Effects of C-Terminal Truncations of the Murine Cytomegalovirus Chemokine Receptor Homologue M33 Upon Signalling and Cellular Localisation

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ABSTRACT

The Murine cytomegalovirus (MCMV) M33 gene is a homologue of a host G protein-coupled receptor conserved throughout the CMVs. Previous studies have demonstrated the importance of viral chemokine receptor homologues in immune evasion, virus dissemination and virus replication. In a previous study of the M33 C-terminus determined a number of regions that were found to be important for cellular localisation and constitutive signalling. In this study we constructed a number of C-terminal truncation mutants of M33 through PCR mutagenesis in order to map regions of M33 that have roles in trafficking and signalling. The results indicate that the region between $C^{321}$and L^{326} contains residues important for trafficking of M33 while region between R^{340} and A^{348} contains residues important for signalling.

INTRODUCTION

Human cytomegalovirus (HCMV), a member of the betaherpesvirus family is characterised by its ability to set up life-long latent infections following a primary infection (10). This ubiquitous pathogen is extremely prevalent with 40-90% (country dependent) of the population infected (15, 17). In a 2006 national serosurvey it was reported that the population-weighted rate of HCMV in Australia was 57% (15). While HCMV infections are most commonly asymptomatic, in immunocompromised hosts they are associated with severe pathogenicity including atherosclerosis, pneumonia, retinitis, colitis and graft rejection (5, 11, 15). The immunologically immature are another risk group with HCMV being the most common cause of congenital infection resulting in hearing and vision impairments along with mental retardation (11, 12, 15, 17).

Herpesviruses, such as HCMV, have co-evolved with their hosts over millions of years. This close association is highlighted by mimicry whereby the virus encodes homologues of host proteins (13, 17). Each of the viruses within the betaherpesvirus family encode homologues of chemokines and chemokine receptors including G protein-coupled receptors (GCPRs) which are believed to have important functions in immune evasion, virus dissemination and virus replication (5, 7, 10, 14, 21).

Four GPCR homologues have been described have been described in HCMV; UL33, U78, US28 and US27(3, 5, 6). While US28 and US27 are believed to be restricted to primate CMVs, UL33 and UL78 are highly conserved across all betaherpesviruses (3, 5). UL33 is of particular interest in current research. Murine cytomegalovirus (MCMV) and rat cytomegalovirus (RCMV) homologues of UL33 have been identified, M33 and R33 respectively (5, 8, 10). Because the M33 homologue is so highly conserved (47% sequence identity) it has been targeted as a model for UL33 (8).

Studies of MCMV indicate that M33 has an essential role in replication in and dissemination to the salivary glands as well as virus-induced smooth muscle migration(5, 8, 10). These results were further confirmed in studies using the RCMV homologue, R33(1). Constitutive signalling, a notable feature of many virally encoded chemokine receptor homologues including M33, is believed to be involved in the dissemination/replication in salivary glands and also contributes to efficient reactivation from latency in a mouse model (4, 5, 13, 16, 17).

In order to identify what portions of the M33 GPCR are important for constitutive signalling and translocation to the membrane, Case et al. (2008) designed a number of mutagenesis experiments that targeted specific regions of the protein. When C-terminal deletions of 38 (M33ΔC38) and 57 (M33ΔC57) amino acids were made, constitutive signalling assessed via CRE- and NFAT-mediated transcription was inhibited (5). Furthermore using confocal microscopy of M33-GFP fusion proteins, cell surface localisation was not detectable with the M33ΔC57 mutant(5).

Through the construction of a series of C-terminal truncation mutants, this experiment aims provide finer mapping of regions contributing to signalling and cell-surface expression. Fluorescence imaging will be used to analyse the trafficking of M33, while CREB-mediated transcription factors will be used to examine signalling properties.

MATERIALS AND METHODS

Primer name	Sequence (5'-3')	Orientation	Restriction site(s)
pcDNA3f	GGAGAGCTCTCTGGCTAACTA	+	
M33CΔ29r	CGGGAATTCAGAGGGCCCCGGCGCGGTGTGGCGTC	–	(EcoRI)/ApaI
M33CΔ34r	CGGGAATTCAGAGGGCCCGCGTCGAGGGCGAGCGC	–	(EcoRI)/ApaI
M33CΔ45r	CGGGAATTCAGAGGGCCCGCATGCGGCGCCGACTG	–	(EcoRI)/ApaI
M33CΔ51r	CGGGAAT TCAGAGGGCCCAGAGCTTGCCAGTTATGCATT	–	(EcoRI)/ApaI

Table 1: PCR primers used in this study for the production of M33 C-terminal truncation mutants for incorporation into both tagged and untagged GFP constructs.

Primer Design. The forward primer (conserved across each of the PCR reactions) binds to pcDNA3 upstream from the start of M33 utilising the EcoRI site for subsequent cloning. The reverse primers used in these experiments were designed to allow for incorporation into both GFP tagged and untagged constructs. This was achieved by incorporating two restriction sites, an ApaI site upstream from the stop codon and an EcoRI site downstream from the stop codon for tagged and untagged constructs respectively. Primers used are shown in Table 1 while the cloning strategy is illustrated in Figure 1. In the case of the untagged construct an additional three amino acids (Gly Ala Leu) were incorporated into the reverse primer as a result of the upstream ApaI site. We don't believe that these amino acids will adversely affect the results.

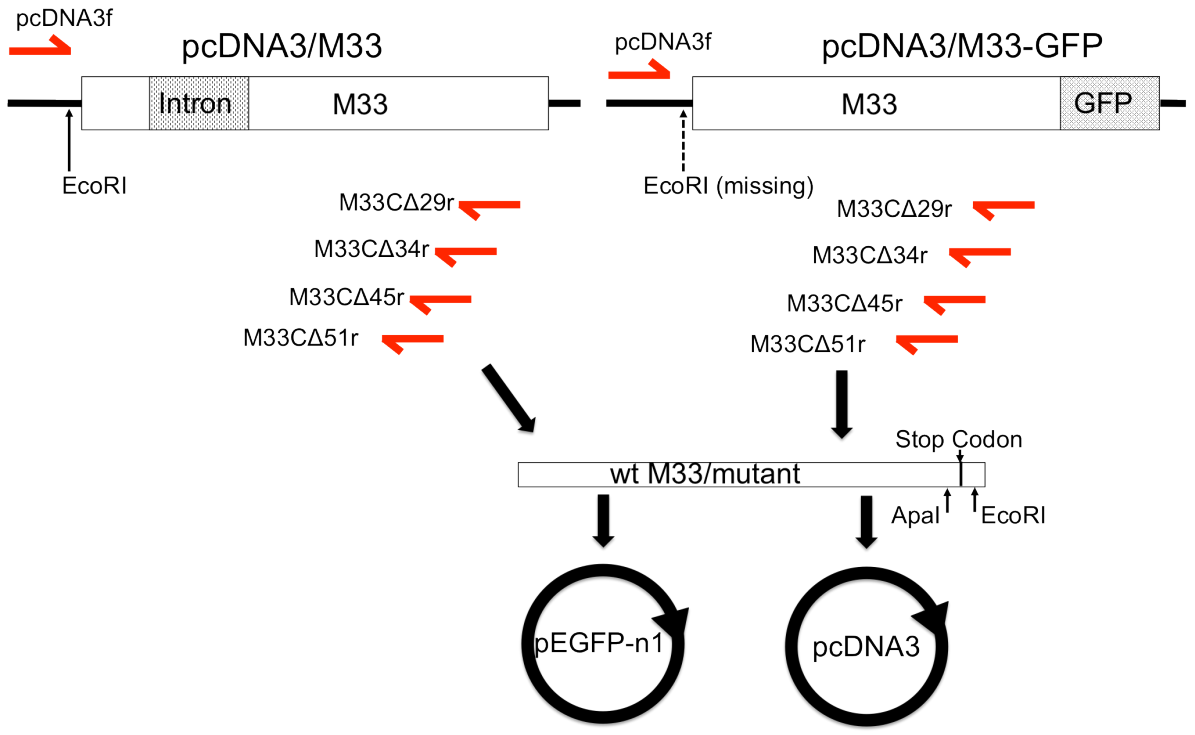

Figure 1. Cloning strategy (not to scale). PCR mutagenesis used to produce M33 C-terminal deletions. The forward primer (pcDNA3f) binds upstream of M33 on the template strands. The reverse primers bind at four different locations amplifying M33ΔC29, M33ΔC34, M33ΔC45 or M33ΔC51 mutants. Incorporated into each of the reverse primers is; an ApaI site upstream of stop codon for incorporation into pEGFP-n1 tagged construct for the cellular localisation experiments and an EcoRI site downstream of the stop codon for incorporation into pcDNA3 untagged construct for signalling assays.

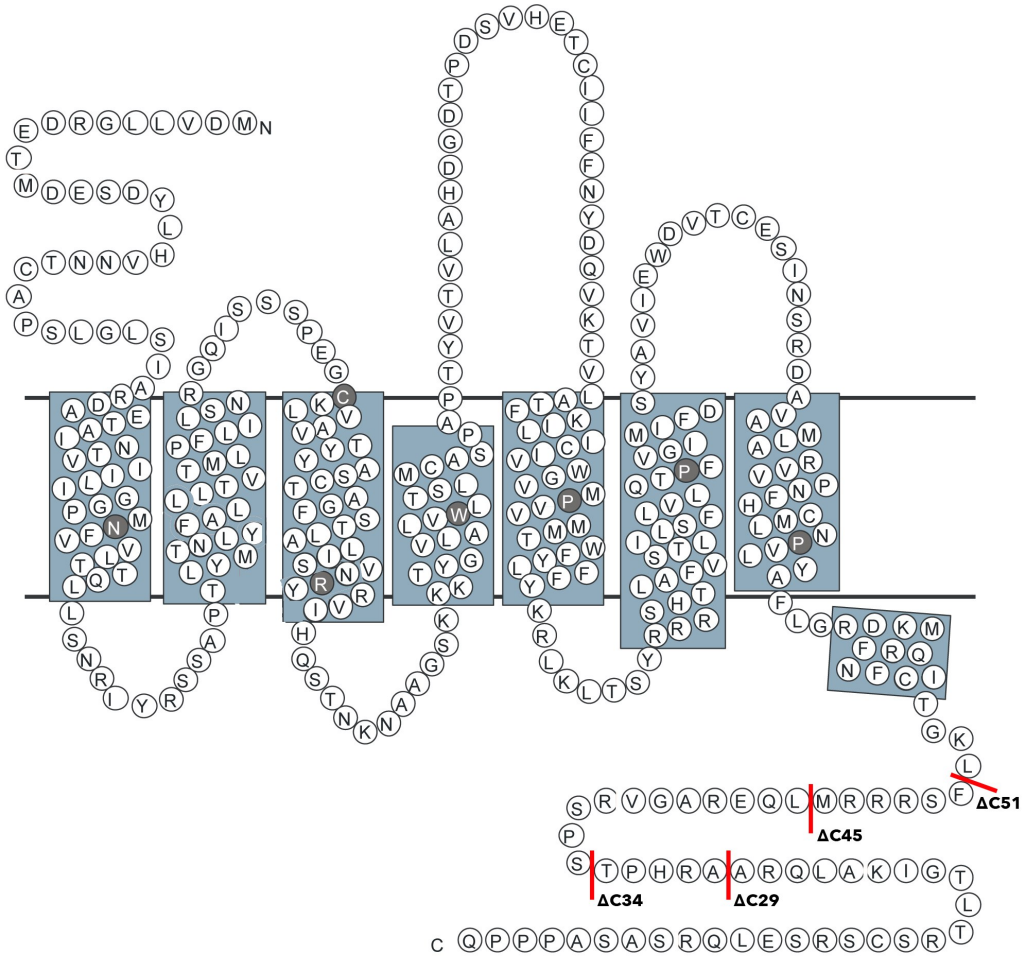

Figure 2. Serpentine model of MCMV M33 indicating positions of C-terminal truncations. Adapted from Case et al. (2008). This represents a prediction of the topology of M33 within the cell membrane. Shading represents the trans membrane regions (I to VII) and the eighth predicted α-helix. The M33 sequence presented is that of the K181 (Perth) strain (GenPept accession number CAP08079). The grey circles with white letters show conserved residues among rhodopsin-like 7TMRs. The red lines indicate positions of C-terminal deletions of M33 mutants used in this study.

Plasmids for signalling assays. PCR mutagenesis using standard PCR procedure was used to produce various M33 C-terminal deletion mutants from a pcDNA3/M33-GFP template strand with the M33 intron spliced out. Refer to Figure 1 and 2 for cloning strategy and M33 mutants. PCR amplified wild-type M33 and mutants were purified using the High Pure PCR Product Purification Kit (Roche). Amplified M33 and mutant fragments were cloned into pcDNA3 (Invitrogen) using the EcoRI site incorporated via the reverse primer at the 3' end and what was believed to be an EcoRI site from the template strand at the 5' end. Subsequent sequence analysis has revealed that the pcDNA3/M33-GFP template did not have an EcoRI site at the 5' end where indicated on the plasmid map. We believe that EcoRI star activity cut the amplified M33 cassettes upstream and by chance ligated into the pcDNA3 plasmid at extremely low efficiency.

Plasmids for fluorescence imaging studies. A similar procedure to the one outlined above in "Plasmids for signalling assays" was used to produce various M33 C-terminal deletion mutants. However in this case a pcDNA3/M33 template strand containing the intron was

used. Refer to Figure 1 and 2 for cloning strategy and M33 mutants. PCR amplified wild-type M33 and mutants were purified using the High Pure PCR Product Purification Kit (Roche). The wt M33 and deletion mutants were cloned into ApaI/EcoRI digested pEGFP-N1 (BD Biosciences, Oxford, United Kingdom) using the ApaI site incorporated via the reverse primer at the 3' end and the EcoRI site from the template strand at the 5' end.

Isolation and purification of recombinant plasmids. Recombinant pcDNA3/M33 and pGEFP-N1/M33 plasmids were transformed into DH5-α derivative *E. coli* cells (NEB) along with pUC and no insert controls and plated on 2TY agar plates overnight. The plates were supplemented with either ampicillin (100μg/ml) or kanamycin (50μg/ml) for selection of pcDNA3 and pEGFP-N1 derived clones respectively.

For the pcDNA3/M33 cloning experiment, the plasmids from randomly selected colonies to be screened were isolated using a bench mini prep method (Refer to Appendix 1). Initial restriction digests with NruI were used to identify possible positive clones, subsequent digests with PstI and SalI were used to confirm. Sequencing of the positive clones identified was conducted to confirm identity of clones and ensure the absence of any potential mutation that could alter the outcomes of the study.

For the pEGFP-N1/M33 cloning experiment, because of good transformation results and unidirectional cloning, no restriction digest checks were believed to be necessary. Sequencing checks of two clones for each was undertaken using NucleoBond Xtra Midi kit (Macherey-Nagel) for plasmid isolation and purification. The same kit was also used for the plasmid purification for use in signalling and fluorescent imaging studies.

CREB-mediated transcription assay. HEK-293 cells were grown in Dulbecco's modified Eagle's medium (DMEM)-GlutaMAX (Invitrogen) supplemented with 10% heat inactivated fetal calf serum, 180 U/ml penicillin, and 45 μg/ml streptomycin at 37°C and 5% CO_2. Cells were seeded (5×10^4 cells/well) in 96-well black walled isoplates (PerkinElmer Life and Analytical Sciences) without antibiotics. One day after seeding, the cells were cotransfected with test plasmids at varying concentrations and 200ng of pCRE-Luc PathDetect cis-reporter plasmid (Stratagene, La Jolla, CA, USA). Four concentrations of the constructed mutants along with two previously constructed mutants, wild type M33 and empty vector (pcDNA3) were tested (0.6, 1.25, 2.5 and 5 ng/μL) along with a no plasmid control for each sample. The no plasmid results were averaged within each plate of samples, and used as the baseline. Four replicates of each sample were tested with averaged calculated. Transfections carried out using OptiMEM and Lipofectamine 2000 (Invitrogen) following guidelines supplied by manufacturer. 24hours post transfection Steady Lite substrate (PerkinElmer Life and

Analytical Sciences) was added to the cells. Luminescence was measured on a Wallac Microbeta Trilux luminometer.

Fluorescent imaging. HeLa cells were seeded onto coverslips in 24 well trays, using 0.5ml at a density of 2×10^5 cells/ml in culture medium comprising MEM-Glutamax (Invitrogen) with 10% foetal calf serum (In Vitro Technologies). After overnight incubation, cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. 24 hours post-transfection coverslips were processed for cell-surface staining using AlexaFluor 594 conjugated Wheat Germ Agglutinin (ImageIt Live (Invitrogen), according to manufacturer's instructions), rinsed twice with HBSS, fixed (4% paraformaldehyde in HBSS (Invitrogen), 15 mins, room temp), rinsed in HBSS then mounted using ProlongGold (Invitrogen). Slides were examined using a Leica TCS SP2 confocal microscope fitted with a 63× objective, and images were acquired with the Leica confocal software using a zoom factor of ×2.5. Images were imported into Adobe Photoshop CS2, version 9, and overlays of the GFP and Alexa Fluor 594 images were produced.

RESULTS

Cloning C-terminal truncation mutants of M33. In order to identify regions on the C-terminus of the M33 critical for cellular localisation and constitutive signalling mutants with C-terminal truncations were constructed as outlined in Figure 2. Two alternative template strands (one containing intron one with intron absent) were used in the formation of these mutants. There were two reasons for this, to protect against unsuccessful PCR amplification and allow for the possibility of further *in vivo* studies where intron is required. The signalling studies were undertaken with M33 mutants from template without intron while the cellular localisation studies used M33 mutants from the template with intron. Previous studies have demonstrated the intron does not have a significant effect upon expression levels in transient transfection. The PCR products appear to be of the correct length indicating successful amplification (see Figure 3). Examination of band sizes reveals distinct variances in size between PCR products from template without intron, correlating to expected truncation lengths. Those amplified from the template with intron are slightly bigger due to presence of intron and thus no distinct size variation between mutants can be observed. A digestion check of the vector plasmids to which the purified inserts were ligated into was also undertaken. As demonstrated in Figure 3, pEGFP-N1 (used in cellular localisation studies) was successfully digested first with EcoRI and then with ApaI and pcDNA3 (used in signalling studies) was successfully digested with EcoRI. Some partial digestion was noted in the pcDNA3 digest but not considered significant.

In the first attempt at transformation extremely poor results were observed with less than 7 colonies grown. A restriction digest check with SalI offered no successful clones (results not shown). A new plasmid/insert preparation was prepared at this time. In subsequent transformation significantly higher colony numbers were observed with >70 in most tests. However similar numbers were observed in the no insert control. An initial NruI digest revealed a number of potential clones (results not shown) but secondary digests with PstI and SalI only confirmed the identity of two positive clones 195-6 (M33CΔ29), 197-18 (M33CΔ45), refer to Figure 3.

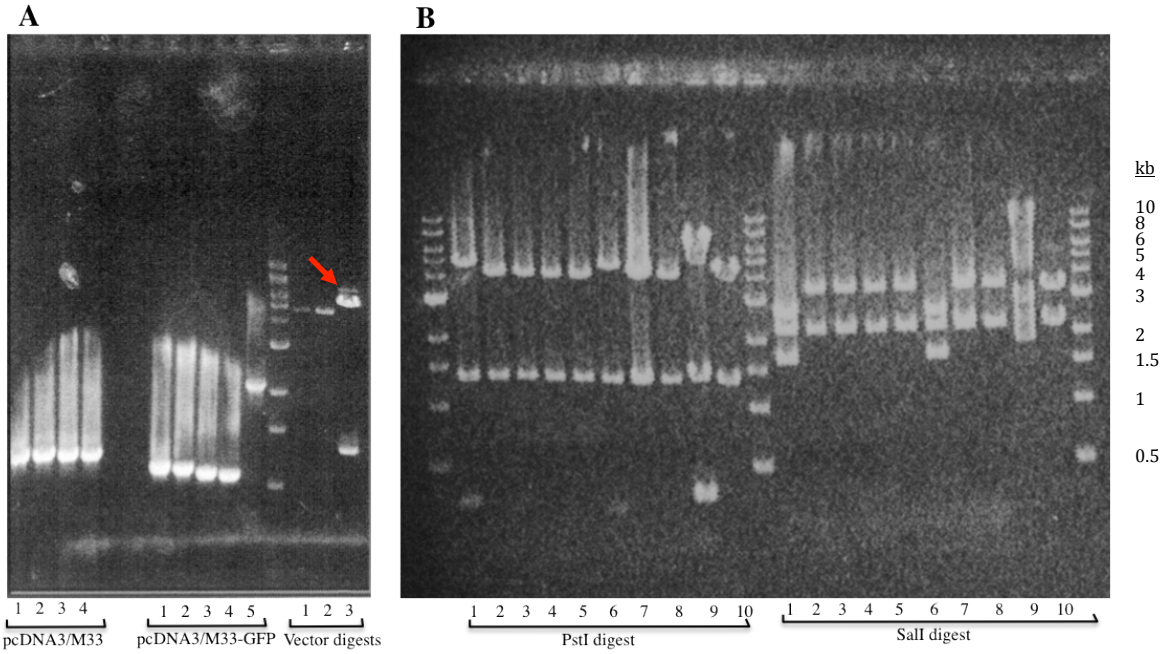

Figure 3. Gel analysis of steps in cloning. A: Amplified mutant M33 PCR products and Digested plasmid vectors. The four lanes in the pcDNA3/M33 group show M33 mutants amplified from template with intron that was used in cellular localisation study. pcDNA3/M33-GFP group contains M33 mutants amplified from template without intron, used in signalling studies. The fifth lane of this group contains a PCR control. Order of mutants in both groups is; M33ΔC29, M33ΔC34, M33ΔC45 and M33ΔC51 respectively. To ensure presence of restriction sties in pEGFP-N1 it was digested with ApaI (lane 1) and EcoRI (lane 2). For pcDNA3 check it was digested with EcoRI (lane 3). Red arrow indicates partial digestion of pcDNA3. Samples run out on ethidium bromide stained agarose gel. B: RE digest check of selected transformants for signalling studies. DH5α derivative cells transformed with pcDNA3/M33 mutants. Plasmids of selected colonies isolated through bench mini prep method (Appendix 1) and digested with PstI and SalI. Clones 1 and 6 (M33ΔC29 and M33ΔC45 respectively) positive by both digests. Lanes 9 and 10 in both digests contain a positive (pcDNA3/M33) and negative (pcDNA3) control respectively. Samples run out on ethidium bromide stained agarose gel. Ladder used in both gels is a 1kb DNA ladder.

Sequence analysis. Sequencing was employed to confirm the identity of each clone and absence of any detrimental mutations. For one of the pEGFP-N1/M33ΔC29 clones a missing residue at the beginning of the EGFP region resulted in a frameshift mutation (refer to Figure 4) inhibiting the expression of green fluorescent protein. The second pEGFP-N1/M33ΔC29 clone was absent of the mutation.

Sequence analysis of the two successful pcDNA3/M33 mutants (pcDNA3/M33ΔC29 and pcDNA3/M33ΔC45) revealed unexpected results. The results show that the M33 mutant inserts amplified from the pcDNA3/M33-GFP template did not contain the 5' EcoRI site expected. It is thought that EcoRI star activity cut the amplified M33 cassettes upstream and by chance ligated into the pcDNA3 plasmid at extremely low efficiency. Subsequent analysis of pcDNA3/M33-GFP template showed that it did not have an EcoRI site upstream of M33 where indicated on the plasmid map (results not shown). Despite this there seems to be no disruption to the start of M33 as outlined in Figure 5.

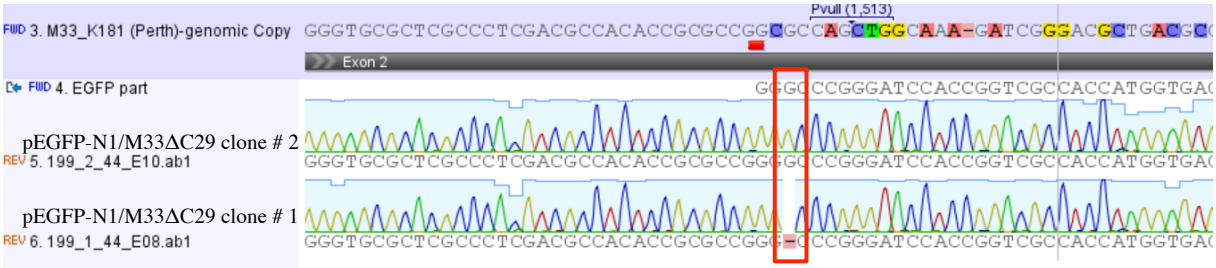

Figure 4. pEGFP-N1/M33ΔC29 sequence data displaying a frameshift mutation. The red box indicates a missing residue in the EFGP region of the first M33ΔC29 clone resulting in frameshift mutation preventing expression of GFP. GENEious software was used to analyse sequence data for each of the clones.

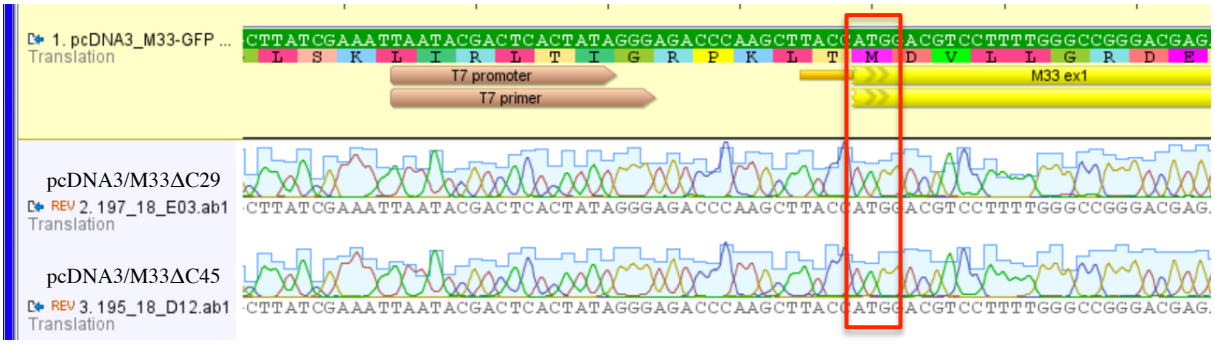

Figure 5. pcDNA3/M33ΔC29 and pcDNA3/M33ΔC45 sequence data. ATG start codon is in frame relative to CMV IE1 promoter of pcDNA3. Red box indicates that the start of pcDNA3/M33ΔC29 and pcDNA3/M33ΔC45 is conserved with pcDNA3/M33-GFP template strand. GENEious software was used to analyse sequence data for each of the clones.

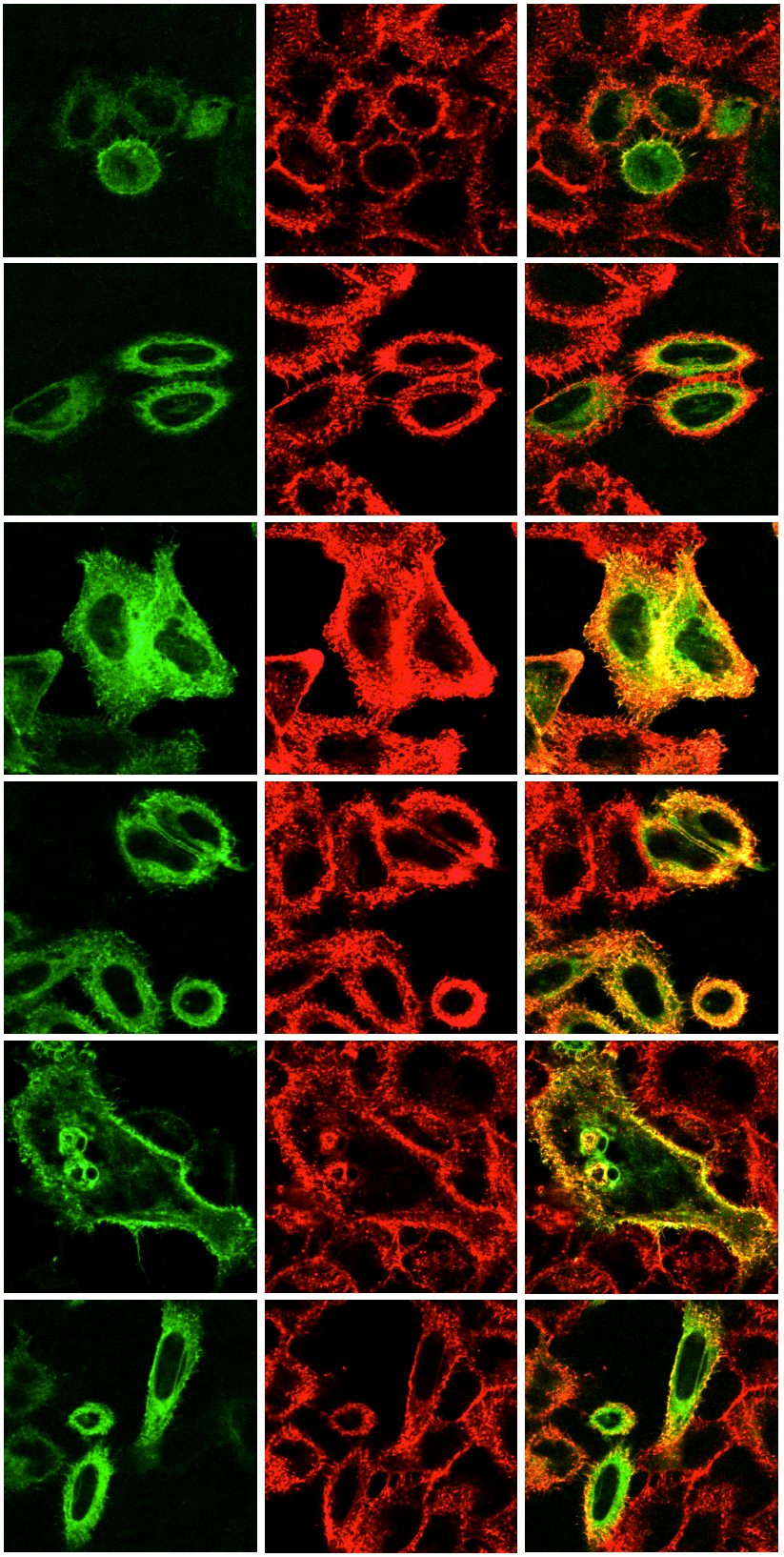

Figure 6. Cellular localisation of wild-type M33 and M33 mutants. HeLa cells were transfected with plasmids expressing GFP-tagged wt M33, a previously constructed M33ΔC57 mutant or new M33 C-terminal truncation mutants. Fluorescence visualised via confocal microscopy 24hrs post transfection. Cells stained then fixed. Left panel: M33 distribution (EGFP fluorescence) represented in green. Middle panel: Cell surface shown by reactivity of Alexa Fluor 594-conjugated wheat germ agglutinin, represented in red. Right panel: Colocalisation of GFP-tagged M33 and cell surface, represented in yellow.

Cellular localisation. Confocal microscopy was used to visualise GFP tagged M33 mutants in transfected cells in order to determine the localisation within the cell. Specifically we were looking for inhibition of cell surface expression. Cell surface expression was detected in each

of the truncation mutants M33ΔC29, M33ΔC34, M33ΔC45 and M33ΔC51 (see Figure 6). C-terminal deletions of 57 amino acids have previously been shown to inhibit cell surface expression. For this reason M33ΔC57 was used as a negative control and supports the previous studies with distinct intracellular retention. This is a qualitative approach allowing only for detection of mutants completely deficient in cell surface expression.

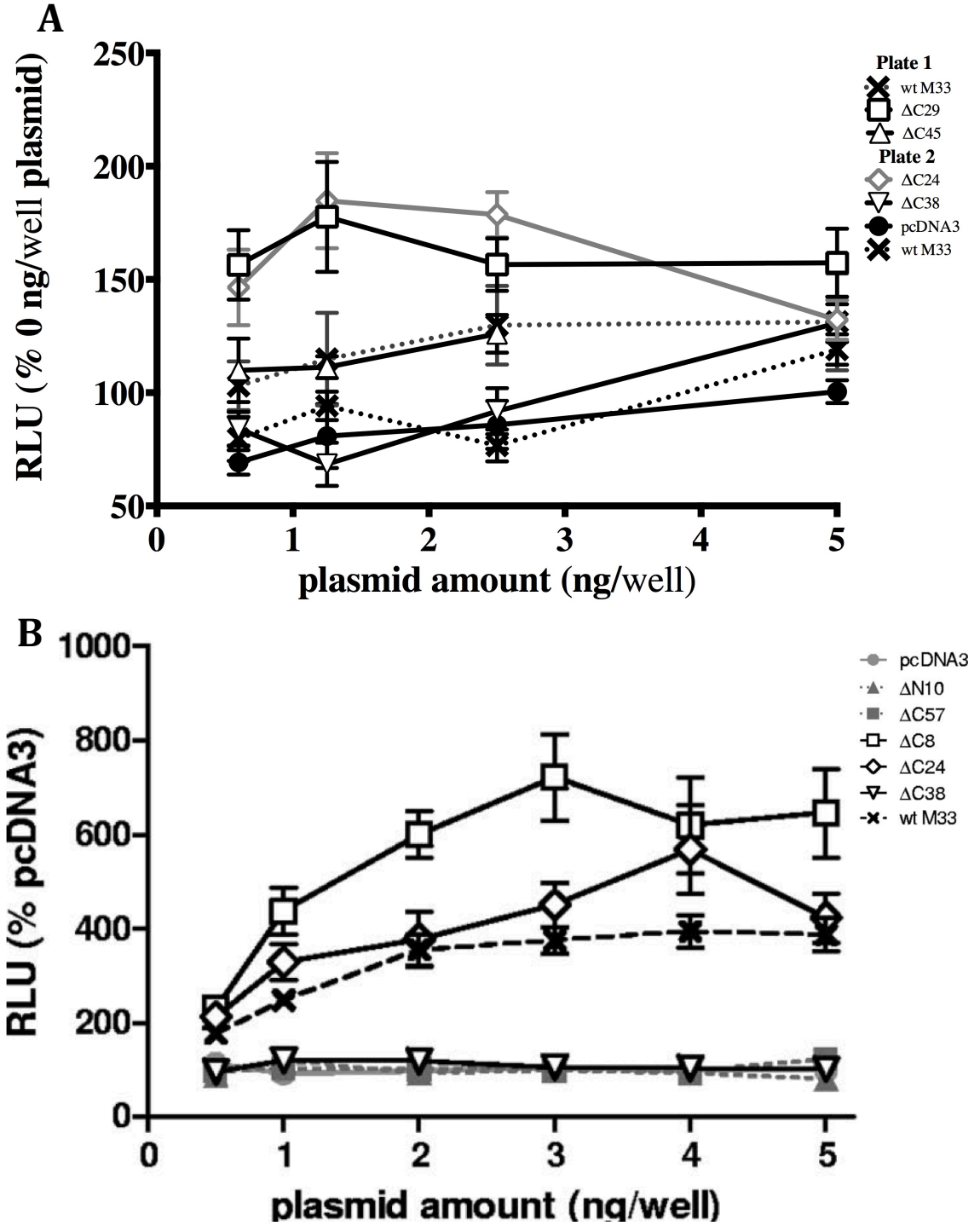

Figure 7. CREB-mediated transcription. A: HEK-293 cells were transfected with wt M33, pcDNA3 or C-terminal truncation mutants at varying doses shown, together with luciferase reporter constructs. Results are shown in relative light units (RLU) compared to the no plasmid control. Experiment conducted over two plates results standardised against the mean 0ng/well plasmid controls for each. wt M33 which was supposed to be positive control was not signalling appropriately and is displayed as a dotted line. B: Results obtained from Case et al (2009), HEK-293 cells were transiently transfected with either the C- or N-terminal M33 truncation mutants at the various doses shown, together with luciferase reporter constructs. Results are expressed as relative light units (RLU) compared to the negative control plasmid pcDNA3. Error bars depict the standard error of the mean in both graphs (n = 4 for A and n = 8 to 16 for B).

Constitutive signalling. M33 is recognised to be constitutively active through CREB signal transduction pathways. So for this study constitutive signalling of the M33 mutants was assessed through a CREB luciferase reporter construct. Unfortunately the results are not as clear as anticipated illustrated in Figure 7. Wild-type M33 is not considered to be a particularly strong signaller through CREB mediated pathways but in this study M33 signalling is regarded to be insignificant for the most part. This is possibly due to a problem with the M33 construct used. A mutant from an earlier study M33ΔC24, previously shown to retain constitutive signalling despite a 24 amino acid C-terminal truncation was used in this study and acts as a positive control. The shorter of the truncation mutants we constructed, M33ΔC29, is constitutively active in CREB signal transduction pathways. The second mutant, M33ΔC45, does not exhibit significant results that indicate signalling. The results for this mutant are somewhat ambiguous. In comparison to the M33ΔC29 and M33ΔC24 mutants however, signalling is insignificant. Results obtained from signalling studies of M33 C-terminal truncation mutants Case et al (2008) have been included. It is shown in these results the expected signalling capability of wild-type M33. Furthermore the results for M33ΔC29 and M33ΔC24 in this experiment confirm our own.

DISCUSSION

The aim of this study was to provide finer mapping of regions in the C-terminus of the M33 GPCR contributing to signalling and cell-surface expression. C-terminal truncation mutants of M33 were constructed and tested for CREB signal transduction and cellular localisation. Using results of this study and results obtained from a previous study conducted by Case and colleagues (2008) we have determined that the region between $C^{321}$and L^{326} contains residues important for trafficking of M33 while region between R^{340} and A^{348} contains residues important for CREB-mediated signalling.

G protein-coupled receptor localisation at the cell surface is required for access to ligands and activation of signal transduction pathways (18). A number of studies have demonstrated that the C-terminus of GPCRs plays a role in the translocation to the cell surface. In a study that looked at C-terminal truncations of CCR5 it was shown that with progressive deletions there was a reduction in cell surface expression (20). Reduction in cell surface expression has also been observed in a study that induced a number of point mutation to arginine and lysine residues on the C-terminus of the melanin-concentrating hormone receptor 1 (MCH1R) (19). Furthermore a deletion of 61 amino acids from the C-terminus of R33 was associated with intracellular retention(9). In contrast to these however, substantial truncations of the CCR2B C-terminus failed to have a significant effect on cell surface expression (20). Likewise the C-terminal deletions of US28 were not found to reduce cell surface expression rather increase it as a result of reduced endocytosis (22).

Our study follows on from a 2008 study where Case and colleagues analysed the cellular localisation and signalling of a number of M33 C-terminal truncation mutants. In their study a deletion of 57 amino acids resulted in ablation of cell surface expression while localisation consistent with wild type was observed deletion mutants of 38 amino acids (5). A cysteine reside (C^{321}) and RR (R^{329}/R^{330}) motif conserved among the UL33 family were subsequently identified as potentially contributing to the loss of expression. In Grijthuijsen and colleagues study of R33 they also identified the conserved RR motif as a contributing factor through C-terminal deletions and point mutations(9). In our study each of the constructed C-terminal truncation mutants including the largest, M33ΔC51 (that had the RR motif deleted) displayed evidence of localisation to the cell surface. This suggests the existence of important residues between $C^{321}$and L^{326}. Furthermore these results support the proposed importance of C^{321}.

Basic residues in the C-terminus of GPCR are believed to be important for cell surface expression as observed in mutational experiments of CCR5, MCH1R and R33 (9, 19, 20). It is

thought that they could mediate an interaction with the negatively charged phospholipids of the membrane while palmitoylated cysteine residues insert into the membrane and act to stabilise the interaction(2, 9, 20). While our results don't directly implicate the RR motif it is possible that removal of this region could have resulted in a decrease of cell surface expression undetectable by the qualitative results obtained through fluorescent imaging. In a future study, point mutations of these important residues could be induced to confirm the hypothesised contribution.

Unfortunately finer mapping of the region involved with signalling was unsuccessful due to complications in cloning. However our results support earlier experiments that indicate specific deletions to the C-terminus of M33 prevent correct CREB signalling. In a study of a different GPCR, C-terminal deletions of MCHR1 were found to have an effect upon signal transduction(19). Likewise a study by Case and colleagues, showed that a C-terminal mutant of 24 amino acids signalled constitutively while CREB signalling could be detected with a deletion of a further 14 amino acids(5). Signalling was detected in the results for the M33ΔC29 mutant suggesting that it is the region between R^{340} and A^{348} that has residues important for signalling. Furthermore no detection of signalling in the M33ΔC45 mutant supports evidence that a deletion of greater than 38 amino acids inhibits signalling.

In contrast to these results extensive C-terminal truncations of US28 have failed to effect signal transduction (22). In an additional study, UL33 activation of CREB-mediated transcription factors was also associated with other regions suggesting that a number of regions could contribute to constitutive signalling of M33(4).

The results of our study have shown that while expression of M33ΔC45 was detected on the cell surface, it failed to stimulate CREB signal transduction pathways. On the other hand M33ΔC29 was detected on the surface and CREB signalling was observed. It is expected that correct cell surface expression is required for signal transduction and previous results have shown evidence that this is true (5). These results indicate that expression on the cell surface is not enough for constitutive signalling and that other residues within the C-terminus are important for signalling. Another potential explanation however is that serial deletions of the M33 C-terminus results in progressive loss of cell surface expression undetectable due to qualitative designs in this experiment resulting in deficient CREB signalling.

The fluorescent imaging used to detect cellular localisation in this experiment is a qualitative approach that allows only for identification of mutants totally deficient in cell surface

expression. In order to test whether there is a progressive loss of cell surface expression, which may result in inhibition of signalling, a quantitative approach must be taken. Previous attempts to tag the N-terminus of M33 with hemagglutinin (HA) and *c-myc* have been unsuccessful (5). It appears that N-terminal modifications of M33 impact on signalling and cell surface expression. For future studies utilisation of a surface biotinylation assay similar to the one described in *Receptor signal transduction protocols* could be used to quantify not only level of surface expression but also total expression and rate of endocytosis(23). This method works through covalent bonding of biotin to surface receptors.

Viral homologues of host chemokine receptors have been described in a number of viruses to have important roles in immune evasion, virus dissemination and virus replication. The MCMV M33 GPCR is one such homologue highly conserved across the CMVs. Here we have provided information that allows for the mapping of regions within the M33 C-terminus important in constitutive signalling and cellular localisation. We conclude that truncations of these regions result in inhibition of signalling and intracellular retention. However the specific residues and the mechanism that confers these outcomes require further research. Ultimately this research suggests that the C-terminus of viral GPCRs could be a potential target for therapeutic drugs.

ACKNOWLEDGEMENTS

I would like to thank the Herpesvirus Molecular Pathogenesis Unit at the Sir Albert Sakzewski Virus Research Centre for providing the facilities, equipment and the samples required to undertake this project. I would also like to thank Associate Professor Nicholas Davis-Poynter and Dr Helen Farrell for their support and guidance.

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