Expression of heterologous genes in oncolytic adenoviruses using picornaviral 2A sequences that trigger ribosome skipping

Garth M. Funston,1 Susanna E. Kallioinen,1 Pablo de Felipe,1 Martin D. Ryan2 and Richard D. Iggo1

1School of Medicine, Biomolecular Sciences Building, University of St Andrews, St Andrews KY16 9ST, UK
2School of Biology, Biomolecular Sciences Building, University of St Andrews, St Andrews KY16 9ST, UK

Insertion of picornaviral 2A sequences into mRNAs causes ribosomes to skip formation of a peptide bond at the junction of the 2A and downstream sequences, leading to the production of two proteins from a single open reading frame. Adenoviral protein IX is a minor capsid protein that has been used to display foreign peptides on the surface of the capsid. We have used 2A sequences from the foot-and-mouth disease virus (FMDV) and porcine teschovirus 1 (PTV-1) to express protein IX (pIX) and green fluorescent protein (GFP) from pIX–2A–GFP fusion genes in an oncolytic virus derived from human adenovirus 5. GFP was efficiently expressed by constructs containing either 2A sequence. Peptide bond skipping was more efficient with the 58 aa FMDV sequence than with the 22 aa PTV-1 2A sequence, but the virus with the FMDV 2A sequence showed a reduction in plaque size, cytopathic effect, viral burst size and capsid stability. We conclude that ribosome skipping induced by 2A sequences is an effective strategy to express heterologous genes in adenoviruses; however, careful selection or optimization of the 2A sequence may be required if protein IX is used as the fusion partner.

INTRODUCTION

Replication-competent oncolytic adenoviruses have been extensively tested in animals, and a virus lacking the E1B 55K gene was recently approved for cancer therapy in China (reviewed by Alemany, 2007). The main factor limiting widespread application of oncolytic adenoviruses is their low efficacy. Many groups have attempted to develop more active viruses, for example by inserting genes for toxic proteins or prodrug-activating enzymes (reviewed by Alemany, 2007). We and others have previously used internal ribosome entry sites (IRESs) to initiate the translation of prodrug-activating enzymes in adenoviruses but the results were disappointing (reviewed by de Felipe, 2002; Fuerer & Iggo, 2004; Lukashev et al., 2005). Ribosome skipping is a recently described mechanism that allows translation of multiple proteins from a single mRNA. It is based on the use of a picornaviral 2A sequence that causes the ribosome to continue translation after skipping the formation of one peptide bond. This process was first characterized in foot-and-mouth disease virus (FMDV) (Ryan & Drew, 1994). The process was originally termed ‘cleavage’ by analogy with the protease-mediated cleavages occurring at other sites in the FMDV polyprotein but this is misleading because the ‘cleavage’ results from failure to form a peptide bond during translation. Unlike reinitiation of translation by IRESs, skipping induced by 2A sequences gives approximately equal expression of the proteins upstream and downstream of the 2A site (de Felipe et al., 2006). 2A and 2A-like sequences have previously been used in biotechnology applications, but not in adenoviruses (reviewed by de Felipe et al., 2006).

Protein IX is a small cement protein located between the hexons in the capsid of the adenovirus (Furcinitti et al., 1989). It is essential for the packaging of full-length viral genomes (Ghosh-Choudhury et al., 1987). Besides its role as a structural protein, it is a transcriptional activator (Rosa-Calatrava et al., 2001; Sargent et al., 2004a) and reorganizes promyelocytic leukaemia (PML) nuclear bodies (Rosa-Calatrava et al., 2003) (reviewed by Parks, 2005). Protein IX has been extensively studied as a platform to express foreign peptides on the surface of the capsid (Dmitriev et al., 2002; Le et al., 2004; Li et al., 2005; Meulenbroek et al., 2004; Vellinga et al., 2004, 2007). Since protein IX has been so well characterized, we selected it as a fusion partner to test whether 2A sequences could be used to express foreign genes in adenoviruses. We compared the 2A sequence from FMDV with the 2A sequence from porcine teschovirus-1 (PTV-1) that has previously been used in biotechnology applications (Holst et al., 2006).
METHODS

Cell lines. 293T and HT29 cells were supplied by ATCC. C7 cells (Amalfitano & Chamberlain, 1997) were provided by Dr J. Chamberlain (Department of Biochemistry, Box 357350, University of Washington Seattle, USA). HER911 cells (Fallaux et al., 1996) were supplied by Dr P. Beard (ISREC, Ch. des Boveresses 155, CH-1066 Épalinges, Switzerland). All cells were cultured in Dulbecco’s modified Eagle’s medium (Invitro) with 10% fetal bovine serum and 1% penicillin/streptomycin.

Antibodies. Rabbit antibody against the last amino acids of the PTV-1 2A sequence (Holst et al., 2006) was provided by Dr D. Vignali (Department of Immunology, St. Jude Children’s Research Hospital, 332 N. Lauderdale, Memphis, USA). Rabbit anti-Ad5 hexon and anti-protein IX antibodies were provided by Professor W. Russell (BMS Building, North Haugh, University of St Andrews, St Andrews, Fife, UK). Rabbit anti-protein IX antibody (Caravokyri & Leppard, 1995) was provided by Dr K. Leppard (Department of Biological Sciences, University of Warwick, Coventry, UK) (this antibody was used in the anti-protein IX immunoblots shown in this report). Monoclonal mouse anti-z-tubulin antibody (clone B-5-1-1) was supplied by Sigma. Mouse anti-green fluorescence protein (GFP) antibody was supplied by Roche. Mouse anti-E1A antibody (MS8) was supplied by BD Biosciences. Peroxidase-conjugated AffiniPure goat anti-mouse IgG (H+L; 115-035-003) and anti-rabbit IgG (H+L; 111-035-003) were supplied by Jackson ImmunoResearch.

Viruses. The plIX–2A–GFP viruses are called vKM11 (FMDV 2A, ‘F2A’) and vKM31 (PTV–1 2A, ‘P2A’). The 2A sequences were inserted into the genome of vKH6 (Homicsek et al., 2002) by two-step gene replacement in yeast (Gagnebin et al., 1999). pPDF2 (unpublished) is a pcDNA3.1(+) (Invitrogen) derived vector with a hybrid cytomegalovirus (CMV)/T7 promoter and deletion of the neo cassette. pRS406 is a yeast integrating vector (Sikorski & Hieter, 1989). Overlapping protein IX–IA2 fragments of Ad5 genomic DNA (ATCC VR5) were amplified by PCR and cloned into pPDF2 (protein IX region: primers oPF3 5’-GGCCGGCTAGCATGACCACTCACTGTTGA-3’ and oPF4 5’-GTACCCTCATATTGACGAAATGCTATTGA-3’ and pRS406 (IA2 region: primers oPF7 5’-ATGCTAGATTCAATAATGTCGAGAAGGCTGACTGACGACGAAAGC-3’ and oPF8 5’-GCCAGCCGACGGGTACCCCATCATTATGGACGAA-3’) to give pPF2 and pPF3, respectively. The PTV–1 2A site was inserted into pPDF2 by inverse PCR with primers oPF5 5’-CACGCTCTTCTTGTGTTTAAACGAGAAGATCGTGTGCTTGCAAGGAAGAAGAAGAAGGCTCCTAGATGACGACCACTCACTGTTGA-3’ and oPF6 5’-TCCGACGGCCGCGCCGCGCAGATTTCCATGTGCTGCAGCTGGATTTAAAACACATTAAATA-3’). pPDF16 is an unpublished derivative of pl–P–1D2A–G from which a run of nine thyminides between FMDV 2A and GFP was deleted (de Felipe & Izquierdo, 2000, 2003). An FMDV 2A–GFP cassette was cloned from pPDF16 into pPF4 on an XbaI–NdeI fragment to give pPF5. The remaining steps in the construction of the gene replacement vectors (pPF13 for F2A, pPF14 for P2A) are shown in Fig. 1b. pPF13 and pPF14 were linearized with Sacl for insertion into vKH6 (Homicsek et al., 2005). The resulting plasmids containing the modified, full-length viral genomes were called vKM11 (F2A) and vKM31 (P2A). They were cut with PacI to liberate the viral DNA and then transfected into C7 cells to produce virus. After plaque purification on SW480 cells the viruses were called vKM11 (F2A) and vKM31 (P2A) respectively. The PTV–1 2A site was inserted into vKH6 (Homicsko et al., 2002) to liberate the viral DNA and used for construction of recombinant viruses, they were cloned downstream

RESULTS

To test whether picornaviral 2A sequences can be used to express foreign genes in adenoviruses, 2A skipping sites were inserted after the protein IX gene in an oncolytic virus that targets colon cancer cells (Fig. 1a). GFP was cloned after the 2A sequence itself (F2A) preceded by a 3 aa spacer that improves cleavage and 22 aa sequence that includes the PTV-1 2A-like sequence (Holst et al., 2001) and 39 aa from the FMDV 1D protein (Donnelly et al., 2001) for insertion into an oncolytic virus that targets colon cancer cells (Fig. 1a). GFP was cloned after the 2A sequence itself (F2A) preceded by a 3 aa spacer that improves cleavage and 22 aa sequence that includes the PTV-1 2A-like sequence (Holst et al., 2006). The size of the plaques was measured using ImageJ (NIH, Bethesda, MD). A minimum of 22 plaques were measured per virus. Error bars are SEM. Photographs of individual plaques were taken with a Nikon Coolpix 990 camera, using mCherry filters (49008; Chroma) for propidium-stained cells and GFP filters (49002; Chroma) for viral GFP-expressing cells, on an Olympus CKX41 microscope.

Expression of protein IX and GFP from plIX–2A–GFP fusion genes

To test the plIX–2A–GFP cassettes in vitro before construction of recombinant viruses, they were cloned downstream

(TNT T7 system; Promega). Translation reactions performed in a total volume of 7 μl were programmed with 0.1 μg plasmid DNA and incubated in the presence of [35S]methionine [5 μCi (185 kBq); GE Healthcare] at 30 °C for 90 min. Reactions were analysed by autoradiography of SDS-PAGE gels.

Immunoblotting. To test the protein IX expression cassette, 293T cells were transfected with pPF2, pPF5 and pPF6, and harvested 24 h later. To test protein expression from viruses, SW480 cells were infected with vKH6, vKM11 and vKM31 at m.o.i. 0.5 p.f.u. per cell, the medium was changed after 4 h and the cells were harvested 12 h later. Nitrocellulose membranes were probed with primary antibodies against E1A, hexon, protein IX, 2A, GFP and tubulin, followed by secondary antibodies coupled to horseradish peroxidase and developed by using enhanced chemiluminescence (GE Healthcare).

Measurement of plaque size. HER911 cells were infected at an m.o.i. of 20 p.f.u. per well in six-well plates and covered with agar. The wells were stained with propidium iodide and pictures were taken 10 days after infection. The size of the plaques was measured using ImageJ (NIH, Bethesda, MD). A minimum of 22 plaques were measured per virus. Error bars are SEM. Photographs of individual plaques were taken with a Nikon Coolpix 990 camera, using mCherry filters (49008; Chroma) for propidium-stained cells and GFP filters (49002; Chroma) for viral GFP-expressing cells, on an Olympus CKX41 microscope.

RESULTS

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Expression of protein IX and GFP from plIX–2A–GFP fusion genes

To test the plIX–2A–GFP cassettes in vitro before construction of recombinant viruses, they were cloned downstream
of a hybrid CMV/T7 promoter in a plasmid expression vector (pPF5 and 6, Fig. 1b). Skipping at the arrows shown in Fig. 1(a) should lead to the formation of protein IX with the 2A site at the C terminus and GFP with a single additional proline at the N terminus. In vitro transcription/translation in the presence of 35S-labelled methionine was used to study in vitro skipping at the 2A sites. Both 2A constructs showed efficient skipping to yield GFP and protein IX isoforms of the expected size (Fig. 2a; the wild-type protein IX, pIX–P2A and pIX–F2A proteins are predicted to be 14, 17.5 and 21 kDa, respectively). Fig. 2(a) also shows protein IX expressed from a construct containing

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**Fig. 1.** (a) Schematic diagram showing the genome organization of the pIX–F2A–GFP and pIX–P2A–GFP viruses (vKM11 and vKM31). The viruses contain Tcf sites in the inverted terminal repeats and the E1B promoter. There is an RGD motif in the fiber HI loop. The protein IX region has been mutated to contain pIX–2A–GFP fusion genes with the indicated 2A sequences. The 2A skipping site is marked with an arrow. (b) Schematic diagram showing the cloning strategy. The final plasmids shown, pPF13 and pPF14, are yeast integrating vectors that were used to insert the pIX–2A–GFP cassettes into plasmids containing the entire viral genome (yeast artificial chromosome/bacterial artificial chromosomes, Gagnebin et al., 1999).
just pIX–P2A without a downstream open reading frame (pPF4, Fig. 1b). The pIX–P2A protein expressed from this construct is the same size as the pIX–P2A protein expressed from the pIX–P2A–GFP construct. Small amounts of pIX–2A–GFP fusion proteins can also be seen (Fig. 2a). We conclude that ribosomal skipping occurs at the 2A site in the absence of exogenous viral proteins yielding proteins of the expected size, as described previously (Donnelly et al., 2001; Holst et al., 2006).

To test whether skipping occurs in vivo, 293T cells were transfected with the fusion constructs, and cell lysates were immunoblotted for protein IX, 2A and GFP. There was a prominent GFP band after Coomassie blue staining of the gel or Ponceau S staining of the membrane (data not shown), indicating that this is an efficient expression strategy. Immunoblotting for 2A confirmed the presence of the 2A sequence in the pIX–2A proteins (Fig. 2b; since the antibody prefers the P2A epitope, the relative abundance of the two forms cannot be assessed from this blot). Skipping at the P2A site was less efficient than at the F2A site, as shown by the presence of more pIX–2A–GFP fusion protein, but in both cases the amount of GFP was far greater than that of the fusion proteins (Fig. 2b). We conclude that the pIX–2A expression strategy gives good expression of GFP.

To test skipping in the context of the virus, the pIX–2A–GFP cassettes were cloned into an oncolytic viral genome by two-step gene replacement in yeast (Gagnebin et al., 1999). The parental virus, vKH6, has an RGD peptide in the HI loop of the fiber protein and Wnt-responsive (Tcf/LEF) transcription factor-binding sites in the E1A, E1B and E4 promoters (Homicsko et al., 2005). The viruses were produced in C7 cells, plaque purified on SW480 colon cancer cells to reduce the risk of unwanted recombination events, expanded on SW480 cells and purified by two rounds of equilibrium density-gradient centrifugation.

Fig. 2. (a) In vitro transcription/translation of protein IX, pIX–P2A, pIX–P2A–GFP and pIX–F2A–GFP proteins. Transcription/translation reactions were programmed with plasmids pPF5 (F2A) and pPF6 (P2A, see Fig. 1b). (b) Expression of protein IX, pIX–P2A–GFP and pIX–F2A–GFP genes after transfection of 293T cells with plasmids expressing protein IX (pPF2), pIX–P2A–GFP (pPF6) and pIX–F2A–GFP (pPF5) from the CMV promoter. Western blots were probed with anti-protein IX, anti-2A and anti–GFP antibodies as indicated. Tubulin was used as a loading control. Note that the anti-2A antibody has a higher affinity for the P2A than the F2A epitope. (c) Western blots showing the presence of pIX–2A proteins in virions. Blots of purified virus were probed with anti-protein IX and anti-2A antibodies. Ad5 hexon was used as a loading control. (d) Expression of protein IX and GFP after infection of SW480 cells with vKM11 (pIX–F2A–GFP), vKM31 (pIX–P2A–GFP) and vKH6 (parental virus). Cells were harvested 16 h after infection. Western blots were probed with anti-protein IX, anti-2A, anti-GFP and anti-E1A antibodies. Tubulin was used as a loading control.
Immunoblotting of the purified virions for 2A (Fig. 2c) confirmed the presence of the 2A epitope in the pIX–F2A and pIX–P2A proteins of the vKM11 and vKM31 viruses, respectively; the weaker F2A signal is expected given the higher affinity of the antibody for the P2A epitope. The ratio of protein IX to hexon was similar for the parental virus, vKH6, and the two progeny viruses, vKM11 (pIX–F2A–GFP) and vKM31 (pIX–P2A–GFP). The pIX–F2A protein in the virion showed signs of degradation, with the appearance of lower bands (Fig. 2c, asterisk). Only trace amounts of the pIX–2A–GFP proteins were present, suggesting that the viruses may preferentially incorporate pIX–2A protein into the capsid.

The ability of the viruses to express the pIX–2A and GFP proteins correctly was tested by infecting SW480 cells at an m.o.i. of 0.5 p.f.u. per cell. Cells were harvested 16 h after infection to avoid losing cells that detached from the plate. Immunoblotting for E1A showed that the early steps of infection were not affected by the presence of the pIX–2A proteins in the capsid (Fig. 2d). There was more pIX–2A–GFP protein present after infection with the P2A virus, again suggesting that skipping is less efficient with the P2A sequence. The level of the pIX–F2A protein was substantially higher than that of either the wild-type or P2A proteins (Fig. 2d). The same trend was visible after transfection of the CMV expression vectors (Fig. 2b). In both cases the ratio of GFP to pIX–P2A–GFP was much higher than the ratio of protein IX to pIX–P2A–GFP, which was close to one. The reason is unclear, since all three proteins initiate from the same ATG and have long half-lives. Despite these differences, both 2A viruses expressed GFP in equal amounts. We conclude that, consistent with findings in other biological systems, 2A sequences allow efficient expression of transgenes in adenoviruses.

**pIX–F2A capsids are unstable**

The pIX–F2A virus was more difficult to produce than the pIX–P2A virus or the parental virus. This suggests that the pIX–F2A protein may be partially defective. Visual inspection showed that pIX–P2A plaques were generally larger than pIX–F2A plaques (Fig. 3a). To pursue this we measured plaque size by staining dead cells with propidium iodide and measuring the surface area of plaques photographed through mCherry filters (Fig. 3b). This showed that the pIX–F2A virus forms significantly smaller plaques than the other viruses (Fig. 3b). To test whether the
pIX–F2A virus is less cytopathic than the other viruses, SW480 cells were infected with log dilutions of virus. SW480 cells are highly permissive for the parental virus, vKH6, because they have high Tcf activity, leading to strong activation of Tcf-regulated promoters. The CPE of the pIX–P2A virus (vKM31) was similar to that of the parental virus, whereas that of the pIX–F2A virus (vKM11) was reduced 10-fold (Fig. 3c). To identify the reason for the reduction in activity of the pIX–F2A virus, a burst assay was performed. SW480 cells were infected at an m.o.i. of 0.1 p.f.u. per cell and virus was harvested after 48 h. The burst size of the pIX–P2A virus was twofold lower than that of the parental virus, whereas that of the pIX–F2A virus was reduced 90-fold (Fig. 3d). The CPE assay was performed in conditions where several cycles of infection were required, so the difference in burst size is a potential explanation for the reduction in CPE. Viruses defective in protein IX function rapidly lose activity at mildly elevated temperatures. To test whether this might have contributed to the reduction in CPE of the pIX–F2A virus, aliquots of virus were heated to 45 °C and plaque assays were performed to measure the decline in titre. This showed a marginal difference in stability of the pIX–P2A virus compared with the parental virus, and a large reduction in stability of the pIX–F2A virus (Fig. 3e). We conclude that addition of the FMDV 2A sequence to the C terminus of protein IX interferes with protein IX function and this leads to a reduction in CPE, burst size and stability of the capsid.

**DISCUSSION**

We have shown that picornoviral 2A sequences can be used to express transgenes in oncolytic adenoviruses. Both 2A sequences tested gave efficient GFP expression, but there were important differences in the activity of the viruses. The fusion partner, protein IX, has been extensively investigated as a site for expression of foreign peptides on the capsid of adenoviruses (Vellinga et al., 2005b). Indeed, entire scFv peptides have been successfully expressed on the capsid as protein IX fusions (Vellinga et al., 2007). It was therefore unexpected that the addition of a 57 aa sequence derived from FMDV to protein IX should attenuate the virus. Several results point to a loss of activity of the pIX–F2A protein. The viral burst size and CPE activity were both reduced at least 10-fold relative to the parental virus. Protein IX acts at multiple stages in the viral life cycle, including transactivation of early promoters and stabilization of the capsid (Parks, 2005). The defect could be a consequence of defective transactivation, but loss of transactivation by protein IX is thought to have only a small effect on the virus compared with the dramatic destabilization of capsids lacking protein IX. The pIX–F2A virus was temperature sensitive, so a capsid defect is a likely explanation for the other defects: the CPE assay and plaque size assay require prolonged incubation of infected cells at 37 °C, during which time an unstable virus could lose activity. The same could be true of the burst assay, which was harvested after 48 h, but the magnitude of the reduction was so great that other effects of protein IX should be considered. The phenotype may be similar to that of a recently reported protein IX deficient virus with a severe defect in viral burst size that could not be solely explained by a defect in capsid stability (Sargent et al., 2004b). The FMDV 2A sequence used was longer than the PTV-1 sequence and included part of the FMDV 1D capsid protein. It is possible that the 1D sequence imposed a quaternary structure on protein IX incompatible with its normal functioning or that the 1D sequence binds to the protein IX N terminus, perturbing the interaction with hexon.

Despite the effect of the FMDV peptide on protein IX function, peptide bond skipping at the F2A site was highly efficient, with only trace amounts of pIX–F2A–GFP protein produced. The PTV-1 2A-like sequence induced less efficient skipping, as shown by the presence of larger amounts of the pIX–P2A–GFP protein. This may not be important, given the apparently equal GFP expression from the two viruses and the fact that the P2A sequence had little, if any, effect on protein IX function. Careful examination of the relative expression levels of the proteins produced by the two protein IX expression cassettes reveals a conundrum. Since translation of both cassettes initiates at the protein IX ATG and the amount of the downstream protein (GFP) is similar, one would expect to see similar amounts of protein IX in both cases. This is clearly not the case. The pIX–F2A protein is present in larger amounts than the pIX–P2A protein, whether it is expressed from the virus or from the CMV promoter in a transfected plasmid. The ratio of pIX–P2A–GFP to pIX–F2A–GFP is the same with anti-protein IX antibody and anti-GFP antibody, so we can rule out differences in antibody affinity as an explanation for the greater amount of the pIX–F2A protein. Despite the lower skipping activity of the P2A sequence, the pIX–2A–GFP to GFP ratio strongly favours GFP in both cases, whereas the pIX–P2A–GFP to pIX–P2A ratio is almost one. In other words, there is much less pIX–P2A than expected. Compared to the parental virus, however, the pIX–P2A level is normal and it is the pIX–F2A protein that is too abundant. These differences cannot be explained by a difference in the half-life of the proteins (data not shown), or an idiosyncrasy of the anti-protein IX antibody, since they are also seen with a different anti-protein IX serum (data not shown). Partial explanations might include premature activation of the protein IX promoter in the pIX–F2A virus or, conceivably, sequestration of the pIX–F2A protein in a form that escapes Western blotting. Despite the strong evidence that protein IX tolerates the addition of many different heterologous peptides to its C terminus (Vellinga et al., 2006, 2007), addition of the F epitope tag has been shown to prevent accumulation of protein IX in nuclear inclusion bodies (Rosa-Calatrava et al., 2001). It is possible that the FMDV 2A site has a similar effect and that this contributes to the increase in pIX–F2A protein level.
Based on these results, future development of this approach, if protein IX is used as the fusion partner, should be based on the PTV-1 sequence. The use of protein IX as a platform for modification of the adenoviral capsid has been extensively studied in the context of pseudotyped replication-defective vectors (Vellanga et al., 2004, 2005a, b, 2006, 2007). Interference with protein IX function did not emerge as a major issue in those studies, perhaps in part because replication-defective vectors are less sensitive to mild defects in viral functions. One important conclusion from that work was that the addition of spacers to lift peptides above the surrounding hexons greatly improves the exposure of epitopes added to the protein IX C terminus. It may be that addition of a long spacer between protein IX and F2A would prevent interference of F2A with protein IX function. We chose not to do this because the goal was to prevent interference of F2A with protein IX function. There are many other potential fusion partners in an adenovirus, so there should be no difficulty using the 2A sequences to express transgenes in adenoviruses if protein IX proves unsatisfactory.

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