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B_1 Bradykinin Receptor Homo-Oligomers in Receptor Cell Surface Expression and Signaling: Effects of Receptor Fragments

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ABSTRACT

The human B_1 bradykinin receptor is an inducible and constitutively active G protein-coupled receptor that is involved in the inflammatory and pain responses to injury. Here, we investigated the role of B_1 receptor homo-oligomerization in cell surface receptor expression. B_1 receptors tagged with either the FLAG or hemagglutinin epitope were monitored immunologically and by radioligand binding, biotinylation, and phosphoinositide hydrolysis in human embryonic kidney 293 cells. Selective immunoprecipitation, immunoblotting, and immunoelectron microscopy with epitope-specific antibodies together provided evidence for constitutively formed cell surface receptor homo-oligomers. Truncation of the receptor from the N- and C-terminal ends indicated that the epitope for oligomerization seems to be located between Leu^{26} on top of transmembrane helix 1 and Val^{71} at the bottom of helix 2. A receptor construct terminating at Asp^{134} at the bottom of helix 3, B1stop135, was expressed in the cell. It is interesting that this construct behaved as a dominant-negative mutant by competitively preventing formation of intact B_1 receptor homo-oligomers, and redistributing B_1 receptors from the cell surface to a common intracellular compartment. In contrast, expression of a construct containing the residues downstream of Asp^{134}, B1del(2-134), was inactive in this regard. Together, these results are consistent with a mechanism where constitutive B_1 receptor homo-oligomerization is required for expression of receptors on the cell surface and subsequent constitutive receptor signaling. This may be a novel mechanism by which the cell regulates the presentation of this constitutively highly active receptor at various stages of injury.

The functional significance of receptor homo-oligomerization has been extensively studied in the receptor tyrosine kinase family. Considerably less is known about oligomerization of G protein-coupled receptors (GPCRs). Nevertheless, recent data seriously challenge the 1:1:1 stoichiometry originally thought to exist in the agonist-GPCR-G protein interaction (Rios et al., 2001; Angers et al., 2002; George et al., 2002). The crystal structure of the metabotropic glutamate receptor mGluR1 N-terminal domain shows that the ligand binding pocket in this domain is a dimer (Kunishima et al., 2000). Furthermore, infrared laser atomic force microscopy of mouse retinal disc membranes illustrates that the native arrangement of rhodopsin is as a dimer and possibly higher order arrays (Fotiadis et al., 2003; Liang et al., 2003).

Coimmunoprecipitation and fluorescence or bioluminescence energy transfer of differentially tagged receptors expressed in transfected cell systems have been useful in identifying GPCR oligomerization (Rios et al., 2001; Angers et al., 2002; George et al., 2002). Some of these studies point toward the importance of receptor homo-oligomers in early receptor biosynthesis and in trafficking of receptors to the cell surface. On the other hand, little is known about how such oligomeric units are regulated structurally and functionally by agonists and other factors during the receptor life cycle.

In the model based on the rhodopsin arrays (Liang et al., 2003), it was concluded that intradimer contacts probably exist between transmembrane helices 4 and 5 with some interactions also provided by helices 1 and 2. Cross-linking studies with the D2 dopamine receptor have directly implicated a symmetric dimer interface in helix 4 (Guo et al.,

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ABBREVIATIONS: GPCR, G protein-coupled receptor; HEK, human embryonic kidney; HA, hemagglutinin; PI, phosphoinositide; PBS, phosphate-buffered saline; PNGase F, peptide-*N*-glycosidase F; PAGE, polyacrylamide gel electrophoresis; DK, desArg^{10}kallidin; BRET, bioluminescence resonance energy transfer.

2003). The same conclusion was drawn from cross-linking in the C5a receptor, which also inferred a symmetric interface in helix 1 and 2 (Klco et al., 2003). A role for helix 1 in receptor dimerization was also provided by fluorescence resonance energy transfer using the α_{1B}-adrenergic receptor (Carrillo et al., 2003) and the yeast α factor receptor (Overton et al., 2003).

Kinins are potent agonists who participate in the inflammatory and pain responses to insults by acting through two receptor subtypes named B_1 and B_2 (Couture et al., 2001). The B_2 receptor is expressed constitutively and thought to mediate kinin responses under nonpathological and short-term responses. Sustained insult leads to an induction of B_1 receptors from essentially a null level, which is triggered by proinflammatory cytokines (Marceau et al., 1998) and important in the long-term and pathological response (Dray and Perkins, 1993; Pesquero et al., 2000). B_1 receptor induction may be necessary to prolong the kinin response because it has been observed that the B_2 receptor desensitizes rapidly, whereas the B_1 receptor response is sustained (Mathis et al., 1996). Indeed, strict regulation of B_1 receptor expression is probably critical because this receptor is constitutively highly active (i.e., active in the absence of agonist) (Leeb-Lundberg et al., 2001; Kang and Leeb-Lundberg, 2002).

The mechanism underlying B_1 receptor induction lays in part at the level of mRNA synthesis (Menke et al., 1994). Changes in mRNA stability may also participate in receptor induction (Zhou et al., 1999). We hypothesized that B_1 receptor induction may in part also involve changes in receptor maturation and trafficking to the cell surface. To begin to address this, we investigated receptor homo-oligomerization. Herein, we provide evidence that B_1 receptors constitutively homo-oligomerize. Using a truncated receptor construct with dominant-negative behavior, we show that homo-oligomerization is apparently necessary for the expression of constitutively signaling B_1 receptors on the cell surface. This may be a novel mechanism of regulating the cellular presentation of this constitutively highly active receptor.

Materials and Methods

Cell Culture, Mutation, and Transfection. HEK293 human embryonic kidney cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum in 10% CO_2 at 37°C. B_1 receptor cDNA in pcDNA3 were mutated using a polymerase chain reaction-ligation-polymerase chain reaction protocol as described previously (Kang and Leeb-Lundberg, 2002). The FLAG and hemagglutinin (HA) epitopes were inserted at the receptor N terminus immediately after the initial methionine using synthesized primers containing the epitope sequences. The cells were transiently transfected with varying amounts of DNA using the calcium phosphate precipitate method as described previously (Kang and Leeb-Lundberg, 2002). The total amount of cDNA transfected was always kept constant by supplementing with pcDNA3 vector.

Cell Fractionation. Cells were lysed in ice-cold hypotonic lysis buffer (20 mM HEPES, pH 7.4, 6 mM $MgCl_2$, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μM leupeptin) using a Dounce homogenizer. The homogenate was then centrifuged at 1000*g* for 5 min at 4°C. Sucrose was then added to the supernatant to obtain a final concentration of 0.2 M, and the lysate (1 ml) was layered on top of a discontinuous sucrose step gradient containing 0.5, 0.9, 1.2, 1.35, 1.5, and 2.0 M, each in a volume of 1.5 ml. The gradient was centrifuged for 16 h at 29,000 rpm in a Beckman SW41 rotor (Beckman Coulter, Fullerton, CA). Fractions (0.5 ml) were collected from the bottom of the tube and then immunoblotted with anti-FLAG or anti-HA antibodies. Anti-Na/K-ATPase α subunit antibodies (Sigma-Aldrich, St. Louis, MO) were used to identify the plasma membrane fraction.

Functional Receptor Assays. The B_1 receptor activity was assayed by monitoring phosphoinositide (PI) hydrolysis in cells transfected with a series of receptor cDNA amounts and labeled with 1 μCi/ml [*myo*-^{3}H]inositol (PerkinElmer Life and Analytical Sciences, Boston, MA) as described previously (Kang and Leeb-Lundberg, 2002). The B_1 receptor density was determined using saturating concentrations (2–3 nM) of [^{3}H]desArg^{10}kallidin (PerkinElmer Life and Analytical Sciences). The slope factors of the increases in basal cellular PI hydrolysis and agonist-stimulated PI hydrolysis as a function of the level of receptor expression were used as parameters of constitutive and agonist-dependent receptor activity.

Cell Surface Biotinylation. Cells were biotinylated with 4 ml of 0.3 mg/ml nonpermeable polyethylene oxide-maleimide-activated biotin (Pierce Chemical, Rockford, IL) for 30 min at 4°C, washed two times with ice-cold phosphate-buffered saline (PBS), and quenched with 1 mM dithiothreitol in PBS for 10 min at 4°C. After washing three times with ice-cold PBS, cells were immunoprecipitated and electroblotted as described below. Biotinylated receptors were detected with streptavidin conjugated to horseradish peroxidase (Vector Laboratories, Burlingame, CA).

Immunoprecipitation and Immunoblotting. Cells were subjected to immunoprecipitation and/or immunoblotting essentially as described previously (Kang and Leeb-Lundberg, 2002). In short, cells were solubilized in 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, 10 μg/ml leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride) for 30 min at 4°C. The lysate was centrifuged at 13,000*g* for 15 min at 4°C. For immunoprecipitation, the lysate was incubated 12 to 18 h at 4°C with anti-FLAG M2 antibody (1:300; Sigma-Aldrich), anti-HA antibody (1:300; Covance, Denver, PA), or polyclonal antibodies raised against the B_1 receptor C-terminal tail residues 317 to 353 [anti-B1R(317-353); Lamb et al., 2002], followed by incubation with protein A-Sepharose beads with or without precoupled rabbit anti-mouse IgG for an additional 2 h at 4°C. The beads were then washed twice with 1 ml of lysis buffer and then with 1 ml of 10 mM Tris-HCl, pH 7.4. For immunoblotting, samples were dissolved in SDS sample buffer and subjected to SDS-PAGE under reducing conditions (6% β-mercaptoethanol) on 12% gels, unless otherwise indicated, electroblotted onto nitrocellulose membranes, and then stained with anti-FLAG M2 antibody (1:1000) or anti-HA antibody (1:1000). 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). In some experiments, blots were sequentially stained with the FLAG and HA antibodies. Stripping of blots between antibody staining was done by washing in 62.5 mM Tris-HCl, pH 6.7, 2% SDS, and 100 mM β-mercaptoethanol for 45 min at 50°C. The blots were then washed three times for 30 min in Tris-buffered saline. Residual immunoreactivity was checked with immunodetection kits in the absence of primary antibody.

Enzymatic Deglycosylation. To determine the presence of *N*-glycosylation in the B_1 receptor, anti-FLAG or anti-HA immunoprecipitates were treated with 2 units of peptide-*N*-glycosidase F (PNGase F; Roche Diagnostics, Indianapolis, IN) in 10 mM Tris-HCl, pH 7.4, for 2 h at 37°C. Partially deglycosylated immunoprecipitates were washed with 1 ml of 10 mM Tris-HCl, pH 7.4.

Immunoeletronmicroscopy. HA antibodies were labeled with 5-nm colloidal gold as described previously (Baschong and Wrigley, 1990). Cells expressing HA-tagged B_1 receptor were incubated with the gold-labeled antibodies for 30 min at 4°C. The cells were fixed for 1 h at room temperature and then fixed overnight at 4°C in 2.5% glutaraldehyde in 0.15 M sodium cacodylate, pH 7.4 (cacodylate buffer). The fixed samples were washed with cacodylate buffer and

postfixed for 1 h at room temperature in 1% osmium tetroxide in cacodylate buffer. The samples were then dehydrated in a graded series of ethanol and embedded in Epon 812 using acetone as intermediate solvent. Specimens were sectioned with a diamond knife into 50-nm-thick ultrathin sections on an LKB ultramicrotome. The sections were then stained with uranyl acetate and lead citrate. Specimens were observed in a Jeol JEM 1230 electron microscope operated at 80-kV accelerating voltage. Images were recorded with a Gatan Multiscan 791 charge-coupled device camera.

Data Analysis. Where indicated, data are presented as the mean ± S.E. and were compared using the Student's *t* test.

Results

Identification of B_1 Receptors. To identify the human B_1 receptor (Fig. 1A), the FLAG or HA epitope was inserted at the N-terminal end of the receptor immediately after the initial methionine. The tagged receptors were expressed in HEK293 cells and monitored using epitope-specific antibodies and antibodies against the human B_1 receptor C-terminal residues 317 to 353 [anti-B1R(317-353)]. Mock-transfected cells were always included to identify receptor-specific species. Epitope-tagged B_1 receptors were expressed and functionally active, as described previously (Leeb-Lundberg et al., 2001; Kang and Leeb-Lundberg, 2002). Immunoprecipitation followed by immunoblotting revealed that both FLAG-tagged B_1 receptor (FB1) (Fig. 1B, lane 1) and HA-tagged B_1 receptor (HB1) (lane 2) expressed as 35-kDa species, which is close to the theoretical molecular mass of the receptor, and consequently thought to be the monomeric form of the receptor. However, HB1 seemed more heterogeneous than FB1 with additional major bands at about 39 to 45 kDa and a weak band at about 28 kDa (Fig. 1B). Treatment of anti-HA immunoprecipitates with 2 units of PNGase F for 2 h at 37°C resulted in the conversion of the 39- to 45-kDa HB1 species to an approximately 37-kDa rather than a 35-kDa species (Fig. 1C, lane 3). Thus, the complex HB1 pattern is caused to a large extent by receptor *N*-glycosylation. That the receptor species remained at 37 kDa is probably due either to incomplete *N*-deglycosylation during the relatively short time period of PNGase F treatment or to the presence of further covalent modification. This receptor contains several serines and threonines in the N-terminal domain and extracellular loops that may be subject to *O*-glycosylation. To further investigate the role of the N-terminal domain in receptor microheterogeneity, residues 2 to 25 in the B_1 receptor were deleted to make B1del(2-25) (Fig. 1A). HB1del(2-25) migrated as a relatively homogeneous species at about the predicted theoretical molecular mass (Fig. 1C, lane 4). On the other hand, HB1stop320, in which the assumed nonglycosylated C-terminal residues 320 to 353 had been deleted in the B_1 receptor (Fig. 1A), retained a significant amount of the microheterogeneity observed in HB1 (Fig. 1C, lane 5). We conclude from these results that *N*-glycosylation occurs in the N-terminal domain, and presumably on Asn^{14} and/or Asn^{22}. Long exposure of FB1 immunoblots revealed that this receptor displayed microheterogeneity similar to HB1 but to a significantly lesser degree (data not shown). We have previously reported that this heterogeneity is not altered by PNGase F treatment, which also shows that the effect on HB1 was not simply caused by proteolytic degradation during the incubation with the enzyme (Kang et al., 2004). The reason for the difference in the degree or detection of *N*-glycosylation between the two epitope-tagged versions of the receptor is currently unknown. Possible explanations are that the two differentially tagged receptors become *N*-glycosylated to different degrees or that the two antibodies exhibit slightly different sensitivity to this type of *N*-glycosylation.

Constitutive B_1 Receptor Homo-oligomers. In addition to a specific apparently monomeric FB1 receptor species at approximately 35 kDa, specific receptor species of relatively higher molecular mass were also present on SDS-PAGE gels (Fig. 2, lanes 4–6). Whereas these species may be receptors tightly associated with some accessory protein, their relative migration suggested that they may be multiples of 35 kDa, and therefore some higher order homo-oligomeric receptor forms, including, e.g., dimers, trimers, etc.

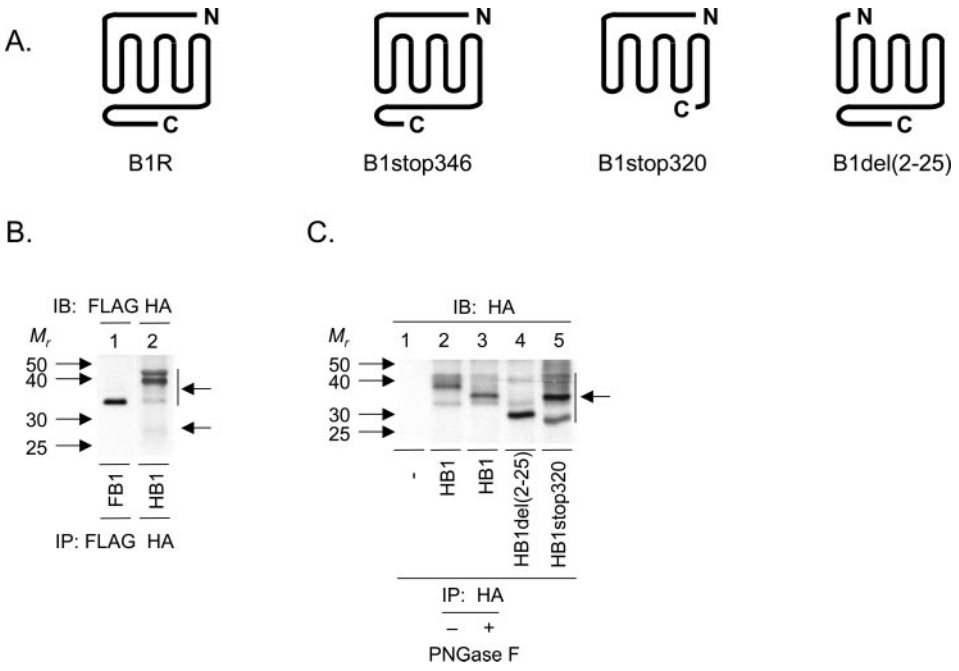

Fig. 1. Identification of B_1 receptors and their level of *N*-glycosylation. A, schematic presentation of wild-type and N- and C-terminally truncated mutant receptors. B, cells transfected with FB1 (lane 1) and HB1 (lane 2) were immunoprecipitated (IP) and immunoblotted (IB) with anti-FLAG and anti-HA antibodies, respectively. C, cells transfected with pcDNA3 (−; lane 1), HB1 (lanes 2 and 3), HB1del(2–25) (lane 4), and HB1stop320 (lanes 5) were IP and IB with anti-HA antibody. The anti-HA immunoprecipitates were treated without (lane 2) and with (lane 3) PNGase F for 2 h at 37°C before immunoblotting. Molecular mass (M_r) standards (left side arrows) and receptor species (right side arrows) are indicated, and the results are representative of experiments performed three times.

Treatment of cells with iodoacetamide to block free sulfhydryl groups had no effect on the receptor migration pattern either in the absence (Fig. 2, lane 4) or presence (lane 5) of reducing agent. Thus, if these B_1 receptor species represent homo-oligomeric complexes in the cell, their formation is disulfide-independent.

To further investigate B_1 receptor homo-oligomerization, FB1 and HB1 were expressed in cells either individually or in combination and then immunoprecipitated and immunoblotted with epitope-specific antibodies. Figure 3A shows that FB1 and HB1 were selectively recognized by FLAG and HA antibodies, respectively, whether the constructs were expressed individually (lanes 2, top, and 5, bottom) or together (lanes 3, top, and 4, bottom). Immunoprecipitation of HB1 with HA antibodies yielded coprecipitation of FB1 (Fig. 3A, lane 4, top), and immunoprecipitation of FB1 with FLAG antibodies yielded coprecipitation of HB2 (lane 3, bottom). FLAG antibody blots of anti-HA immunoprecipitates always contained a band at about 25 kDa. Because this band was also present in samples of mock-transfected cells (Fig. 3A, lane 6), it was clearly nonspecific. The varying intensity of this band in different blots depended on the exposure time of the blot. Coprecipitation of the HB1 and FB1 constructs was cell-dependent because mixing lysates from cells expressing each tagged receptor individually did not yield any FB1 reactivity in the HB1 precipitate (Fig. 3B, lane 3). Incubation of cells with the B_1 receptor agonist desArg^{10}kallidin (DK) at 0.1 μM for various lengths of time had no apparent effect on the amount of FB1 coprecipitated with HB1 (Fig. 3C). These results are consistent with the constitutive formation of B_1 receptor homo-oligomers.

Immunoelectronmicroscopy was used as a detergent-free technique to provide additional evidence in support of B_1 receptor homo-oligomeric complexes in intact cells. To this end, intact cells expressing HB1 were first incubated with 5-nm gold-labeled HA antibodies at 4°C to selectively detect cell surface receptors and then fixed and prepared for microscopy. As shown in Fig. 4, surface B_1 receptors were readily detected by this method. Furthermore, clusters of labeling were clearly visible, including dimers and trimers. Higher order structures were also apparent but not represented in the figure. Because the specimens were cut only in one plane, additional apparently invisible or only partially visible particles may be present in some clusters. Closer inspection of some single particles revealed that they were not perfectly round, suggesting additional particles in proximity. These results provide evidence that constitutively formed higher order B_1 receptor complexes are present on the cell surface.

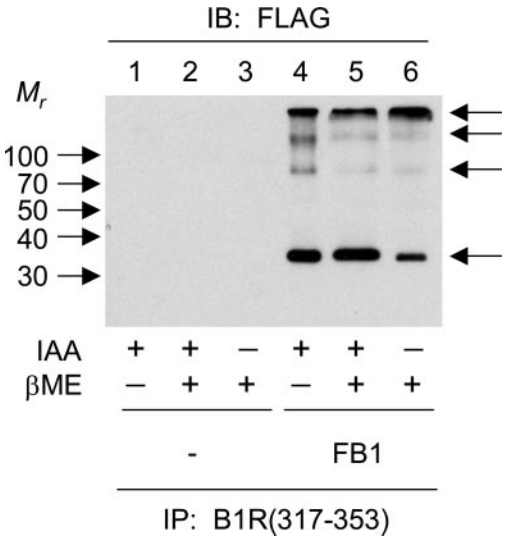

Fig. 2. Identification of SDS-resistant homo-oligomeric B_1 receptor complexes. Cells transfected with pcDNA3 (−; lane 1–3) and FB1 (lanes 4–6) were treated without (lanes 3 and 6) and with (lanes 1, 2, 4, and 5) 10 mM iodoacetamide (IAA) before lysis and during immunoprecipitation (IP) with anti-B1R(317-353) antibody. The precipitates were loaded on SDS-PAGE in the absence (lanes 1 and 4) and presence of β-mercaptoethanol (β-ME) (lanes 2, 3, 5, and 6) and then immunoblotted (IB) with anti-FLAG antibody. Molecular mass (M_r) standards (left side arrows) and receptor species (right side arrows) are indicated, and the results are representative of experiments performed three times.

Mapping of the B_1 Receptor Homo-oligomerization Epitope. Truncating the B_1 receptor by 8 and 34 residues from the C-terminal end to make B1stop346 and B1stop320, respectively, (Fig. 1A), followed by tagging with either FLAG or HA, did not significantly interfere with receptor expression as determined by both radioligand binding (Kang and Leeb-Lundberg, 2002) and immunoblotting (Fig. 5A). Figure 5A further shows that FB1stop346 coprecipitated with its HA-tagged counterpart HB1stop346 (lane 5), and FB1stop320 coprecipitated with its HA-tagged counterpart HB1stop320 (lane 6), when the two epitope-tagged versions were expressed together. Furthermore, the degrees of coprecipitation of the truncated constructs were very similar to that observed with the intact receptors (compare Fig. 5A, lanes 4–6). In addition, all the truncated constructs readily "hetero"-oligomerized with FB1 and HB1 (data not shown).

The same approach was used to determine the role of the N-terminal tail of the receptor. Deletion of the 25 N-terminal residues to make FB1del25 and HB1del25 had no apparent effect on receptor expression as determined by both radioligand binding (data not shown) and immunoblotting (Fig. 5B, lanes 2, top and 5, bottom). As discussed above, truncation of the N-terminal end erased the majority of the difference in the banding patterns of the two tagged receptors. HB1del25 coprecipitated with its FLAG-tagged counterpart FB1del25 (Fig. 5B, lanes 3 and 4) as well as with FB1 (data not shown). In addition, incubation of cells coexpressing FB1 and HB1 with 1.25 mM of a peptide comprised of N-terminal residues 2 to 37 for 2 h had no effect on the ability of these constructs to coprecipitate (Fig. 5C, lanes 3 and 4). These results reveal that, as determined by coimmunoprecipitation, neither the C-terminal nor the N-terminal tails are critical for B_1 receptor homo-oligomerization.

To further search for the epitope(s) involved in B_1 receptor homo-oligomerization, the receptor was divided in two parts after Arg^{134}, which is located in the receptor DRY sequence at the bottom of transmembrane helix 3, to make B1stop135 and B1del(2-134) (Fig. 6A). HA-tagged versions of both constructs were readily expressed in cells as determined both by anti-HA immunoprecipitation of HB1stop135 (Fig. 6B, lane 5, bottom) and HB1del(2-134) (Fig. 6C, lane 1). Considering that HB1del(2-134) contains an intact C-terminal domain, this construct was also detected by immunoprecipitation with anti-B1R(317-353) (Fig. 6C, lane 2). No immunoreactivity was detected in lysates of mock-transfected cells (Fig. 6C, lane 3). HB1del(2-134) expressed as two species, at about 22 and 26 kDa, that did not seem to be multiples of each other. The reason for this is currently unknown. HB1stop135, of

which the monomer migrated at <15 kDa, readily formed detergent-resistant higher order structures (Fig. 6B, lane 5, bottom). This construct also readily coimmunoprecipitated with FB1 (Fig. 6B, lanes 3, bottom, and 4, top). In contrast, HB1del(2-134) did not seem to form higher order structures (Fig. 6C, lanes 2 and 3) or coimmunoprecipitate with FB1 (Fig. 6D, lane 4). HB1stop135 was further truncated after Val^{71} at the C-terminal end of intracellular loop 1 to make HB1stop72. Monomeric HB1stop72 migrated at <15 kDa and readily formed higher order structures (Fig. 6E, lane 5, bottom). Again, this construct also coimmunoprecipitated with FB1 (Fig. 6E, lanes 3, bottom, and 4, top). Together, these results show that, as determined by coimmunoprecipitation, the epitope for B_1 receptor homo-oligomerization seems to reside somewhere between residues 25 and 71.

B1stop135 Exhibits Dominant-Negative Behavior on B_1 Receptor Cell Surface Expression. Because the B1stop135 fragment apparently oligomerize with intact B_1 receptor, we next determined the effect of this fragment on B_1 receptor homo-oligomers. Expression of FB1 and HB1 together with different concentrations of tag-less B1stop135 led to a concentration-dependent decrease in the amount of FB1 that coprecipitated with intact HB1 (Fig. 7, A and B). This may be caused by the ability of B1stop135 to disrupt B_1 receptor homo-oligomers and form hetero-oligomers with the intact receptor. If so, this behavior may be used to determine the role of homo-oligomers in receptor expression and signaling.

To study the effect of B1stop135 on B_1 receptor expression, we first used a membrane-impermeable maleimide-activated biotin derivative that reacts only with extracellular cysteines, which are present on both constructs. Incubation of the cells with the biotin derivative resulted in the detection of 35-kDa FB1 (Fig. 8A, lane 2). On the other hand, no specific biotinylated band was present in lysates expressing FB1stop135 (Fig. 8A, lane 4). Furthermore, and as expected, expression of B1stop135 did not introduce specific cellular [^{3}H]DK binding (data not shown). Thus, it is seems that FB1 is expressed on the cell surface, whereas FB1stop135 is not. On the other hand, FB1stop135 may express on the surface but with cysteines not available for reaction with the biotin reagent. It is interesting that B1stop135 decreased the amount of 35-kDa FB1 available for biotinylation (Fig. 8A, lane 3, and C). On the other hand, B1stop135 did not decrease significantly the expression of the 35-kDa FB1 species (compare Fig. 8B, lanes 2 and 3; C). An increase in the 35-kDa FB1 product could have occurred and existed in higher order complexes. However, this was difficult to assess because such complexes would have been hidden by the immunoglobulin bands present on the immunoblot at >50 kDa. That B1stop135 decreased the number of intact cell surface B_1 receptors was also evident by the dose-dependent decrease in the total cellular binding of the peptide agonist [^{3}H]DK assayed at 4°C, which selectively measures cell surface receptors (Fig. 9A). In addition, B1stop135, which did not exhibit any intrinsic signaling activity on its own, dose dependently decreased expression of constitutive B_1 receptor activity as measured by receptor-dependent and agonist-independent basal PI hydrolysis (Fig. 9B). In contrast, the C-terminal half of the receptor B1del(2-134), which was expressed but apparently did not hetero-oligomerize with intact B_1 receptor (Fig. 6D), also did not influence either [^{3}H]DK binding (Fig. 9A) or the expression of constitutive B_1 receptor activity (Fig. 9B).

To investigate in more detail whether B1stop135 interfered with the plasma membrane targeting of the B_1 receptor

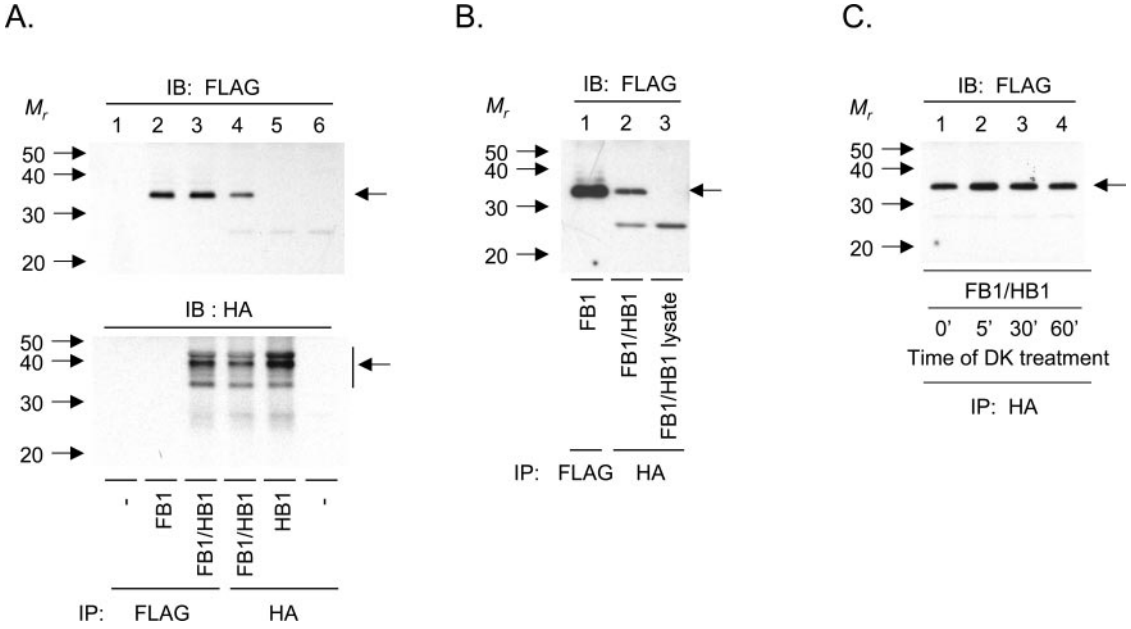

Fig. 3. Identification of intact homo-oligomeric B_1 receptor complexes. A, cells transfected with pcDNA3 (−; lanes 1 and 6), FB1 (lane 2), and HB1 (lane 5) either individually or together (lanes 3 and 4) were immunoprecipitated (IP) and immunoblotted (IB) with anti-FLAG antibody (top) or anti-HA antibody (bottom). B, cells transfected with FB1 without (lane 1) or with HB1 (lane 2) were IP with anti-FLAG antibody (lane 1) or anti-HA antibody (lanes 2 and 3) and IB with anti-FLAG antibody. In lane 3, lysates of cells transfected with FB1 and HB1 individually were mixed, immunoprecipitated with anti-HA antibody and immunoblotted with anti-FLAG antibody. C, cells transfected with FB1 and HB1 were incubated in the absence (lane 1) and presence (lanes 2–4) of 0.1 μM DK for the indicated periods at 37°C, IP with anti-HA antibody and IB with anti-FLAG antibody. pcDNA3 was used to equalize the amount of DNA transfected. Molecular mass (M_r) standards (left side arrows) and receptor species (right side arrows) are indicated, and the results are representative of experiments performed three times.

or simply reduced the total B_1 receptor expression, cells expressing HB1 and FB1stop135 either individually or in combination were lysed hypotonically and fractionated on a sucrose density gradient. The fractions were then directly immunoblotted with either HA or FLAG antibodies. Figure 10A shows that HB1 immunoreactivity migrated primarily in the plasma membrane fractions (fraction 14–16). On the other hand, the distribution of FB1stop135 was broad with immunoreactivity in fractions 14 to 20. Fractions 17 and 18 contained a <15-kDa species, which is presumed to be the monomer of this construct, whereas fractions 18 and 19 contained an approximately 20-kDa species and an approximately 30-kDa species. Fractions 14 and 15, the plasma membrane fractions, contained a ~40-kDa species and some higher order forms. Thus, the various oligomeric forms of B1stop135 seem to be targeted to different intracellular compartments as well as the plasma membranes. Coexpression of HB1 and FB1stop135 resulted in the redistribution of HB1 from fractions 14 to 16 to fractions 17 to 19. The nature of the compartment to which B1stop135 redistributed HB1 is unknown other than that it is of less density than the plasma membrane fraction and also targeted by some forms of B1stop135. Thus, B1stop135 may accomplish its dominant-negative behavior by forming hetero-oligomers, which are unable to properly target to cell surface.

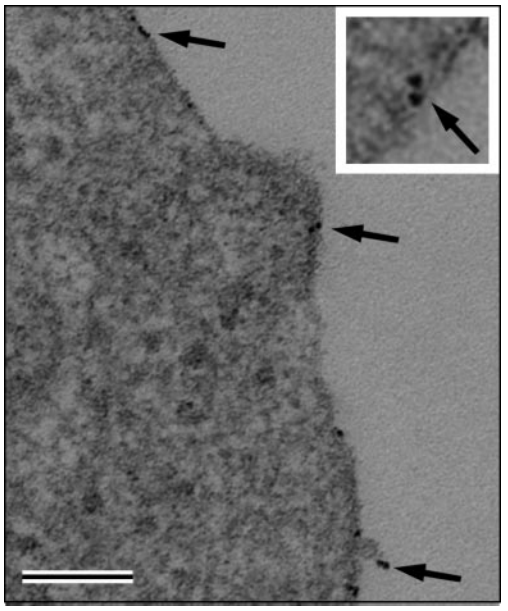

Fig. 4. Identification of plasma membrane homo-oligomeric B_1 receptor complexes by immunoelectron microscopy. Cells were incubated with 5-nm gold-labeled HA antibodies for 30 min at 4°C. The cells were then processed for electron microscopy as described under *Materials and Methods*. Scale bar, bottom, 0.2 μm. The arrows indicate some 5-nm gold homo-oligomeric complexes. Inset, magnified area containing complexes.

Discussion

Together, the results from this study provide evidence for B_1 receptor homo-oligomerization and the importance of this mechanism in the expression of the receptor on the cell surface of HEK293 cells. This mechanism directly translates into the presentation of a constitutive mechanism for cellular signaling through $G_{q/11}$-mediated phospholipase Cβ activity. Such homo-oligomers may be a regulatory point in B_1 receptor maturation, and their disruption could provide a unique

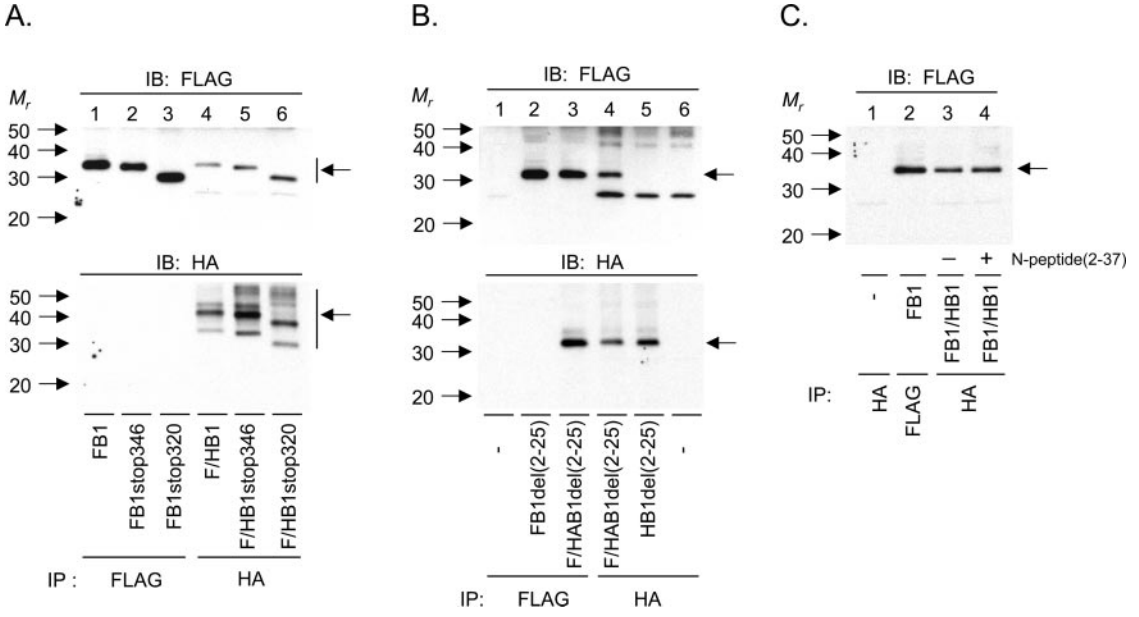

Fig. 5. Effect of N- and C-terminal truncation on B_1 receptor homo-oligomerization. A, cells transfected with FB1, FB1stop346, and FB1stop320 either individually (lanes 1–3) or together with their respective HA-tagged counterparts HB1, HB1stop346, and HB1stop320 (lanes 4–6) were immunoprecipitated with anti-FLAG antibody (lanes 1–3) or anti-HA antibody (lanes 4–6) and immunoblotted with anti-FLAG antibody (top) or anti-HA antibody (bottom). B, cells transfected with pcDNA3 (−; lanes 1 and 6), FB1del(2-25) (lane 2), and HB1del(2-25) (lane 5) either individually or together (lanes 3 and 4) were immunoprecipitated (IP) with anti-FLAG antibody (lanes 1–3) or anti-HA antibody (lanes 4–6) and immunoblotted (IB) with anti-FLAG (top) or anti-HA antibody (bottom). C, cells transfected with pcDNA3 (−; lane 1), FB1 without (lane 2) or with HB1 (lanes 3 and 4) were incubated in the absence (lane 3) or presence (lane 4) of 1.25 mM *N*-peptide(2-37) for 2 h at 37°C and then immunoprecipitated with anti-HA antibody (lanes 1, 3, and 4) or anti-FLAG antibody (lane 2), and immunoblotted with anti-FLAG antibody. pcDNA3 was used to equalize the amount of DNA transfected. Molecular mass (M_r) standards (left side arrows) and major receptor species (right side arrows) are indicated and the results are representative of experiments performed three times.

way for silencing signaling through this and other B_1 receptor-stimulated pathways during inflammation.

Multiple methods were used to investigate whether B_1 receptors form homo-oligomers in HEK293 cells. Although neither of these methods alone is sufficient to prove oligomerization, together they are consistent with such a mechanism. Higher order oligomeric receptor forms were readily apparent on immunoblots of SDS-PAGE gels. Such complexes were also indicated by coimmunoprecipitation of differentially tagged receptors. Immunoelectron microscopy of intact cells using gold-labeled antibodies enabled the visualization of oligomeric structures on the cell surface. Finally, the redistribution of the B_1 receptor after coexpression with the C-terminally truncated construct B1stop135 from the plasma membrane to an apparently common intracellular compartment as determined by sucrose-density gradient fractionation of hypotonically lysed cells is supportive of complex formation between these two receptor constructs.

Although various methods were used to provide evidence for B_1 receptor homo-oligomerization, it is not absolutely clear whether the oligomeric complexes detected by each method are the same. For example, complexes detected after detergent treatment may not strictly reflect those observed in the absence of detergent. In this regard, it was interesting to observe that the various detergent-resistant forms of B1stop135 fractionated differently on a sucrose-density gradient. The B1stop135 monomer and some lower order detergent-resistant forms were present in relatively lighter fractions, possibly containing Golgi structures, whereas higher order forms were present in the plasma membrane fractions. The fractionation pattern of B1stop135 suggests that it is capable of targeting the plasma membrane but only in certain higher order homo-oligomeric form. The B_1 receptor apparently also exists as a higher order structure in the plasma membrane fraction, which is in agreement with the immunoelectronmicroscopy results.

Analysis of the crystallographic rhodopsin structure and the rhodopsin dimer rows in rod outer segments identified by atomic force laser microscopy has led to a model suggesting the involvement of multiple GPCR interfaces in different domains in receptor oligomerization (Liang et al., 2003). Mutagenesis studies in several different GPCR systems also suggest multiple points of receptor contacts. Truncation of the B_1 receptor revealed that homo-oligomerization of this receptor, as determined by coimmunoprecipitation of differentially tagged receptors, is dependent on an epitope(s) lo-

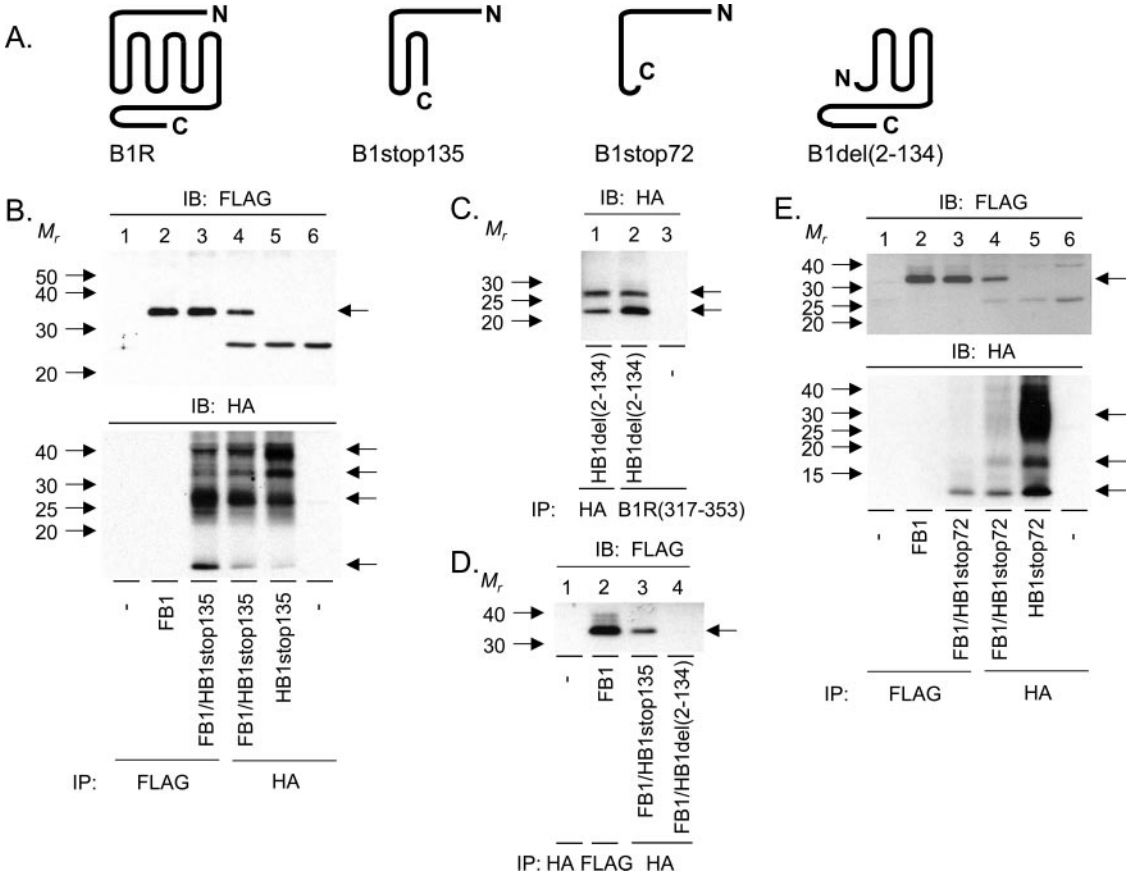

Fig. 6. Effect of N- and C-terminal fragments on B_1 receptor homo-oligomerization. A, schematic presentation of wild-type B_1 receptors and N- and C-terminal receptor fragments. B, cells transfected with pcDNA3 (−; lanes 1 and 6), FB1 (lane 2), and HB1stop135 (lane 5) either individually or together with FB1 (lanes 3 and 4) were immunoprecipitated (IP) with anti-FLAG (lanes 1–3) or anti-HA antibody (lanes 4–6) and immunoblotted (IB) with anti-FLAG (top) or anti-HA antibody (bottom). C, cells transfected with pcDNA3 (−; lane 3) and HB1del(2-134) (lanes 1 and 2) were IP with anti-HA antibody (lane 1) or anti-B1R(317-353) antibody (lanes 2 and 3) and immunoblotted with anti-HA antibody. D, cells transfected with pcDNA3 (−; lane 1) and FB1 without (lane 2) or with HB1stop135 (lane 3) or HB1del(2-134) (lane 4) were IP with anti-HA antibody (lanes 1, 3, and 4) or anti-FLAG antibody (lane 2) and IB with anti-FLAG antibody. E, cells transfected with pcDNA3 (−; lanes 1 and 6), FB1 (lane 2), and HB1stop72 (lane 5) either individually or together with FB1 (lanes 3 and 4) were IP with anti-FLAG (lanes 1–3) or anti-HA antibody (lanes 4–6) and IB with anti-FLAG (top) or anti-HA antibody (bottom). pcDNA3 was used to equalize the amount of DNA transfected. Molecular mass (M_r) standards (left side arrows) and major receptor species (right side arrows) are indicated and the results are representative of experiments performed three times.

cated between residue Leu[26] on top of transmembrane helix 1 and Val[71] at the C-terminal end of intracellular loop 1. This epitope may be similar to that in the yeast α-factor receptor (Overton et al., 2003), C5a receptor (Klco et al., 2003), and CCR2 receptor (Mellado et al., 2001), which apparently require helix 1 for homo-oligomerization, or the CXCR2 receptor, which requires helix 3 (Trettel et al., 2003). On the other hand, the dopamine D2 receptor forms a symmetric dimer interface involving helix 4 (Guo et al., 2003), and the B_2 bradykinin receptor subtype apparently requires an epitope in the N-terminal domain (AbdAlla et al., 1999). B_1 and B_2 bradykinin receptors heterodimerize (Kang et al., 2004), suggesting that these receptor subtypes do indeed contain multiple epitopes through which they interact with GPCR.

It is interesting that coexpression of B1stop135 with the B_1 receptor led to the redistribution of the intact receptor from the plasma membrane fraction to a lighter density fraction containing the B1stop135 monomer and some lower order

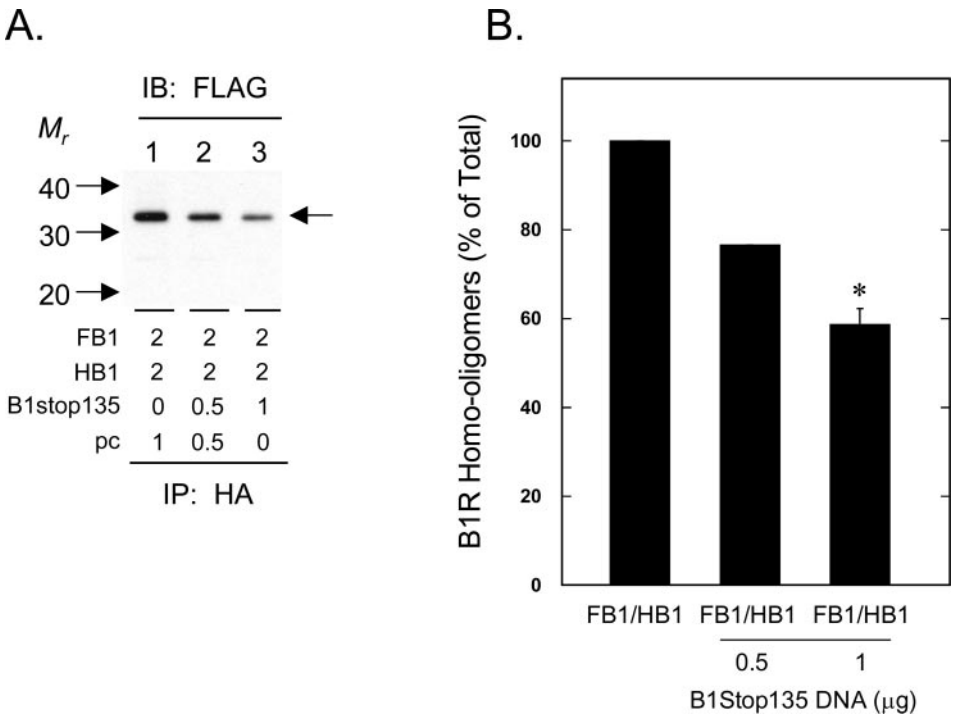

Fig. 7. Concentration-dependent effect of B1stop135 on intact B_1 receptor homo-oligomerization. A, cells cotransfected with FB1 and HB1 without (lane 1) or with increasing amount of B1stop135 (lanes 2 and 3) as indicated were immunoprecipitated (IP) with anti-HA antibody and immunoblotted (IB) with anti-FLAG antibody. pcDNA3 was used to equalize the amount of DNA transfected. B, densitometric analysis (Scion Corporation, Frederick, MD) of homo-oligomeric B_1 receptors as determined in A. Results are shown as percentage of total where 100% is the amount of oligomers in cells transfected with FB1 and HB1 only. Data are presented as mean ± S.E. of at least three experiments. *, $p < 0.05$ compared with in the absence of B1stop135. Molecular mass (M_r) standards (left side arrows) and receptor species (right side arrow) are indicated.

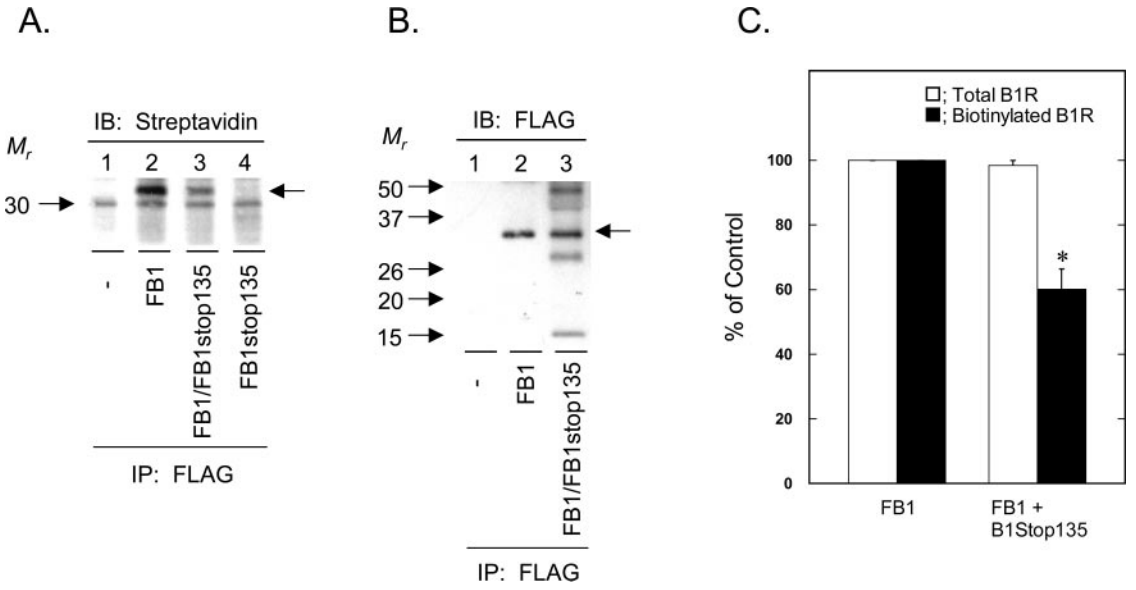

Fig. 8. Effect of B1stop135 on cell surface B_1 receptor expression. A, cells transfected with pcDNA3 (−; lane 1), FB1stop135 (lane 4), and FB1 without (lane 2) or with FB1stop135 (lane 3) were biotinylated, IP with FLAG antibody, and stained with streptavidin conjugated to horseradish peroxidase. B, cells transfected with pcDNA3 (−; lane 1) and FB1 without (lane 2) or with FB1stop135 (lane 3) were immunoprecipitated (IP) and immunoblotted (IB) with anti-FLAG antibody. C, densitometric analysis (Scion Corporation) of total and biotinylated FB1 detected as described in A and B, respectively. Results are shown as percentage of total where 100% is the amount of total FB1 and biotinylated FB1 in cells transfected with FB1 alone. Data are presented as mean ± S.E. of at least three experiments. *, $p < 0.05$ compared with in the absence of B1stop135. pcDNA3 was used to equalize the amount of DNA transfected. In A and B, molecular mass (M_r) standards (left side arrows) and major receptor species (right side arrows) are indicated and the results are representative of experiments performed three times.

detergent-resistant homo-oligomeric B1stop135 forms. Because B1stop135 and the B_1 receptor coimmunoprecipitate, one possibility is that this redistribution is caused by the hetero-oligomerization of the B_1 receptor with some form of B1stop135, possibly monomer, which traps the B_1 receptor in some compartment in the receptor maturation pathway. This explanation would be indicative of the formation of receptor oligomers early during receptor maturation. The best support for such a mechanism is provided by the $GABA_B$ receptor system, where both the type 1 and 2 are required for proper trafficking of a functional hetero-oligomerized receptor to the cell surface (Jones et al., 1998; Kaufmann et al., 1998; White et al., 1998). Furthermore, coexpression of splice variants of the dopamine D3 receptor, gonadotropin-releasing hormone receptor, and V2 vasopressin receptor, or truncated V2 and CCR5 receptor mutants with their wild-type counterparts resulted in mislocalization of the wild-type receptor in the cell (Benkirane et al., 1997; Grosse et al., 1997; Zhu and Wess, 1998; Karpa et al., 2000). Our results show that receptor fragments can indeed have pronounced effects on receptor expression. It is interesting that bradykinin receptor fragments can be generated in vivo as a direct consequence of oligomerization. Formation of B_1 and B_2 receptor heterodimers is associated with partial degradation of the participating B_2 receptor, with the N-terminal fragment remaining associated with the B_1 receptor (Kang et al., 2004). Depending on where this fragmentation occurs, it could have dramatic effects on receptor expression and localization.

Because B1stop72 and B1stop135 readily homo-oligomerized and hetero-oligomerized with the intact receptor, oligomerization does not seem to be dependent on either agonist or G protein binding. In this regard, B_1 receptor oligomers may be similar to, for example, CCR5 and CXCR4 receptor oligomers, which were reported to form constitutively and not to change in response to agonist treatment as determined by BRET (Issafras et al., 2002; Babcock et al., 2003), but different from constitutively formed β_2-adrenergic receptor oligomers, which increased in response to agonist treatment as determined by both coimmunoprecipitation (Hebert et al., 1996) and BRET (Angers et al., 2002), and δ-opioid receptor oligomers, which decreased in response to agonist treatment as determined by coimmunoprecipitation (Cvejic and Devi, 1997). Although these results may reflect inherent differences in oligomerization of these receptors, differences in the techniques used to measure the agonist effect may also be the reason. Coimmunoprecipitation is a relatively insensitive method and requires detergent, which may partially perturb the native agonist-sensitive structure. Considering the likely presence of multiple interfaces, coprecipitation may also rely on a receptor interface that is not agonist sensitive, or agonists may modulate the availability of critical immunoreactive epitopes. For fluorescence resonance energy transfer and BRET, receptors are often fused to relatively large donor and acceptor fluorophore proteins that themselves may dimerize independently of the receptor. Furthermore, the energy transfer between the interacting fluorophores is highly sensitive to the distance between them and their orientation. Thus, agonist-induced conformational changes may occur that moves these fluorophores without disrupting the dimer. The immunoelectron microscopy protocol used here is an attractive additional method that involves minimal cellular perturbation and monitors the receptors in their natural state. On the other hand, this method does not provide direct proof for a physical interaction and cannot be used to dynamically detect rapid agonist-promoted effects.

The best evidence for a functional role of oligomers comes from the crystal structure of the N-terminal domain of mGluR1 showing that the ligand binding pocket of this receptor is a dimer (Kunishima et al., 2000). In the β_2-adrenergic receptor, a peptide derived from transmembrane helix 6 that inhibited agonist-stimulated receptor dimerization also inhibited receptor activity (Hebert et al., 1996). On the other hand, in the B_2 bradykinin receptor a peptide derived from the N-terminal domain that inhibited agonist-stimulated receptor dimerization had no effect on receptor activity (AbdAlla et al., 1999). However, this peptide attenuated agonist-promoted receptor internalization. A considerable amount of research is clearly still needed to understand the functional consequence of GPCR monomer-monomer and higher order interactions.

In conclusion, our results present a scenario in which B_1 receptor oligomers form early in the biosynthesis or maturation of the B_1 receptor and their existence is required for the presentation of a functional receptor in the plasma mem-

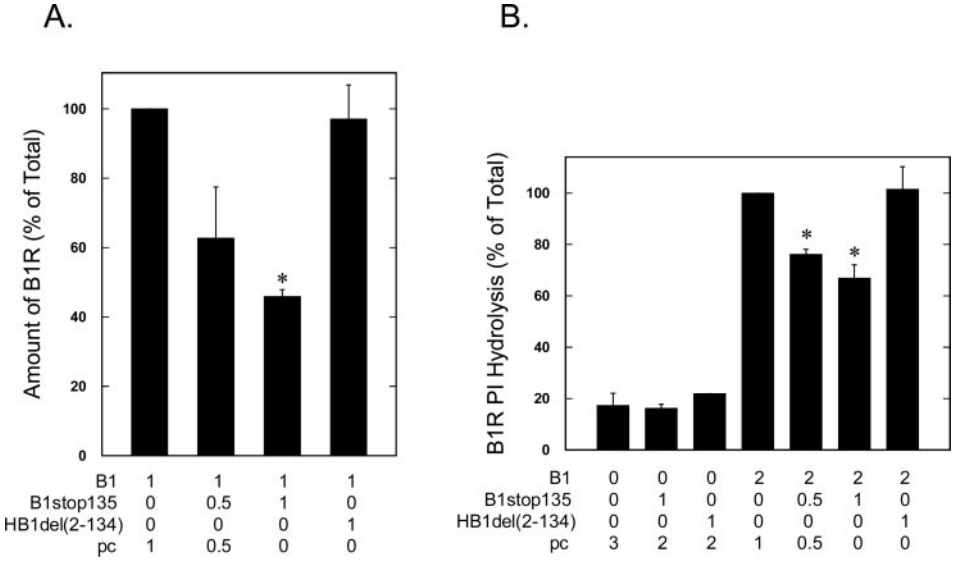

Fig. 9. Effect of B1stop135 on cell surface agonist binding and constitutive B_1 receptor signaling. In A and B, cells were transfected with 2 μg of wild type B_1 receptor DNA in the absence and presence of varying amounts of B1stop135 or 1 μg of HB1del(2-134) cDNAs. pcDNA3 was used to equalize the amount of DNA transfected. In B, the cells were assayed for basal PI hydrolysis, and in A the cells were assayed for specific cell surface [^{3}H]DK binding. Results are shown as percentage of total where 100% is the amount of PI hydrolysis (B) and binding (A) in cells transfected with B_1 receptor alone. Data are presented as the mean ± S.E. of at least three experiments. *, $p <$ 0.05 compared with in the presence of B_1 receptor but in the absence of B1stop135.

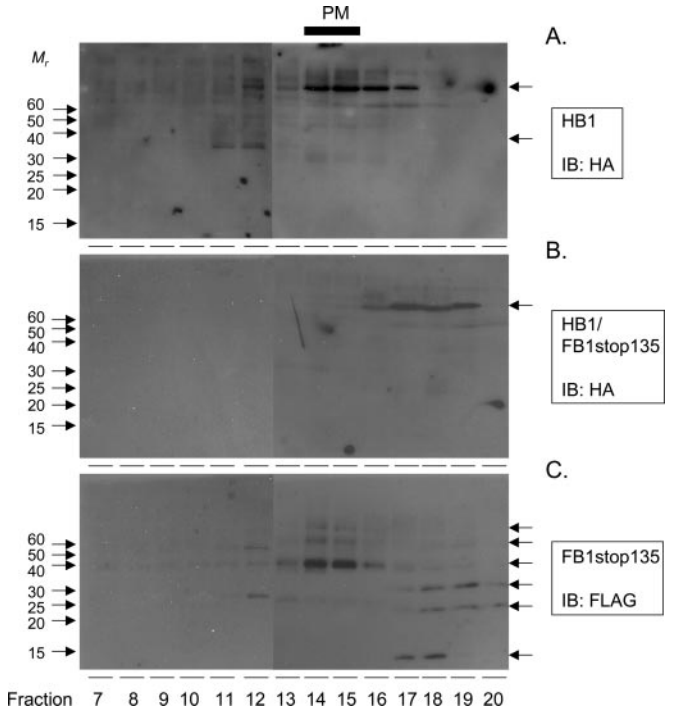

Fig. 10. Effect of B1stop135 on the subcellular distribution of the B_1 receptor. Cells transfected with HB1 (A) and FB1stop135 (C) either individually or in combination (B) were lysed in ice-cold hypotonic lysis buffer and subjected to sucrose-density gradient on a step gradient as described under *Materials and Methods*. Each fraction was then subjected to immunoblotting with HA antibody (A and B) or FLAG antibody (C). Molecular mass (M_r) standards (left side arrows) and major receptor species (right side arrows) are indicated and the result is representative of experiments performed twice.

brane. Induction of this receptor by proinflammatory stimuli involves changes in mRNA synthesis (Menke et al., 1994) and possibly mRNA stability (Zhou et al., 1999). Regulation at the level of receptor homo-oligomerization and maturation may be an additional more rapid mechanism by which the expression of this receptor is regulated by some stimuli.

Acknowledgments

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References

AbdAlla S, Zaki E, Lother H, and Quitterer U (1999) Involvement of the amino terminus of the B_2 receptor in agonist-induced receptor dimerization. *J Biol Chem* **274:**26079–26084.

Angers S, Salahpour A, and Bouvier M (2002) Dimerization: an emerging concept for G protein-coupled receptor ontogeny and function. *Annu Rev Pharmacol Toxicol* **42:**409–435.

Babcock GJ, Farzan M, and Sodroski J (2003) Ligand-independent dimerization of CXCR4, a principal HIV-1 coreceptor. *J Biol Chem* **278:**3378–3385.

Baschong W and Wrigley NG (1990) Small colloidal gold conjugated to Fab fragments or to immunoglobulin G as high-resolution labels for electron microscopy: a technical overview. *J Electron Microsc Technol* **14:**313–323.

Benkirane M, Jin DY, Chun RF, Koup RA, and Jeang KT (1997) Mechanism of transdominant inhibition of CCR5-mediated HIV-1 infection by CCR5delta32. *J Biol Chem* **272:**30603–30606.

Carrillo JJ, Pediani J, and Milligan G (2003) Dimers of class A G protein-coupled receptors function via agonist-mediated trans-activation of associated G proteins. *J Biol Chem* **278:**42578–42587.

Couture R, Harrisson M, Vianna RM, and Cloutier F (2001) Kinin receptors in pain and inflammation. *Eur J Pharmacol* **429:**161–176.

Cvejic S and Devi LA (1997) Dimerization of the δ opioid receptor: implication for a role in receptor internalization. *J Biol Chem* **272:**26959–26964.

Dray A and Perkins M (1993) Bradykinin and inflammatory pain. *Trends Neurosci* **16:**99–104.

Fotiadis D, Liang Y, Filipek S, Saperstein DA, Engel A, and Palczewski K (2003) Atomic-force microscopy: rhodopsin dimers in native disc membranes. *Nature (Lond)* **421:**127–128.

George SR, O'Dowd BF, and Lee SP (2002) G-protein-coupled receptor oligomerization and its potential for drug discovery. *Nat Rev Drug Discov* **1:**808–820.

Grosse R, Schöneberg T, Schultz G, and Gudermann T (1997) Inhibition of gonadotropin-releasing hormone receptor signaling by expression of a splice variant of the human receptor. *Mol Endocrinol* **11:**1305–1318.

Guo W, Shi L, and Javitch JA (2003) The fourth transmembrane segment forms the interface of the dopamine D2 receptor homodimer. *J Biol Chem* **278:**4385–4388.

Hebert TE, Moffett S, Morello JP, Loisel TP, Bichet DG, Barret C, and Bouvier M (1996) A peptide derived from a β2-adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation. *J Biol Chem* **271:**16384–16392.

Issafras H, Angers S, Bulenger S, Blanpain C, Parmentier M, Labbe-Jullie C, Bouvier M, and Marullo S (2002) Constitutive agonist-independent CCR5 oligomerization and antibody-mediated clustering occurring at physiological levels of receptors. *J Biol Chem* **277:**34666–34673.

Jones KA, Borowsky B, Tamm JA, Craig DA, Durkin MM, Dai M, Yao W-J, Johnson M, Gunwaldsen C, Huang L-Y, et al. (1998) $GABA_B$ receptors function as a heteromeric assembly of the subunits $GABA_BR1$ and $GABA_BR2$. *Nature (Lond)* **396:**674–679.

Kang DS and Leeb-Lundberg LMF (2002) Negative and positive regulatory epitopes in the C-terminal domains of the human B1 and B2 bradykinin receptor subtypes determine receptor coupling efficacy to G_q-mediated phospholipase Cβ activity. *Mol Pharmacol* **62:**281–288.

Kang DS, Ryberg K, Mörgelin M, and Leeb-Lundberg LMF (2004) Spontaneous formation of a proteolytic B1 and B2 bradykinin receptor complex with enhanced signaling capacity. *J Biol Chem* **279:**22102–22107.

Karpa KD, Lin R, Kabbani N, and Leverson R (2000) The dopamine D3 receptor interacts with itself and the truncated D3 splice variant D3nf: D3–D3nf interaction causes mislocalization of D3 receptors. *Mol Pharmacol* **58:**677–683.

Kaufmann K, Malitschek B, Schuler V, Heid J, Froestl W, Beck P, Mosbacher J, Bischoff S, Kulik A, Shigemoto R, et al. (1998) $GABA_B$ receptor subtypes assemble into functional heteromeric complexes. *Nature (Lond)* **396:**683–687.

Klco JM, Lassere TB, and Baranski TJ (2003) C5a receptor oligomerization. I. Disulfide trapping reveals oligomers and potential contact surfaces in a G protein-coupled receptor. *J Biol Chem* **278:**35345–35353.

Kunishima N, Shimada Y, Tsuji Y, Sato T, Yamamoto M, Kumasaka T, Nakanishi S, Jingami H, and Morikawa K (2000) Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. *Nature (Lond)* **407:**971–977.

Leeb-Lundberg LMF, Kang DS, Lamb ME, and Fathy DB (2001) The human B1 bradykinin receptor exhibits high ligand-independent, constitutive activity: roles of residues in the fourth intracellular and third transmembrane domains. *J Biol Chem* **276:**8785–8792.

Liang Y, Fotiadis D, Filipek S, Saperstein DA, Palczewski K, and Engel A (2003) Organization of the G protein-coupled receptors rhodopsin and opsin in native membranes. *J Biol Chem* **278:**21655–21662.

Lamb ME, Zhang C, Shea T, Kyle DJ, and Leeb-Lundberg LMF (2002) Human B1 and B2 bradykinin receptors and their agonists target caveolae-related lipid rafts to different degrees in HEK293 cells. *Biochemistry* **41:**14340–14347.

Marceau F, Hess JF, and Bachvarov DR (1998) The B1 receptors for kinins. *Pharmacol Rev* **50:**357–386.

Mathis SA, Criscimagna NL, and Leeb-Lundberg LM (1996) B1 and B2 kinin receptors mediate distinct patterns of intracellular Ca^{2+} signaling in single cultured vascular smooth muscle cells. *Mol Pharmacol* **50:**128–139.

Mellado M, Rodríguez-Frade JM, Vila-Coro AJ, Fernández S, de Ana AM, Jones DR, Torán JL, and Martínez AC (2001) Chemokine receptor homo- or heterodimerization activates distinct signaling pathways. *Eur Mol Biol Organ (EMBO) J* **20:**2497–2507.

Menke JG, Borkowski JA, Bierilo KK, MacNeil T, Derrick AW, Schneck KA, Ransom RW, Strader CD, Linemeyer DL, and Hess FJ (1994) Expression cloning of a human B1 bradykinin receptor. *J Biol Chem* **269:**21583–21586.

Overton MC, Chinault SL, and Blumer KJ (2003) Oligomerization, biogenesis and signaling is promoted by a glycophorin A-like dimerization motif in transmembrane domain 1 of a yeast G protein-coupled receptor. *J Biol Chem* **278:**49369–49377.

Pesquero JB, Araujo RC, Heppenstall PA, Stucky CL, Silva JA, Walther T, Oliveira SM, Pesquero JL, Paiva AC, Calixto JB, et al. (2000) Hypoalgesia and altered inflammatory responses in mice lacking kinin B_1 receptors. *Proc Natl Acad Sci USA* **97:**8140–8145.

Rios CD, Jordan BA, Gomes I, and Devi LA (2001) G-protein-coupled receptor dimerization: modulation of receptor function. *Pharmacol Ther* **92:**71–87.

Trettel F, Di Bartolomeo S, Lauro C, Catalano M, Ciotti MT, and Limatola C (2003) Ligand-independent CXCR2 dimerization. *J Biol Chem* **278:**40980–40988.

White JH, Wise A, Main MJ, Green A, Fraser NJ, Disney GH, Barnes AA, Emson P, Foord SM, and Marshall FH (1998) Heterodimerization is required for the formation of a functional $GABA_B$ receptor. *Nature (Lond)* **396:**679–682.

Zhou X, Prado GN, Chai M, Yang X, Taylor L, and Polgar P (1999) Posttranscriptional destabilization of the bradykinin B1 receptor messenger RNA: cloning and functional characterization of the 3′-untranslated region. *Mol Cell Biol Res Commun* **1:**29–35.

Zhu X and Wess J (1998) Truncated V2 vasopressin receptors as negative regulators of wild-type V2 receptor function. *Biochemistry* **37:**15773–15784.

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