosphatase (lane 3). Untreated tau was run as a control in lane 2. In lane 1 the four bands from the cAMP-PK-treated tau obtained positions intermediate to those of the control in lane 2, whereas the four bands from the alkaline phosphatase-treated tau in lane 3 are parallel to the lower four bands in the control but have a different distribution of protein concentration. Coomassie-stained gels, as shown in Fig. 4B, produced identical results. The same samples were also run on SDS-polyacrylamide gels containing 6 M urea to determine if the differential rates of migration were due to charge or conformational changes. Fig. 4C demonstrates that the differences in the band migration patterns due to altered phosphorylation of tau that were identified on SDS-polyacrylamide gels were not apparent on SDS-polyacrylamide gels containing 6 M urea. The absence of phosphorylation-induced differences in the migration of the tau bands in the presence of urea suggests that differential migration of the tau protein bands on SDS-polyacrylamide gels after phosphorylation is probably due to SDS-resistant domains which are unfolded by phosphorylation and thereby slow the migration of the protein in the gel (Billings et al., 1979). Control tau, which separated into five bands on SDS-polyacrylamide gels, migrated as four bands on SDS-polyacrylamide gels containing 6 M urea. The bottom band appeared to merge with one of the slower migrating bands. Alkaline phosphatase appears as a faint band above the top tau band in lane 3 of the Coomassie-stained gels (Fig. 4, B and C). Interestingly, all the tau bands migrated at a faster rate in SDS-polyacrylamide gels containing 6 M urea compared with the rates at which they migrated on SDS-polyacrylamide gels without urea (Fig. 4, B and C).

Two-dimensional Analysis—Untreated tau, subjected to NEPHGE in the first dimension, SDS-PAGE in the second dimension (6–12.5% gradient SDS-polyacrylamide gels), and immunoblotted with Tau-2 (1:20,000) (A) or Coomassie-stained (B or C). The Coomassie-stained SDS-polyacrylamide gel in C contained 6 M urea. Alkaline phosphatase (AP) migrated at approximately 65 kDa and is visible in the Coomassie-stained SDS-polyacrylamide gels in B and C. The positions at which molecular mass standards of 49 and 66 kDa migrated on the SDS-polyacrylamide gels are indicated.

Phosphorylation of tau by cAMP-PK significantly altered the isoforms separated by two-dimensional NEPHGE. Phosphorylation reduced the number of isoforms by approximately 50% compared with the control. In addition, the isoforms shifted to more acidic positions on immunoblots, and many of the most acidic spots were darker, indicating a greater
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protein concentration in those acidic isoforms (Fig. 5B).

Tau dephosphorylated by alkaline phosphatase also showed decreased heterogeneity and an altered two-dimensional NEPHGE pattern, although the changes were not as extensive as those induced by phosphorylation (Fig. 5C). The most basic isoforms from the top two bands and the most acidic isoforms from the lower two bands appeared to shift into position coincident with the remaining isoforms, which created a diagonal clear zone through the tau pattern. This is evidenced by the presence of larger, more intensely stained spots. This contrasts with the results obtained after cAMP-PK phosphorylation of tau where all the isoforms that shifted moved to more acidic positions.

Proteolysis of Phosphorylated and Dephosphorylated Tau by Calpain—Phosphorylation of tau with the catalytic subunit of cAMP-PK significantly inhibited calpain-induced proteolysis compared with untreated controls. Representative immunoblots of the calpain-induced hydrolysis of control and phosphorylated tau are shown in Fig. 6, A and B, respectively. Interestingly, phosphorylation of tau not only inhibits calpain-induced degradation but also alters the pattern of immunoreactive proteolytic products (compare Fig. 6, A and B). The calpain-induced proteolysis of untreated tau and tau incubated with heat-killed cAMP-PK was the same (data not shown). Quantitative analysis of the data is shown in Fig. 7. Tau phosphorylated by cAMP-PK (Figs. 6B and 7) was more resistant to calpain-induced proteolysis than the control. The rate of proteolysis of phosphorylated tau was slower as indicated by the initial slopes (Fig. 7). The extent of proteolysis was also significantly different, with 22% of phosphorylated tau remaining intact after 60 min, compared with 3% of the control tau (Fig. 7). Tau dephosphorylated by alkaline phosphatase was proteolyzed by calpain at the same rate and to the same extent as control tau, with no differences in the degradation products (Figs. 6A, 6C, and 7). In addition, the phosphorylation-induced inhibition of tau proteolysis was reversible. Calpain-induced hydrolysis of phosphorylated tau that was subsequently treated with alkaline phosphatase prior to degradation was not significantly different from the proteolysis of untreated tau (Fig. 8). It also should be noted that the calpain-induced proteolytic fragments of phosphorylated tau that has been dephosphorylated (Fig. 8B) are similar to the proteolytic fragments of control tau (Fig. 8A).

**DISCUSSION**

Tau, like other cytoskeletal proteins, has a number of sites available for phosphorylation by several kinases. The phosphorylation state of tau may regulate its interaction with other cytoskeletal components (Lindwall and Cole, 1984; Selden and Pollard, 1983) and also may be a regulatory mechanism in the determination of molecular stability. The data presented here demonstrate that phosphorylation of tau by cAMP-PK affects the heterogeneity of the population, the isoelectric points of the isoforms, and its susceptibility to calpain-induced proteolysis.

CAMP-PK is found in high levels in the brain (Nestler and Greengard, 1984) and has a broad protein substrate specificity (Edelman et al., 1987). The limiting requirement for substrate specificity of cAMP-PK is the presence of one or two basic residues located two or three residues away from the target Ser on its N-terminal side (Reed et al., 1985), a motif that occurs several times within bovine tau (Himmler, 1989). Previously, Pierre and Nunez (1983) demonstrated that tau is phosphorylated by cAMP-PK; however, other investigators did not observe substantial in vitro phosphorylation of tau by the kinase (Baudier and Cole, 1987). In this report we demonstrate that tau isolated from twice-cycled microtubules is phosphorylated by the catalytic subunit of cAMP-PK, with 1.4 mol of phosphate/mol of tau being incorporated. Tau isolated from twice-cycled microtubules has been reported to contain 1.5–2.2 mol of phosphate/mol of protein (Selden and Pollard, 1983). When tau is treated with alkaline phosphatases prior to being phosphorylated by cAMP-PK, a maximum...
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of 1.7 mol of phosphate/mol of tau was incorporated. However, this increase in phosphorylation of the dephosphorylated tau was not significant. This finding was not unexpected since the rate and extent of "P-phosphorylation by the catalytic unit of cAMP-PK of twice-cycled microtubule MAP-2 and dephosphorylated MAP-2 were not significantly different (Theurkauf and Valee, 1983). These results indicate that the phosphorylation sites that are exposed by phosphatase treatment are probably not primary targets for cAMP-PK in vitro. Tau that has been phosphorylated by cAMP-PK is readily dephosphorylated by alkaline phosphatase; at 180 min only 11% of the "P remained associated with the tau (Fig. 2). The phosphorylation of control (or dephosphorylated) tau by cAMP-PK results in a decrease in the number of tau bands from five to four and a decrease in the mobility of the proteins on SDS-polyacrylamide gels. The four bands of the cAMP-PK-treated tau migrated to positions intermediate to those of the control tau (Fig. 4, A and B). Phosphorylation of tau by calcium/calmodulin-dependent protein kinase previously has been reported to result in similar altered electrophoretic mobilities (Baudier and Cole, 1987), but phosphorylation of tau by protein kinase C did not change its mobility on SDS-polyacrylamide gels (Baudier et al., 1987). These findings suggest that phosphorylation of tau by cAMP-PK or calcium/calmodulin-dependent protein kinase may cause similar conformational changes in situ, resulting in similar functional or metabolic changes, in contrast to phosphorylation by protein kinase C which does not alter electrophoretic mobility (Baudier et al., 1987; Hoshi et al., 1987; Johnson et al., 1989b).

Recently, Steiner et al. (1990) demonstrated that the cloned and bacterially expressed bovine tau isoform, Tau4, was phosphorylated by cAMP-PK; however, this phosphorylation did not result in decreased mobility on SDS-polyacrylamide gels. It is possible that the differences between the studies are due to posttranslational modifications of tau that occur in vivo and not when the protein is bacterially expressed, which are required for the cAMP-PK-induced structural changes and slowed migration on SDS-polyacrylamide gels. Alternatively, intermolecular interactions between the different tau isoforms may be required for the putative structural changes to occur. The presence of urea in the SDS-polyacrylamide gels abolished alterations in the electrophoretic patterns of tau induced by phosphorylation with cAMP-PK or dephosphorylation by alkaline phosphatase. These results suggest that the shifts in the migration of the tau isoforms may be due to phosphate groups unfolding SDS-resistant domains which would slow the migration on polyacrylamide, or conversely, the removal of phosphates which may refold these domains and increase the migration rate (Billings et al., 1979). The decrease in band number from five to four of control tau could indicate that two of the isoforms are the same size but differ in phosphorylation state. The anomalously slow migration of tau on SDS-polyacrylamide gels has been suggested to be due to the extended configuration of tau (Lee et al., 1988; Lichtenberg et al., 1988). However, on SDS-polyacrylamide gels containing 6 M urea the tau isoforms migrate at approximately their predicted molecular masses (37-46 kDa) (Lee et al., 1988; Goedert et al., 1989; Himmerl, 1988).

Dephosphorylation of control or phosphorylated tau by alkaline phosphatase resulted in four distinct bands which appear to migrate on SDS-polyacrylamide gels at the same positions as the fastest four bands of untreated tau but with a different distribution of protein concentration. These findings are in agreement with results reported by other investigators (Lindwall and Cole, 1984; Butler and Shelanski, 1986).

The two-dimensional separation of tau revealed multiple isoelectric variants within each of the bands obtained by one-dimensional SDS-PAGE. Since phosphorylation and dephosphorylation significantly reduced the number of isoforms, much of the heterogeneity of tau can be attributed to phosphorylation state. The results of the phosphatase experiments agree with those reported by Butler and Shelanski (1986). Specific combinations of phosphorylation and dephosphorylation by multiple kinases and phosphatases may occur in the formation of functional tau isoforms.

The most important and significant finding of this study is that phosphorylation of tau by cAMP-PK inhibits calpain-induced hydrolysis. Previous investigators have demonstrated that phosphorylation of actin-binding protein by cAMP-PK significantly increased its resistance to calpain-induced proteolysis (Chen and Stracher, 1989). Interestingly, dephosphorylation of tau by alkaline phosphatase did not alter the rate or extent of calpain-induced proteolysis. This indicates that the increased resistance of tau to calpain hydrolysis, after treatment with cAMP-PK, is due to the phosphorylation of specific site(s) that are not phosphorylated on the control tau, and that the phosphate groups on the untreated tau which are removed by alkaline phosphatase are not involved in altering the sensitivity of tau to calpain. In addition, the phosphorylation-induced inhibition of tau proteolysis by calpain is reversible. Phosphorylated tau treated with alkaline phosphatase prior to calpain hydrolysis was degraded at a rate and extent similar to untreated tau.

Tau protein in Alzheimer’s disease brain is believed to be abnormally phosphorylated (Grundke-Iqbal et al., 1986b; Wood et al., 1986), and it appears that the presence of abnormally phosphorylated tau may precede the formation of NFTs (Bancher et al., 1989). PHFs, which are a major structural component of NFTs, are extremely insoluble and protease-resistant (Goedert et al., 1988; Wischik et al., 1988). Tau is a major antigenic component of PHFs (Grundke-Iqbal et al., 1986a), and this tau appears to be abnormally phosphorylated (Grundke-Iqbal et al., 1986b). This abnormal or excessive phosphorylation of tau could result in a more stable, protease-resistant molecule and contribute to the formation of the PHFs. Interestingly, the two-dimensional NEPHGE pattern observed after the cAMP-PK phosphorylation of tau has similarities to two-dimensional patterns of aberrant tau proteins from Alzheimer’s disease brain. The altered proteins (A68), which are probably derivatized forms of normal tau (Ksiezak-Reding et al., 1990; Greenberg and Davies, 1990; Lee et al., 1991), show retarded migration on polyacrylamide gels and are observed to be more acidic than normal tau. It is also of interest that two studies suggest that cAMP production may be elevated in Alzheimer’s disease tissue (Baker et al., 1988; Danielsson et al., 1988) and that aluminum, which some investigators believe may be associated with the etiology of Alzheimer’s disease (Martyn et al., 1989; Crapper McLachlan et al., 1989), increases cAMP levels in mammalian brain (Johnson and Jope, 1987; Johnson et al., 1989a) and in neuroblastoma × glioma (NG108-15) cells (Singer et al., 1990).

In summary, we have demonstrated for the first time that phosphorylation of tau by cAMP-PK inhibits degradation by calpain. These findings suggest that an abnormal increase in the phosphorylation of tau may play a role in the pathological accumulation seen in Alzheimer’s disease.

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