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Labeling Neural Cells Using Adenoviral Gene Transfer of Membrane-Targeted GFP

Neurotechnique

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Summary

We describe an experimental system to visualize the soma and processes of mammalian neurons and glia in living and fixed preparations by using a recombinant adenovirus vector to transfer the jellyfish green fluorescent protein (GFP) into postmitotic neural cells both in vitro and in vivo. We have introduced several modifications of GFP that enhance its fluorescence intensity in mammalian axons and dendrites. This method should be useful for studying the dynamic processes of cell migration and the development of neuronal connections, as well as for analyzing the function of exogenous genes introduced into cells using the adenovirus vector.

Introduction

A number of studies on the development and maturation of neuronal circuits have been performed using tracers such as horseradish peroxidase and lipophilic fluorescent dyes (O'Leary and Koester, 1993). These techniques have limitations for the analysis of the dynamic changes occurring during the development of neuronal networks or the molecular mechanisms underlying the formation of mature neuronal circuits. Recently, a bioluminescent protein, green fluorescent protein (GFP), originally isolated from the jellyfish Aequorea victoria, has been shown to have ideal characteristics for use as an expression marker in living cells (Chalfie et al., 1994). GFP emits green light when exposed to blue light without the use of a substrate and may thus be viewed in living or fixed tissue using a fluorescence microscope and a standard fluorescein filter. GFP is also a relatively small protein of 238 amino acids, making it especially useful as a marker for other gene products, whether used as a fusion construct or in a dicistronic expression vector.

The aim of this study was to develop a recombinant adenovirus to express efficiently an exogenous GFP gene product in postmitotic mammalian neural cells and to trace axons and dendrites arising from the infected neurons. Here we describe the successful expression of GFP in cortical neurons and glia by adenovirus-mediated gene transfer in vitro and in vivo, and the effects of some modifications of GFP that extend its usefulness in the mammalian nervous system to study mechanisms of normal neural development and plasticity.

Results

Efficient Gene Transfer of GFP into Neurons

The structures of recombinant adenoviruses containing the wild-type and modified GFP proteins are schematically illustrated in Figure 1. Infection of the recombinant adenovirus carrying the wild-type GFP cDNA under the control of the strong and ubiquitous CAG promoter (Niwa et al., 1991; the virus clone AdV-CA-GFP) resulted in a green fluorescence in COS cells (Figure 1A). The fluorescence was preferentially localized to the nucleus, which could be detected as early as 12 hr after infection, and continuously increased up to 48 hr after infection. The fluorescence was stable and resistant to formaldehyde fixation, as reported previously (Chalfie et al., 1994). However, when the AdV-CA-GFP virus was infected into cultured cortical neurons, the fluorescence of GFP was faint even 72 hr after infection, probably due to either a less efficient infection of the adenovirus vector in cortical neurons or a relatively low rate of protein synthesis in neurons compared with COS cells.

In an effort to increase the intensity of the GFP fluorescence, we synthesized fusion constructs of two GFP cDNAs in tandem, either fused directly or separated by linker sequences, to enable proper folding of the protein. However, when transiently transfected into COS cells, none of these constructs produced a visually brighter fluorescence than the single wild-type GFP (data not shown). In addition, we introduced mutations into the serine 65 residue of the GFP chromophore that were recently reported to enhance the fluorescence intensity of GFP (Heim et al., 1995). We constructed adenovirus vectors containing either of two mutant GFP sequences that replaced serine 65 with either alanine or threonine, referred to as AdV-CA-GFP(S65A) and AdV-CA-GFP(S65T), respectively (Figure 1). When the AdV-CA-GFP(S65A) virus was infected into COS cells, the resulting GFP fluorescence was significantly brighter than that of the wild-type GFP (Figures 1A and 1B). In contrast to the report by Heim et al. (1995), however, GFP(S65T) did not show a significant increase in the fluorescence intensity (Figure 1C). As with wild-type GFP, both mutant GFPs accumulated in the nucleus of COS cells (Figures 1B and 1C). In dissociated cortical neurons, the GFP(S65A) fluorescence was easily detected 24 hr after infection and was mainly localized in cell bodies and proximal neurites (Figure 2A). In neurons, a 10-fold increase in virus titer was required to induce a GFP fluorescence intensity comparable to that observed in COS cells. At a multiplicity of infection of 100, the AdV-CA-GFP(S65A) virus induced GFP expression in >90% of neurons without detectable toxicity (Figure 2A).

Fusion of a Membrane-Anchoring Signal to GFP Targets GFP to Neuronal Processes

Although we could detect GFP expression in cortical neurons, neuronal processes were visible only in a minority of cells expressing higher overall levels of GFP

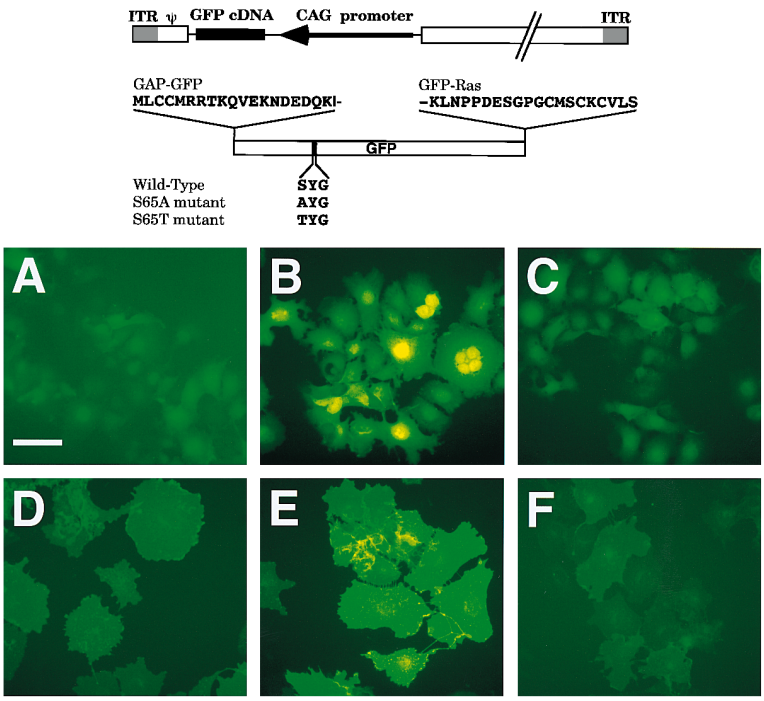

Figure 1. COS Cells Expressing Various GFP Constructs

The recombinant adenovirus and various GFP constructs are indicated above the GFP fluorescence analysis. The schematic diagram of the structure of the recombinant adenovirus shows the relative positions of the inverted terminal repeat (ITR) on both ends of the adenovirus genome, the packaging signal (ψ), the GFP cDNA (black box), the CAG promoter (arrow), and the adenovirus genome (white box). The membrane-anchoring signal sequence of either GAP-43 or Ras attached to GFP and point mutations introduced into the chromophore are shown above and below the schematic diagram of the GFP structure, respectively. COS cells were infected with a recombinant adenovirus carrying one of the various GFP constructs at a multiplicity of infection of 20. Cells were fixed 48 hr after infection, and photographs were taken and printed with the same exposure time. The panels show GFP (A), GFP(S65A) (B), GFP(S65T) (C), GAP-GFP (D), GAP-GFP-(S65A) (E), and GAP-GFP(S65T) (F). Bar, 100 μm.

(Figure 2A). To visualize GFP more efficiently in axonal and dendritic processes, we made a modification that targeted GFP to the plasma membrane rather than to the nucleus and cytoplasm. Growth-associated protein-43 (GAP-43) and c-Ha-Ras are known to contain the ~20 amino acid sequences that serve as a signal for targeting these proteins to the plasma membrane (Aronheim et al., 1994; Liu et al., 1994). We made two fusion GFP constructs, referred to as AdV-CA-GAP-GFP and AdV-CA-GFP-Ras, in which the membrane anchoring signals of GAP-43 and c-Ha-Ras, respectively, were fused to GFP (Figure 1). We made adenovirus vectors carrying these fusion constructs in combination with the serine 65 mutation.

In COS cells, the GAP-GFP constructs were largely expressed in association with the plasma membrane (see Figures 1D–1F). Fine cellular details, such as pseudopodial-like processes, were clearly visible in COS cells expressing these GFP constructs. The expression of the fusion GFP constructs had no apparent effect on cell shape or cell viability. The GFP(S65A) mutant produced a much brighter fluorescence than the wild-type GFP, regardless of the presence of the membrane-anchoring signal. Results obtained with the AdV-CA-GFP-Ras virus were similar to those observed for the GAP fusion constructs.

We next examined the ability of the membrane-anchoring signal to distribute GFP in neuronal processes. When cortical neurons in primary cultures were infected with either AdV-CA-GFP(S65A) or AdV-CA-GAP-GFP(S65A), GFP fluorescence was easily detected within 24 hr, reaching a peak intensity 48–72 hr after infection. The fusion of the GAP-43 sequence greatly enhanced the visibility of the GFP fluorescence in neuronal processes (Figures 2B, 2C, and 2E). The GFP(S65A) without a membrane-anchoring signal, however, was mainly located within the nucleus and soma (Figure 2A). Immunostaining of the same GFP-expressing cells with the neuronal dendrite marker microtubule-associated protein 2 (MAP2; Pennypacker et al., 1991) (Figure 2D) or the axonal marker, phosphorylated neurofilament (Pennypacker et al., 1991) (Figure 2F) showed that GAP-GFP(S65A) was distributed in both dendritic and axonal processes. Within axons, GFP fluorescence was highest in the growth cone (Figures 2G and 2H, arrow). Fine processes, such as filopodia extended from the axonal growth cone, were well labeled with GAP-GFP(S65A) (Figure 2G). Results obtained with the AdV-CA-GFP-(S65A)-Ras virus were similar to those described here for GAP-GFP(S65A).

Figures 2I and 2J show an elongating GFP-labeled axonal process by video imaging GAP-GFP(S65A)-labeled neurons at multiple time points 2 days postinfection using low light levels and a silicon-intensified target (SIT) camera. Labeling was sufficient to visualize changes in growth cone morphologies (compare Figures 2I and 2J). The infection of these cells with the adenovirus and the expression of GAP-GFP did not appear to impede cellular growth, indicating that GAP-GFP can be used as a marker for studying process extension by living neurons.

GFP Fluorescence in Living Slice Preparations

To assess further the utility of these constructs, we tested the possibility of infecting cells in living brain slices. The AdV-CA-GAP-GFP(S65A) virus was injected into a limited portion of the CA3 region of the pyramidal cell layer of an organotypic slice culture of the postnatal day 6 (P6) rat hippocampus. GFP labeling was assessed 3 days after infection. The green fluorescence at the

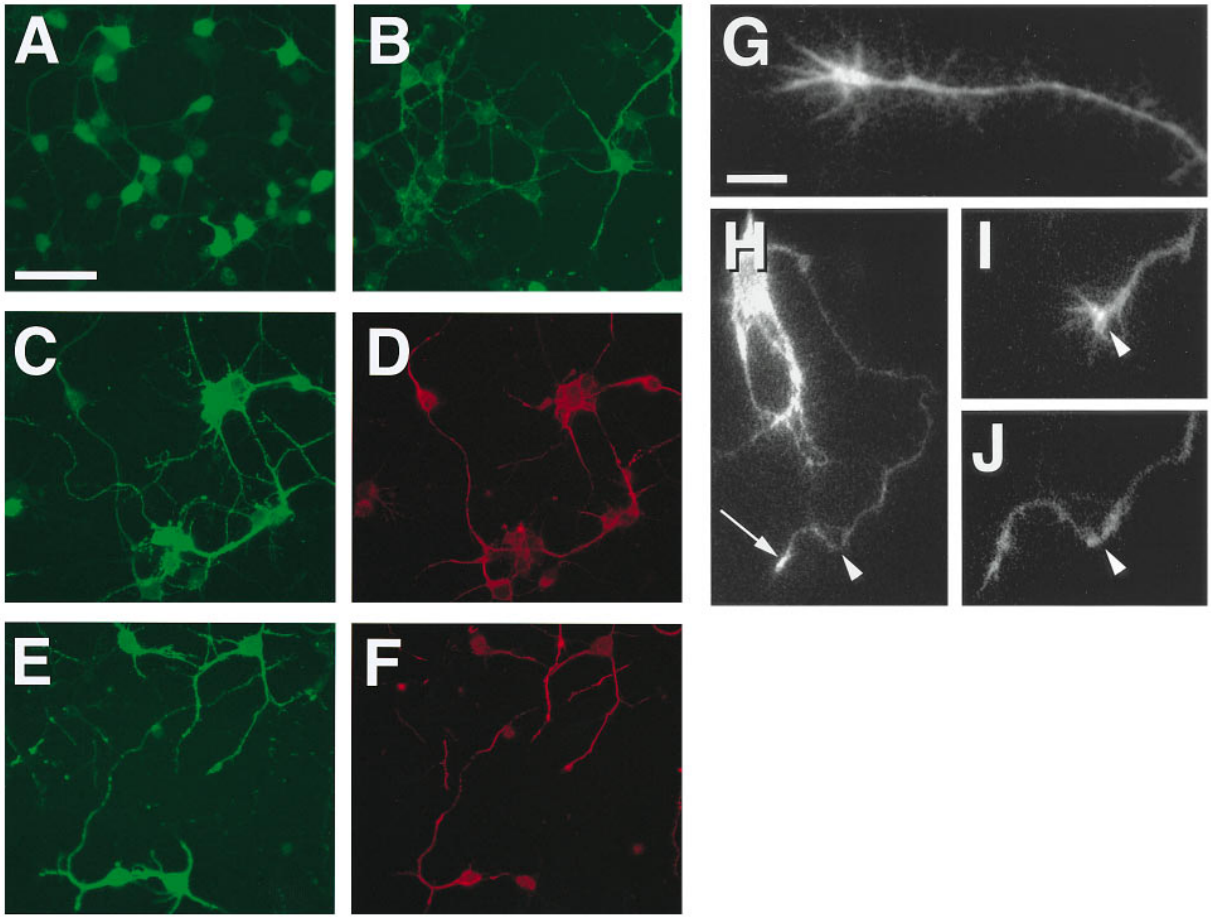

Figure 2. Modified GFP Expressed in Cortical Neurons

(A–F) Dissociated cultures of rat cortical neurons were infected with recombinant adenoviruses carrying GFP(S65A) (A) or GAP-GFP(S65A) (B–F) at a multiplicity of infection of 100. Cells were fixed 3 days after infection and immunostained with the anti-MAP2 (C and D) or anti-phosphorylated neurofilament (E and F) antibody. GFP and immunostaining were visualized under a fluorescence microscope using a fluorescein filter (A–C and E) and a rhodamine filter (D and F), respectively.

(G–J) Dissociated cortical neurons were infected with the GAP-GFP(S65A) recombinant adenovirus at the time of plating and then imaged with a SIT camera 2 days later. (G and H) GFP was present throughout the infected cells, including their growth cones (arrow) and filopodia emanating from the growth cones. (H–J) The video images showing axon extension. The images in (H) and (J) were taken 1 hr after that in (I). The arrowheads mark the same point in (H)–(J). Bar in (A), 50 μm for (A)–(F); bar in (G), 10 μm for (G), 30 μm for (H), and 15 μm for (I) and (J).

injection site was easily visible even at a low magnification, but the density of intensely labeled cells precluded the identification of individual fluorescence-positive cells (data not shown). Many of the infected cells were likely to be glia since infection studies in primary cultures indicated that glia are more susceptible to adenoviral infection than neurons, and that GFP fluorescence is stronger in glia than in neurons (data not shown). However, at higher magnification of infected slices, apical dendrites and axons were clearly visualized as fluorescently labeled long processes emanating from the virus-injected region (Figures 3A–3C), which were easily distinguished from the background in living and fixed slice preparations, and the intensity was unchanged throughout the length of the processes. In some cases, the tips of the labeled processes were more brightly fluorescent (Figure 3C, arrows), which probably correspond to an accumulation of GFP in the growth cone, as we observed in dissociated cortical neurons (see Figure 2G).

Injections of the Adenovirus-Expressing GFP In Vivo

To determine whether the adenovirus/GFP system can be used to label cells in vivo, we made injections of the AdV-CA-GAP-GFP(S65A) virus into the neocortex of early postnatal rats, well after the completion of cortical neurogenesis (Bayer and Altman, 1991). As observed in vitro, GFP expression was high at 2 days postinjection (Figure 4). Small injections made into the cortical gray matter labeled discrete groups of cells near the injection site (Figure 4A) confined to the cortical layers intensely stained with the neuron-specific marker MAP2 (Figure 4B). At higher magnification, individual cells could be clearly resolved, and GFP fluorescence was distributed in both the cell body and processes (Figure 4C). Multiple, larger injections infected larger regions of the cortex, but the zone of GFP expression remained relatively confined (Figure 4E). Although many of the GFP-labeled cells had morphologies that closely resembled those of cortical

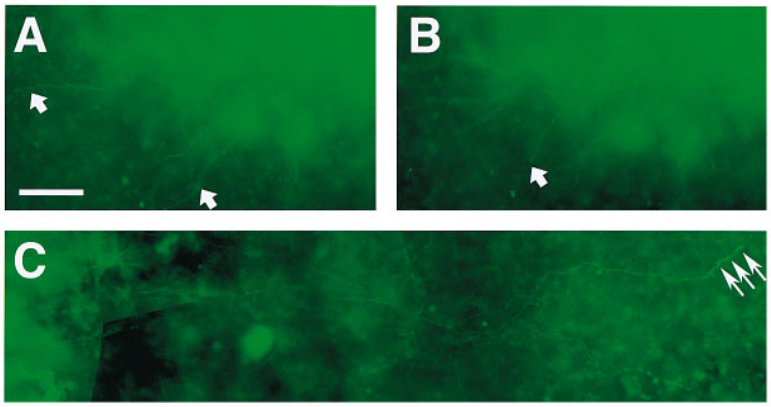

Figure 3. Neuronal Processes Labeled with the Modified GFP

The GAP-GFP(S65A) recombinant virus was injected into the pyramidal cell layer of the CA3 region of the hippocampal slice culture. (A and B) Several long processes were clearly visible at the stratum radiatum (thick arrows) next to the injection site. (A) and (B) were taken from the same visual field at different focal planes. (C) Another example indicating the accumulation of GFP fluorescence at the tip of an axon (arrows). The images shown were taken after fixation for photographic presentation, but images were unchanged before and after fixation. Bar, 100 μm.

neurons (e.g., Figure 4D), their definitive identification as neurons was not possible due to the high density of MAP2-positive cells and processes. We therefore utilized the neuron-specific nuclear marker NeuN (Mullens et al., 1992) to show definitively the localization of GFP in cortical neurons (Figures 4F–4H). In addition, a proportion of the GFP-labeled cells could be identified as glia. The GFP-labeled processes and cell bodies of some cells infected with virus injected into the cortical gray matter (Figures 4I and 4J) were colabeled with the

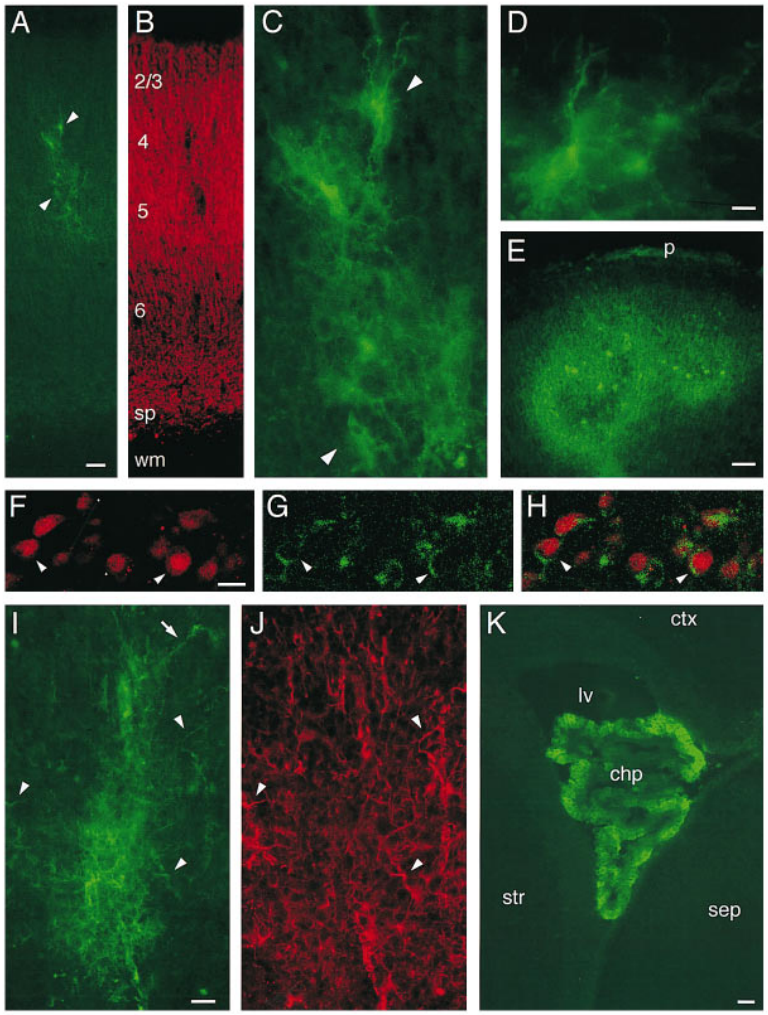

Figure 4. In Vivo Labeling with GFP Recombinant Adenovirus

Injections of the GAP-GFP(S65A) recombinant virus were made in P1 rats perfused at P3.

(A) Small, localized injection of the virus labeled a discrete cluster of cortical cells in the cortical plate.

(B) Immunostaining of the same section shown in (A) with the neuron-specific marker MAP2. Numbers refer to cortical layers.

(C) Magnified region of (A) showing individual cell labeling (same cells are marked with arrowheads in [A] and [C]).

(D) Higher magnification of a cortical cell labeled with GFP.

(E) Multiple injections of larger volumes of the virus-labeled cells over a greater extent of the cortex.

(F–H) Confocal images of a section through the cortical subplate of a rat infected with GAP-GFP(S65A). (F) Neurons identified by immunostaining with the neuron-specific nuclear marker NeuN. (G) Same field as in (F), showing GFP-labeled cells. (H) Overlaid confocal images presented in (F) and (G) to show GFP/NeuN colocalization. Some of the cells double-labeled with NeuN and GFP are marked with arrowheads in (F)–(H).

(I) GFP-labeled cells in the cortical plate.

(J) The same section as in (I) was immunostained for GFAP, a marker for astrocytes. Some of the GFP-labeled processes were colabeled with GFAP (I and J, arrowheads), while others were GFAP negative (I, arrow).

(K) GFP-labeled cells of the choroid plexus infected with the virus injected into the lateral ventricle.

Bar in (A), 50 μm for (A) and (B), 10 μm for (C); bar in (D), 10 μm; bar in (E), 100 μm; bar in (F), 20 μm for (F)–(H); bar in (I), 25 μm for (I) and (J); bar in (K), 50 μm.

ctx, cortex; chp, choroid plexus; lv, lateral ventricle; p, pia; sep, septum; sp, subplate; str, striatum; wm, white matter.

astrocyte-specific marker glial fibrillary acidic protein (GFAP). The density of cells within the cortex precluded a definitive quantitation of the percentage of GFP-labeled cells that were neurons or glia. Although a sizable proportion of infected cells were neurons, the majority were likely to be glia. Deeper injections into the lateral ventricle beneath the cortex infected cells of the choroid plexus, which displayed intense GFP fluorescence (Figure 4K).

Discussion

We have utilized a replication-defective recombinant adenovirus as a vector for the expression of wild-type and modified forms of the bioluminescent GFP in neural cells. As first described by Chalfie et al. (1994), GFP has great potential as an expression marker and will prove to be an invaluable tool in many fields of neurobiology (Lo et al., 1994; Marshall et al., 1995; Wu et al., 1995). However, in contrast to the report of Marshall et al. (1995), which showed the cytoplasmic distribution of GFP in HEK 293 cells, our findings indicate that the adenovirus-mediated, unmodified GFP protein is mainly confined to the cell bodies, especially to the cell nucleus, in both COS cells and neural cells. We therefore engineered several modifications in the GFP coding sequence to expand its usefulness as a research tool for the mammalian nervous system. After the reports by Heim et al. (1994, 1995), we introduced two different point mutations into the GFP chromophore to enhance its fluorescence intensity in mammalian cells. However, the GFP(S65T) mutant showed little improvement in fluorescence intensity over wild-type GFP. This discrepancy between our finding and the report by Heim et al. (1995) may be due to differences used for the analysis of GFP fluorescence (the GFP protein purified from its expressing E. coli extracts and the GFP product in mammalian cells) or to differences in the terminal amino acid sequences used for the GFP constructs. In contrast to GFP(S65T), the GFP(S65A) mutant showed much brighter fluorescence than the wild-type GFP in COS cells and neural cells and greatly improved the visualization of GFP in vivo. We also constructed GFP fusion proteins with the membrane-anchoring signals. This modification targeted GFP to the cell membrane, distributed GFP throughout the cell, and allowed the visualization of fine cellular processes. These modifications of GFP expand its usefulness, especially as a marker for cell movement or growth in the developing and mature mammalian nervous system.

Several properties of the adenovirus used here make it an excellent vector for the expression of GFP. In addition to its ability to infect differentiated neurons, an advantage of adenovirus over commonly used retrovirus vectors is that a high titer of replication-incompetent virus can be readily obtained. The replication-defective adenovirus is highly infectious, able to infect a wide host range (Le Gal La Salle et al., 1993), and virtually free of virally expressed proteins because it lacks the E1 region (Becker et al., 1994). Transcription from an ectopic promoter in the adenovirus vector also occurs using the host cell machinery, and it will be possible to restrict the expression of GFP and other genes to specific brain regions or cell types by selecting a tissue- or cell-specific promoter. Furthermore, since the membrane-anchoring signals used here consist of 20 amino acids and GFP is only ~0.7 kb, the ~8 kb capacity of the adenovirus vector still allows for the addition of large gene inserts. Thus, it is possible to express simultaneously both the cDNA encoding for the modified GFP and another gene of interest, to analyze its function in the developing or mature mammalian nervous system.

Experimental Procedures

Construction of the Recombinant GFP Adenoviruses

The adenovirus shuttle vector plasmid pAdV was constructed by inserting two DNA fragments corresponding to base numbers 1–457 and 3328–8919 of the adenovirus type 5 genome (Chroboczek et al., 1992) into the modified pBluescript II (Stratagene) plasmid. The E1 region was missing in this construct, and the resultant recombinant viruses were replication defective and free from expression of the viral proteins in COS cells and neuronal cells. The whole coding region of GFP was amplified by PCR using the plasmid pGFP10.1 (Chalfie et al., 1994) as a template. The primer sequences used were CGGAATTCTCGCCACCATGGGGATCCTGAGTAAAGGAGAA and CGGAATTCTTAATCGATTTTGTATAGTTCATC. The former contains the BamHI site followed by the sequence encoding Kozak's consensus sequence (Kozak, 1987), while the latter possesses the ClaI site. As a consequence of introducing these restriction sites, glycine–isoleucine–leucine was contained in the N-terminus, following the initiation methionine, and isoleucine–aspartic acid was contained in the C-terminus of GFP. GFP tandem repeats were generated by subcloning and constructed as direct fusion proteins or separated by either a short (GGSGGS) or a longer (GGSGGSLVPRGSGGSGGS) linker sequence. GAP-GFP and GFP-Ras were constructed by inserting the oligonucleotides corresponding to the membrane-anchoring signals of GAP-43 (Liu et al., 1994) at the N-terminus and c-Ha-Ras (Aronheim et al., 1994) at the C-terminus of GFP (Figure 1). Mutants at the position of serine 65 of GFP were constructed by the PCR method. In the course of the construction of these mutants, an unexpected mutation that replaced aspartic acid 76 with glutamic acid was introduced, but this mutation had no effect on the fluorescence intensity of GFP. All these constructs were inserted into the adenovirus shuttle vector pAdV under the control of the CAG promoter (Niwa et al., 1991).

HEK 293 cells (ATCC CRL 1573) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Recombinant viruses were obtained in HEK 293 cells by homologous recombination between the linearized pAdV and the right part of the viral genomic DNA, which lacks the E3 region. Adenoviral genomic DNA was prepared by the modified Hirt's method (Hirt, 1967) and purified by CsCl density gradient centrifugation. The linearized pAdV (up to 1 μg) and the viral genome fragment (0.2 μg) were cotransfected into subconfluent HEK 293 cells on 6-well plates using LipofectAMINE (GIBCO). The recombinant viruses were amplified, and either the supernatant from infected HEK 293 cells or a CsCl-purified viral preparation (Kanegae et al., 1994) was used as a source of the infectious viruses. A viral titer was determined by the standard end-point cytopathic effect assay (Precious and Russell, 1985).

Optics and Image Preparation

GFP fluorescence was visualized using a Nikon fluorescence microscope with filter set B-2E (EX 450–490, DM 505, BA 525–560) and a 20× or 40× objective lens (NA 0.75); an Olympus fluorescence microscope with filter set #32000 (EX 425–460, DM 480, BA 505–540) and a 40× objective lens (NA 1.0); a Zeiss laser confocal microscope (EX 488, EM 515–560) with a 63× objective lens (NA 1.25); or a Bio-Rad MRC 1000 confocal microscope (Hemel Hempstead, UK) equipped with a krypton/argon laser (EX 488, EM 522–532 for GFP; EX 568, EM 605–632 for NeuN) and coupled to a Zeiss Axiovert 135M microscope with a 63× oil, DIC objective (NA 1.40). Figures

were assembled using Adobe photoshop 3.0 and printed using a Fujix pictography 3000 digital photographic printer.

Primary Cortical Cell Cultures

Embryos were obtained from Sprague–Dawley rats between gestational days 15 and 18. The cerebral cortex was isolated, dissociated, plated on poly-D-lysine-coated glass coverslips (Matsunami) and cultured in DMEM supplemented with 10% fetal bovine serum. Cultures were infected with the virus either at the time of plating or up to 3 days after plating. GFP fluorescence was observed in living cells or after fixation with 4% paraformaldehyde in phosphate-buffered saline for 5 min at room temperature. Immunostaining was performed by incubating fixed cells with anti-MAP2 (Sigma) or anti-phosphorylated neurofilament (SMI) monoclonal antibody, followed by the subsequent staining with the Texas red–conjugated anti-mouse secondary antibody (Cappel). Texas red fluorescence was visualized using either a fluorescence microscope (Nikon, Olympus) or a laser confocal microscope (Zeiss). For video imaging, cortical cells prepared from P1 rats were dissociated and plated at 1×10^5 cells/ml onto poly-D-L—lysine-coated, 35 mm glass bottom microwells (MatTek Corp.) and infected with 5–10 μl of virus ($\sim 10^9$ PFU/ml). Two days after infection, the cultures were placed in an incubation box mounted on an inverted fluorescence microscope (Nikon Optophot) for video imaging. Cells were located using phase-contrast microscopy, and video imaging was performed under low light level conditions using a SIT camera (Hamamatsu). To minimize photo damage to GFP-labeled cells, neutral density filters and an electronic shutter (Uniblitz) were placed in the light path. The shutter was controlled by Image-1 software (Universal Imaging Corp.) to open for about 200 ms. Eight frames per image were captured and averaged at 1 hr intervals over periods ranging from 1 to 12 hr.

Slice Cultures

Hippocampal organotypic slice cultures were made according to the procedure of Stoppini et al. (1991). In brief, the hippocampus was dissected from P6 rats and cut to 350 μm slices by a McIlwain tissue chopper. Slices were placed on a porous membrane (Millipore, Millicell-CM) and cultured using standard procedures. A virus solution ($\sim 10^6$ PFU) was injected into slices, which were then analyzed 3 days after infection.

In Vivo Experiments

P1 rats were anesthetized with hypothermia. The skin overlying the skull was opened, and a fine glass micropipette was inserted through the skull and into the cortex. Closely or distantly spaced injections of $\sim 10^6$ PFU were made into the cortex using a picospritzer. The skin was then sutured and the pups were allowed to recover. Two days later, they were perfused with 4% paraformaldehyde. Brains were placed in fixative overnight, equilibrated in a 30% solution of sucrose in phosphate-buffered saline, and then sectioned on a freezing microtome at 40 μm. The sections were stained with anti-MAP2 (Boehringer-Mannheim), anti-NeuN (a gift from R. Mullens), or anti-GFAP (Chemicon) monoclonal antibodies, followed by either a rhodamine-conjugated anti-mouse secondary antibody (Jackson Immunoresearch) for MAP2 or GFAP labeling, or a biotinylated donkey anti-mouse secondary antibody (Jackson Immunoresearch) and then a Texas red–conjugated streptavidin (Jackson Immunoresearch) for NeuN labeling.

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