S100β Interaction with Tau Is Promoted by Zinc and Inhibited by Hyperphosphorylation in Alzheimer’s Disease

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The zinc-binding protein S100β has been identified as an interacting partner with the microtubule-associated protein tau. Both proteins are individually affected in Alzheimer’s disease (AD). S100β, is overexpressed in the disease, whereas hyperphosphorylated tau constitutes the primary component of neurofibrillary tangles. In this study, we examine factors that modulate their binding and the potential role the complex may play in AD pathogenesis. Zinc was identified as a critical component in the binding process and a primary modulator of S100β-associated cellular responses. Abnormally phosphorylated tau extracted from AD tissue displayed a dramatically reduced capacity to bind S100β, which was restored by pretreatment with alkaline phosphatase. In differentiated SH-SY5Y cells, exogenous S100β was internalized and colocalized with tau consistent with an intracellular association. This was enhanced by the addition of zinc and eliminated by divalent metal chelators. S100β uptake was also accompanied by extensive neurite outgrowth that may be mediated by its interaction with tau. S100β-tau binding may represent a key pathway for neurite development, possibly through S100β modulation of tau phosphorylation and/or functional stabilization of microtubules and process formation. S100β-tau interaction may be disrupted by hyperphosphorylation and/or imbalances in zinc metabolism, and this may contribute to the neurite dystrophy associated with AD.

Key words: S100β; tau; Alzheimer’s disease; zinc; binding; colocalization; neuronal development

S100β is a small molecular weight (10 kDa) zinc–calcium binding protein produced by astrocytes (Donato, 1991; Mrak et al., 1995). In addition to metal binding, S100β has several functions that include a role in the cytokine cycle, inhibition of selected phosphokinasas, including phosphokinase C (PKC), and the stimulation of neurite outgrowth (Kligman and Marshak, 1985; Baudier and Cole, 1988; Marshak and Pena 1992; Zimmer et al., 1995; Griffin et al., 1998; Heizmann and Cox, 1998). S100β is located on chromosome 21 and is increased in Down’s syndrome and Alzheimer’s disease (by as much as 20-fold) (Griffin et al., 1989, 1998; Marshak et al., 1992; Castets et al., 1997). In AD, the pathology is defined by amyloid plaques and neurofibrillary tangles (NFT). Alzheimer’s disease has been identified as an interactor of the amyloid precursor protein (APP), the presenilins (PS1 and PS2) were also assessed. Among the proteins we evaluated, tau was the only significant binding protein and furthermore, based on immunofluorescence studies, colocalized with S100β after internalization by neuronal cells. Zinc has also been implicated in some aspects of AD pathology, such as promotion of amyloid fibril formation (Bush et al., 1994) and, when examined in the current system, it
significantly affected the relationship between S100β and tau. This may be attributable to zinc-induced conformational changes that result in the exposure of a hydrophobic domain and could represent a key site for tau binding (Fujii et al., 1986; Baudier and Cole, 1988; Baudier et al., 1992). In addition, changes to tau also regulated this interaction, as shown by the altered binding of S100β to the AD-related hyperphosphorylated NFT-tau. Based on our observations, S100β-tau binding, overexpression of S100β, and tau hyperphosphorylation in Alzheimer’s disease pathology suggest that S100β-tau interactions may contribute to neuronal development as well as neuronal dysfunction.

MATERIALS AND METHODS

**Purification of S100β.** Extracts containing S100β were prepared from fresh bovine brains using the method described by Isobe et al. (1977). A 20% homogenate was made in a potassium phosphate buffer (0.1 M KPO₄, pH 7.1, 1 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM polymethylene sulphate) with 2.66 M (or 50%) ammonium sulfate (AmSO₄). Cell debris was removed by centrifugation at 10,000 × g, and the supernatant was adjusted to 85% AmSO₄ at pH 4.2 and incubated at 4°C for 2 h. Precipitated proteins were recovered by centrifugation, dialyzed against phosphate buffer, and stored at −20°C in lyophilized form. From this crude material, S100β was purified using a modified method as described by Baudier et al. (1982). Crude extracts were dissolved in the elution buffer (50 mM Tris-Base, pH 7.4) with 1 mM ZnSO₄ and applied to a Phenyl Sepharose 650 m column (TosoPearl, Montgomeryville, PA). S100β was eluted using a step gradient containing 300 mM NaCl, 0.25 mM ZnSO₄, or 2 mM EDTA. Protein purity was assessed by SDS-PAGE with Coomassie staining and by Western blotting with an S100β monoclonal antibody (clone SH-B1; Sigma, St. Louis, MO).

**Electrophoresis and Western blotting.** S100β (1 μg) was dissolved in Laemmli buffer and separated on a 10–20% Tricine gel (Novex, Carlsbad, CA). Gels were either stained with 2% Coomasie blue or transferred to a polyvinylidene difluoride membrane. The membrane was washed in Tris-buffered saline (200 mM Tris-base, pH 7.4, 150 mM NaCl) and nonspecific binding proteins were removed by washing with the initial buffer. A high salt (100 mM HEPES, 1 M NaCl, 0.25 mM ZnSO₄ or 2 mM EDTA) wash was used to elute proteins with weak S100β binding. High-affinity S100β binding proteins were subsequently eluted with 1 mM EDTA, and any remaining bound elements were removed with 1 m urea. All samples were collected and dialyzed then stored at −20°C in their lyophilized form. Eluted proteins were analyzed on 4–20% Tricine gels (Novex) and examined by silver staining and by Western blotting. Antibodies corresponding to S100β, amyloid-β (clone 6F3D; Dako, Carpinteria, CA), tau (Dako), and a presenilin antisera (Yu et al., 1998) were used to determine if they were capable of binding to S100β.

**Formation of S100β complexes with normal and AD tau.** AD and control brain were homogenized (10% w/v) in 0.1 M KHPO₄, 2 mM EDTA, 2 mM EGTA, and protease inhibitors. Samples were centrifuged for 45 min at 20,000 × g, and the supernatant was fractionated using 35 and 55% ammonium sulfate to produce a tau-enriched fraction. Crude proteins containing complexes were resuspended in 20 mM Tris and 0.5 mM NaCl, pH 7.6, with protease inhibitors. Samples were boiled, centrifuged at 25,000 × g for 30 min, and control aliquots were collected. To assess the effects of phosphorylation on S100β binding, samples were also treated with alkaline phosphatase (Sigma) for 30 min at 37°C. Binding of S100β with tau from these enriched samples was assessed by immunoprecipitation. Aliquots of the brain extracts (50 μg of total protein) were combined with 1 μg of purified bovine S100β and 10 μl of S100β monoclonal antibody. The mixture was incubated overnight at 4°C and the S100β-containing complexes were recovered by immunoprecipitation by protein-G sepharose. Beads were washed with buffer containing 50 mM Tris with 150 mM NaCl and 0.5% NP-40, and S100β with bound proteins was eluted with 500 mM NaCl with 1 mM EDTA. Samples were collected, dialyzed, and examined by Western blotting using tau antibodies.

**RESULTS**

**Identification and analysis of S100β binding proteins**

Interactions of brain-derived proteins, such as tau, were initially examined by affinity chromatography using immobilized S100β as the primary substrate. A native S100β secondary structure was maintained in the presence of calcium and zinc to obtain physiologically relevant conditions for the evaluation of binding proteins (Baudier et al., 1982). A series of increasing elution stringencies were used to determine the relative affinities of S100β binding proteins. Proteins that failed to bind to the S100β substrate were recovered in the initial wash. This was followed by a high salt elution to isolate proteins with weak ionic binding properties. High-affinity S100β-associated proteins were removed by the addition of zinc chelators, which caused a structural rearrangement of S100β. Previous studies have shown that zinc exposes a hydrophobic domain, which represents a potential binding site for its cellular partners (Isobe et al., 1977). Finally, any remaining proteins bound to the affinity column were removed with a denaturing urea wash, and each of these fractions was examined by direct silver staining as well as Western blotting.

Immunoblotting of the various elutions demonstrated that tau constituted a principal S100β binding protein. All other AD-related proteins such as APP, amyloid-β, PS1, and PS2 did not show any significant S100β binding and were recovered in the initial elution. Tau binding was particularly evident in the samples obtained from control cases in which strong signals were observed for all brain regions (Fig. 1A). The control tau was only eluted

![Figure 1. Affinity chromatography using immobilized S100β for identification of binding proteins (A). Immunoblotting of zinc (lanes 1, 3, 5, 7)- and EDTA (lanes 2, 4, 6, 8)-eluted fractions indicated a significant amount of S100β-associated tau in control samples from both frontal (lanes 1, 2) and temporal cortices (lanes 3, 4). Comparable affinity analysis with AD-extracted proteins from frontal (lanes 5, 6) or temporal (lanes 7, 8) cortex indicated only weak tau immunoreactivity consistent with a reduced interaction with S100β. Zinc-treated samples did not elute any proteins with tau immunoreactivity. Immunoblotting of total brain homogenates from AD and control indicating the elevated levels of S100β, as has been previously demonstrated by Griffin et al. (1989) (B).](https://example.com/figure1.png)
after zinc chelation with EDTA, suggesting that the observed conformation changes are important for binding. In contrast, in the comparable elutions, there was a marked decrease in the amount from the AD tau fraction (Fig. 1A). The lack of tau was not attributable to loss of immunoreactivity caused by changes in the AD-related protein because a polyclonal, nonphosphorylation-dependent antibody was used. To confirm this, additional antisera were used (e.g., phosphorylation epitopes detected by the antibody AT8), which demonstrated a similar lack of tau binding. Examination of the complete range of elutions revealed that AD-tau was found in both the flowthrough and salt washes. Based on this finding, it was determined that tau from AD samples had a significantly lower affinity for S100β.

To examine potential changes in the S100β levels between AD and control cases, Western blotting of comparable tissue samples was investigated. In the AD cases that showed the loss of tau binding to S100β, appreciable increases in the S100β levels were observed in all AD brain samples (Fig. 1B). The reason for the increased expression is unclear but does suggest an imbalance in S100β levels that may represent a compensatory mechanism for reduced activity. For example, if S100β does modulate tau function and/or metabolism, then the loss of this interaction in AD may induce the elevated expression.

Identification of S100β and tau complex
To assess further the binding of S100β to tau, immunoprecipitation of in vitro complexes was examined using both AD and control extracted samples. To accomplish this, a tau-enriched fraction was obtained from the brain homogenates through ammonium sulfate precipitation and incubated with purified S100β. The effects of tau phosphorylation on S100β–tau binding were also examined by immunoprecipitation with untreated extracts as well as after incubation with alkaline phosphatase. Because AD-tau is heavily phosphorylated, this may be one reason for the observed reduction in its binding to S100β.

Immunoprecipitation of untreated AD extracts using an anti-S100β antibody yielded very low or undetectable levels of associated tau in all tissues examined (Fig. 2). This finding is consistent with the affinity chromatography results and suggests an impaired binding. In contrast, similar immunoprecipitation control samples produced a robust level of binding of tau to S100β. The high level of tau immunoreactivity reflects the amount of binding to S100β in immunoprecipitation samples relative to the same amount of protein used in the AD samples. The formation of the S100β–tau complex in the control extracts was also zinc-dependent. This event was demonstrated by the removal of zinc with EDTA, followed by the subsequent release of tau from immunoprecipitated S100β. This observation is consistent with the elution profile from the affinity column, which facilitated the removal of tau from the immobilized S100β. Dephosphorylation of tau by alkaline phosphatase restored the normal, possibly functional, binding of tau to S100β (Fig. 2). In all AD cases, we observed a significantly higher level of binding after tau dephosphorylation. There was little or no change in the amount of tau that could be immunoprecipitated in the comparable control samples after alkaline phosphatase treatment. Restoration of binding after dephosphorylation of tau indicates a possible mechanism for the lack of S100β–tau interaction in the AD cases.

Internalization and subcellular distribution of S100β in neuronal cells
S100β has a stimulatory activity on neurite outgrowth that may result from metal influx (calcium), cytokine activation, and acti-

![Figure 2](image_url) Immuno precipitation of S100β complexed with brain-extracted tau from control and AD cases (3 separate tissue samples). Purified S100β incubated with tau-enriched and precipitated with an S100β polyclonal antibody indicated significant interacted evidenced by the coprecipitating tau. Untreated AD extracts displayed reduced tau binding to S100β under comparable conditions. The association was restored by dephosphorylation of the tau-containing extracts using alkaline phosphatase (Alk-Phos).
Figure 3. Time course of S100β internalization and clearance from differentiated SH-SY5Y neuroblastoma cells that were preincubated with S100β for 4 or 24 hr. Lysates were examined at different time points (0, 15, 30, and 60 min and 4 and 24 hr) after the removal of S100β from the culture medium. Readily detectable S100β (monomeric and dimeric forms) were observed after the 24 hr pulse and to a lesser extent after 4 hr preincubation.

Figure 4. Immunofluorescence of SH-SY5Y cells that were preincubated with S100β for various lengths of time. Untreated cells displayed very low levels of S100β (A), which were increased after addition of S100β to the medium and incubations for 4 (B) and 12 hr (C). The S100β levels were significantly increased after 24 hr of incubation (D). S100β was distributed within the cell body and processes consistent with the internalization of the protein rather than cell surface association. Scale bar, 10 μm.

Figure 5. Immunofluorescence of differentiated SH-SY5Y cells demonstrating the effects of zinc on S100β internalization. Samples exposed to untreated S100β showed an easily detectable level of protein uptake at 24 hr (A). Elevation of the culture medium zinc concentration to 10 μM resulted in a substantial increase in the intracellular S100β levels (B). This zinc-induced enhancement of S100β internalization could be reversed with addition of metal chelators such as EDTA (C). Scale bar, 10 μm.

amounts of intracellular S100β staining after exposures for 4 and 12 hr (Fig. 4). Consistent with the Western blotting data, substantial levels of S100β were found after the 24 hr incubation (Fig. 4D). S100β immunoreactivity was distributed within the cell body and extended into the processes but was absent from the nuclear region.

The degree of S100β internalization was also affected by zinc, as shown by the increased level of staining within cells, as compared with control, when zinc was added to the medium and coincubated for 24 hr (Fig. 5). The effect of zinc (and possibly other divalent metals) was supported by EDTA treatment that has a higher affinity for the metal as compared with S100β. Under these metal-depleted conditions, the level of S100β was markedly reduced in the SH-SY5Y cultures as compared with controls (Fig. 5C). To examine the effects of other divalent cations, the calcium-specific chelator EGTA was added to our cultures to block free and extracellular calcium. Low EGTA concentrations were used because they were not toxic and do not block neuritic sprouting but were sufficient to bind a significant proportion of free calcium. EGTA-treated cells exhibited comparable S100β staining, providing additional support for the specific role of zinc (data not shown). Cumulatively, the Western blotting and immunofluorescence studies suggest that S100β is actively internalized by the cells as opposed to surface association. This uptake has a number of implications for the mechanism of S100β activity in neuronal systems and its possible relationship to tau function.

Colocalization of S100β with tau and enhanced neurite outgrowth

To investigate the relationship between S100β-tau binding and neurite outgrowth, differentiated SH-SY5Y cells were allowed to internalize S100β, and its subcellular distribution with respect to tau was examined by immunofluorescence. Under control conditions, S100β was broadly distributed within the cell body and some processes. Furthermore, in the double-labeled cells, the staining overlaps to some degree with tau (Fig. 6A). However, a zinc-induced increase in the level of S100β within the cell produced a much more defined colocalization with tau. This is particularly evident within the processes in which the S100β and tau coincided as punctate staining that was observed in virtually all neurites (Fig. 6B, arrows). Colocalization of S100β was also time-dependent because 24 hr of incubation produced higher levels of overlapping signals when compared with the 4 or 12 hr samples. To ensure that there were no significant changes in tau, the S100β-treated cells were also analyzed for changes in phosphorylation using the paired helical filament (PHF)-tau AT8 antibody. AT8 immunoreactivity was not detected in any of the treated cells, at any time points (data not shown). The effects of zinc and the enhanced colocalization may reflect simply an increased cellular uptake of S100β, or metal binding may promote a preferred conformation that facilitates tau binding. This latter possibility would be consistent with our affinity chromatography and immunoprecipitation results. These findings suggest that internalized S100β may be associated with tau and thereby affect tau function and/or metabolic events such as phosphorylation.
Figure 6. Colocalization of internalized S100β with tau in differentiated neuroblastoma cells. Under control conditions, S100β (red) that was taken up by the cells showed partial overlap with tau (green), suggesting a possible intracellular association (A). The colocalization was more pronounced with the addition of zinc to the culture medium (B). Zinc elevated levels of S100β resulted in increased neurite outgrowth and frequent overlap of S100β with tau in these processes, which appear as discrete, punctate staining within the cell processes (B). Addition of EDTA to the culture medium before incubation of the cells with S100β eliminated the tau colocalization pattern caused by reduced protein uptake (C). Scale bar, 10 μm.

Figure 7. Stimulation of neurite outgrowth in SH-SY5Y cells after S100β internalization. Retinoic acid differentiated cells displayed a neuron-like morphology but with only a limited number of extensions (A, arrow). With the addition of untreated S100β, the number and length of the processes were enhanced (B). Addition of zinc to the medium and the accompanying increase in S100β uptake resulted in widespread increase in neurite outgrowth, leading to the formation of dense networks of cell processes (C). Cells and processes were visualized by immunofluorescence staining of the cell surface cadherins. Scale bar, 10 μm.

Tau is one of the key elements that control axonal growth and may be modulated, to some degree, by interactions with S100β. This hypothesis is supported in our experimental system by the response of the SH-SY5Y cells to S100β and zinc. Even with retinoic acid differentiation, SH-SY5Y cells do not produce extensive process formation and have a predominantly “spindle-type” morphology (Fig. 7A). With the addition of S100β, a greater number of neurites were observed when visualized using an antibody staining for cadherins on the cell surface (Fig. 7B). Neurite outgrowth was even more pronounced in the presence of zinc in which enhanced S100β uptake resulted in increased number of neurites with extensive outgrowth that produced both longer networks of processes (Fig. 7C). Under these conditions, abnormal neuritic sprouting was also observed with processes emanating from the cell body. Stimulation of neurites and colocalization of S100β with tau provides additional evidence for a physiological role for their interaction.

**DISCUSSION**

These studies were performed to establish the binding of S100β to tau and the chemical properties involved, as well as to identify its relevance to Alzheimer’s disease. Our findings demonstrate that S100β binds to tau. In addition, this interaction is enhanced by zinc and inhibited by tau hyperphosphorylation. The functional aspects of S100β–tau binding may impact on several different pathways that are regulated by the two proteins. For example, S100β may provide a scaffolding structure for tau to stabilize microtubules and possibly contribute to the abnormal neuritic dystrophy that is observed in AD (Baudier and Cole, 1988; Tam, 1990; Azmitia et al., 1995). This is illustrated by our observation that nonphysiological sprouting of processes are from the cell body, which is not normally seen in differentiated neuronal cultures. The second possibility is that S100β is a modulator of tau phosphorylation and that any changes in their interaction could be a factor in the AD-related hyperphosphorylation, as has been previously suggested (Baudier et al., 1987a; Sorci et al., 2000). Furthermore, the ability of S100β to inhibit PKC may potentiate the aberrant phosphorylation at key sites [e.g., residues 262 and 313 (Correas et al., 1992; Singh et al., 1996b)]. However, in our in vitro studies, S100β did not appear to promote aberrant phosphorylation, as indicated by the lack of AT8 staining that identifies PHF–tau related phosphorylated epitopes (Biernat et al., 1992). Neuritic development, although beneficial in the short term to rejuvenate lost neuronal connections, can also be detrimental in the chronic stages of AD because it increases cellular metabolic requirements and exposes the neurons to external insults.

Initially, our finding that S100β failed to bind AD-derived tau was attributed to the reduced number of neurons, which is associated with the progression of AD. This did not appear to be the case because the normal binding could be restored after alkaline phosphatase treatments. Although this may suggest that all phosphate groups on tau hinder S100β binding, this is not evident because tau is naturally phosphorylated, and this does not affect binding of the control sample tau to S100β. In these studies, it is only with abnormal hyperphosphorylation of tau present in AD that prevents S100β–tau binding activity. Our study has also demonstrated that zinc is an important factor in the internalization of S100β into neurons and enhances tau binding. In addition, we observed an increase in neuritic sprouting in SH-SY5Y cells treated with S100β and zinc, which suggests that metal binding may be critical to this outgrowth activity.

S100β has been demonstrated to have several biological functions in AD. This is reflected by its ability to bind zinc and calcium, as well as inhibit certain phosphorylation pathways. In addition, S100β has been shown to activate the complement pathway through interleukin-6 activation (Stanley et al., 1994; Mrak et al., 1995; Sheng et al., 1996a,b; Hays, 1998). S100β itself is activated by interleukin-1 and may also participate in a positive feedback loop, thereby inducing its own production through the promotion of astrocytic activity (Mrak et al., 1995). In AD, the observed increase in S100β production appears to be related to some of the physiological changes associated with interleukins and to the increase in neuritic sprouting. The uptake of S100β may represent a key role in its ability to alter the neuronal activity. Our immunofluorescence data suggests that S100β uptake by cells is enhanced by the addition of zinc. As stated previously, zinc causes S100β to undergo a conformational change, exposing a hydrophobic domain that facilitates neuronal internalization. Within the cell, S100β may alter many cellular processes, including binding to tau.

The metal-binding capacity of S100β appears to be a crucial functional element and may have some bearing on other disease pathways. S100β–calcium effects have been extensively examined by Baudier and Cole (1987a,b, 1988), in which they found evidence of S100β–calcium binding to microtubule-associated proteins, including tau, and calcium–calmodulin-dependent protein.
kinase II. Calcium is also thought to be excitotoxic in AD (Kim et al., 2000). In AD, both calcium and zinc have been implicated in the amyloid toxicity pathway. Zinc, as well as copper, is believed to accelerate the formation of amyloid fibrils (Bush et al., 1994; Yang et al., 2000). Amyloid is implicated as a potential membrane protein that may promote the influx of calcium across the plasma membrane. The increase of $\text{S}100\beta$ in AD may contribute to the shuttle of these metals to points of interaction, thereby accelerating the pathogenic process.

Zinc does not normally appear in the cell as a free, or unbound, form. It is believed to be toxic in this state. This may be related to the ability of free zinc to enter via AMPA channels (Sensi et al., 1997, 1999; Yin et al., 1998), promoting excitotoxicity. Proteins such as metallothionein and $\text{S}100\beta$ are induced by astrocytes to compensate for the extrusion of zinc into the extracellular space to block its toxic effects. In the case of $\text{S}100\beta$, the effect may detrimentally alter the disease process.

The role of zinc in AD has generated several interesting and pathogenically significant hypotheses. The potential role that it may play with $\text{S}100\beta$ on the effect on neuritic sprouting is another important addition to this metals role in the disease process. Finally, our observations suggest that, in addition to its activation of cytokines, $\text{S}100\beta$ may also play a more direct role in tau-related pathways that are associated with neurodegeneration in Alzheimer’s disease.

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