

Research report

## $\beta$ -Amyloid regulates gene expression of glial trophic substance S100 $\beta$ in C6 glioma and primary astrocyte cultures

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### Abstract

S100 $\beta$ , a calcium-binding protein synthesized by CNS astrocytes, has trophic effects in vitro (neurite extension and glial proliferation). In Alzheimer's disease and Down's syndrome, severely afflicted brain regions exhibit up to 20-fold higher levels of S100 $\beta$  protein, and astrocytes surrounding neuritic plaques exhibit highly elevated levels of S100 $\beta$  immunostaining. A major constituent of plaques,  $\beta$ -amyloid, has been reported to have neurotoxic and neurotrophic effects in vitro. In our study we examined the responses of CNS glia to  $\beta$ -amyloid. C6 glioma cells and primary rat astrocyte cultures were treated with  $\beta$ A(1–40) peptide at doses up to 1  $\mu$ M. Weak mitogenic activity, measured by [<sup>3</sup>H]thymidine incorporation, was observed. Northern blot analysis revealed increases of S100 $\beta$  mRNA within 24 h in a dose-dependent manner. Nuclear run-off transcription assays showed that  $\beta$ A(1–40) specifically induced new synthesis of S100 $\beta$  mRNA in cells maintained in serum, but under serum-free conditions, there was a general elevation of several mRNA species. Corresponding increases of S100 $\beta$  protein synthesis were observed by immunoprecipitation of <sup>35</sup>S-labeled cellular proteins. To evaluate whether this effect of  $\beta$ -amyloid was mediated via neurokinin receptors or by calcium fluxes, various agonists and antagonists were tested and found to be ineffective at stimulating S100 $\beta$  synthesis. In sum, these in vitro data suggest that in neuropathological conditions,  $\beta$ -amyloid itself is an agent which may provoke chronic gliosis and the production of trophic substances by astrocytes.

*Keywords:* S100 $\beta$ ; S-100;  $\beta$ -Amyloid; Alzheimer's disease; Trophic factor; Gliosis; Astrocytes; Gene regulation; Transcription; Calcium-binding protein

### 1. Introduction

The neuropathologies of Alzheimer's disease (AD) and Down's syndrome (DS), among other neurodegenerative disorders, are marked by an accumulation of senile plaques containing  $\beta$ -amyloid, surrounded by reactive astrocytes whose cell processes frequently penetrate the plaques, contacting the amyloid core and neuritic tangles [14,22,30,33,35,48,61,63,69,76,78,79]. A sign of gliosis, the number of astrocytes appears to be increased in AD [59]. However, unlike the acute, transient nature of gliosis following traumatic brain injury, gliosis in AD and DS is chronic and persistent for decades. While acute and chronic gliosis were recognized by the earliest neuroscientists including Alzheimer (1910), Ramon y Cajal (1913) and Weigert (1895), it was Penfield (1932) who made the definition explicit [47]. The terms 'reactive astrocytes,'

'astrocytosis,' or 'gliosis' refer to the glial changes that accompany neuropathologies or injuries to the brain, namely hypertrophy, hyperplasia, proliferation, and a variety of metabolic changes [47,60]. As acute reactions subside, glial scars which inhibit axonal regeneration may form at the site of CNS lesions. Thus, factors which contribute to chronic gliosis deserve attention in light of attempts to understand and intervene in neurodegenerative conditions such as AD.

One measure of gliosis is the elevation of astrocyte-specific proteins, such as glial fibrillary acidic protein (GFAP) and S100 $\beta$ . Reactive astrocytes exhibit increased GFAP mRNA [20,23], and increased GFAP immunostaining [22,35,48], with protein levels estimated to be 8–16-fold higher than normal in AD and DS [19,35]. Some small plaques, due to a lack of amyloid or neuritic-type staining, are identifiable only by the presence of enveloping, GFAP-positive cells [48]. This suggests that changes in astrocytes may precede the neuritic alterations seen in plaques, implicating astrocyte involvement in the early stages of these disorders.

Similarly, S100 $\beta$  shows highly elevated levels of

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mRNA in AD [51], and increased S100 $\beta$  immunostaining AD and DS [30,35] with protein levels estimated to be 4–20 times higher than normal in the temporal lobe [35,51]. Astrocytic changes in DS occur decades before the appearance of neuritic plaques and neurofibrillary tangles [63,79], and excessive expression of S100 $\beta$  is detectable from middle age to as early as postnatal day 2 in DS temporal lobe [30]. Again, these examples illustrate that reactive astrocytes, with chronically high levels of S100 $\beta$ , may precede or be coincident with deposition of  $\beta$ -amyloid plaques (amyloidosis) and neuronal degeneration.

The over-expression of S100 $\beta$  is additionally interesting because this calcium-binding protein exhibits trophic activity *in vitro*. S100 $\beta$  homodimers have neurotrophic and gliotrophic effects (for reviews, see [9,21,49]). Recently it has been suggested to have neurotoxic effects as well [24]. This activity has led to the hypothesis that S100 $\beta$  plays a contributory, perhaps causal role in neuronal degeneration of the AD and DS type [7,51].

In this report, we draw a connection between two trophic substances over-produced in AD and DS:  $\beta$ -amyloid and S100 $\beta$ . A great deal of work has implicated amyloidosis and  $\beta$ -amyloid directly in neuronal degeneration (for reviews, see [28,39,53,66]). In cell culture experiments  $\beta$ -amyloid, such as the bioactive  $\beta$ A(1–40) peptide, has exhibited both neurotrophic and neurotoxic effects (for review, see [18]). Until recently, the effect of  $\beta$ -amyloid on astrocytes had not been extensively studied. We report here that exogenous  $\beta$ -amyloid treatment leads to an increase in S100 $\beta$  mRNA and protein, in both C6 glioma and cultured rat astrocytes. These cells are known to synthesize S100 $\beta$  [11], amyloid precursor protein (APP) [44,71], and  $\beta$ -amyloid [13], and all three of these molecules are known to exert various trophic effects. This suggests the possibility that S100 $\beta$  and  $\beta$ -amyloid may interact to contribute to the state of chronic gliosis in AD and DS, and perhaps to neuronal degeneration as well.

## 2. Materials and methods

### 2.1. Cell culture

C6 glioma cells, originally obtained from American Type Culture Collection (#CCL 107), were maintained between passage 45–55 in Dulbecco's modified eagle medium (DMEM) (Gibco BRL) plus 10% fetal bovine serum (FBS). Primary astrocyte cultures were prepared from 1 day postnatal rat cerebral cortices and purified by the method of McCarthy and de Vellis as described elsewhere [17]. Astrocytes were maintained in 1:1 DMEM/F-12 (Gibco BRL) plus 10% FBS, and were used only up to the third passage. In addition, SH-SY5Y neuroblastoma cultures (gift from June Biedler, Memorial-Sloan Kettering Institute) were used as non-glia controls and were maintained in RPMI-1640 plus 5% FBS and 10% heat-in-

activated horse serum (Gibco BRL). For individual experiments, subconfluent cultures were plated onto 10 cm dishes (Costar), synchronized by serum deprivation for 24 h, changed to fresh media with or without serum (see figure legends), and then incubated 24 h before peptides were added directly to the culture medium.

All peptides were obtained from Bachem California, and were solubilized and administered to cultures according to Yankner et al. [81]. Lyophilized peptides were dissolved in a vehicle of 35% acetonitrile, 0.1% trifluoroacetic acid and diluted 1:50 directly into the culture medium. Control cultures received vehicle alone. For experiments with tromethamine (Sigma), Tris buffer pH 7.2 was prepared in water; control cultures received an equal volume of water.

### 2.2. Northern blot and nuclear run-off transcription assay

Total RNA was isolated from glial cultures by acid guanidinium thiocyanate/phenol/chloroform extraction [16], separated on a 1.1% agarose-formaldehyde gel in the presence of ethidium bromide, transferred to nitrocellulose, and then photographed [5]. A cDNA insert was excised and purified from a plasmid containing rat S100 $\beta$  [51] and labeled with [<sup>32</sup>P]dCTP (NEN/DuPont) to probe the blots. Hybridizations were carried out overnight at 45°C, washed up to 0.1  $\times$  SSC/0.2% SDS at 50°C. Autoradiograms were scanned and analyzed on an Ultrascan XL laser densitometer controlled by GelScan XL software (Pharmacia LKB); photographed rRNA bands on the blots were similarly scanned. S100 $\beta$  mRNA levels were expressed as the ratio AU S100 $\beta$ /AU 18S rRNA.

Further transcriptional analysis was carried out in C6 cells with nuclear run-off assays, described elsewhere [5] with modifications [43]. Briefly, from lysed cells,  $4 \times 10^7$  nuclei were incubated with 0.5 mCi [<sup>32</sup>P]CTP (NEN/DuPont), reactions were disrupted with guanidinium thiocyanate, and RNA was extracted with acid phenol/chloroform as described above. Linearized cDNA probes of rat S100 $\beta$ , GFAP [45], glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and pBR322 were prepared as slot blots and were hybridized with nuclear RNA (total cpms equal for each sample). GAPDH, constitutively expressed in mammalian cells [25], and pBR322 were employed as positive experimental and negative internal controls, respectively [43].

### 2.3. Immunoprecipitation

$\beta$ A(1–40) was added 18–20 h in advance of metabolic labeling of the cultures. Cells were incubated for 3–4 h in 3 ml methionine-free DMEM, supplemented with 0.5–1 mCi [<sup>35</sup>S]Met/Cys labeling mix (Trans<sup>35</sup>S-Label, ICN; Express-Label, NEN/DuPont). At various timepoints, cells were harvested, lysed, and aliquots of equal cpm were immunoprecipitated for S100 $\beta$ , as described previously

[49], with several modifications. For these experiments, antiserum originally raised against bovine S100 $\beta$ , but specific also to rat S100 isoforms (L.A. Peña and D.R. Marshak, unpublished observations), was used at a dilution twice as concentrated (1:100). S100 $\beta$ -immunoglobulin complexes were immobilized with protein A Sepharose (Pierce), boiled, and subjected to polyacrylamide gel electrophoresis using the Tris-tricine buffer system of Shäggar and Von Jagow [67]. Gels were soaked in Enhance (NEN/DuPont), a fluorography intensifier, before drying and autoradiography.

#### 2.4. [<sup>3</sup>H]Thymidine cell proliferation assay

Cells were seeded onto 24 well plates (Costar) at  $5 \times 10^4$  cells/cm<sup>2</sup>, incubated for 2 days, then serum deprived for 24–48 h. Cells were then treated with various factors, and 1 mCi/ml [<sup>3</sup>H]thymidine (ICN) was added 12 h prior to harvesting. S100 $\beta$  dimers were purified and the activity of the lot was determined by bioassay, as described previously [49]. Cells were rinsed with PBS, precipitated with 10% TCA, rinsed with 0.1 N potassium acetate, and solubilized in 2 N NaOH. Aliquots were combined with Ecolume scintillation fluid (ICN) and counted. Tests for significant differences (ANOVA) were performed using SigmaStat computer software (Jandel Scientific).

### 3. Results

Glial cell cultures respond to  $\beta$ -amyloid treatment by upregulating the synthesis of S100 $\beta$ . Transcription of the S100 $\beta$  gene is increased by  $\beta$ -amyloid in a time-dependent fashion, as determined by Northern blot analysis (Fig. 1). A high dose of a bioactive  $\beta$ -amyloid peptide, 1  $\mu$ M  $\beta$ A(1–40), causes a substantial increase and peak in S100 $\beta$  mRNA after 24 h of treatment in C6 glioma cells (Fig. 1b) and normal rat primary astrocytes (Fig. 1c). This is typical for structural genes, as opposed to early response genes which can peak within 1 h after treatment with trophic factors and then decline [2]. S100 $\beta$  mRNA levels remain significantly elevated 48 h after treatment (Fig. 1a). These cultures were deprived of serum 48 h before treatment and during exposure to  $\beta$ A(1–40), thus the induction and elevation of S100 $\beta$  mRNA occurred over a baseline which was declining (Fig. 1b). The baseline remained relatively stable in cultures maintained in serum, but the relative increase of S100 $\beta$  was less (data not shown).

The data from this experiment suggested that the lower dose (1 nM) might also be significantly different from the untreated, control cultures, thus a complete examination of the dose-response characteristics was performed separately (Fig. 2). Serum-deprived C6 cells were treated with a wide concentration range of  $\beta$ A(1–40), from 1 pM to 1  $\mu$ M,

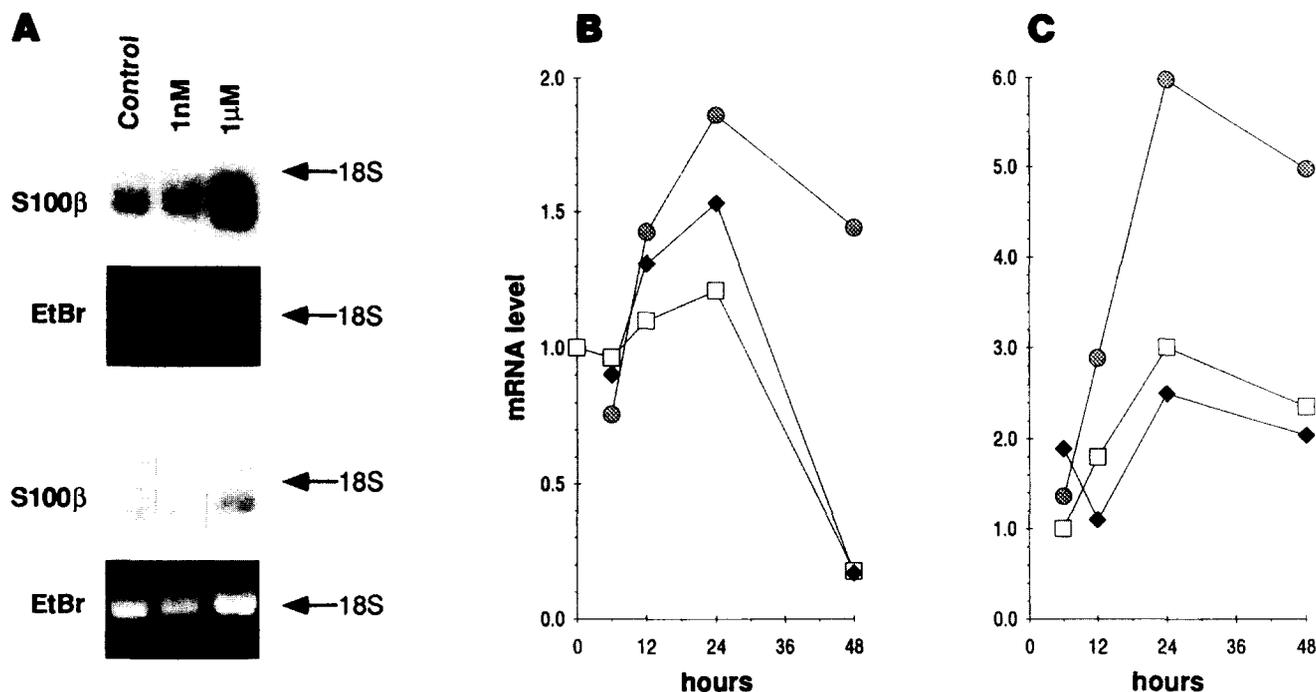


Fig. 1. Northern blot analysis of S100 $\beta$  mRNA induction in glial cell cultures by  $\beta$ -amyloid peptide  $\beta$ A(1–40). A: Northern blots of total RNA (20  $\mu$ g/lane) using a <sup>32</sup>P-labeled full length rat S100 $\beta$  cDNA probe, hybridized and washed under identical conditions, and autoradiographed for equal amounts of time. Lane 1, untreated controls (vehicle alone); lane 2, 1 nM  $\beta$ A(1–40); lane 3, 1  $\mu$ M  $\beta$ A(1–40). Upper panels depict autoradiogram of S100 $\beta$  mRNA bands and corresponding ethidium bromide-stained 18S rRNA bands on nitrocellulose blot from C6 cell cultures after 48 h treatment. Lower panels depict RNA from rat primary astrocyte cultures. B and C: Relative S100 $\beta$  mRNA levels determined by densitometric analysis of Northern blots (see Materials and methods) as in Panel A. □ = untreated control, ◆ = 1 nM  $\beta$ A(1–40), ● = 1  $\mu$ M  $\beta$ A(1–40). B: C6 cultures; C: primary astrocyte cultures.

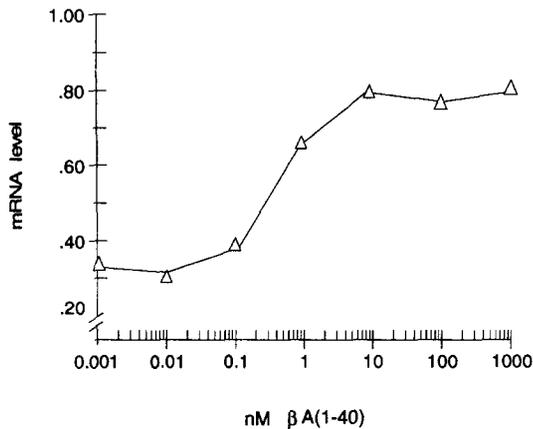


Fig. 2. Dose response of S100 $\beta$  mRNA in C6 cells to varying concentrations of  $\beta$ A(1–40) peptide. C6 cultures were treated for 24 h. Relative mRNA levels determined by densitometric analysis of Northern blots (see Materials and methods).

and harvested at 24 h. The dose-response relationship showed half-maximal activation at 1–5 nM  $\beta$ A(1–40) and saturated at 10 nM. This is in agreement with data on the positive trophic effects of  $\beta$ -amyloid, such as the bioactive  $\beta$ A(1–40), in neuronal cultures [77,81]. The absolute value of mRNA appears to be lower here than in Fig. 1, however the *relative difference* between the highest and lowest value at 24 h in Fig. 1b is roughly equivalent – the apparent discrepancies in signal strength may be due to variation in hybridization washes and length of autoradiogram exposures.

To distinguish whether the increase in S100 $\beta$  mRNA was due to increased *de novo* synthesis or an accumulation of mRNA by other mechanisms, run-off transcription assays from C6 cell nuclei were performed. The  $\beta$ A(1–40) peptide stimulated new transcription of the S100 $\beta$  gene in both serum-free and serum-supplemented cultures (Fig. 3). Densitometric analysis revealed that the S100 $\beta$  signal was about 60% greater than controls from the cultures grown in serum, but about 150% greater than controls in the serum-starved cultures. This demonstrates that at least part of the increase of S100 $\beta$  mRNA was due to the production of new mRNA, but does not rule out the possibility of changes in the rate of mRNA degradation.

The  $\beta$ -amyloid effect does not appear to be specific to S100 $\beta$  gene induction.  $\beta$ -Amyloid seems to increase the transcription of several other mRNA species, at least in serum-starved conditions. Synthesis of GFAP, a glial-specific marker [45], was increased, as was GAPDH, normally considered to be a constitutively expressed transcript [25,43]. On the other hand, in serum-supplemented cultures, there was no obvious induction of GFAP and the constitutive marker, GAPDH.

To determine whether an increase in the transcription of S100 $\beta$  was followed by translation into protein, immunoprecipitation using a specific S100 antiserum was done with  $^{35}$ S-labeled cells.  $\beta$ A(1–40) treatment increased the

amount of S100 $\beta$  protein in the C6 cells (Fig. 4a) and astrocytes (Fig. 4b). For both cell cultures, the increase was clearly apparent after 24 h and persisted to 48 h, which is consistent with the mRNA results shown in Fig. 1. Earlier timepoints and shorter labeling times did not reveal consistent elevations of S100 $\beta$  protein (data not shown).

With these basic parameters established, we sought to address the mechanisms which might account for  $\beta$ -amyloid trophic effects. Pharmacological evidence from cell culture experiments and injections into the brain suggests that  $\beta$ -amyloid stimulates the substance P/neurokinin/tachykinin class of neuropeptide receptors [40,81]. However, direct receptor binding studies failed to show that this was a specific receptor-ligand interaction [56]. Another hypothesis based on the finding that  $\beta$ -amyloid aggregates in lipid bilayers and forms ion channels [3] suggests that chronic  $Ca^{2+}$  influx from  $\beta$ -amyloid ion channels renders neurons susceptible to excitatory amino acid neurotoxicity [53,54]. We attempted to address these potential mechanisms in the glial cell cultures. Astrocytes and astrocytomas are known to possess tachykinin receptors of the NK1 subclass [10,34]. Therefore we tested a variety of tachykinins and tachykinin antagonists [41,57] to the glial cultures, alone or in combination with  $\beta$ -amyloid. Also, tromethamine (Tris) is known to block ion pores and is non-toxic to cultured cells [3]. However, none of the compounds listed in Table 1 showed any effect on S100 $\beta$  synthesis in C6 cells. They were applied singly or in combination with 20  $\mu$ M  $\beta$ A(1–40), and apart from the

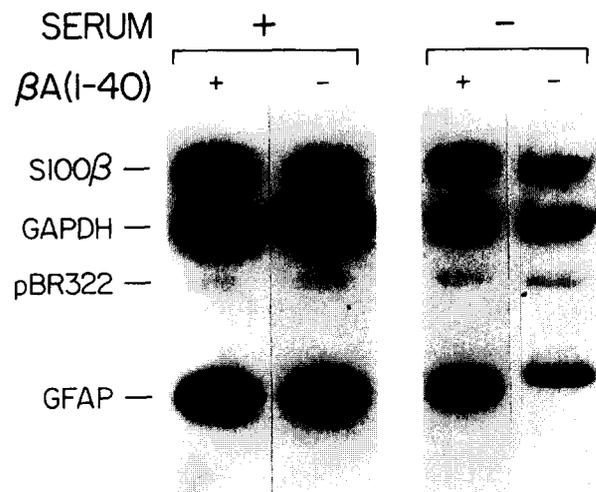


Fig. 3. Nuclear transcription assay depicting stimulation of *de novo* S100 $\beta$  mRNA synthesis in C6 cells exposed to  $\beta$ -amyloid. Cultures were serum-deprived 24 h, changed to fresh media with serum, left half (+), or without serum, right half (–), for an additional 24 h. Cultures were then treated with 1  $\mu$ M  $\beta$ A(1–40) peptide (+), or with vehicle alone (–) and harvested after 18 h. Nuclear RNA was labeled with [ $^{32}$ P]dCTP and hybridized with previously prepared slot blots of S100 $\beta$  and GFAP cDNAs, including GAPDH and pBR322 as positive and negative controls.

Table 1  
Compounds tested for ability to alter S100 $\beta$  mRNA synthesis

	Alone	With $\beta$ A(1–40)
Substance P	–	+
[D-Arg <sup>1</sup> ,D-Trp <sup>7,9</sup> ,Leu <sup>11</sup> ]-substance P	–	+
Neurokinin A	–	+
Neurokinin B	–	+
Physalaemin	–	+
Eledoisin	–	+
Tromethamine (10 $\mu$ M–10 mM)	–	+

The compounds (20  $\mu$ M each, except where noted) were added to C6 cultures alone or in combination with 20  $\mu$ M  $\beta$ A(1–40).

effect of  $\beta$ A(1–40), no change in S100 $\beta$  synthesis was attributable to the addition of these compounds.

Considering that S100 $\beta$  is weakly glial mitogenic [65], that the larger APP parent molecule of  $\beta$ A(1–40) has been shown to have mitogenic effects [64], and that there may be an increased number of astrocytes surrounding senile plaques [59], we sought to test  $\beta$ A(1–40) for mitogenic effects and interactions with S100 $\beta$ . Indeed,  $\beta$ A(1–40)

Table 2  
Increase in cell proliferation stimulated by  $\beta$ -amyloid in C6 cultures measured by [<sup>3</sup>H]thymidine incorporation

Treatment	cpm/well	SEM	Stimulation (% control)
Control	38 881	± 1560	100
$\beta$ A(1–40)	48 505	± 834	125 <sup>a,b</sup>
S100 $\beta$	49 691	± 1163	128 <sup>a,b</sup>
$\beta$ A(1–40)+S100 $\beta$	46 649	± 837	120 <sup>a,b</sup>
Serum	92 893	± 1048	239 <sup>a</sup>

Cells grown on a 24-well dish were synchronized by serum deprivation, then treated with 1  $\mu$ M  $\beta$ A(1–40), 30 ng/ml S100 $\beta$  dimer, 10% FBS, or vehicle alone ( $n = 6$ ).

One-way ANOVA, Bonferroni's multiple comparison procedure. <sup>a</sup> Significant difference compared to control,  $P \leq 0.001$ ; <sup>b</sup> not significant differences, comparison to each other,  $P \geq 0.06$ .

does exhibit modest mitogenic activity for C6 cells (Table 2). Compared to serum-treated cultures whose [<sup>3</sup>H]thymidine incorporation was almost 2.5-fold above control levels,  $\beta$ A(1–40)-treated cultures were about 25% above

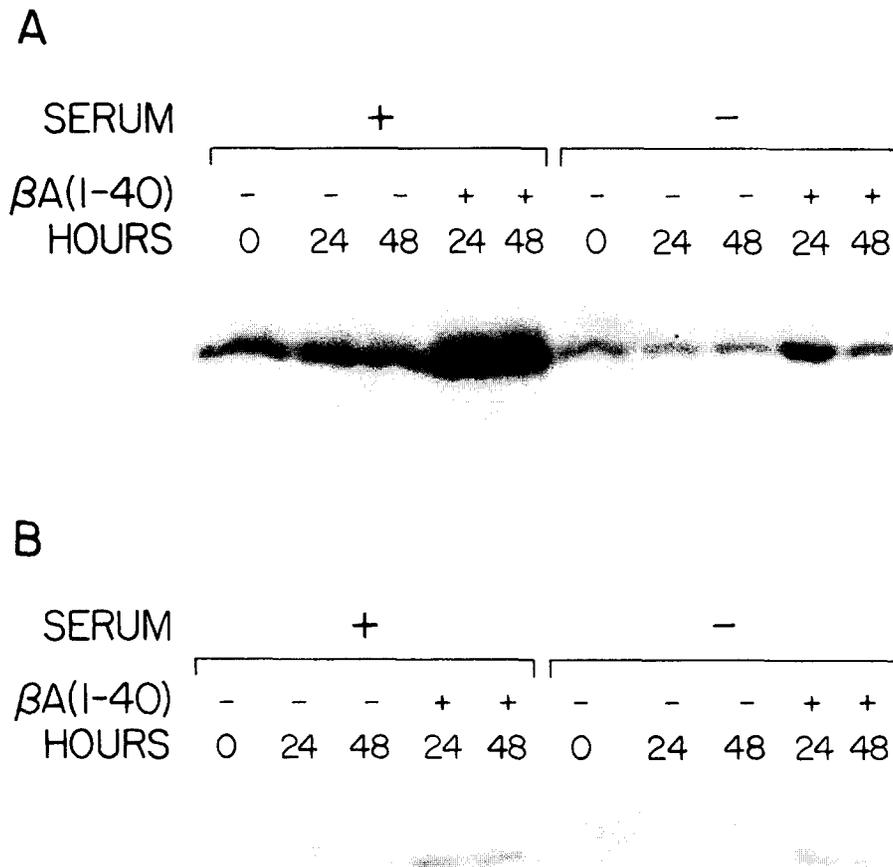


Fig. 4. Immunoprecipitation of S100 $\beta$  from glial cell cultures treated with  $\beta$ -amyloid. Cultures were serum-deprived 1 day, then preincubated an additional 1 day with serum, left half (+), or without serum, right half (–). At  $t = 0$  h, cells were treated with 1  $\mu$ M  $\beta$ A(1–40) (+), or with vehicle alone (–). 4 h prior to harvesting at  $t = 0$ ,  $t = 24$  h, or at  $t = 48$  h, cultures were labeled with 500  $\mu$ Ci [<sup>35</sup>S]Met/Cys per dish. A: C6 glioma cultures; B: rat primary astrocyte cultures.

control – a statistically significant difference. A  $\beta A(1-40)$  dose-response experiment (data not shown) resulted in a curve nearly identical to the S100 $\beta$  mRNA dose response curve in Fig. 2. Stimulation by  $\beta A(1-40)$  has also been observed in astrocytes cultures at a similar magnitude [32]. For comparison, S100 $\beta$  elicited a characteristic stimulation of nearly 28% higher than control in this experiment. This is a magnitude similar to the  $\beta A(1-40)$  effect and also statistically different from the control, but not statistically different from each other. Under these conditions, however, no synergistic effect was observed with the application of a maximally stimulatory dose of S100 $\beta$  combined with  $\beta A(1-40)$ . It is possible that these molecules, all synthesized by astrocytes and elevated in AD and DS, may contribute to the state of chronic gliosis in vivo by virtue of their modest mitogenic activity.

#### 4. Discussion

Astrocytes have been of interest to Alzheimer researchers because the production, processing, and secretion of amyloid is recognized to occur in glia as well as neurons [13,44,71]. However, the *direct effects* of exogenous  $\beta$ -amyloid on astrocytes have only recently become a focus of investigation. The present study suggests a link between biochemical components of AD and trophic activity ( $\beta$ -amyloid and S100 $\beta$ ), and a link to a neuropathological characteristic of AD (gliosis). This is in line with the earliest study to examine direct effects of  $\beta$ -amyloid on astrocytes, which found that astrocytes were stimulated to produce trophic agents, namely the cytokine interleukin-1 (IL-1) and the growth factor basic fibroblast growth factor (bFGF) [1]. Other recent data include proliferative effects of  $\beta$ -amyloid on astrocytes, with a magnitude similar to what we observed ([32], see Table 2). The deposition of a form of chondroitin sulfate proteoglycan (CSPG), which inhibits neurite extension [15], was reported for astrocytes treated with  $\beta$ -amyloid, and different forms of CSPG, depending on the age and origin of the astrocyte, e.g., cortical vs. cerebellar vs. spinal cord cultures [31]. Finally,  $\beta$ -amyloid was reported to induce cytokine secretion in astrocytes, and to potentiate IL-1 $\beta$  induced cytokine secretion [27]. This supports the idea of an interaction between  $\beta$ -amyloid and S100 $\beta$  in astrocytes, since intracranial IL-1 stimulates S100 $\beta$  and APP production in reactive astrocytes [70,80].

There are several interesting parallels between  $\beta$ -amyloid and S100 $\beta$ . Both are overexpressed in AD [29,51].  $\beta$ -Amyloid has neurotrophic effects in some in vitro culture systems [77,81] but shows neurotoxicity in others [38,54,81]. Similarly, S100 $\beta$  can be neurotrophic [6,12,46,49,71], but it has recently been shown to be toxic to PC12 cells as well [24]. They both seem to raise intracellular  $Ca^{2+}$  levels [9,53], and they both are weakly mitogenic for glial cells ([65], see Fig. 4).

It is worth noting that in the experiments reported here, the effects of  $\beta A(1-40)$  were more apparent in quiescent, serum-deprived cells than in actively dividing, serum-supplemented cells. One possibility is that serum in the cultures stimulated the dependent measures (S100 $\beta$  synthesis, GFAP and GAPDH transcription, [ $^3H$ ]thymidine incorporation) to maximal levels, thereby masking any small increment contributed by  $\beta A(1-40)$  treatment. Another possibility is that the cellular signal triggered by  $\beta$ -amyloid in astrocytes is weak and/or non-specific, thus only quiescent cells exhibit strong responses to it. Evidence for the latter is suggested by the increased transcription of several mRNA species (Fig. 3) in the quiescent cultures.

A candidate for such a signal is elevation of intracellular  $Ca^{2+}$ . It is known that the dose of S100 $\beta$  sufficient to stimulate glial proliferation (1–50 ng/ml) causes  $Ca^{2+}$  fluxes into the cells [9] and that S100 toxicity in PC12 cells (0.5–5  $\mu M \approx 5250$ –52500 ng/ml S100 $\beta$  monomer) is also  $Ca^{2+}$ -dependent [24].  $\beta A(1-40)$  disrupts  $Ca^{2+}$  homeostasis in cells [53,54] and causes  $Ca^{2+}$  fluxes across lipid bilayers [3]. Thus, judging from the time frame and magnitude of the responses measured here, it is plausible that  $\beta A(1-40)$  causes a common non-specific signal, such as the elevation of  $Ca^{2+}$ . The similar magnitude of cell proliferation induced by maximally stimulating doses of S100 $\beta$  and  $\beta A(1-40)$  and the lack of synergy between the two (Table 2) are consistent with this idea. Elevated  $Ca^{2+}$  in turn may lead to a diverse set of effects of small or modest magnitude. Small changes in astrocytic  $Ca^{2+}$  may provoke larger effects in both astrocytes and neurons since  $Ca^{2+}$  waves have been shown to propagate from astrocyte to astrocyte, and from astrocyte to neuron via physical intercellular connections such as gap junctions [58]. The role of  $Ca^{2+}$  in this hypothesis can be evaluated in further studies in a relatively straightforward manner in vitro.

Several hypotheses have now been advanced for an astrocytic role in AD and DS type neurodegeneration. Frederickson [26] reviewed various possibilities for a causal role of astrocytes in AD, including excitotoxicity, tachykinins, immunoinflammatory responses, monoamines, and proteoglycans. Azmitia et al. [7] suggested a role for S100 $\beta$  in AD and DS, also implicating serotonin, citing evidence that astrocytes release S100 $\beta$  upon stimulation of their serotonergic 5HT $_{1A}$  receptors and that S100 $\beta$  exerts trophic effects on cultured serotonergic neurons. The S100 $\beta$  hypothesis considered here incorporates the specific phenomena that (i) S100 $\beta$  has measurable trophic effects, (ii) S100 $\beta$  is overexpressed in the gliosis attending neurodegenerative disorders like AD and DS, and in brain injury, and (iii) S100 $\beta$  escapes from astrocytes and enters the CSF of Alzheimer's disease patients [52]; therefore S100 $\beta$  may contribute to the cascade of events that lead to neuropathology. While the coincidence of these factors is suggestive, the nature of an S100 $\beta$  contribution is unknown. Assuming that S100 $\beta$ 's neurotrophic and

gliotrophic activity [9,21,49] observed *in vitro* also occurs in the brain, the additional S100 $\beta$  originating from reactive astrocytes may contribute to the maintenance of surviving neurons. Therefore an S100 $\beta$  contribution could be beneficial. On the other hand, since S100 $\beta$  is known to increase intracellular Ca<sup>2+</sup> influxes into cells [8] and S100-mediated Ca<sup>2+</sup> influxes kill PC12 cells [24], an excess S100 $\beta$  might have the deleterious effect of promoting the loss of neuronal Ca<sup>2+</sup> homeostasis, thereby contributing to neuronal degeneration in Alzheimer's disease [37,53]. As a trophic substance, excess S100 $\beta$  could provoke inappropriate cellular responses leading to cell death [50] as is the case for other trophic substances which are toxic at high concentrations [81]. Either way, additional S100 $\beta$  originating from reactive astrocytes may contribute to neuronal death. At their simplest, these formulations suggest a causal involvement of S100 $\beta$  in neurodegenerative disorders.

The importance of confirming these *in vitro* findings *in vivo* is very clear. Several studies of  $\beta$ -amyloid have utilized *in vivo* models (for reviews, see [18,62]) but most demonstrations of  $\beta$ -amyloid activity have been done in cell cultures. *In situ* hybridization analysis of the brain has shown that  $\beta$ -amyloid precursor protein (APP) is localized mainly in neurons with widespread and abundant transcription, but an occasional low-level expression in oligodendrocytes and astrocytes is also detectable [55,72]. It is after injury to the brain that astrocytes begin to produce APP and  $\beta$ -amyloid in significant amounts [36,73]. Furthermore, in terms of astrocyte density, the severity of astrogliosis in AD is more strongly correlated with  $\beta$ -amyloid deposition than other histopathological indicators [14]. Like  $\beta$ -amyloid or APP, S100 $\beta$  can be found in the cerebrospinal fluid of rats and humans [4,42,53,68], and cultured cells can release it into the surrounding medium [74,75]. Our demonstration of a regulation of S100 $\beta$  by  $\beta$ A(1–40) provides the first direct evidence that exogenous  $\beta$ -amyloid can stimulate the production of a bioactive molecule released by astrocytes with potential trophic or toxic effects. This provides additional impetus for *in vivo* studies of the active role of astrocytes and the consequences of chronic gliosis in neurodegenerative disorders.

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