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Kinin B1 Receptor Homo-oligomerization Is Required For Receptor Trafficking To the Cell Surface

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**Abstract**

The kinin B1 receptor (B1R) is a G protein-coupled receptor with pro-inflammatory activity that is latent in healthy tissues but induced by tissue insult. Here, we investigated if B1R homo-oligomerization is a possible mechanism regulating the presentation of this receptor at the level of maturation and trafficking to the cell surface. To this end, we used HEK293 cells stably expressing N-terminal FLAG and HA epitope-tagged wild-type human B1R and an N-terminal receptor fragment, B1stop135, which terminates at the C-terminal end of the third transmembrane domain and has previously been shown to oligomerize with B1R. Receptors were monitored by immunoblotting and immunoprecipitation, receptor function by agonist binding and agonist-promoted phosphoinositide hydrolysis, and receptor trafficking by confocal immunofluorescence microscopy. When expressed alone, B1R is core N-glycosylated and forms oligomers localized intracellularly and on the cell surface. B1stop135 also exists as core N-glycosylated oligomers but is localized exclusively intracellularly. When co-expressed, B1stop135 prevents specifically B1R homo-oligomerization by forming nonfunctional B1R-B1stop135 hetero-oligomers, retains B1R intracellularly at least in part in the endoplasmatic reticulum (ER), increases calnexin binding to the receptor, and increases receptor degradation. We conclude that B1R homo-oligomerization is necessary for B1R maturation and trafficking to the cell surface. Modulating this mechanism may be a novel therapeutic avenue in inflammatory disease.

*Keywords:* Kinins, G protein-coupled receptors, Kinin B1 receptors, Oligomerization, Membrane trafficking
1. Introduction

Kinins are potent vasoactive peptides that are produced upon inflammatory insult and act through two subtypes of kinin receptors, B1 (B1R) and B2 (B2R), to promote inflammatory responses including vasodilatation, increased vascular permeability, and pain [1,2]. B2R is constitutively and ubiquitously expressed, signals in response to bradykinin (BK) and Lys-BK [1,2], rapidly desensitizes and internalizes upon agonist binding, and recycles to the plasma membrane following internalization [3-6]. In contrast, B1R expression is induced by sustained insult [1,2], signals in response to the carboxypeptidase products desArg⁹-BK and Lys-desArg⁹-BK [1,2], slowly desensitizes upon agonist binding [3], internalizes constitutively [5,7] in a manner that is impeded by the agonist [5], and the internalized receptor targets lysosomes for degradation [5].

It is now well documented that the emergence of B1-subtype responsiveness in most tissues occurs only during stages of sustained inflammatory insult through induction of B1R gene transcription by pro-inflammatory cytokines such as interleukin-1β [1,2]. For example, B1R induction is part of the proinflammatory phenotype promoted by low laminar shear stress and may participate in responses including vasodilatation and leukocyte recruitment in atherogenesis [8]. Furthermore, B1R is coupled to both proliferation [9] and the release of metalloproteinases in breast cancer cells [10] suggesting an involvement in cancer cell invasiveness. Thus, B1R is a promising therapeutic target in both renal and cardiovascular disease [11] and cancer [12].

Considering that B1R is expressed only under conditions of inflammatory insult, this receptor likely harbors activity that has to be restricted to these conditions. B1R is constitutively active, at least in recombinant HEK293 cells [13], which may be detrimental under healthy conditions.
Recent studies have shown that a relatively large portion of the cellular B1R pool in HEK293 cells is intracellular, in part localized in the endoplasmatic reticulum (ER), with relatively few receptors at the cell surface [5,14]. Indeed, B1R exits ER slowly and is subject to significant degradation via ER-associated degradation (ERAD) [14]. Thus, in addition to induction of gene transcription, mechanisms may exist at the level of receptor maturation and trafficking to the cell surface to regulate B1R presentation.

Considerable evidence now exist that G protein-coupled receptor (GPCR) homo-oligomerization is required for receptors to exit the ER and traffic to the plasma membrane [15,16]. Indeed, GPCR oligomerization is emerging as a novel therapeutic target [17,18]. B1R was recently shown to homo-oligomerize in HEK293 cells and exist as oligomers on the cell surface [19]. In this study, we wanted to further analyze the concept of B1R homo-oligomerization as a regulatory mechanism in receptor maturation and trafficking to the cell surface. To this end, we constructed HEK293 cell model systems in which epitope-tagged wild-type B1R and an N-terminal B1R fragment (B1stop135), terminating at the C-terminal end of the third transmembrane domain, were stably expressed either individually or in combination. Our results show that B1R homo-oligomerization is necessary for receptor trafficking to the cell surface. Considering that B1R is an emerging new drug target in inflammatory disease, increased understanding of B1R oligomerization will aid in the search for novel therapeutic opportunities in such disease.
2. Materials and methods

2.1. Cell culture, transfections, and constructs

HEK293 cells (American Type Culture Collection) were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco BRL) supplemented with 10% fetal calf serum (FCS) (HyClone) in 10% CO$_2$ at 37°C. Human B1R and B2R cDNAs were subcloned into a pcDNA3.1 vector containing a zeocin or hygromycin selection marker. An artificial signal sequence and the FLAG tag were added in series to make FLAG-tagged B1R (FB1) and B2R (FB2) as described previously [5]. To make HA-tagged B1R (HB1), HA-tagged B1stop135 (HB1stop135), and HA-tagged B2R (HB2), receptor cDNA in pcDNA3.0, containing a G418 selection marker, was mutated using a polymerase chain reaction-ligation-polymerase chain reaction protocol and the HA epitope was inserted at the receptor N terminus as described previously [19,20]. Transient transfections were done with the calcium phosphate precipitate method. Single colonies were then chosen and propagated in the presence of selection-containing media to generate clonal HEK293 cell lines stably expressing the receptors.

2.2. B1R antibodies

Polyclonal antibodies against human B1R (B1N) were raised in New Zealand white rabbits using a peptide corresponding to N-terminal residues 2-38 as the antigen. The antibodies were purified using affinity chromatography using a SulfoLink coupling resin (Pierce) to which the peptide antigen was immobilized.
2.3. Immunoprecipitation and immunoblotting

Confluent cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in 1 ml lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 10 mM sodium phosphate, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS) with a complete protease inhibitor cocktail (Roche) for 30 min at 4°C. Lysates were cleared by centrifugation at 13,000 x g for 15 min at 4°C. Receptors were immunoprecipitated by either incubating the cleared lysates with B1N antibodies (1:1000), followed by incubation with protein A-Sepharose beads (Sigma-Aldrich) for an additional 2 h at 4°C, or by incubating with mouse anti-M2 FLAG antibody affinity resin (Sigma-Aldrich) overnight at 4°C. The precipitates were washed extensively and sequentially in the lysis buffer and in 10 mM Tris-HCl, pH 7.4. Proteins were denatured in sodium dodecylsulphate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer including 6% β-mercaptoethanol for 30 min at 37°C, fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, and the membrane was blocked for at least 45 min in Tris-buffered saline (TBS) and 10% nonfat milk. The proteins were stained by incubating with mouse anti-M2 FLAG antibody (Sigma-Aldrich; 1:1000), anti-HA.11 antibody (Nordic Biosite; 1:1000), or anti-calnexin antibody (Sigma-Aldrich; 1:2000) for 1 h at 22°C. Immunoreactive bands were visualized with a chemiluminescence immunodetection kit using peroxidase-labeled sheep anti-mouse antibody according to the procedure described by the supplier (PerkinElmer Life and Analytical Sciences). Some blots were then stripped by washing in 62.5 mM Tris-HCl, pH 6.7, 2% SDS, and 100 mM β-mercaptoethanol for 30 min at 50°C, washed three times for 10 min each in TBS, and restained with primary antibodies.
2.4. **Enzymatic deglycosylation**

To determine the presence of N-glycosylation in FB1, HB1, and HB1stop135, immunoprecipitates were treated with 500 units PNGase F (New England Biolabs) in 10 mM Tris-HCl, pH 7.4 for 2 h at 37ºC.

2.5. **Immunofluorescence microscopy**

Cells were propagated to 50% confluency in growth media on glass coverslips coated with poly-D-lysine (Sigma-Aldrich). For live staining, to detect cell surface receptors, live cells were incubated in growth media containing primary mouse anti-M1 FLAG antibody (Sigma-Aldrich; 1:1000) or anti-HA.11 antibody (Covance; 1:1000) for 30 min at 37ºC. Cells were fixed using 3.7% formaldehyde in PBS and permeabilized with blotto (3% dry milk, 0.1% Triton X-100, 1 mM CaCl$_2$, 50 mM Tris-HCl pH 7.4). For fixed staining, to detect total cellular receptors, cells were fixed and permeabilized followed by incubation in blotto containing primary mouse anti-M1 FLAG antibody (1:500) or anti-HA.11 antibody (1:500) for 1 h at 22ºC. In all experiments, cells were then washed with PBS and receptors visualized by incubation with secondary Alexa488- or Alexa555-labeled anti-mouse IgG2b or anti-mouse IgG1 antibodies (Invitrogen). For receptor colocalization, primary mouse anti-M1 FLAG and anti-HA.11 antibodies were incubated together in live or fixed and permeabilized cells. To investigate whether the receptors are located in the ER, fixed and permeabilized cells were incubated with primary mouse anti-FLAG- or anti-HA.11-antibodies together with primary rabbit anti-calnexin antibodies (Sigma-Aldrich; 1:200).
for 1 h at 22ºC. Alexa488- or Alexa555-conjugated anti-mouse IgG1 or IgG2b or anti-rabbit antibodies were then used as secondary antibodies. Images were collected using a Nikon Eclipse confocal microscope.

2.6. Phosphoinositide hydrolysis

Cells were seeded in six-well dishes and labeled with 1 μCi/ml myo-[³H]inositol (PerkinElmer Life and Analytical Sciences) in growth medium for 20 to 24 h at 37ºC, washed four times in DMEM, and then further incubated in DMEM for 1 h at 37ºC. This was followed by incubation in the absence or presence of various concentrations of the B1R agonist Lys-desArg⁹-BK in DMEM supplemented with 50 mM LiCl for 30 min at 37ºC. Cells were then lysed with 0.1 M formic acid for 20 min at 4ºC, and lysates were cleared by centrifugation at 16,100 x g for 5 min at 4ºC. The supernatants were added to anion exchange columns, which were washed two times with a low-salt solution (60 mM ammonium formate and 5 mM sodium borate). Inositol phosphates were then eluted with a high-salt solution (1 M ammonium formate and 0.1 M formic acid) and counted for radioactivity in a Beckman LS6000 liquid scintillation counter.

2.7. Particulate preparation

Confluent cells in 10-cm petri dishes were scraped with a rubber policeman, resuspended in buffer A (25 mM TES, pH 6.8, 0.5 mM EDTA, 0.2 mM MgCl₂, and 1 mM phenanthroline) and homogenized using an Ultra-Turrax homogenizer at 20,500 rpm for 10 s. Membranes were
isolated by centrifugation at 45,000 x g for 30 min at 4°C, and the pellets resuspended in 4 ml binding buffer (buffer A supplemented with 0.1% BSA and 0.014% bacitracin).

2.8. Radioligand binding

For radioligand binding on membrane preparations, membranes were mixed with about 1 nM [³H]Lys-desArg⁹-BK, in the absence and presence of 1 µM Lys-desArg⁹-BK to determine non-specific binding of [³H]Lys-desArg⁹-BK, in binding buffer in a total volume of 0.5 ml. After incubation for 60 min at 22°C, binding was terminated by addition of 4 ml ice-cold PBS with 0.3% BSA, followed by rapid vacuum filtration on Whatman GF/C filters previously soaked in 1% polyethyleneimine. The trapped membranes were then washed with an additional 2 x 4 ml of ice-cold PBS, 0.3% BSA, and the filters counted for radioactivity in an LS6000 liquid scintillation counter.

For intact cell binding, cells were grown in 6-well dishes and washed 3 times with DMEM containing 1 mM 1,10-phenanthroline and 140 µg/ml bacitracin, and then incubated with about 2 nM [³H]Lys-desArg⁹-BK, with or without 1 µM Lys-desArg⁹-BK, in the same medium for 90 min at 4°C. Binding was terminated by rinsing the cells with 2 x 2 ml of ice-cold PBS with 0.3% BSA, followed by addition of 0.5 ml solubilization buffer (1% SDS, 1 M NaOH, 0.1 M Na₂CO₃) for 4 min at 22°C. The lysates were then counted for radioactivity in an LS6000 liquid scintillation counter.
3. Results

3.1. Cellular distribution of B1R and B1stop135

To address the role of B1R homo-oligomerization in receptor maturation, we made HEK293 cell lines stably expressing N-terminal FLAG- (FB1) or HA-tagged (HB1) wild-type human B1R either alone or together with HA-tagged truncated human B1R terminating at Asp134 at the C-terminal end of the third transmembrane domain (HB1stop135), which has previously been shown to oligomerize with B1R [19] (Fig 1). Total cellular receptors were visualized by confocal immunofluorescence microscopy of cells stained with primary epitope-specific antibodies in fixed and permeabilized cells (fixed staining), and cell surface receptors were visualized in cells stained live prior to fixation and permeabilization (live staining). When FB1 was expressed alone, it was localized both at the cell surface, as determined by live staining, and intracellularly, both in a tubular network and in distinct puncta, as determined by fixed staining (Fig. 2A). Live FB1 staining also revealed distinct intracellular puncta, which represents spontaneous receptor endocytosis, as described previously [5]. On the other hand, HB1stop135 expressed alone was localized exclusively intracellularly in a tubular network (Fig. 2A). As a reference, we used HEK293 cells stably expressing HA-tagged B2R (HB2) (Fig. 1), which was localized almost exclusively at the cell surface as determined by both live and fixed staining (Fig. 2A).

3.2. B1stop135 partially colocalizes with B1R intracellularly and specifically inhibits B1R trafficking to the cell surface
When expressed together, HB1stop135 apparently completely retained FB1 intracellularly without any receptors detected at the cell surface (Fig. 2B). Under these conditions, the intracellular puncta observed in fixed FB1 staining (Fig. 2B) do not represent constitutive receptor endocytosis because they still appeared when live staining was preceded by hyperosmotic sucrose treatment of the cells (data not shown), which blocks spontaneous receptor endocytosis [5]. HB1stop135 remained intracellular in the presence of FB1, and the two proteins partially co-localized in distinct puncta (Fig. 2B, arrow). As a control, we used HEK293 cells stably co-expressing FB1 and HB2 (Fig. 2C). In this case, HB2 apparently did not perturb FB1 trafficking. On the other hand, clear colocalization of HB2 and FB1 was observed at the cell surface. We also co-expressed FLAG-tagged B2R (FB2) (Fig. 1) with HB1stop135, which apparently did not perturb FB2 trafficking (Fig. 2D). Thus, B1stop135 specifically inhibits B1R trafficking to the cell surface.

3.3 B1stop135 inhibits B1R agonist binding and signaling

The B1R agonist Lys-desArg⁹-BK bound with high affinity to FB1 (Fig. 3A) and stimulated receptor-mediated phosphoinositide (PI) hydrolysis with high potency in live cells expressing FB1 alone (Fig. 3B), which indicates that functional B1R exist on the surface under these conditions. On the other hand, Lys-desArg⁹-BK was unable to bind to live cells expressing HB1stop135 (Fig. 3A), which is expected since this construct is localized intracellularly and lacks critical ligand binding epitopes [21,22]. Co-expressing FB1 with HB1stop135 completely prevented Lys-desArg⁹-BK binding (Fig. 3A) and Lys-desArg⁹-BK-promoted PI hydrolysis in live cells (Fig. 3B). Notably, HB1stop135 also completely prevented high affinity Lys-desArg⁹-
BK binding to FB1 in a total particulate preparation of the cells (Fig. 3C). Thus, B1stop135 expression not only blocks functional B1R from reaching the cell surface but also disrupts the high affinity agonist binding site in the receptor.

3.4. B1stop135 disrupts B1R homo-oligomers and increases receptor degradation

To address the mechanism by which B1stop135 influences B1R, we analyzed the molecular structure of B1R in cells with and without B1stop135 co-expression. When expressed alone, FB1 migrated in gel electrophoresis as detergent-resistant multimers of about 40 kDa (a), the expected size of the receptor monomer, 80 kDa (b) and 160 kDa (c), in approximately equal proportions (Fig. 4A, lane 2), which were absent in non-transfected control cells (lane 1). Essentially the same receptor species were present in cells stably expressing HB1, even though the 160-kDa species was not clearly identified (Fig. 4B, lane 2). Treatment of samples with PNGase F prior to gel electrophoresis caused similar small decreases in the masses of the 40-kDa and 80-kDa species (Fig. 4A, compare lanes 2 and 3; Fig. 4B, compare lanes 2 and 3). Even though not clearly apparent at this mass, a similar decrease seemed to occur in the 160-kDa species. The doublet HB1 species at about 40-kDa (Fig. 4B, lane 2) has been observed previously and may be explained by micro-heterogeneity in N-glycosylated FB1 and HB1 [19]. Thus, the 80-kDa and 160-kDa receptor species are not increasingly N-glycosylated forms of the 40-kDa monomer. Instead, they are most likely homo-oligomeric forms of the monomer, as previously proposed [19].

Several HB1stop135 species were identified ranging in masses from about 15 kDa, the expected size of the fragment monomer, to ≥100 kDa (Fig. 4B, lane 4). Many of the species
decreased only slightly in mass following PNGase F treatment including the 19 kDa species \((d)\), which is most likely a glycosylated form of the 15-kDa monomer \((e)\) (Fig. 4B, lane 5). Thus, B1stop135 is core N-glycosylated and the higher mass species are likely homo-oligomeric forms of the monomer.

Fig. 4A, lane 4 and Fig. 4C, lane 3 show that when FB1 and HB1stop135 were co-expressed, the relative amount of the 40-kDa monomeric FB1 species increased dramatically at the expense of the 80-kDa and 160-kDa species, and with few if any unique multimeric structures. An additional approximately 22-kDa FB1 species \((a')\) also appeared, which is likely a receptor degradation product (Fig. 4A, lane 4; Fig 4C, lane 3). Both the 40-kDa monomer and the 22-kDa degradation product remained core N-glycosylated in the presence of HB1stop135 (Fig. 4A, compare lanes 4 and 5). Under these conditions, immunoprecipitation of FB1 co-precipitated HB1stop135 exclusively as a major 19 kDa species and a minor 15 kDa species (Fig. 4D, lane 3), again the former species likely being an N-glycosylated form of the latter (Fig. 4B, compare lanes 4 and 5). These results provide direct evidence that B1stop135 forms primarily a heterodimeric complex consisting of a 40-kDa glycosylated FB1 monomer and a 19-kDa glycosylated B1stop135 monomer, and that this event is associated with increased B1R degradation.

**3.5. B1stop135 traps B1R in the ER and increases calnexin binding**

Calnexin is a chaperone localized in the ER where it serves in protein quality control by promoting proper folding of immature receptors and preventing the transport of terminally misfolded receptors [24-26]. To investigate the role of calnexin in B1R maturation, the relative distribution and physical interaction of these proteins were investigated. Immunofluorescence
microscopy showed that calnexin is expressed intracellularly in a tubular network (Fig. 5A and B). FB1 co-localized significantly with calnexin both in the absence (Fig. 5A and B), which was particularly apparent in the presence of HB1stop135 (Fig. 5B). As shown in Fig. 6A, immunoprecipitation of FB1 from cells expressing FB1 alone resulted in co-precipitation of calnexin suggesting that the two proteins physically interact in the ER. A trend towards an increase in the amount of calnexin in the FB1 immunoprecipitate relative to the total cellular calnexin level appeared to occur when FB1 was co-expressed HB1stop135 (Fig. 6B). Thus, B1stop135 appears to physically trap B1R in the ER by terminally misfolding it, increasing its interaction with calnexin, and increasing receptor degradation by ERAD.
4. Discussion

Here, we report that the normal exit of B1R from ER and receptor trafficking to the cell surface is dependent on B1R homo-oligomerization. This was shown by disrupting intact receptor homo-oligomers with a truncated N-terminal B1R fragment, which dimerizes with B1R, retains the receptor in the ER, disrupts the receptor agonist binding site, and increases receptor degradation (Fig. 7). Thus, receptor homo-oligomerization may be a mechanism in addition to receptor induction to regulate the expression of this constitutively active pro-inflammatory receptor.

The induction of B1R in response to pro-inflammatory cytokines has evolved as an important pathophysiological regulatory mechanism to express this receptor primarily during conditions of inflammatory insult. Considering that this receptor also exhibits significant constitutive activity, at least in the recombinant HEK293 cell model system, additional cellular mechanisms may exist to control the presentation of this receptor at the cell surface. A relatively large portion of the cellular B1R pool in HEK293 cells is localized intracellularly in the ER with only few receptors at the cell surface [5,14]. Slow receptor folding and ER exit, yielding increased degradation, has been reported as one cause for this distribution [14]. The steady-state cell surface receptor level is further regulated by constitutive B1R endocytosis that targets the receptor to lysosomes for degradation [5,7]. This is in sharp contrast to the B2R subtype, which trafficks efficiently to the plasma membrane, and signals and internalizes only in response to agonist binding [5,13]. The reason for slow trafficking of receptors to the plasma membrane may be to provide the cell with a pool of receptors that can be rapidly mobilized following an appropriate stimulus and/or to tightly control constitutive receptor activity.
Receptor sequences have been identified that promote receptor retention in the ER [26]. In addition, recent studies show that GPCR need to homo-oligomerize to efficiently exit ER [16]. Thus, homo-oligomerization may be one mechanism by which cells regulate the ER exit and cell surface presentation of B1R. The receptor is core N-glycosylated and forms homo-oligomers of the 40-kDa monomer in the cell, which is consistent with our prior observations that differentially epitope-tagged B1R co-immunoprecipitate and receptor oligomers are present on the cell surface as determined by immunoelectron microscopy and sucrose density gradient fractionation [19].

The exit of B1R from ER is inefficient and receptors are subject to significant degradation by ERAD [14], which suggest that receptor misfolding occurs to some degree in the ER despite receptor homo-oligomerization. ER exit is also dependent on the expression levels of various more or less specific chaperones that promote proper folding of immature receptors and prevent the transport of terminally misfolded receptors [24-26]. B1R was found to interact with calnexin, a chaperone that promotes both of the above functions, which suggests that this chaperone participates in B1R folding control. This is consistent with work on the D1 and D2 dopamine receptors, which were also found to interact with calnexin in HEK293 cells [27]. The same study showed that overexpression of calnexin resulted in decreased cell surface receptor expression, and the receptors mostly appeared in discreet punctate oval-like clusters within the cell [27].

B1stop135 is core-N-glycosylated and forms multimers of the 15-kDa monomer in HEK293, which is consistent with our prior observation that B1stop135 co-immunoprecipitates with differentially epitope-tagged B1stop135 and with intact B1R [19]. Indeed, an N-terminal B1R fragment terminating at residue 72 in the first intracellular loop of the receptor (B1stop72) also forms multimers and co-immunoprecipitates with itself and with intact B1R [19]. While these
observations suggest that receptor homo-oligomerization requires an epitope(s) within the first 72 residues of the receptor, more specific mutations and refined assay methods, such as bioluminescence energy transfer, will be necessary to delineate precisely the receptor epitope(s) involved in homo-oligomerization. In contrast to B1R, B1stop135 remained in the ER. Thus, the absence of residues beyond Asp134 yields either nonfunctional oligomers or the loss of a critical protein interaction epitope(s) necessary for receptor maturation.

Co-expression with B1stop135 apparently completely retained B1R intracellularly at least in part in the ER. This effect was specific for B1R because HB1stop135 did not retain FB2, and HB2 did not retain FB1. This observation suggests that the cellular localization of B1R homo-oligomerization, and the epitope(s) necessary for this event, is different from B1R-B2R hetero-oligomerization [28]. Co-immunoprecipitation showed that monomeric core-N-glycosylated B1stop135 apparently forms a dimeric complex with monomeric core-N-glycosylated B1R. This complex also contained a limited amount of a 22-kDa B1R degradation product, which indicates that B1stop135 promotes B1R degradation while remaining associated with B1R. Under these conditions, B1R completely lost high affinity agonist binding. This loss cannot be accounted for by receptor degradation, which was only limited. Thus, agonist binding likely depends on a native homo-oligomeric structure. B1R is associated with calnexin, and this association may increase in the presence of B1stop135, which in turn may make the receptor more prone to ERAD degradation.

Whereas B1stop135 apparently completely prevented the appearance of cell surface B1R, only partial co-localization of the two constructs was observed, raising a question about the fate of the B1R not co-localized with B1stop135. While this question remains to be fully addressed, it is possible that some B1R-B1stop135 dimers dissociate easily therefore avoiding B1R
degradation but misdirecting trafficking of the receptor. Alternatively, B1R-B1stop135 dimers may dissociate during B1R degradation hence separating B1stop135-derived HA and B1R-derived FLAG immunoreactivities. Also, immunofluorescence microscopy may not be sufficiently sensitive to the appearance of a small number of B1R that escape B1stop135 dimerization and mature to the cell surface. However, the last explanation may be less likely because both agonist receptor binding and signaling was also absent in cells co-expressing B1R and B1stop135.

In summary, we show that B1R needs to homo-oligomerize in order to exit ER and traffick to the plasma membrane. We suggest that this mechanism may be regulated e.g. at the level of chaperone binding, in order to carefully control the presentation of this pro-inflammatory receptor. Considering that GPCR oligomerization is an emerging therapeutic target, B1R homo-oligomerization may therefore be an attractive option to therapeutically modulate B1R signaling in inflammatory disease.
Acknowledgements

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Abbreviations

B1R, kinin B1 receptor; B2R, kinin B2 receptor; GPCR, G protein-coupled receptor; ER, endoplasmatic reticulum; BK, bradykinin; ERAD, ER-associated degradation; HEK293, human embryonic kidney 293; FCS, fetal calf serum; DMEM, Dulbecco’s modified Eagle’s medium; HA, hemaglutinin; FB1, FLAG-tagged B1R; FB2, FLAG-tagged B2R; HB1, HA-tagged B1R; HB2, HA-tagged B2R; FB1stop135, FLAG-tagged B1stop135; SDS, sodium dodecylsulphate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; EDTA, ethylenediamine tetra-acetic acid; BSA, bovine serum albumin; PI, phosphoinositide.
References


Figure legends

**Fig. 1.** Schematic presentation of the molecular structures of FLAG and HA epitope-tagged and truncated B1R and B2R described in the text.

**Fig. 2.** Cellular distribution of B1R, B1stop135, and B2R. (A), HEK293 cells expressing FB1, HB1stop135 (H135), or HB2 alone were subjected to live (Live) and fixed staining (Fixed) with epitope-specific antibodies as described in Materials and Methods. (B), HEK293 cells expressing FB1 and HB1stop135 (H135) together were subjected to live (Live) and fixed staining (Fixed) with epitope-specific antibodies. The specific epitope-tagged construct stained is indicated (underlined), and individual and merged (Merge) images were collected. (C), HEK293 cells expressing FB1 and HB2 together were subjected to live (Live) and fixed staining (Fixed) with epitope-specific antibodies. The specific epitope-tagged construct stained is indicated (underlined), and individual and merged (Merge) images were collected. (D), HEK293 cells expressing FB2 and B1stop135 (H135) together were subjected to fixed staining (Fixed) with epitope-specific antibodies. The specific epitope-tagged construct stained is indicated (underlined), and individual and merged (Merge) images were collected. The results are representative of experiments performed at least three times, and the individual and merged images were collected using a Nikon Eclipse confocal microscope, 60x objective, 50 µm zoom. Arrow in merged images indicates obvious co-localized staining (yellow).

**Fig. 3.** Effect of B1stop135 expression on B1R-dependent Lys-desArg⁹-BK binding and stimulation of PI hydrolysis. (A), intact HEK293 cells expressing FB1 or HB1stop135 alone or
together were subjected to $[^3\text{H}]$Lys-desArg$^9$-BK binding as described under Materials and Methods. (B), Intact HEK293 cells expressing FB1 alone or together with HB1stop135 were subjected to Lys-desArg$^9$-BK-promoted PI hydrolysis. (C), Particulate preparations of HEK293 cells expressing FB1 or HB1stop135 alone or together were subjected to $[^3\text{H}]$Lys-desArg$^9$-BK binding. Values are means ± SEM with each data point performed in triplicates. ND, not detectable.

**Fig. 4.** Effect of B1stop135 on the molecular B1R structure. (A), HEK293 cells (Mock) and cells expressing FB1 alone or together with HB1stop135 were lysed, immunoprecipitated with polyclonal B1N antibodies, treated with (+) or without (-) PNGase F for 2 h, and then immunoblotted with mouse M2 FLAG antibodies. (B), HEK293 cells (Mock) and cells expressing HB1 or HB1stop135 alone were lysed, immunoprecipitated with polyclonal B1N antibodies, treated with (+) or without (-) PNGase F for 2 h, and then immunoblotted with mouse HA.11 antibodies. (C), HEK293 cells (Mock) and cells expressing FB1 or HB1stop135 alone or together were lysed, immunoprecipitated with Sepharose-linked mouse M2 FLAG antibodies, and then immunoblotted with mouse M2 FLAG antibodies. (D), the blot in (C) was stripped and rebotted with mouse HA.11 antibodies. Molecular mass ($M_r$) standards (*left side arrows*) and receptor species referred to in Results (*right side arrows*) are indicated, and the results are representative of experiments performed at least three times.

**Fig. 5.** Effect of B1stop135 on the association of B1R with ER. (A,B), HEK293 cells expressing FB1 alone (A) or FB1 and HB1stop135 (H135) together (B) were subjected to fixed staining for FB1 (*underlined; green*) or calnexin (*Calnexin; red*). Individual and merged (*Merge*) images are
shown. The results are representative of experiments performed at least three times, and the individual and merged images were collected using a Nikon Eclipse confocal microscope, 60x objective, 50 μm zoom. *Arrow* in merged images indicates obvious co-localized staining (*yellow*).

**Fig. 6.** Effect of B1stop135 on B1R and calnexin interaction. (*A,B*), HEK293 cells (*Mock*) and cells expressing FB1 alone or together with HB1stop135 were lysed. Lysates were then either directly immunoblotted (IB) with calnexin antibodies (bottom image), or first immunoprecipitated (IP) with Sepharose-linked mouse M2 FLAG antibodies and then immunoblotted (IB) with calnexin antibodies (top image) or M2 FLAG antibodies (middle image). (*B*), the images in (*A*) were analyzed by densitometry, and the amount of calnexin in the FB1 immunoprecipitate relative to total calnexin in the lysate was calculated. 100% refers to the relative amount of calnexin in the FB1 immunoprecipitate from cells expressing FB1 alone. The results are typical of three independent experiments.

**Fig. 7.** Proposed intracellular events depicting B1R maturation from the ER to the plasma membrane based largely on results obtained in the study. (1a) Calnexin interacts with receptors to aid in correct receptor folding and homo-oligomerization. (1b) Normal folding of B1R homo-oligomers leads to dissociation of the receptor-calnexin complex. (1c) B1R homo-oligomers exit the ER through COPII-coated vesicles, traffick through Golgi, and incorporate into the plasma membrane. (2a) B1stop135 competes for normal B1R homo-oligomerization yielding misfolded receptor hetero-dimers. (2b) Misfolded B1R targets ER-associated degradation (ERAD)
involving calnexin dissociation and ER export, and receptor ubiquitination and proteosomal degradation in the cytosol.
Fig. 1
Fig. 3
**Fig. 4**

A. 

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**B.**

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**C.**

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**D.**

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**Legend:**
- **IB:** Immunoblot
- **IP:** Immunoprecipitation
- **PNGase:** Protein N-glycosidase F
- **Mock:** Control sample
- **FB1:** Experimental sample
- **HB1 stop135:** Sample with mutation

**M_{r} (kDa):**
- 150
- 100
- 75
- 50
- 37
- 25
- 20
- 15
- 10
- 5
- 2.5
- 1.5
- 1

**Notes:**
- a, b, c, d, e are labeled bands on the blots.
Fig. 5

A. FB1 Calnexin Merge

B. FB1/H135 Calnexin Merge
Fig. 6

A.

IP: FLAG
IB: calnexin

IP: FLAG
IB: FLAG

IB: calnexin

Mock  FB1  FB1+HB1stop135

B.

FB1-Calnexin Association (%)

0  50  100  150  200

FB1  FB1+HB1stop135

IP: FLAG
IB: calnexin
Fig. 7

ER

1b. Calnexin dissociation

Homo-oligomer

1a. Folding

2a. Misfolding

Calnexin

B1stop135 monomer

B1R monomer

1c. Export

Proteasome

Ubiquitin

2b. ERAD

Plasma membrane

Golgi