RESEARCH COMMUNICATION

High-efficiency Semliki Forest virus-mediated transduction in bovine adrenal chromaffin cells

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Adrenal chromaffin cells are commonly used in studies of exocytosis. Progress in characterizing the molecular mechanisms has been slow, because no simple, high-efficiency technique is available for introducing and expressing heterologous cDNA in chromaffin cells. Here we demonstrate that Semliki Forest virus

(SFV) vectors allow high-efficiency expression of heterologous protein in chromaffin cells.

Key words: acidic vesicle, confocal, double C2 protein β -isoform, exocytosis, SFV.

INTRODUCTION

Adrenal chromaffin cells have long been a model system of choice for studies of exocytosis. These neuroendocrine cells, like neurons, fire action potentials, exhibit calcium-dependent exocytosis, and express many proteins closely related to synaptic proteins.

An important barrier to the advance in understanding of the molecular mechanisms underlying exocytosis is the lack of a high-efficiency technique for introducing heterologous DNA or RNA into chromaffin cells. 'Traditional' methods for mammalian cell transfection, such as calcium phosphate precipitation, lipid-mediated transfection and electroporation have met with limited success and have variable efficiencies [1–4].

A number of viral transduction systems have been developed to permit the efficient transduction of cDNA into eukaryotic cells. The ideal viral-vector system would be easy to use, have a broad host range but low human pathogenicity, and permit efficient infection of target cells with high-level protein expression and low cytotoxicity. Of the existing expression systems for higher eukaryotic cells, perhaps the most efficient in terms of protein production is the baculovirus system for insect cell hosts [5]. Efficient viral transduction systems for mammalian cells based on a recombinant vaccinia virus, adenovirus or defective herpes simplex virus-1 have been developed (for a review, see [6]), but these require considerable expertise for use and can be time-consuming and expensive. In addition, adequate containment facilities may not be available.

The recently introduced Semliki Forest virus (SFV) transduction system [7,8] has several potential benefits over other viral transduction systems, in particular, minimal containment requirements and ease of use. The gene of interest may be ligated directly to a DNA cloning vector encoding non-structural SFV genes for replicase, reverse transcriptase and helicase. After *in vitro* transcription, resultant capped RNA is co-electroporated alongside structural gene RNA into a permissive host cell line,

commonly baby-hamster kidney (BHK)-21 cells. Attenuated, non-infectious packaged viral particles are assembled in the host cells, secreted into the extracellular medium and can be stored for future use. Treatment of these viral particles with a protease renders them infectious [7–8].

The SFV transduction system has already been used to express a number of proteins in a broad range of mammalian cells, including cultured rat hippocampal neurons, BHK-21, HeLa, and Madin–Darby canine kidney cells [7–10,11]. This broad host cell range, the ability to sub-clone directly into the expression vector without the need for *in vivo* recombination steps, and the excellent attenuation of recombinant SFV together make this system a particularly attractive candidate for transducing cDNA into chromaffin cells. In the present study, we examine the efficacy of SFV transduction of green fluorescent protein (GFP) mut3 and double C2 protein β -isoform-enhanced GFP (DOC2 β -EGFP) in chromaffin cells. DOC2 β is a protein that has been shown to associate with secretory vesicles previously [12], and which may play a role in exocytosis [13]. Efficient SFV transduction of chromaffin cells is obtained with the modification of previously published procedures.

MATERIALS AND METHODS

Isolation of a cDNA encoding mouse brain DOC2 β

A reverse-transcription PCR strategy was used to amplify a cDNA encoding the open reading frame of mouse brain DOC2 β . mRNA (1 μ g) was used as a template in a first-strand cDNA synthesis directed from an anchored deoxyoligo d(T) (5'-TTCTAGAATTCAGCGGCCGC(T)₃₀N₁N₂) primer, using Superscript II reverse transcriptase (Gibco BRL Life Technologies). The resultant cDNA was diluted and used in a PCR reaction between forward (5'-CTGCCTGCATGACCCTCCG-GC) and reverse (5'-TCAGTCGCTGAGYACAGCCCCTG-

Abbreviations used: BHK, baby-hamster kidney; DOC2 β , double C2 protein β -isoform; GFP, green fluorescent protein; EGFP, enhanced GFP; SFV, Semliki Forest virus.

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GG) deoxyoligonucleotides, using Expand polymerase cocktail (Boehringer Mannheim). The PCR product(s) were ligated to a T/A vector (pCR2.1, Invitrogen) and completely sequenced on both strands (Oswel DNA Services, Southampton, U.K.).

Cell culture

Bovine adrenal chromaffin cells were prepared from adrenal glands freshly obtained from an abattoir. The standard approach [14] involved collagenase digestion and mechanical dispersion. The dispersed cells were maintained in culture for up to 5 days in 30-mm diameter plastic culture chambers and on glass coverslips in Dulbecco's modified Eagle's medium supplemented with 1% (v/v) $1 \times$ insulin/transferrin/selenium supplement (ITS-X), 0.1% penicillin/streptomycin and 1% (w/v) sodium pyruvate (all from Gibco BRL Life Technologies) at 37 °C in 95% air, 5% CO₂. BHK-21 cells were cultured in Glasgow Modified Eagle's medium supplemented with 10% (v/v) tryptose phosphate broth, 10% (v/v) foetal bovine serum, 2 mM glutamine and 100 units/ml of penicillin/streptomycin (all from Gibco BRL Life Technologies) at 37 °C in 95% air, 5% CO₂, and used between passage numbers 5 and 15.

Generation of recombinant SFV particles

PCR was used to generate an *EcoRI*-*Bam*HI flanked *DOC2 β* fragment for ligation to pEGFPN1 (Clontech). *DOC2 β* -EGFP cDNA was sub-cloned into the *Sma*I site of pSFV1 expression vector (Life Technologies) as a T4 DNA polymerase blunt-ended *EcoRI*-*Not*I fragment from pEGFPN1. A GFPmut3 insert was amplified by PCR using forward (5'-CGGAGATCTATGAGT-AAAGGAGAAGAACTTTTCACT) and reverse (5'-GCCGG-ATCCCATATGTTTGTATAGTTCATCCATGCCATGTGT-AAT) deoxyoligonucleotides, and a GFPmut3 plasmid as a template. The PCR product was treated with Klenow enzyme and ligated to the *EcoRV* site of pBluescript II KS (Stratagene). The insert was subcloned as a *Bam*HI-*Bgl*II fragment into pSFV1. The orientation and integrity of the cDNA inserts were confirmed by DNA sequencing. Capped mRNA was generated from these constructs and pSFV2 helper vector (Gibco BRL Life Technologies) by linearizing both vectors with *Spe*I and transcribing *in vitro* using SP6 RNA polymerase according to the manufacturer's instructions. Five to ten micrograms of each *in vitro*-transcribed mRNA were electroporated into approx. 1×10^7 BHK-21 cells in Glasgow modified Eagle's medium supplemented with 2 mM glutamine at 2125 V/cm, 25 μ F and pulsed twice using a Bio-Rad GenePulser apparatus. Cells were allowed to recover for 5 min, resuspended in 24 ml of complete medium and plated for culture for a further 48 h. Medium was recovered from these plates, and filtered through a 0.25 μ m-pore-size filter before storage at -20 °C.

Infection of chromaffin cells with recombinant SFV particles

Approx. 1×10^6 freshly prepared chromaffin cells were plated in 90-mm diameter tissue-culture plates containing sterile glass coverslips. After 24 h of culture at 37 °C, the conditioned culture medium was removed and stored for later use. Virus stock was activated by the addition of chymotrypsin A4 (250 μ g/ml; Sigma-Aldrich) and digestion for 10 min on ice. Proteolysis was halted by the addition of aprotinin (0.67 mg/ml; Sigma-Aldrich). A 1:10 dilution of the virus stock was made in conditioned chromaffin cell medium, and approx. 1 ml of this was overlaid on to the cells. The cells were incubated with the virus for 2 h, then the medium was removed and replaced again with conditioned

chromaffin cell medium. The infected cells were cultured for specific times post-infection before harvesting for immunoblot analysis and confocal fluorescence microscopy.

Immunoblotting

Cells were removed from culture at appropriate time points after infection and washed three times with ice-cold PBS, pH 7.4 (Gibco BRL Life Technologies). Cells were homogenized in the plate by the addition of 500 μ l of lysis buffer [10 mM Tris/HCl, 0.1% (w/v) SDS, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, 0.2 mM 4-(2-aminoethyl)benzenesulphonyl fluoride, pH 7.4] and scraping into a fresh tube. The protein concentration was determined by assaying a 1 in 10 dilution of the mixture using the Bradford method according to the manufacturer's instructions (Bio-Rad). Approx. 10 μ g of protein per sample were electrophoresed in an SDS/10% polyacrylamide gel using the method of Laemmli [15]. Proteins were transferred to poly(vinylidene difluoride) (Bio-Rad) using a Trans-Blot SD (Bio-Rad) semi-dry electrobloater at 20 V for 30 min. Following transfer, excess binding sites on the membrane were blocked by incubating in blotting buffer (PBS, 5% (w/v) non-fat skimmed milk) for 1 h. Monoclonal anti-synaptotagmin, anti-rSEC8, anti-rabphilin-3A, anti-munc-18 (Transduction Laboratories), monoclonal anti-GFP (Clontech) or polyclonal anti-dopamine- β -hydroxylase (D- β -H) were diluted in blocking buffer and incubated with the membranes for 1 h. Membranes were washed extensively with PBS before incubation with horseradish peroxidase-conjugated anti-mouse IgG (Transduction Laboratories, for monoclonals) or horseradish-peroxidase conjugated anti-rabbit IgG (Amersham-Pharmacia Biotech, for polyclonal), diluted in blocking buffer as before. Decorated proteins were revealed by ECL[®] (Amersham-Pharmacia Biotech) according to the manufacturer's instructions.

Single-cell fluorescence and electrophysiology

Cells expressing GFP or EGFP fusion proteins were identified by their green fluorescence. The cells attached to glass coverslips were mounted in a perfusion chamber located on the stage of an inverted microscope (Zeiss Axiovert 100), and illuminated via the epi-illumination pathway by a monochromated light source (TILL Photonics, Planegg, Germany). Excitation was at 488 nm for EGFP and 395 nm for the mut3.1GFP. The filter combination was a dichroic mirror Q500L and the emission filter HQ500LP (Chroma Technology, Brattleboro, VT, U.S.A.).

Fluorescent cells were subjected to perforated-patch recording with an EPC-9 patch clamp system (HEKA Elektronik, Germany); a system which performs automated on-line measurements of ionic currents, membrane capacitance, membrane conductance and series conductance. Membrane capacitance is proportional to cell-membrane-surface area and serves as a measure of secretion, since the surface area increases upon exocytotic addition of vesicular membrane and decreases upon endocytosis [14]. The patch pipette solution contained 135 mM caesium glutamate, 9 mM NaCl, 1 mM triethylammonium chloride, 10 mM Hepes (pH 7.3, titrated with CsOH). For perforation of the membrane patch, standard protocols were followed [16] and the amphotericin (Sigma-Aldrich) concentration was 200 μ g/ml. After seal formation, the series resistance was measured continuously and recordings were started when the series resistance dropped below 20 M Ω . The external bath solution contained 145 mM NaCl, 2.8 mM KCl, 5 mM CaCl₂, 1 mM MgCl₂, 10 mM Hepes (pH 7.3, titrated with NaOH) and 10 mM glucose. The external bath solution was constantly perfused at 0.5–1 ml/min. Recordings were made at room

temperature (approx. 20 °C). The values of membrane current and capacitance responses are reported as the mean $\pm$ S.E.M. in the text.

Confocal microscopy

Cells cultured on glass coverslips were washed twice with PBS supplemented with Ca^{2+} and Mg^{2+} before fixation for 5 min with ice-cold 4% (w/v) buffered paraformaldehyde, or living cells were imaged using a Leica TCS NT Confocal System (Leica Lasertechnik GmbH, Heidelberg, Germany) with a PL APO 63 $\times$ /1.32–0.6 oil immersion lens, also made by Leica. If required, cells were counter-stained using the acidotropic dye LysoTracker Red according to the manufacturer's instructions (Molecular Probes).

RESULTS

Highly efficient SFV infection of adrenal chromaffin cells

Previously published procedures for SFV transduction yielded low efficiencies of infection/expression (10–20%) in chromaffin cells. Adding the step of inactivating the chymotrypsin A (used to activate the viral particles) increased the number of cells that survived viral incubation, but the numbers of cells expressing GFP still remained low (< 30%). The further modification using conditioned chromaffin cell medium rather than PBS or BHK-21 medium for both the virus incubation and recovery stages dramatically improved the final percentage of cells

observed to have a fluorescent phenotype 48–72 h post-infection. Approx. 90–100% of all cells were observed to fluoresce, estimated by directly counting the fluorescent cells compared with the total number of cells counted under visible light (Figure 1B). Immunoblot studies of viral-infected chromaffin cells demonstrated that DOC2 β -EGFP could be detected using a monoclonal anti-GFP antibody between 16–19 h post-infection. These observations were supported by the development of a fluorescent-chromaffin-cell phenotype in infected cells between these time-points. The levels of detectable decorated DOC2 β -EGFP increased steadily up to 72 h post-infection, with no apparent change in cellular morphology (Figures 1A and 1B).

In this study, we used a 1:10 dilution of one-sixtieth of the virus stock (approx. 8×10^6 infectious units) to infect approx. 1×10^5 cells. This provided a multiplicity of infection of approx. 8, assuming that all the viral particles were competent to infect chromaffin cells. In practice, the dilution of the viral stock could have been greater.

Chromaffin cell biology after SFV infection

Immunoblot studies of the endogenous chromaffin cell proteins synaptotagmin, D- β -H, rSEC8, rabphilin-3A and munc-18 demonstrated that the steady-state levels of these proteins did not decrease throughout this time course (Figure 1A). The stabilities of synaptic proteins are variable [11], although no data is available relating to these proteins in bovine adrenal chromaffin

Figure 1 (A) Immunoblot analysis of DOC2 β -EGFP fusion protein expression in SFV-infected bovine adrenal chromaffin cells, and (B) low-power confocal images superimposed on bright-field images of SFV-infected bovine adrenal chromaffin cells

(A) DOC2 β -EGFP fusion protein was first detected between 16 h and 19 h post-infection (p.i.), with the level of detectable protein increasing with time up to 72 h post-infection. The levels of synaptotagmin, dopamine- β -hydroxylase (D- β -H), rSEC8, rabphilin-3A and munc-18 did not decrease over the time of the study. The time course of expression is in contrast with the time course of SFV-transduced expression, reported previously, where host-cell protein synthesis was sequestered as early as 9 h post-infection (see text for references). (B) Cells grown on sterile glass coverslips were removed from the same cultures used for immunoblot analysis and examined under 488 nm illumination as described in Materials and methods section. DOC2 β -EGFP could be seen from 19 h post-infection, and increased in intensity up to 72 h post-infection. No EGFP fluorescence could be detected in non-infected cells. (a) At 16 h post-infection, no fluorescence is visible. (b) At 19 h post-infection, some EGFP fluorescence is just visible. This correlates with immunoblot analyses, which first detected decorated DOC2 β -EGFP between 16 h and 19 h. (c) and (d) The intensity of the EGFP-fluorescence appeared to increase between 24 h and 72 h post-infection. The relative percentage of cells with the green fluorescent phenotype can be judged by comparing fluorescent cells with non-infected cells, revealed by the superimposition of the bright-field image. At 24 h post-infection, a single cell (arrow) that is not expressing DOC2 β -EGFP can be seen in this field. In these experiments, we judged the efficiency of infection and expression to be 90–100% by 48–72 h post-infection. These images are representative of several independent experiments, where the observed transduction efficiencies were comparable ($n > 25$ experiments). Scale bars: 10 μ m.

Figure 2 Confocal microscopy and electrophysiology of DOC2 β -EGFP-expressing chromaffin cells

(A) High-power confocal images of a group of three DOC2 β -EGFP-expressing chromaffin cells 48 h post-infection. A single 1 μ m-thick confocal optical section, illuminated at 488 nm and 577 nm, was used to produce these images. (a) The EGFP fluorescence is seen distributed throughout the chromaffin cell in a punctate distribution with nuclear sparing, reminiscent of a vesicular localization. This coincides with the distribution of LysoTracker Red in the same cell (b), which also shows a punctate distribution with nuclear sparing. (c) Superimposition of the two images revealed the fluorescent spots to be overlapping, as indicated by yellow. A yellow line AB shown on (a) and (b), was measured for fluorescence intensity. (d) Shows the normalized intensity under the line proceeding in arbitrary distance units from point A to point B. Similar vertical lines were drawn through 55 vesicular areas in 5 cells. DOC2 β -EGFP peaks were coincident with LysoTracker Red peaks in 98% of cells. Conversely LysoTracker Red peaks were coincident with DOC2 β -EGFP peaks in 76% of cells. The arrows mark peaks that are representative of vesicular fluorescence. Scale bar: 10 μ m with bold inset (yellow square on left hand end of scale bar in panel c; 0.39 μ m) representing the approximate size of a large dense core vesicle. Membrane currents and capacitance responses in response to depolarizations of 100-ms duration to +10 mV, from a holding potential of -70 mV for (B) non-infected cells and (C) infected cells. The solutions have been designed to isolate the inward currents – an initial rapidly inactivating sodium current, followed by a maintained calcium current. The membrane capacitance increases in response to the injections of calcium due to the depolarizations. There is a gap during the depolarizations, during which the capacitance measurements are not calculated.

cells. Interestingly, the half-life of synaptotagmin I in neurons has recently been shown to be within 8–22 h during 1–3 days of culture [11]. The stable synaptotagmin level in our experiments suggest that the ability of SFV-infected cells to synthesize new proteins remains unaffected over the course of this experiment. Biophysical studies of cells expressing GFP and DOC2-EGFP constructs 48 h after SFV infection revealed that the cell mem-

branes were intact, with no significant increase in the leak current [2.8 ± 0.4 pA ($n = 18$) compared with 3.2 ± 0.5 pA ($n = 18$) in non-infected cells], and the cells displayed significant calcium currents and capacitance increases, indicative of exocytosis (Figures 2B and 2C). When subjected to 100-ms depolarizations to +10 mV, the chromaffin cells displayed a mean calcium current amplitude of 297 ± 23 pA ($n = 18$), which is approx.

25% less than the current measured in non-infected chromaffin cells at 407 ± 37 pA ($n = 18$). In response to the injection of calcium current, the transduced cells responded with an increase in membrane capacitance of 14 ± 2 fF ($n = 18$), compared with 31 ± 4 ($n = 18$) in non-infected cells. Thus, 48 h after viral infection, all the infected cells are expressing heterologous protein, and they continue to carry out normal physiological processes of calcium current conduction and exocytosis.

DOC2 β is localized to an acidic, vesicular compartment in chromaffin cells

Confocal microscopy revealed that DOC2 β -EGFP fluorescence was distributed throughout the cell in a characteristic punctate pattern with nuclear sparing, reminiscent of a vesicular localization. Cells that were loaded with the acidic compartment probe LysoTracker Red showed a similar fluorescence distribution. The vesicular distribution of DOC2 β -EGFP shows 98% coincidence with LysoTracker Red in double-labelled cells, suggesting that the DOC2 β -EGFP fusion protein was directed to an acidic compartment in these cells (Figure 2A). In contrast, transduced GFPmut3 displayed a homogeneous cytoplasmic distribution (results not shown).

DISCUSSION

We have developed a modification of the SFV transduction approach that efficiently introduces heterologous cDNA into bovine adrenal chromaffin cells. The steady-state levels of several endogenous chromaffin cell proteins remain unaffected up to 72 h post-infection. This result is surprising because SFV has been shown to sequester host-cell protein-synthesis machinery after infection, effectively halting endogenous protein synthesis in favour of viral protein production; however, the observation suggests that there may be a time window over which meaningful biophysical studies of exocytosis can be carried out. Indeed, our preliminary results with patch-clamp measurements have demonstrated that the cells still have significant calcium currents and secretion, though somewhat depressed compared with non-infected cells. These data are in agreement with a recent paper describing the use of SFV vectors to express synaptic proteins in cultured neurons without cytotoxic effects [11].

The time course of heterologous protein expression in chromaffin cells appeared to be slower than in cultured cell-lines such as BHK-21 cells, COS-7 cells and Chinese hamster ovary cells (e.g. [17]), where it has been reported that expressed protein can be detected as early as 9 h post-infection, at which point host endogenous protein synthesis is already depressed. Furthermore, the levels of heterologous protein expression, judged by im-

munoblotting and GFP fluorescence intensity, are relatively low compared with those in other cell types reported previously [8–10].

The observation here that DOC2 β -EGFP is directed to an intracellular acidic compartment, in support of earlier work on DOC2 intracellular localization (e.g. [12]), provides evidence that proteins expressed from SFV-based vector systems in chromaffin cells can be correctly processed and targeted. Furthermore, this is the first direct visualization of DOC2-EGFP *in vivo*. Additional experiments using SFV-GFPmut3 alone confirmed the high-efficiency of transduction using these conditions, and the ability of SFV-expressed proteins to be directed to the appropriate intracellular localization.

SFV transduction systems have, until now, been used for large-scale, high-efficiency protein production in mammalian cells. The slow time course, high-efficiency of infection and expression, and reduced expression levels seen in bovine chromaffin cells should make the SFV transduction system a valuable tool in the study of chromaffin cell biology.

We thank Dr. David Apps for his gift of the rabbit polyclonal anti-bovine dopamine- β -hydroxylase antibody. We also thank Ms. Linda Sharp for her expert assistance with the confocal microscopy. This work was funded by a Wellcome Trust Project grant to R.H.C. and M.J.S.

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