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Molecular Analysis of the Function of the Neuronal Growth-Associated Protein GAP-43 by Genetic Intervention

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Abstract

GAP-43 is a presynaptic membrane phosphoprotein that has been implicated in both the development and the modulation of neural connections. The availability of cDNA clones for GAP-43 makes it possible to examine with greater precision its role in neuronal outgrowth and physiology. We used Northern blots and *in situ* hybridization with GAP-43 antisense RNA probes to show that GAP-43 is expressed selectively in associative regions of the adult brain. Immunocytochemical analyses showed alterations in the pattern of GAP-43 expression in the hippocampus during reactive synaptogenesis following lesions of the perforant pathway. Genetic intervention methodology was used to analyze the molecular nature of GAP-43 involvement in synaptic plasticity. GAP-43-transfected PC12 cells displayed an enhanced response to nerve growth factor, suggesting that GAP-43 may be directly involved in neurite extension and in the modulation of the neuronal response to extrinsic trophic factors. Studies of PC12 cell transfectants, in which the synthesis of GAP-43 was blocked by expression of GAP-43 antisense RNA, showed that evoked dopamine release was significantly attenuated in these cells. The use of gene transfer into neurons with the HSV-1 vector is presented as a method of analyzing the interaction of GAP-43 with signal transduction systems during neurotransmitter release.

Index Entries: Growth-associated protein GAP-43; F1; B-50; transfection; neurite outgrowth; antisense RNA; dopamine release; herpes virus vector; adenylate cyclase.

Introduction

GAP-43 (B-50, F1, pp46, neuromodulin) is a phosphoprotein of the presynaptic membrane that has been implicated in both the development and the modulation of neural connections. In developing (Skene and Willard, 1981a) or regenerating (Skene and Willard, 1981b; Benowitz et al., 1981) neurons, this protein is synthesized and transported to the nerve terminals at levels 20–100 times higher than in neurons that have established mature synaptic relationships. GAP-43 is an integral constituent of the growth cone in growing neurons (De Graan et al., 1985; Katz et al., 1985; Meiri et al., 1986; Skene et al., 1986). Although overall levels of GAP-43 fall considerably in the brain as it matures, significant amounts continue to be associated with the presynaptic terminals of certain neurons in the adult brain (Oestreicher and Gispen, 1986; Benowitz et al., 1988).

The correlation between the phosphorylation of GAP-43 and both neurotransmitter release in vitro (Dekker et al., 1989,1991) and the establishment and maintenance of long-term potentiation (Akers and Routtenberg, 1985; Lovinger et al., 1985) suggests that it plays a role in synaptic plasticity. However, the molecular mechanisms by which GAP-43 continues to modulate synaptic function in the mature brain have not been precisely delineated. Evidence from several laboratories has defined several functional domains within GAP-43 (Fig. 1); the data indicate that it is probably implicated in more than one signal transduction pathway within the neuron. It has been implicated in the regulation of free calmodulin levels in neurons by binding calmodulin in the absence of calcium (Andreasen et al., 1983; Alexander et al., 1987; Liu and Storm, 1990). It has also been shown to inhibit phosphatidyl inositol phosphate kinase (Gispen et al., 1985) and to stimulate GTP hydrolysis by the G protein G_o (Strittmatter et al., 1990). In addition, it is a substrate for protein kinase C (Nelson and Routtenberg, 1985; Aloya et al., 1983) and possibly other protein kinases (Schuh et al., 1989; Pisano et al., 1988).

The presence of GAP-43 in the growth cone indicates that it must be anchored to the presynaptic membrane. Nevertheless, the amino sequence derived from GAP-43 cDNA sequences (Cimler et al., 1987; Basi et al., 1987; Rosenthal et al., 1987; Kosik et al., 1988; Ng et al., 1988) revealed no obvious transmembrane domains. Subsequent work showed that palmitic acid is incorporated into GAP-43, probably at cysteines 3 and 4, in cultured rat cortical neurons (Skene and Virag, 1989).

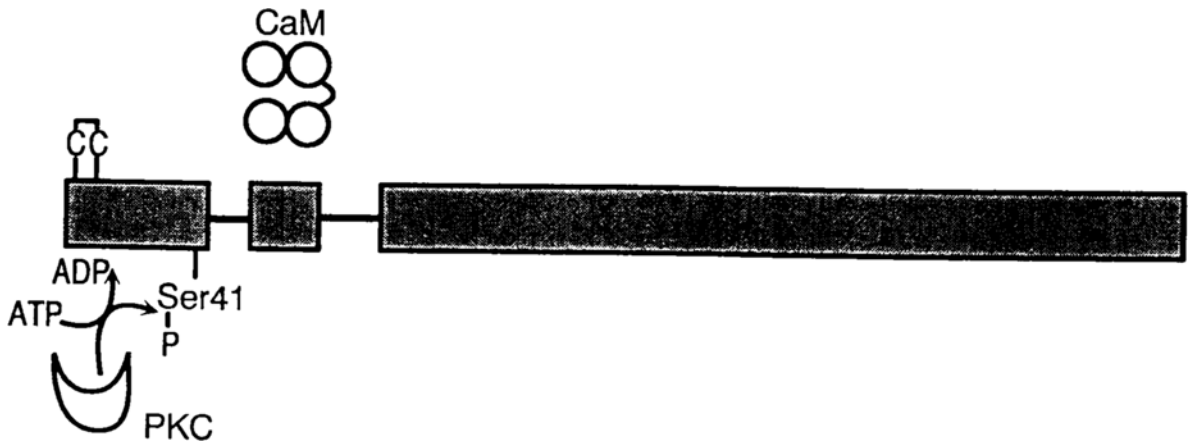

Fig. 1. Illustration of functional domains of GAP-43. The first 10 amino acids, containing cysteines three and four, are thought to be involved in the attachment of GAP-43 to the membrane. It is phosphorylated at serine 41 by protein kinase C; immediately downstream of this phosphorylation site is the putative calmodulin binding domain.

The membrane association of GAP-43 was abolished by substitution of these two cysteines with serines by site-directed mutagenesis (Zuber et al., 1989), suggesting that palmitylation at these residues is required for membrane association of GAP-43.

The availability of cDNA clones for GAP-43 makes it possible to examine with greater precision its role in neuronal outgrowth and physiology. We used RNA blot analyses and *in situ* hybridization to show that GAP-43 is expressed selectively in associative regions of the adult human brain. We then turned to genetic intervention methodology to analyze the molecular nature of GAP-43 involvement in synaptic plasticity. Transfection of the GAP-43 cDNA as a retroviral recombinant into the rat PC12 cell line revealed a dramatically increased response to nerve growth factor (NGF) in stably transfected cells overexpressing this protein, suggesting that GAP-43 may be directly involved in neurite extension and in the modulation of the neuronal response to extrinsic trophic factors. Studies of additional PC12 cell transfectants in which synthesis of GAP-43 was blocked by stable expression of the antisense RNA indicated that evoked dopamine release was significantly attenuated in these cells.

To define the domain of the GAP-43 protein that targets it to the growth cone membrane, we fused the aminoterminus of the protein to β-galactosidase in a defective Herpes Simplex Virus One (HSV-1) vector, and showed that the fusion protein was directed preferentially to neuronal processes in differentiated PC12 cells and in superior cervical ganglion (SCG) neurons in primary culture. We discuss the use of gene transfer into neurons with the HSV-1 vector to analyze the interaction of GAP-43 with signal transduction systems during neurotransmitter release.

Selective Expression of GAP-43 in Associative Regions of the Adult Brain

It has been proposed that, by analogy to the events of development, nerve terminals at which GAP-43 levels remain high throughout life may be specialized sites that can undergo functional or even structural changes in response to patterns of physiological activity (Pfenninger, 1986; Benowitz and Routtenberg, 1987). Hence, we sought to determine which neurons of the human

brain express the GAP-43 mRNA. We initially showed with Northern blots (Neve et al., 1987) that, whereas GAP-43 is expressed at high levels throughout the human brain during fetal development, it is expressed selectively in neurons of the associative neocortex in the adult brain. We subsequently (Neve et al., 1988) confirmed and extended this finding using RNA blots coupled with *in situ* hybridization. GAP-43 gene expression is low in primary sensory areas of the cortex (e.g., Brodmann areas [A] 1 and 17) and high in associative cortical regions, including the frontal, inferior temporal, perisylvian, and parietal cortices. Modest levels of GAP-43 mRNA were observed in cerebellum, and low levels in caudate-putamen and thalamus. At the cellular level, neurons expressing GAP-43 mRNA are concentrated in layer 2 of the neocortex and in hippocampal pyramidal cells. Many of the layer 2 neurons that express the GAP-43 mRNA appear to coincide with the small pyramidal neurons that give rise to local intracortical associations. Thus, GAP-43 is expressed selectively in a subset of neurons that are particularly abundant in the hippocampus and in associative cortical areas. These neurons may be specialized for ongoing functional or structural plasticity.

Altered Expression of GAP-43 in the CNS During Reactive Synaptogenesis

To determine whether GAP-43 may be involved in synaptic remodeling in the mature brain, we examined its pattern of expression during the reactive synaptogenesis that occurs in the rat hippocampal formation following lesions of the perforant pathway (Benowitz et al., 1990). If the perforant pathway from the entorhinal cortex to the hippocampal formation is lesioned, additional neurons are recruited to sprout axon collaterals that synapse onto segments of the dentate granule cell dendrites denervated by the lesion (Steward et al., 1974; Lynch et al., 1976). Using this model system, we investigated whether GAP-43 levels change when new synapses arising from hippocampal pyramidal cells and from the septum are forming.

We found that, between 2–4 d after lesioning of the perforant pathway, levels of GAP-43 increased markedly in the inner molecular layer of the dentate gyrus, coincident with the time at which commissural-associational (CA) fibers begin to sprout from collaterals into dendritic portions denervated by the lesion. GAP-43 immunoreactivity in the inner molecular layer began to diminish by 8 d postlesion, but continued to define an expanded CA projection for at least 1 mo. In contrast, GAP-43 levels declined in the outer molecular layer after the perforant pathway lesion and remained low for 2–3 wk, during which time sprouting of septal inputs into this layer could be visualized by cholinesterase histochemistry. These data demonstrate that alterations of GAP-43 levels are associated with reactive synaptogenesis in the CNS, and also suggest that there are differences among neural systems in their expression of the protein during synaptic remodeling.

Increased Response to NGF in Cells Transfected with the GAP-43 Gene

The circumstantial involvement of GAP-43 in the development and modulation of neural connections has been heavily documented; however, the precise molecular nature of its role in these processes is not yet known. We have begun to use genetic intervention methodology to begin to understand the molecular mechanisms by which GAP-43 participates in neuronal outgrowth and physiology. As a first step, we transfected the human GAP-43 cDNA under the control of the Moloney murine leukemia virus long terminal repeat (MMLV LTR) into PC12 cells (Yankner et al., 1990).

We inserted a cDNA comprising the entire human GAP-43 coding sequence into the retroviral expression vector DOJ. The GAP-43-DOJ construct, and the control nonrecombinant DOJ vector, were transfected into undifferentiated PC12 cells. Stably transfected cells were subcloned, expanded, and confirmed by RNA blot analysis to be expressing significant levels of transfected GAP-43 mRNA under the control of the MMLV LTR.

No significant neurite outgrowth occurred in either transfected or nontransfected PC12 cells in the absence of NGF. Following the addition of NGF, GAP-43-transfected cells showed a marked acceleration of neurite outgrowth relative to vector transfected controls. At any given time-point, GAP-43-transfected cell neuritic processes were, on the average, longer and more abundant than those of vector-transfected control cells (Fig. 2). Moreover, the GAP-43-transfected cells displayed significantly increased sensitivity to NGF. These cells displayed maximal neurite outgrowth response to NGF at an NGF concentration of 1 ng/mL, whereas the maximal dose for control cells was in the range of 20–100 ng/mL NGF. The half-maximal dose of NGF was about 10-fold lower for the GAP-43-transfected cells than for control PC12 cells.

We also used these cell lines to investigate effects of GAP-43 on neurite regeneration. When the neurites of GAP-43- and vector-transfected PC12 cells were mechanically removed after incubation of the cells with NGF for 1 wk, the subsequent regeneration of neurites after replating of the cells with NGF was considerably greater (in terms of numbers of neurite-bearing cells) for the GAP-43-transfected cells than for the control cells. Furthermore, when GAP-43-transfected cells were washed in NGF-free medium and then replated in the absence of NGF, the cells displayed significant regeneration 1 d later, whereas control cells showed virtually no regeneration under these conditions.

These results provide evidence for the involvement of GAP-43 in neurite extension. They also suggest that GAP-43 increases the sensitivity of the cell to NGF, either by direct interaction with the NGF signal transduction mechanism or by induction of a more generalized "primed" state resulting in increased NGF responsiveness. GAP-43 may affect the NGF response by altering phosphoinositide metabolism, which has been shown by GAP-43 (Jolles et al., 1980). Alternatively, GAP-43 may have a more direct effect on neurite outgrowth that is not confined to specific interactions with NGF.

Expression of GAP-43 Antisense RNA in Transfected PC12 Cells

Our data derived from overexpressing GAP-43 in transfected PC12 cells show that GAP-43 is involved in neurite extension. These results do not, however, prove that GAP43 is required for neurite outgrowth in these cells. To investigate this question, we expressed a GAP-43 cDNA in the antisense orientation under control of the mouse mammary tumor virus (MMTV) promoter (Fig. 3) in PC12 cells (Fidel et al., 1990; Ivins et al., 1991). We also transfected into the PC12 cells vector carrying the GAP-43 cDNA in the sense orientation and vector alone. We were able to show that the antisense GAP-43-transfected cells, although expressing very low levels of GAP-43 relative to controls, extended neurites in response to NGF. This suggests that GAP-43 is not necessary for process outgrowth in PC12cells. Recent corroboration of these results was reported by Baetge and Hammang (1991), who showed normal NGF-, bFGF-, and cAMP-mediated neurite outgrowth in PC12 cells shown to be deficient in GAP-43.

Because GAP-43 has been implicated in the modulation of neurotransmitter release (Dekker et al., 1989,1991), we measured the spontaneous and evoked release of dopamine from the transfected cells. Spontaneous release of dopamine as

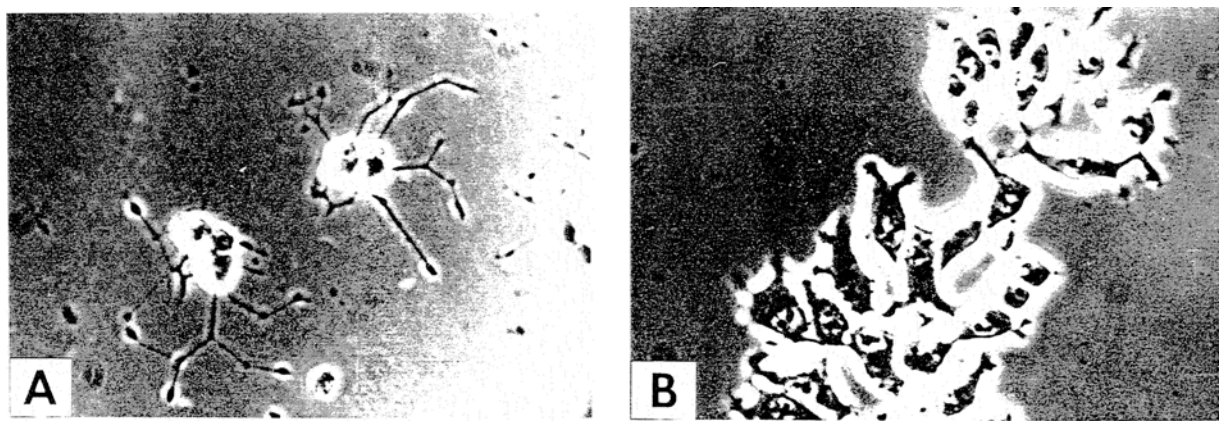

Fig. 2. GAP-43-transfected and control PC12 cells. **A** depicts GAP-43-transfected cells after 2 d of treatment with 50 ng/mL NGF; **B** shows control PC12 cells after the same treatment with NGF. Note enhanced outgrowth of the GAP-43-transfected PC12 cell line (**A**) relative to the control (**B**).

a percentage of intracellular dopamine was not altered in the transfected cells, but intracellular dopamine was greatly diminished in antisense GAP-43 transfected PC12 cells relative to controls and to sense GAP-43-transfected PC12 cells. Depolarization of cells with high K^+ caused a significant stimulation in dopamine release from control cells and from cells transfected with GAP-43, but evoked release of dopamine from cells transfected with antisense GAP-43 was greatly reduced or absent. These results indicate that GAP-43 is involved causally in the release of dopamine from PC12 cells, and that it may be required for evoked dopamine release.

The data are particularly interesting in light of a recent report that GAP-43 is expressed at high levels, specifically in monoaminergic neurons in the brainstem of adult rats (Bendotti et al., 1991). The robust expression of GAP-43 in serotonergic, dopaminergic, and noradrenergic neurons may at least partially account for the regenerative sprouting and synaptic reorganization observed following lesions of these systems. To determine whether GAP-43 indeed modulates neurotransmitter release not only in PC12 cells, but also in neurons, we have placed the sense and antisense GAP-43 cDNAs in a defective HSV-1 vector (*see below*) for stable expression in sympathetic and cortical neurons.

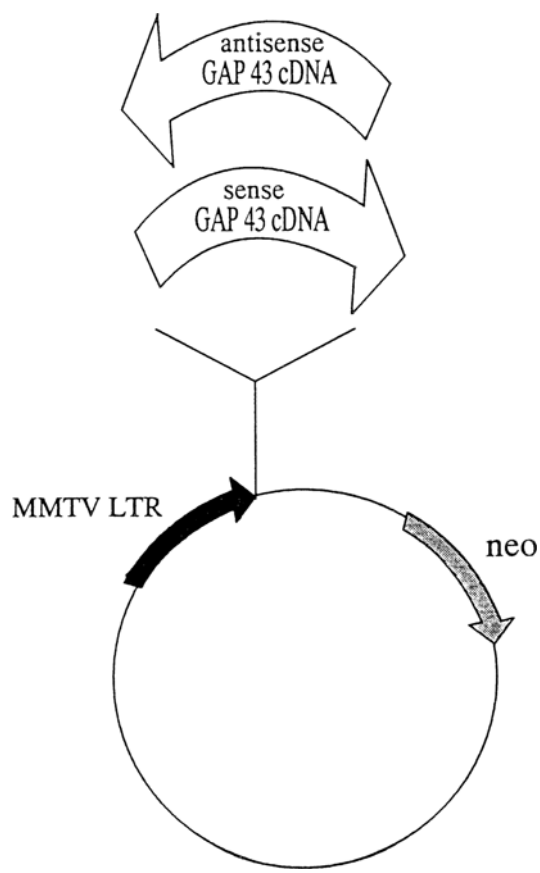

Fig. 3. Illustration of the scheme for constructing sense and antisense GAP-43 cDNA recombinants for expression in PC12 cells.

Use of an HSV-I Vector to Manipulate the Expression of GAP-43 in Neurons

We have developed a defective HSV-1 vector system to introduce genes into neurons (Freese et al., 1990). In the prototype vector pHSVlac (Geller and Breakefield, 1988; Geller and Freese, 1990), the *LacZ* gene is under the control of the HSV-1 immediate early (IE) 4/5 promoter, a constitutive promoter. pHSVlac stably expresses β-galactosidase for at least 2 wk in cultured rat peripheral (Geller and Breakefield, 1988) or central (Geller and Freese, 1990) nervous system neurons. β-Galactosidase in pHSVlac can be replaced with any cloned cDNA. Thus, stable modification of gene expression in neurons can be attained by introducing exogenous genes into these nondividing cells by infection with a recombinant defective HSV-1 virus. We are using this system to identify molecular domains of GAP-43 and to study its role in neurotransmitter release.

Fusion of the GAP-43 Aminoterminus to Recombinant Proteins to Target Proteins to Neuronal Processes

GAP-43 is thought to be attached to the growth cone membrane via fatty acylation of the protein's only two cysteine residues (Skene and Virag, 1989). These cysteines (residues three and four) are found within a ten-amino acid domain at the amino terminus of the molecule (Fig. 1). To determine whether this domain alone is capable of transporting a protein to the neuronal process membrane, and to develop a method for targeting molecules to the neuronal growth cone, we constructed a chimeric clone in which the first ten amino acids of human GAP-43 were fused to β-galactoidase (GAPlac) in the defective HSV-1 (Geller and Breakefield, 1988) vector (Neve et al., 1990). PC12 cells were infected with GAPlac virus and with pHSVlac virus, which expresses the unmodified s-galactosidase. β-Galactosidase immunoreactivity was detected predominantly in the cell body of PC12 cells infected with pHSVlac virus (Fig. 4A), whereas it was detected prominently in the cell processes as well as the soma of cells infected with pHSVGAPlac virus (Fig. 4B). When these recombinant viruses were used to infect primary cultures of rat superior cervical ganglion (SCG) cells, β-galactosidase activity was observed primarily in the cell body of neurons infected with pHSVlac, whereas it was seen in both processes and soma of SCG neurons infected with pHSVGAPlac. These results suggest that the first ten amino acids of GAP-43 are sufficient to direct the protein to the cell membrane and provide a means of targeting recombinant molecules to the neuronal growth cone. Liu and Storm (1991) reported that fusion proteins carrying the entire GAP-43 sequence, but not those carrying the aminoterminal ten amino adds were transported to growth cones; however, their data were based on transient transfections with an expression vector that is not stably expressed in neurons.

Future Directions: Expression of Unregulated Signal Transduction Enzymes in HSV-1 and Analysis of Their Interaction with GAP-43 in Mechanisms of Neurotransmitter Release

To study the interaction of GAP-43 with specific signal transduction pathways during neurotransmitter release, we will use a strategy that we have developed to express unregulated signal transduction enzymes in neuronal cell lines and in primary neurons (Geller, Durling, et al., 1991). Signal transduction enzymes are a particularly attractive target for genetic manipulation and analysis of synaptic function, since signal transduction pathways play critical roles in effecting both transient and stable changes in invertebrate neuronal physiology and short-term changes in mammalian physiology. Many signal transduction enzymes are composed of a domain structure that lends itself to mutational analysis. Some enzymes involved in cyclic nucleotide metabolism contain such a domain structure: For

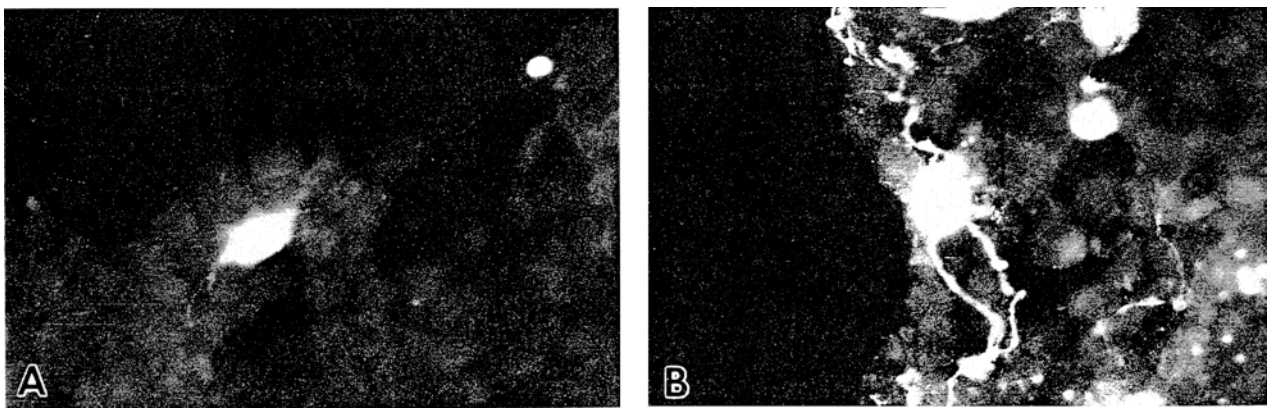

Fig. 4. **A** and **B**, β-galactosidase immunoreactivity in PC12 cells stably expressing pHSVlac (**A**) or pHSVGAPlac (**B**).

example, the yeast adenylate cyclase (cyr) has distinct catalytic and regulatory domains (Kataoka et al., 1985). We have expressed the catalytic domain alone in mammalian neurons with a HSV-1 vector, and have shown that it results in dominant positive unregulated activity, thereby increasing cAMP levels in the cell (Geller et al., 1990, in press). Expression of the yeast adenylate cyclase catalytic domain in neurons has specific effects on neuronal function: we observed not only increased cAMP levels in the neuron, but also stimulation of protein phosphorylation and increased spontaneous neurotransmitter release. These changes in neuronal physiology are stable for at least 1 wk.

We have expressed additional unregulated signal transduction enzymes in neurons from the HSV-1 vector (Geller, Bryan, et al., 1991). We inserted a cDNA encoding the rat protein kinase C βII (PKC βII) catalytic domain into pHSV; preliminary data indicate that stable expression of this recombinant in cultured neurons resulted in increased evoked monoamine or excitatory amino acid neurotransmitter release from sympathetic or cortical neurons, respectively, relative to controls. The increase in release required calcium. The catalytic domain of rat Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) expressed from the HSV-1 vector also caused a long-term, activity-dependent increase in neurotransmitter release, but of smaller magnitude.

We now have the potential to begin to analyze some of the events involved in neurotransmitter release with respect to the involvement of GAP-43. An example is illustrated in Fig. 5. It has been hypothesized that GAP-43 may act as a local calmodulin store in vivo, based on both in vitro (Andreasen et al., 1983; Alexander et al., 1987, 1988) and in vivo (De Graan et al., 1990) evidence that it binds calmodulin with a higher affinity in the absence of Ca^{2+} than in its presence. Perhaps GAP-43 holds calmodulin locally at the active zone for neurotransmitter release, so that intracellular rises in Ca^{2+} induced by depolarization, for example, will cause GAP-43 to be dissociated from calmodulin, which is then available for Ca^{2+}/calmodulin-dependent enzymes involved in neurotransmitter release. If such a scenario is correct, then the absence of GAP-43 in antisense GAP-43-transfected cells implies an absence of local stores of calmodulin at the site of neurotransmitter release. Infection of antisense GAP-43-transfected cells with recombinant HSV-1 expressing unregulated CaM kinase II, which is independent of regulation by calmodulin, might be expected to restore neurotransmitter release. Although speculative, such hypotheses and scenarios provide exciting possibilities for the dissection of the role of GAP-43 in neuronal physiology.

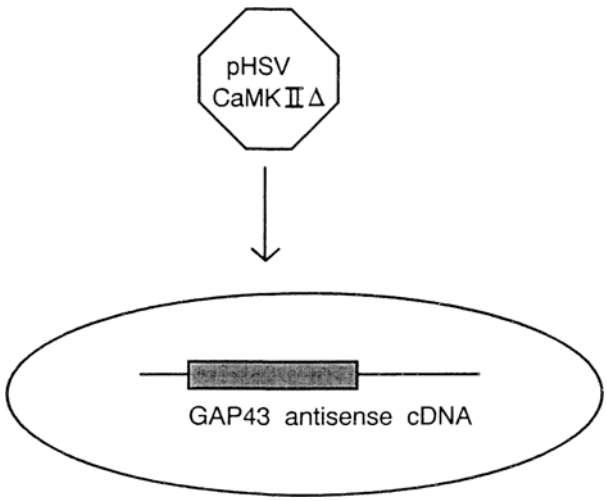

Fig. 5. Scheme for introducing a recombinant HSV-1 virus expresssing unregulated CaM kinase II (pHSVCaMKIIΔ) into antisense GAP-43-transfected cells.

Acknowledgments

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