Applications
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Microinjection of plasmid DNA constructs for rapid expression in mammalian cells

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Abstract

Microinjection is one of the core methods to introduce foreign DNA and other non-permeable molecules into cells. Nuclear injection of plasmid DNA enables rapid expression of proteins in specific cells within a population. The major advantage of this approach over other transient transfection methods is the rapid burst of expression that follows. Recently we have used this approach to introduce function-blocking mutants of proteins, inhibitory antibodies and cell impermeable chemical inhibitors to inhibit specific functions within the secretory pathway.

Introduction

Work in my lab investigates the mechanisms operating in the mammalian secretory pathway. In particular, our work focuses on the molecular machinery involved in the transport of secretory cargo from its site of synthesis and assembly (endoplasmic reticulum (ER)) to the Golgi apparatus. The major protein complexes involved in this step are the COPI and COPII complexes [1]. The COPII complex mediates the selection and packaging of cargo at the ER membrane and results in the formation of transport vesicles [2]. These vesicles fuse to form a vesicular tubular transport carrier (VTC), which itself becomes coated with the COPI complex. Assembly of COPII on the ER membrane is triggered by GDP:GTP exchange on the small GTPase Sar1p in a reaction catalyzed by the guanine nucleotide exchange factor, Sec12p. Sar1p-GTP subsequently recruits two protein complexes, Sec23p-Sec24p and Sec13p-Sec31p. Respectively, these complexes are believed to form the major cargo binding and structural roles within the coat. Sec23p also acts as the guanine nucleotide exchange factor for Sar1p, triggering disassembly of the complex. Our recent work has used microinjection of DNA and chemical inhibitors that are not cell permeable to investigate the role of the GTPase cycle of COPII in more detail.

Fig. 1: Workstation for semiautomatic microinjection into adherent cells: Olympus microscope with Eppendorf InjectMan NI 2 and microinjector FemtoJet

Materials and methods

1 Cell preparation

HeLa cells (ATCC CCL-2) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) on live cell dishes (MatTek, Ashland, MA) or gridded coverslips (Bellco Biotechnology, Vineland, NJ). Cells were plated 24 - 48 hours before microinjection to ensure that they were well spread (this makes injection easier).

2 Devices

Microinjection was performed using an Eppendorf FemtoJet /InjectMan NI 2 system (Fig. 1) using either pre-pulled capillaries (Eppendorf Femtotips) or borosilicate glass capillaries pulled using a Sutter P-97 pipette puller.

3 Consumables

GTP-γ-S stock was diluted in ddH_2O to a final concentration of 50 mM. Plasmid DNA was diluted to a final concentration of 50 ng/ml in ddH_2O. Solutions for injection were centrifuged at 4ºC in an Eppendorf 5417R refrigerated microcentrifuge for 30 minutes. The supernatant was removed and used immediately to load capillaries for injection. Plasmids encoding inhibitors of COPII function (Sar1p(H79G)), dynactin function (p50dynamitin), Rab function (RabGDIβ) were co-injected along with reporter constructs for secretory cargo proteins (tsO45-G-GFP, lumFP or GPI-FP); all plasmids are described in [3].

4 Procedure
Cells grown on live cell dishes were microinjected (into the nucleus) with plasmids encoding combinations of secretory cargo proteins and inhibitory constructs. In other experiments, either GTP-γ-S or a function blocking antibody directed against the COPI complex (anti-EAGE) was injected following expression of secretory cargo proteins. Microinjection capillaries were loaded with 2 ml of plasmid mixes using Eppendorf GELoader. Typical pressure settings for the FemtoJet microinjector were compensation pressure (Pc) 50 hPa, injection pressure (Pi) 120 hPa, injection time (Ti) 0,3 sec. For experiments in which the injected molecules are non-fluorescent (antibodies or GTP-γ-S) we included a co-injection marker, typically Alexa-568 dextran. This allows easy identification of injected cells at a later point.

5 Downstream applications
Following expression of proteins (typically 4 hours expression time following injection), cells were either imaged live using a TILL Photonics widefield imaging system (described in [4]) or fixed and processed for immunofluorescence using standard procedures [4]. Localization and motility of secretory markers was quantified to determine any differential sensitivity. Use of gridded live cell dishes and coverslips permits us to find cells that have been analyzed by live cell imaging after they have been fixed and processed for immunofluorescence. This is useful for several reasons including (i) confirmation of expression of non-fluorescent proteins from inhibitor plasmids, and (ii) analysis of cell organization and morphology (e.g. the structure of the Golgi). Using a 40 x UPlanApo NA 1.00 phase contrast oil immersion lens (Olympus microscopes) we are able to locate and inject cells using phase contrast microscopy followed immediately by high resolution fluorescence imaging without the need to change lenses. In particular this has proven useful for analysis of membrane dynamics immediately after injection of function blocking antibodies [5].

Results

Expression of small soluble GFP within the lumen of the ER (lumFP) or of GPI-anchored GFP (GPI-FP), followed by microinjection of the poorly hydrolysable GTP analogue, GTP-γ-S, was used to determine the requirement for GTPase activity in the export of these secretory cargo proteins. Under these conditions, we have shown that GPI-FP is selectively retained and evenly distributed within the ER, while lumFP becomes accumulated into punctate structures distributed throughout the cytoplasm (see figure 2). These punctate structures co-label with antibodies directed against components of the COPII complex identifying them as ER export sites (pre-budding complexes). These data were indistinguishable from those obtained following expression of these cargo proteins and a GTP-restricted mutant of Sar1p (Sar1p-(H79G)). This reveals that distinct cargo proteins show differential requirements for GTPase activity of Sar1p.

Further to this, we shows that both cargo proteins exhibit the same dependence on downstream components of the secretory pathway, namely the COPI complex and dynactin complex. Inhibition of COPI function using microinjection of anti-EAGE, or expression of p50dynamitin to inhibit dynactin function, inhibited the transport of both cargo proteins through the secretory pathway to the same extent.

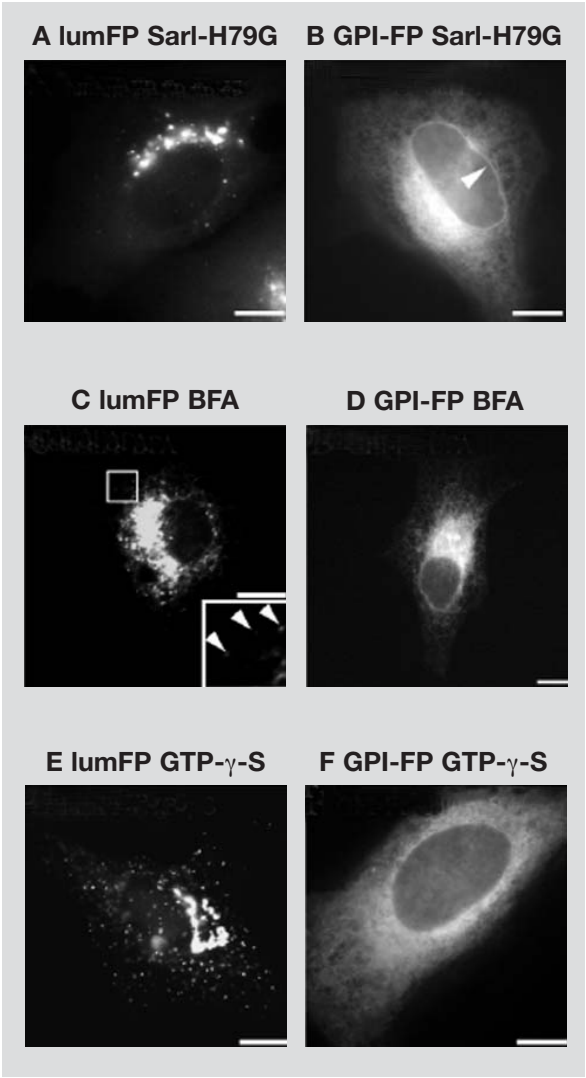

Fig. 2: Export of GPI-FP from the ER requires GTP hydrolysis by the Sar1p GTPase. (A,B) Cells co-injected with plasmids encoding Sar1p(H79G) and either lumFP (A) or GPI-FP (B). Arrowhead shows localization to the nuclear envelope. (C,D) Cells expressing lumFP (C) or GPI-FP (D) were incubated in BFA for 2 hours. Subsequently, cells were microinjected with GTP-γ-S, and BFA was washed-out for 1 hour (E,F). LumFP exits the ER in the absence of GTP hydrolysis by Sar1p (compare A and E) but GPI-FP remains exclusively within the ER (compare B and F). In addition to clear labelling of the ER, lumFP showed accumulation into punctate structures distributed throughout the cells but concentrated in the juxtanuclear region (C, enlargement, arrowheads). Bars, 10 µm.

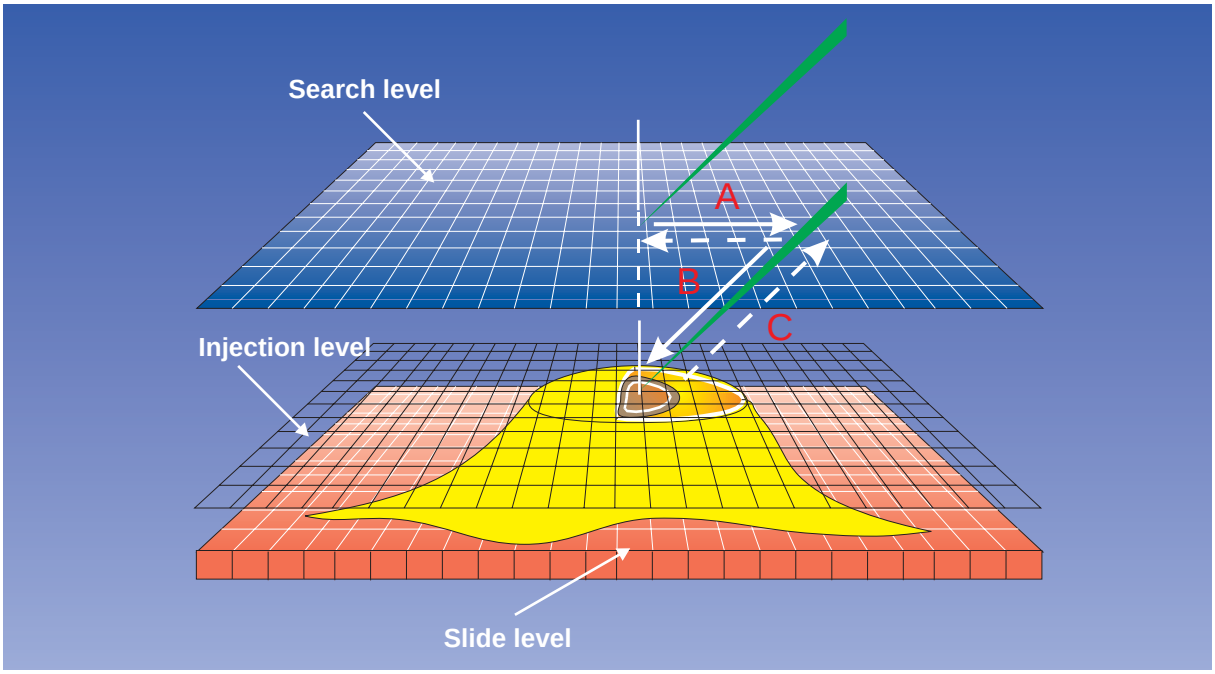

Fig. 3: Semi-automatic microinjection into adherent cells

Discussion

Microinjection of DNA constructs and cell impermeable chemicals has enabled us to dissect the GTPase requirements for the ER export event in more detail. By perturbing the function of protein complexes known to be involved in the export of proteins from the ER and their transport to the Golgi, we were able to reveal differential effects of these inhibitors on the ER export event. Our major finding was that the export of different classes of secretory cargo exhibits differential sensitivity to perturbation of the GTPase cycle of Sar1p. Expression of a Sar1p mutant that is restricted to the GTP-bound state, or direct microinjection of GTP-γ-S, both result in inhibition of export of GPI-anchored GFP from the ER while permitting the accumulation of soluble GFP expressed in the lumen of the ER into nascent export complexes, coated with COPII.

Remarks

We routinely use microinjection to facilitate the expression of proteins, notably GFP fusions, from plasmid DNA. Microinjection allows a degree of control and precision regarding expression level that we have not been able to obtain using lipid-based transfection methods. In particular, we are able to look at earlier time points after introduction of DNA, enabling us to use GFP-tagged proteins as “tracers” at the lowest possible level of expression that we are still able to image. In the experiments described here, we were also able to introduce both purified antibody fragments and chemicals that are not cell permeable. In our current work we are also using microinjection to inject a specific subset of cells, such as those close to the wound edge in scratch-wound cell polarization assays.

Semi-automatic microinjection into adherent cells

Electronic connection of the InjectMan NI 2 to the FemtoJet microinjector makes microinjection into adherent cells easy, quick and reliable. By a coordinated sequence of manipulation and injection a “semi-automatic” microinjection can be performed (Fig. 3).

First, the injection parameters (compensation pressure, injection pressure, injection time) are set on the FemtoJet microinjector. Subsequently, the capillary is lowered to the level of the cells where the injection level is determined by pressing a button (Limit) on the InjectMan NI 2. The capillary is then brought back to the search level.

By activating the joystick key, the semi-automatic injection (Fig.3, A, B) starts with an axial movement to penetrate the cells. When the capillary has reached the injection level, the microinjector automatically switches from compensation pressure to injection pressure. The injection pressure is applied to the capillary according to the preset injection time. After injection has taken place, the pressure is switched back to the compensation pressure and the capillary returns along the same path (Fig.3, C) to its starting position.

The capillary tip thus serves as a ”pointer” for the localization of the injection point in the cell. At the same time, the exact axial injection movement secures the highest survival rate. With the help of this technology it is possible to process more than 40 cells per minute simply, efficiently and reproducibly.

Literature

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Ordering information

Article	Description	Order no. international	Order no. North America
InjectMan®NI2 1)	Dynamic micromanipulator for microinjection	5181 000.017	920000029
Microscope adapter	Adapter for any inverse microscopes	available on request	
FemtoJet 1)	Programmable microinjector with integrated pressure supply	5247 000.013	920010504
Femtotip	20 sterile glass capillaries for microinjection into adherent cells	5242 952.008	930000035
Femtotip II	20 sterile glass capillaries for microinjection into adherent and suspension cells	5242 957.000	930000043
Microloader	Pipette tip for filling the microinjection capillaries, set of 2 x 96 pcs.	5242 956.003	930001007
GELoader	pipette tip for loading samples onto polyacrylamide gels, set of 2 x 96 pcs	0030 001.222	022351656
Pipette Research	0.5 – 10 µl	3111 000.122	022471902
Eppendorf 5417 R 1)	without rotor, refrigerated centrifuge	5407 000.317	022621840

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