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Characterisation of the adiponectin receptors: The non-conserved N-terminal region of AdipoR2 prevents its expression at the cell-surface

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ABSTRACT

Adiponectin is a beneficial adipokine with insulin-sensitizing, anti-inflammatory and anti-atherogenic effects. These effects are mediated by two poorly characterised, closely related, atypical seven-transmembrane receptors. In the current report we have used C-terminal, epitope-tagged AdipoR1 and AdipoR2 constructs to monitor cell-surface expression by indirect immunofluorescence microscopy and quantitative plate-based analysis. We demonstrate that only AdipoR1 is constitutively expressed on the cell-surface. Further investigations, involving characterisation of a number of chimeric and truncated constructs, show the non-conserved region of AdipoR2 (residues 1–81) restricts its cell-surface expression. Introduction or deletion of this region, into AdipoR1 or AdipoR2, resulted in inhibition or promotion of cell-surface expression, respectively. We also confirmed that AdipoR1 and AdipoR2 can form heterodimers when co-expressed and that co-expression leads to the cell-surface expression of AdipoR2. Collectively these studies demonstrate that the non-conserved region of AdipoR2 restricts its cell-surface expression and raise the possibility that the majority of cell-surface AdipoR2 may be present in the form of heterodimers.

1. Introduction

Since their discovery in 2003 [1] the adiponectin receptors, AdipoR1 and AdipoR2, have been the subject of extensive investigations. A large body of evidence has accumulated which indicates that these receptors mediate many of the salutary effects of adiponectin, a key adipokine produced by adipocytes [2], and thereby defining them as attractive therapeutic targets [3]. Early characterisation suggested the receptors represented an atypical form of seven-transmembrane domain receptor (7TMR) that showed reverse topology to the classic GPCRs, with intracellular and extracellular N-termini and C-termini, respectively [1]. They were subsequently recognised as prototypical members of a 7TMR family, termed the PAQR family, that show conserved structural and topological organisation with some, albeit limited, invariant intracellular amino acids [4]. Recent evidence suggests the receptors may have intrinsic ceramidase activity [5].

Classic loss and gain of function studies showed AdipoR1 and AdipoR2 transduce the effects of adiponectin to activate a number of intracellular signalling networks including AMPK and PPARα [1,6]. Evidence from knockout mice suggests that both receptors are important for metabolic homeostasis however there are striking differences between the different mouse lines generated by three independent groups making detailed mechanistic interpretation difficult [6–8]. Perhaps surprisingly our understanding of the basic biology of AdipoR1 and AdipoR2 is relatively limited. Like GPCRs [9], they have been reported to form homo- and hetero-dimers [1,10,11] although the functional and physiological significance of this is unclear. A dimerization motif ($G(X)_3G$) has been identified in the fifth transmembrane domain of AdipoR1 [11] and a recent report has also identified two motifs ($D(X)_3LL$ and $F(X)_3F(X)_3F$) within the intracellular N-terminal region of AdipoR1 that are required for anterograde trafficking to the cell-surface [12]. Interestingly, all three motifs are conserved in AdipoR2. In addition, a number of proteins have been shown to interact with the intracellular N-terminal regions of AdipoR1 and or AdipoR2 and modulate signal transduction [13]. The best characterised of these is APPL1 [14], which interacts with both AdipoR1 and AdipoR2 [15]. We recently identified ERp46 as an AdipoR1-specific interacting protein and demonstrated that knockdown of ERp46 increased the enrichment of AdipoR1, and AdipoR2, in the plasma membrane (PM) and altered adiponectin signalling [16]. In the current report we have extended these studies by further characterisation of AdipoR1 and AdipoR2 at the level of cell-surface expression.

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2. Materials and methods

2.1. Reagents and antibodies

Reagents were from Sigma–Aldrich (Castle Hill, Australia) unless otherwise stated. Tissue culture reagents were from Invitrogen (Mount Waverley, Australia). Primary antibodies against FLAG (Sigma–Aldrich), HA (Covance), and Calnexin (Affinity Bioreagents) were from the indicated suppliers. AdipoR1 and AdipoR2 antibodies were as described [16]. Secondary antibodies were from Molecular Probes (Invitrogen) or Rockland (PA, USA). Molecular biology reagents were from New England Biolabs (Ipswich, NA, USA) or Promega Corporation (Madison, WA, USA).

2.2. Molecular biology

Original constructs encoding C-terminally epitope-tagged (HA or FLAG) human AdipoR1 and AdipoR2 were as described [16]. Standard PCR-based approaches were used to generate chimeric and truncated receptor constructs. Integrity of all constructs was confirmed by direct sequencing. Chinese Hamster Ovary (CHO) cells or Human Embryonic Kidney (HEK) cells were transfected using Lipofectamine PLUS (Invitrogen) according to the manufacturer's instructions. Cells were typically analysed 24 h after transfection.

2.3. Generation of plasma membrane (PM) and ER fractions

Preparation of cell lysates for biochemical analysis of the subcellular distribution of AdipoR1 and AdipoR2 was essentially as described [16].

2.4. Immunofluorescence microscopy

Immunofluorescence microscopy of permeabilised cells was performed as described [17]. For microscopy of non-permeabilised cells, cells were washed in ice-cold PBS then blocked in 0.2% BSA and 0.2% fish skin gelatin in CO_2-independent medium for 35 min on ice. Cells were incubated with primary antibody in blocking solution for 45 min followed by 4×5 min washes in PBS on ice. After washing, cells were fixed in 4% PFA in PBS on ice for 20 min and quenched in 0.3 M glycine for 15 min. Following this step cells were processed as described [17]. The percentage of cells expressing detectable levels of total or cell-surface expression of AdipoR1 or AdipoR2 was determined by scoring HA-positive permeabilised (total) or non-permeabilised (cell-surface) cells, respectively. For each independent experiment at least 100 cells were counted per condition.

2.5. Plate-based determination of cell-surface expression of AdipoR1 and AdipoR2

Quantitative measurement of total and cell-surface expression of AdipoR1 and AdipoR2 was performed using a plate-based assay, which was based on the methods outlined above for immunofluorescence microscopy. Signals were detected using the Odyssey infrared imaging system (LICOR).

2.6. Electron microscopy

CHO cells were fixed with 0.2% glutaraldehyde/2% PFA in 0.1 M phosphate buffer and processed for EM as described [18]. Sections were labelled with α-HA antibodies followed by 10 nm protein A-gold.

2.7. Statistical analysis

Data are presented as mean ± SEM. Significance was determined using a Student's t test with statistical significance defined as $p < 0.05$.

3. Results

3.1. AdipoR1 but not AdipoR2 is expressed at the cell-surface

We previously observed that the biochemical subcellular fractionation properties of AdipoR1 and AdipoR2 differ, with the bulk of AdipoR1 resident in the PM whilst the majority of AdipoR2 is present in the ER in HeLa cells [16]. We confirmed this differential subcellular distribution following subcellular fractionation of HEK cells (Fig. 1A), suggesting this is a general phenomenon.

In order to investigate this further we employed indirect immunofluorescence microscopy. Attempts to detect total cellular and cell-surface expression of endogenous AdipoR1 and AdipoR2 proved unsuccessful in a range of cell types and this probably reflects the relatively low levels of expression of these proteins. To circumvent this problem and allow detailed mechanistic studies we performed experiments involving transient transfection of C-terminal, HA-tagged AdipoR1 and AdipoR2 constructs (Fig. 1B). Cell-surface expression of the C-terminal-tagged constructs results in exposure of the epitope-tag, providing a straightforward method for determination of cell-surface expression. In permeabilised cells, the distribution of AdipoR1-HA and AdipoR2-HA appeared similar, with both showing reticular staining patterns characteristic of the ER (Fig. 1C). Immunoelectron microscopy also revealed similar intracellular distribution profiles for AdipoR1-HA and AdipoR2-HA, with both proteins found in the rough ER as well as a range of morphologically-diverse membranous structures including tubular and vesicular profiles (Fig. 1E). In contrast, in non-permeabilised cells AdipoR1-HA was readily detected at the cell-surface but AdipoR2-HA was undetectable (Fig. 1C). Semi-quantitative analysis was performed by scoring cells expressing detectable total (permeabilised) or cell-surface (non-permeabilised) expression of AdipoR1-HA or AdipoR2-HA. Around 50% of cells expressed detectable levels of AdipoR1-HA or AdipoR2-HA (Fig. 1D). Cell-surface expression of AdipoR1-HA was evident in around 45% of cells (Fig. 1D). A plate-based assay was employed to provide more quantitative analysis (Fig. 1F and G). This approach revealed that around 50% of AdipoR1-HA was present at the cell-surface, whilst cell-surface AdipoR2-HA was undetectable (Fig. 1F and G). These complementary approaches indicate that around 90% of cells expressing AdipoR1-HA have readily detectable cell-surface expression and that this represents around 50% of total cellular AdipoR1-HA. Similar results were obtained using AdipoR1-FLAG and AdipoR2-FLAG tagged constructs (data not shown). These results reveal a surprising difference in steady-state, cell-surface expression of AdipoR1 and AdipoR2.

3.2. Characterisation of adiponectin receptor chimera suggests a key role for the non-conserved N-terminal region of the AdipoRs

We next sought to determine the molecular basis for the observed differences. AdipoR1 and AdipoR2 share 68% identity at the amino acid level. However, the cytoplasmic domains can be split into two regions that differ in their degree of sequence homology. The N-terminal regions, consisting of $AdipoR1_{(1-70)}$ and $AdipoR2_{(1-81)}$, show only 17% homology whilst the remaining sequences, comprising $AdipoR1_{(71-136)}$ and $AdipoR2_{(82-147)}$, show 95% homology (Fig. 2A). To test whether the non-conserved, N-terminal regions underpinned the differences between the AdipoRs

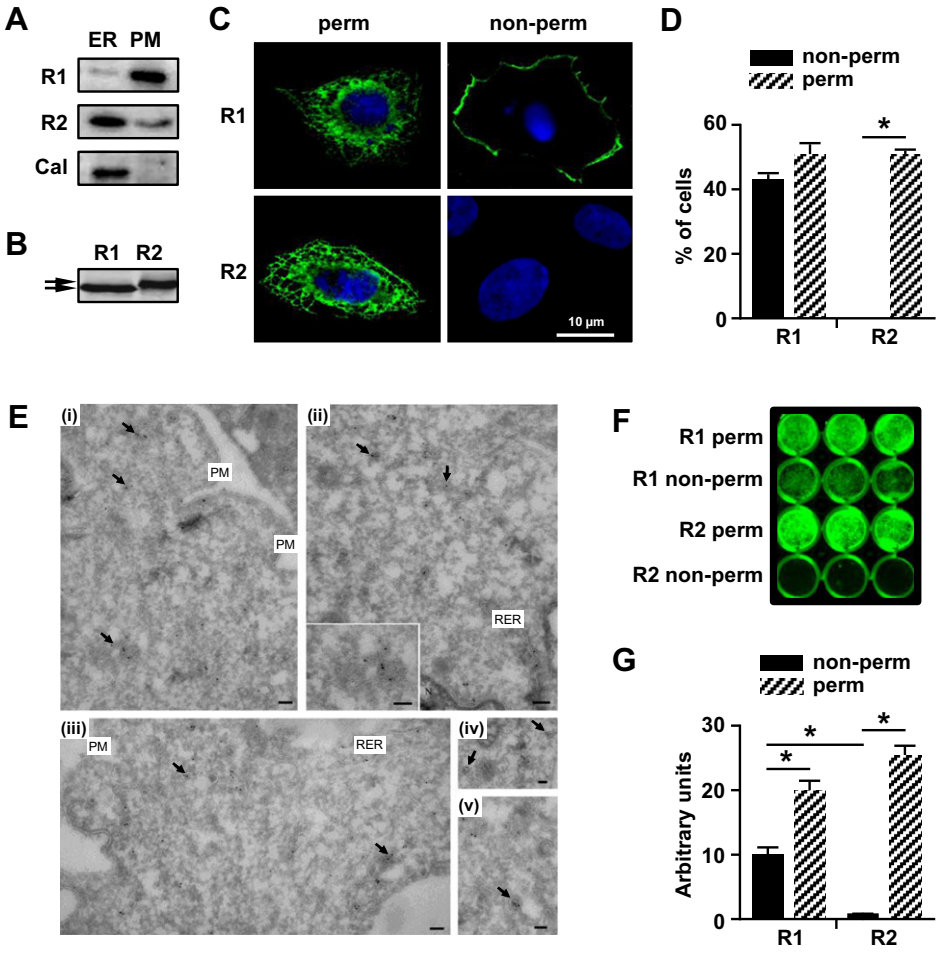

Fig. 1. Differences in cell-surface expression of AdipoR1 and AdipoR2. (A) HEK cells were fractionated and the distribution of endogenous AdipoR1, AdipoR2 and Calnexin in the ER and PM fractions was determined by Western blot. (B) Western blot of CHO cells transiently transfected with AdipoR1-HA or AdipoR2-HA probed with α-HA antibody. (C) Immunofluorescence microscopy of transiently expressed AdipoR1-HA or AdipoR2-HA constructs in permeabilised and non-permeabilised CHO cells. Nuclei are counterstained with DAPI. (D) Semi-quantitative analysis of CHO cells expressing detectable receptors in non-permeabilised and permeabilised cells (data are from four independent experiments with ⩾100 cells counted for each condition, per experiment; $^{*}p < 0.05$). (E) Immunogold localization showing intracellular distribution of transiently expressed AdipoR1-HA (i, ii, iv and v) and AdipoR2-HA (iii). Note the labelling of the RER as well as a range of morphologically-diverse membranous structures including tubular and vesicular profiles (arrows); inset shows higher magnification of labelled elements in panel (i). PM, plasma membrane; N, nucleus. Bars, 100 nm. (F) Plate-based analysis of cell-surface (non-permeabilised) and total (permeabilised) receptor expression. (G) Quantitation of plate-based analysis from four independent experiments ($^{*}p < 0.05$).

we generated two chimeras, swapping the non-conserved, N-terminal domains to produce an AdipoR2$_{(1-81)}$/R1$_{(71-375)}$-HA chimera (R2/R1) and an AdipoR1$_{(1-70)}$/R2$_{(82-386)}$-HA chimera (R1/R2) that expressed at similar levels (Fig. 2B). Semi-quantitative microscopy indicated that 40–45% of permeabilised cells expressed detectable levels of the two chimeras but only the R1/R2-HA chimera was detectable at the cell-surface, and this was present in around 40% of cells (Fig. 2C). Quantitative plate-based analysis confirmed these results, with around 40% of total cellular R1/R2-HA detected at the cell-surface (Fig. 2D). These results indicate that the difference in cell-surface expression of AdipoR1 and AdipoR2 can be explained entirely by the non-conserved, N-terminal regions.

3.3. Characterisation of truncated adiponectin receptors demonstrates that AdipoR2$_{(1-81)}$ inhibits cell-surface expression

We next examined whether AdipoR1$_{(1-70)}$ drives expression at the cell-surface or AdipoR2$_{(1-81)}$ prevents cell-surface expression by generating and characterising truncated receptors lacking the non-conserved regions, $_{(\Delta 1-70)}$AdipoR1-HA (ΔR1) and $_{(\Delta 1-81)}$AdipoR2-HA (ΔR2). Western blot showed the truncated constructs were expressed at similar levels (Fig. 2E). Semi-quantitative microscopy and quantitative plate-based analysis demonstrated that ΔR1 and ΔR2 expressed at the cell-surface with similar efficiency (Fig. 2F and G). These results indicate that residues 1–81 in AdipoR2 interfere with cell-surface expression. It is also noteworthy that the efficiency of cell-surface expression of ΔR1 and ΔR2 was lower than that observed for full-length AdipoR1-HA, or the R1/R2-HA chimera, at around 20% of total cellular ΔR1 and ΔR2. This may reflect a positive role for residues 1–70 of AdipoR1 or simply a limitation of the truncation approach (akin to an artefact). Further truncation of the entire cytoplasmic domains $_{(\Delta 1-127)}$AdipoR1 or $_{(\Delta 1-138)}$AdipoR2 abolished cell-surface expression of either construct (data not shown).

3.4. Co-expression of AdipoR1 with AdipoR2 promotes cell-surface expression of AdipoR2

Next we investigated the effects of co-expression of AdipoR1 and AdipoR2 on cell-surface expression. Co-transfection followed by co-immunoprecipitation demonstrated that AdipoR1 and AdipoR2 can form homo- and hetero-oligomers (Fig. 3A), consistent with previous reports [1,10,11]. Around 50% of AdipoR2 was present in hetero-oligomers when co-expressed with AdipoR1. Control

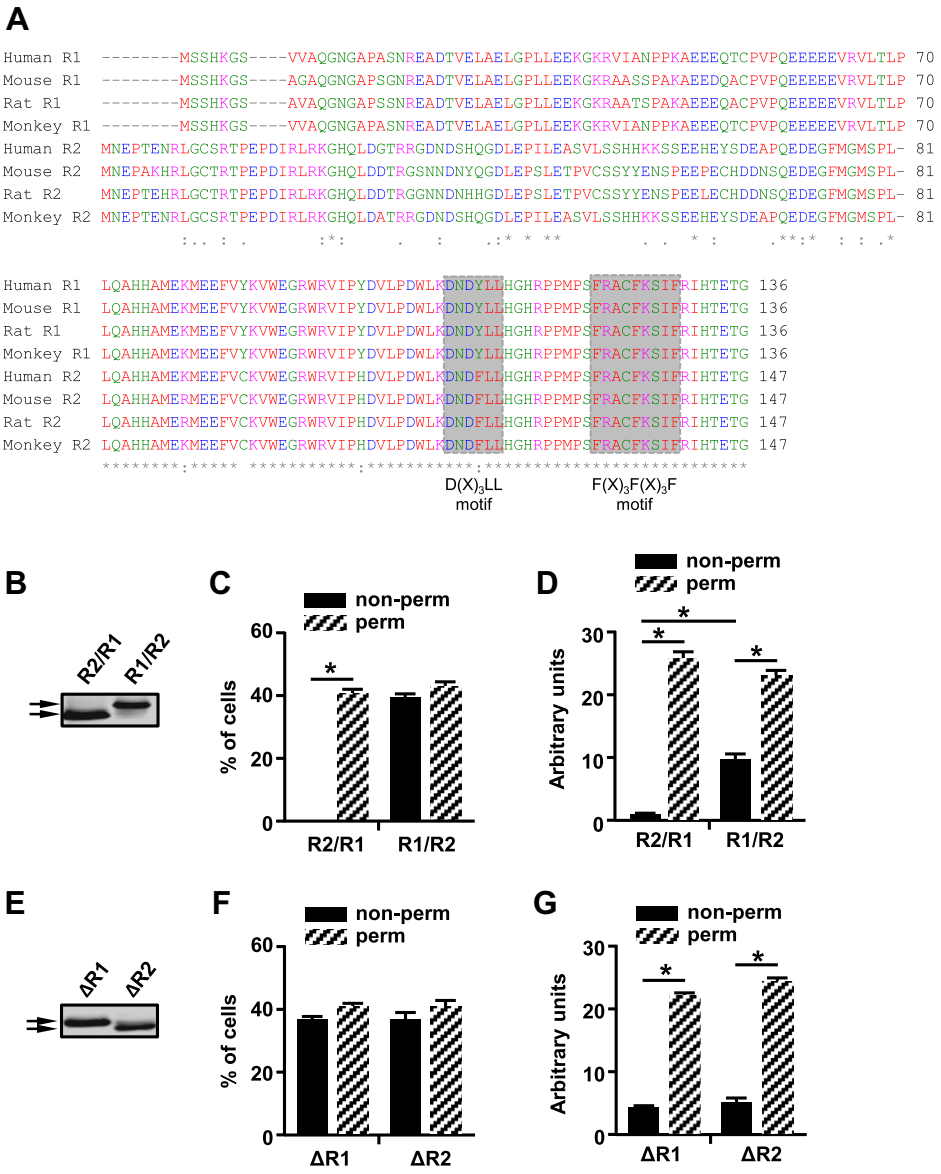

Fig. 2. Characterisation of cell-surface expression of AdipoR1 and AdipoR2 chimera and truncated constructs. (A) Alignment of the cytoplasmic, N-terminal domains of AdipoR1 and AdipoR2 showing the non-conserved (residues 1–70 and 1–81) and conserved regions (residues 71–136 and 82–147), respectively. The recently described anterograde trafficking motifs ($D(X)_3LL$ and $F(X)_3F(X)_3F$) are highlighted. (B) Western blot of transiently expressed $AdipoR2_{(1-81)}/R1_{(71-375)}$-HA chimera (R2/R1) or $AdipoR1_{(1-70)}/R2_{(82-386)}$-HA chimera (R1/R2). (C) Semi-quantitative analysis of CHO cells expressing detectable receptors in non-permeabilised and permeabilised cells (data are from four independent experiments with $\geqslant$100 cells counted for each condition, per experiment; $^{*}p < 0.05$). (D) Quantitative, plate-based analysis of cell-surface (non-permeabilised) and total (permeabilised) receptor expression from four independent experiments ($^{*}p < 0.05$). (E) Western blot of transiently expressed $_{(\Delta 1-70)}$AdipoR1-HA (ΔR1) or $_{(\Delta 1-81)}$AdipoR2-HA (ΔR2). (F) Semi-quantitative analysis of CHO cells expressing detectable receptors in non-permeabilised and permeabilised cells (data are from four independent experiments with $\geqslant$100 cells counted for each condition, per experiment). (G) Quantitative, plate-based analysis of cell-surface (non-permeabilised) and total (permeabilised) receptor expression from four independent experiments ($^{*}p < 0.05$).

experiments, where lysates of singly transfected cells were mixed prior to immunoprecipitation, indicated that formation of such oligomers was dependent on co-expression and did not reflect an artefact of the approach. Cell-surface expression of AdipoR1-HA was unaffected by co-expression with either AdipoR1-FLAG or AdipoR2-FLAG (Fig. 3B). Importantly, cell-surface expression of AdipoR2-FLAG was readily detected in cells co-transfected with AdipoR1-HA (Fig. 3B) demonstrating that co-expression of AdipoR1 promotes cell-surface expression of AdipoR2.

4. Discussion

This report establishes major differences in the steady-state, cell-surface levels of the adiponectin receptors, AdipoR1 and AdipoR2, and indicates that these differences can be explained entirely by differences in the non-conserved, N-terminal cytoplasmic domains. Cell-surface expression of AdipoR2 is restricted by amino acids 1–81. Co-expression with AdipoR1 can overcome this. These findings have major implications, in particular when considering cell-surface accessibility of the receptors and the potential for enhancing adiponectin sensitivity by increasing cell-surface expression of AdipoR2.

Our observations showing restricted cell-surface expression of AdipoR2 under steady-state conditions may appear somewhat surprising. However, it has been proposed that the AdipoRs and PAQR3 may be derived from a shared evolutionary protein [4,19]. PAQR3, which has been reported to bind adiponectin and referred to as AdipoR3 [20,21], is also known as Raf kinase trapping to Golgi

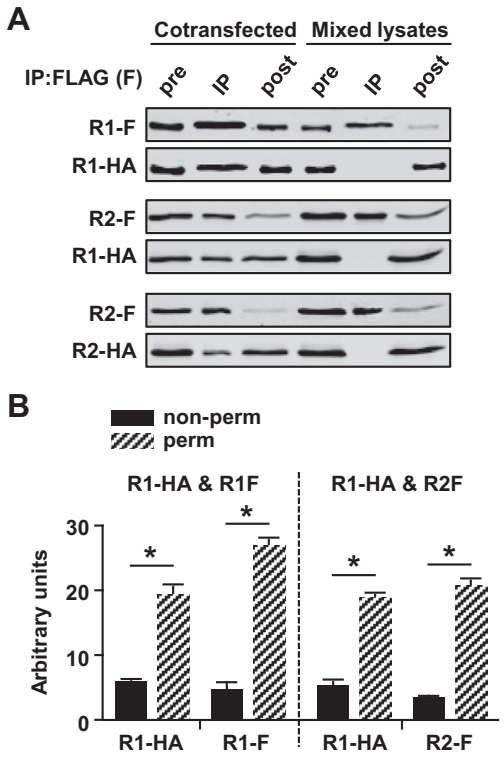

Fig. 3. Co-expression of AdipoR1 promotes cell-surface expression of AdipoR2. (A) CHO cells were either co-transfected or singly transfected with AdipoR constructs as indicated and lysates were immunoprecipitated using α-FLAG antibody. Western blots show AdipoRs in starting material (pre), immunoprecipitated material (IP) and post-IP supernatant (post) probed with α-FLAG (F) and α-HA antibodies, respectively. (B) Cells were co-transfected with AdipoR1-HA and either AdipoR1-FLAG (F) or AdipoR2-F. Cell-surface (non-permeabilised) and total (permeabilised) AdipoR1-HA and AdipoR1-F or AdipoR2-F were measured using quantitative, plate-based analysis. The graph shows results from four independent experiments ($^*p < 0.05$).

(RKTG) and is a Golgi-resident membrane protein with a cytoplasmic N-terminus that is known to interact with, and provide spatial regulation of, Raf kinase [22,23]. Collectively, these findings highlight our limited understanding of the basic biology of the AdipoRs. Although the original description and preliminary characterisation of transiently expressed AdipoR1 and AdipoR2 proteins suggested both receptors presented at the PM with exposed C-termini [1], subsequent anecdotal evidence from independent groups suggested AdipoR2 may not be expressed at the cell-surface [19,24]. We have shown that endogenous AdipoR1 and AdipoR2 display different subcellular fractionation properties, with AdipoR1 enriched in the PM and AdipoR2 enriched in the ER in HEK cells (in this report) and in HeLa cells [16]. Moreover, in this study we have provided rigorous quantitative assessment of cell-surface expression of transiently expressed, epitope-tagged AdipoR1 and AdipoR2 and a range of chimeric and truncated constructs which provide evidence that the cell-surface expression of AdipoR2 is restricted by the non-conserved residues 1–81. Indeed, our observations showing cell-surface expression of a $_{(\Delta 1-81)}$AdipoR2-HA (ΔR2) construct are entirely consistent with those from the original report by Kadowaki and colleagues, who inadvertently characterised a truncated form of AdipoR2 lacking this N-terminal region [1,4].

We also demonstrated that co-expression of AdipoR1 with AdipoR2 results in increased cell-surface expression of AdipoR2. Several groups have shown that AdipoR1 and AdipoR2 can form homo and hetero-dimers [1,10,11]. Our data suggests that formation of hetero-oligomers occurs with the expected frequency (around 50%) when AdipoR1 and AdipoR2 are co-expressed, prompting us to speculate that the appearance of AdipoR2 at the cell-surface when co-expressed with AdipoR1 reflects the cell-surface expression of AdipoR1/AdipoR2 dimers. Such a model is also consistent with our previous observations, showing that ERp46 restricts the localisation of endogenous AdipoR1 and AdipoR2 at the PM [16]. ERp46 interacts specifically with AdipoR1, via the non-conserved, N-terminal region of AdipoR1 [16], suggesting that this interaction precludes the AdipoR1/AdipoR2 interaction. Thus, knockdown of ERp46 would be expected to facilitate increased interactions between endogenous AdipoR1 and AdipoR2, hence increased cell-surface expression of both receptors. The importance of dimerization, particularly hetero-dimerization, and interacting proteins in the regulation of GPCR trafficking and signalling is now widely recognised [9,25,26] and it seems likely that similar principles will apply to the AdipoRs.

A major determinant of the extent of hetero-dimerization is the relative expression levels of AdipoR1 and AdipoR2, as well as other modulators such as ERp46. Current information detailing the relative levels of AdipoR1 and AdipoR2 is restricted to mRNA. Whilst this is a clear limitation, such information provides a basis to estimate the likely extent of hetero-dimerization in different tissues. For example, in mice the mRNA levels of AdipoR1 are 5–6-fold higher than AdipoR2 in skeletal muscle whereas expression of the two receptors is comparable in liver [27]. Although it would follow that a greater proportion of AdipoR2 would be present in hetero-dimers in muscle, the hetero-dimers would still represent a minor species in this tissue where AdipoR1 appears to be functionally dominant [28]. In contrast, the hetero-dimers represent a potentially major species in the liver, and macrophages, where AdipoR2 has been shown to be of functional importance [29,30]. Intriguingly, recent evidence suggests the formation of AdipoR1 dimers is reduced by adiponectin [11]. Considering the above, future, more-comprehensive studies to characterise the effects of adiponectin on the dynamics of both homo- and hetero-AdipoR dimers are warranted.

Emerging evidence supports the notion that adiponectin resistance contributes to the aetiology of obesity related disease [2,27]. Increasing cell-surface expression of the receptors, most notably AdipoR2, may provide a novel therapeutic approach to help improve adiponectin sensitivity. Indeed, several lines of evidence suggest that increased transduction of the adiponectin signal from the PM leads to enhanced coupling to AMPK [14,16,24], which itself represents a major target for therapeutic intervention [31].

The current report reveals fundamental differences between AdipoR1 and AdipoR2 and, in combination with earlier studies of AdipoRs and GPCRs, suggest that the trafficking and signalling properties of AdipoRs are likely to be determined by receptor composition (homo- or hetero-dimer/oligomer) and interacting proteins. Our current findings suggest that the majority of cell-surface AdipoR2 may be present in the form of hetero-dimers, which are likely to have unique characteristics when compared with AdipoR1 or AdipoR2 homo-dimers, respectively. Increased understanding may provide new opportunities to selectively enhance adiponectin's key, beneficial effects.

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