Gene Therapy (2004) 11, 1673–1674

www.nature.com/gt

Multiple gene products from a single vector: 'self-cleaving' 2A peptides

PA Radcliffe and KA Mitrophanous

Gene Therapy (2004) **11,** 1673–1674. doi:10.1038/sj.gt.3302361

In a recent issue of Nature Biotechnology, Andrea Szymczak[1] and co-workers report the remarkable achievement of reconstituting the T-cell receptor (TCR):CD3 complex by expressing six proteins from two retroviral vectors utilising 'self-cleaving' 2A peptides.

Most strategies for expressing more than one protein from a gene therapy vector involve the use of internal ribosome entry sites (IRES) or additional promoters. There are a number of limitations with using these elements, including their size and variability of expression. In addition, internal promoters can suffer from downregulation. Thus, these approaches are likely to result in differing amounts of the encoded proteins, which may vary significantly depending on the target cell. For some applications, the stoichiometric production of proteins is advantageous or even essential. While it may be possible to achieve this using conventional means, it would necessitate optimisation of the system by testing several combinations of promoters and/or IRESs as well as the order of genes within the expression cassette.

Szymczak *et al* set themselves the ambitious task of reconstituting the TCR:CD3 multichain complex consisting of at least eight different subunits made up from six different proteins (TCRα:TCRβ, CD3ε:CD3γ, CD3ε:CD3δ, CD3ζ:CD3ζ). This was achieved by using self-processing peptides, such as the 2A peptide of Foot-and-Mouth Disease Virus (FMDV) shown in Figure 1. These peptides allow multiple proteins to be encoded as polyproteins, which dissociate into component proteins on translation. The 2A peptide sequence impairs normal peptide bond formation through a mechanism of ribosomal skipping.[2] Szymczak *et al* demonstrate expression of the four proteins that are required to make up CD3 and the two proteins required to make up TCR using two retroviral vectors. To achieve this, four 2A sequences were used from four different viruses. All these share a conserved sequence (shown in red in Figure 1). As duplicated sequences within retroviruses can recombine, resulting in deletions of the intervening sequences,[3] the authors changed the codon usage by making silent mutations within the 2A sequences.

In vitro transcription/translation was used to demonstrate almost 100% cleavage of a polyprotein made up of CD3γ and CD3δ separated by various 2A sequences. By utilising a noncleaving 2A peptide sequence (containing two amino-acid changes in the positions underlined in Figure 1), they were able to demonstrate that the 2A peptide sequence was required for efficient generation of the two proteins. The authors also suggest in a recent addendum that the efficiency of cleavage appears to be affected by the sequences that are N-terminal to the 2A peptide.[4] This implies that the 2A peptide, when used, will require some degree of evaluation with any new proteins used. The authors suggest that this problem may be overcome by using a Gly–Ser–Gly linker between the protein N-terminal to the 2A-peptide.

Transient transfections of 293T cells were used to demonstrate that two plasmids were enough for efficient reconstitution of the TCR:CD3 complex. This requires the expression of all the six chains. The authors went on to transduce 3T3 cells with two retroviral vectors: one retroviral vector expressed all four CD3 chains (CD3δγεζ–2A.MIG) and a second vector expressed TCRα and TCRβ. Each of these retroviruses contained an IRES GFP cassette to allow for GFP sorting/analysis. Surface biotinylation was used to confirm surface localisation of all the expressed proteins.

To evaluate whether the chains generated by 2A cleavage were active, they were used to transduce haematopoietic stem cells, which were subsequently transferred into sublethally irradiated recipient mice. Stem cells were obtained from either C57BL/6 or $CD3^{\Delta P/\Delta P}$ crossed with CD3ζ−/− mice. $CD3^{\Delta P/\Delta P}$ mice lack CD3ε and have low-level gene expression of CD3γ and CD3δ, leading to an arrest in T-cell development at the $CD44^{-}CD23^{+}$ double-negative ($CD4^{-}CD8^{-}$) stage.[5] The mice generated by the $CD3^{\Delta P/\Delta P}$ crossed with CD3ζ−/− mice cross lack all four CD3 chains. Stem cells from C57BL/6 or $CD3^{\Delta P/\Delta P} \times$ CD3ζ−/− mice were transduced with retroviral vectors and transferred to $CD3^{\Delta P/\Delta P} \times$ CD3ζ−/− mice.

T-cell development was restored when stem cells from C57BL/6 transduced with empty vector or from $CD3^{\Delta P/\Delta P} \times$ CD3ζ−/− mice transduced with CD3δγεζ–2A.MIG were used. Although the total number of GFP^{+} cells were fewer in the mice receiving CD3δγεζ–2A.MIG transduced $CD3^{\Delta P/\Delta P} \times$ CD3ζ−/− stem cells compared to the empty vector transduced C57BL/6 stem cells, the proportion of GFP^{+} T cells relative to the total number of GFP^{+} cells was the same. The level of T-cell receptor surface expression, as measured by FACS analysis, was about half that of normal T cells, presumably a reflection of the activity of the retroviral MSCV LTR. The reconstituted TCR was shown to be active as stimulation with an anti-TCR antibody led to T-cell proliferation.

Finally, to determine the degree of cleavage *in vivo*, FRET analysis was carried out using cyan fluorescent protein (CFP) and yellow florescent protein (YFP). FRET is a way of assessing the proximity of two proteins within 60–100 Å. Using FRET, the authors were able to show that CFP and YFP linked with 2A peptide were efficiently cleaved in all cell types tested including T and B cells, macrophages and NK cells. Western blot analysis demonstrated equal amounts of the two proteins.

In summary, this is a comprehensive demonstration of the utility of the 2A peptide in allowing the stoichiometric production of up to four proteins from a single vector.

Figure 1 *The FMDV 2A peptide sequence. Conserved residues are shown in red. Underlined residues were mutated to alanine to make the noncleaving form.[1] The red arrow indicates the site of cleavage. The 2A and 2B arrows indicate the sequences retained in the N- and C-terminal chains, respectively.*

One outstanding question is the effect of the additional 17–20 amino acids left on the C-terminus of the upstream protein. This extra peptide may affect the activity of this protein, although this is not the case in the present study, but more importantly may cause an immune response to the transduced cells. Such concerns may not be an issue for certain applications such as generating transgenics, targeting cells in immunoprivileged sites or where the recipient is immunosuppressed. However, this will be an important area for future investigation.

The ability to express more than one protein facilitates the use of vectors for the treatment of such conditions as Parkinson's disease where up to three genes may be required for the effective production of dopamine.[6,7] In addition, other conditions such as cancer may be treatable, with a single vector expressing a number of suicide, antiangiogenic and immunogenic proteins. The ability to synthesise stoichiometric amounts of protein may be of particular value when trying to express heteromultimeric proteins such as immunoglobins.[1] ■

Dr PA Radcliffe and Dr KA Mitrophanous are at the Oxford BioMedica plc, The Oxford Science Park, Medawar Centre, Oxford OX4 4GA, UK.
E-mail: K.Mitrophanous@oxfordbiomedica.co.uk

1 Szymczak AL *et al*. Correction of multi-gene deficiency *in vivo* using a single 'self-cleaving' 2A peptide-based retroviral vector. *Nat Biotechnol* 2004; **22**: 589–594; Epub 2004, April 4.
2 Donnelly ML *et al*. The 'cleavage' activities of foot-and-mouth disease virus 2A site-directed mutants and naturally occurring '2A-like' sequences. *J Gen Virol* 2001; **82**: 1027–1041.
3 Julias JG, Hash D, Pathak VK. E-vectors: development of novel self-inactivating and self-activating retroviral vectors for safer gene therapy. *J Virol* 1995; **69**: 6839–6846.
4 Szymczak AL *et al*. Addendum: correction of multi-gene deficiency *in vivo* using a single 'self-cleaving' 2A peptide-based retroviral vector. *Nat Biotechnol* 2004; **22**: 760.
5 Wang B *et al*. T lymphocyte development in the absence of CD3 epsilon or CD3 gamma delta epsilon zeta. *J Immunol* 1999; **162**: 88–94.
6 Shen Y *et al*. Triple transduction with adeno-associated virus vectors expressing tyrosine hydroxylase, aromatic-L-amino-acid decarboxylase, and GTP cyclohydrolase I for gene therapy of Parkinson's disease. *Hum Gene Ther* 2000; **11**: 1509–1519.
7 Azzouz M *et al*. Multicistronic lentiviral vector-mediated striatal gene transfer of aromatic L-amino acid decarboxylase, tyrosine hydroxylase, and GTP cyclohydrolase I induces sustained transgene expression, dopamine production, and functional improvement in a rat model of Parkinson's disease. *J Neurosci* 2002; **22**: 10302–10312.