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Interactions between the Mas-related receptors MrgD and MrgE alter signalling and trafficking of MrgD

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Running title: MrgD-MrgE interactions

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[1]**Abbreviations**: GPCR, G protein-coupled receptor; Mrg, Mas-related gene; Tr-FRET, time-resolved fluorescence resonance energy transfer

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ABSTRACT

When expressed via an inducible promoter in HEK293 cells the rat Mas-related gene D (rMrgD) receptor responded to β-alanine but not L-alanine by elevating intracellular [Ca^{2+}], stimulating phosphorylation of the MAP kinases ERK1 and ERK2 and translocating from the plasma membrane to punctate intracellular vesicles. By contrast, the related rat Mas-related gene E (rMrgE) receptor did not respond to β-alanine. Co-expression of rMrgD with rMrgE, which occurs in peripheral noiceptive neurons, allowed co-immunoprecipitation of the two receptors and resulted in the detection of cell surface rMrgD-rMrgE hetero-dimers via time-resolved fluorescence resonance energy transfer. These interactions increased the potency of β-alanine to phosphorylate ERK1 and ERK2 as well as maintaining the capacity of β-alanine to elevate intracellular [Ca^{2+}], which was reduced in magnitude and slowed in response with increasing times of expression of rMrgD in isolation. Associated with these effects, the presence of rMrgE restricted β-alanine-induced internalization of rMrgD. This is the first report of hetero-dimeric interactions between members of the Mrg receptor family and indicates that interactions between rMrgD and rMrgE modulate the function of rMrgD. As the Mrg receptors are potential therapeutic targets in pain these results suggest that efforts to understand the function and regulation of individual Mrg family receptors may require co-expression of relevant pairs.

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G protein-coupled receptors (GPCRs[1]) of the Mas-related gene (Mrg) family (Dong et al., 2001) are selectively expressed in subpopulations of sensory neurons involved in the perception of pain (Dong et al., 2001; Lembo et al., 2002). This has resulted in them also being named sensory neuron-specific G protein-coupled receptors (Lembo et al., 2002) and in suggestions that they might represent attractive targets for therapeutic intervention in pain. In mouse the family is large, consisting of more than 50 members (Dong et al., 2001; Zylka et al., 2003) but the complement of related receptors in man, macaque and rat is significantly less extensive (Zhang et al., 2005) although true orthologues are difficult to identify. In rat, for example, only single members of each of the MrgA and MrgC subfamilies have been identified (Zylka et al., 2003; Zhang et al., 2005), although, as in mouse, a substantial number of both true MrgB receptor encoding- and pseudo-genes have been identified (Zylka et al., 2003). A single gene appears to encode the MrgD receptor in all rodent and primate species and this GPCR, which has also been named TGR7 (Shinohara et al., 2004), is activated selectively by β-alanine (Shinohara et al., 2004). By contrast, many of the other Mrg receptors have been shown to respond to relatively high concentrations of a range of peptide ligands (Dong et al., 2001; Lembo et al., 2002; Han et al., 2002; Grazzini et al., 2004; Robas et al., 2003) or remain orphans.

It is now widely accepted that GPCRs can form dimers and it appears that this may be integral to function (Angers et al., 2002; Milligan et al., 2003; Breitwieser, 2004; Milligan, 2004). There is also growing evidence that certain GPCRs can form hetero-dimers when they are co-expressed (Milligan, 2004; George et al., 2002) and that such hetero-dimers may have pharmacology (Rocheville et al., 2000), function (Jordan and Devi, 1999) and regulation (Hansen and Sheikh, 2004) distinct from the

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corresponding homo-dimers. This has not been examined for members of the Mrg family and hence, as an initial effort to explore this issue, we selected the rat (r) MrgD and MrgE receptors because they are closely related and, as shown previously, are co-expressed in individual dorsal root ganglion neurons from a number of species (Zhang et al., 2005). However, whilst β-alanine acts as an agonist for MrgD, selective agonist ligands for MrgE have not yet been identified. Using cell lines able to express either rMrgD or rMrgE in an inducible manner or to induce expression of rMrgD in the face of constitutive expression of rMrgE we confirm the agonist actions of β-alanine only on rMrgD, demonstrate that rMrgD and rMrgE interact directly upon co-expression and that interaction with rMrgE alters both the ability of β-alanine to internalize rMrgD and the potency of β-alanine to induce intracellular signals. These results demonstrate that Mrg receptors can form hetero-dimers and by so doing may alter their functionality and response to appropriate stimuli in sensory neurons.

Materials and Methods

Materials/Ligands

β-alanine, L-alanine, doxycycline and biotin-conjugated anti-c-myc antibody were supplied by Sigma (Gillingham, Dorset, UK) and all materials for tissue culture were from Invitrogen (Paisley, UK). Oligonucleotides were purchased from ThermoElectron (Ulm, Germany). Antibodies recognizing c-myc, ERK1/2 and their phosphorylated forms were from Cell Signalling (Hitchin, Herts., UK). Reagents for the time-resolved fluorescence resonance energy transfer (Tr-FRET) studies were from Perkin Elmer Life and Analytical Sciences (Boston, MA).

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Plasmid construction

rMrgD receptor

The rMrgD receptor was used as a PCR template for all rMrgD receptor constructs. For the N-terminally modified forms of the receptor, primers encoded the appropriate epitope tag sequence and introduced a stop codon after the last amino acid of the receptor sequence. For the C-terminally modified forms of the receptor, primers were designed to amplify the sequence and remove the stop codon.

FLAG-rMrgD

Sense:5'GATAAGCTTGCCACCATGGACTACAAGGACGACGATGATAAGAA
CTACACTCCTTATAGCAGCCCAGCCCCAGGT 3'

Anti-sense:

5'CGCGGCTCGAGTCAGACCCAATCATTAGTACATGTGGATGGCGT
CTC 3'

A *HindIII* site present in the sense primer and a *XhoI* site present in the anti-sense primer are underlined and the amplified fragment digested and ligated into pcDNA3 or pcDNA5/FRT/TO.

c-myc-rMrgD

Sense:5'GATAAGCTTGCCACCATGGAACAAAAACTTATTTCTGAAGAAGAT
CTG AACTACACTCCTTATAGCAGCCCAGCCCCAGGT 3'

Anti-sense: same as for FLAG-MrgD

A *HindIII* site present in the sense primer and a *XhoI* site present in the anti-sense primer are underlined and the amplified fragment digested and ligated into pcDNA3 or pcDNA5/FRT/TO.

c-myc-rMrgD-eYFP

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Sense: same as for c-myc-rMrgD

Anti-sense:

5'CGGCCGGTACCGACCCCATCATTAGTACATGTGGATGGCGTCTCCCTG 3'

A *HindIII* site present in the sense primer and a *KpnI* site present in the anti-sense primer are underlined and the amplified fragment digested and ligated into pcDNA3 upstream of and in frame with eYFP ligated between *KpnI* and *NotI*.

rMrgE receptor

The rat MrgE receptor was used as a PCR template for all rMrgE receptor constructs. For the N-terminally modified forms of the receptor, primers encoded the appropriate epitope tag sequence and introduced a stop codon after the last amino acid of the receptor sequence. For the C-terminally modified forms of the receptor an anti-sense primer was designed to remove the stop codon.

FLAG-rMrgE

Sense:5'GATAAGCTTGCCACCATGGACTACAAGGACGACGATGATAAGTCC

CTGAGAGTGCACACGCATTCTCCCAGCACC 3'

Anti-sense:

5'CGCGGCTCGAGTTAGACAGTCATGTCCACAAGTCCCCCTTGGGA

AGC 3'

A *HindIII* site present in the sense primer and a *XhoI* site present in the anti-sense primer are underlined and the amplified fragment digested and ligated into pcDNA3.

c-myc-rMrgE

Sense:5'GATAAGCTTGCCACCATGGAACAAAAACTTATTTCTGAAGAAGAT

CTGTCCCTGAGAGTGCACACGCATTCTCCCAGCACC 3'

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Anti-sense: same as for FLAG-MrgE

A *HindIII* site present in the sense primer and a *XhoI* site present in the anti-sense primer are underlined and the amplified fragment digested and ligated into pcDNA3 or pcDNA5/FRT/TO.

FLAG-rMrgE-eYFP

Sense: same as for FLAG-rMrgE

Anti-sense:

5'CTAATGCGGCCGCTGACAGTCATGTCCACAAGTCCCCCTTGGGAA

GCCTCT 3'

A *HindIII* site present in the sense primer and a *NotI* site present in the anti-sense primer are underlined and the amplified fragment digested and ligated into pcDNA3 upstream and in frame with eYFP ligated between *NotI* and *XhoI*.

rMrgE-eYFP

Sense:

5'GATAAGCTTGCCACCATGTCCCTGAGAGTGCACACGCATTCTCCCAGC

ACC 3'

Anti-sense: same as for FLAG-rMrgE-eYFP

A *HindIII* site present in the sense primer and a *NotI* site present in the anti-sense primer are underlined and the amplified fragment digested and ligated into pcDNA3 upstream and in frame with eYFP ligated between *NotI* and *XhoI*.

Generation of stable Flp-In T-REx HEK293 cells

Cells were maintained in Dulbecco`s modified Eagle's medium without sodium pyruvate, 4500 mg/L glucose and L-glutamine, supplemented with 10% (v/v) foetal

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calf serum, 1% antibiotic mixture and 10 μg/ml blastacidin at 37°C in a humidified atmosphere of air/CO_2 (19:1). To generate Flp-In T-REx HEK293 cells able to inducibly express c-myc-rMrgD, c-myc-rMrgE, FLAG- rMrgE-eYFP or FLAG-rMrgD receptors, the cells were transfected with a mixture containing the desired receptor cDNA in pcDNA5/FRT/TO vector and the pOG44 vector (1:9) using LipofectAMINE (Invitrogen), according to the manufacturers' instructions. After 48 h, the medium was changed to medium supplemented with 200 μg/ml hygromycin B to initiate selection of stably transfected cells. To constitutively stably co-express a second receptor of the rMrg family in inducible cell lines, the appropriate cells were further transfected with the desired receptor cDNA in pcDNA3 as described above and resistant cells were selected in the presence of 1 mg/ml G418. Resistant clones were screened for receptor expression by Western blotting. Cells were treated with 1 μg/ml doxycycline 24-96 h prior to assays to induce expression of receptors cloned into the Flp-In locus.

Receptor internalization

Monolayers of cells in 96-well plates were induced with 1 μg/ml doxycycline and incubated with growth medium containing vehicle or varying concentrations of β-alanine for 30 min at 37°C. Afterwards cell surface receptors were labelled with anti-c-myc antibody (1:500) in growth medium for 30 min at 30°C. The cells were washed once with 20 mM Hepes/Dulbeccos' modified Eagles' medium and then incubated for another 30 min at 37°C in growth medium supplemented with anti-rabbit horseradish-peroxidase-conjugated IgG as secondary antibody and 1 μM Hoechst nuclear stain (Sigma) to determine cell number. The cells were then

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washed twice with phosphate buffered saline and incubated with SureBlue (Insight Biotechnology) for 5 min in the dark at room temperature and the absorbance read at 620 nm in a Victor^2 plate reader (Packard Bioscience). Receptor internalization was determined as loss of cell surface receptors in agonist-treated cells.

Immunostaining for N-terminal c-myc-tagged rMrgD and rMrgE receptors

Immunostaining was performed essentially according to the method of Cao *et al.* (1999). Cells were plated on to coverslips and induced with 1 μg/ml doxycycline. After 24-72 h, the medium was changed for 20 mM Hepes/Dulbeccos' modified Eagles' medium containing the anti-c-myc antibody diluted 1:100 and incubated for 40 min at 37°C in 5% CO_2. Where required, 20 mM Hepes/Dulbeccos' modified Eagles' medium containing the desired concentration of agonist was added and incubated for 30 min at 37°C in 5% CO_2. Coverslips were washed three times with phosphate buffered saline and then cells fixed with 4% paraformaldehyde in phosphate buffered saline/5% sucrose for 10 min at room temperature followed by three more phosphate buffered saline washes. Cells were then permeabilized in 0.15% Triton X-100/3% non-fat milk/ phosphate buffered saline (TM buffer) for 10 min at room temperature. The coverslips were subsequently incubated with an Alexa™ 594-labelled goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR) at a dilution of 1:400 (1–4 mg/ml), upside down on Nescofilm, for 1 h at room temperature, then washed twice in TM buffer and three times with phosphate buffered saline. Finally, coverslips were mounted on to microscope slides with 40% glycerol in phosphate buffered saline.

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Confocal laser-scanning microscopy

Cells were observed using a confocal laser-scanning microscope (Zeiss LSM 5 Pascal) using a Zeiss Plan-Apo 63×1.40 NA oil immersion objective, pinhole of 20 and electronic zoom 1 or 2.5 (Milasta et al., 2005). eYFP was excited using a 488 nm argon laser and detected with a 505–530 nm band-pass filter. The Alexa™ 594 label was excited using a 543 nm helium/neon laser and detected with a 560 nm long-pass filter. The images were analysed with MetaMorph software. For the receptor internalization studies fixed cells were used. Cells on glass coverslips were washed with phosphate buffered saline and fixed for 10 min at room temperature using 4% paraformaldehyde in phosphate buffered saline/5% sucrose, pH7.4. After three washes with phosphate buffered saline coverslips were mounted on to microscope slides with 40% glycerol in phosphate buffered saline.

Co-immunoprecipitation studies

Cells were harvested 24-72 h following induction with 1 µg/ml doxycycline and resuspended in RIPA buffer (50 mM HEPES, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 10 mM NaF, 5 mM EDTA, 0.1 mM $NaPO_4$, 5% ethylene glycol). The cell pellet was placed on a rotating wheel for 1 h at 4°C. Samples were then centrifuged for 1 h at 100, 000 x g at 4°C and the supernatant transferred to a fresh tube containing 200 µl of RIPA and 5 0µl of Protein G beads (Sigma) to pre-clear the samples. Following incubation on a rotating wheel for 1 h at 4°C the samples were re-centrifuged at 20800 x g at 4°C for 1 min and the protein concentration of the supernatant determined. Samples containing equal

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amounts of protein were incubated overnight with 40 µl Protein G beads and 5 µg M2 anti-FLAG antibody (Sigma) at 4°C on a rotating wheel and fractions reserved to monitor protein expression in the cell lysates. Samples were centrifuged at 20, 800 x g for 1 min at 4°C and the Protein G beads washed 3 times with RIPA buffer. Following addition of 2 x reducing loading buffer and heating to 85°C for 4 min, both immunoprecipitated samples and cell lysate controls were revolved by SDS-PAGE using pre-cast 4-12% acrylamide Novex Bis-tris gels (Invitrogen BV). Proteins were transferred onto PVDF membrane. These membranes were incubated in 5% (w/v) low fat milk, 0.1% Tween 20/Tris buffered saline (TBS) (v/v) solution at room temperature on a rotating shaker for 1 h and then with primary antibody overnight in 5% (w/v) low fat milk, 0.1% Tween 20/TBS (v/v) solution at 4°C. The membrane was washed 3 x in TBS/0.1% Tween 20 before addition of secondary antibody. Following further washes the membrane was subsequently developed using ECL solution (Pierce, Cramlington, Northumberland, UK).

ERK1/2 phosphorylation and immunoblots

Cells were grown in 6-well plates and serum starved overnight prior to stimulation with ligands as indicated. Cells were then placed on ice, washed twice with cold phosphate buffered saline and lysed in RIPA buffer. After 1 h at 4 °C, the lysates were centrifuged for 15 min at 20, 800 x *g* at 4°C to remove the insoluble material. The samples were mixed with 2 × reducing loading buffer and heated for 3 min at 95°C. ERK1/2 phosphorylation was detected by protein immunoblotting using phospho-ERK1/2-specific antibodies and anti-rabbit horseradish-peroxidase-

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conjugated IgG as secondary antibody for immunodetection. After visualizing the level of ERK1/2 phosphorylation, the PVDF membranes were stripped and reprobed using the anti-ERK1/2 antibody.

$[Ca^{2+}]_i$ imaging

Cells induced or not to express receptors were loaded with the Ca^{2+}-sensitive dye Fura-2 (Sigma) by incubation (15-20 min, 37°C) under reduced light in Dulbeccos' modified Eagles' medium containing the dye's membrane-permeant acetoxymethyl ester form (1.5 μM). Details of the imaging studies and their analysis have been described previously (Liu et al., 2002).

Time-resolved fluorescence resonance energy transfer

Was performed using a combination of an Eu^{3+}-labelled anti-c-myc antibody, as a long lived energy donor, and allophycocyanin-labelled anti-FLAG antibody as a potential energy acceptor (McVey et al., 2001; Wilson et al., 2005) to cells constitutively expressing c-myc-rMrgE that were induced or not to express FLAG-rMrgD.

Gene expression analysis

100 ng RNA from each sample of the rat total RNA panel (Clontech) was reverse transcribed in triplicate using a GeneAmp RNA PCR core kit (Applied Biosystems) as per manufacturers instructions. Quarter of this cDNA (25 ng) was used as a template for quantitative PCR using the 7900HT Sequence Detection System (Applied

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Biosystems). Absolute quantitation was achieved by means of a 5-log (10 to 10^5 C-value) standard curve of rat genomic DNA (Clontech) assuming that 1 C-value (2.65 pg) contains 1 copy of the target gene. The reaction was run as per manufacturers instructions for absolute quantitation, with primer conditions determined from primer and probe optimization studies performed as per Applied Biosystems protocol. 300 nM forward primer (CAGCCTCGGCGGCTCTA), 900 nM reverse primer (CCAACGGCAGAGAACAGGTAAG) and 175 nM (5'FAM-TGGTCATCCTGACTTCCGTCCTTGTCTTC-TAMRA 3') dual labeled probe were used for rMrgD. Identical primer concentrations were also used for rMrgE (forward TGGCACACACCCCTCTACTTCT; Reverse AGGCTTGGCCGCACTGT) with 150 nM of probe (5'FAM-TCACTTCAGCTTCTTCATGGCCAGTGTG-TAMRA3'). Error bars represent the Standard Deviation of the triplicate samples.

Results

Distribution patttern of rMrgD and rMrgE receptor messenger RNA

The distribution of mRNA encoding the MrgD and MrgE receptors in rat tissues was assessed via quantitative reverse transcription-polymerase chain reaction following isolation of RNA. As anticipated from previous work (Zhang et al., 2005), a substantial amount of mRNA encoding each of MrgD and MrgE was present in dorsal root ganglion tissue. By contrast, levels of mRNA encoding MrgD were essentially undetectable in all other tissues, apart from testis, examined. MrgE mRNA could also be detected in a crude brain sample, spinal cord and sciatic nerve but at levels no more than 20% of that in dorsal root ganglion (Figure 1). Such results confirmed the

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expression of both MrgD and MrgE mRNA, and thus presumably protein, in dorsal root ganglia.

Homo-dimeric interactions of rMrgD and rMrgE receptors

It is becoming widely accepted that GPCRs are able to form homo-dimers/oligomers and that this may be important for cellular trafficking and function. As this has not been reported previously for any member of the Mrg receptor family we expressed N-terminally FLAG and c-myc tagged forms of rat (r)MrgD either individually or in combination in HEK293 cells. Samples were immunoprecipitated with an anti-FLAG antibody, resolved by SDS-PAGE and then immunoblotted to detect the presence of c-myc reactive polypeptides in these immunoprecipitates. Only following co-expression of FLAG and c-myc rMrgD was a c-myc reactive polypeptide of some 33kDa present in the anti-FLAG immunoprecipitates (Figure 2). This is consistent with the two co-expressed forms of rMrgD being present within a dimeric/oligomeric complex. Equivalent studies with FLAG and c-myc-tagged forms of rMrgE also resulted in the presence of a c-myc reactive 28kDa polypeptide in anti-FLAG immunoprecipitates only when the two forms of rMrgE were co-expressed (Figure 2).

Stable expression of rMrgD and rMrgE receptors in HEK293 cells

To investigate function and potential physical interactions between the rMrgD and rMrgE receptors, we utilized HEK293 Flp-In T-REx cells with the capacity to stably express either receptor alone or both receptors together. In all the single receptor expressing cell lines produced for these studies the receptor of interest was cloned

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into the Flp-In locus to allow inducible expression under the control of a tetracycline-on promoter. Clones for expression of each of N-terminally FLAG- and c-myc-tagged forms of rMrgD and N-terminally c-myc-tagged rMrgE as well as rMrgE C-terminally tagged with enhanced yellow fluorescent protein (eYFP) were generated. Immunoblots of cell lysates using anti-FLAG and anti-c-myc antibodies confirmed expression of the anticipated receptor polypeptides in a manner that was entirely dependent upon addition of doxycycline as inducing agent (Figure 3A). In this system c-myc-rMrgE migrated through SDS-PAGE as an apparent 28kDa polypeptide with some evidence of heterogeneity that may reflect differential glycosylation (Figure 3A) whereas both FLAG- and c-myc-tagged forms of rMrgD migrated predominantly as polypeptides of some 33kDa (Figure 3A).

Clones capable of co-expressing rMrgD and rMrgE were subsequently obtained by transfection of the above cell lines using conventional transfection and selection with a second antibiotic resistance marker. This resulted in the production of clones in which one receptor was expressed constitutively whilst the second could be produced upon treatment with doxycycline. As shown in Figures 3B and 3C, in appropriate clones rMrgE tagged at the C-terminus with eYFP or N-terminally c-myc-tagged rMrgE receptors could be detected in whole cell lysates in the absence of doxycycline treatment (Figures 3B and 3C) whilst differentially tagged forms of rMrgD were only present following treatment of the cells with doxycycline. Induced expression of the various rMrgD receptor constructs did not modulate the levels of constitutively expressed rMrgE receptors since the various forms of rMrgE could be detected at similar levels in cell lysates with and without treatment with doxycycline to induce

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expression of rMrgD (Figures 3B and 3C). Multiple clones had equivalent characteristics (Figure 3 and data not shown).

In cells expressing c-myc-rMrgE constitutively, FLAG-rMrgD was expressed in a time-dependent manner after addition of doxycycline (Figure 4A). Although immuno-detectable levels were present within 6 h, treatment with doxycycline for between 24-48 h was required to achieve maximal levels and this was maintained for up to 72-96 h (Figure 4A). In contrast, and as expected, levels of c-myc-rMrgE were largely maintained throughout this time period (Figure 4A). Co-expressed c-myc-rMrgE and FLAG-rMrgD were able to interact with one another, because immunoprecipitation of FLAG-rMrgD resulted in co-immunoprecipitation of c-myc-rMrgE (Figure 4A). Co-immunoprecipitation of c-myc-rMrgE in anti-FLAG immunoprecipitates only occurred with detectable expression of FLAG-rMrgD and the amount of co-immunoprecipitation of c-myc-rMrgE mirrored the time-course of induction and extent of production of FLAG-rMrgD (Figure 4A). In induced cells expressing only FLAG-rMrgD, this receptor was expressed to similar levels as in the cells able to co-express FLAG-rMrgD and c-myc-rMrgE but, although the anti-FLAG antibody was effective in immunoprecipitating FLAG-rMrgD from lysates of these cells, no c-myc reactive polypeptides were co-immunoprecipitated (Figure 4A). Confirmation of the specificity and requirement for co-expression to allow co-immunoprecipitation of c-myc-rMrgE and FLAG-rMrgD was obtained by mixing cell lysates of induced cells expressing only FLAG-rMrgD with those from cells constitutively expressing c-myc-rMrgE but not induced to express FLAG-rMrgD. No co-immunoprecipitation of c-myc-rMrgE was obtained from such mixtures of lysates although FLAG-rMrgD reactivity was present in the anti-FLAG immunoprecipitates

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(Figure 4A). Delivery of FLAG-rMrgD and c-myc-rMrgE to the cell surface and interactions between FLAG-rMrgD and c-myc-rMrgE at the surface of living cells were monitored via time-resolved fluorescence resonance energy transfer (Tr-FRET). Addition of a combination of Eu^{3+}-labelled anti-c-myc antibody, as a long lived energy donor, and allophycocyanin-labelled anti-FLAG antibody as a potential resonance energy acceptor to cells constitutively expressing c-myc-rMrgE and induced to express FLAG-rMrgD resulted in a significant Tr-FRET signal (Figure 4B). No signal was detected in these cells if FLAG-rMrgD expression was not induced (Figure 4B) and there was also no Tr-FRET signal obtained when cells individually expressing c-myc-rMrgE or FLAG-rMrgD were mixed and then exposed to the combination of antibodies (Figure 4B). Controls that simply measured fluorescence of the allophycocyanin-labelled anti-FLAG antibody bound to cells demonstrated similar levels of cell surface FLAG-rMrgD receptors in the cells co-expressing the two receptors and in the mixed cell populations (Figure 4C).

Internalization of rMrgD but not rMrgE in response to β-alanine

β-alanine has been described as an agonist for MrgD, able to cause internalization of the receptor (Shinohara et al., 2004). Cells induced to express c-myc-rMrgD only were pre-labelled with anti-c-myc antibody and treated with increasing concentrations of β-alanine for 30 min. In the absence of agonist the majority of the immunostained c-myc-rMrgD receptor detected following cell permeabilization was localized at the plasma membrane (Figure 5A), however, a small proportion of the receptor could be detected in intracellular vesicles suggesting that c-myc-rMrgD may partially internalize independently of agonist stimulation (Figure 5A). β-alanine caused

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substantial internalization of the c-myc-rMrgD receptor into punctate intracellular vesicles with maximum internalization being observed after treatment with 1 mM ligand (Figure 5A). When expressed alone c-myc-rMrgE was also expressed predominantly at the plasma membrane following induction of receptor expression (Figure 5B). However, treatment with up to 10 mM β-alanine did not result in detectable internalization of the rMrgE receptor (Figure 5B).

Recent reports indicate that hetero-dimerization of GPCRs can affect the internalization properties of the individual receptors (Jordan et al., 2001; Breit et al., 2004). To determine whether co-expression with rMrgE altered the internalization properties of rMrgD, HEK293 Flp-In T-REx cells constitutively expressing FLAG-rMrgE-eYFP were induced for 72 h with doxycycline to also express c-myc-rMrgD and to allow hetero-dimer formation as shown in Figure 4. In doxycycline-induced but unstimulated cells both receptors were expressed at the plasma membrane and displayed overlapping distributions (Figure 6A upper panels). In response to β-alanine, internalization of c-myc-rMrgD receptor could be observed, whereas no extra FLAG-rMrgE-eYFP could be detected in intracellular vesicles (Figure 6A lower panels). Unlike when c-myc-rMrgD expression was induced in the absence of rMrgE, where little cell surface staining could be observed following β-alanine treatment and most of the detectable rMrgD receptor was apparently localized in endocytic vesicles (Figure 6A), co-expression of FLAG-rMrgE-eYFP appeared to impair β-alanine-induced sequestration of c-myc-rMrgD. This was suggested because significant amounts of immunostained c-myc-rMrgD receptor could still be detected at the plasma membrane (Figure 6A lower panels). Such observations are entirely

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qualitative and thus to quantify the extent of internalization of c-myc-rMrgD in the absence or presence of FLAG-rMrgE-eYFP, enzyme-linked immunosorbent assays were performed. Cells induced to express c-myc-rMrgD with or without constitutive expression of FLAG-rMrgE-eYFP were treated with or without β-alanine for 30 min and then immunostained with anti-c-myc-antibody and a horseradish peroxidase secondary antibody to allow detection of c-myc-rMrgD receptors at the cell surface. The extent of rMrgD receptor endocytosis was reduced significantly ($p < 0.01$) in the presence of rMrgE, reaching only 48% ± 2% compared to 63% ± 2% in cells expressing only c-myc-rMrgD (Figure 5B). These results demonstrate that co-expression of rMrgE impairs internalization of rMrgD in response to β-alanine and are consistent with the concept that the rMrgD-rMrgE hetero-dimer is either unable or less able to internalize in response to agonist stimulation than the rMrgD homo-dimer. By contrast, and in agreement with the confocal images, enzyme-linked immunosorbent assays confirmed no significant internalization of FLAG-rMrgE-eYFP in response to β-alanine whether FLAG-rMrgE-eYFP was expressed alone or when c-myc-rMrgD was co-expressed by treatment with doxycycline (Figure 6C).

β-alanine is an agonist at rMrgD but not rMrgE

The observation that treatment with β-alanine caused sequestration of rMrgD but not of rMrgE from the plasma membrane to intracellular vesicles did not exclude the possibility that rMrgE might generate downstream signals in response to β-alanine because ligand-stimulated receptor internalization is not an infallible surrogate marker for agonism of downstream signalling events (Roettger et al., 1997; Whistler et al., 2002). To explore signal transducing effects of β-alanine at the rMrgD and rMrgE

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receptors, β-alanine (100 μM)-mediated phosphorylation of the ERK1/2 MAP kinases was examined initially in cells induced to express only either rMrgD or rMrgE receptors. In cells expressing only rMrgD, β-alanine stimulated ERK1/2 phosphorylation in a transient manner with maximal effects observed after 5 min and with signal returning to basal levels within 15 min (Figure 7A). No effect of β-alanine was observed in these cells if rMrgD expression had not been induced by treatment with doxycycline (not shown). By contrast, β-alanine was unable to cause phosphorylation of ERK1/2 in cells induced to express rMrgE (Figure 7B). β-alanine was also able to cause phosphorylation of ERK1/2 in cells that constitutively expressed rMrgE and in which co-expression of rMrgD was induced by treatment with doxycycline. The time course of ERK1/2 phosphorylation was similar to that observed in cells expressing only rMrgD (Figure 7C). To further examine potential effects of rMrgD-rMrgE receptor hetero-dimerization on ERK1/2 activation, β-alanine-concentration-response curves were performed. The maximal response of ERK1/2 phosphorylation in rMrgD only expressing cells was observed with addition of 1mM β-alanine (Figure 8A), a concentration of ligand similar to that necessary to cause maximal receptor internalization (Figure 5A). To ascertain that this rMrgD receptor response was specific for β-alanine and not due to a non-specific effect reflecting the high concentration of β-alanine required, rMrgD receptor expressing cells were treated with the same concentrations of L-alanine. No ERK1/2 activation could be detected (Figure 8B). Cells expressing either rMrgD or rMrgE alone or co-expressing both receptors were treated with concentrations of β-alanine ranging from 0.01 to 10 mM to examine whether the formation of rMrgD-rMrgE receptor hetero-dimers has an effect on the sensitivity of ligand-induced ERK1/2 phosphorylation.

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Interestingly, a significant ($p < 0.01$) 2.5 fold increase in potency of β-alanine was observed in cells co-expressing rMrgE and rMrgD (Figure 9) although cells expressing rMrgE alone did not respond to β-alanine at any concentration tested (Figure 9). To extend these observations we also measured changes in $[Ca^{2+}]_i$ in response to β-alanine treatment of rMrgD and rMrgE receptor expressing cells. Stimulation of single rMrgD receptor expressing cells induced a rapid and transient elevation of $[Ca^{2+}]_i$, whereas no elevation in intracellular $[Ca^{2+}]$ could be observed after addition of β-alanine to un-induced cells harbouring the rMrgD receptor at the Flp-In locus or cells induced to express rMrgE (Figure 10). The capacity of β-alanine to elevate $[Ca^{2+}]_i$ in cells in which induction of rMrgD expression was maintained for varying times prior to analysis showed both a reduction in the maximal signal and a slower kinetic of onset in cells that had been expressing rMrgD for 72 h compared to those expressing this receptor for 24 h (Figure 11). Intriguingly, this time-dependent loss of rMrgD-mediated function of β-alanine was completely absent in cells in which rMrgD was induced for similar time periods but in the presence of constitutive expression of rMrgE (Figure 11).

Discussion

Most members of the Mrg family of GPCRs are expressed predominantly or exclusively in dorsal root ganglion neurons that are key for perception of pain (Dong et al., 2001; Lembo et al., 2002). The family is substantial in number in both primates and rodents (Dong et al., 2001; Lembo et al., 2002; Zylka et al., 2003). However, although marked expansion (Zylka et al., 2003) of the number and adaptive evolution (Choi and Lahn, 2003) of genes encoding members of the MrgA and MrgC

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subfamilies in mice (Zylka et al., 2003) has raised questions as to the identity of orthologues in other species, including man, there appear to be only single genes encoding the MrgD and MrgE receptors in different species and these are co-expressed in individual dorsal root ganglion cells (Zhang et al., 2005). A number of reports have indicated the ability of a range of peptide ligands to activate various Mrg family receptors from different species (Dong et al., 2001; Lembo et al., 2002; Han et al., 2002; Grazzini et al., 2004; Robas et al., 2003; Kamohara et al., 2005). However, a small number of Mrg family receptors have been shown to respond to more simple, non-peptide ligands (Shinohara et al., 2004, Bender et al., 2002). MrgD (also named TRG7) from various species is activated by β-alanine (Shinohara et al., 2004) but to date, the closely related MrgE remains an orphan GPCR.

In recent years, the concept that GPCRs can form in transfected cell systems, and exist in physiological settings, as homo-dimers or homo-oligomers (Angers et al., 2002; Milligan et al., 2003; Breitwieser, 2004; Milligan, 2004) has been tested widely using approaches that range from co-immunoprecipitation of differentially epitope-tagged polypeptides to atomic force microscopy (Milligan and Bouvier, 2005). In many cases such interactions appear to occur during protein synthesis and to be important for delivery of functional receptors to the surface of cells (Salahpour et al., 2004; Terrillon et al., 2003; Bulenger et al., 2005). It has been claimed that greater than 90% of the entire family of non-chemosensory GPCRs is expressed to some level in the central nervous system (Vassilatis et al., 2003), and gene chip analysis of GPCR expression in regions of brain, including key small nuclei, suggests that many GPCRs are likely to be co-expressed in specific neurons (Hakak et al., 2003). As such, demonstrations that certain GPCR pairs can form hetero-dimers/oligomers

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(Milligan, 2004; George et al., 2002; Bulenger et al., 2005) as well as homo-dimers/oligomers, even in physiological settings (AbdAlla et al., 2001; Kostenis et al., 2005) have attracted considerable attention. Key issues that are currently being addressed include whether such hetero-dimers display distinct pharmacology and function and if so, whether they might provide novel sets of targets for therapeutic intervention in disease (Devi et al., 2001; Milligan, 2004; George et al., 2002). Recent identification of a ligand that is able to selectively activate a hetero-dimer between KOP and DOP opioid receptor monomers and demonstration that this acts as a spinally-selective analgesic (Waldhoer et al., 2005) has significantly raised both interest and expectation in this field.

Initial studies using the HEK293 Flp-In T-REx cell system demonstrated the absolute requirement for addition of the inducing agent to allow expression of rMrgE and rMrgD receptors cloned into the Flp-In locus of these cells and that β-alanine functioned as an agonist at rMrgD but not rMrgE. Cells in which rMrgD was induced in response to treatment with doxycycline whilst rMrgE was expressed constitutively allowed detection of direct rMrgD/rMrgE interactions at the cell surface whilst combinations of cells individually expressing rMrgD or rMrgE and those constitutively expressing rMrgE and which harboured rMrgD at the Flp-In locus but where expression of rMrgD protein was not induced, provided important and clear cut negative controls.

Although β-alanine caused substantial internalization of rMrgD both in cells expressing only this receptor and in those in which its expression was induced in the face of constitutive expression of rMrgE, visual inspection suggested this to be less

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effective in cells co-expressing rMrgE. Visual inspection of such images can, at best, provide qualitative indications. However, quantitation of the extent of β-alanine-induced internalization via cell surface ELISA confirmed significantly lower levels of rMrgD internalization in the presence of rMrgE and confirmed a lack of internalization of rMrgE whether expressed alone or in combination with rMrgD. Other studies have indicated a lack of internalization of the β_2-adrenoceptor in response to agonist ligands when co-expressed with the KOP opioid receptor that is largely resistant to internalization when occupied by its own selective agonist ligands (Jordan et al., 2001). Such observations have been interpreted as an indication of hetero-dimerization between these two GPCRs when co-expressed (Jordan et al., 2001) although it has also been argued that whilst such interactions can be observed in transfected cells, this receptor pair does not form high affinity hetero-dimers (Ramsay et al., 2002) and thus may be of limited importance in a physiological context. In similar studies, co-expression of the β_2-adrenoceptor with the closely related β_3-adrenoceptor has also been shown to hinder agonist-induced internalization of the β_2-adrenoceptor (Breit et al., 2004), presumably because it has been long appreciated that the β_3-adrenoceptor is internalized very poorly in response to agonists (Breit et al., 2004), and that interaction with the β_3-adrenoceptor limits internalization of a β_2-adrenoceptor-β_3-adrenoceptor hetero-dimer because the-β_3-adrenoceptor element is dominant in this phenotype. The internalization and β-arrestin-interaction phenotype of co-expressed GPCRs has also been examined for receptor pairs that respond to the same or similar ligands but individually display distinct β-arrestin-interaction affinities (Hanyaloglu et al., 2002; Terrillon et al., 2004). Therefore, the altered internalization characteristics of rMrgD in the presence of rMrgE are certainly

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compatible with their hetero-dimerization. The fact that a substantial fraction of rMrgD was still able to internalize in response to β-alanine in the presence of rMrgE may, at first sight, seem inconsistent with this model. However, it must be anticipated that when two GPCRs are co-expressed, the corresponding homo-dimers will also be generated and that the proportion of homo- and hetero-dimers will reflect the absolute expression level of each GPCR as well as the relative propensity to form homo- and hetero-dimers and this is likely to be determined by their relative interaction affinity for a homo-monomer and the potential hetero-partner. As such, it is certainly possible that the rMrgD internalized in the presence of rMrgE is actually the fraction that represents rMrgD homo-dimers and that the difference in extent of internalization in the presence and absence of rMrgE expression actually represents the fraction of rMrgD present as the rMrgD-rMrgE hetero-dimer. These are enormously challenging questions to address directly and quantitatively but it may be that differential 2 protein and 3 protein FRET imaging techniques with associated photo-bleaching protocols will be able to provide insights.

To assess the functional relevance of rMrgD-rMrgE interactions we examined two distinct signalling endpoints. β-alanine promoted ERK MAP kinase phosphorylation via rMrgD but not rMrgE. However, in rMrgD-rMrgE co-expressing cells, although the transient nature of ERK1/2 phosphoryation was not different from cells expressing only rMrgD, there was a clear and statistically significant increase in potency of β-alanine to produce this effect. It is impossible at this stage to provide clear evidence for the mechanism responsible. The pharmacology of a number of GPCR hetero-dimers has been shown to be distinct from the corresponding homo-dimers (Maggio et al., 2005). However, as the available ligands at rMrgD are essentially restricted to β-

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alanine and rMrgE remains an orphan GPCR this cannot be addressed at this point. As noted earlier, the presence of rMrgE limited the extent of β-alanine-mediated rMrgD internalization. A substantial literature has examined the importance or otherwise of receptor internalization for ERK1/2 phosphorylation and activation (Kramer and Simon, 2000; Pierce et al., 2000). However, studies with GPCRs modified to prevent internalization in response to agonist occupancy or those which are naturally resistant to agonist-induced internalization have confirmed that receptor internalization is not a pre-requisite (Hislop et al., 2001; Budd et al., 1999). It is anticipated that interactions between the two elements of a GPCR hetero-dimer will produce allosteric effects on ligand binding (Durroux, 2005) and *vice-versa* and such effects may also contribute to the different potency of β-alanine observed.

One unexpected but very obvious difference in function of β-alanine in cells co-expressing rMrgD and rMrgE compared to cells expressing only rMrgD was in the regulation of $[Ca^{2+}]_i$. Although rMrgD expression was maintained at similar levels over a period of induction of expression of between 24-72 h it was obvious both that the maximal elevation of $[Ca^{2+}]_i$ was reduced at the latter time point and that the kinetics of elevation were considerably slower. As this was initially surprising, we analyzed this effect in more than 100 individual cells at each time point and thus this difference is highly significant ($p < 0.001$). However, in cells co-expressing rMrgD and rMrgE, β-alanine-mediated elevation of $[Ca^{2+}]_i$ was not different at different periods of rMrgD expression. Although the basis for this difference is unclear these observations further indicate the importance of examining co-expressed pairs or indeed groups of receptors in concert rather than in isolation following expression.

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These studies provide the first demonstration of hetero-dimerization between members of the Mrg family of GPCRs and highlight that many aspects of receptor function, including agonist-mediated internalization, and hence potentially desensitization, and the details of agonist potency and extent of function can be altered by co-expression and hetero-dimerization between distinct but related GPCRs. The current studies are limited, however, by a number of potential issues. Firstly, as MrgE is an orphan GPCR and no high affinity ligands of MrgD are available, it has not been possible to quantitate the expression levels of the two receptors used in these studies. It is thus unclear how these relate to expression levels within dorsal root ganglia. Secondly, the lack of Mrg receptor-subtype specific antibodies limits efforts to explore direct protein-protein interactions involving MrgD and MrgE in native tissues. Finally, although the addition of a wide range of both N and C-terminal tags frequently has little effect on the basic pharmacology and function of many GPCRs (Wilson et al., 2005) the extremely limited pharmacology currently available to explore Mrg receptor function means that we cannot state with certainly that this has not modified receptor function in the current studies. The physiological significance of in-vivo oligomerization of MrgD and MrgE receptors, therefore, remains to be determined. Interestingly it has been demonstrated that expression of MrgD is restricted to nociceptive neurons (Shinohara et al., 2004; Zylka et al., 2005), and this receptor is up-regulated in animal models of neuropathic pain (Shinohara et al., 2004). Based on these limited data it is interesting to speculate that the physiological interaction of these receptors in-vivo could provide a level of control or fine tuning of nociceptive processing. Future work using all available genetic

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and chemical tools will address these interesting and fundamental questions.

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Figure Legends

Figure 1. Co-expression of MrgD and MrgE receptor mRNA in rat dorsal root ganglia

RNA from various rat tissues was used for quantitative reverse transcriptase-PCR as detailed in Experimental to detect mRNA encoding MrgD (**A**) or MrgE (**B**). Only dorsal root ganglia contained high copy number of mRNA for each receptor. Error bars represent the S.D. of 3 samples.

Figure 2. rMrgD and rMrgE form constitutive homo-dimeric/oligomeric complexes

FLAG (1,3) and c-myc (2,3)-tagged forms of rMrgD (left hand panel) or rMrgE (right hand panel) were expressed transiently in HEK293 cells either individually (1-2) or in combination (3). In (4), samples individually expressing FLAG or c-myc-tagged receptors were mixed. Anti-FLAG antibody was used to immunoprecipitate samples and these were resolved by SDS-PAGE and subsequently immunoblotted with anti-c-myc.

Figure 3: Constitutive and inducible expression of rMrgD and rMrgE receptor constructs in Flp-In T-REx HEK293 cells

***A*:** Flp-In T-REx HEK293 cell lines were generated to allow inducible expression of N-terminally c-myc-tagged-rMrgE, FLAG-tagged rMrgD or c-myc-tagged-rMrgD. Cells were grown with or without 1 μg/ml doxycycline for 24 h. Whole cell lysates were prepared and samples containing 20 μg protein were resolved by SDS-PAGE,

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transferred onto a PVDF membrane and immunoblotted with anti-c-myc (upper panel) or anti-FLAG (lower panel) antibodies.

B,C: Cells as in **A**, with the capacity to inducibly express FLAG- (**B**) or c-myc- (**C**) tagged rMrgD, were further transfected to stably and constitutively express c-myc-rMrgE (**B**) or FLAG-rMrgE-eYFP (clone 1) or rMrgE-eYFP (clone 21) (**C**). Following growth with or without 1 μg/ml doxycycline for 24 h whole cell lysates were prepared and the individual receptors detected as in **A** using anti-c-myc, anti-FLAG or anti-GFP antibodies.

Figure 4: Constitutive hetero-dimerization between co-expressed rMrgD and rMrgE receptors revealed by co-immunoprecipitation and Tr-FRET

A. Flp-In T-REx HEK293 cells either harbouring FLAG-rMrgD at the inducible locus only or constitutively expressing c-myc-rMrgE and harbouring FLAG-rMrgD at the inducible locus were induced with 1 μg/ml doxycyline for varying times. In the 'mix' samples, lysates from FLAG-rMrgD induced for 96 h and of un-induced FLAG-rMrgD-c-myc-rMrgE cells were mixed before analysis.

Upper panels: Cell lysates were immunoprecipitated with anti-FLAG antibody, samples resolved by SDS-PAGE and then immunoblotted with either anti-c-myc or anti-FLAG antibodies.

Lower panels: Western blot analysis of cell lysates using anti-FLAG and anti-c-myc antibodies demonstrated maintained levels of c-myc-rMrgE over time and the time course of induction of FLAG-rMrgD.

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B. Flp-In T-REx HEK293 cells constitutively expressing c-myc-rMrgE and harbouring FLAG-rMrgD at the inducible locus were treated with (black bars) or without (open bars) 1 μg/ml doxycyline for 24 h. A combination of Eu^{3+}-labelled anti-c-myc antibody and APC-labelled anti-FLAG antibody was added and Tr-FRET measured as in Experimental. The pair of antibodies was also added to a mixture of cells induced to express either c-myc-rMrgE or FLAG-rMrgD (gray bar). Data represent means ± S.E.M. from 3 independent experiments.

C Flp-In T-REx HEK293 cells constitutively expressing c-myc-rMrgE and harbouring FLAG-rMrgD at the inducible locus were induced (induced) or not (control) to express FLAG-rMrgD. A mix control was generated as in **B**. APC-labelled anti-FLAG antibody was added and, after washing, fluorescence corresponding to APC bound to the cells was measured. Equivalent signal above background was present in the induced and mixed control, confirming the lack of Tr-FRET in the mix control in **B** did not reflect poor induction of expression of FLAG-rMrgD. Data represent means ± S.E.M. from 3 independent experiments.

Figure 5: Internalization of rMrgD but not rMrgE receptors in response to β-alanine

Flp-In T-REx HEK293 cell lines were induced with 1 μg/ml doxycycline for 72 h to express either c-myc-rMrgD (**A**) or c-myc-rMrgE (**B**) and then immunostained with anti-c-myc antibody prior to stimulation with varying concentrations of β-alanine for 30 min at 37°C. Confocal images monitoring the location of receptor-associated anti-c-myc were taken following permeabilization of untreated cells (**a**) and cells treated

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with 0.1 mM (**b**), 0.3 mM (**c**), 1 mM (**d**), 3 mM (**e**) or 10 mM (**f**) β-alanine. Similar results were produced in 3 independent experiments.

Figure 6: Co-expression with rMrgE impairs β-alanine-induced internalization of rMrgD

***A*:** c-myc-rMrgD receptor (**(i)**, **Red**) expression was induced by treatment with 1 μg/ml doxycycline for 72 h in cells constitutively expressing FLAG-rMrgE-eYFP (**(ii)**, **Green**). Cell surface c-myc-rMrgD receptors were labelled with anti-c-myc antibody and the cells treated with vehicle (**Upper panels**) or 3 mM β-alanine (**Lower panels**) for 30 min at 37°C. The overlay of the two signals (**Upper panel (iii)**, **Yellow**) in the absence of β-alanine was partially resolved (**Lower panel (iii)**) by internalization of c-myc-rMrgD (**Red**).

***B*:** Cells as in **A** were untreated or stimulated with 3 mM β-alanine for 30 min at 37°C, incubated with rabbit polyclonal anti-c-myc and anti-rabbit horseradish peroxidase-conjugated antibodies and cell surface receptor immunoreactivity measured by colorimetry. Internalization was defined as the loss of cell surface immunoreactivity and is expressed as the percentage of the immunoreactivity present in the absence of agonist. Data represent means ± S.E.M. of 4 independent experiments. A lower percentage of c-myc-rMrgD was internalized when rMrgE was co-expressed ($p<0.01$).

C. Two distinct clones constitutively expressing c-myc-rMrgE and harbouring FLAG-rMrgD at the inducible locus were induced (filled bars) or not (open bars) to express FLAG-rMrgD. Cells were stimulated with 3 mM β-alanine for 30 min at

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37°C and cell surface levels of c-myc-rMrgE measured as in **B** following addition of rabbit polyclonal anti-c-myc and horseradish peroxidase-conjugated anti-rabbit antibodies. Induction of FLAG-rMrgD expression did not result in internalization of c-myc-rMrgE in response to β-alanine. Data represent means ± S.E.M. of 4 independent experiments

Figure 7: β-alanine stimulates phosphorylation of ERK1/2 via rMrgD but not rMrgE

Cells were induced to express FLAG-rMrgD (**A**), c-myc-rMrgE (**B**), or FLAG-rMrgD in the presence of constitutively expressed c-myc-rMrgE (**C**). Following overnight serum-starvation cells were stimulated with 100 μM β-alanine for the indicated times at 37°C. ERK1/2 phosphorylation was then detected using phospho-specific anti-ERK1/2 antibodies (**P-ERK1/2**). Expression levels of ERK1/2 were monitored using antibodies directed against the total population of ERK1/2 (**ERK1/2**). Expression of FLAG-rMrgD and c-myc-rMrgE was also monitored. Similar results were produced in three separate experiments.

Figure 8: Selectivity of β-alanine-induced ERK1/2 phosphorylation

Flp-In T-REx HEK293 cells were treated with 1 μg/ml doxycyline to induce expression of c-myc-rMrgD. 24 h later the cells were serum starved and then treated with varying concentrations of β-alanine (**A**) or L-alanine (**B**) for 5 min at 37°C. Phosphorylation of ERK1/2 was evaluated by immunoblot analyses using anti-phospho specific ERK1/2 antibodies (**P-ERK1/2**). Total ERK1/2 levels (**ERK1/2**) and the presence of the receptors (**c-myc**) were monitored as in Figure 7.

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Figure 9: Co-expression of rMrgE enhances the potency of rMrgD-β-alanine-induced ERK1/2 phosphorylation

Flp-In T-REx HEK293 cells were treated with 1 μg/ml doxycyline for 96 h to induce expression of FLAG–rMrgD in the absence (**A**, **triangles in D**) or presence (**B**, **diamonds in D**) of constitutive expression of c-myc-rMrgE. In **C** cells constitutively expressing c-myc-rMrgE were not induced. Data represent means of 4 independent experiments. Errors bars are within the size of the symbols. * Significantly different ($p < 0.01$).

Figure 10: Stimulation of rMrgD but not rMrgE evokes elevation of intracellular [Ca^{2+}]

Flp-In T-REx HEK293 cells were treated with doxycycline for 24 h to induce expression of c-myc-MrgD (red) or c-myc-MrgE (blue) receptors. As a control, cells harbouring c-myc-MrgD were not induced (black). FLAG-MrgD was also induced in the presence of the constitutive expression of c-myc-MrgE (green). . The effect of 1 mM β-alanine on cellular $[Ca^{2+}]_i$ was then assessed in individual cells. The data (means ± S.E.M.) are pooled from 62 cells for the non-induced samples and 43 cells in the case of each of the induced cell lines.

Figure 11. Co-expression with rMrgE maintains the capacity β-alanine to elevate intracellular [Ca^{2+}] via rMrgD

Flp-In T-REx HEK293 cells were induced to express FLAG-MrgD receptors for 24 (**A**), 48 (**B**) or 72 (**C**) h in the absence (red) or constitutive presence of c-myc-MrgE

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receptors (black). Cells were loaded with Fura-2/AM and $[Ca^{2+}]_i$ levels imaged over time following exposure to 1 mM β-alanine. Data represent means ± S.E.M from analysis of 115 cells for each condition at each time point. * Significantly different ($p < 0.001$).

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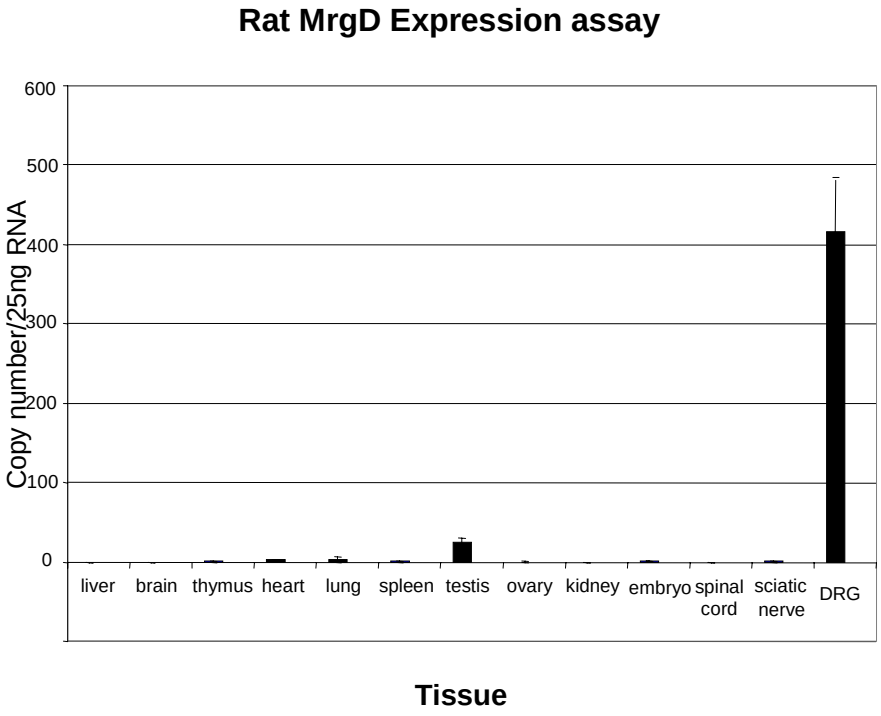
Figure 1

A



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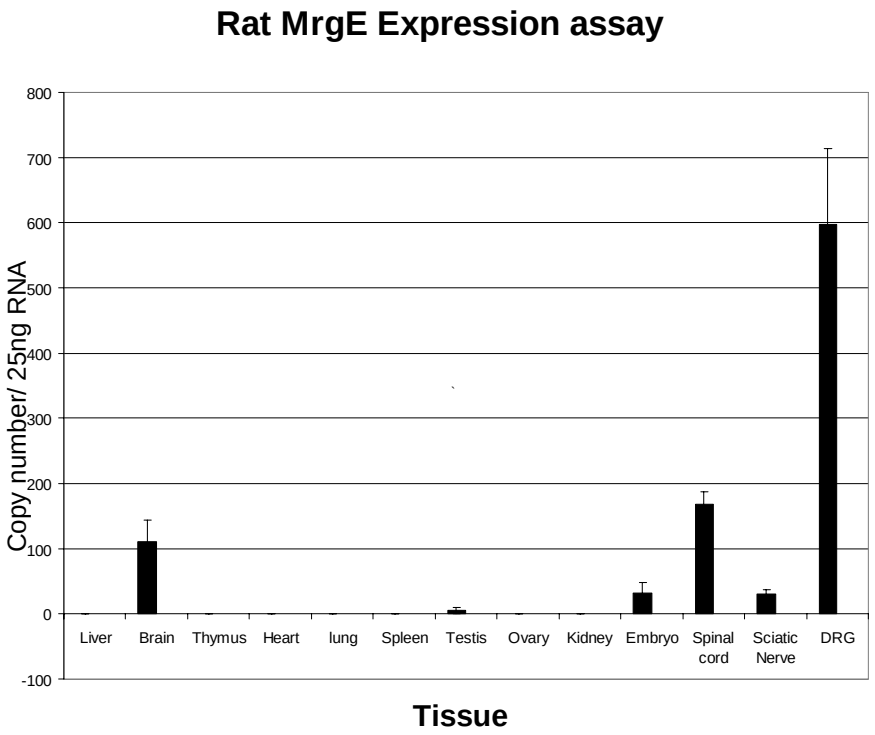

Figure 2

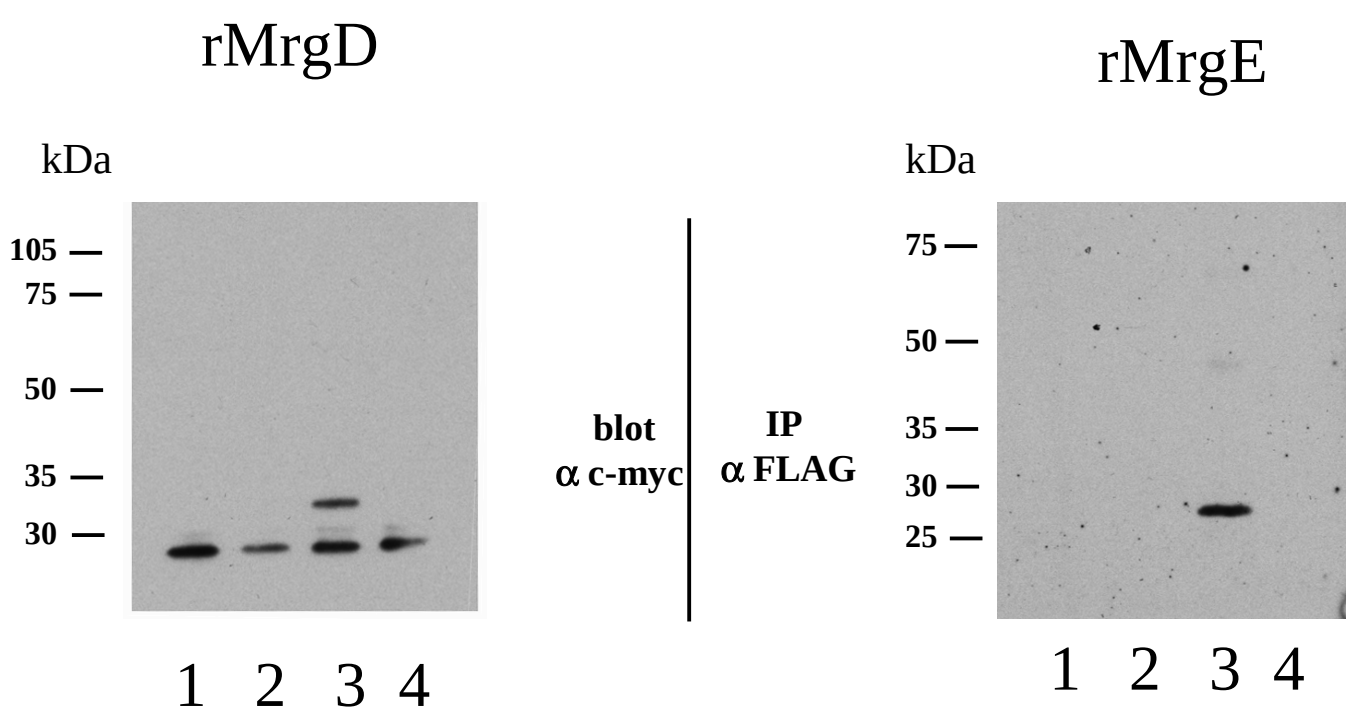

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Figure 3

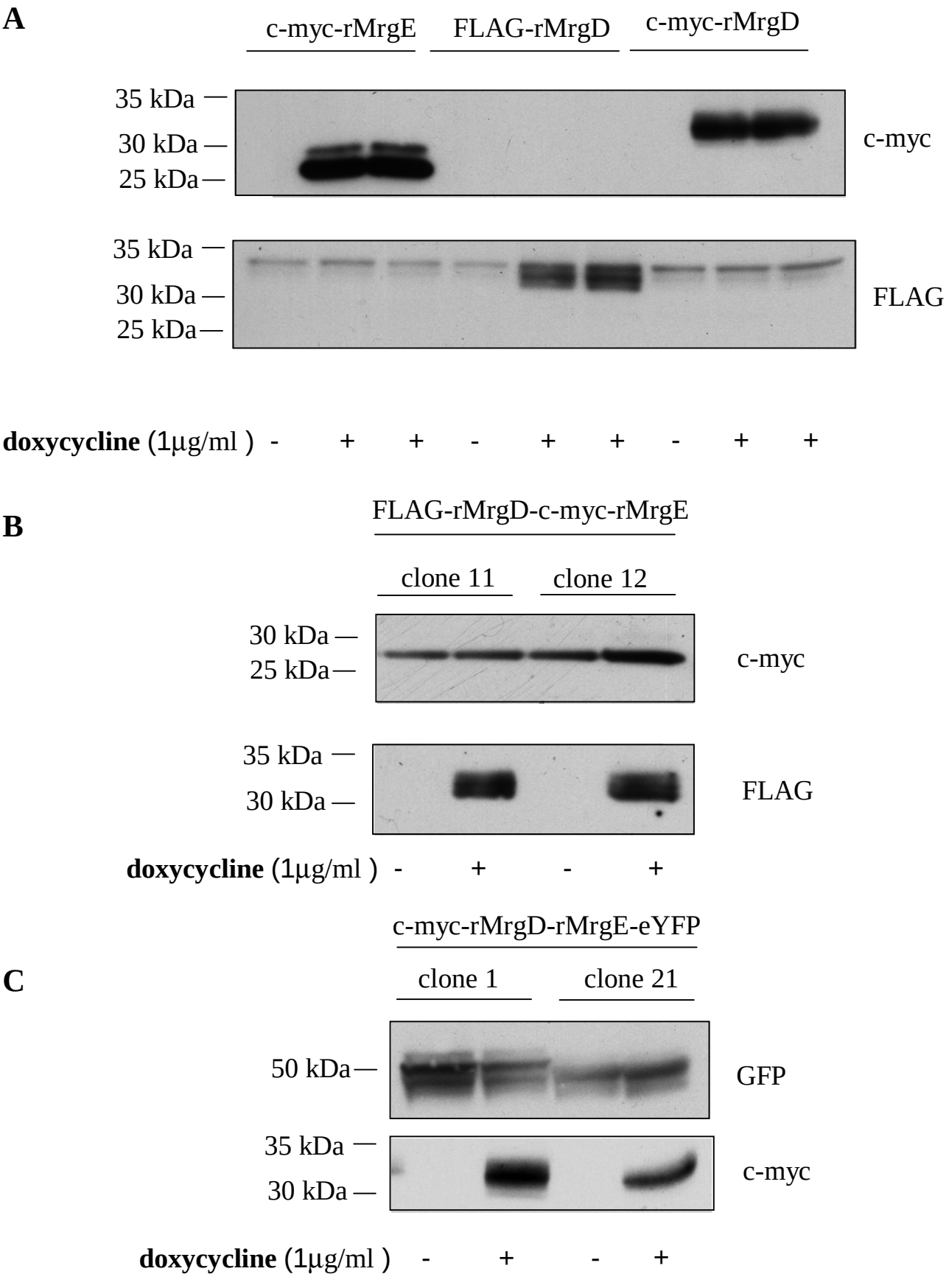

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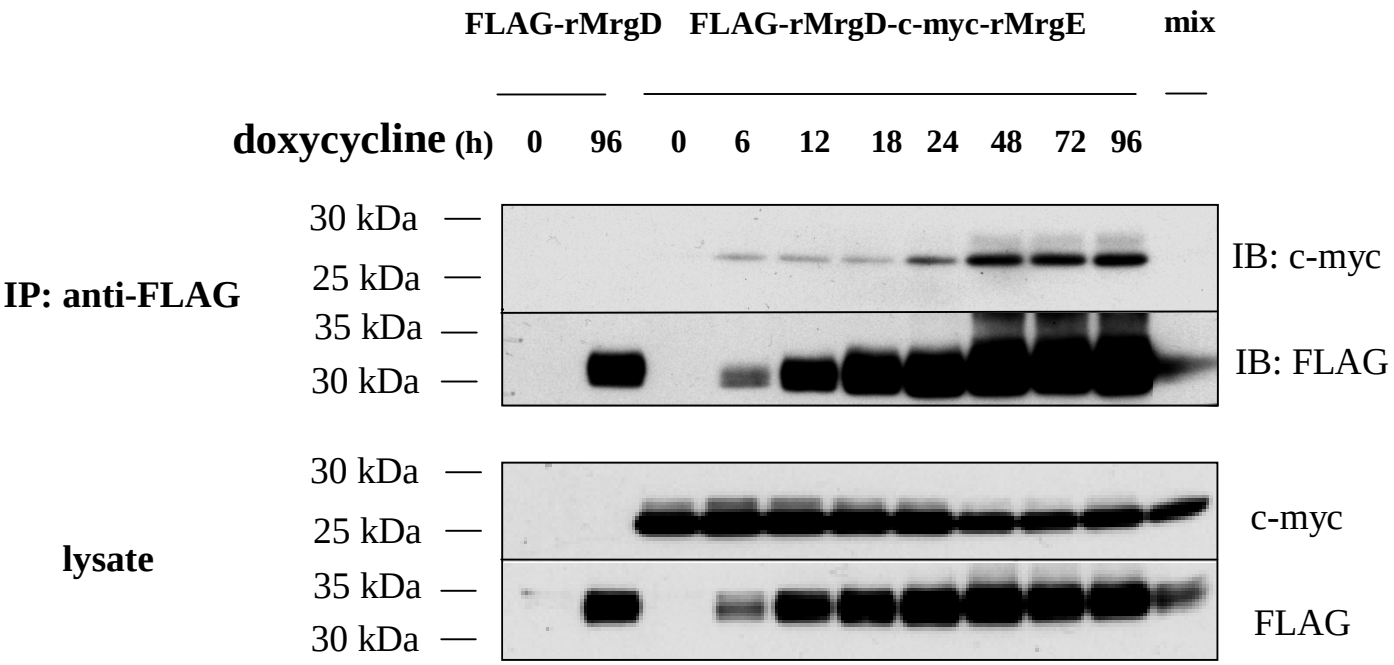
Figure 4A



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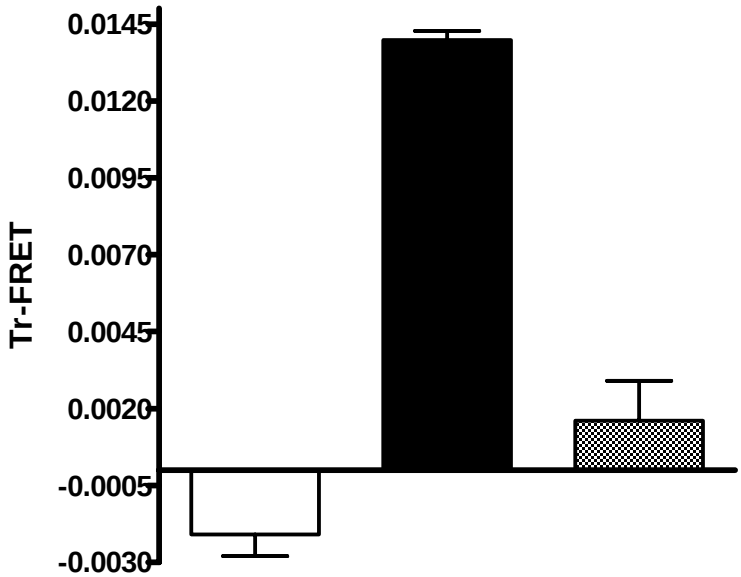
Figures 4B,C

(B)



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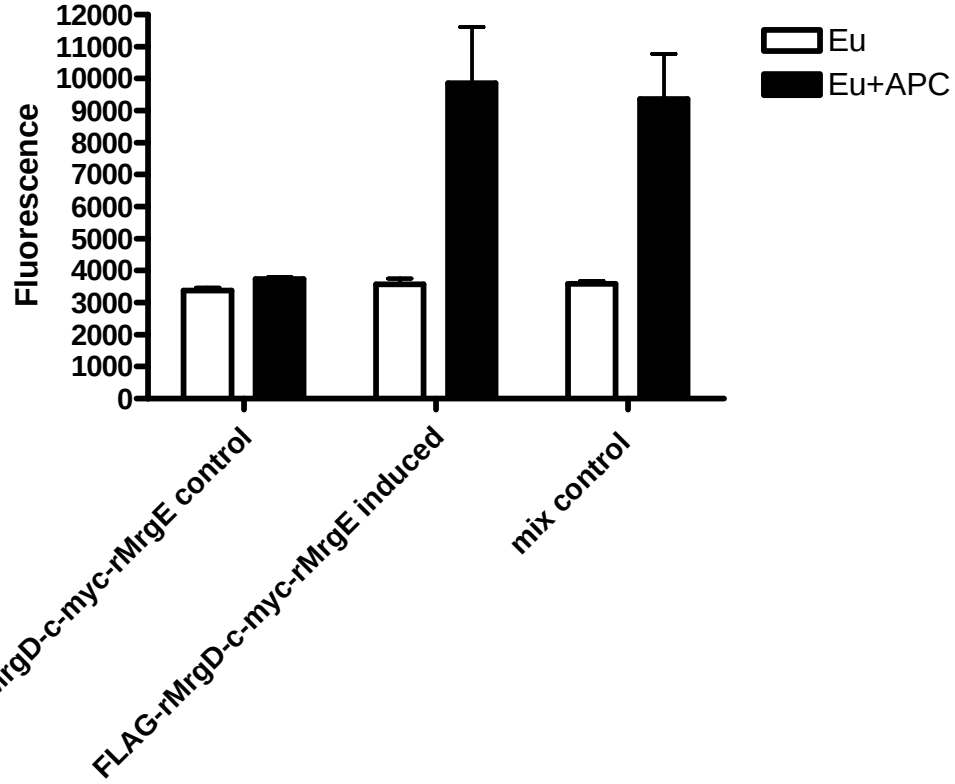

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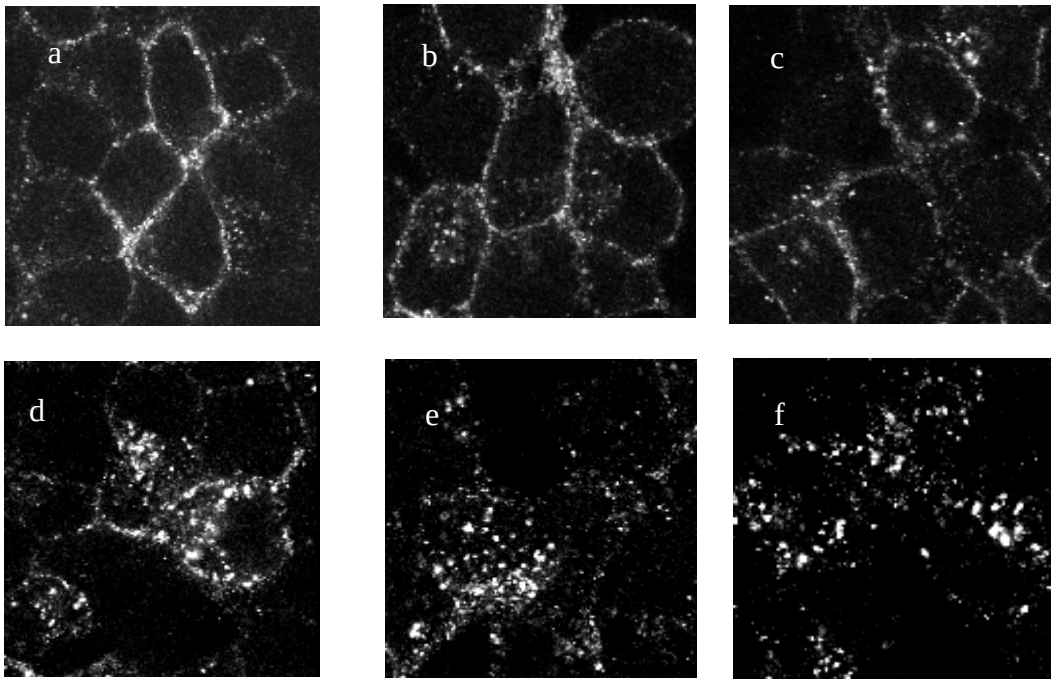
Figure 5

A



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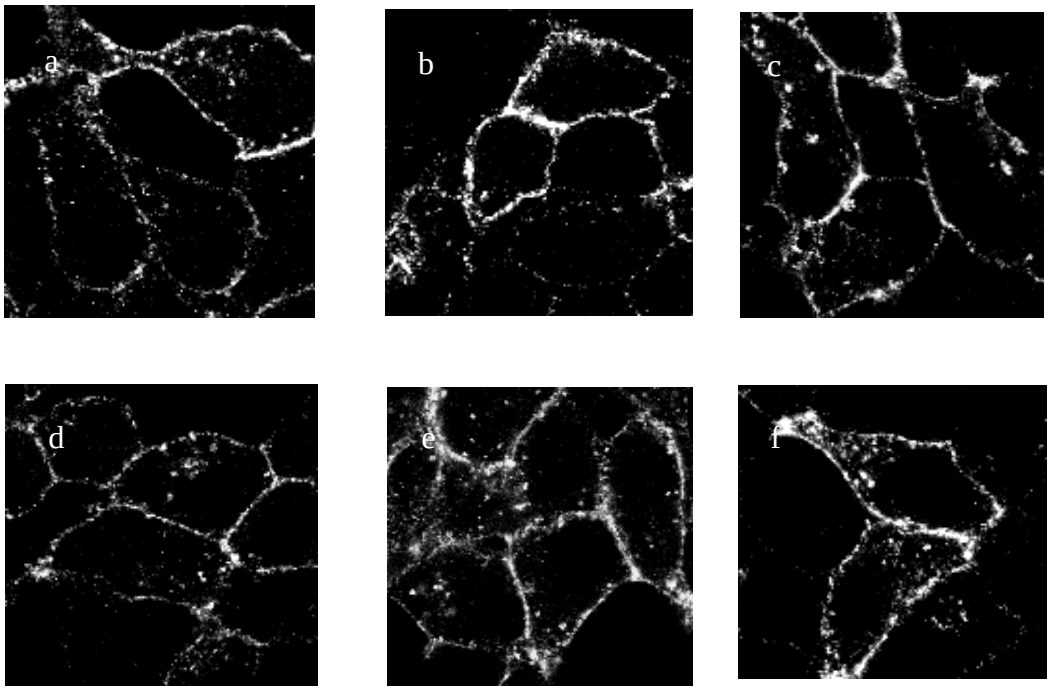

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Figure 6A

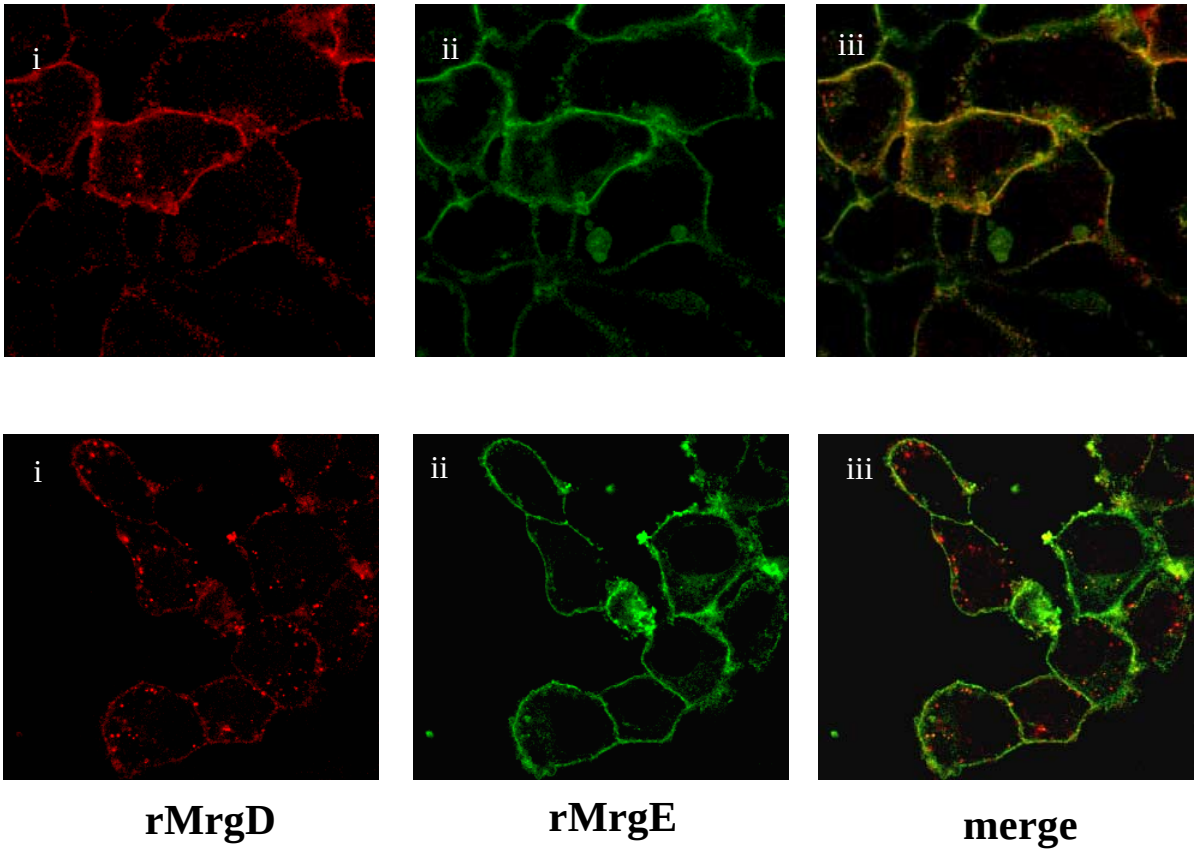

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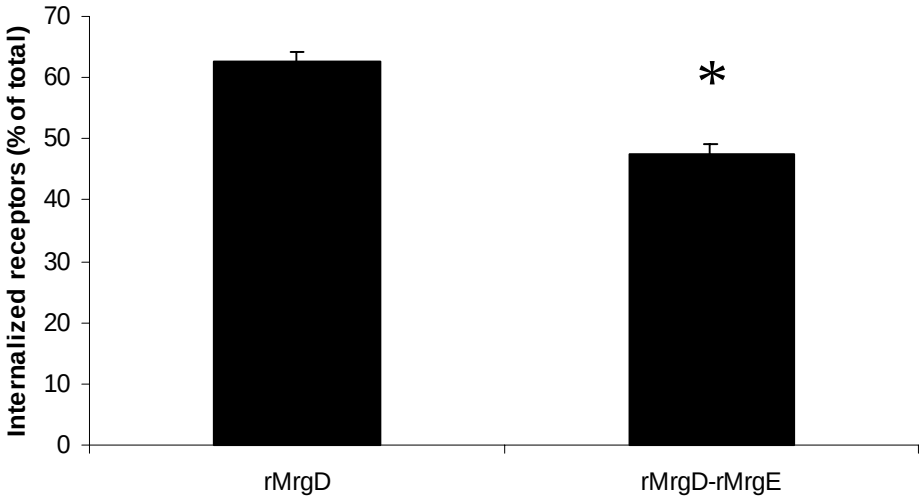
Figure 6B,C

(B)



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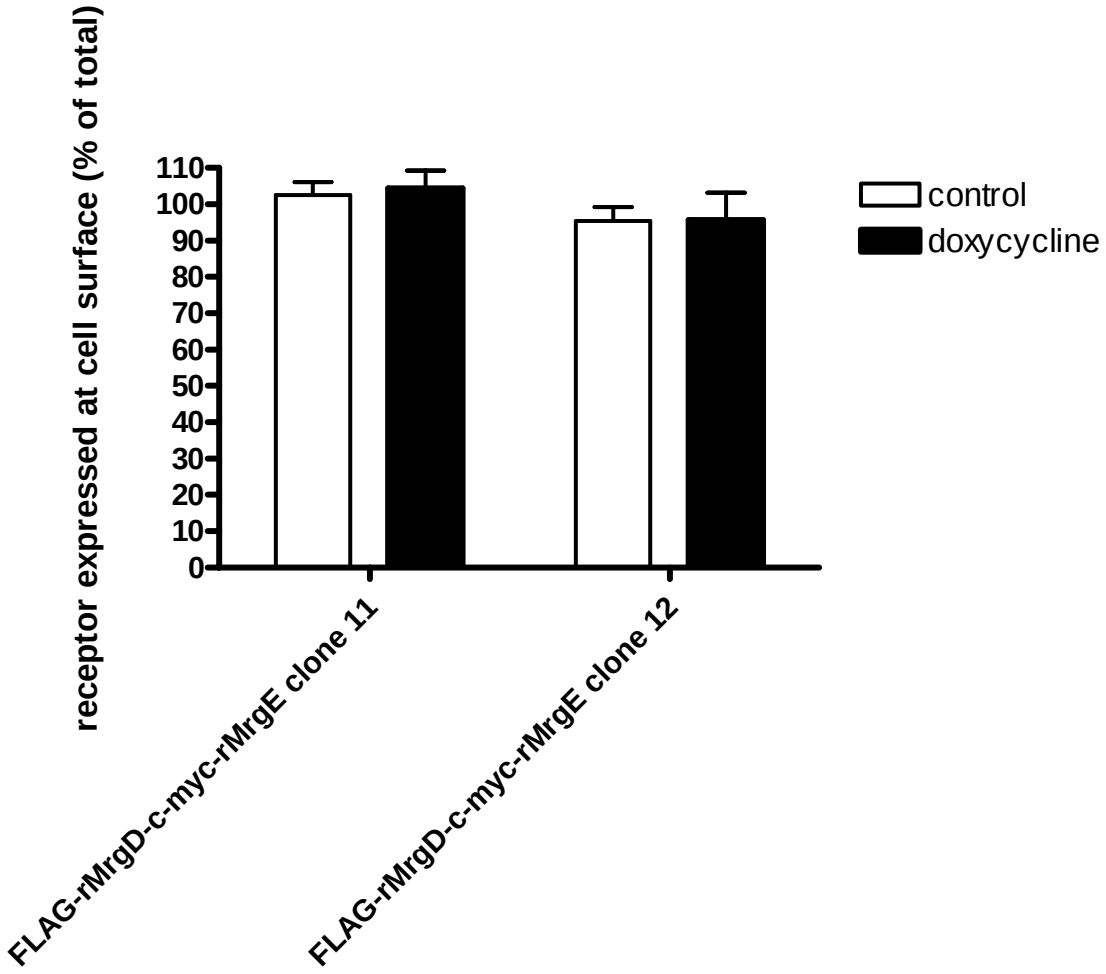

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Figure 7

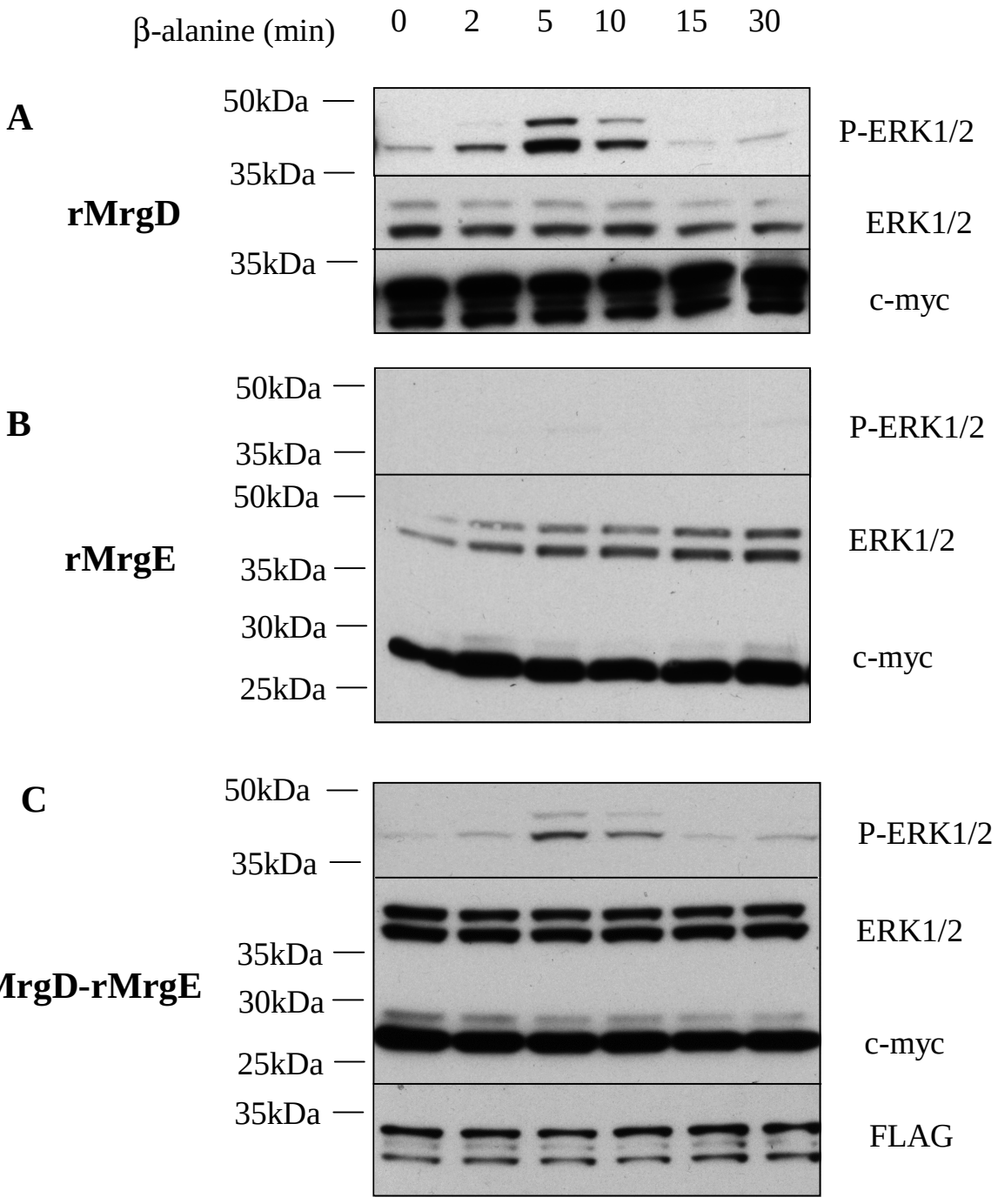

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Figure 8

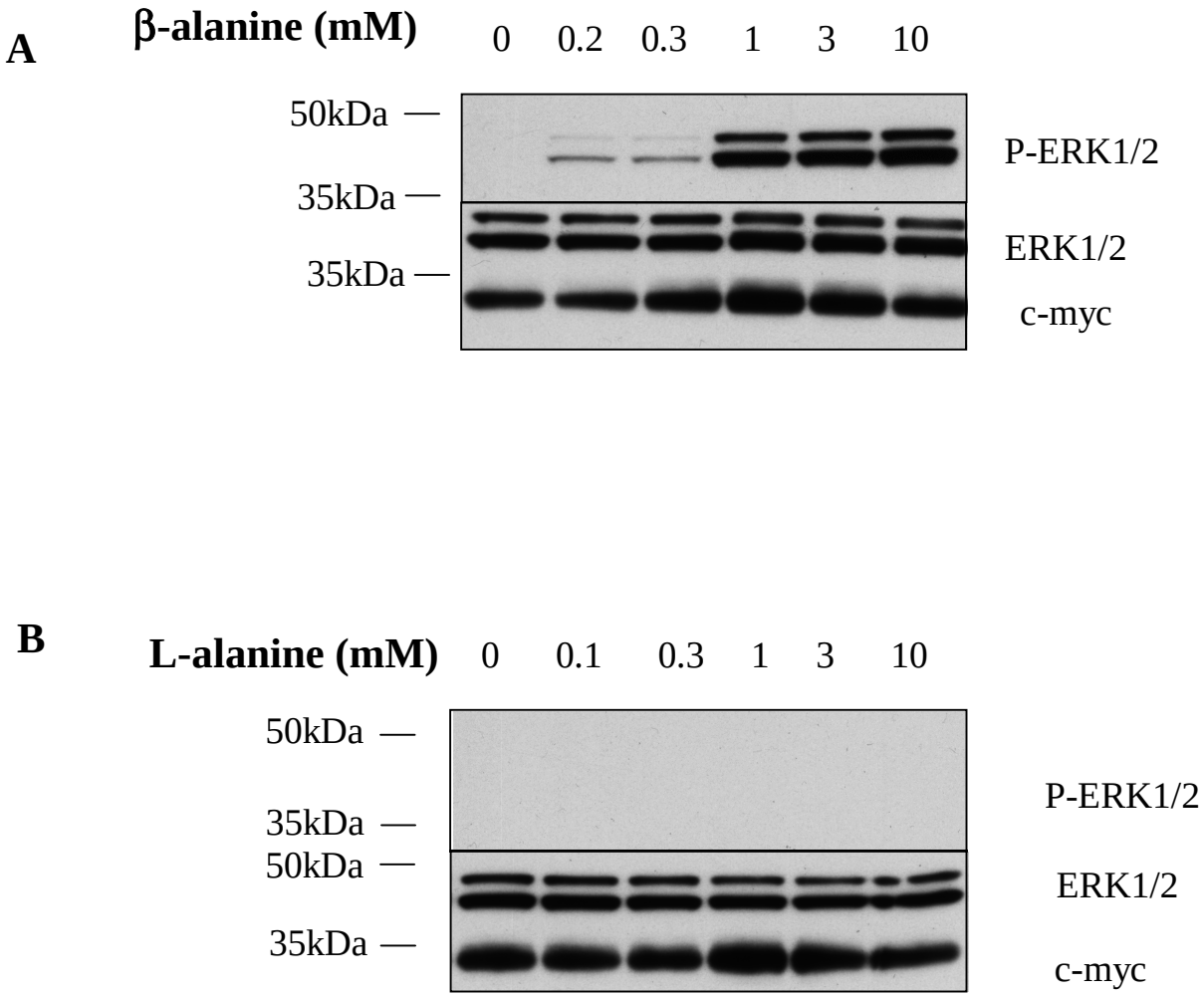

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Figure 9A,B,C

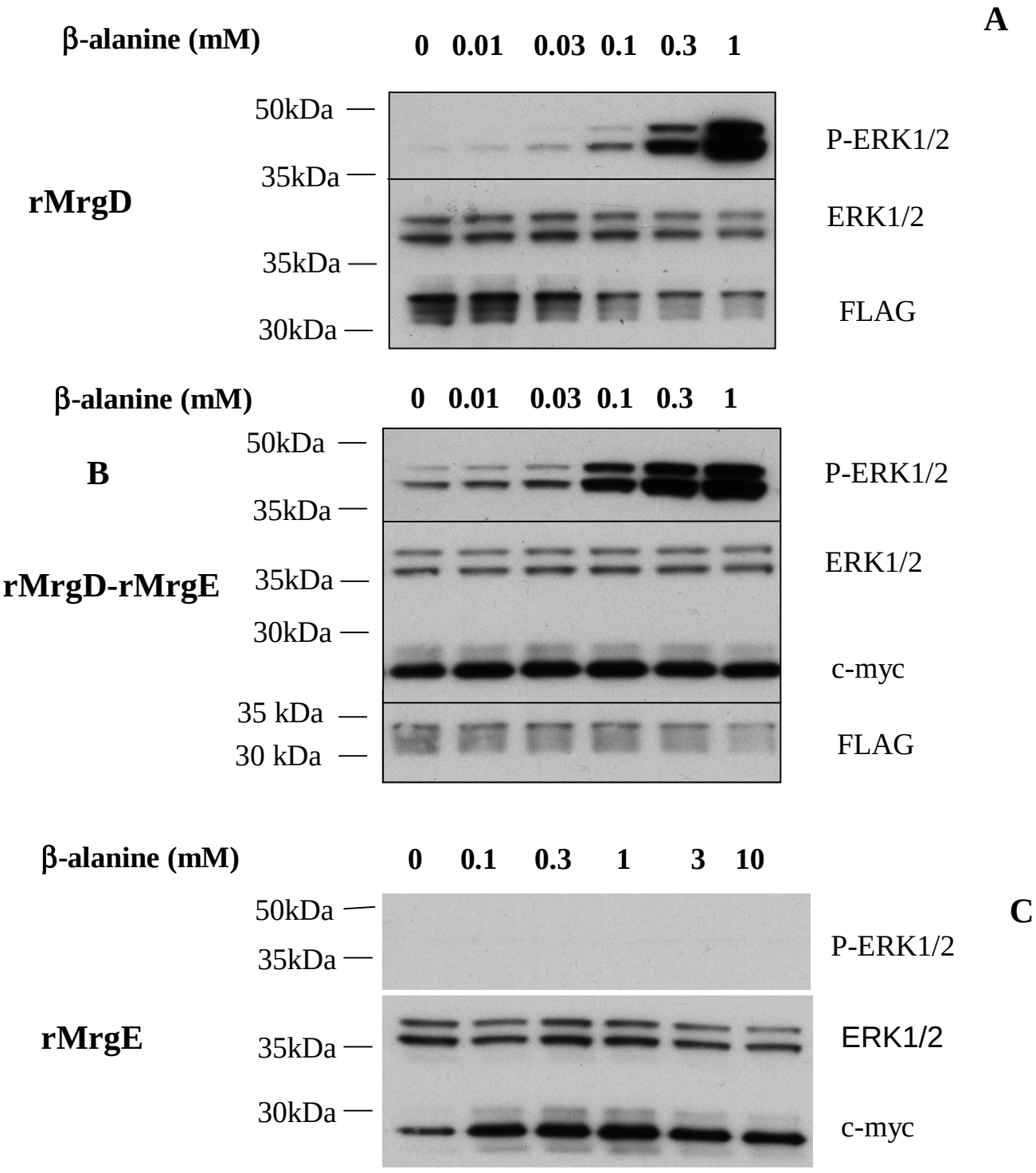

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Figure 9D

D

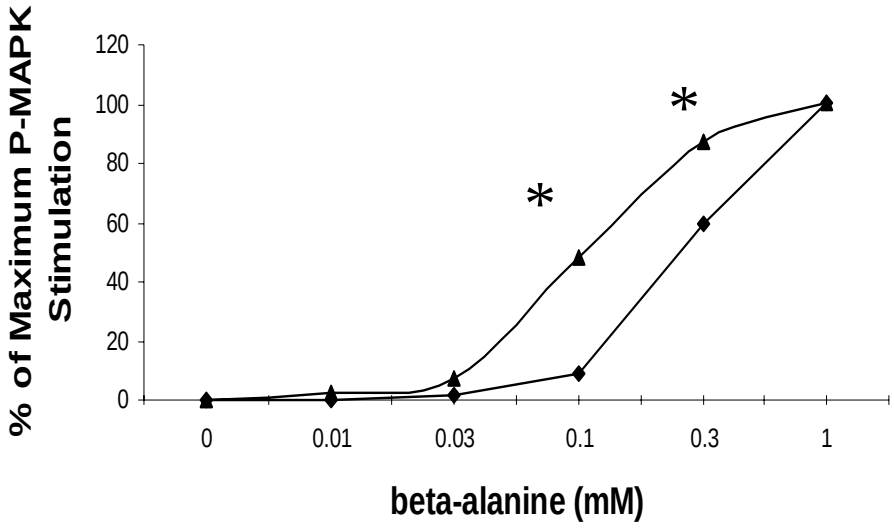

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Figure 10

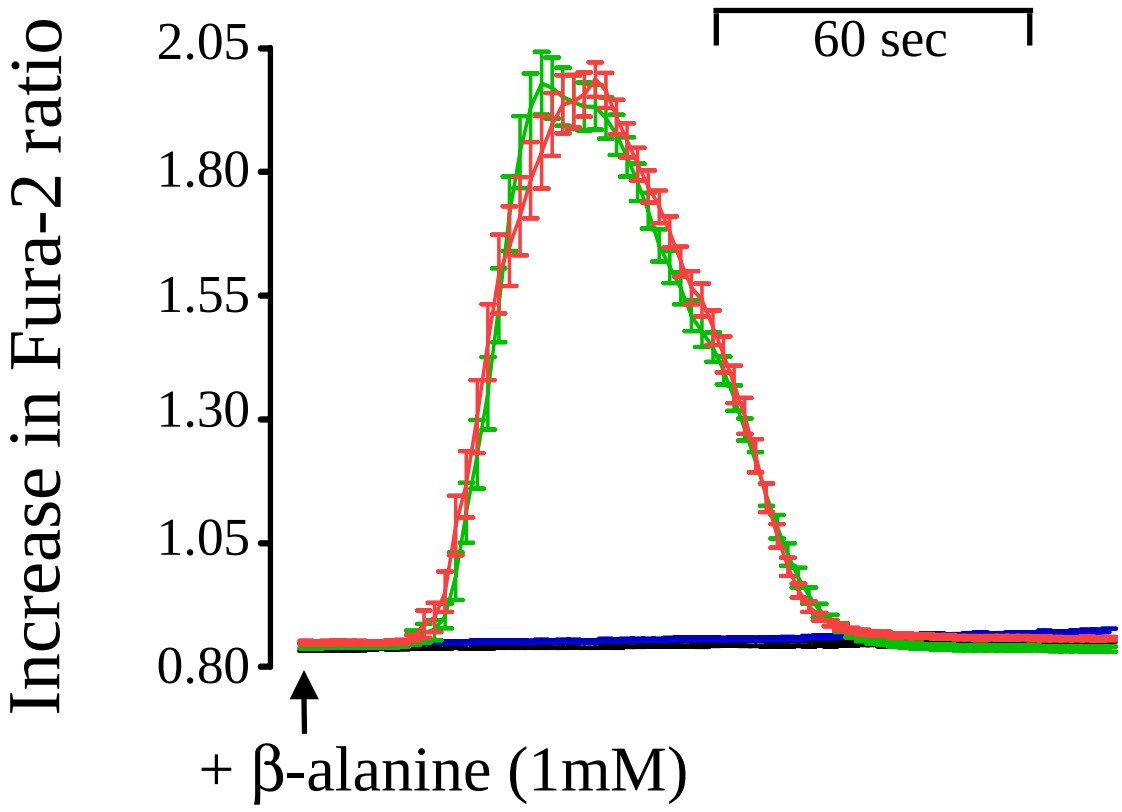

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Figure 11