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Presynaptic phosphoprotein B-50/GAP-43 in neuronal and synaptic plasticity

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B-50/GAP-43 is a growth-associated phosphoprotein enriched in growth cones and in the presynaptic terminal. The expression of the protein is restricted to the nervous system and is highest in the first week after birth. In adult brain, B-50 is enriched in areas with high plasticity. The regulation of expression of the B-50 gene occurs both at the transcriptional and post-transcriptional level by unknown mechanisms. The gene contains 2 regions displaying promoter activity, the most 3' of which (P2) is the active one *in vivo*. Expression of B-50 in non-neuronal cells results in filopodial extensions whereas antibodies or antisense oligo's to B-50 prevent neurite outgrowth. The protein is important for neuronal pathfinding. Several post-translational modifications have been described, ADP-ribosylation and palmitoylation in the membrane binding domain, phosphorylation by PKC, casein kinase II and phosphorylase kinase, and dephosphorylation by several phosphatases, among which is calcineurin. Interactions of B-50 have been described with calmodulin, PIP kinase, F-actin, and phospholipids. Recent studies indicate that the phosphorylation state and amount of calmodulin bound to B-50 regulate the rate of transmitter release. Induction of long-term potentiation by high frequency stimulation of hippocampal slices results in an increased state of B-50 phosphorylation. This will increase the amount of free calmodulin in the presynaptic terminal and increase the amount of transmitter released. Although B-50 is involved in seemingly unrelated forms of neuronal plasticity, neurite outgrowth and transmitter release, our unifying hypothesis is that the protein plays an (unknown) essential, modulatory role in membrane expansion.

B-50 has originally been characterized as a phosphoprotein in adult rat brain [1], where it is located at the presynaptic membrane [2]. The identical protein GAP-43 was described as a member of the family of growth-associated proteins (GAPs) in the nervous system. These represent a relatively small subset of proteins synthesized at strikingly high levels during neurite outgrowth [3, 4]. The best characterized member of the family is B-50/GAP-43. It is consistently expressed during both developmental and regenerative axonal growth, and delivered by fast axonal transport to the extending neurite, especially to the growth cones [3, 4]. The presumed mediating role of B-50 in neurite growth is documented by a large number of

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Abbreviations: CAT, chloramphenicol acyltransferase; CaM, calmodulin; dbcAMP, dibutyryl cyclic AMP; DRG, dorsal root ganglia; EPSP, excitatory postsynaptic potential; GAPs, growth associated proteins; GABA, γ-amino-butyric acid; GNRP, guanine nucleotide releasing protein; LTP, long-term potentiation; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; UTR, untranslated region.

studies in different neuronal systems during the last decade (see reviews by Benowitz & Routtenberg [5] and Skene [6]). Most recent studies in transgenic mice indicate that overexpressed B-50 modifies neurite pathfinding [7, 8].

EXPRESSION OF B-50 IN THE NERVOUS SYSTEM

During embryogenesis B-50 is expressed in developing axons of virtually all systems of the brain [9, 10]. Although B-50 is predominantly expressed in neurons, under certain circumstances B-50 is also present in other tissue [11] and in other cell types of the nervous system [12–15].

In mature non-damaged neurons of the adult brain B-50 expression is mostly low. High levels of B-50 expression are detected after nerve injury in regenerating axons [16–18] and in neurons which are believed to be involved in ongoing synaptic remodelling, e.g. in human associative brain areas [19, 20] and rat hippocampal areas (i.e. the pyramidal cells) and olfactory areas [21–23].

REGULATION OF B-50 mRNA EXPRESSION

The expression of B-50 mRNA as studied by Northern blotting or *in situ* hybridization is increased during development, neuritogenesis and regeneration. During development B-50 is expressed at a very early stage, before expression of the neurofilaments, and coincides with neuritogenesis and pathfinding but not with synaptogenesis [24].

Extracellular regulation

In vivo, factors that can regulate the B-50 mRNA expression during development are sex hormones. During early postnatal life a sex dimorphism exists in the cortex, the bed nuclei of the stria terminalis and the medial preoptic nuclei, indicated by higher levels of B-50 mRNA in males compared to females [25]. B-50 mRNA expression is upregulated by estrogen in the cortex and ventromedial hypothalamus (VMH) [26]. Levels of B-50 expression in the cortex, the bed nuclei of the stria terminalis and the medial preoptic nuclei are differentially regulated by estrogen and androgen [27].

Many studies have investigated the regulation of B-50 mRNA expression after nerve or brain injury [17, 18, 28, 29]. From studies on peripheral or central lesioning it was demonstrated that B-50 is upregulated after central injury only when the injury is close to the cell body, while in peripheral lesioning distance does not influence B-50 expression [16, 30]. Therefore it is clear that the event of axonal injury or disruption of target contact *per se* is not sufficient for the induction of B-50 mRNA expression. Although high B-50 mRNA expression colocalizes with nerve growth factor (NGF) receptor expression in uninjured dorsal root ganglia, NGF does not change B-50 expression upon axotomy [31], excluding it as the main trigger for B-50 induction after injury. Factors that can influence B-50 mRNA expression *in vivo* after neuronal damage are glucocorticoids [32], cAMP after hypoxia [33] and myelinization [34, 35]. These *in vivo* studies, however, do not provide clear indications of what molecule(s) are decisive for B-50 induction and what is the underlying mechanism.

More information is available from *in vitro* studies. In general, neuronal differentiation of cells in culture induces B-50 expression at mRNA and protein level [36–39]. Not all factors that modulate neurite outgrowth, however, also change B-50 expression (for instance electrical activity [40]). It has been suggested that in these situations the B-50 protein that is already present is modified. It seems that the induction of B-50 mRNA expression is less dependent on the sort of factor used to obtain neuronal differentiation, since B-50 induction has been demonstrated using NGF in sensory neurons [38] and PC12 cells [41], IGF-I in SH-SY5Y neuroblastoma cells [39], retinoic acid in P19-EC cells [36] and DMSO in mouse N1E-115 neuroblastoma cells [42]. Possibly the factors initiate a similar cellular mechanism of induction or alternatively increase B-50 expression at a different level of transcription or translation.

Intracellular regulation

Several intracellular mechanisms of regulation of B-50 expression has been reported. Stimulation of PC12 or N1E-115 neuroblastoma cells with low phorbol ester concentrations stimulates B-50 mRNA levels, similar to that observed with NGF, whereas down regulation of PKC by addition of high phorbol ester concentrations prevents the NGF induced rise in B-50 mRNA [42, 43]. In agreement with this finding, TPA was found to increase B-50 mRNA levels in the human neuroblastoma cell line

SH-SY5Y [44] and to a lesser extent in another PC12 clone [45]. In the hybrid mouse neuroblastoma x rat glioma cells NG 108-15 a synergistic effect of phorbol esters and dibutyryl cyclic AMP (dbcAMP) was observed on B-50 mRNA expression as well as neurite outgrowth [46]. The upregulation of B-50 mRNA in Schwann cells during demyelination is mimicked by forskolin or dbcAMP [15, 47], suggesting that both PKC and cAMP are intracellular regulators of B-50.

Transcriptional and post-transcriptional control

Direct analysis of B-50 transcription in isolated nuclei from neonatal and adult cortex showed that the developmental regulation of B-50 expression is largely controlled at the transcriptional level [9]. In PC12 cells the B-50 mRNA upregulation by NGF treatment and down-regulation by corticosteroids cannot be blocked by cycloheximide, indicating that these two modulators have a direct action on the B-50 mRNA expression. The inhibition of B-50 mRNA expression by corticosteroids is by a direct action on the basal rate of B-50 transcription, whereas the effect of NGF is post-transcriptional [48].

Detailed transcriptional analysis of developing brain cortex, NGF treated PC12 cells and regenerating goldfish retinas demonstrated that the change in B-50 mRNA levels was 5–10 fold higher than the change in the rate of B-50 transcription [49], indicating that B-50 mRNA levels are determined by both transcriptional and post-transcriptional mechanisms. Further analysis of the stimulating effect of NGF on B-50 mRNA levels showed that NGF increased the stability of B-50 mRNA [41]. Unlike other mRNAs regulated by stability, the induction and stabilization of the B-50 mRNA is independent of translation [43]. It has been suggested that NGF-induced increases in B-50 mRNA expression are mediated *via* PKC activated mRNA stabilization independent of translation [43]. Recently, three brain cytosolic RNA-binding proteins were found to interact with the 3′ untranslated region (UTR) of B-50 mRNA [50]. The half-life of B-50 mRNA strongly correlates to the presence of this 3′ UTR region.

B-50 GENE STRUCTURE AND PROMOTER ANALYSIS

The human and the rat B-50 gene are single copy genes that span at least 50 kb and contain 3 exons (see for an overview Fig. 1) [51, 52]. The first exon contains the 5′ untranslated region and encodes the first 10 amino acids, which contains the membrane binding domain of the protein [53]. The second exon encodes the bulk of the protein and includes a calmodulin-binding and a PKC phosphorylation site [54–57]. The third exon encodes the carboxy terminus part of the protein that contains an F motif for interaction with cytoskeletal components [58] and contains two polyadenylation signals [51, 59, 60].

The 5′ UTR of the rat and the human B-50 gene are very homologous [51, 52, 60, 61]. At least two different transcription initiation sites are used, the most 5′ is located –411 nt upstream of the translation initiation codon, the most 3′ is located at about –50 nt [51, 52, 60–62]. Extensive probing of Northern blots containing 8 day old rat brain mRNA and *in vitro* B-50 transcripts demonstrated that two mRNAs can be distinguished of 1450 and 1650 bases in length.

Promoter analysis of the 5′ UTR of the rat gene by Nedivi *et al.* [52] indicated that the single B-50 promoter is a 386 bp fragment located directly upstream of the (GA)-repeat. This "core promoter" drives the neuron-specific expression of a chloramphenicol acetyltransferase (CAT) construct in primary cortical cultures. The (GT)- and the (GA)-repeat flanking this core promoter stabilize the expression of the promoter activity. The core promoter could restrict gene expression to neural cells in developing zebrafish [63]. An additional part of the intron 1 sequence reduced ectopic expression in transgenic mice [64]. In contrast to these findings it was shown that in PC12, C6, and Rat2 cells a much shorter fragment of 600 bp flanking the start codon was sufficient for neuronal specific expression, while 230 bp restricted expression to neural cells [65]. It was demonstrated that in the rat B-50 gene there is a second promoter region (P2) present in the 5′ UTR, located between –233 and –1 bp, directly upstream the start codon [61]. Transfection of this promoter construct into differentiated P19-EC cells was sufficient for a nearly complete neurospecific expression of reporter gene [66]. Transcripts derived from P1 and P2 correspond to the earlier described long and short transcripts. Nothern blot analysis of early postnatal rat brain revealed that most of the transcripts are derived from P2 promoter activity [67]. In the human B-50 gene similar promoter regions

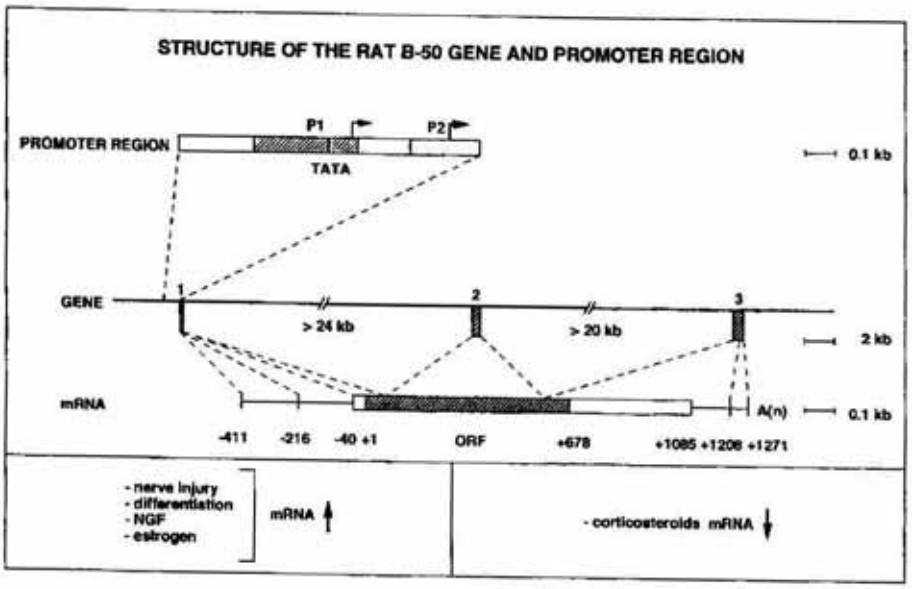

Fig. 1. Organization of the B-50 gene and the 5′ UTR.
The B-50 gene contains 3 exons represented by hatched black boxes, numbered 1, 2 and 3 and the minimal size of the introns, derived from the papers of Nedivi *et al.* [52] and Grabczyk *et al.* [51], is indicated. In the mRNA the open reading frame is represented by a hatched box and the 5′ and 3′ UTR by an open box. The dashed lines represent exon borders derived from literature, 5′ ends of the cDNAs were found at: –411 nt [52], –216 nt [59], –40 nt [56]; 3′ ends of the cDNAs were found at: +1085 nt [56], +1208 [9], +1271 [59]. At the left top of the figure the position of promoters P1 and P2 with their respective transcription starts, represented by arrows and the putative TATA box are depicted. At the bottom of the figure, the modulators of B-50 mRNA levels as discussed in the section "Regulation of B-50 mRNA expression" are indicated.

have been described [62, 68], with a preference for the short transcript [60]. The presence or absence of the (GT)- and the (GA)-repeat did not significantly influence the activity of either P1 or P2. *In vitro* translation of B-50 *in vitro* transcripts mimicking those derived from P1 and P2 showed that P1 derived B-50 transcripts are translated with very low efficiency in rabbit reticulocyte lysates, when compared with transcripts containing the P2 leader [67].

Taking together our data suggest that although the B-50 gene has the potency to transcribe two different mRNAs, only the ones derived from P2, containing a short leader sequence, are actively transcribed *in vivo* and can be translated *in vitro*, indicating that *in vivo* P2 is the most active B-50 promoter.

Expression of the B-50 gene is largely restricted to neural cells and regulated by glucocorticoids and may be by estrogen. Comparison of the 5′ UTR of both the human and the rat B-50 gene revealed no known binding sites for previously described neural tissue specific transcription factors, nor does it contain glucocorticoid or estrogen response elements, although it does contain a sequence motif that is common to several neural-selective genes [52]. The 5′ UTR also does not contain perfect consensus elements for immediate early genes, cyclic AMP or protein kinase C [52]. Several different explanations can be given for the absence of consensus elements in the known part of the B-50 gene, i.e. (i) the B-50 gene is not primarily responding to factors inducing neuronal differentiation, but that the expression of the gene is a secondary response; (ii) the neural preferred expression is regulated by elements present in the large introns; (iii) the transcription of the B-50 gene is constitutive and the neural preferred expression is due to regulation of the mRNA stability; (iv) the expression of the B-50 gene is regulated by unknown B-50 specific transcription factors binding to sequences that are contained within the regions of the gene that have been identified to direct B-50 specific transcription, i.e. P1 and/or P2.

POSTTRANSLATIONAL MODIFICATIONS: CELL MORPHOLOGY, NEUROTRANSMITTER RELEASE AND LTP

Several posttranslational modifications of B-50 have been described. The conservation between species of several domains of B-50 that are involved in these modifications imply their importance in the function of the protein [69].

One of the most characteristic sites of B-50 that is subjected to posttranslational modification is the so-called "GAP-module" [69]. This domain spans 18 amino acids (31–40 in man) and has been shown to bind calmodulin (CaM) at amino acid 39–41 [54]. Unlike other CaM-binding proteins B-50 binds CaM only at relatively low intracellular Ca^{2+} levels [70, 71]. When CaM is not bound to B-50, the Ser^{41} site can be phosphorylated by PKC both *in vitro* and *in vivo* [55, 57]. Conversely, phosphorylation by PKC can inhibit CaM binding [54, 70]. Another site for PKC has been identified at Ser^{209} [72]. This site is less well conserved. Close to this second PKC site, at Ser^{192}, a potential casein kinase II (CKII) phosphorylation site was found [73]. CKII phosphorylation of B-50 is only possible when no phosphate group is present at the Ser^{41} position [74]. S100 was shown to inhibit phosphorylation by PKC and CKII [75]. Gangliosides could also inhibit phosphorylation, but not the PKC site at Ser^{41} [76]. Dephosphorylation of B-50 can be mediated by several phosphatases *in vitro*, among which is the calmodulin-dependent phosphatase calcineurin [77–79]. Proteolytic digestion of *in situ* phosphorylated B-50 resulted into the same phospho-fragments as *in vitro*, indicating that this phosphorylation site is used *in vivo* [80–82].

A second domain that is highly conserved and presumably involved in several functional interactions is the N-terminus, especially the first 10 amino acids. The two cysteines present at position 3 and 4 are involved in membrane attachment [83] possibly through palmitoylation [84]. In addition, several reports indicate that this N-terminal domain is involved in interaction between G_0, a GTP binding protein, and B-50, both major components of the growth cone membrane. B-50 has been shown to stimulate the binding of GTPγS to G_0. The interaction between B-50 and G_0 is located in the amino terminal domain of B-50 (amino acids 1–24, [85]). This domain is homologous to the cytoplasmic tail of G-linked receptors, and it was therefore suggested that in the growth cone B-50 mimics the cytosolic tail of transmembrane receptors thereby triggering an intracellular second messenger system by stimulating the binding of GTP to the α-subunit of G_0 [85]. The interaction between B-50 and G_0 is different from that normally observed in G protein-coupled receptors. B-50 enhances the release of GDP from G_0, by increasing the initial rate of GTPγS binding to G_0 and by increasing the GTPase activity. This B-50 effect is, however, not blocked by pertussis toxin nor affected by the G-protein βγ subunits or phospholipids. Thus it was concluded that B-50 is a novel guanine nucleotide releasing protein (GNRP) [86]. The minimal B-50 N-terminal fragment that is still active in stimulating the binding of GTPγS to G_0 is the 1–10 peptide. Mono-palmitoylation reduced and di-palmitoylation abolished that activity of N-terminal peptides to stimulate G_0. The same results were seen with intact B-50 isolated from brain. These data suggest that palmitoylation of B-50 controls a cycle between membrane-bound, inactive B-50 and cytosolic active B-50 [87]. Both forms of B-50 are still subject to phosphorylation by protein kinase C, indicating that phosphorylation and calmodulin binding and the interaction with the membrane or G-protein are regulated independent mechanisms and may therefore serve different functions in the cell [88].

Not all of the posttranslational modifications have been demonstrated directly in *in vivo* experiments. Many studies have made use of mutant B-50 to disrupt physiological interactions. Using these techniques a role for posttranslational modifications has been implied in cell morphology, transmitter release and long term potentiation.

Cell morphology

Since B-50 is thought to be involved in neural plasticity, numerous studies have investigated the role of posttranslational modifications of the protein in morphological changes of the cell. It was found that after lesioning not only the amount of B-50 increases, but also its phosphorylation by PKC [89]. During axonal growth phosphorylation by PKC increases in the vicinity of the target [90], possibly through stabilization of the growth cone. In cultured dissociated dorsal root ganglia (DRG)s the antibody specific for the phosphorylated form of B-50 demonstrated that also in this culture neuritogenesis begins in the absence of phosphorylated B-50. Increased B-50 phosphorylation correlated with a reduced extent of neurite outgrowth. Motile growth cones contained very low levels of phosphorylated B-50, whereas stationary growth cones showed much more immunoreactivity. Down regulation of PKC by

phorbol ester prevented the phosphorylation of B-50 thereby inducing growth cone collapse [90]. Two rat B-50 phosphorylation mutants (Ser^{41} to Ala^{41} preventing and Ser^{41} to Asp^{41} mimicking phosphorylation by PKC) had prominent effects on the cell morphology. Non-neuronal COS-7 and L6 cells transfected with the Ser^{41} mutant that prevents PKC phosphorylation (Ala^{41}) spreaded poorly and contained numerous filopodia, whereas cells that were transfected with the PKC phosphorylation mimicking mutation spreaded extensively and displayed large irregular membranous extensions with little filopodia [91]. Recently, the typical Ala^{41} mutant phenotype was confirmed in B-50 deficient PC12 cells and attributed to the uncoupling of plasma membrane and its underlying cytoskeleton [92]. The role of CaM in these morphological changes of these B-50 mutant cells is still unclear. In organotypic culture of a carp eye CaM and CaMKII inhibitors prevented dark/light morphological adaptation while affecting the phosphorylation of B-50 [93].

Mutations of the first ten amino acids also resulted in morphological changes. Both in the nonneuronal COS-7 and L6 cells and in PC12 cells mutation of the Cys^3Cys^4 into Ala^3Ala^4 [91] or into Ser^3Gly^4 [83] prevented the association of B-50 with the plasma membrane and the Golgi-apparatus. Fusion of this N-terminal domain of the B-50 gene to a β-galactosidase gene resulted in membrane sorting [94], while mutation of Cys^3Cys^4 disrupted membrane targetting.

The direct involvement of G-protein in neurite outgrowth has been studied in culture sympathetic neurons. Electroporation of GTPβS into these cells inhibits neurite outgrowth, whereas introduction of GTPγS promotes neurite formation. This implies that G protein stimulation inhibits neurite formation [95]. G protein-coupled receptors are involved in growth cone collapse caused by solubilized embryonic chick membranes or myelin components [96]. Whether this is caused by G_0 activation or mediated through another G-protein is at present unclear, but the mechanism by which this collapse is mediated is pertussis toxin sensitive arguing against a role for B-50 in this process, since the interaction of B-50 with G_0 is pertussis toxin insensitive [97]. Since B-50 and G_0 are the major components of the growth cone and B-50 promotes neurite outgrowth may imply that in the growing tip of the axon B-50 is present in an acylated form, and therefore bound to the membrane in order to prevent stimulation of G_0 which would cause growth arrest. Indeed, administration of N-terminal B-50 peptides to dorsal root ganglion neurons aggrevated the growth cone collapse induced by myelin and serotonin [98].

Neurotransmitter release

The involvement of B-50 in neurotransmitter release was suggested after the observation that PKC-mediated protein phosphorylation is involved in regulation of synaptic transmitter release [99] and B-50 is partly associated with synaptic vesicles [100]. In adult brain B-50 immunoreactivity was localized mainly in monaminergic systems [100–102]. In the cerebellum B-50 is absent from GABAergic neurons, but involved in the outgrowth of the nonGABAergic parallel fibers [103]. B-50 immunoreactivity in the postnatal spinal cord is present in both noradrenergic [104] and serotonergic neurons [105]. Similarly in the postnatal adrenal gland B-50 is expressed by noradrenergic, but not by adrenergic chromaffin cells [106].

Molecular tools that prevent B-50 expression in PC12 cells reduce the dopamine release [107, 108]. The introduction of Ser^{41} specific, but not C-terminal antibodies into permeated synaptosomes inhibited Ca^{2+}-induced noradrenaline release [109, 110], demonstrating that the CaM binding/PKC phosphorylation site is vital for B-50's function in release. Earlier studies, however, indicated that PKC, the main kinase phosphorylating B-50, is not important for steps in release following the calcium trigger [111]. The state of phosphorylation of B-50, determined by both kinase (PKC) and phosphatase (calcineurin) activity, rather than its phosphorylation by PKC seems to be involved in modulating neurotransmitter release, possibly by modulating B-50's capacity to store CaM.

Long term potentiation

The role of PKC in the induction of LTP has been well studied (reviewed by Pasinelli *et al.* [112]). Tetanic stimulation in hippocampal slices revealed an increase in phosphorylation of several proteins, one of which has been identified as B-50 [113]. Indeed, a correlation between the phosphorylation of B-50 and LTP has been established in several studies [5, 114–117]. Since these earlier studies were performed on *post-hoc* obtained material, a new technique

was introduced to examine phosphorylation during LTP *in situ* [118]. Using this technique it was demonstrated that the phosphorylation state of B-50 raised transiently during induction of LTP in hippocampal slices and that the increase in phosphorylation is highly correlated with the increase in the evoked responses (EPSP) at each time-point [119]. Long term memory induced by training and ACTH was also preceeded by an increase in B-50 phosphorylation [120].

CONCLUDING REMARKS

The function of B-50, as it was found *in vivo* in transgenic mice [7, 8] and *in vitro* in neuronal and nonneuronal cell culture systems [91, 121], has been specified to a mediating role in neurite pathfinding during development and axonal regeneration, and in neuronal plasticity processes like transmitter release and LTP. Since this function requires a tight spatial and temporal restriction of gene expression, the protein is regulated by both transcriptional and posttranscriptional control. Regulation of the B-50 protein is controlled by two promoter regions for gene expression, by regulation of mRNA stability in its 3' UTR region and by posttranslational modifications.

The functional alterations of the B-50 protein after posttranslational modifications indicate that molecular interactions may play an important role in activation of B-50 dependent processes. The translocation to the membrane mediated by the two N-terminal cysteines was shown to be essential in morphological changes [83], implying that B-50 can only perform its function when it is associated to the plasma membrane. This also enforces the possibility that G-protein interaction may be involved [86]. In addition, extensive studies have revealed that phosphorylation by PKC and CaM-binding are decisive for most, if not all, of B-50's plastic functions.

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