Effective expression of the green fluorescent fusion proteins in cultured *Aplysia* neurons

I. Sahly¹, H. Erez, A. Khoutorsky, E. Shapira, Micha E. Spira^{*}

Department of Neurobiology, Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

Received 13 January 2003; received in revised form 14 March 2003; accepted 14 March 2003

Abstract

The green fluorescent fusion protein and its isoforms are extensively used to monitor gene expression, protein localisation and their dynamics in relations to fundamental cellular processes. However, it has not yet been effectively applied to *Aplysia* neurons that serve as a powerful model to study the mechanisms underlying neuroplasticity. We report here the development of a procedure combining in vitro transcription of mRNA encoding fluorescent-tagged proteins and its subsequent injection into the cytoplasm to image, in real-time, protein dynamics in cultured *Aplysia* neurones. To illustrate the efficiency of the procedure we report here the visualisation of actin, microtubules and vesicle trafficking. The results presented here introduce a reliable and effective method to express green fluorescent protein (GFP) fusion proteins in cultured *Aplysia* neurons.

© 2003 Elsevier Science B.V. All rights reserved.

Keywords: Green fluorescent protein; GFP; mRNA; Actin; Tubulin; *Aplysia* neurons

1. Introduction

The green fluorescent protein (GFP) emits green light when excited by blue light without the use of a substrate and may thus be viewed in living or fixed tissue (Lippincott and Smith, 1997; Tsien, 1998; Baumann and Reyes, 1999; Chamberlain and Hahn, 2000). Since its discovery and isolation from the jellyfish *Aequorea victoria* (Chalfie et al., 1994), it has been efficiently used to image gene expression, to monitor cellular protein localisation and its dynamics (for review Stearns, 1995; Gerdes and Kaether, 1996; Day et al., 2001; Van Roessel and Brand, 2002). The growing family of fluorescent protein variants enables more sophisticated studies of protein function within living cells (Ellenberg et al., 1999; Periasamy and Day, 1999; Day et al., 2001).

GFP fusion proteins have been used in neurobiological studies as a tracer for individual neurons during development of neuronal networks and as markers

for proteins distribution within single neurons in relation to various physiological functions (Spergel et al., 2001).

Aplysia neurons have been extensively used to study the biophysical, cellular and molecular mechanisms that underlie simple forms of behaviour, and in particular the mechanisms underlying short and long-term neuronal plasticity (for review see the Nobel lecture of Kandel, 2001a,b). In addition, cultured *Aplysia* neurons are extensively used to study the mechanisms of growth cones motility, navigation, regeneration and synaptogenesis (for review see Spira et al., 1996, 2001, 2003; Suter and Forscher, 2000). The relationship between these processes and protein dynamics is mainly based on retrospective immunolabelling of various proteins, microinjections of fluorescent probes and electron microscopy (for example see Bailey and Kandel, 1993; Gitler and Spira, 1998, 2002; Spira et al., 2001; Scheafer et al., 2002).

The studies performed using *Aplysia* to elucidate fundamental mechanisms underlying neuroplasticity could be significantly facilitated by the use of GFP fusion proteins. Nevertheless, attempts to express GFP fusion proteins in *Aplysia* neurons were only partially successful. In 1996, Kaang introduced the gene encoding

^{*} Corresponding author. Tel.: +972-2-658-5091; fax: +972-2-563-7033.

E-mail address: spira@cc.huji.ac.il (M.E. Spira).

¹ Present address: Institute of Biology, College de France, 11 place Marcelin Berthelot, 75231 Paris Cedex 5, France.

GFP using a pNEX derivative vector (Kaang, 1996a,b). The pNEX3 vector was reported as a plasmid fostering the most efficient expression of β -galactosidase from the *lacZ* gene in *Aplysia* neurons. However, the rate of successful expression of GFP following DNA injection of pNEX3-GFP plasmid was low, approximately 20% (Kim and Kaang, 1998). Furthermore, no significant fluorescent signal was detected in the axon or in the neurites.

Here we report the development of an effective procedure for the expression of EGFP-tagged protein in *Aplysia* neurones. The method consists of in vitro transcription of mRNA encoding EGFP-fusion proteins and its subsequent intracellular microinjection into the cytoplasm. On-line confocal imaging of EGFP tagged actin, tubulin and EGFP-labelled vesicle used to demonstrate the efficiency of the method.

2. Materials and methods

2.1. Solutions

2.1.1. L-15 supplemented for marine species (msL-15)

Leibovitz's L-15 Medium (GibcoBRL, Paisley, Scotland) was supplemented for marine species according to Schacher and Proshansky (1983) by the addition of 12.5 g/l NaCl, 6.86 g/l D(+) Glucose·H₂O, 3.15 g/l anhydrous MgSO₄, 344 mg/l KCl, 192 mg/l NaHCO₃, 5.7 g/l MgCl₂·6H₂O and 1.49 g/l CaCl₂·2H₂O. Penicillin, streptomycin and amphotericin B (Biological Industries, Kibbutz Beit Haemek, Israel) were added up to final concentrations of 100 U/ml, 0.1 mg/ml and 0.25 µg/ml, respectively.

2.1.2. Culture medium

Consists of 5–10% filtered hemolymph obtained from *Aplysia faciata* (specimens were collected along the Mediterranean coast) diluted in msL-15.

2.1.3. Artificial sea water (ASW)

NaCl 460 mM, KCl 10 mM, CaCl₂ 10 mM, MgCl₂ 55 mM, HEPES 10 mM, adjusted to pH 7.6.

2.2. Cell culture

Neurons B1 and B2 from buccal ganglia, MCn neurons from the metacerebral ganglion or LUQs from the abdominal ganglion of *Aplysia californica* were isolated and maintained in culture as previously described (Schacher and Proshansky, 1983; Spira et al., 1993, 1996). Briefly, specimens were anaesthetised by injection of isotonic MgCl₂ solution (380 mM) into the animal's body cavity. The ganglia were dissected and incubated in msL-15 containing 1% protease (type IX, Sigma, Rehovot, Israel) at 34 °C for 1.5–2.5 h. Follow-

ing the protease treatment the ganglia were washed with msL-15, pinned and desheated. The identified neurons were manually pulled out along with their original axon with the aid of a sharp glass microelectrode. The neurons were immediately plated in glass-bottom dishes coated with poly-L-lysine (Sigma) containing culture medium. Microinjections were performed 8–42 h from plating, at room temperature (21–25 °C).

2.3. Microscopy

The system used for imaging consisted of an Olympus microscope IX70 and a BioRad Radiance 2000/AGR-3 confocal imaging system. The objective used was an Olympus planApo 60 ×, 1.4 NA oil objective. Images of YGFP and EGFP-actin or tubulin shown in this paper were collected by excitation at 488 nm and emitted fluorescent collected at 500–560 nm.

2.4. Axotomy

Axonal transection was performed by applying pressure on the axon with the thin shaft of a micropipette under visual control, as has been previously described (Benbassat and Spira, 1993; Spira et al., 1993, 1996).

3. Results

3.1. Plasmid constructions

3.1.1. EGFP-actin construct

EGFP cDNA was amplified from pEGFP-N1 vector (Clontech) by polymerase chain reaction (PCR) with two specific primers: 5' GGCCATGGTGAGCAAGG3' and 5' CTTGTACAGCTCGTCCATG3' containing HindIII and SmaI restriction sites, respectively, in their 5' ends. The PCR product was digested with HindIII and SmaI and subcloned into corresponding sites of pBluscript II SK (Stratagen). *Aplysia* actin, (provided by Dr DesGrosillier, Montreal University) was amplified by PCR from pBluscript containing the actin cDNA using two specific primers: 5'ATGTGTGACGACG-ATGTT3' and 5'TTAGAAGCACTTGCGGTCG3' with SmaI and XbaI restriction sites at their 5' ends. Following digestion with SmaI and XbaI, the PCR product was subcloned in-frame with EGFP into the previously prepared pBluscript-EGFP vector linearized with SmaI and XbaI. The EGFP-Actin fragment was then cut out from pBluscript with ClaI and XbaI and subcloned into corresponding sites of pCS2+vector (Rupp et al., 1994; Turner and Weintraub, 1994).

Fig. 1. Expression of EYFP. EYFP mRNA was injected 20 h before imaging. A and C are differential interference contrast images of the neuron. C and D are enlargements of the neuronal segment within the rectangle in A. B and D are confocal images. Note that the fluorescent signal is evenly distributed in the axoplasm. The confocal images were produced by the following setting: laser intensity 5%, iris setting 1.6, gain 100 and Zero offset.

3.2. Other DNA constructs

EYFP, EBFP, ECFP and RFP constructs were prepared as described for EGFP-actin. EYFP, EBFP and ECFP cDNAs were amplified, respectively, from pEYFP, pEBFP and pECFP (Clontech) by PCR with the same primers used for EGFP cDNA amplification. The RFP cDNA was amplified from pDsRed1-N1 vector (Clontech) using the primers: 5'GGCCACCATGGTGCGCTCCT 3' and 5'CAGGAACAGGTGGTGGCGG3'.

Aplysia's SNAP-25 cDNA (provided by Dr W. Sossin, University of Montreal) was amplified by PCR with two primers containing SmaI and SpeI restriction sites and replaced the actin gene from pCS2-EGFP-actin that was excised by SmaI and XbaI.

pCS2-EGFP- α -tubulin was prepared from Clontech pEGFP-Tub (cat. 6117-1). This plasmid encodes a fusion protein consisting of EGFP and human α -tubulin. The plasmid was digested with BamHI, filled in with T4 DNA Polymerase, digested with NheI and ligated to pCS2 cut by XbaI and SnaBI.

3.3. In vitro transcription of mRNAs

mRNAs were in vitro transcribed using recombinant transcription system. Briefly, 10 μ g of the different pCS2+ constructs were linearized with NotI and purified using DNA clean-up system (Promega). 1–3 μ g of linearized DNA were transcribed using RiboMax-sp6 kit (Promega P1280). Typical reaction contain 8 μ l Transcription Buffer, 8 μ l rNTPs mix containing 25 mM CTP, ATP, UTP and 12 mM GTP, 4 μ l of 15 mM Cap analog (Roche 85846029), 1 μ l rNasin (Promega N251A), 4 μ l enzyme mix. Final volume is 40 μ l and the incubation time is 2–4 h at 37 °C. RNA purification is done by RNeasy mini kit (Qiagen 74104) and the clean RNA is eluted to final volume of 25–40 μ l and kept at –80 °C until use.

3.4. Microinjection of mRNAs

The transcribed mRNAs were pressure injected into the cytoplasm of cultured *Aplysia* neurons 8–48 h after

Fig. 2. EGFP-actin bundles at the leading edge of a growth cone lamellipodium formed after axotomy. A B1 neuron injected with mRNA encoding EGFP-actin fusion protein was axotomized 28 h later. Transection is followed by rapid extension of a growth cone's lamellipodium. (A) An image of the growth cone's lamellipodium taken at the level of the glass substrate 29 min after axotomy. (B) An image taken from the same region 3 μ m above the substrate. In A, actin puncta are seen along the axonal plasma membrane facing the substrate. The perimeters of the growth cone's lamellipodium contain radially oriented actin bundles. In B, 3 μ m above the substrate, the actin polymerise along the axon's plasma membrane. The core of the axoplasm does not contain clear actin network.

plating. For the injections 0.5–5 μ g/ μ l mRNA was prepared in 80 mM KCl.

Injection was performed by pressure using Medical system Corp microinjector under visual control. We estimate the injected volume to be approximately 10% of the cell's body volume. The micropipette used for injection was also used to continuously monitor the trans membrane potential and input resistance (Benbasat and Spira, 1993). At the end of the injection the

micropipette was gently pulled out of the cell. Injected cells were imaged for protein expression 12–48 h later.

3.5. EGFP mRNA injection

To determine whether mRNA injection lead to successful and sufficient protein expression in cultured *Aplysia* neurones, in vitro transcribed mRNA encoding EGFP or EYFP were injected and examined 12–24 h

Fig. 3. Cytochalasin B induces disassembly of actin bundles at the leading edge of growth cones. Shown are two growth cones formed by a cultured B1 neuron injected with mRNA encoding EGFP-actin fusion protein. (A) The leading edges of the growth cones contain bundles of EGFP labelled actin. (B) Nine minutes after bath application of 2 μ M cytochalasin B the actin bundles disassemble and the dissociated actin form small aggregates within the collapsed growth cones and the axoplasm.

later. EGFP or EYFP expression was observed in close to 100% of the injected neurones within the cell body, the axons and neurites (Fig. 1). The fluorescent signal is evenly distributed in the cytoplasm.

3.6. Expression of EGFP-fusion proteins

Because of the particular interest that our laboratory have in visualising on-line cytoskeletal element restructuring and vesicle trafficking during growth cone formation, after mechanical injury of cultured *Aplysia* neurones (Ziv and Spira, 1995, 1997, 1998; Ashery et al., 1996; Spira et al., 1996, 2001, 2003; Gitler and Spira,

1998, 2002), we chose to demonstrate here the translational efficiency of the mRNAs encoding EGFP tagged actin and tubulin.

Injection of a solution containing mRNA encoding EGFP-actin fusion protein resulted in a high fluorescent signal in the cell body, axons, and neurites. The fluorescent signal appears to be distributed homogeneously in the cell body cytoplasm, main axon and neurites but is not detected within the nucleus. Fluorescent hot spots, most likely representing adhesion plaques, were seen along the plasma membrane facing the substrate. To determine whether the observed fluorescent signal corresponds to EGFP-tagged actin,

Fig. 4. Depolymerisation of microtubules within a growth cone formed after axotomy. A cultured B1 neuron injected with mRNA encoding EGFP at tubulin fusion protein. (A) EGFP labelled microtubules radiate from the growth cone centre towards the growth cone's lamellipodium perimeters. (B) Twenty three minutes after bath application of 5 μ M nocodazole the microtubules depolymerise.

rather than EGFP alone the main axon was transected and the fluorescent signal distribution was imaged during the formation and extension of the growth cone's lamellipodium. Axonal transection of cultured *Aplysia* neuron leads to the rapid formation of a growth cone's lamellipodium at the tip of the cut axon (Ziv and Spira, 1995; Ashery et al., 1996; Gitler and Spira, 1998, 2002; Spira et al., 2001). It is well documented that the growth cone's lamellipodium perimeters are rich with actin filaments that are central to its motility (Forscher and Smith, 1988; Lin et al., 1994; Tanaka and Sabry, 1995; Scheafer et al., 2002).

Following axotomy of neurons injected with EGFP-actin, a growth cone in the form of large lamellipodium is formed within 10–15 min of axotomy. The perimeters of the growth cone are rich with fluorescent actin bundles while the central region exhibit fluorescent puncta (Fig. 2). The fluorescent bundles depolymerise within minutes of cytochalasin B application (Fig. 3). Therefore, it is safe to assume that these bundles are EGFP labelled actin bundles. These results demonstrate that the fluorescent signal corresponds to expressed EGFP-actin fusion protein, which incorporates into the actin skeleton and allows the visualisation of the dynamic behaviour of this skeletal component.

3.7. Expression of EGFP/EYFP-tagged microtubules and synaptic vesicle proteins

To establish that the method described above can be used as a reliable tool to express various types of proteins in cultured *Aplysia* neurons we injected several other mRNAs encoding proteins tagged with either EGFP or EYFP. This included a tubulin and a member of the SNARE complex, the synaptosome associated protein of 25 kDa (SNAP-25). On-line confocal microscope imaging of neurons injected by a solution containing mRNA encoding EGFP- α tubulin fusion protein resulted in incorporation of the tagged tubulin into microtubules that extended into an axotomy induced growth cone's lamellipodium (Fig. 4A). Bath application of the microtubules depolymerizing agent nocodazole (5 μ M) for 5 min resulted in depolymerisation of most microtubules (Fig. 4B).

The fluorescent signal of SNAP-25 revealed the presence of fluorescent spots in the intact axon as well as in the growth cone (not shown).

4. Discussion

In the present study we report the development of an efficient method for expression of GFP fusion proteins in cultured *Aplysia* neurons.

Functional genes have been expressed in *Aplysia* neurons following the injection of DNA plasmids

(Kaang, 1996a,b; Manseau et al., 2001; Lee et al., 2001), nevertheless, these proteins were expressed at low levels and visualised only within the domain of the cell body.

Attempts to express mRNA encoding the *lacZ*, gene in *Aplysia* neurons were not very successful (Kaang, 1996a,b). Nevertheless, it is important to note that successful expression of mRNA encoding tetanus toxin (TeTx), and the light chain of botulinum neurotoxin (BoNT) has been reported already by Mochida et al. (1990). It is reasonable to assume that the positive results reported by these authors are due to the proteolytic nature of the toxins and the sensitive functional test to detect it. Thus, even very small amounts of expressed TeTx and BoTx are sufficient to totally block synaptic transmission.

The examples presented in Section 3 demonstrate the effectiveness of the procedures developed by us to express GFP fusion proteins in *Aplysia* neurons. Online imaging of the distribution of EGFP-tagged cytoskeletal elements, molecular motors, enzymes, kinases, lipases and other proteins are expected to facilitate the studies of fundamental mechanisms underlying various forms of neuroplasticity by the large community that uses *Aplysia* neurons as a model system.

Acknowledgements

This study was supported by grants from the Israel Science Foundation (No. 620/98), the US-Israel Bi-National Research Foundation (No. 97-00297-1) and the Charles E. Smith Family Laboratory for Collaborative Research in Psychobiology, the Hebrew University of Jerusalem. M.E. Spira is the Levi DeVial Professor in Neurobiology.

References

- Ashery U, Penner R, Spira ME. Acceleration of membrane recycling by axotomy of cultured *Aplysia* neurons. *Neuron* 1996;16:641–51.
- Bailey CH, Kandel ER. Structural changes accompanying memory storage. *Annu Rev Physiol* 1993;55:397–426.
- Baumann CT, Reyes IC. Tracking components of the transcription apparatus in living cells. *Methods* 1999;19:353–61.
- Benbassat D, Spira ME. Survival of isolated axonal segments in culture: morphological, ultrastructural and physiological analysis. *Exp Neurol* 1993;122:295–310.
- Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. Green fluorescent protein as a marker for gene expression. *Science* 1994;263:802–5.
- Chamberlain C, Hahn KM. Watching proteins in the wild: fluorescence methods to study protein dynamics in living cells. *Traffic* 2000;1:755–62.
- Day RN, Periasamy A, Schaufele F. Fluorescence resonance energy transfer microscopy of localized protein interactions in the living cell nucleus. *Methods* 2001;25:4–18.

- Ellenberg J, Lippincott SJ, Presley JF. Dual-colour imaging with GFP variants. *Trends Cell Biol* 1999;9:52–6.
- Forscher P, Smith SJ. Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. *J Cell Biol* 1988;107:1505–16.
- Gerdes HH, Kaether C. Green fluorescent protein: applications in cell biology. *FEBS Lett* 1996;389:44–7.
- Gitler D, Spira ME. Real time imaging of calcium-induced localized proteolytic activity after axotomy and its relation to growth cone formation. *Neuron* 1998;20:1123–35.
- Gitler D, Spira ME. Short window of opportunity for calpain induced growth cone formation after axotomy of *Aplysia* neurons. *J Neurobiol* 2002;52:267–79.
- Kaang BK. Parameters influencing ectopic gene expression in *Aplysia* neurons. *Neurosci Lett* 1996a;221:29–32.
- Kaang BK. Neuronal expression of reporter genes in the intact nervous system of *Aplysia*. *Mol Cell* 1996b;6:285–95.
- Kandel ER. The molecular biology of memory storage: a dialog between genes and synapses. *Biosci Rep* 2001a;21(5):565–611.
- Kandel ER. The molecular biology of memory storage: a dialog between genes and synapses. *Science* 2001b;294:1030–8.
- Kim HK, Kaang BK. Truncated green fluorescent protein mutants and their expression in *Aplysia* neurons. *Brain Res Bull* 1998;47:35–41.
- Lee JA, Kim HK, Kim KH, Han JH, Lee YS, Lim CS, Chang DJ, Kubo T, Kaang BK. Overexpression of and RNA interference with the CCAAT enhancer-binding protein on long-term facilitation of *Aplysia* sensory to motor synapses. *Learn Mem* 2001;8:220–6.
- Lin CH, Thompson CA, Forscher P. Cytoskeletal reorganization underlying growth cone motility. *Curr Opin Neurobiol* 1994;4:640–7.
- Lippincott SJ, Smith CL. Insights into secretory and endocytic membrane traffic using green fluorescent protein chimeras. *Curr Opin Neurobiol* 1997;7:631–9.
- Manseau F, Fan X, Hueflein T, Sossin W, Castellucci VF. Ca²⁺-independent protein kinase C Apl II mediates the serotonin-induced facilitation at depressed *Aplysia* sensorimotor synapses. *J Neurosci* 2001;21:1247–56.
- Mochida S, Poulain B, Eisel U. Exogenous mRNA encoding tetanus or botulinum neurotoxins expressed in *Aplysia* neurons. *Proc Natl Acad Sci USA* 1990;87:7844–8.
- Periasamy A, Day RN. Visualizing protein interactions in living cells using digitized GFP imaging and FRET microscopy. *Methods Cell Biol* 1999;58:293–314.
- Rupp RAW, Snidel L, Weintraub H. *Xenopus* embryos regulate the nuclear localization of XmyoD. *Genes Dev* 1994;8:1311–23.
- Schacher S, Proshansky E. Neurite regeneration by *Aplysia* neurons in dissociated cell culture: modulation by *Aplysia* hemolymph and the presence of the initial axonal segment. *J Neurosci* 1983;3:2403–13.
- Scheafer AW, Kabir N, Forscher P. Filopodia and actin arcs guide the assembly and transport of two populations of microtubules with unique dynamic parameters in neuronal growth cone. *J Cell Biol* 2002;158(1):139–52.
- Spergel DI, Kruth U, Shimshek DR, Sprengel R, Seeburg PH. Using reporter genes to label selected neuronal populations in transgenic mice for gene promoter, anatomical and physiological studies. *Prog Neurobiol* 2001;63:673–86.
- Spira ME, Benhassat D, Dormann A. Resealing of the proximal and distal cut ends of transected axons: electrophysiological and ultrastructural analysis. *J Neurobiol* 1993;24:300–16.
- Spira ME, Dormann A, Ashery U, Gabso M, Gitler D, Benbassat D, Oren R, Ziv NE. Use of *Aplysia* neurons for the study of cellular alterations and the resealing of transected axons in vitro. *J Neurosci Methods* 1996;69:91–102.
- Spira ME, Oren R, Dormann A, Llouz N, Lev S. Calcium, protease activation and cytoskeleton remodeling underlie growth cone formation and neuronal regeneration. *Cell Mol Neurobiol* 2001;21(6):591–03.
- Spira ME, Oren R, Dormann A, Gitler D. Critical calpain-dependent ultrastructural alterations underlie the transformation of an axonal segment into a growth cone after axotomy of cultured *Aplysia* neurons. *J Comp Neurol* 2003;457(3):293–312.
- Stearns T. Green fluorescent protein. The green revolution. *Curr Biol* 1995;5:262–4.
- Suter DM, Forscher P. Substrate-cytoskeletal coupling as a mechanism for the regulation of growth cone motility and guidance. *J Neurobiol* 2000;44:97–113.
- Tanaka E, Sabry I. Making the connection: cytoskeletal rearrangements during growth cone guidance. *Cell* 1995;83:171–6.
- Tsien RY. The green fluorescent protein. *Annu Rev Biochem* 1998;67:509–44.
- Turner DL, Weintraub H. Expression of achaete-scute homolog 3 in *Xenopus* embryos converts ectodermal cells to a neural fate. *Genes Dev* 1994;8:1434–47.
- Van Roessel P, Brand AH. Imaging into the future: visualizing gene expression and protein interactions with fluorescent proteins. *Nat Cell Biol* 2002;4:E15–20.
- Ziv NE, Spira ME. Axotomy induces a transient and localized elevation of the free intracellular calcium concentration to the millimolar range. *J Neurophysiol* 1995;74:2625–37.
- Ziv NE, Spira ME. Localized and transient elevations of intracellular Ca²⁺ induce the dedifferentiation of axonal segments into growth cones. *J Neurosci* 1997;17:3568–79.
- Ziv NE, Spira ME. Induction of growth cone formation by transient and localized increases of intracellular proteolytic activity. *J Cell Biol* 1998;140:223–32.