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G protein-coupled receptors directly bind Filamin A with high-affinity and promote Filamin phosphorylation

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Abbreviations and Textual Footnotes:

G protein-coupled receptors, GPCRs; Protein kinase A, PKA; Filamin A, FLNa; Immunoglobulin repeat, Ig repeat: Angiotensin II type receptor I, AT1R; Filamin binding motif, FBM; D3 dopamine receptor, D3R; Isothermal titration calorimetry, ITC; Nuclear magnetic resonance, NMR .

Abstract

Although interaction of a few G protein coupled receptors (GPCRs) with filamin A, a key actin cross linking and biomechanical signal transducer protein, has been observed, a comprehensive structure-function analysis of this interaction is lacking. Through a systematic sequence-based analysis, we found that a conserved filamin binding motif is present in the cytoplasmic domains of >20% of the 824 GPCRs encoded in the human genome. Direct high-affinity interaction of filamin binding motif peptides of select GPCRs with the Ig domain of Filamin A was confirmed by nuclear magnetic resonance spectroscopy and isothermal titration calorimetric experiments. Engagement of filamin binding motif with Filamin A Ig domain induced the phosphorylation of filamin by protein kinase A in vitro. In transfected cells, agonist activation as well as constitutive activation of representative GPCRs dramatically elicited recruitment and phosphorylation of cellular Filamin A– a phenomenon long known to be crucial for regulating structure and dynamics of the cytoskeleton. Our data suggests a molecular mechanism for direct GPCR-cytoskeleton coupling via filamin. Until now GPCR signaling to the cytoskeleton was predominantly thought to be indirect, through canonical G protein-mediated signaling cascades involving GTPases, adenylyl cyclases, phospholipases, ion channels and protein kinases. We propose that the GPCR-induced filamin phosphorylation pathway is a conserved, novel biochemical signaling paradigm.

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G protein-coupled receptors (GPCRs) initiate wide-ranging responses including the integrin-regulated processes such as cell migration, cell survival, growth, chemotaxis and the associated cell morphological changes such as membrane ruffling, formation of filopodia, focal adhesions and lamellipodia.[1,2] These fundamental changes in cells are contingent upon engaging cytoskeletal proteins upon activating the conventional G protein-dependent and -independent signaling mechanisms involving proteins such as β-arrestin, small GTPases and PDZ containing proteins.[3] At a molecular level cell morphological changes or cytoskeletal responses to GPCRs are thought to be an indirect outcome of signaling cascades, and as a consequence direct binding and activation of a major cytoskeletal protein by GPCRs is not considered a mechanism at present. Bio-physical and bio-chemical studies presented here indicate that a substantial number of GPCRs may directly bind and activate a major actin cross linking protein, filamin A (FLNa), and that the binding triggers filamin phosphorylation by cellular protein kinases.

The relationship between GPCRs and cytoskeletal modulation of cellular phenotype is widely evident in physiological and pathological paradigms. Apart from their well-established role as transducers of neuro-endocrine hormone and sensory signals[4], GPCRs are expressed at high levels in some breast[5], gynecological[6], neurological[7] and prostate cancers[8] and alter invasive properties of tumor cells[9]. Indeed, GPCR blockade is currently being explored as a cancer therapy and majority of drugs used in clinical practice are ligands for GPCRs[9]. Anti-psychotic drugs target reorganization of nerve cytoskeletal components which is critical for neuronal morphology, plasticity, and the synaptic architecture in the adult brain, via GPCR-antagonism.[10] However, definitive mechanisms for direct communication between GPCRs and cytoskeleton remain undefined.

Filamin is an actin binding dimeric cytoskeletal protein with 24 immunoglobulin (Ig) repeats that engages the cytoplasmic regions of many transmembrane proteins.[11,12] A few genetic and biochemical studies have reported interaction of GPCRs with FLNa. In these studies, the role of FLNa in augmenting the membrane expression, subcellular localization, trafficking and signaling of GPCRs is emphasized. For example, the intracellular loop 3 (ICL3) of D2 and D3 dopamine receptors was shown to interact with immunoglobulin-like repeat (Ig) 19 of FLNa to promote proper cell surface expression and signaling.[13-15] The C-terminal tail (Ct) of calcitonin receptor and C-C chemokine receptor type 2 were shown to interact with FLNa Ig20-21 and Ig21-24, respectively and play key roles in endocytic sorting and internalization of the receptor. [16,17]

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Similarly, the interaction of μ-type opioid receptor with FLNa Ig24[18-20], calcium-sensing receptor with FLNa Ig14-15[21-26], metabotropic glutamate receptor subtype 7b with FLNa Ig21-22, and somatostatin receptor type 2 with FLNa Ig19-20, are reported to play scaffolding and functional roles in GPCR signaling.[27-30] Filamin is a key mediator of epithelial defense against intrusion of transformed cells[31]; therefore, GPCR signal regulation through FLNa is an important unexplored mechanism.

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FLNa Ig4, Ig9, Ig12, Ig17, Ig19, Ig21 and Ig23 are class A repeats which possess a conserved binding site capable of engaging several proteins such as platelet glycoprotein Ib alpha (GPIbα), integrins and migfilin.[32] Recently these repeats have been shown to reduce integrin activation when overexpressed in a platelet integrin model through a two site binding mechanism.[33] Among the class A repeats, Ig21 was shown to have the highest binding affinity for the filamin ligands.[32] Interestingly, Ig21 exists in an auto-inhibited form by engaging the N-terminal portion of Ig20 repeat as an intra-molecular inhibitory ligand.[34] Recruitment of filamin binding proteins, such as integrins and migfilin to filamin relieves this auto-inhibition and promotes structural reorganization of filamin[35]. Recently it is revealed that disrupting the Ig21 auto-inhibition by FLNa ligands promoted protein kinase A (PKA)-dependent phosphorylation of S2152 site[36,37] on Ig20. The ligand-dependent S2152 phosphorylation by a variety of FLNa ligands may be a novel pathway for activating filamin function in diverse filamin-mediated cellular processes.

Overall, the current scientific literature suggests a potential involvement of GPCRs in the engagement of different FLNa Ig repeats during a variety of biological responses. Nevertheless, the mechanisms for direct communication between GPCRs and FLNa remain poorly defined at the molecular level. In this study we found that >20% of human GPCRs are endowed with a likely FLNa binding motif (FBM). We demonstrate high affinity physical interaction of predicted FBMs for three different GPCRs with FLNa Ig21: (i) angiotensin II type 1 receptor (AT1R), a prototypical agonist activated GPCR with important roles in cardiovascular physiology (ii) proto-oncogene MAS, a constitutively active GPCR with a cardio-protective role, and (iii) α_{1D}-adrenoreceptor (α_{1D}-AR), a neuro-hormone GPCR with cardiovascular roles. As a consequence of high affinity binding we demonstrate enhanced PKA mediated filamin phosphorylation of Ig16-24 in vitro. We further determined that GPCRs, AT1R and MAS directly recruited FLNa and promoted its phosphorylation by cellular S/T kinases in an agonist dependent manner. Our studies thus provide a structural framework for filamin in GPCR signaling, potentially regulating a variety of cellular responses.

Experimental procedures

Peptides and reagents: The following peptides were synthesized by the Biotechnology Core at the Lerner Research Institute of the Cleveland Clinic: (1) D3R: VRKLSNGRLSTSLKLGPLQPRGV (2) MAS: KKKRFKESLKVVLTRAFK, (3) AT1R: LGKKFKRYFLQLLKYIPPKA and (4) α_{1D}-AR: KGHTFRSSLSVRLLKFSR. All peptides were HPLC purified and were >95% pure. Peptide concentrations were estimated using their predicted extinction coefficient at A_{280} using the protein parameters tool on ExPASY server (http://web.expasy.org/pro/). In cases where the peptide did not have a UV-signature, the thoroughly lyophilized peptide was weighed carefully and 85% purity was assumed to estimate concentration.

Human Filamin A (Uniprot P21333) immunoglobulin domains Ig16-24 (1772-2647) were cloned into pGST-parallel vectors and purified as described earlier.[32,35] Phospho filamin A (S2152, #4761), and filamin A(#4762) polyclonal antibodies were obtained from Cell Signaling Technology (Danvers, MA) while antibody for GAPDH was obtained from Life Technologies (Grand Island, NY). Filamin A monoclonal antibody to detect immunoprecipitated filamin was from Millipore (MAB1680). Agonist peptide angiotensin II (Ang II) for stimulation was purchased from Bachem and AT1R antagonist candesartan was a gift from AstraZeneca. Specific MAS activating and inhibiting ligands AR234960 (AR-*agonist*) and AR244555 (AR-*inverse*), respectively were an unrestricted gift from Arena Pharmaceuticals, Inc. (San Diego, CA). PKA-inhibitor, H-89 was purchased from Sigma-Aldrich (St. Louis, MO). MAS ligands and other inhibitors were dissolved in DMSO as 10mM stocks. The pH of the buffer in the experiments was verified to be neutral (7-7.5) after adding the ligands at desired concentrations of 10-50µM.

Identifying filamin Class A repeat binding motif (FBM) in GPCRs. Based on the sequence alignment of peptides from peptide bound filamin structures (PDB IDs: 2BRQ, 2J3S, 2K9U, 2W0P, 2BP3, 2JF1 and 3ISW), homologous sequences and predicted alignment of peptides from previous study[38] , we rationally defined the FBM as: $[X_{-a}/R/K]_{-3}$-$[X'_{-a}/R/K]_{-2}$-$[\Omega/\Phi/T]_{-1}$-

$[R/K/\underline{\Phi/S/F}]_0$-$[\zeta/E/D]_1$-$X'_2$-$[\Psi/F]_3$-$X'_4$-$[\Psi/R/K/\Omega]_5$-$X'_6$-$[\Psi/\Phi/\Omega/M/R/K/S/P]_7$-$X_8$ wherein Ψ = (L, V, I, T), Ω = (F, W, Y), Φ = (A,V, I, L), ζ = (S, T, N, Q), X represents any amino acid, X_{-a} represents any amino acid except acidic residues (D and E) and X' represents any amino acid except P. In the FBM, the position (denoted as subscripts) of the most conserved residue is numbered '0' and usually contains a basic residue (K or R). In addition, in migfilin and CFTR peptide bound filamin structures we observed that lack of basic residues at position 0 (underlined residues) appears to be compensated at position -2 or -3. The basic residues at these positions appear to be important for peptide interactions with FLNa Ig21 which has a complementary acidic patch. Therefore, in our search algorithm we introduced following additional conditions for residues at positions -2 and -3: (1) presence of K or R is a must in case they are absent at position 0 and (2) no acidic residue (D or E) is allowed. Furthermore, in positions -2 through 6 the residue P was avoided as it is not a preferred residue in a beta strand. A curated list of G protein-coupled receptors (GPCR) was downloaded from the UniProt Knowledgebase.[39] All 824 human GPCR sequences in this list were scanned for (i) the FBM without any mismatches and (ii) a 100% overlap of FBM in the intracellular halves of trans-membrane helices, intracellular loop and C-terminal regions as annotated in the downloaded sequence files. The Ig21 structure from 2J3S is represented by the electrostatic potential on the surface calculated using the program APBS[40] and contoured at ±12kT/e.

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***In vitro* kinase assays:** The kinase assay reaction conditions were 50mM Tris (pH 7.5), 10mM $MgCl_2$, 10µM FLNaIg16-24 as substrate, 10µM/50µM/200 µM filamin binding peptides (to release auto-inhibition), 500µM ATP. For each 100µl reaction 1000units of murine PKA (from NEB) was used. Protein and peptide phosphorylation was detected by western blotting using phospho-filamin A antibody (see materials).

Nuclear magnetic resonance (NMR) spectroscopy: ^{15}N labeled proteins were purified and the HSQC spectra were recorded in a Bruker Avance 600MHz spectrometer at 30^0C. Spectral processing and analysis was done using nmrPipe and NMRView.[41]

Isothermal titration calorimetry (ITC): MicroCal iTC200 calorimeter from GE Healthcare was used for determining ligand affinities to FLNa Ig repeats. Purified proteins were extensively buffer exchanged into 25mM sodium phosphate (pH 6.4), 5mM NaCl and 1mM DTT. Peptide ligands were dissolved in the same buffer and estimated as described earlier. 50µM protein in the sample cell was titrated against 1mM peptide in the syringe at 30^0C in 1µl increments at a stirring speed of 1000rpm.Solubility of α_{1D}-AR peptide was limited and hence this peptide was at an effective concentration of 0.4mM in the syringe. Affinities were determined by fitting the heat changes to a one site binding model using the associated Origin package.

Expression of FBM containing GPCRs, AT1R and MAS constructs and cell culture: The cloning of wild-type (WT) MAS with an N-terminal *myc*-tag and establishment of tetracycline/doxycycline inducible stable cell lines in HEK293 cells was described previously (HEK-MAS).[42] These stable cell lines were maintained in a humidified incubator at 37°C and 5% CO_2 and grown in complete media (DMEM supplemented with fetal bovine serum (10%), penicillin/streptomycin (100units/ml) with blasticidin (5µg/ml) and hygromycin (300µg/ml). For experiments, the cells were induced with complete media containing doxycycline (100ng/ml) for 26-28h for the expression of MAS. Un-induced cells were used as negative controls in the experiments. Expression and characterization of HA tagged rat AT1R (HA-AT1R) in the HEK293 cell line was described previously (HEK-AT1R).[43]

Co-immunoprecipitation of filamin with FBM containing GPCRs:
HEK293 and HEK-AT1R cells were used for these experiments. For filamin-GPCR interaction experiment, HEK293 and HEK-AT1R cells were serum starved for 4h. The cells were lysed in Triton X-100 lysis buffer (0.8% Triton X-100, 20 mM Tris-Cl pH 7.4, 300 mM NaCl, 1 mM EDTA, 20% glycerol, 0.1 mM PMSF, 10 µg ml^{-1} each of Leupeptin, and Aprotinin). 2mg of total protein was used to immunoprecipitate AT1R using the anti-HA affinity matrix (Roche). Immunoprecipitates were resolved using 8% SDS-PAGE and immunoblotted for filamin using the anti-filamin A monoclonal antibody MAB1680 (Millipore). Blots were stripped and reblotted for HA to confirm immunoprecipitation of HA-AT1R.

Evaluating filamin phosphorylation levels in cells expressing FBM containing GPCRs:
HEK-AT1R and HEK-MAS cells were used for these experiments. For the analysis of filamin phosphorylation, HEK-AT1R cells were grown until they reached 80-90% confluency. The cells

were serum starved for 2h and were pre-treated with either vehicle or AT1R antagonist candesartan (10μM) for 30min. Following this, AT1R expressing cells were treated with either vehicle or agonist Angiotensin II (1μM) for 1h. Whole cell protein lysates were prepared from these cells in Mammalian Protein Extraction Reagent buffer (M-PER from Thermo Scientific, Rockford, IL) with protease and phosphatase inhibitors. Equal quantities (~25μg) of these lysates were separated on 4-12% SDS-PAGE and then transferred onto nitrocellulose membrane for western blot analysis. The membranes were probed with pFLNa, FLNa, and GAPDH antibodies and suitable near-infrared (IR) dye conjugated secondary antibodies (LI-COR, Lincoln, NE). The ratios of phospho FLNa (pFLNa) to FLNa were calculated based on the fluorescence values and expressed as fold increase over uninduced/untreated controls. GAPDH was used as an additional control to confirm equivalent total protein loaded in the lanes. Data for AT1R is presented as an average (mean±SEM) of two independent experiments (N=2). Significance levels of unpaired Student's t-test are: *$p<0.05$. The HEK-MAS cells were induced for 24h with or without 10μM AR-*inverse agonist*. This was followed by 2h serum starvation of cells without any inhibitors or with a combination of 10μM AR-*inverse agonist* or 10μM PKA-inhibitor (H-89) depending on the experimental design. During induction and serum starvation AR-*inverse agonist* was added to the HEK-MAS cells to prevent constitutive activation of MAS. Following serum starvation and pre-treatment with inhibitors the cells were treated with 10μM AR-*agonist* along with PKA-inhibitor H-89 for 1h. FLNa phosphorylation in whole cell protein lysates was assayed on western blots as described for HEK-AT1R. Data for MAS is presented as an average (mean±SEM) of 3 independent experiments (N=3). Significance levels of unpaired Student's t-test are: *$p<0.05$.

Results

Frequency of FBMs in GPCRs: In a recent study[37] we recognized that filamin ligand peptides from integrins and migfilin enhance PKA mediated phosphorylation of S2152 in filamin by relieving auto-inhibition of the Ig21 repeat by Ig20. Since some GPCRs are known to interact with filamin[13-15], we parsed the literature for GPCR derived peptides that were reported to bind filamin. The D3 dopamine receptor (D3R) peptide from ICL3 region was shown to bind Ig19 of filamin. Many such peptides derived from cell adhesion related proteins bind class A Ig repeats of filamin as reported earlier.[32] Similar to these peptides, the D3R peptide increased the rate of PKA mediated phosphorylation at S2152 in the purified 100kDa FLNa Ig16-24, a filamin surrogate as described earlier[36,37] (Fig 1A). The effect though was not as pronounced as strong binding migfilin, integrinβ7 and GP1bα derived peptides.[37.] The stronger ligands relieve auto-inhibition more effectively thereby enhancing S2152 phosphorylation by PKA. The result suggested that D3R binds not only to Ig19 but may also bind Ig21.[32] We confirmed that this ICL3 peptide from D3 dopamine receptor (D3R) binds FLNa Ig19 and Ig21(Figure 1B and 1C) just as other filamin binding peptides by HSQC NMR spectroscopy. The affinity of the D3R peptide to Ig21 could not be assessed accurately by ITC owing to its lower affinity. However, this observation lead us to explore the possibility that occurrence of FBM sequence in GPCRs might be a conserved feature essential to directly engage filamin in cells and activate FLNa phosphorylation. We therefore used sequence and structural information of known binders for class A Ig domain of FLNa including D3R and defined a consensus FBM for GPCRs (see Methods and Figure 2). A bioinformatics screening of the sequences of 824 human GPCRs uncovered conservation of FBMs in the cytoplasmic regions of 116 non-olfactory GPCRs (Supplementary Table S1) and 73 olfactory GPCRs (Supplementary Table S2). Of particular significance to the cell biological function of GPCRs is the finding that FBMs were predominantly located in the functionally significant regions of GPCRs, the cytoplasmic tail (Ct) followed by the intracellular loop 3 (ICL3). Several novel candidate GPCRs that can potentially engage FLNa with high affinity were found in our search.

We selected AT1R and MAS from this list for experimental verification given their importance in cardiovascular physiology. The FBM in AT1R and MAS is present in the Ct. In the recently solved structure of AT1R[44], the position of FBM overlaps with the functionally important and structurally flexible 'helix 8' region. The α_{1D}-adrenoreceptor (α_{1D}-AR) with cardiovascular and neuronal roles was chosen as representative GPCR with predicted FBM in ICL3.[45,46] The ICL3 loop is a very important determinant for G protein activation by GPCRs. To the best of our knowledge there are no studies reporting the direct interaction of these three GPCRs with FLNa.

FLNa Ig 21 binds to predicted FBMs from AT1R, MAS and α_{1D}-AR: FLNa Ig21 is the representative filamin Ig repeat used extensively to test ligand binding.[32, 35] We therefore performed NMR spectroscopy and ITC experiments on the binding of this repeat with the GPCR derived FBM-peptides. Binding of all the three FBM-peptides to Ig21 resulted in significant changes in the ^{15}N HSQC spectra (Figure 3A, C, E). In the spectra; peaks for G2267, G2270 and S2279 that occupy the top and bottom of the "CD" ligand binding groove showed changes that are typical of high-affinity binders.[32, 36] Hence, these peptides most likely bind in a mode similar to the known filamin Ig-peptide complex structures through a β-strand augmentation. ITC experiments estimated the binding affinities of peptide motifs of AT1R, MAS, and α_{1D}-AR to FLNa Ig21 with K_d values of 0.8µM, 0.3µM and 0.8µM, respectively. These binding affinities are 50-100 times higher compared to that of the FBM peptides from cell adhesion proteins, the integrins, which are the most common filamin binding proteins in a majority of cell types (see discussion). The β7 integrin Ct peptide binds Ig21 with a Kd of 40µM.[47] These binding affinities are among the tightest known to bind filamin with the exception of the platelet specific protein, GPIbα[32] which has an affinity of 0.1µM.

Given that the filamin-adhesion receptor FBM interaction is tightly coupled to filamin phoshphorylation at S2152 by PKA[37], we tested the effect of these GPCR FBM-peptides on FLNa phosphorylation at S2152 in the filamin surrogate, FLNa Ig16-24 by PKA in an *in vitro* kinase assay. As expected, binding of these FBM-peptides enhanced rate of phosphorylation of the 100kDa surrogate of FLNa at S2152 by PKA in vitro (Figure 4) similar to the conventionally known FLNa ligands, integrins and migfilin.[32] This enhancement of filamin S2152 phosphorylation by high-affinity FBM-peptides derived from AT1R, MAS and α_{1D}-AR were much more robust than that compared to the D3R derived peptide which binds with lower affinity (Fig 1A vs Fig 4). This observation further emphasizes our premise that tighter binding ligands drive filamin Ig21 domain conformation towards a PKA compliant state.[32] These findings validate that the predicted FBMs in GPCRs are *bona fide* functional units and therefore may confer the ability to recruit FLNa to agonist activated GPCRs in cells.

AT1R binds to filamin and agonist treatment of AT1R promotes filamin phosphorylation. To demonstrate ligand regulation of a native GPCR interaction with filamin in cells, we selected ATIR as a model receptor. Immunoprecipitation of HA tagged rat AT1R from detergent solubilized HEK-AT1R cell lysates using anti-HA monoclonal antibody showed co-immunoprecipitation of endogenous Filamin A (280 kDa) with HA-AT1R (≈78kDa). HEK cells not expressing HA-AT1R were used as a negative control (Fig 5A). Immunoprecipitation of HA tagged AT1R is shown in the lower panel (Fig 5A). We next examined whether ligand modulated receptor activity is associated with phosphorylation of filamin in cells. Treatment of the cells with the agonist peptide, Ang II led to a 5-fold higher filamin phosphorylation and treatment with the inverse agonist, candesartan reduced filamin phosphorylation to basal levels (Figure 5B). Together these results demonstrate that endogenous filamin A binds AT1R and agonist activation of AT1R enhances phosphorylation of cellular filamin at S2152, suggesting a direct role for AT1R in actin remodeling through filamin.

Physical and functional interaction of MAS with FLNa: We studied MAS, to substantiate GPCR linked filamin phosphorylation results, as MAS is a constitutively active oncogenic GPCR with promiscuous G protein coupling ability.[42] In addition it contains a *bona fide* FBM which binds Ig21 with very high affinity as measured by robust biophysical techniques (figure 3C and D). For these studies we used the tetracycline-inducible system of myc-tagged human MAS expression in HEK293 cells as previously described.[42] We examined functional coupling between MAS and endogenous FLNa in tetracycline-induced cells by monitoring FLNa-phosphorylation at S2152. The un-induced cells served as control. Tetracycline-induction of MAS expression alone caused a 7-fold increase in the phosphorylation of FLNa (Figure 6A). This is not surprising as we and others have reported high constitutive activity of MAS.[42,48-49] FLNa phosphorylation induced by constitutive activity of MAS was abolished in cells treated with a specific inhibitor of the constitutive activity of MAS, AR-*inverse agonist* (Figure 6B). Treatment of MAS expressing cells with a specific MAS agonist, AR-*agonist*, further increased the phosphorylation of FLNa to 10-fold (Figure 6A). Agonist-stimulation of cells pre-treated with AR-*inverse agonist* (during induction) resulted in 9-fold increase in FLNa phosphorylation (Figure 6B). Unlike AT1R, MAS activates all major G proteins in cells including the G_s protein-coupled cAMP pathway leading to the activation of PKA. Consistent with this finding, treatment of MAS agonist activated cells with the PKA-inhibitor, H-89, resulted in reversal of the phosphorylated FLNa (pFLNa) levels to those of constitutive levels (Figure 6A). Furthermore, treatment with H-89 decreased the pFLNa levels by 60% in cells pre-treated with AR-*inverse agonist.* These experiments demonstrate that only a part of FLNa phosphorylation in cells by both constitutive- and agonist-activation of MAS is PKA-dependent. The remaining phosphorylation takes place perhaps by other cellular Ser/Thr kinases.

Experiments in Figures 5 and 6 suggest that the FBM in AT1R and MAS likely adopts a FLNa binding conformation only upon receptor activation. AT1R does not signal constitutively and needs agonist binding to activate the filamin binding mode, however, MAS likely binds filamin constitutively and hence leads to constitutive filamin phosphorylation. These results emphasize that it is the active receptor that mediates filamin phosphorylation by PKA or other cellular S/T kinases. Overall, our data suggests (i) conservation of FBM in GPCRs, and (ii) receptor activation-dependent phosphorylation of FLNa which is most likely a consequence of FLNa-GPCR interactions.

Discussion

Impetus for (i) the bioinformatics discovery of FBM in >20% of GPCRs and (ii) experimental validation of GPCRs as *bona fide* ligands for relieving auto-inhibited state of FLNa, is based on our findings reported recently.[37] It was found that phosphorylation of S2152 in filamin was dependent on relieving auto-inhibition of Ig20 by engagement of Ig21 by receptors harboring an FBM. Conventionally studied filamin activators were peptides derived from cell adhesion molecules. Migfilin, β7 integrin and GPIbα peptides enhanced PKA mediated filamin phosphorylation *in vitro*, indicating that functionally diverse ligands can promote filamin phosphorylation. Regulation of homeostatic equilibrium between FLNa and pFLNa by these conventional activators is known to be critical in cell cytoskeletal dynamics leading to changes in cell adhesion responses.[33] Though there is literature on filamin-GPCR interaction at the functional and biochemical level, broad generalizations were obscure at best. The D2R and D3R peptides were the most clearly recognized class A filamin binders[38, 32]. The extent of involvement of FBMs in GPCRs, the super-family of cell surface receptors to carry out FLNa-mediated cytoskeletal signaling is a major gap in the current knowledge base. To bridge this gap, we report here for the first time a consensus FBM in GPCRs and identified several GPCRs to contain FBMs in their intracellular regions. We noticed several important GPCRs that are part of this select GPCR list (Table S1). For example, lysophosphatidic acid treatment was reported to increase FLNa phosphorylation in previous studies.[50] However, the role of any

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particular receptor in the process was not addressed. Our predicted hits contain lysophosphatidic acid receptor 2 suggesting possible role for this receptor in directly engaging FLNa. Similarly, kinin treatment was shown to alter filamin translocation in endothelial cells.[51, 52] The roles for B1 and B2 bradykinin receptors (B1R and B2R) was alluded to in this process, but never proven. In our predictions we identified B2R to contain the FBM; and most interestingly a pair-wise sequence alignment between B1R and B2R reveals a gap in the sequence of B1R at the homologous region spanning the entire length of the FBM, thus, providing evidence for the role of B2R but not B1R in the process. Similar, differences in the FBM regions were seen in neuropeptide FF receptors 1 and 2, and dopamine receptors.

Along with the known filamin binders such as dopamine receptors, we also identified several important GPCRs that respond to serotonin, acetylcholine, opioids, angiotensin, chemokines, eicosanoids and fatty acid ligands. Our list also included the recently identified CXCR4 FBM as a positive hit although the FBM in this receptor spans both the cytoplasmic and transmembrane regions. The list also has several orphan, olfactory and taste receptors. Few of the GPCRs that were previously reported to interact with filamin are absent from our list. This is expected as in this study we only attempted to define FBM for class A repeats in FLNa with very strict criteria. We speculate that by expanding the definition of FBM to include proteins binding to (1) repeats other than class A and (2) other filamin isoforms (B and C) the predicted list of GPCRs to interact with filamin would be substantially large. More crystal structures of peptide motifs with FLN Ig domains would further aid in improving these predictions. Since our predictions are based on a limited number of crystal structures with peptides, the potential for false positive hits as well as missing more potent filamin binding GPCRs cannot be excluded. Our search criteria were stringent and all the 12 residues needed for filamin engagement were filtered to be part of cytoplasmic regions (see methods).

From the predicted list we picked three different GPCRs, MAS, AT1R and α_{1D}-AR, which are important in cardiovascular physiology for further experimental validation. These three receptor peptides have no known topological constraints to bind filamin. Using NMR and ITC we show unambiguous tight binding of the FBM peptides to FLNa Ig21. The affinities in the nano molar range by ITC are extremely rare for filamin binders. Only the platelet specific GP1bα receptor peptide shows a tighter binding than the above three GPCRs.[32] Compared to integrins,[47] the 3 GPCR peptides tested here have 50-100 fold higher affinity and are likely to be very potent biologically. We are not aware of any report of GPCR peptides showing such high affinity by ITC for any known GPCR binder. The only study we came across is the binding of the cannabinoid receptor peptide to β arrestin with an affinity of 2μM.[54] Furthermore, the bound FBM peptides promoted rapid phosphorylation of FLNa at S2152 *in vitro* conditions validating our biophysical studies.

In strong support of the *in vitro* findings that ligand binding induces FLNa phosphorylation at S2152, we observed robust phosphorylation of FLNa on S2152 site in cells expressing the FBM containing GPCRs, AT1R and MAS. We note that unlike AT1R there was strong basal/constitutive (agonist-independent) filamin phosphorylation induced by MAS. This was expected as the cAMP levels are significantly higher at basal levels in MAS expressing cells[42,48] causing PKA activation that could be inhibited by H-89. To tease out acute or short-term signaling effects of MAS and its consequences in cells we inhibited constitutive activity by adding AR-*inverse agonist* to the cells at the time of induction. This allowed us to demonstrate the receptor-activation-dependent increase in the phosphorylation of FLNa. The lack of robust reagents prevented us from extending our results to α_{1D}-AR.

Intriguingly, only 60% of the increase in pFLNa in MAS expressing cells was inhibited by H-89 suggesting involvement of kinases other than PKA in the process.[12] The residual component of pFLNa is most likely generated by kinases that are activated in the cells by other G protein pathways.[50,55] Constitutive or ligand-dependent activation of AT1R and MAS likely introduces conformational changes in the FBM that is suitable for FLNa engagement. Thereby AT1R and MAS directly binds to Ig21 and prevents auto-inhibition of FLNa Ig21 by Ig20. The resultant GPCR bound FLNa becomes a substrate for kinases (not limited to PKA) that are active in cells through signaling or independently by other mechanisms. Ser/Thr kinase such as ribosomal S6 kinase activated upon AT1R activation is a known FLNa phopsphorylating kinase.[50, 56] Overall, kinases increase pFLNa levels in the cells and likely promote cytoskeletal assembly. Taken together these two receptors seem to couple with filamin during or immediately after agonist activation. Antagonist binding prevents phosphorylation of filamin with both these receptors validating our agonist data. The details of this process most likely involve a helix/loop to βstrand change in the Ct regions of the receptors that transiently enhances filamin binding only in the presence of the agonists. The cytoplasmic regions of GPCR also bind β-arrestin following agonist activation. In a previous proteomic study FLNa was shown to interact with β-arrestin [57]. Whether β-arrestin assembly plays a role in FLNa interaction with GPCRs and subsequent FLNa phosphorylation is currently unknown and is an interesting area for future research. The enhanced engagement of filamin to an activated receptor is seen in some recent work though not recognized as such.[53] Our model provides molecular explanation of the previously reported G protein signaling mediated filamin phosphorylation where direct FLNa-GPCR interaction was not known.[50,58]

This is the first report to demonstrate filamin phosphorylation tied to activation status of GPCRs. Filamin phosphorylation has been linked to defects in neuronal migration, actin binding and cytoplasmic localization[59] through the guanine exchange factor ARFGEF2. Interestingly agonist dependent filamin phosphorylation appears to be not limited to G_s coupled GPCRs ase observed in this study and in other reports. This implies that a cAMP independent activation of PKA may play a role in case of those GPCRs. Evidence for such a phenomenon is available for mouse AT1A and endothelin-1 receptors.[60] Filamin phosphorylation might reposition filamin with respect to the plasmamembrane due to charge repulsion and in this new microenvironment its binding partners may change thereby bridging a different set of proteins. Filamin seems necessary for optimal coupling of G protein to D3R[14] and dopamine treatment has been shown to reduce filamin binding to the receptor.[61] Filamin has been shown to form a complex with the β-arrestin and GPCR in at least three cases, i.e. D3R[61], AT1R and muscarinic M1 receptor (M1MR).[62] The Ig22 domain of filamin was characterized as the most likely docking site for β-arrestin and a complex of β-arrestin-FLNa-receptor regulate ERK activation and membrane ruffling.[62] However, direct binding of filamin to the AT1R and M1MR was not considered a mechanism in the previous work. However, our bioinformatics search identified a potetnial FBM in M1MR (Supplementary Table S1). More structural information by NMR or crystallography will help in robust hypothesis generation to unravel the precise molecular details. In AT1R and MAS the FBM is part of the cytoplasmic helix 8 that plays crucial but imprecisely defined role in various GPCRs.[63] In AT1R and other GPCRs this region was also shown/predicted to bind to tubulin.[64] In most crystal structures of GPCRs this region is a helix or part of a helix or unstructured or with missing electron density (PDB: 4DAJ, 2R4S, 3ODU, 3VW7 and 4YAY). We envisage an agonist/antagonist dependent conformational switch in this region of many GPCRs that facilitate filamin engagement. Given our data, the role that filamin and its phosphorylation plays in GPCR function will have to be pursued with more rigor.

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On a final note, the class A Ig repeats of filamin mainly binds unstructured segments of membrane receptors. It is also interesting to note that the cytoplasmic regions especially the ICL3 and Ct regions of GPCRs are highly variable in length and are predicted to be intrinsically disordered.[65,66] Given that greater than 30% of the eukaryotic proteomes contains disordered segments,[67,68] it is tempting to speculate that filamin may bind many more proteins than currently recognized. Moreover, Filamin Ig repeats have expanded in number during eukaryotic evolution[69] and this may have some correlation with the expansion of disordered segments in many signaling proteins. There is speculation in the literature that millions of such disordered motifs may exist[70] and large proteins like filamin with their ability to bind these motifs may have co-evolved. FLNa is also alternately spliced resulting in an isoform that lacks autoinhibtion and the S2152 phosphorylation site.[71,72] Filamin isoform switching may therefore regulate both mechanistic and signaling outcomes during animal development.[73]

Field Code Changed

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Supporting Information Available: Table S1 and S2 contain a list GPCRs having predicted filamin binding motifs.

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Figure Legends

Figure 1. Filamin binding D3 dopamine receptor peptide increases PKA mediated filamin phosphorylation and binds ClassA Ig repeats. (A) Time-dependent phosphorylation of 100kDa band of FLNa Ig16-24; in the free form (lanes 1-4) and with 200µM dopamine receptor3 peptide (lanes 5-8). (B and C) HSQC spectra of FLNa Ig19 and Ig21 in the free form (Black) and in the presence of 2 fold excess dopamine receptor peptide (red) showing that both these repeats bind the peptide.

Figure 2. Sequence and structural analysis of FLNa binding motifs. **(A)** Sequence alignment of known FLNa ligands. The peptide ligands are derived from various sources: †X-ray structures (chain annotation), §NMR structure, ‡Homologous sequences of integrin β1 and integrin β3, and predicted alignment of dopamine receptor peptides from previous study (37). The basic residues are in blue, acidic residues in red, uncharged polar residues in green and hydrophobic residues are shown in black. The most conserved residue position is designated 0 and all residues are labeled accordingly (numbering above the alignment). The cartoon of beta sheet on the top of the alignment depicts the secondary structure adopted by the FLNa ligand in the PDB structures. The residues that are important for defining the FLNa binding motif (FBM) are also highlighted by shading. **(B)** Overlay of FLNa ligands on FLNA Ig21 upon structural alignment of Ig21 and Ig17 from different PDB structures (2BRQ, 2J3S, 2W0P, 2BP3, 2JF1 and 3ISW). The Ig21 structure from 2J3S is represented by the electrostatic potential on the surface calculated using the program APBS (54) and contoured at ±12kT/e. Acidic and basic charged surface areas are colored red and blue, respectively. The internal FBM peptide from Ig20 (2J3S) is shown as cartoon and colored in cyan. The FBMs from integrin β7, integrin β2, migfilin, CFTR and GPIbα are shown as sticks. The acidic patch on Ig21 and the complementary basic residues of the FBMs are circled. The N-terminus of the bound peptide (cyan) is in the circled part (left side of the protein) and the C-terminal end of the bound peptide (cyan) is sticking out of the Ig repeat on the right side of the Ig repeat.

Figure 3. Interaction of AT1R, MAS and α_{1D}-AR FBMs with FLNa Ig21. HSQC spectra of ^{15}N labeled FLNa Ig21 repeat with **(A)** AT1R, **(C)** MAS and **(E)** α_{1D}-AR peptides show extensive spectral changes indicating a strong binding event. The peptides used in the experiments are also shown with the residues colored as described in Figure 2. **(B, D, F)** Corresponding ITC measurements with calculated binding affinities are shown. These affinities are tighter than any know GPCR peptide and protein partners by isothermal calorimetry. The α_{1D}-AR peptide was at a concentration of 0.4mM in the syringe unlike 1mM for AT1R and MAS and hence the slope of the titration is different, though the affinities are comparable.

Figure 4. Time-dependent phosphorylation of FLNa Ig16-24 (~100kDa) in the absence (the first two lanes from the left in each blot) and presence of FBMs (+: 10µM and ++: 50µM,the 2 lanes on the right in each blot) of AT1R (left panel), MAS (center panel) and α_{1D}-AR (right panel).

Figure 5. Physical and functional interactions of full-length AT1R with FLNa. **(A)** AngII receptor AT1R complexes with filamin (280kDa) in HEK cells **(B)** AT1R mediated FLNa phosphorylation upon stimulation with AngII and the lack of FLNa phosphorylation when treated with antagonist, candesartan. The data is a representation of two independent (N=2) experiments.

Figure 6. Functional interaction of MAS with FLNa. **(A)** MAS mediated FLNa phosphorylation upon stimulation with AR-*agonist* and its inhibition by PKA inhibitor, H-89 in MAS expressing (induced; IN) and control (un-induced; UI) cells. **(B)** FLNa phosphorylation under inhibition of constitutive activity. Inhibition of constitutive activation of MAS by AR-*inverse agonist* (AR-*inv*) abolished constitutive FLNa phosphorylation. Under these conditions treatment with AR-*agonist* stimulated maximal FLNa phosphorylation. PKA inhibitor, (H-89), partially inhibited AR-*agonist* induced FLNa phosphorylation. Data is presented as an average (mean±SEM) of independent experiments (N=3).

Figure 7. Model for select GPCR mediated filamin A phosphorylation. Agonist bound receptor couples to filamin thereby releasing autoinhibition of filamin and this in turn promotes PKA mediated S2152 phosphorylation.

Figures

Figure 1.

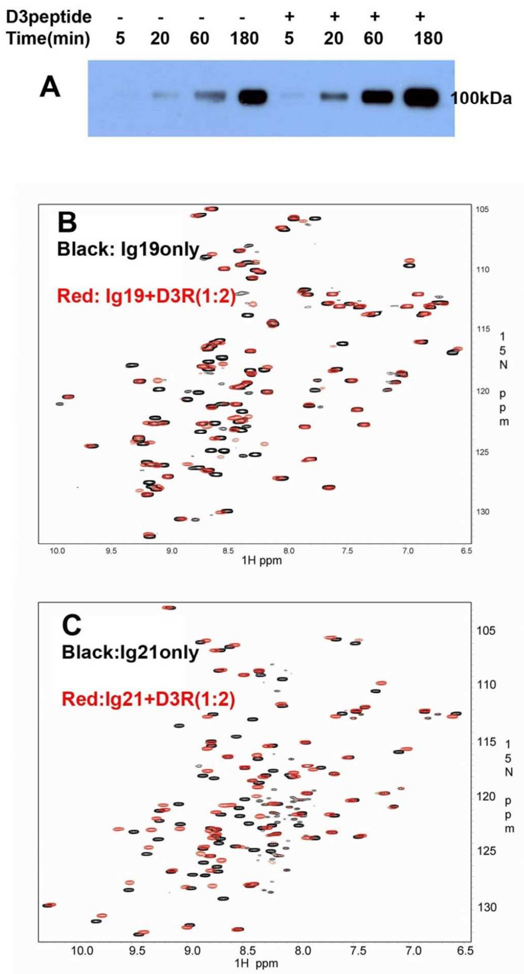


Figure 2.

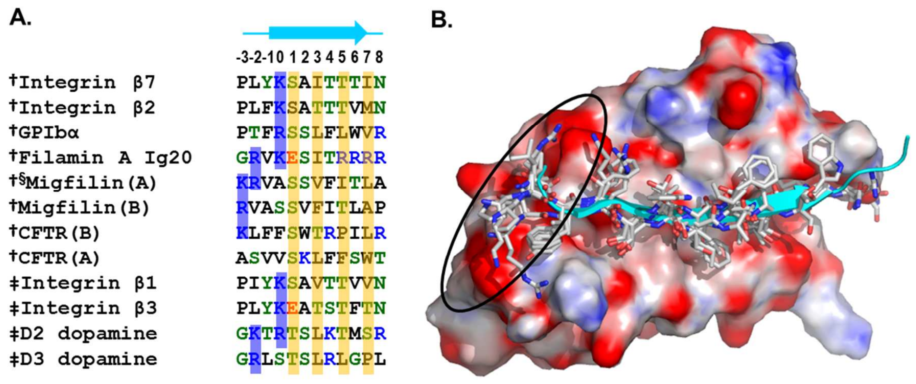

A.
B.
-3-2-1 0 1 2 3 4 5 6 7 8
†Integrin β7 PLYKSAITTTIN
†Integrin β2 PLFKSATTTVMN
†GPIbα PTFRSSLFLWVR
†Filamin A Ig20 GRVKESITRRRR
†§Migfilin(A) KRVASSVFITLA
†Migfilin(B) RVASSVFITLAP
†CFTR(B) KLFFSWTRPILR
†CFTR(A) ASVVSKLFFSWT
‡Integrin β1 PIYKSAVTTVVN
‡Integrin β3 PLYKEATSTFTN
‡D2 dopamine GKTRTSLKTMSR
‡D3 dopamine GRLSTSLRLGPL

Figure 3.

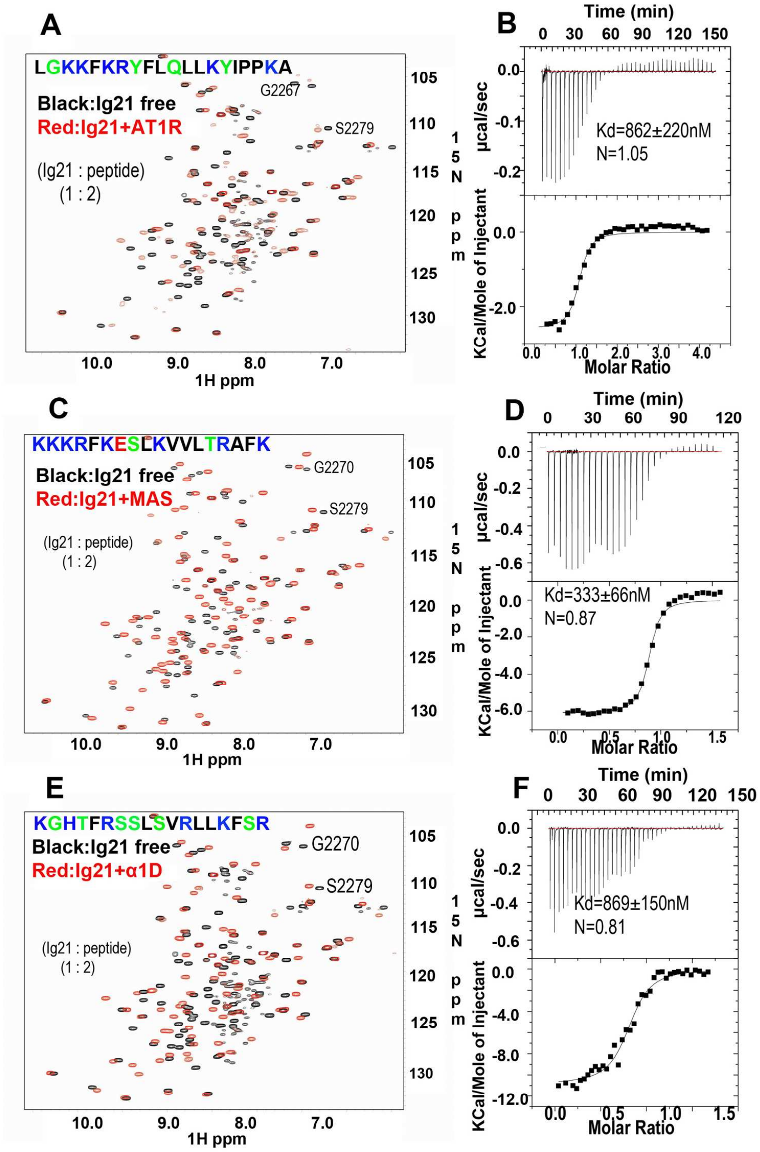

A
LGKKFKRYFLQLLKYIPPKA
Black:Ig21 free
Red:Ig21+AT1R
(Ig21 : peptide)
(1 : 2)
G2267
S2279
105
110
115
120
125
130
15N ppm
10.0
9.0
8.0
7.0
1H ppm
B
Time (min)
0 30 60 90 120 150
µcal/sec
0.0
-0.1
-0.2
Kd=862±220nM
N=1.05
KCal/Mole of Injectant
0.0
-2.0
0.0 1.0 2.0 3.0 4.0
Molar Ratio
C
KKKRFKESLKVVLTRAFK
Black:Ig21 free
Red:Ig21+MAS
(Ig21 : peptide)
(1 : 2)
G2270
S2279
D
Time (min)
0 30 60 90 120
-0.2
-0.4
-0.6
Kd=333±66nM
N=0.87
-4.0
-6.0
0.0 0.5 1.0 1.5
E
KGHTFRSSLSVRLLKFSR
Black:Ig21 free
Red:Ig21+α1D
(Ig21 : peptide)
(1 : 2)
G2270
S2279
F
Time (min)
0 30 60 90 120 150
Kd=869±150nM
N=0.81
-8.0
-12.0

Figure 4.


FBM peptide
Time (min)
AT1R
MAS
α1D-AR
– – ++ ++ – – + + – – ++ ++
5 20 5 20 5 20 5 20 5 20 5 20
100kDa

Figure 5.

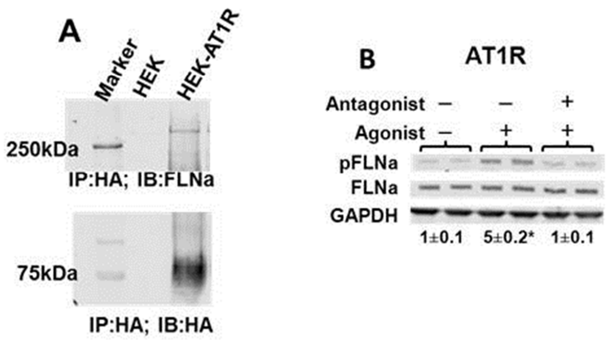

A
Marker
HEK
HEK-AT1R
250kDa
IP:HA; IB:FLNa
75kDa
IP:HA; IB:HA
B
AT1R
Antagonist – – +
Agonist – + +
pFLNa
FLNa
GAPDH
1±0.1
5±0.2*
1±0.1

Figure 6.

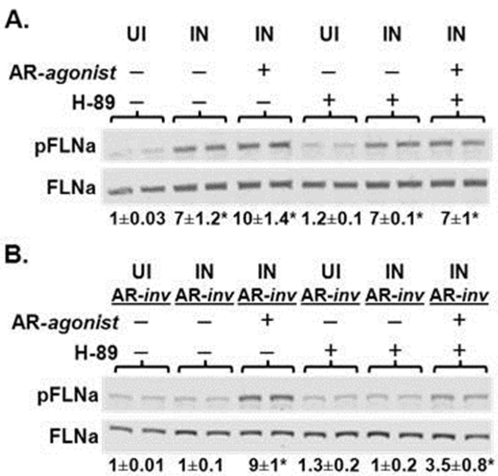
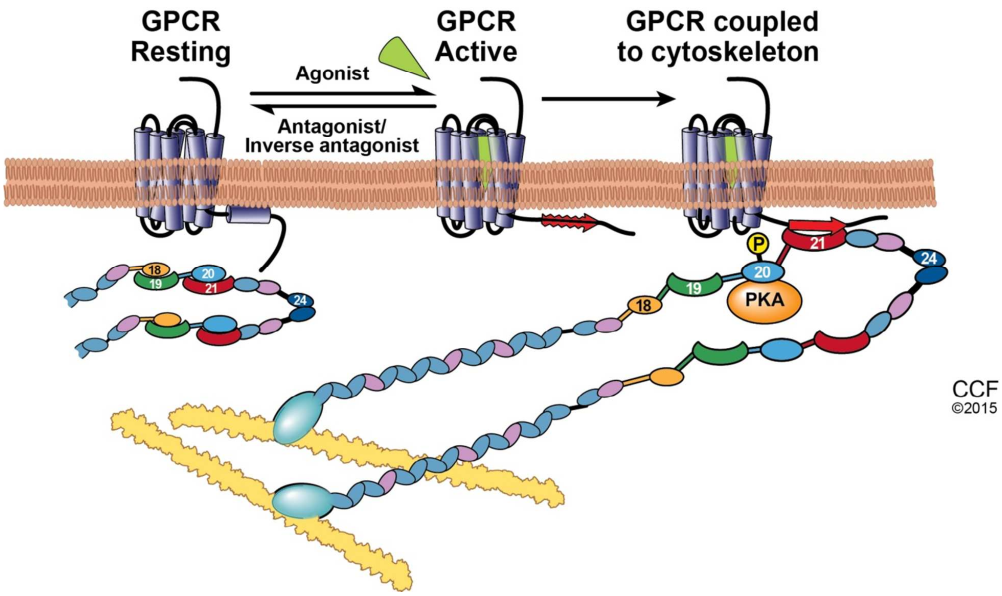

A.
UI IN IN UI IN IN
AR-agonist – – + – – +
H-89 – – – + + +
pFLNa
FLNa
1±0.03 7±1.2* 10±1.4* 1.2±0.1 7±0.1* 7±1*
B.
UI IN IN UI IN IN
AR-inv AR-inv AR-inv AR-inv AR-inv AR-inv
AR-agonist – – + – – +
H-89 – – – + + +
pFLNa
FLNa
1±0.01 1±0.1 9±1* 1.3±0.2 1±0.2 3.5±0.8*

Figure 7.


GPCR
Resting
GPCR
Active
GPCR coupled
to cytoskeleton
Agonist
Antagonist/
Inverse antagonist
18
19
20
21
24
P
PKA
CCF
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Filamin A phosphorylation by direct engagement of G protein-coupled receptors: Scaffolding mechanism for coupling GPCR-cytoskeletal signaling

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