Journal of Immunological Methods 397 (2013) 61–65

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Technical Note

Co-expression of a scFv antibody fragment and a reporter protein using lentiviral shuttle plasmid containing a self-processing furin-2A sequence

Sarah L. Appleby [a,1], Yazad Irani [a,1], Lauren A. Mortimer [a], Helen M. Brereton [a], Sonja Klebe [a], Miriam C. Keane [a], Peter J. Cowan [b], Keryn A. Williams [a,*]

[a] Department of Ophthalmology, Flinders University, Adelaide, SA 5042, Australia
[b] Immunology Research Centre, St Vincent's Hospital, Melbourne, VIC 3065, Australia

ARTICLE INFO

Article history:
Received 7 June 2013
Received in revised form 17 August 2013
Accepted 19 August 2013
Available online 30 August 2013

Keywords:
scFv
Reporter protein
Co-expression
2A self-processing sequence
Furin cleavage site
Lentiviral vector

ABSTRACT

It is often desirable to co-express a reporter protein with a potential therapeutic protein, to verify correct targeting of an expression strategy. Vectors containing a viral self-processing 2A sequence have been reported to drive equimolar expression of two or more transgenes from a single promoter. Here, we report on the co-expression of a secreted antibody fragment and an intracellular reporter protein, enhanced yellow fluorescent protein from lentiviral shuttle plasmids by inserting a furin-2A (F2A) sequence between the two cDNAs, in two different orientations, in the expression cassette. We show that the order of these two transgenes relative to the F2A sequence affects expression levels. Reduced expression of each transgene positioned downstream of F2A, compared with upstream of F2A, was observed ($p < 0.05$). Moreover, protein expression from double-cDNA plasmids was significantly lower than from their corresponding single transgene counterparts ($p < 0.05$).

1. Introduction

Lentiviral gene therapy vectors possess desirable characteristics for use in the eye, including their ability to transduce non-dividing cells such as corneal endothelium (Parker et al., 2007), and their demonstrably low inflammatory potential in ocular tissues. Lentiviral vectors have a relatively large capacity (Vigna and Naldini, 2000) and can carry more than one transgene in the expression cassette, so that much attention has been focused on the development of multigenic vectors. The benefit of such an approach is that the eventual MOI at which the vector is applied to target cells or tissues can be reduced, compared with the use of a vector cocktail. One approach to construction of multigenic lentiviral vectors is the use of a single promoter, and a viral self-processing 2A sequence positioned between each transgene (Chinnasamy et al., 2006, reviewed in de Felipe et al., 2006). The self-processing activity of the 2A sequence, which cleaves at its C-terminus through a ribosomal "skip" mechanism (Donnelly et al., 2001), has been reported to allow essentially equimolar expression in mammalian cells of all transgenes in a string, and was selected for the current study (de Felipe et al., 2006). A furin cleavage site, inserted between the upstream cDNA and the 2A sequence, has been used to cleave residual 2A amino acids from the distal end of the upstream transgenic protein (Fang et al., 2005).

Our application is gene therapy to modulate the immune response in the eye to improve experimental corneal allograft survival. Our initial approach was to transduce donor corneas, prior to transplantation, with a lentiviral vector carrying cDNA

Abbreviations: scFv, single chain antibody fragment; eYFP, enhanced yellow fluorescent protein; GFP, green fluorescent protein; MOI, multiplicity of infection; F2A, furin-2A composite sequence; HEK-293A, human embryonic kidney-293A cell line; HSD, honestly significant difference test.

* Corresponding author at: Department of Ophthalmology, Flinders University, Flinders Medical Centre, Bedford Park, SA 5042, Australia. Tel.: +61 8 8204 5047; fax: +61 8 8277 0899.
E-mail address: keryn.williams@flinders.edu.au (K.A. Williams).

[1] Contributed equally to this manuscript.

0022-1759/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jim.2013.08.012

of an immunomodulatory antibody fragment (anti-CD4 scFv) (Appleby et al., 2013). In experiments designed to confirm that the scFv was correctly expressed from the corneal endothelium, the secreted scFv was co-expressed with an intracellular reporter protein, enhanced yellow fluorescent protein (eYFP). The two transgenes, separated by a furin-2A (F2A) composite sequence (Fang et al., 2005) in different upstream–downstream orders were cloned into lentiviral shuttle plasmids (Anson and Fuller, 2003), and single gene shuttle plasmids were used for comparison. Prior to generating lentiviral vectors, transgene expression was tested by transfection of the shuttle plasmids in mammalian cells.

2. Materials and methods

2.1. Construction of shuttle plasmids

cDNAs for the two transgenes were cloned individually or in pairs into the lentiviral shuttle plasmid pHIV-1SDmSV-empty-ΔLTR, the gift of Dr D.S. Anson, Department of Paediatrics, University of Adelaide, Adelaide, Australia (Anson and Fuller, 2003). A foot-and-mouth disease virus-derived 2A sequence (APVKQTLNFDLLKLAGDVESNPGP) (Fang et al., 2005) with a furin cleavage site (RAKR) (Thomas, 2002) immediately upstream of 2A (F2A) was used to link two cDNAs by slice-overlap extension polymerase chain reaction (SOE PCR) (Fig. 1 and Supplementary Information). Plasmids were constructed with each transgene upstream or downstream of the F2A sequence. Single-transgene plasmids encoding enhanced Yellow Fluorescent Protein (eYFP) (Parker et al., 2007) or anti-rat CD4 scFv antibody fragment (scFv) (Thiel et al., 2002; Appleby et al., 2013) were used for comparative purposes (Fig. 1). For each plasmid, the integrity and orientation of the insert sequences were confirmed by DNA sequencing.

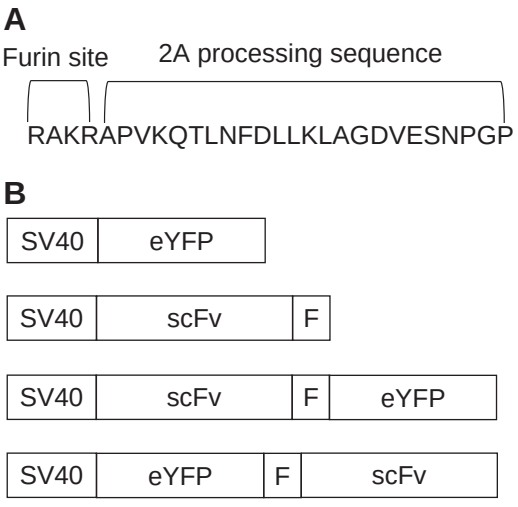

Fig. 1. The constructs. A: F2A peptide sequence composed of a furin cleavage site upstream of a foot-and-mouth disease virus (FMDV)-derived 2A self-processing sequence. B: Schematic of the open reading frame (ORF) used for construction of single transgene and double transgene plasmids. The first open reading frame was positioned upstream of the F2A sequence (F). The second open reading frame was positioned downstream of the F2A sequence (F). Transgene expression was controlled by the internal SV40 promoter in an HIV-1-based lentiviral shuttle plasmid (pHIV). Transgenes: scFv = antibody fragment in scFv format with specificity for rat CD4; eYFP = enhanced Yellow Fluorescent Protein.

2.2. Transfection of cell lines with plasmid constructs

HEK-293A cells (Qbiogene Inc., Carlsbad, CA, USA) were transfected with plasmid constructs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Four biological replicates were performed for each construct in a 6-well plate. Cells were cultured for 5 days in HEPES-buffered RPMI 1640 medium (ICN, Costa Mesa, CA, USA) supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin sulphate, 2 mM L-glutamine and 10% v/v heat-inactivated (56 °C, 30 min) foetal calf serum (all from Invitrogen, Rockville, MD, USA). The supernatant was sterile-filtered prior to assay for the secreted protein.

2.3. Quantification of cell numbers

Following removal of culture supernatant, transfected cells were incubated with fresh medium (1 ml/culture well) for 30 min at room temperature, then 100 μl MTS reagent (AQueous One Solution Cell Proliferation Assay, Promega, Madison, Wisconsin, USA) was added for 1 h. Absorbance at 490 nm of the MTS supernatant was directly proportional to the number of live cells per well and was used to normalise transgene expression levels amongst wells.

2.4. Quantification of protein expression

Levels of intracellular eYFP expression were visualised by fluorescence microscopy and quantified by flow cytometry (Parker et al., 2007). Following removal of the MTS reagent, cells were washed with Dulbecco's A phosphate buffered saline, pH 7.2 (PBS), detached from wells with 500 μl 0.05% w/v trypsin-0.02% w/v EDTA for 3 min at 37 °C, transferred to tubes, washed, fixed and counted on a FACScan flow cytometer using Cell-Quest software v3.01f (Becton Dickinson, Franklin Lakes, NJ, USA). Levels of anti-rat CD4 scFv secreted into the culture medium were measured by functional binding to CD4 epitopes on rat thymocytes, detected by flow cytometry using a 6-HIS tag on the scFv (Thiel et al., 2002). A serial dilution of bacterially-produced anti-rat CD4 scFv was used to quantify the amount of scFv present in each supernatant.

2.5. Western blotting

TRIzol-extracted protein samples (40 μg) were subjected to polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. eYFP was detected with a mixture of two anti-GFP monoclonal antibodies (Roche), followed by peroxidase-conjugated anti-mouse IgG (Chemicon) and enhanced chemiluminescence reagents (Pierce), and was visualised on a Fujifilm LAS-4000 Imager with Multi Gauge v3.0 software. Sample loading was verified using an anti-human α-tubulin monoclonal antibody (Abcam).

2.6. Statistical analysis

To identify differences in transgenic protein expression from transfected cells, analysis of variance (ANOVA) with