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Molecular determinants on extracellular loop domains that dictate interaction between β-arrestin and human APJ receptor

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The human APJ receptor (APJR), activated by apelin isoforms, regulates cardiovascular functions and fluid homeostasis. Understanding its structure-function relationship is crucial for a comprehensive knowledge of signalling aberrations that cause several physiological disorders. Here, we demonstrate the influence of extracellular loop (ECL) domains in the mechanism of β-arrestin-mediated signalling from human APJR: Apelin system. Alanine mutations of evolutionarily conserved residues were characterized using receptor internalization, β-arrestin pull-down, Akt phosphorylation and cell migration assay. C281A and ^{268}KTL^{270}-AAA in ECL3 were deficient in all assays, whereas ^{183}MDYS^{186}-AAAA mutant in ECL2 showed impaired β-arrestin-mediated signalling but demonstrated G_i-dependent cell migration. Our findings establish that conserved residues in the extracellular domain play a prominent role in modulating receptor interactions with the β-arrestin signalling cascade.

Keywords: β-arrestin; Akt phosphorylation; apelin; APJ receptor; cell migration

Human APJ receptor is a 'Rhodopsin family' cardiac G-protein-coupled receptor (GPCR), that despite sharing 31% sequence similarity to angiotensin II type I receptor is not activated by angiotensin II [1]. Apelin-13 (a 13 residue peptide product from proteolytically processed 77 residue precursor peptide), one of APJRs cognate ligands, is the predominant form in heart and brain, whereas apelin-36 is found in kidney, lungs and reproductive organs [2]. Understanding the pathophysiology of APJR is of paramount importance owing to its multifarious functions like positive 'inotropic' effect required for cardiovascular functions, fluid homeostasis, energy metabolism and angiogenesis and the process of HIV-recognizing its host cell, causing infection [3–5].

Earlier reports have demonstrated that the canonical signalling pathway of APJR on activation by apelin-13 is G_i-and G_q-dependent [6]. More recent studies have proposed that APJR activates different G-proteins and/or β-arrestins depending on the cell type and microenvironment [7,8]. This accounts for the varied intracellular downstream effectors triggered by APJR such as extracellular regulated kinases 1/2 (ERK1/2), protein kinase B (PKB/Akt), MAP kinases, etc., that are involved in cell division, survival and metabolism [6]. Another report revealed that C-terminal truncation of the Apelin-17 peptide (K16P, deletion of F^{17}) jeopardized the ability of rat APJR to reduce arterial blood pressure and also receptor internalization (recruitment by

Abbreviations
APJR, human APJ receptor; DMEM, Dulbecco's modified eagle medium; ECL, extracellular loop domain; eGFP, enhanced green fluorescent protein; ERK1/2, extracellular regulated kinases 1/2; GPCR, G-protein-coupled receptor; GST, glutathione-*S*-transferase; PIC, Protease Inhibitor Cocktail.

β-arrestin1/2) despite maintaining its G_i-mediated signalling. This enables us to hypothesize that rat APJR, on activation by apelin executes its hypotensive activity in a β-arrestin1/2-dependent pathway [8].

β-arrestins, on the other hand, are scaffolding proteins that sterically inhibit heterotrimeric G-proteins from coupling to activated GPCRs, thereby preventing G-protein-mediated second messenger pathways [6]. They are constitutively phosphorylated and become dephosphorylated to interact with the phosphorylated C-terminal tail of the receptor. This process of dephosphorylation is crucial for β-arrestin functionality including receptor internalization and signalling. β-arrestins adopt various conformations based on the distinct phosphorylation pattern generated by receptor kinases [9].

In this study, we have delineated the role of highly conserved motifs in the extracellular domains of APJR [^{183}MDYS^{186} (in ECL2) and ^{268}KTL^{270} motifs, in addition to Cys^{281} (in ECL3)]. Mutating these motifs affected apelin-mediated β-arrestin signalling by the receptor. These motifs dictate the complex pluridimensional signalling of the receptor and hence cause biased signalling of APJR. The signal transduction pathway initiated by apelin binding was monitored by receptor internalization, β-arrestin1/2 pull down, cellular migration and activation of PKB/Akt. We provide insight into the molecular mechanisms of β-arrestin signalling of Apelin: APJR complex and domains that act as 'effector discriminator'. Taken together, these results are helpful in understanding the structure-function relationship, mode of activation and signalling mechanism of the β-arrestin pathway of human APJ receptor.

Materials and methods

Multiple sequence alignment of APJR

A total of 42 APJR sequences of different species were retrieved from the NCBI protein database (Table S1). Few organisms such as zebrafish (*Danio rerio*), atlantic salmon (*Salmo salar*) frog (*Xenopus laevis*), red jungle fowl (*Gallus gallus*) and croaker fish (*Larimichthys crocea*) had two isoforms each. Sequence alignment was done using MAFFT tool [10] and the conserved amino acid sequences were identified in extracellular domains.

Cloning of APJR-'enhanced green fluorescent protein (eGFP)' chimera and generation of mutants

APJ gene was PCR amplified using pCDNA3.0 APJ clone as template and the amplicon was cloned in *pMJ* and *pEGFPN1* vectors. The mutations ^{183}MDYS^{186}-AAAA in ECL2 and ^{268}KTL^{270}-AAA in ECL3 and the double mutant S^{345}S^{348}-AA were generated by Polymerase Incomplete Primer Extension method [11] using APJ clone in the pMJ vector as a template. The point mutant C281A in ECL3 and ^{103}KL^{104}-AA double mutation in ECL1 were generated using a set of two complementary primers, by site directed mutagenesis [12]. For our study, ^{268}KTL^{270}-AAA mutant was generated in pEGFPN1-APJ clone.

Maintenance of HEK293 cells

HEK293 cells were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM) with high glucose containing 1 mM pyruvate and 1 mM L-glutamine and 10% foetal bovine serum (FBS-South American origin). The cells were grown at 37 °C in a humidified chamber containing 5% CO_2. The cell culture growth media also contained 1× antibiotic and antimycotic agents (Anti-Anti, Gibco, Life Technologies, Mumbai, India) to prevent bacterial and fungal contamination. HEK293 cells, stably expressing wild-type and all other mutants in the pMJ vector were maintained in the same media along with 0.2 μg·mL^{-1} puromycin. Cells expressing ^{268}KTL^{270}-AAA mutant in the pEGFPN1 vector were maintained with 1 mg·mL^{-1} G418 for further experiments.

Radiolabelling of apelin-13 peptide

Apelin peptide (purchased from GenScript, Piscataway, NJ, USA) was resuspended in HPLC grade water at a concentration of 10 μg·μL^{-1}. Radiolabelling was performed by adding ~ 200 μCi of radioactive iodine (Na^{125}I) and 30 μg of Chloramine-T to 100 μg of apelin peptide with 0.5 M phosphate buffer (pH-7.5) and incubated for 90 s at room temperature. The reaction was terminated by adding 90 μg of sodium metabisulfite and incubated for 60 s followed by addition of an excess of cold potassium iodide (~ 100 μg). The quality and efficiency of labelling were checked using HPLC (JASCO 2850 PLUS, JASCO, Easton, MD, USA) with reverse phase C18 column and a suitable solvent (Gradient mixing of water + acetonitrile + 0.1% TFA) at a flow rate of 1 mL·min^{-1}. The labelled peptide was stored at 4 °C until further use.

APJR ligand binding

HEK293 cells in 100 mm dishes were transfected (24 h after seeding) with plasmids (pMJ-APJ receptor and its ECL mutants) using lipofectamine 2000, Invitrogen, Mumbai, India as per manufacturer's protocol. The cells were then allowed to grow for 24 h in complete media so that they were 80–90% confluent. The cells were collected using PBS and centrifuged at low speed (~ 150 ***g*** for 5 min at 4 °C). The cell pellet was then resuspended in HEPES based buffer (20 mM HEPES, 10 mM EDTA, pH-7.4) and sonicated for a brief period on ice (30 s with 29% amplitude and 2 s 'ON' and 4 s 'OFF' pulse). The cell lysate was then centrifuged (450 ***g*** for 10 min at 4 °C) to remove

nuclear fraction and cell debris. The supernatant was then centrifuged at very high speed (100 000 ***g*** for 1 h at 4 °C) and the resulting membrane protein pellet was resuspended in low EDTA buffer (20 mM HEPES, 0.5 mM EDTA, pH-7.4) for further storage at −80 °C until future studies.

Membrane preparations expressing APJ receptor and its mutants were treated with varying concentrations of ^{125}I labelled apelin-13 radioligand (0.5–50 nM) in a suitable binding buffer (20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, pH-7.4) and incubated for 2 h to achieve equilibrium binding. The reaction was then stopped by centrifuging at 10 500 ***g*** for 5 min and the supernatant containing free radioligand was removed. The pellet was then washed twice with ice-cold 1× PBS to remove excess unbound free ligand. Radioactivity of the ligand: receptor complex was then determined by scintillation counting. Nonspecific binding was determined in the presence of 500 nM cold apelin-13 ligand to the above mentioned reaction mixture. All data were fit to nonlinear regression analysis with one site specific binding model using GRAPHPAD PRISM 6 software (GraphPad, San Diego, CA, USA). Affinity constants (K_d) was calculated from the best-fit values of specific binding.

Internalization assay

HEK293 cells expressing wild-type receptor and its mutants were seeded in 35 mm plates over coverslips. The cells were allowed to grow for 24 h in complete media so that they were 60–80% confluent. The cells were treated with 10 μM of ligand (apelin-13 peptide) and incubated further at 37 °C in a humidified chamber with 5% CO_2 for 1 h. The cells were then fixed on glass slides and receptor internalization was imaged by a confocal microscope Leica DMi8, Leica Microsystems, Wetzlar, Germany by monitoring the fluorescence signal from the enhanced green fluorescent protein (eGFP) tag.

β-arrestin1/2 pull down assay

HEK293 cells expressing wild-type receptor and its mutants were treated with 1 μM apelin-13 and incubated at 37 °C in a humidified chamber with 5% CO_2 for different time periods of 5, 10 and 40 min. Receptor activation was stopped by removing the media and adding ice-cold 1× PBS with Protease Inhibitor Cocktail (PIC) and phosphatase inhibitors (PTI-1 mM Na_3VO_4 and 1 mM NaF). The cells were mechanically scraped and washed with 1 mL of 1× PBS (with PTI). The cells were then resuspended in radioimmunoprecipitation buffer (RIPA buffer with PTI and PIC) for cell lysis. Cell debris was removed by centrifugation and total protein of the lysate was estimated using Bradford method with BSA as standard [13].

The lysate (40 μg) was incubated with 1 μg of anti-APJ receptor antibody for 3 h at 4 °C under gentle rotation. Later, 20 μL of Protein A/G agarose beads (Santa Cruz, Santa Cruz Biotechnology Inc, Dallas, TX, USA, sc-2003) was added to the mixture and incubated for 1 h at 4 °C under gentle rotation. The unbound protein was removed by centrifugation and the beads were washed twice with RIPA buffer to remove nonspecific binding. Elution was then done by adding 50 μL of 2× SDS loading buffer and later heating at 95 °C for 5 min. This elute was loaded on 12% SDS/PAGE [14] and later transferred to nitrocellulose membrane. The blot was probed with a suitable primary antibody (Cell Signaling Technology, Danvers, MA, USA – 1 : 2500 dilution for both β-arrestin 1/2 in 5% BSA) overnight at 4 °C and followed by incubation with suitable secondary antibody after washing. The blot was developed using 'Enhanced chemiluminescence method' and documented in VersaDoc (Bio-Rad, Hercules, CA, USA). The lysate which was used for pull down was probed with APJR antibody (rabbit polyclonal – 1 : 2000 dilution in 5% BSA) and it served as internal control for protein concentration. Data were analysed in GRAPHPAD PRISM 6 using ANOVA with Dunnett's *post hoc* test for multiple comparisons. Results were considered significant if $P < 0.05$.

Akt phosphorylation assay and western blot

HEK293 cells expressing wild-type receptor and its mutants were treated with 1 μM apelin-13 as in earlier assay for different time periods (5, 10, 40 min). Akt Phosphorylation was stopped by removing the media and adding ice-cold 1× PBS (with PIC and PTI). The cell lysate was prepared as above and the total protein was estimated using Bradford method. Western blot was done as previously mentioned and probed with primary antibody (Cell Signaling) generated in rabbit (1 in 2500 dilution for both Akt and phopsho-Akt antibody in 5% BSA) overnight at 4 °C and documented in VersaDoc using enhanced chemiluminescence method. Total Akt served as internal control for protein concentration.

Cell migration assay

HEK293 cells expressing wild-type receptor and its mutants were grown to confluence in 35 mm plates. A scratch was made in the monolayer using a sterile 10 μL pipette tip after incubating the cells in serum free DMEM media for 16 h and mitomycin C (10 $\mu g \cdot mL^{-1}$, MilliporeSigma, St. Louis, MO, USA) treatment, 3 h before introducing a wound. The initial scratch was imaged and the cells were then grown for 24 h in serum-free DMEM media containing 10 μM apelin-13 peptide. The cells without apelin-13 peptide treatment acted as a control. After 24 h, the scratch was reimaged to understand the cell migration due to apelin-13 treatment [15].

Expression and purification of ligand

For our study, apelin-13 was expressed and purified as described previously [16]. Briefly, the apelin-13 peptide was expressed with glutathione-*S*-transferase (GST) tag in *Escherichia coli*. ER2566 cells. It was affinity purified using

GST resin. The fusion protein was cleaved by 'PreScission protease' to release peptide from GST tag in volatile buffer (0.15 M Ammonium bicarbonate, pH-7.8). The tag was removed by acetonitrile precipitation and ligand in soluble form was recovered by centrifugation and was concentrated by freeze-drying method.

Results

Multiple sequence alignment of APJR

A multiple sequence alignment of APJRs from various organisms revealed conservation in several regions. This study focuses on the ECL regions of the receptor. The mutants in ECL domains generated for studying APJR interaction with β-arrestin are marked in Fig. 1A. A conserved motif in ECL1 is K(L/I/V/A)SSY, with leucine and isoleucine in most organisms, whereas valine and alanine are in some birds and pacific oyster. The Cys, at the juncture of TM3 and ECL1, that most likely forms a disulfide bond with Cys in ECL2 are both completely conserved in APJR sequences similar to members of Class A GPCRs. (M/L)D(Y/F)S motif in ECL2 domain is a highly conserved motif, with few exceptions like Zebra fish type B and desert mole rats, where 'Asp' is 'Asn' and 'Ser' is 'Asn' respectively. Another characteristic feature of peptide receptors and chemokine receptors is the conserved Cys in ECL3 domain that might form disulfide linkages with Cys of the N-terminal domain. Similar to other peptide receptors, APJR also has a highly conserved Cys281 in ECL3. The other motif in ECL3 domain, K(T/S)(L/I/M/A) is fairly conserved across various species of APJR (Fig. 1A).

Apelin peptide ligand binding to APJR

Wild-type APJR and its ECL mutants were compared for their ligand binding efficiency by saturation binding experiments using radiolabelled (I^{125}) apelin peptide (0.5–50 nM). ^{103}KL^{104}- AA (K_d – 4.53 nM) mutant in ECL1 showed similar binding efficiency as that of wild-type (K_d – 2.86 nM). ^{268}KTL^{270}-AAA in ECL3 exhibited ~ 4 fold decrease compared to wild-type peptide binding. But, ^{183}MDYS^{186}-AAAA and C281A in ECL2 and ECL3 demonstrated ~ 7 fold decrease in apelin binding affinity compared to wild-type (Fig. 1B, Table 1).

Agonist dose-dependent modulation of internalization of APJR and its ECL mutants

The receptor internalization was studied using fluorescence-based imaging of eGFP tagged APJR and its mutants to confirm activation by the apelin peptide. Activation of receptor and its mutants by apelin-13 (0.1–10 μM) was monitored after 1 h of ligand addition by tracking the eGFP. Unlike wild-type, ECL domain mutants failed to exhibit punctate appearance at lower concentration (0.1 and 1 μM) of apelin-13 (data not shown). APJR wild-type exhibited punctate appearances convincingly on activation by 10 μM of apelin-13, whereas the untreated cells did not. Ligand treatment (10 μM) of ^{268}KTL^{270}-AAA and C281A in ECL3 resulted in scarcely distributed punctated images and hence, demonstrated partial activation. ^{183}MDYS^{186}-AAAA mutant in ECL2 and S^{345}S^{348}-AA did not elicit any punctate spots even though apelin-13 was added in higher amounts (Fig. 2).

APJR activation leads to β-arrestin2 binding

A pull-down assay was carried out using anti-APJR antibody in order to identify the β-arrestin isoform that binds to the phosphorylated C-terminus of the receptor causing downstream signalling. Total cell lysate, obtained from apelin-13 (1 μM) treated HEK 293 cells, stably expressing APJR and its ECL mutants, was incubated with APJR antibody. Later, protein A/G agarose beads were added and β-arrestin bound receptor was eluted and probed for both β-arrestin1 and β-arrestin2. With time, the wild-type receptor showed a pronounced change in β-arrestin2 activation (Fig. 3, $P < 0.001$) compared to β-arrestin1. ^{103}KL^{104}-AA (that causes internalization similar to wild-type) exhibited β-arrestin2 activation (Fig. 3, $P < 0.001$), independent of the time at which the interaction was probed (starting at 5 min). But ^{268}KTL^{270}-AAA and C281A (with poor internalization) demonstrated marginal β-arrestin2 activation, only at 40 min of apelin-13 treatment (Fig. 3, $P < 0.001$). On comparing ligand treated with untreated (0 min), ^{183}MDYS^{186}-AAAA mutant (that was deficient in receptor internalization) failed to significantly activate β-arrestin2 with time (Fig. 3, $P < 0.01$). The wild-type or the mutant receptors, on ligand treatment, did not alter β-arrestin1 levels significantly with time (Fig. 3A, Fig. S2). This led to the deduction that APJR activates β-arrestin2 to initiate the signalling cascade. Receptor expression levels were found to be similar from 0 to 40 min for APJR and its ECL mutants (Fig. S1, internal control).

APJR and β-arrestin2 coupling activates PKB/Akt

Wild-type APJR and all ECL mutants caused variable extents (rate and amounts) of phosphorylation of PKB/Akt upon activation by 1 μM apelin-13

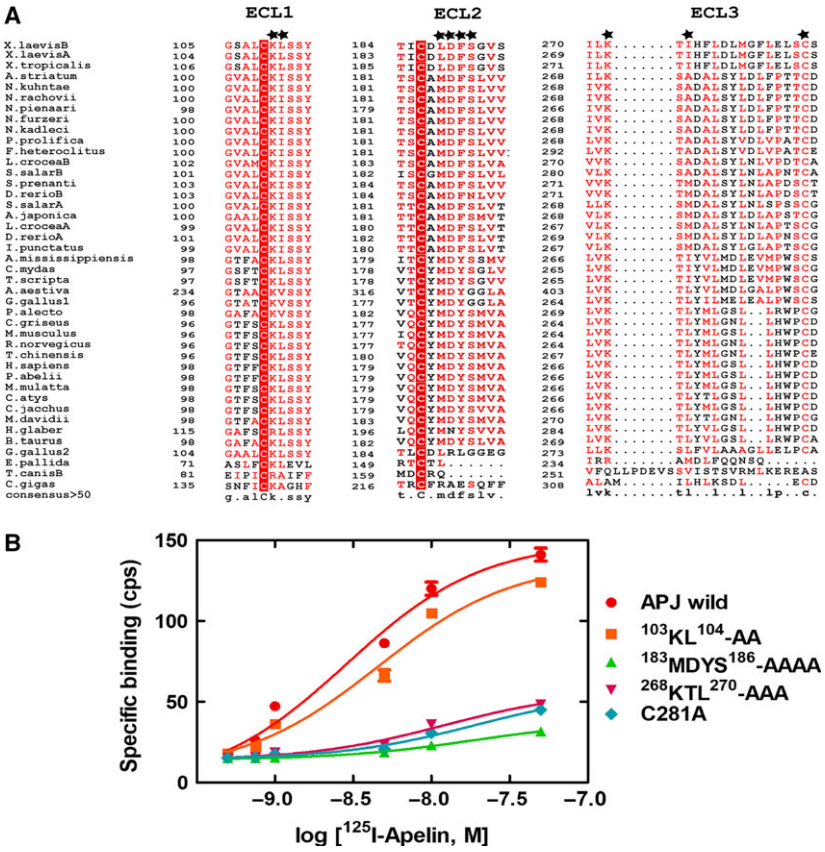

Fig. 1. ^{125}I-Apelin-13 peptide binding to APJ wild-type receptor and its ECL domain mutants. (A) The multiple sequence alignment of amino acids sequence of APJ Receptor across various species showed several conserved residues. The three ECL domains are denoted as ECL. Mutations in the receptor are marked by star (★). Highly conserved residues are displayed in red, whereas the variable residues are in black. Completely conserved residues are shaded in red. ECL1 domain has conserved Cys residue, and KL/I/V and SSY motifs. ECL2 domain has conserved Cys residue and conserved MDY(F)/S motif. ECL3 domain has a highly conserved Cys residue and KT(S)L(I/M) motif. (B) Membrane preparations prepared from HEK293 cells overexpressing APJR and its ECL mutants were treated with varying concentrations of ^{125}I-Apelin-13 (0.5–50 nM) for 2 h. After incubation and subsequent washing, the bound radioactive peptide to the receptor membranes was measured. Mean of the triplicate data was fit using nonlinear regression analysis and affinity constant was calculated. On comparing with wild-type (●), ^{183}MDYS^{186}-AAAA mutant in ECL2 (▲), ^{268}KTL^{270}-AAA (▼) and C281A (◆) in ECL3 showed several folds decreased binding affinity. ^{103}KL^{104}-AA mutant in ECL1 (■) had similar binding affinity as that of APJ wild-type receptor.

(Fig. 4). Wild-type APJR and ^{103}KL^{104}-AA mutant elicited Akt phosphorylation at 5 min with peak phosphorylation happening at 40 min. The ^{268}KTL^{270}-AAA and C281A mutants caused Akt phosphorylation only at 40 min though not as significantly as wild-type. Although ^{183}MDYS^{186}-AAAA mutation caused phosphorylation of Akt, there was no significant alteration with time when compared to untreated cells (0 min).

Effect of mutations on APJR signalling resultant cell migration

APJR signalling activates PKB/Akt pathway, a key regulator of cell motility. In order to gain insight into the functional role of the receptor and its domains, we performed the scratch assay. The experiment was carried out in the presence of mitomycin C to inhibit cell proliferation-mediated scratch closure. On examining

Table 1. Peptide binding constants of APJ wild-type and its ECL domain mutants. APJR effectively binds to apelin-13 peptide, whereas ECL2 and ECL3 mutants have reduced binding (20–35% binding). The table is a representation of three independent experiments in triplicates.

Type	K_d (nM)
APJR-wild-type	2.86
^{103}KL^{104}-AA	4.53
^{183}MDYS^{186}-AAAA	18.66
^{268}KTL^{270}-AAA	11.99
C281A	19.34

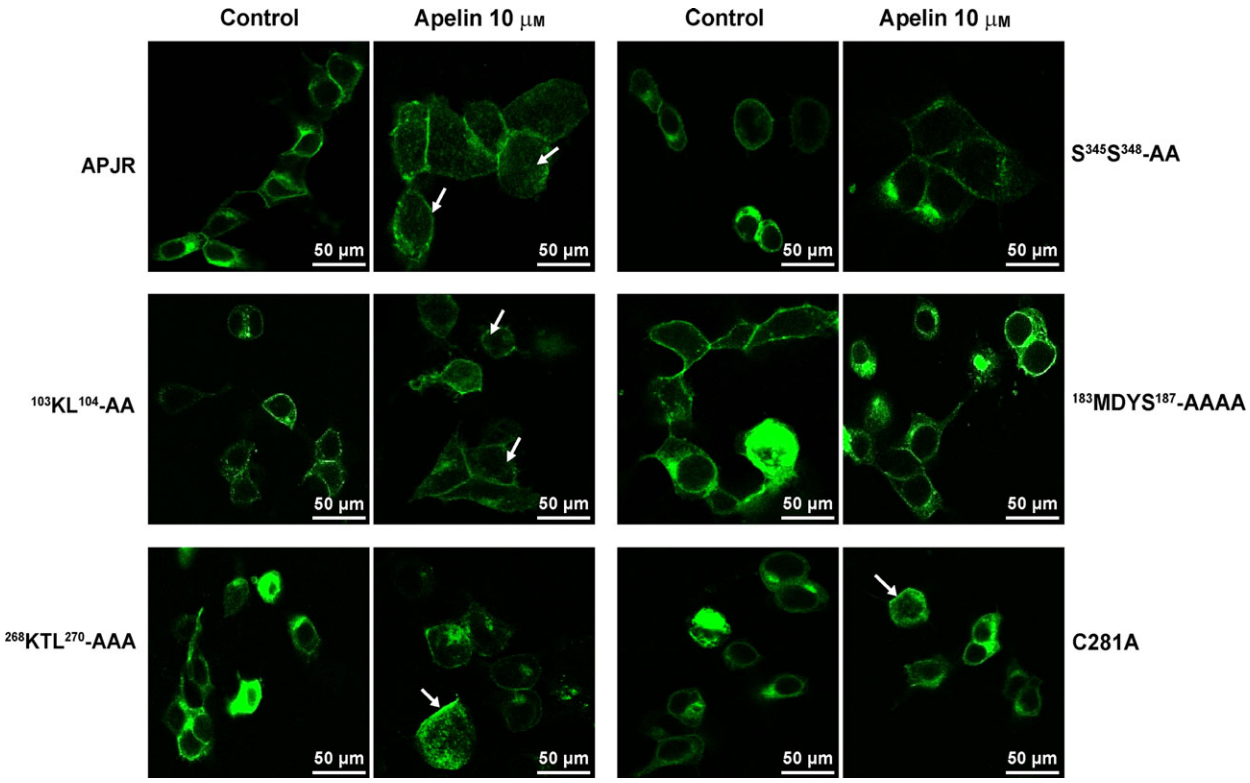

Fig. 2. Mutation dictated receptor internalization upon apelin binding: HEK293 cells, expressing eGFP tagged APJR and mutants were treated with apelin-13 (10 μM) for 1 h. On comparison with untreated cells, APJR showed distinct punctate appearance (indicating receptor internalization) in response to apelin-13 binding. ^{183}MDYS^{186}-AAAA mutant in ECL2 domain failed to show any punctate appearance, whereas ^{268}KTL^{270}-AAA and C281A mutants in ECL3 domain elicited very few punctate spots when induced by 10 μM apelin-13. S^{345}S^{348}-AA, a positive control (deficient in β-arrestin binding), also failed to show receptor internalization. The punctate appearances due to receptor internalization are indicated by the arrows.

the scratch at 0 and 24 h with apelin-13 treatment, we found that there was a prominent scratch closure in cells stably expressing wild-type APJR (Fig. 5A). ^{103}KL^{104}-AA mutant also showed satisfactory cell migration with apelin-13 activation (Fig. 5B). But ^{268}KTL^{270}-AAA and C281A mutants, devoid of any β-arrestin-dependent signalling demonstrated no scratch closure even after 24 h (Fig. 5D,E). Surprisingly, ^{183}MDYS^{186}-AAAA mutant, that did not elicit β-arrestin-mediated receptor internalization or β-arrestin2 activation showed prominent scratch closure similar to APJR wild-type (Fig. 5C).

Discussion

In recent times, our understanding of GPCRs has taken a paradigm shift from the receptors being simple 'on' and 'off' switches to their activation resulting in an ensemble of conformations. These structural intermediates, in turn, activate diverse signalling cascades leading to varied physiological responses [3,17]. Given the repertoire of physiological functions carried out by Apelin: APJR system, understanding the molecular mechanisms of receptor activation and downstream signalling becomes mandatory. This provides better insight into the structure-function relationship of the receptor and enables targeted drug discovery. β-arrestins are important signalling partners of APJR, responsible for the hypotensive function, as demonstrated in rats [8]. Not many reports deal with the important relationship between extracellular residues/domain (ligand binding domain) and the intracellular domains, leading to the consequent specific selection of one or the other downstream effectors (G-proteins or β-arrestins) [3,17]. These residues may play a modulating role in APJR functions such as angiogenesis and vasodilation. Hence, this study was aimed at identifying the residues in extracellular domains of human APJR that are critical for causing structural changes required for β-arrestin-mediated signalling. On the basis of multiple sequence alignment, we chose

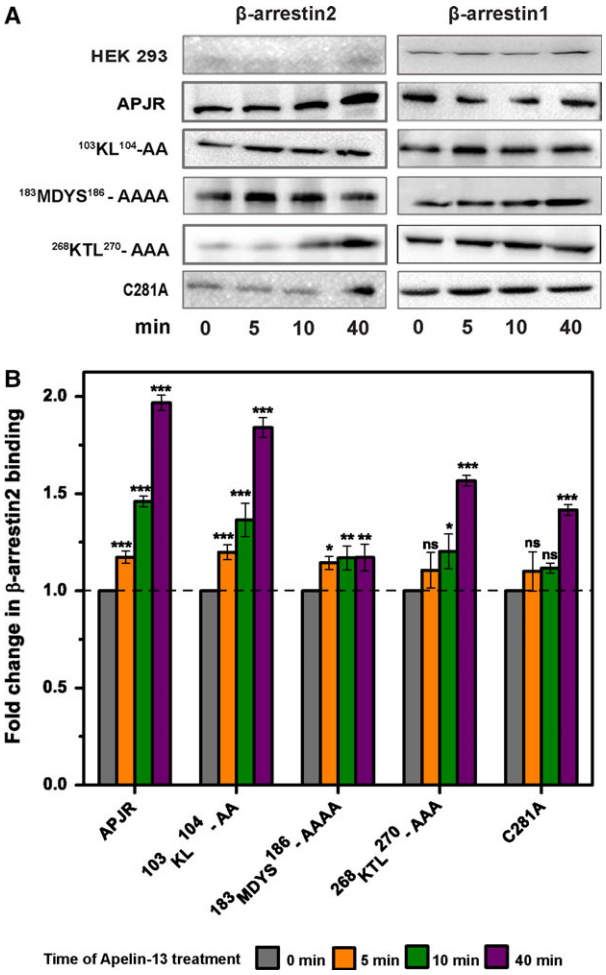

Fig. 3. β-arrestin1/2 binding upon apelin induced receptor activation. HEK293 cells stably expressing APJR and its mutants were treated with 1 μM apelin-13 for 5, 10 and 40 min. A pull down assay was carried out to monitor β-arrestin1/2 binding. (A) APJR showed increased binding of β-arrestin2 with time, compared to untransfected cells. ^{103}KL^{104}-AA mutant in the ECL1 domain also exhibited β-arrestin2 activation similar to wild-type. ^{183}MDYS^{186}-AAAA mutations in ECL2 domain failed to achieve β-arrestin2 binding with time. ^{268}KTL^{270}-AAA and C281A mutants in ECL3 domain exhibited futile β-arrestin2 binding except at 40 min. APJR and its ECL mutants ceased to activate β-arrestin1 with time on apelin treatment. (B) Quantification of the western blot showing β-arrestin2 binding on ligand interaction with receptor was done using ImageLab software and normalized with corresponding receptor expression levels. Data represented in graph are mean and SEM of three independent experiments (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns: not significant). Dashed line represents the basal β-arrestin2 binding of APJR and its ECL mutants observed without ligand addition (0 min).

conserved motifs and performed alanine scanning mutagenesis to understand their implication in β-arrestin-dependent signalling by human APJR.

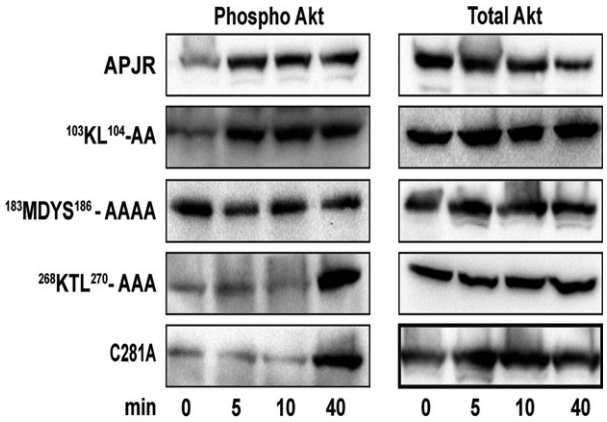

Fig. 4. Phosphorylation of Akt due to signaling from APJR and its ECL mutants. HEK293 cells stably expressing APJR and its mutants were treated with 1 μM apelin-13 for 5, 10 and 40 min. Phosphorylation was stopped by adding ice-cold PBS containing PIC and PTI. Cells were lysed in RIPA buffer (with PTI and PIC) and total cellular proteins were separated on 12% SDS/PAGE and change in Phopsho-Akt levels were measured by western blot. Receptor activation by apelin-13 led to significant phosphorylation of Akt as observed in APJR and ^{103}KL^{104}-AA mutant from 5 min. ^{183}MDYS^{186}-AAAA mutant, on the other hand, did not show a manifold increase in Akt phosphorylation with time on apelin treatment. C281A and ^{268}KTL^{270}-AAA mutants showed phosphorylation only at 40 min.

G-protein-coupled receptor activation by its cognate ligand causes phosphorylation at its C-terminus by receptor kinase leading to clathrin-mediated receptor endocytosis [18]. In a fluorescence-based cell imaging assay, receptor internalization (at 10 μM apelin-13) was monitored for wild-type and mutant receptors. $S^{345}S^{348}$-AA was used as positive control for inadequate β-arrestin binding due to the absence of phosphorylation crucial for β-arrestin recruitment [7]. While the wild-type receptor internalized upon activation significantly, ^{183}MDYS^{186}-AAAA, ^{268}KTL^{270}-AAA and C281A mutants in ECL3 did not elicit the same response probably due to their inept coupling to β-arrestin as seen by poor punctate appearance (Fig. 2). These mutants demonstrated inefficient internalization similar to the phosphorylation-deficient receptor mutant ($S^{345}S^{348}$-AA) (Fig. 2). Hence, our results emphasize the role of ^{183}MDYS^{186}, ^{268}KTL^{270} and C^{281} residues in causing structural changes in the receptor that enable arrestin binding.

In this work, we delineate the receptor residues involved in ligand binding that lead to differential binding to arrestin isoforms. Investigation of the β-arrestin isoform which might be involved in signalling response led to the outcome that β-arrestin2 binding happens in greater proportion than β-arrestin1

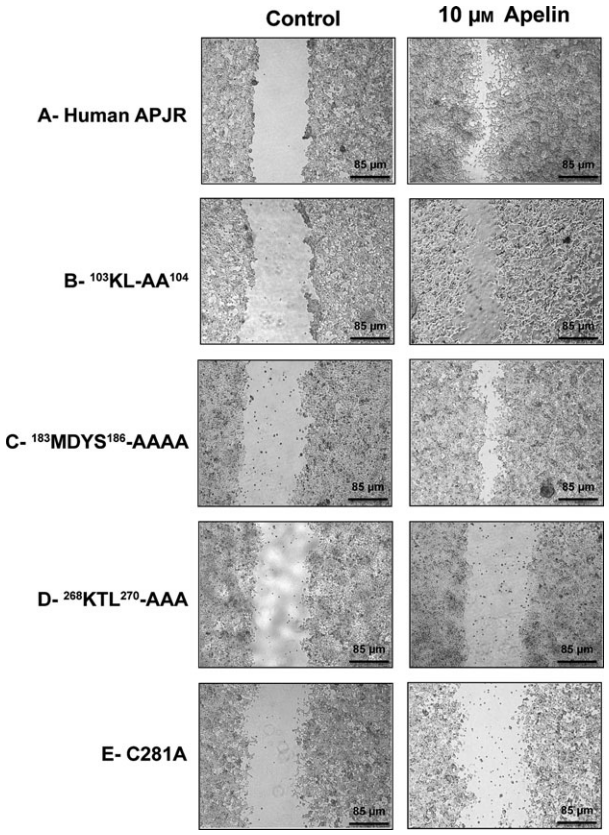

Fig. 5. APJR and its mutants initiated cellular migration. Cell migration, resulting from apelin-13 treatment to HEK293 cells stably expressing APJR and its mutants for 24 h was investigated using the scratch assay. (A, B) APJR and ^{103}KL^{104}-AA mutant revealed significant scratch closure compared to untreated cells at 24 h. (C) ^{183}MDYS^{186}-AAAA mutant that did not elicit β-arrestin-mediated activity showed prominent scratch closure similar to APJR wild-type. (D, E) ^{268}KTL^{270}-AAA and C281A mutants with poor β-arrestin-mediated activity were deficient in causing cell motility.

upon activation by apelin-13. Ceraudo *et al.* [8], demonstrated that activation of rat APJR in HEK293T cells by apelin-17 peptide led to increased recruitment of β-arrestin2 compared to β-arrestin1. GPCRs that undergo clathrin-mediated endocytosis are classified as Class A or B and differ in their ability to bind β-arrestin isoforms. Class A GPCRs like β_2-adrenergic receptor bind with increased affinity to β-arrestin2 than β-arrestin1 [18]. Our results demonstrate that human APJR, a well-known Class A GPCR, behaves in accordance to this concept and activates β-arrestin-2. The internalization-deficient mutants, namely, ^{268}KTL^{270}-AAA and C281A in ECL3 and ^{183}MDYS^{186}-AAAA in ECL2 also failed to activate β-arrestin2, as a function of time, in a significant manner (Fig. 3B). Although, ^{183}MDYS^{186}-AAAA mutant displayed substantial basal β-arrestin-2 binding, the levels did not significantly increase with ligand treatment. This phenomenon brings up the proposal that ^{183}MDYS^{186} motif could be an allosteric site that regulates constitutive activity of APJR and its mutation has led to loss of tight control causing considerable basal β-arrestin-2 binding.

Akt/PKB, a serine/threonine kinase, is a major player in cell survival, migration, proliferation and nutrient metabolism acting downstream of GPCRs [19]. A detailed physiological study in mice confirmed that apelin-36 prevented ischaemic brain injury and reduced apoptosis by activating PI3K/Akt signalling pathway as a protective mechanism [20]. Hosoya *et al.* [21], had also demonstrated that apelin-13-mediated chemotaxis is Akt phosphorylation-dependent in CHO cells overexpressing rat APJR.

All these studies formed the basis for identifying the molecular determinants in human APJR that govern β-arrestin2-mediated Akt phosphorylation and cellular migration. Monitoring of downstream Akt phosphorylation on apelin addition revealed that ^{268}KTL^{270}-AAA and C281A mutants were deficient in causing phosphorylation compared to wild-type and ^{103}KL^{104}-AA in ECL1. This study further illustrates the physiological function of these domains in the receptor. This result was complimented by the data from scratch assay as the same mutants (^{268}KTL^{270}-AAA and C281A) failed to induce pronounced cell mobility when compared to wild-type. These results are further reinforced by affinity constants derived from radioactive peptide binding analysis as the K_d for ^{268}KTL^{270}-AAA and C281A is much reduced than wild-type (11.99 and 19.34 nM vs. 2.86 nM). ^{183}MDYS^{186}-AAAA mutant in ECL2 did not display marked difference in Akt phosphorylation with time and its affinity constant (18.66 nM vs. 2.86 nM) was ~ 7 fold attenuated compared to wild-type. Akt phosphorylation observed for this mutant could be due to the sizeable basal β-arrestin-2 binding as seen earlier. But, in contrast to all the above data, ^{183}MDYS^{186}-AAAA exhibited conspicuous scratch closure, analogous to wild-type. This might be possible due to the fact that ^{183}MDYS^{186}-AAAA elicits G_i-mediated ERK1/2 phosphorylation [12] which is involved as a scaffolding protein in many anti-apoptotic pathways. The variation in β-arrestin2 activation for mutants could be due to the differences in receptor conformation among the mutants that are favourable for β-arrestin2 coupling [9].

Discerning the residues essential for β-arrestin-mediated signalling would help us to synthesize biased agonists, which are capable of discriminating between the GPCR desensitization and signal scaffolding conformations of β-arrestin. This report also extends our

results wherein ^{183}MDYS^{186} motif continued to exhibit Gi-mediated activity in spite of poor β-arrestin-mediated signalling. In conclusion, we establish that APJR: apelin interactions modulate β-arrestin-mediated signalling differentially. From our earlier study, it was observed that G-protein-mediated activity of ^{268}KTL^{270}-AAA and C281A mutants is fairly comparable to that of wild-type receptor [12]. The ^{268}KTL^{270} motif and Cys281 in ECL3 might be the 'discriminator' residues (enabling specific conformational changes) in activating β-arrestin over G-protein coupling. But the ^{103}KL^{104} motif in ECL1 remains neutral throughout signalling cascade. Our binding analysis of the apelin ligand to the D184A receptor mutant corroborates well with the crystal structure of the receptor-ligand complex [10]. Thus, these motifs exemplify the regions required for human APJR physiological functions, emphasizing the possibilities of multiple activated conformations of the receptor.

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Author contributions

AA and GKA conceived and designed all the experiments. AA performed all the experiments and prepared figures in the manuscript. AA and GKA analysed data and prepared the manuscript. Both authors declare no conflict of interest. MK contributed in peptide radiolabeling and binding studies.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Expression levels of APJR and EC domain mutants.

Fig. S2. β-arrestin1 binding upon apelin-induced receptor activation.

Fig. S3. APJR and its mutants induced cellular migration.

Table S1. List of organisms and their accession number used for multiple sequence alignment to design mutations.