Activation-dependent Conformational Changes in β-Arrestin 2*

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β-Arrestins are multifunctional adaptor proteins, which mediate desensitization, endocytosis, and alternative signaling pathways of seven membrane-spanning receptors (7MSRs). Crystal structures of the basal inactive state of visual arrestin (arrestin 1) and β-arrestin 1 (arrestin 2) have been resolved. However, little is known about the conformational changes that occur in β-arrestins upon binding to the activated phosphorylated receptor. Here we characterize the conformational changes in β-arrestin 2 (arrestin 3) by comparing the limited tryptic proteolysis patterns and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) profiles of β-arrestin 2 in the presence of a phosphopeptide (V2R-pp) derived from the C terminus of the vasopressin type II receptor (V2R) or the corresponding nonphosphopeptide (V2R-np). V2R-pp binds to β-arrestin 2 specifically, whereas V2R-np does not. Activation of β-arrestin 2 upon V2R-pp binding involves the release of its C terminus, as indicated by exposure of a previously inaccessible cleavage site, one of the polar core residues Arg394, and rearrangement of its N terminus, as indicated by the shielding of a previously accessible cleavage site, residue Arg396. Interestingly, binding of the polyamion heparin also leads to release of the C terminus of β-arrestin 2; however, heparin and V2R-pp have different binding site(s) and/or induce different conformational changes in β-arrestin 2. Release of the C terminus from the rest of β-arrestin 2 has functional consequences in that it increases the accessibility of a clathrin binding site (previously demonstrated to lie between residues 371 and 379) thereby enhancing clathrin binding to β-arrestin 2 by 10-fold. Thus, the V2R-pp can activate β-arrestin 2 in vitro, most likely mimicking the effects of an activated phosphorylated 7MSR. These results provide the first direct evidence of conformational changes associated with the transition of β-arrestin 2 from its basal inactive conformation to its biologically active conformation and establish a system in which receptor-β-arrestin interactions can be modeled in vitro.

Seven membrane-spanning receptors (7MSRs),¹ also referred to as G protein-coupled receptors (GPCRs), constitute the largest known family of cell surface receptors (1, 2). The human genome encodes ~1,000 7MSRs, which function primarily in the transmission of diverse signals (including light, odors, chemotactants, neurotransmitters, and hormones) from the extracellular environment to the interior of the cell (1, 2). The dynamic sensitivity of 7MSR function is in large part a function of their regulation by the G protein-coupled receptor kinase (GRK)/β-arrestin system (1, 3). This regulation is accomplished by a two-step process involving the phosphorylation of the receptor, usually at its C terminus, by GRKs, and the subsequent binding of β-arrestins, which prevents further receptor activation of G proteins (desensitization) (1, 4). β-Arrestin binding to the receptor also facilitates clathrin-mediated endocytosis (internalization) of the receptor (5–7).

In stark contrast to the multiplicity of 7MSRs, there are only four known isoforms of arrestin: visual arrestin (arrestin 1), cone arrestin, β-arrestin 1 (arrestin 2), and β-arrestin 2 (arrestin 3) (3, 8). Visual arrestin and cone arrestin are expressed primarily in the eye and are almost exclusively involved in visual signaling processes. β-Arrestins 1 and 2 are ubiquitously expressed and are fundamental to the regulation of 7MSR-mediated signaling throughout the body. In addition to their well characterized role in 7MSR desensitization and internalization, recent evidence suggests that the β-arrestins also function as signaling transducers, which interact directly with a variety of effectors, such as non-receptor tyrosine kinases of the c-Src family and extracellular-regulated kinases (ERK1/2) and c-Jun N-terminal kinase (JNK3), in a receptor activation-dependent manner (3, 5–7, 9–11). These receptor activation-dependent interactions of β-arrestins suggest that the conformations of free (basal or inactive) and receptor-bound (active) β-arrestins are different, and that conformational changes in β-arrestin occur upon binding to activated phosphorylated receptors.

Multiple lines of evidence, including mutagenesis and biochemical and biophysical studies, suggest that visual arrestin undergoes substantial conformational changes upon binding to light-activated phosphorylated rhodopsin (12, 13). However, to date, there has been no direct evidence of conformational changes in β-arrestins. For example, the solved crystal structure of bovine β-arrestin 1 is of the molecule in its basal inactive conformation (14, 15). Thus, the structural and molecular basis for β-arrestin activation as well as the associated conformational changes remains largely unknown.

7MSRs can be divided into two broad classes, “Class A” and “Class B,” based on the nature of their interaction with β-arrestin (16). Class A receptors, such as the β2-adrenergic receptor, interact with β-arrestin transiently, whereas Class B receptors, such as the V2R, form relatively stable receptor/β-arrestin interactions.

MALDI-TOF MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; m/z, mass (m) to charge (z) ratio; TPCK, L-tosylamido-2-phenyl ethyl chloromethyl ketone; DTT, dithiothreitol; GST, glutathione-S-transferase.

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‡ The abbreviations used are: 7MSR, seven membrane-spanning receptor; GRK, G protein-coupled receptor kinase; V2R, vasopressin type II receptor; V2R-np and V2R-pp, synthetic nonphosphopeptide and phosphopeptide derived from the C terminus of V2R; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; m/z, mass (m) to charge (z) ratio; TPCK, L-tosylamido-2-phenyl ethyl chloromethyl ketone; DTT, dithiothreitol; GST, glutathione-S-transferase.
arrestin complexes. This increased stability of the interaction between the receptor and β-arrestin makes Class B 7MSRs, like V1R, ideal for studying the ability of a receptor to induce conformational changes in β-arrestin. Accordingly, in an effort to investigate the activation mechanism of β-arrestins, we used limited proteolysis and MALDI-TOF MS to characterize conformational changes in β-arrestin 2 upon binding a synthetic phosphopeptide (V1-R pp) derived from the C terminus of the Class B V1R to mimic receptor binding to β-arrestin 2.

**Experimental Procedures**

**Peptide Synthesis**—The phosphopeptide (V1-R pp) and the corresponding nonphosphopeptide (V1-R np) derived from the C terminus of the human V1R were synthesized by the Protein Chemistry Core Laboratory of Baylor College of Medicine. The C-terminal sequence of V1R is ARGR-PFSLGQPDESCTASSLARDD. The C-terminal sequence of V2R is ARGRT-PDFGLQPSKSFLKSSRRS (termed 28-mer peptide) and a 30-mer NWVRDPQMPD-DMKGVSEQAPPSGKSCMC (termed 30-mer peptide), were synthesized by the laboratory.

**Mutagenesis and Purification of Rat β-Arrestin 2**—Wild-type rat β-arrestin 2 was cloned in a pGEX4T3 expression vector (expresses GST-β-arrestin 2, i.e. GST-β-arrestin 2 or a PET29 expression vector (expresses S-protein-tagged β-arrestin 2, i.e. S-β-arrestin 2). All mutations discussed here were constructed with a QuikChange® II Mutagenesis kit (Stratagene). All constructs were confirmed by DNA sequencing and transferred into Escherichia coli strain BL21 (DE3) pLYS S.

To overexpress GST-β-arrestin 2 in *E. coli*, 800 ml of LB medium were inoculated with 10 ml of overnight cell cultures and grown initially at 37 °C until the OD600 reached 0.6–0.8. The temperature was then lowered to 25 °C, and the expression of GST-β-arrestin 2 was induced with 100 μM isopropyl-1-thio-β-D-galactopyranoside. After overnight incubation, cells were harvested by centrifugation at 4,500 × g, and lysed by freeze-thaw followed by sonication in binding buffer (25 mM Tris-HCl, pH 8.5, 150 mM NaCl, 2 mM DTT, 2 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 0.2 mg/ml benzamidine). The lysate was centrifuged at 18,000 × g for 30 min and the supernatant filtered through a 0.8-μm membrane. The clarified supernatant was then loaded on a glutathione-Sepharose column (Amersham Biosciences) by gravity and washed with 20 column volumes of binding buffer. The GST-β-arrestin 2-bound column was washed with binding buffer (25 mM Tris-HCl, pH 8.5, 150 mM NaCl, 2 mM CaCl2, 2 mM DTT). Thrombin protease (Sigma) (10 unit/liter of cell culture) was added to cleave the GST protein tag (seven extra amino acids [GSPNSRV] remain after thrombin cleavage). The thrombin digestion was performed at 4 °C overnight, and flow through was collected followed by two additional washes with the digestion buffer. Washes were pooled with the initial flow through and diluted to a final NaCl concentration of 50 mM. The sample was then loaded on a heparin-Sepharose column and eluted with a 50–350 mM NaCl gradient. Fractions containing β-arrestin 2 were pooled and dialyzed overnight against 25 mM Tris-HCl, pH 8.5, 50 mM NaCl, 2 mM DTT, 2 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 0.2 mg/ml benzamidine. After dialysis, the sample was loaded onto a SP-Sepharose high performance column, and β-arrestin 2 was eluted with a salt gradient of linear NaCl gradient in the dialysis buffer. Fractions were analyzed by SDS-PAGE and pooled based on purity, concentrated to 5–10 mg/ml, flash frozen in liquid nitrogen, and stored at −80 °C. Protein purity was more than 95% based on SDS-PAGE analysis, and typical yield was 1–2 mg of purified β-arrestin 2/liter of cell culture.

Cell growth conditions for the S-β-arrestin 2 was the same as that for the GST-β-arrestin 2. An S-Tag™ Thrombin purification kit (Novagen) was used to purify the S-β-arrestin 2. Cells were lysed under the same conditions as that for the GST-β-arrestin 2, and the cell lysate was incubated with S-protein beads for 2 h with agitation at 4 °C, followed by washing with 20 column volumes of binding buffer. The β-arrestin 2-bound S-protein beads were then divided into two portions. One portion of the beads was digested with thrombin (10 unit/liter of cell culture), and the β-arrestin 2 was eluted and subjected to limited tryptic proteolysis. The other portion of the beads was used directly for in vitro clathrin binding assays.

**Limited Tryptic Proteolysis**—A 5:1 molar ratio of ligand (V1-R pp, V2-R np, the other two nonspecific peptides or heparin) to β-arrestin 2 (0.5–1 mg/ml) was used to reveal the effects of ligand on limited tryptic proteolysis of β-arrestin 2 in all experiments except where otherwise indicated. An average molecular mass of 12,000 Da was used to calculate the concentration of heparin (Sigma). Prior to proteolysis, β-arrestin 2, in the absence or presence of ligand, was incubated at room temperature for 30 min. An appropriate amount of TPCK-treated trypsin (Sigma) was added to the mixture for limited proteolysis. The samples were incubated at 37 °C for indicated time points. At each time point, 5 μl (5–50 μg of β-arrestin 2) was removed except from each reaction. For SDS-PAGE analysis, each sample was transferred to a new microcentrifuge tube containing 5 μl of 2× SDS-PAGE buffer, and boiled for 5 min to quench the tryptic digestion. The samples were run on 4–20% SDS-PAGE gels (Invitrogen) to determine the effects of ligands on the digestion pattern of β-arrestin 2. For MALDI-TOF MS analysis, the samples were transferred to new empty microcentrifuge tubes and flash frozen for MALDI-TOF analysis to measure the molecular masses for the proteolytic fragments.

**MALDI-TOF MS**—MALDI-TOF mass spectra were acquired on a Voyager DE Biospectrometry Work Station (Applied Biosystems) in the linear mode using a nitrogen laser (337 nm). Mass spectra were collected in the positive ion mode using an acceleration voltage of 25 kV and a laser power of 900 ns. The acceleration voltage, guide voltage, guide mass gate, and laser intensity were set to 92.5%, 0.15%, 10,000.0 m/z, and 2580, respectively. Each mass spectrum collected represents the data from the data sets from 75 laser shots.

Sinapinic acid (SA) (Sigma) was used as the matrix and the SA matrix solution was prepared as a saturated, aqueous solution that contained 45% acetonitrile and 0.1% trifluoroacetic acid. During MALDI-TOF sample preparation, 1 μl of flash frozen limited tryptic proteolysis products was mixed with 9 μl of SA matrix solution before depositing 1 μl (1–2 μl final protein concentration) of the sample mixture on the MALDI-TOF sample plate. The sample was allowed to air-dry at room temperature and then subjected to MALDI-TOF analysis. Cytochrome c and carbonic anhydrase were used as internal calibrants for data calibration. For each limited tryptic proteolysis fragment, the mean of at least three independent samples was used to determine the molecular mass, and a standard deviation was calculated from six independent experiments. A β-arrestin 2 was also subjected to theoretical MALDI-TOF MS analysis of the molecular masses for all potential tryptic proteolytic fragments using a ProteinProspector program (prospector.ucsf.edu). The experimental molecular mass (m/z) of each limited proteolytic fragment was compared with the theoretical masses (m/z) predicted by ProteinProspector program, and all the candidate fragments with theoretical masses within the standard deviations were selected (Table 1). The limited proteolytic fragments with only one possible theoretical mass (m/z) within standard deviation were assigned to individual proteolytic fragments directly, with or without further confirmation of the assignments by N-terminal sequencing or by other mass spectrometrical techniques (m/z), which could not be distinguished by MALDI-TOF MS, were designated by coupling the MS data with N-terminal sequencing, Western blot analysis with antibodies that recognize different domains of β-arrestin 2, and limited tryptic proteolysis of truncated β-arrestin 2 mutants.

**N-terminal Sequencing**—The limited proteolytic products were subjected to SDS-PAGE analysis. The proteolytic fragments of β-arrestin 2 were then electrotransferred to a polyvinylidene difluoride membrane. Proteins bound to polyvinylidene difluoride membrane were stained by Coomassie Blue R-250, and the membrane was washed with Millipore water for 5 min and air-dried. The stained protein bands were excised with a clean razor blade, placed in a 1.5-mL conical centrifuge tube and subjected to N-terminal sequencing. The N-terminal sequencing was performed by the Molecular Biology Resource Facility of University of Oklahoma Health Science Center.

**Clathrin Binding**—To measure clathrin binding to β-arrestin 2, 25 μl of β-arrestin 2 S-protein beads (containing 20 μg of S-β-arrestin 2), in the absence or presence of 5:1 (ligand:β-arrestin 2) molar ratio of ligand (V1-R pp, V2-R np, 28-mer peptide or heparin) were incubated in binding buffer (25 mM Tris-HCl, pH 8.5, 50 mM NaCl, 2 mM DTT) at room temperature for 30 min. After the incubation, the volume of each mixture was brought up to 500 μl with binding buffer, followed by the addition of 5 μg of clathrin heavy chain (Sigma) and agitated for 2 h at 4 °C. The β-arrestin 2 bound S-protein beads were then centrifuged at 20,000 × g in a benchtop microcentrifuge, washed with 1 ml of binding buffer 5 times, and incubated with 25 μl of 2× SDS-PAGE buffer. Clathrin binding to β-arrestin 2 was measured by Western blot analysis using antibodies specific for the clathrin light chain (Sigma).
FIG. 1. Sequence of the recombinant rat \( \beta \)-arrestin 2 derived from GST-\( \beta \)-arrestin 2 fusion protein by thrombin cleavage. Sequence of rat \( \beta \)-arrestin 2 with an extra fragment GSPNSRV at the N terminus left from thrombin cleavage and a His\(_6\) tag at the C terminus. The first amino acid Met (M) of \( \beta \)-arrestin 2 is replaced by Asp (D) in the thrombin recognition sequence and is numbered as the first position. The amino acids are numbered in reference to the first Asp (D), which is bold and italic. All the potential tryptic cleavage sites are underlined, with those identified in this study in bold.

| Amino Acid | Position |
|------------|----------|
| GSPNSRV    | 1        |
| R           | 2        |
| K            | 3        |
| G            | 4        |
| P            | 5        |
| N            | 6        |
| S            | 7        |
| V            | 8        |
| L            | 9        |
| G            | 10       |
| P            | 11       |
| N            | 12       |
| V            | 13       |
| R            | 14       |
| D            | 15       |
| G            | 16       |
| S            | 17       |
| P            | 18       |
| N            | 19       |
| V            | 20       |
| L            | 21       |
| R            | 22       |
| P            | 23       |
| L            | 24       |
| N            | 25       |
| V            | 26       |
| R            | 27       |
| S            | 28       |
| V            | 29       |
| L            | 30       |
| R            | 31       |
| P            | 32       |
| L            | 33       |
| R            | 34       |
| S            | 35       |
| V            | 36       |
| R            | 37       |
| P            | 38       |
| L            | 39       |
| R            | 40       |
| G            | 41       |

FIG. 2. Limited tryptic proteolysis patterns of rat \( \beta \)-arrestin 2 in the absence or presence of V\(_{2R}\)-np and V\(_{2R}\)-pp. A, SDS-PAGE analysis of the limited tryptic proteolytic products of rat \( \beta \)-arrestin 2 without peptide (panel I), with V\(_{2R}\)-np (panel II), and with V\(_{2R}\)-pp (panel III). 1:5,000 (trypsin/\( \beta \)-arrestin 2) w/w ratio of TPCK-treated trypsin was incubated with \( \beta \)-arrestin 2 for the indicated time points at 37 °C. STD is the MagicMark™ XP Molecular Weight Standards. The apparent molecular masses of the tryptic digestion fragments are labeled on the left. B, schematic patterns for limited tryptic proteolysis of rat \( \beta \)-arrestin 2. Control pattern represents the tryptic proteolysis pattern of \( \beta \)-arrestin 2 without peptide or in the presence of V\(_{2R}\)-np. V\(_{2R}\)-pp pattern represents the tryptic proteolysis pattern of \( \beta \)-arrestin 2 in the presence of V\(_{2R}\)-pp.

RESULTS AND DISCUSSION

Conformational Changes in \( \beta \)-Arrestin 2 in Vitro upon Its Specific Association with Phosphopeptide V\(_{2R}\)-pp—Rat \( \beta \)-arrestin 2 was expressed and purified from E. coli strain BL21 (DE3) pLysS as described under “Experimental Procedures.” The primary sequence of the purified recombinant rat \( \beta \)-arrestin 2 cleaved from GST-\( \beta \)-arrestin 2 is shown in Fig. 1. To investigate the \( \beta \)-arrestin 2 activation mechanism by 7MSR and the associated conformational changes, the purified rat \( \beta \)-arrestin 2 was subjected to limited proteolysis in the absence or presence of a synthetic phosphopeptide, V\(_{2R}\)-pp, or the corresponding nonphosphopeptide, V\(_{2R}\)-np, derived from the C terminus of the Class B receptor V\(_{2R}\). Among several proteases tested in this study, TPCk-treated trypsin yields the best resolution of the digestion fragments. The optimal w/w ratio of trypsin to \( \beta \)-arrestin 2 for resolution of digestion fragments by SDS-PAGE was determined to range from 1:2,000 to 1:5,000 at 37 °C.

The limited proteolysis of \( \beta \)-arrestin 2 in the absence of peptide and in the presence of 5:1 (peptide/\( \beta \)-arrestin 2) molar ratio of V\(_{2R}\)-pp results in identical digestion patterns on SDS-PAGE (panels I and II in Fig. 2A), which we term the “control pattern.” As illustrated in the control pattern panel of Fig. 2B, full-length \( \beta \)-arrestin 2 (residues Gly\(_{7}^{–}\)–His\(_{116}^{+}\)) appears at 48 kDa and digestion with trypsin results in fragments with the apparent molecular masses of 42, 33, and 32 kDa (this study focused only on these major fragments). However, in the presence of 5:1 (peptide/\( \beta \)-arrestin 2) molar ratio of V\(_{2R}\)-pp, the limited proteolysis pattern of \( \beta \)-arrestin 2 is altered and represented as “V\(_{2R}\)-pp pattern” (panel III of Fig. 2A). Within 15 min of proteolysis in the presence of V\(_{2R}\)-pp, a new fragment with an apparent molecular mass of 45 kDa is generated, whereas the prominent 32-kDa fragment seen in the control pattern is not generated (V\(_{2R}\)-pp pattern of Fig. 2B). An identical V\(_{2R}\)-pp pattern is also obtained when \( \beta \)-arrestin 2 cleaved from S-\( \beta \)-arrestin 2 is used (data not shown). Limited tryptic proteolysis of \( \beta \)-arrestin 2 was also conducted in the presence of two other non-specific peptides, a 28-mer peptide and a 30-mer peptide (the sequences are shown under “Experimental Procedures”). Neither peptide had any effect on the limited proteolysis pattern of \( \beta \)-arrestin 2.

Trypsin is a pancreatic serine protease that cleaves peptide bonds in proteins that have carboxyl groups donated by arginine or lysine. A change in protein conformation can mask or unmask such cleavage sites, and an alteration in the limited proteolysis pattern of a protein can be indicative of conformational changes (17–23). Thus, the change in digestion pattern of \( \beta \)-arrestin 2 in the presence of V\(_{2R}\)-pp implies that V\(_{2R}\)-pp binds to \( \beta \)-arrestin 2 specifically in vitro and induces conformational changes. V\(_{2R}\)-np does not alter the digestion pattern of \( \beta \)-arrestin 2, suggesting that V\(_{2R}\)-np either cannot induce conformational changes upon binding or cannot bind \( \beta \)-arrestin 2 (see below).

Requirement for Phosphorylation on Residues in Synthetic Peptide for Inducing Conformational Changes in \( \beta \)-Arrestin 2 in Vitro—We have shown that V\(_{2R}\)-pp cannot induce changes in the pattern of limited tryptic proteolysis of \( \beta \)-arrestin 2 at a 5:1 (peptide/\( \beta \)-arrestin 2) molar ratio. This could be caused by a lower affinity of V\(_{2R}\)-np for \( \beta \)-arrestin 2. We therefore examined the effect of different peptide to \( \beta \)-arrestin 2 molar ratios on the limited tryptic proteolysis pattern of \( \beta \)-arrestin 2. As shown in the upper panel of Fig. 3A, V\(_{2R}\)-pp alters the control pattern (lane labeled with 0 in the upper panel) of \( \beta \)-arrestin 2.
at a 1:1 (peptide/β-arrestin 2) molar ratio to a V2R-pp pattern (lane labeled with 1:1 in the upper panel), whereas V2R-np does not change the proteolysis pattern even at a 100:1 (peptide/β-arrestin 2) molar ratio (lane labeled with 100:1 in the lower panel). Thus, even a 100-fold molar excess of V2R-np cannot induce conformational changes in β-arrestin 2.

We next performed a competition binding experiment of V2R-pp and V2R-np to β-arrestin 2 (Fig. 3B). β-Arrestin 2 was incubated, simultaneously, with a 1:1 (peptide/β-arrestin 2) molar ratio of V2R-pp and with different molar ratios of V2R-np (lanes 4–6) at room temperature for 30 min prior to addition of trypsin. STD is the rainbow standard (Amersham Biosciences). In both A and B, a 1:5,000 (trypsin/β-arrestin 2) w/w ratio of TPCK-treated trypsin was added to the sample, and the limited proteolysis was performed at 37 °C for 15 min. The apparent molecular masses of the tryptic digestion fragments are labeled on the left of each panel.

We also tested the effects of salt concentration ([NaCl]) on limited tryptic proteolysis of β-arrestin 2 in the presence of V2R-pp (data not shown). Our data indicate that the association of V2R-pp with β-arrestin 2 is salt concentration-dependent. At low NaCl concentration, V2R-pp binds to β-arrestin 2 and causes a V2R-pp pattern of limited proteolysis. When the NaCl concentration increases to 550 mM, the association of V2R-pp with β-arrestin 2 is disrupted and the proteolysis pattern returns to a control pattern, indicating that electrostatic interactions may play a crucial role in the association of V2R-pp with the positively charged residues in β-arrestin 2. Thus phosphorylation of the synthetic peptide is required for inducing conformational changes in β-arrestin 2 in vitro, consistent with the notion that β-arrestin 2 binds to 7MSRs in a phosphorylation-dependent fashion (3, 8, 11, 24, 25).

Involvement of Polar Core Residue Arg394 and Both N Terminus and C Terminus of β-Arrestin 2 in the Conformational Changes Induced by V2R-pp—The above SDS-PAGE analysis of limited tryptic proteolysis products clearly indicates that V2R-pp induces conformational changes in β-arrestin 2 upon its

**Fig. 3.** The effects of different peptide to β-arrestin 2 molar ratios on the limited tryptic proteolysis of rat β-arrestin 2 (A) and competitive binding of V2R-pp and V2R-np to β-arrestin 2 (B). A, β-arrestin 2 was incubated without (labeled 0) or with different peptide to β-arrestin 2 molar ratios (labeled 1:1 to 100:1) of V2R-pp (upper panel) or V2R-np (lower panel) for 30 min at room temperature before limited proteolysis. STD is the MagicMark™ XP Molecular Weight Standards. B, β-arrestin 2 was simultaneously incubated with 1:1 (peptide:β-arrestin 2) molar ratio of V2R-pp and different molar ratios of V2R-np (lanes 4–6) at room temperature for 30 min prior to addition of trypsin. STD is the rainbow standard (Amersham Biosciences). In both A and B, a 1:5,000 (trypsin/β-arrestin 2) w/w ratio of TPCK-treated trypsin was added to the sample, and the limited proteolysis was performed at 37 °C for 15 min. The apparent molecular masses of the tryptic digestion fragments are labeled on the left of each panel.
In order to assign each proteolytic fragment of β-arrestin 2, the full-length recombinant β-arrestin 2 (residues Gly7–His416) was subjected to a theoretical limited tryptic proteolysis using a ProteinProspector program as described under “Experimental Procedures.” The theoretical masses (m/z) of the proteolytic fragment candidates within the standard deviation of the experimental mass (m/z) of each fragment were selected and listed in Table I. The fragments (residues Gly7–His416, Gly7–Arg364, Val9–Arg364, and Val9–Arg287) with only one possible theoretical mass (m/z) were assigned directly, with or without further confirmation of the assignments by N-terminal sequencing or by other methods (Table I and Fig. 5). The fragments with more than one possible theoretical mass (m/z), which could not be distinguished by MALDI-TOF MS, were designated by coupling the MS data with N-terminal sequencing, Western blot analysis with antibodies that recognize different domains of β-arrestin 2, and limited tryptic proteolysis of truncated β-arrestin 2 mutants. The fragment with m/z value of 45,158 ± 30, generated in the presence of V2R-pp, could be assigned to two possible theoretical proteolytic fragments, residues Gly7–Arg394 (C-terminal cleavage) or Asp19–His316 (N-terminal cleavage) (Table I). The five N-terminal amino acids of this proteolytic fragment were sequenced as GSPNS, indicating that this fragment is the C-terminal cleavage fragment, residues Gly7–Arg394, from full-length β-arrestin 2 (residues Gly7–His416). This assignment was further confirmed by the limited proteolysis of two C-terminal-truncated mutants, β-arrestin 2 (residues Gly7–Thr293) and β-arrestin 2 (residues Gly7–Arg294) (Fig. 6). In the presence of V2R-pp, no protein band, with an apparent molecular mass 3 kDa less than those of the truncated β-arrestin 2 bands, was detected on SDS-PAGE. This result supports that the fragment with m/z value of 45,158 ± 30 (corresponding to the 45-kDa band on SDS-PAGE) from the limited proteolysis of wild-type β-arrestin 2 is a proteolytic fragment Gly7–Arg394. Appar-

![Fig. 4. MALDI-TOF MS spectra (between m/z 38,000 to 52,000) of limited tryptic proteolytic products of β-arrestin 2 without peptide or with V2R-np or V2R-pp at digestion time points of 15 and 60 min. The MALDI-TOF MS spectra were collected as described under “Experimental Procedures.” The molecular mass (m/z) of each peak was labeled as mean ± S.D., which was calculated from six independent experiments.](image-url)
ently, in the presence of V2R-pp, a previously hidden tryptic cleavage site at residue Arg294 is exposed and becomes accessible to trypsin (Fig. 5). The fragment with m/z 41,039 ± 27 (with m/z 41,042 ± 23 without peptide and 41,062 ± 15 in the presence of V2R-pp, respectively) has two possible assignments, amino acid Val7–Arg287 and Val7–Lys398. With our experimental conditions, we were not able to distinguish these two possibilities. However, this fragment is most likely a derivative from fragment Gly7–Arg287 because of a possible cleavage site at position Arg287. The fragment with m/z 33,315 ± 12 without peptide and 33,317 ± 15 in the presence of V2R-pp, respectively) has two possible assignments, amino acid Gly7–Arg287 and Leu101–Arg396, which have very close molecular masses (m/z) and cannot be differentiated by MALDI-TOF MS. However, this fragment was recognized by a mouse monoclonal antibody (26) (F4C1) directed against the fragment Asp23–Asp33 (sequence DGVVLVD) of β-arrestin 2 (data not shown), indicating that this fragment is residues Gly7–Arg287.

The two peaks with m/z values of 40,102 ± 21 and 31,759 ± 24, which are not generated in the presence of V2R-pp, were assigned directly as proteolytic fragments residues Val9–Arg287 and Val9–Arg364, respectively (Table I and Fig. 5). These data suggest that a previously accessible cleavage site, residue Arg287, is protected from trypsin digestion upon V2R-pp binding (Fig. 5). Protection of residue Arg287 could result either from direct masking by the bound V2R-pp or from ligand-induced changes in conformation of the β-arrestin 2 N terminus and subsequent change in solvent accessibility of residue Arg287. The latter explanation seems more rational for two reasons. First, in the crystal structures of visual arrestin and β-arrestin 1 and the structural model of β-arrestin 2 (Fig. 5), Arg287 (or the corresponding residue in visual arrestin and β-arrestin 1) is located on the convex side, away from the concave surface, which is believed to be the docking site for receptor. This location is not favorable for the interaction of Arg287 with V2R-pp. Second, evidence suggests that Lys11 and Lys12 of visual arrestin (equivalent to Lys23 and Lys24 of rat β-arrestin 2, which are only 2 and 3 residues away from Arg287) are the major possible “phospho-sensors” and directly interact with receptor-attached phosphate moieties. Charge reversal and elimination of residues Lys14 and Lys15 of visual arrestin dramatically reduces arrestin binding to light-activated phosphorylated rhodopsin (27). Thus, it is most likely that V2R-pp directly interacts with Lys11 and Lys12 in β-arrestin 2, forcing a rearrangement of its N terminus. This rearrangement moves Arg287 away from its original solvent accessible position to a solvent inaccessible position.

Arrestin family members are comprised of two domains (N-domain and C-domain) and an extended C-terminal tail (14, 15, 28, 29). In a recently proposed model, the inactive conformation of arrestins is stabilized by an intact polar core, which includes residues Asp26, Arg169, Lys170, Asp290, Asp297, and Arg393 of bovine β-arrestin 1 (Asp27, Arg170, Lys171, Asp279, and Arg292) and Arg394 of rat β-arrestin 2, as well as three element hydrophobic interaction involving β-strand I of the N terminus, the last β-strand XX of the C terminus, and α-helix I (15). Upon activation-dependent phosphorylation of a 7MSR, multiple phosphate moieties at the C terminus of the receptor would disrupt the delicate charge network within the polar core, thus releasing the constraints that keep arrestin in the basal state. Our data indicate that, without V2R-pp binding, the β-arrestin 2 molecule is in an inactive conformation in which the residue Arg394 is an integral part of the polar core and therefore inaccessible to trypsin. Binding of V2R-pp induces conformational changes in β-arrestin 2 and the previously hidden tryptic cleavage site Arg394 is exposed and becomes accessible to trypsin (Fig. 5). Because the polar core residue Arg394 is located in the C terminus of β-arrestin 2, the exposure of residue Arg394 prompts us to hypothesize that the C terminus is released from the rest of the protein molecule during the transition of β-arrestin 2 from its inactive conformation to the active conformation. The findings of: 1) exposure of the polar core residue Arg394 and 2) involvement of conformational changes in both N terminus and C terminus of β-arrestin 2 during its activation by V2R-pp provide the first direct evidence for the conformational changes associated with β-arrestin 2 activation.

**Comparison of V2R-pp-induced Conformation versus Heparin-induced Conformation of β-Arrestin 2—Polyanions, like heparin, have previously been shown to induce conformational changes in visual arrestin similar to those induced by the photoactivated phosphorylated rhodopsin as well as by a synthetic phosphopeptide comprising the fully phosphorylated C-terminal region of rhodopsin (20, 30, 31). This tempted us to examine the effect of heparin on the conformational changes in β-arrestin 2. Limited proteolysis of β-arrestin 2 in the absence or presence of V2R-pp, V2R-pp or heparin was performed in parallel, and the digestion patterns were compared (Fig. 7). Heparin binding changes the digestion pattern of β-arrestin 2, inducing conformational changes in β-arrestin 2. Addition of heparin, leads to release of the C terminus of β-arrestin 2, as indicated by the generation of the 45-kDa fragment. In this aspect, heparin acts similar to V2R-pp. However, striking differences are apparent in the overall digestion patterns induced by V2R-pp and heparin (Fig. 7). The 42 kDa protein is dramatically protected upon V2R-pp binding, whereas the digestion of the 42 kDa protein as well as the full-length β-arrestin 2 are accelerated in the presence of heparin. Possible explanations for this difference are that V2R-pp and heparin may have

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**Table I**

| Assignment of the limited tryptic proteolytic fragments of β-arrestin 2 |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|------------------|-----------------|
| Candidate fragments (theoretical) | Predicted tryptic fragments (amino acid) | Predicted m/z within standard deviations | MALDI-TOF m/z (experimental) | Five amino acids of N-terminal sequence | Recognition by antibody F4C1 | Molecular masses of tryptic fragments on SDS-PAGE |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|------------------|-----------------|
| Gly7–His416 | Gly7–His416 | 47846 | 47854 ± 8 | 47857 ± 23 | 47855 ± 17 | GSPNS | + | 48 |
| Gly7–Arg294 | Gly7–Arg294 | 45139 | — | — | 45158 ± 30 | GSPNS | + | 45 |
| Asp19–His16 | Asp19–His16 | 46517 | — | — | — | — | — | — |
| Gly7–Arg287 | Gly7–Arg287 | 41650 | 41636 ± 24 | 41646 ± 18 | 41646 ± 21 | GSPNS | + | 42 |
| Val1–Arg294 | Val1–Arg294 | 41652 | 41042 ± 23 | 41099 ± 27 | 41092 ± 15 | / | — | — |
| Arg294 | Arg294 | 41090 | — | — | — | — | — | — |
| Val2–Arg294 | Val2–Arg294 | 40117 | 40123 ± 31 | 40102 ± 21 | — | / | — | — |
| Gly7–Arg287 | Gly7–Arg287 | 33307 | 33315 ± 12 | 33318 ± 22 | 33317 ± 15 | / | — | — |
| Leu110–Arg396 | Leu110–Arg396 | 33327 | — | — | — | — | — | — |
| Val7–Arg287 | Val7–Arg287 | 31768 | 31758 ± 14 | 31759 ± 24 | — | / | — | — |

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different binding site(s) on β-arrestin 2 and/or induce different conformational changes in the β-arrestin 2 molecule. These data indicate that both polyanions (heparin) and polyphosphates (V₂R-pp) can evoke structural changes in β-arrestin 2. Nonetheless, V₂R-pp proves to have specific and unique effects that are not obvious with heparin.

The Release of the C Terminus of β-Arrestin 2 Is Sufficient to Enhance Clathrin Binding—Previous studies have demonstrated that β-arrestins function as adaptor proteins in clathrin-mediated endocytosis to promote agonist-induced internalization of 7MSRs (5). β-Arrestins have been reported to directly interact with clathrin heavy chain with high affinity via in vitro binding methods (5). The clathrin binding domain was localized to the C terminus of the β-arrestins, and the predominant binding sites were further demonstrated to lie between residues 371 and 379 of β-arrestin 2 (a clathrin binding motif,
The apparent molecular masses of the fragments are labeled on the left of each panel.

addition of clathrin heavy chain, S-β-arrestin 2 was incubated in the presence or absence of 5:1 (ligand/β-arrestin 2) molar ratio of 28-mer control peptide, V$_2$R-np, V$_2$R-pp or heparin. We found that V$_2$R-pp binding enhanced clathrin binding to S-β-arrestin 2 by 10-fold (Fig. 8), whereas 28-mer control peptide and V$_2$R-Rp had no effect. Interestingly, heparin binding also enhanced clathrin binding to S-β-arrestin 2 to 89 ± 15.5% of that observed with V$_2$R-pp. This result not only supports our hypothesis that the C terminus is released from the rest of the β-arrestin 2 molecule upon its activation by V$_2$R-Rp and heparin, but also implies that the release of the C terminus of β-arrestin 2 is sufficient to enhance clathrin binding.

Previous studies indicate that although heparin and the phosphopeptide from the C terminus of rhodopsin can induce similar conformational changes in visual arrestin, they have distinct effects on the interactions between arrestin and rhodopsin (23, 30). Heparin inhibits visual arrestin binding to rhodopsin (30), whereas the phosphopeptide from rhodopsin enhances light-activated binding of arrestin to both unphosphorylated rhodopsin in disk membranes as well as to endoproteinase Asp-N-treated rhodopsin (deletion of 330–348) (23). Our data reveal that both V$_2$R-pp and heparin-induced conformations of β-arrestin 2 are functionally active in terms of clathrin binding. However, based on the proteolysis data (Fig. 7), we believe that the conformation induced by heparin and V$_2$R-pp are not identical. The V$_2$R-pp-induced conformation is more likely to mimic the conformation induced by the activated receptor.

The findings in our study on β-arrestin 2 activation suggest that a similar activation mechanism is likely conserved among different members of the arrestin family. Based on our data and other mutagenesis, biochemical studies, and the crystal structures of the basal inactive state of bovine visual arrestin and β-arrestin 1, we propose a possible β-arrestin 2 activation model (Fig. 9), which is similar to the model proposed for visual arrestin and β-arrestin 1 (14, 15). Upon binding to V$_2$R-pp or agonist-occupied phosphorylated receptor, the multiple phosphate moieties of V$_2$R-pp or the C terminus of the receptor would disrupt the delicate charge network within the polar core, thus releasing the constraints that keep β-arrestin 2 in the basal inactive state. This induces the dissociation of the N terminus and C terminus away from their original positions and dislodges Arg$_{394}$ from the polar core. Additional global rearrangements of both N-domain and C-domain are most likely involved in this activation process. The release of the C terminus of β-arrestin 2 upon its activation exposes the clathrin binding site, and thus promotes clathrin binding to the active β-arrestin 2, favoring clathrin-mediated endocytosis of 7MSRs (Fig. 9).
In summary, our study reports the activation of \( \beta \)-arrestin 2 by the binding of a phosphopeptide (V\(_2\)R-pp) derived from the C terminus of V\(_2\)R. This active conformation is different from the basal conformation and heparin-induced conformation, as indicated by the different limited tryptic proteolysis patterns. It is also functional, indicated by the enhancement of clathrin binding. The conformational changes of \( \beta \)-arrestin 2 upon activation by V\(_2\)R-pp are consistent with the predicted changes in conformation of visual arrestin and \( \beta \)-arrestin 1 upon binding to receptor as suggested from previous mutagenesis, biochemical, and biophysical studies. This, in conjunction with the clathrin binding data, implies that the active conformation of \( \beta \)-arrestin 2 induced by V\(_2\)R-pp binding may closely mimic the functionally and physiologically active conformation of \( \beta \)-arrestin 2 which is induced upon binding to activated phosphorylated 7MSRs. It seems possible that \( \beta \)-arrestin 1 might display
similar conformational changes. Recent research has suggested that the two β-arrestin isoforms are functionally non-redundant. Our study provides a way to delineate the conformational changes of different isoforms of arrestin induced by the C termini of different 7MSRs. Future studies using this in vitro approach should provide valuable information regarding the activation mechanism of β-arrestins and shed new light on their shared as well as distinctive molecular features.

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