Neurite Extension and Neuronal Survival Activities of Recombinant S100β Proteins That Differ in The Content and Position of Cysteine Residues

Faith Winningham-Major,* Jeffrey L. Staecker,* Steven W. Barger,‡ Steven Coats,‡ and Linda J. Van Eldik*‡

Departments of *Pharmacology and ‡Cell Biology, Vanderbilt University, Nashville, Tennessee 37232

Abstract. S100β produced in *Escherichia coli* from a synthetic gene (Van Eldik, L. J., J. L. Staecker, and F. Winningham-Major. 1988. *J. Biol. Chem.* 263:7830–7837) stimulates neurite outgrowth and enhances cell maintenance in cultures of embryonic chick cerebral cortex neurons. In control experiments, the neurite extension activity is reduced by preincubation with antibodies made against bovine brain S100β. When either of the two cysteines in S100β are altered by site-directed mutagenesis, the resultant proteins maintain the overall biochemical properties of S100β, but lose both the neurite extension and neuronal survival activities. However, another S100β mutant, in which the relative position of one of the two cysteines was changed, had neurotrophic activity similar to that of the unmodified protein. These and other results indicate that (*a*) specific neurite extension activity and neuronal survival activity are two related activities inherent to the S100β molecule; (*b*) a disulfide-linked form of S100β is required for full biological activity, and (*c*) the relative position of the cysteines can be modified. These data suggest potential in vivo roles for S100β in the development and maintenance of neuronal function in the central nervous system, and demonstrate the feasibility of the longer term development of selective pharmacological agents based on the S100β structure.

THE early development of the vertebrate nervous system involves a complex set of events, including neuronal and glial cell proliferation, migration and differentiation; neurite outgrowth and guidance; and establishment of appropriate synapses. This program of development is further complicated by interactions among different components of the nervous system. For example, specific extracellular signals function as neurotrophic factors by stimulating neurite outgrowth and/or enhancing neuronal survival. Identification and characterization of factors that have neurotrophic activity are important steps in understanding the molecular mechanisms by which neuronal development and axonal growth are controlled. Many types of neurotrophic factors have been described, including ions, hormones, extracellular matrix proteins, cell–cell adhesion proteins, growth factors, and oncogenes (for reviews, see Greene, 1982; Berg, 1984; Thoenen et al., 1987; Walicke, 1989). One of the best characterized neurotrophic factors is nerve growth factor (NGF),[1] whose effects include maintenance of the differentiated state of mature sympathetic and sensory neurons, enhancement of survival, promotion and guidance of neurite outgrowth, and regulation of levels of neurotransmitter synthesizing enzymes (for review, see Misko et al., 1987).

S100β, a protein found in high levels in glial cells, has recently been found to have neurotrophic activity on central nervous system (CNS) neurons (Kligman and Marshak, 1985; Van Eldik et al., 1988). S100β belongs to a family of proteins with primary sequence similarities (for reviews, see Donato, 1986; Van Eldik and Zimmer, 1988). The S100 family includes: S100α and S100β (Isobe and Okuyama, 1981); calpactin light chain (Gerke and Weber, 1985; Glenney and Tack, 1985; Hexham et al., 1986); S100L (Glenney et al., 1989); proteins that are elevated in patients with cystic fibrosis and rheumatoid arthritis (CF antigen [Dorin et al., 1987], MRP-8 and MRP-14 [Odink et al., 1987]); and predicted protein sequences deduced from RNAs that are expressed in high levels in differentiated, transformed, or growth factor-treated cells (calcyclin [Calabretta et al., 1986; Ferrari et al., 1987; Murphy et al., 1988], 18A2 [Jackson-Grusby et al., 1987], 42A and 42C [Masiakowski and Shooter, 1988], p9Ka [Barraclough et al., 1987], pEL98 [Goto et al., 1988]). The observation that expression of members of the S100 family is altered during cell growth, differentiation, and in certain diseases, suggests that they may play regulatory roles in these processes.

Dr. F. Winningham-Major's present address is Merck Pharmaceutical Division, Merck, Sharp, and Dohme, West Point, PA 19486. Dr. J. L. Staecker's present address is Abbott Laboratories, Abbott Park, IL 60064.

1. *Abbreviations used in this paper*: CNS, central nervous system; NEF, neurite extension factor; NGF, nerve growth factor; VUSB-1, recombinant S100β.

Although little information is available about the in vivo roles of many of the members of the S100 family, a disulfide-linked, probably dimeric form of S100β has been reported to stimulate neurite outgrowth in primary cultures of cerebral cortex neurons (Kligman and Marshak, 1985; Van Eldik et al., 1988), in a neuroblastoma cell line (Kligman and Hsieh, 1987), and in organotypic cultures of spinal cord/ganglia (our unpublished observations). The observation that a dimeric form of S100β has neurite extension factor (NEF) activity, coupled with the presence of extracellular S100β in brain (Shashoua et al., 1984) and glial cell cultures (Suzuki et al., 1987; Van Eldik and Zimmer, 1987), raises the possibility that during development of the nervous system, S100β might be released from glial cells and act in a paracrine fashion to stimulate neurite outgrowth. The potential importance of S100β as a neurotrophic factor is also indicated by its localization in the developing nervous system during the time of elongation of neuronal processes (Zomzely-Neurath and Walker, 1980; Van Hartsveldt et al., 1986; our unpublished observations).

To develop the necessary reagents for addressing the long term question of how S100β may be involved in neuronal development and maintenance, we previously synthesized a gene coding for S100β, expressed the gene in *Escherichia coli*, and produced protein (termed VUSB-1) by recombinant DNA technology (Van Eldik et al., 1988). We found that VUSB-1 preparations have NEF activity on embryonic chick cerebral cortex neurons, whereas preparations of *E. coli* transformed with the vector lacking the S100β gene do not have NEF activity. We also showed that VUSB-1 preparations lose NEF activity when treated with reducing agents, or when purifications are done in the presence of reducing agents, consistent with previous data (Kligman and Marshak, 1985) that indicated that the NEF activity of bovine brain S100β requires a disulfide form of the protein. This apparent requirement for a disulfide linkage to function as a neurotrophic factor is intriguing in light of the fact that S100β proteins isolated from different species and tissues (Isobe and Okuyama, 1981; Kuwano et al., 1984; Jensen et al., 1985; Marshak et al., 1985) have invariant cysteines at residues 68 and 84. In addition, S100β is the only member of the S100 family of proteins that has cysteines at both of these positions in the amino acid sequence; all other members have a valine at the position analogous to cysteine 68.

The mechanisms by which S100β induces neurite extension are not known, nor is it known what relative importance each cysteine has for formation of the biologically active dimeric species. Previous studies (Kligman and Marshak, 1985; Van Eldik et al., 1988) examined NEF activity under only one set of specific conditions: addition of S100β at the time the cells are placed into culture, and examination of the neurite extension response 20–24 h later. No data are available about the effect of varying the time of exposure of cells to S100β. It is also unknown if purified S100β affects survival of CNS neurons, similar to the effects of NGF on peripheral neurons. Combining recombinant DNA-based methods for the production of S100β proteins that differ in their cysteine content with selective assays for the analysis of NEF activity, we have begun to address some of these questions. In this report, we described studies that: (*a*) analyze the effects of VUSB-1 on neurite extension under defined sets of conditions; (*b*) examine the ability of VUSB-1 to enhance neuronal maintenance in cerebral cortex cultures; and (*c*) test directly, by site-directed mutagenesis/protein engineering experiments, the contribution of the cysteine residues to neurotrophic activity.

Materials and Methods

NEF Activity Assays

Chick cerebral cortex neurons were prepared and NEF activity of VUSB-1 constructs was assayed as previously described (Van Eldik et al., 1988) except for the following modifications: (*a*) cells were plated at a density of 11,500 cells/cm^2; and (*b*) cells were examined by phase-contrast microscopy after 24 h and scored positive if the length of the neurite(s) was equal to or greater than the diameter of the cell body. The percent neurite extension was calculated as the percentage of neurite-bearing cells. Results are expressed as the mean ± SEM of the percentage of neurite-bearing cells from eight fields (4 fields/well; duplicate wells scored).

The time dependence of the NEF assay was analyzed by two types of experiments. In the first experiments, the cells were placed into culture for various lengths of time in bioassay medium (Ham's F12 containing insulin [5 μg/ml], transferrin [5 μg/ml], progesterone [20 nM], sodium selenite [30 nM], putrescine [100 μM], penicillin [100 U/ml], and streptomycin [100 μg/ml]), and then fresh medium with or without VUSB-1 was added. NEF activity was scored 24 h after the addition of fresh medium. In the second experiments, cells were allowed to attach to the tissue culture dish for 2 h, at which time (time 0) VUSB-1 was added to the cultures. At various time points thereafter, the VUSB-1 was removed by adding fresh medium without VUSB-1 to the cultures. The controls did not receive VUSB-1 at time 0, but received a medium change at each of the time points. NEF activity was scored 24 h after each medium change.

Neuronal Cell Survival Assay

Cerebral cortex neurons were prepared from 7-d chick embryos as described above and placed into culture in the presence or absence of VUSB-1. 2 h later (to allow for cell attachment), the number of cells in duplicate wells (4 fields/well) were counted, and this number represented the 100% value. The number of cells present at 24, 48, 72, and 96 h after plating was determined, and the ratio of cells present at each time point to the number of cells present at 2 h was calculated. Medium was not changed during the 96-h assay period.

Preparation of VUSB-1 for NEF Assays

Preparation of VUSB-1 with retention of NEF activity was done as previously described (Van Eldik et al., 1988), except that samples from the DE52 column were filter-sterilized but not dialyzed before storage at −80°C. In all experiments comparing the activity of VUSB-1 and mutant constructs, the proteins were prepared and analyzed for activity on the same day.

Site-directed Mutagenesis of VUSB-1

Site-directed mutagenesis was done by the cassette mutagenesis procedure essentially as described by Craig et al. (1987) by taking advantage of the unique restriction enzyme sites that had been designed into the synthetic S100β gene (Van Eldik et al., 1988). We cleaved the gene (while resident in the vector) by using two different restriction enzyme sites flanking cysteine 68 and cysteine 84. Synthetic, double-stranded oligonucleotide cassettes containing the desired nucleotide changes and containing ends that were complementary to the restriction enzyme sites used to cleave the gene were then prepared. The oligonucleotide cassettes used in these experiments are shown in Fig. 6 *A*. Mutants containing single cysteine changes (C68A, C68V, C68S, C84A, C84S) were prepared by ligating the phosphorylated cassettes into the pVUSB-1 vector (Van Eldik et al., 1988) that had been cleaved with either StyI/SfiI (C68A, C68S, C68V) or Sfi I/Hind 3 (C84A, C84S). Mutants lacking both cysteine residues (C68V84A, C68V84S) were prepared by using an Eco RI/Sfi I fragment isolated from the plasmid containing the C68V gene (pC68V). This fragment was inserted into Eco RI/Sfi I–cleaved plasmids containing either the C84A or C84S genes. The S62C68V mutant was prepared by ligating the phosphorylated cassette into pC68V cut with Sty I and Sal I. This approach allowed insertion of cassettes in the correct orientation and reading frame.

Transformation of *E. coli* was done as previously described (Van Eldik et al., 1988), and colonies were screened for the presence of the mutations by restriction mapping. To facilitate screening of clones, the cassettes for C68A, C68S, and C68V contained a new Dde I site, while the cassette for S62C68V had the Dde I site deleted. The cassettes for C84A and C84S contained a new Pst I site and Fok I site, respectively. After selection of positive constructs, the mutant constructs were characterized by detailed restriction enzyme mapping and/or nucleic acid sequencing as described (Van Eldik et al. 1988). Fig. 6 *B* shows the nucleotide differences between VUSB-1 and the eight mutants used in this study.

Proteins expressed from the mutant constructs were purified as previously described (Van Eldik et al., 1988). Mutations in the proteins were verified by amino acid analysis, and some constructs were also characterized by limited amino acid sequence analysis as described (Van Eldik et al., 1988). The mutant preparations analyzed by amino acid sequence were composed of a mixture of molecules that had either retained or lost the initiator methionine at the amino terminus, similar to what we had previously found (Van Eldik et al., 1988) with VUSB-1 preparations (results not shown). Also, the mobility of the mutant proteins in SDS gels and the levels of expression of the mutant proteins (~4 mg/liter of culture) were similar to those previously found for VUSB-1 (Van Eldik et al., 1988).

Immunochemical Analyses

Rabbit antisera against bovine brain S100β were characterized (Zimmer and Van Eldik, 1987) and immunoblot analyses of SDS-polyacrylamide gels were done (Van Eldik and Wolchok, 1984) as previously described. For immunoprecipitation experiments, VUSB-1 was incubated for 1 h on ice with buffer A alone (20 mM Tris-HCl, pH 7.4, 0.3 M NaCl), with normal rabbit IgG (1:10 dilution), or with rabbit anti-S100β IgG (1:10 dilution). Protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) was then added (100 μl of a 1:1 slurry of resin and buffer A), and the mixtures were incubated on ice for 30 min. After centrifugation at 13,000 *g* for 5 min, the supernatant was assayed for NEF activity as described above.

Protein Determinations

Protein concentrations were determined by the method of Lowry et al. (1951) using BSA as a standard.

Results

Effects of VUSB-1 on Neurite Extension

Fig. 1 shows a representative example of the effect of VUSB-1 on chick cerebral cortex neurons. Cerebral cortex cells from 7-d chick embryos were placed into culture in bioassay medium in poly-L-lysine–coated, 24-well plates. The cells attached to the dish surface by 2–3 h after plating, and cell viability was >98% by trypan blue exclusion. After 24 h, cells cultured in bioassay medium alone (Fig. 1 *A*) showed minimal neurite extension (only 2–12% of the cells were positive for neurite extension). In contrast, cells cultured in the presence of VUSB-1 for 24 h (Fig. 1 *B*) showed a vigorous neurite extension response. The morphology of the cells

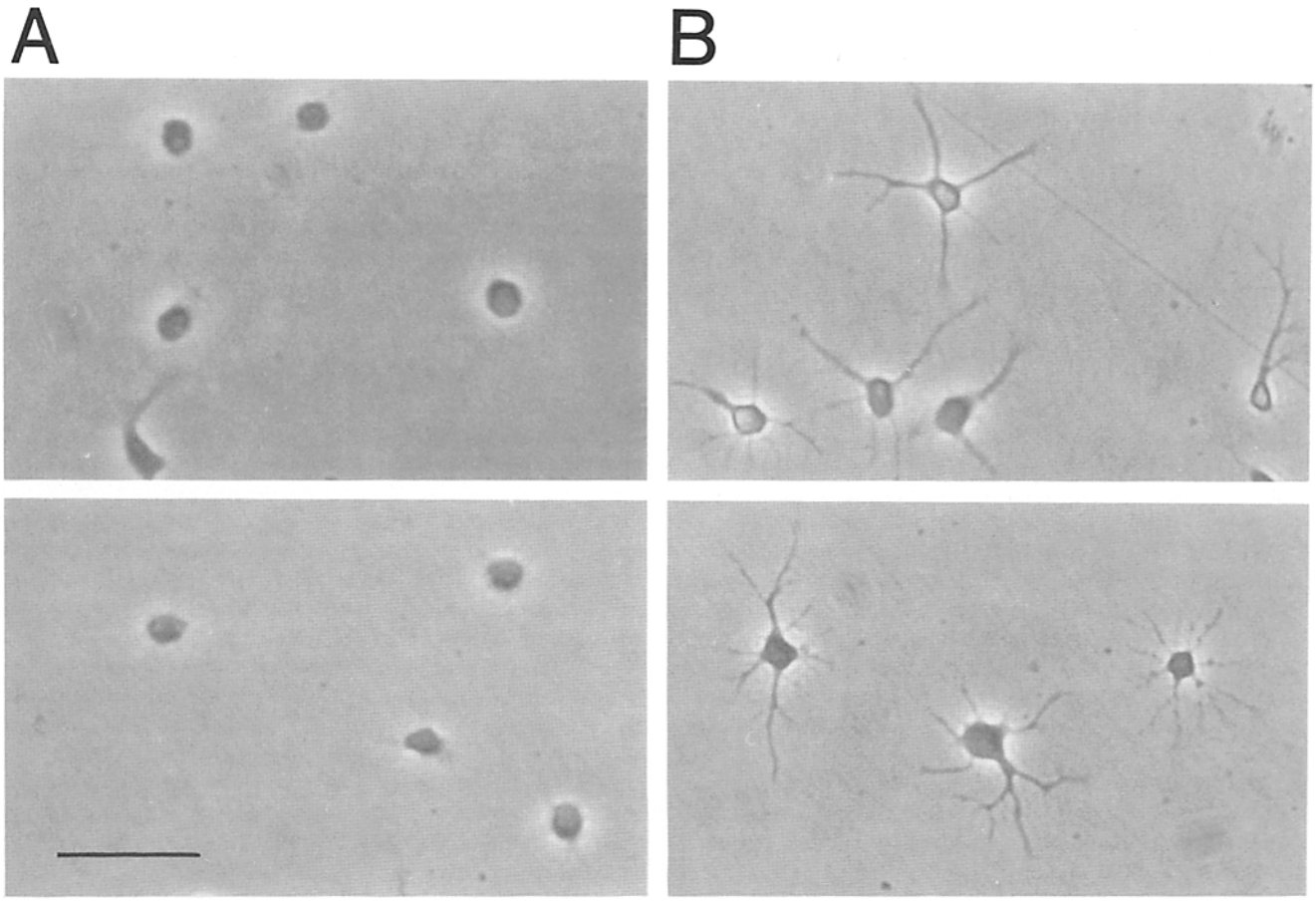

Figure 1. Effect of VUSB-1 on chick cerebral cortex neurons. *A* shows two examples of negative controls, where cells were cultured for 24 h in bioassay medium alone. Similar results are seen when cells are cultured in the presence of extracts of *E. coli* transformed with the vector lacking the S100β gene. *B* shows two examples of cells cultured in the presence of VUSB-1 preparation (142 μg/ml) for 24 h. Note that most of the cells respond by elaborating long neurites. Bar, 50 μm.

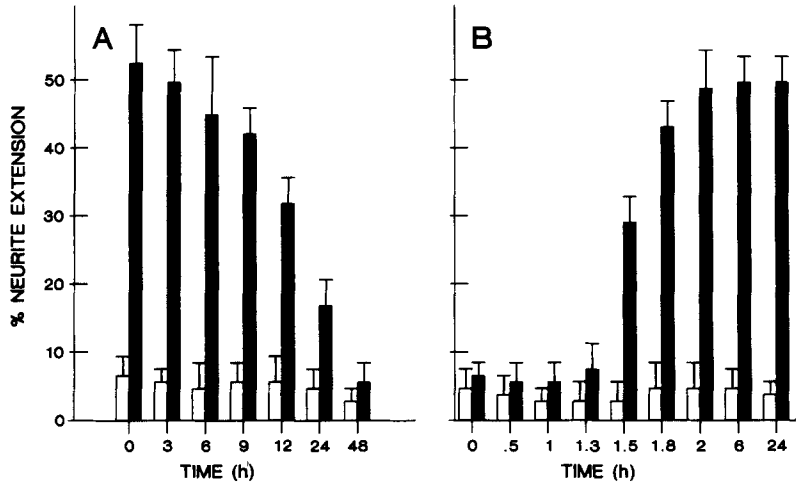

Figure 2. Time dependence of the NEF activity of VUSB-1. In *A*, the effect of adding VUSB-1 to the cultures at various times was examined. Cells were cultured for various lengths of time in bioassay medium, and then fresh medium without (*open bars*) or with 96 μg/ml VUSB-1 (*solid bars*) was added at each time point. The percentage of cells with neurites was determined 24 h after each medium change. In *B*, the effect of removing VUSB-1 from the cultures at various times was examined. Cells were cultured for 2 h in bioassay medium, at which time (time 0) medium without (*open bars*) or with 96 μg/ml VUSB-1 (*solid bars*) was added. At each time point thereafter, fresh bioassay medium was added and the percent neurite extension was determined 24 h after the medium change.

was heterogeneous, with some cells exhibiting monopolar or bipolar processes and others exhibiting multipolar and/or branching processes.

The time dependency of the NEF activity was examined by adding or removing VUSB-1 to or from the cultures at various times, and analyzing the subsequent effects on neurite extension (Fig. 2). When VUSB-1 was added at the time of cell plating (time 0) or at 3, 6, or 9 h after plating, there was no apparent difference in the neurite extension response scored 24 h after VUSB-1 addition (Fig. 2 *A*). However, when VUSB-1 was added at 12, 24, or 48 h after the cells were plated and then scored 24 h after VUSB-1 addition, there was a decrease in the percentage of cells with neurites. We also examined the length of time that cells must be exposed to VUSB-1 to respond in the NEF assay. In these experiments (Fig. 2 *B*), cells were allowed to attach to the culture dishes for 2 h, at which time (time 0) VUSB-1 was added. At various time points thereafter, the VUSB-1 was removed by addition of fresh medium, and neurite extension scored 24 h later. The results show that cells exposed to VUSB-1 for <1.5 h show a neurite extension response similar to control cells. However, continuous exposure of cells to VUSB-1 for 24 h is not required for a maximal response; in fact, the response of cells exposed to VUSB-1 for 2, 6, or 24 h was indistinguishable. These data indicate that there is an early period during which the presence of VUSB-1 is essential for maximum neurite extension, and that once the response is initiated, the presence of VUSB-1 in the cultures is no longer required.

As shown in Fig. 3, addition of VUSB-1 resulted in a dose-dependent increase in the percent of cells with neurites, reaching a plateau value of 51% in this experiment. We generally see maximum responses of ~50–60% positive cells. The difference in the neurite extension response between control cultures and cultures treated with VUSB-1 (at the highest concentrations) was determined to be significant at the 1%

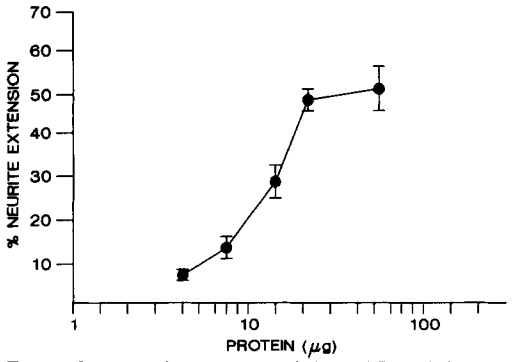

Figure 3. Dose dependence of the NEF activity of VUSB-1. The percentage of cells with neurites was determined 24 h after the addition of increasing amounts of VUSB-1 preparation to the cultures.

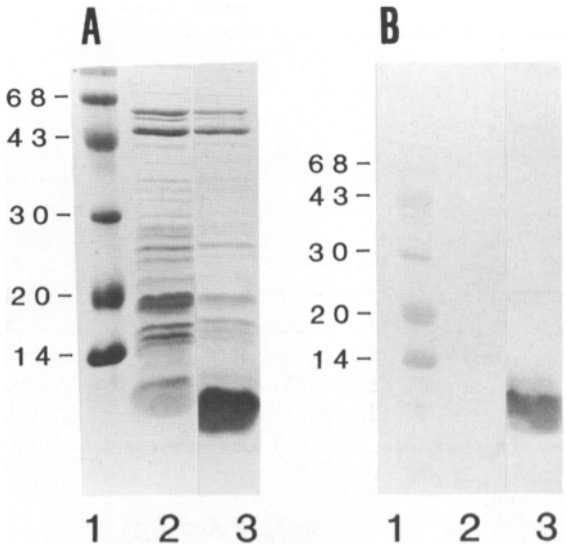

Figure 4. Analysis of VUSB-1 preparation by gels and immunoblots. *A* shows a Coomassie Blue-stained, 15% (wt/vol) acrylamide-SDS gel containing 1 mM EDTA. Samples were run in the absence of reducing agent. Lane *1*, molecular weight standards of 68,000, 43,000, 30,000, 20,000, and 14,000. Lane *2*, DE52 eluate of *E. coli* preparations transformed with the vector lacking the S100β gene. Lane *3*, DE52 eluate of *E. coli* preparations transformed with pVUSB-1 (containing the S100β gene). *B* shows an immunoblot analysis of a similar gel that had been transferred to nitrocellulose and incubated with antibodies to S100β. Lane *1* contains prestained molecular weight standards of 68,000, 43,000, 30,000, 20,000, and 14,000. No reactivity with anti-S100β antibody is observed in the DE52 eluate of *E. coli* preparations transformed with the vector lacking the S100β gene (lane *2*). The VUSB-1 preparation (lane *3*) shows reactivity with anti-S100β antibody, with intense staining of monomeric VUSB-1 and faint staining of dimeric forms of VUSB-1.

level ($n = 12$) using an unranked sign test. The percent positive cells obtained with bioassay media alone or with extracts of *E. coli* transformed with a vector lacking the VUSB-1 gene ranged from 2 to 12%. In the experiment shown in Fig. 3, the concentration of the VUSB-1 preparation required for half-maximal stimulation was ~15 μg/ml. Among different experiments and different VUSB-1 preparations, we have seen a two- to threefold variation in the concentration of VUSB-1 required for half-maximal stimulation. While this concentration of protein is high compared to other neurotrophic factors, it should be noted that the VUSB-1 preparation used for NEF assays is a partially purified, DE52 eluate that contains primarily monomeric VUSB-1 and only a small proportion of dimeric VUSB-1, as analyzed by immunoblots (Fig. 4).

The specificity of the NEF activity was addressed further by examining the activity of VUSB-1 preparations after incubation with antibodies to S100β. As described in Materials and Methods, VUSB-1 was incubated with anti-S100β IgG, complexes were precipitated with Protein A-Sepharose, and the resultant supernatant was assayed for NEF activity. Table I shows that incubation of VUSB-1 with antibodies directed against S100β reduced the neurite extension response, whereas incubation of VUSB-1 with normal rabbit IgG did not reduce the response. These data provide further evidence that the active NEF is VUSB-1 and not a contaminant in the preparation.

Effect of VUSB-1 on Cell Maintenance

We found that VUSB-1 enhanced the maintenance of neurons in addition to stimulating neurite extension (Fig. 5). For these experiments, chick cerebral cortex neurons were cultured in the presence or absence of VUSB-1 for 24, 48, 72, and 96 h, and the number of neurons present were counted after each time period. During the first 24 h in culture, the number of cells present in the control cultures and the cultures containing VUSB-1 were similar (Fig. 5). The cells appeared healthy with little or no cell degeneration or cellular debris. However, after 96 h, over 80% of the cells in control cultures had degenerated. In contrast, addition of VUSB-1 to the cultures increased the precentage of cells remaining in the culture dishes. The ability of VUSB-1 to enhance cell maintenance was dose-dependent over a similar range as the NEF activity (data not shown). Other proteins tested, such as calmodulin or α-lactalbumin, did not enhance cell maintenance (data not shown).

Effect of Cysteine Mutants on NEF Activity

Previous data (Kligman and Marshak, 1985; Van Eldik et al., 1988) suggested that the NEF activity of S100β requires a disulfide form of the protein. S100β contains cysteines at residues 68 and 84 (see Fig. 6), but how the disulfide bonds are arranged to produce functional NEF is unknown. We have examined the relative contribution of cysteine 68 and cysteine 84 to the NEF activity of VUSB-1 as an initial step to address the structural requirements for NEF activity. As described in Materials and Methods, we prepared by site-directed mutagenesis seven mutant VUSB-1 proteins (termed C68A, C68S, C68V, C84A, C84S, C68V84A, C68V84S), in which cysteine 68 was changed to alanine, serine, or valine; cysteine 84 was changed to alanine or serine; or both cysteines were changed (cysteine 68 to valine and cysteine 84 to alanine or serine). The location and description of the mutations in the seven proteins are in Fig. 6. The presence of the appropriate mutation was confirmed by one or more of the following: restriction enzyme mapping, DNA sequence analysis, or amino acid compositional analysis.

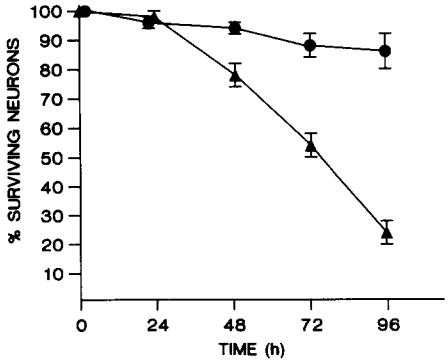

Figure 5. The effect of VUSB-1 on survival of chick cerebral cortex neurons. Cells were cultured in the presence of 163 μg/ml VUSB-1 preparation (circles) or bioassay medium alone (*triangles*), and the percentage of cells remaining in the cultures at various time points was determined.

The mutant proteins were purified for NEF activity assays exactly as VUSB-1, and tested for their ability to stimulate neurite outgrowth. Fig. 7 shows that mutants lacking either cysteine 68 or cysteine 84 (C68A, C68S, C68V, C84A, C84S) showed a significant decrease in NEF activity, with little or no activity at any dose tested (significant at the 5% level for the highest protein concentrations [$n = 6$ or 7 depending on the mutant] using an unranked sign test). Similarly, the mutants lacking both cysteine 68 and cysteine 84 (C68V84A, C68V84S) were inactive compared to VUSB-1. The absence of a cysteine at position 68 or 84 also resulted in a loss in the ability to enhance cell survival (data not shown). These data suggest that the presence of both cysteine residues is necessary for full activity.

To address whether there is flexibility in the exact location of the cysteine residues for NEF activity, we prepared another mutant designated S62C68V. In this mutant, serine 62 was changed to cysteine, and cysteine 68 was changed to valine (cf., Fig. 6). Thus, this mutant still has two cysteine residues, but the invariant cysteine that is normally found at residue 68 has been moved to residue 62. We found that S62C68V stimulated neurite extension (Fig. 8) and enhanced cell maintenance (data not shown) similar to VUSB-1. These data suggest that the structural requirements for NEF activity are somewhat flexible in that the relative position of the two

Table I. NEF Activity is Inhibited by Anti-S100 IgG

	Percentage of cells with neurites	
	Experiment 1	Experiment 2
VUSB-1 Alone	45 ± 6	49 ± 7
VUSB-1 + Normal rabbit IgG	47 ± 3	51 ± 3
VUSB-1 + Anti-S100β IgG	16 ± 4	9 ± 4

Immunoprecipitations were done as described in Materials and Methods. The results from two separate experiments are shown.

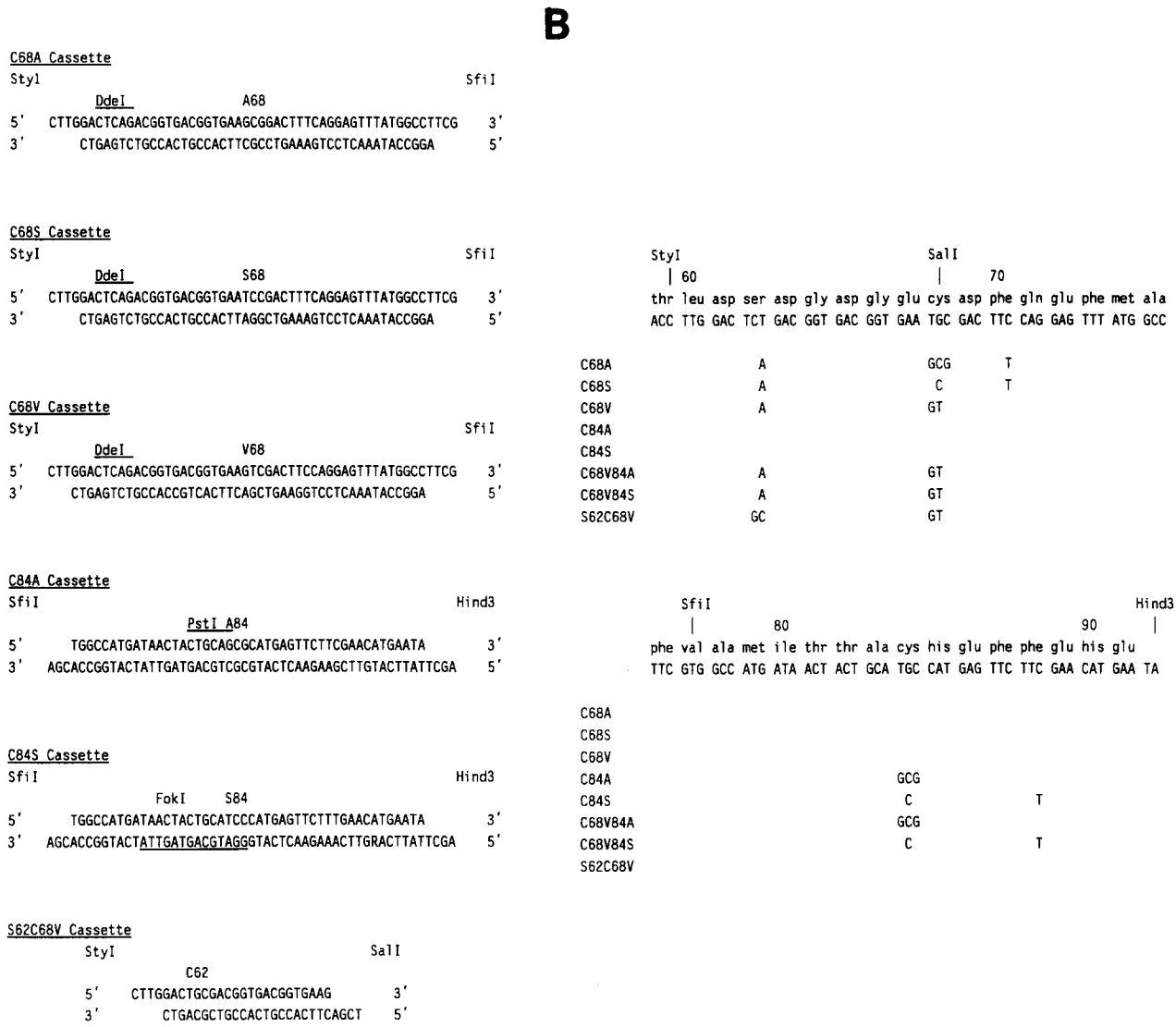

Figure 6. Site-directed mutagenesis of VUSB-1. *A* shows the nucleotide sequences of the double-stranded, oligonucleotide cassettes used for the preparation of VUSB-1 mutants. The positions of the codon changes at residues 68, 84, and 62, and the resultant changes in amino acids are indicated (*A*, alanine; *S*, serine; *V*, valine; *C*, cysteine). The positions of the new restriction enzyme sites incorporated for screening purposes are shown, with the sites delineated by solid lines above or below the sequence. The restriction enzymes listed on the left and right of the cassettes are the enzymes that are complementary to the ends of the cassettes. *B* shows the nucleotide sequence changes of the eight VUSB-1 mutants used in this study. The relevant portion (residues 59–91) of the amino acid sequence of VUSB-1 is shown on the top line, with the corresponding nucleotide sequence on the second line. For the mutants, only nucleotide residues that are different from the VUSB-1 sequence are shown. The positions of selected restriction enzymes used in the construction of the mutants are shown.

cysteine residues can be altered without significantly diminishing the neurotrophic activity.

Discussion

In this report, we have analyzed the ability of a recombinant S100β (VUSB-1) to stimulate neurite outgrowth from chick embryo cerebral cortex neurons, and have demonstrated that VUSB-1 also enhances neuronal maintenance in culture. In addition, we have shown by site-directed mutagenesis/protein engineering approaches that both cysteine residues of VUSB-1 are important for neurite extension and cell survival activity, and that the relative position of the two cysteines can be altered without loss of activity. These data provide further evidence to support the hypothesis that a disulfide-linked form of S100β may act as a neurotrophic factor in the CNS, and indicate the potential for development of pharmacologically useful reagents based on the S100β structure. These results also provide a foundation for future studies into the molecular mechanisms by which S100β affects neuronal development and maintenance.

We previously reported the synthesis and expression of a gene coding for S100β, and showed that the expressed protein (VUSB-1) had NEF activity on embryonic chick cerebral cortex neurons in culture (Van Eldik et al., 1988). The studies reported here extend this previous work by further characterizing the effects of VUSB-1 on neurite extension, and by analyzing the ability of VUSB-1 to enhance cell maintenance in this bioassay system. The bioassay we used is similar to that established by Kligman (1982), whose labora-

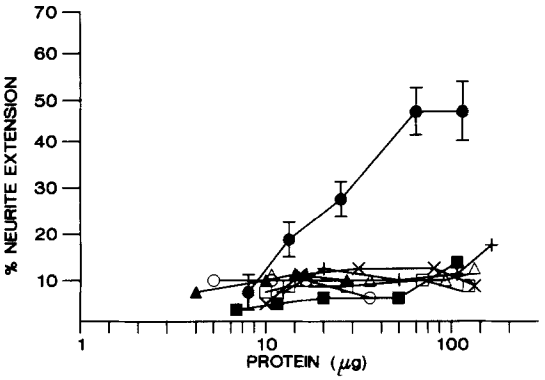

Figure 7. NEF activity of cysteine mutants. The NEF activity of VUSB-1 and of mutants with changes at either cysteine 68 (C68A, C68S, C68V), cysteine 84 (C84A, C84S), or both cysteines (C68V84A, C68V84S) was determined. VUSB-1 (*solid circles*), C68A (X), C68S (*open triangles*), C68V (*open squares*), C84A (*solid squares*), C84S (+), C68V84A (*solid triangles*), C68V84S (*open circles*). For clarity, the SEM bars are indicated only for the VUSB-1. The SEM for the mutants ranged from 3 to 8%.

tory subsequently showed its utility for analysis of the NEF activity of bovine brain S100β preparations (Kligman and Marshak, 1985). Similar to these previous studies with brain S100β, we find that VUSB-1 preparations stimulate neurite outgrowth in a dose-dependent manner. However, it should be noted that the effective concentration of the VUSB-1 preparation required for half-maximal stimulation is higher than the effective concentration of purified brain NEF (125 ng/ml; cf., Kligman and Marshak, 1985); in addition, the concentrations of both VUSB-1 and brain S100β preparations required for NEF activity are high compared to other neurotrophic factors. This apparent discrepancy can be explained in part by the fact that the VUSB-1 preparation used in the present studies, as well as the brain NEF used by Kligman and Marshak (1985), are mixtures of monomeric and dimeric S100β forms, as analyzed by SDS-polyacrylamide gels (cf., Fig. 4). Because S100β can form both covalent and noncovalent dimers, and because the structure of the active disulfide-linked species has not been demonstrated, it is not known how much of the protein migrating as a dimer exists in the active conformation. Thus, the biologically functional molecule may comprise only a fraction of the VUSB-1 preparation, and determination of the effective concentration will require future studies to purify to homogeneity the active disulfide-linked species of VUSB-1.

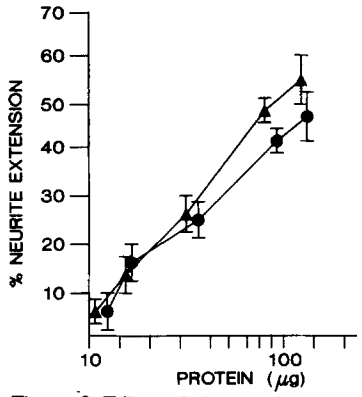

Figure 8. Effect of changes in the position of the cysteine residues of VUSB-1. A mutant (S62C68V) where cysteine 68 was moved to position 62 was constructed as described in Materials and Methods. The NEF activity of VUSB-1 (*triangles*) and S62C68V (*circles*) was determined.

Even though the VUSB-1 used in these studies is a partially purified protein fraction, several lines of evidence demonstrate that the NEF activity is a property of the VUSB-1 molecule and not a result of a contaminant in the preparation. First, NEF activity is diminished after incubation of VUSB-1 with antibodies against S100β, but not by incubation of VUSB-1 with normal rabbit IgG. Second, extracts of *E. coli* transformed with a vector lacking the VUSB-1 gene do not contain NEF activity. Third, preparation of VUSB-1 in the presence of reducing agents results in a loss of NEF activity. Fourth, alteration of a single amino acid by site-directed mutagenesis of either of the cysteine residues results in a decrease in NEF activity.

The molecular mechanisms by which VUSB-1 stimulates neurite extension are not known. VUSB-1 could act as an attachment or adhesion factor, similar to extracellular matrix proteins or cell adhesion molecules (for reviews, see Edelman, 1986; Walicke, 1989). However, this possibility appears unlikely since the neurotrophic effects of VUSB-1 in the cerebral cortex bioassay are not mimicked by addition of laminin, fibronectin, collagen, or FCS (data not shown).

Removal of VUSB-1 from cultures at various times showed that the presence of VUSB-1 for the initial 2 h of culture was sufficient to elicit a maximum response 24 h later (cf., Fig. 2 *B*). This temporally limited dependency is consistent with a potential receptor-mediated mechanism. A mechanism involving VUSB-1 interaction with a specific cell surface receptor would be analogous to the mechanism of action of several other neurotrophic factors (for examples, see Misko et al., 1987; Rodriquez-Tebar and Barde, 1988; Sutter et al., 1979; Walicke, 1989). Although no data are yet available on whether S100 receptors are present on chick cerebral cortex neurons, there have been reports of S100 binding sites on various types of neuronal membranes (Donato, 1977; Donato et al., 1975). Defining the mechanisms by which S100β interacts with the neuronal cell, and the critical molecular events within the neuronal cell that mediate neurite outgrowth are areas of research to be addressed in future studies.

In addition to stimulating neurite outgrowth, VUSB-1 also increases the percentage of cells remaining in the culture dishes as a function of time in culture. While cell viability was not measured directly in these long-term (96-h) culture experiments, our data strongly suggest that VUSB-1 enhances survival of the neuronal cells in culture. These results are consistent with studies of several other neurotrophic factors that stimulate both neurite extension and cell survival (for examples, see Thoenen et al., 1987; Hefti and Will, 1987; Walicke, 1989). However, our results are in disagreement with those of Kligman (1982), who reported that a partially purified preparation of bovine brain NEF did not enhance cell survival in a cerebral cortex bioassay. The reasons for this discrepancy are not clear, although it may result from differences in purification protocols or the source of the protein. We have shown here that VUSB-1 and S62C68V preparations that stimulate neurite extension also enhance cell maintenance, and mutant constructs that are inactive in the

NEF assay are also inactive in the cell survival assay. These data provide further evidence that the ability to stimulate neurite extension and enhance cell survival are both properties of the VUSB-1 molecule, and suggest that there are similar structural or mechanistic constraints on the protein for both activities.

To begin analysis of the structural features of VUSB-1 that are required for neurotrophic activity, we tested the relative contribution of cysteine 68 and cysteine 84. To minimize the potential effects of mutation on indirect, conformational aspects of structure, conservative substitutions were made: cysteines were replaced with serine or alanine (C68S, C68A, C84S, C84A). Serine was chosen because of its similarity to cysteine in structure and solvation properties, and alanine was chosen because of its small size and neutral charge. In addition, because all the members of the S100 family (except S100β) contain a valine at the position analogous to cysteine 68, we also constructed a mutant with cysteine 68 changed to valine (C68V). While a detailed structural analysis of the proteins would be required to determine how particular mutations affect localized conformational changes, the mutant proteins maintained the overall biochemical properties of S100β, allowing their isolation by the same protocol used for the unmodified protein. However, these mutants with changes at either cysteine 68 or cysteine 84 were unable to stimulate neurite extension or enhance neuronal survival. These data indicate that the linear amino acid sequence alone is not sufficient for neurotrophic activity, but that secondary structural features; i.e., disulfide bonds, are important for both neurite extension and neuronal survival activities. In addition, the fact that both cysteine residues are necessary for biological activity suggests an involvement of both residues in the disulfide linkage of the active species of the protein. Three possible active disulfide forms that would be consistent with our results are an interchain dimer with one disulfide linkage between heterologous cysteines (cysteine 68/cysteine 84) or two disulfide linkages between heterologous (cysteine 68/cysteine 84) or homologous (cysteine 68/cysteine 68 and cysteine 84/cysteine 84) cysteines.

To begin to analyze the secondary structural constraints on the protein for neurotrophic activity, we tested the effect of changing the relative position of the two cysteines in the linear amino acid sequence. Because a three-dimensional structure for S100β is not available, we used the structure of the vitamin D-dependent calcium binding protein (Szebenyi and Moffat, 1986) as a model in the design of the S62C68V mutant (in which cysteine 68 was changed to valine and serine 62 was changed to cysteine). The amino acid residues of S100β postulated to be calcium liganding residues (aspartic acid 61, aspartic acid 63, aspartic acid 65, glutamic acid 67, aspartic acid 69, and glutamic acid 72) based on the EF hand model (Kretsinger, 1980) and on homology with the vitamin D-dependent calcium binding protein structure (Szebenyi and Moffat, 1986) encompass the region around residues 61–72. While serine 62 and cysteine 68 are thought not to be involved in direct calcium binding, they comprise part of the loop structure of the postulated EF hand calcium binding site of S100β. The probable proximity of serine 62 and cysteine 68 in three-dimensional structure, as well as the structural similarity between cysteine and serine, made the positioning of a cysteine at residue 62 a logical first choice. When S62C68V was analyzed for neurotrophic activity, we found that the protein stimulated neurite extension and enhanced cell maintenance in a manner similar to the unmodified VUSB-1. These data suggest that, although two cysteines are required for activity, there is flexibility in the relative positioning of the cysteine residues.

The observation that the position of the cysteine residues in the linear sequence can be altered without significantly diminishing the neurotrophic activity provides additional options for generating a population of VUSB-1 molecules with a more homogeneous secondary structure. In addition, this observation has important pharmacological implications. The ability to design synthetic neurotrophic agents based on the S100β structure may allow the future development of reagents potentially useful for nerve regeneration or selective maintenance of neuronal function. Altogether, our data provide new insight into a potential role for S100β in the development and maintenance of neuronal function in the central nervous system.

We thank Paul Matrisian, Augustine Smith, Wendy Gunnarsen, Rebecca Miller, Sandra Wolchok, and Scott Nottingham for their assistance in various aspects of these studies. We are grateful to D. Martin Watterson and Thomas Lukas for their helpful discussions and suggestions.

These studies were supported in part by funds from the Muscular Dystrophy Association and the Alzheimer's Disease and Related Disorders Association (L. Van Eldik), National Cancer Institute predoctoral training grant CA09592 (S. W. Barger and S. Coats), and National Cancer Institute postdoctoral training grant CA09592 and a Pharmaceutical Manufacturer's Association postdoctoral fellowship (both to F. Winningham-Major).

Received for publication 1 June 1989 and in revised form 7 August 1989.

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