Homo- and Hetero-oligomerization of β-Arrestins in Living Cells*

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Arrestins are important proteins, which regulate the function of serpentine heptahelial receptors and contribute to multiple signalizing pathways downstream of receptors. The ubiquitous β-arrestins are believed to function exclusively as monomers, although self-association is assumed to control the activity of visual arrestin in the retina, where this isoform is particularly abundant. Here the oligomerization status of β-arrestins was investigated using different approaches, including co-immunoprecipitation of epitope-tagged β-arrestins and resonance energy transfer (BRET and FRET) in living cells. At steady state and at physiological concentrations, β-arrestins constitutively form both homo- and hetero-oligomers. Co-expression of β-arrestin2 and β-arrestin1 prevented β-arrestin1 accumulation into the nucleus, suggesting that hetero-oligomerization may have functional consequences. Our data clearly indicate that β-arrestins can exist as homo- and hetero-oligomers in living cells and raise the hypothesis that the oligomeric state may regulate their subcellular distribution and functions.

Arrestins play a central role in the regulation and signaling of serpentine heptahelial G protein-coupled receptors (GPCRs).2 Arrestin 1 and 4 are restricted to retinal rods and cones where they regulate rhodopsin (1). In contrast, arrestin 2 and 3, also referred to as visual arrestin, are restricted to retinal rods and cones where they regulate rhodopsin (2). Full-length β-arrestin 1 and 2 (βarr1 and 2 (βarr2), respectively, are ubiquitous and translocate to a large variety of ligand-activated GPCRs. Originally identified as negative regulators of GPCR function, promoting desensitization (2), βarrs were subsequently shown to be adaptor proteins connecting GPCRs to the endocytic machinery (3, 4). βarrs also serve as signaling scaffolds linking receptors to a growing number of effector pathways (5). For example, βarrs act as scaffolds for the activation of ERK and JNK3 (5). In addition, βarr2 redistributes the ubiquitin ligase Mdm2 and the kinase JNK3 from the nucleus to the cytoplasm, a property related to the presence of a leucine-rich nuclear export signal (NES) in βarr2 (6, 7). This signal is absent from βarr1, determining some differences in both subcellular distribution and functional roles between the two isoforms (6–8). Crystal structures of visual arrestin (9, 10) revealed that this molecule contains two globular domains and an extended COOH-terminal tail locking the molecule into an inactive state. Upon binding to receptors, the arrestin C-tail is released, leading to an open active conformation (11). In crystals, visual arrestin is a tetramer composed of two asymmetric dimers (9, 10). In vitro experiments showed that, in solution, tetramers are in equilibrium with monomers at physiological concentrations (12, 13), and it was proposed that self-association might regulate arrestin activity by limiting availability of active monomeric species (13). The crystal structure of βarr1 is very similar to that of visual arrestin, but unlike visual arrestin, full-length βarr1 was found to be monomeric (14). In addition, because of their lower intracellular concentration, falling far below that leading to equilibrium between arrestin monomers and tetramers in solution, it was postulated that βarr1 and βarr2 only exist as monomers in cells (14, 15). However, βarr1 truncated of its COOH-terminal tail was found to form dimers in crystals (15). Therefore, the possibility that βarrs oligomerize in vivo cannot be excluded based on the existing evidence. This hypothesis was investigated here using a combination of biochemical and biophysical approaches in vitro and in living cells.

EXPERIMENTAL PROCEDURES

Materials—If not otherwise specified, all chemicals and reagents were from Sigma-Aldrich (Saint-Quentin Fallavier, France). Polyclonal anti-βarr2 antibodies were a generous gift from Prof. J. L. Benovic (Thomas Jefferson University, Philadelphia, PA). Leptomycin B was a kind gift from Minoru Yoshida (University of Tokyo, Japan). The anti-Myc polyclonal antibody was from Santa Cruz Biotechnology, monocular and polyclonal anti-FLAG antibodies were from Sigma. Alexa-594-conjugated goat anti-mouse immunoglobulin was from Molecular Probes (Molecular Probes Europe, Leiden, The Netherlands).

Expression Vectors—The construction of pβarr2-FLAG, pβarr2-YFP, pβarr2-GEF, pβarr2-L395A-GEF, and pβarr2-R396A-GEF have been described previously (6). The plasmid pβarr2-GEF was generated by subcloning the βarr2 coding region into ECFP31 (Clontech Europe, Erembodegem, Belgium). βarr2 and βarr1 cDNAs were amplified by PCR and subcloned in pCMV-Tag3A (Stratagene) to create pMyc-βarr2 and pMyc-βarr1. To obtain Rluc-βarr1 and Rluc-βarr2-Rluc, appropriate forms of βarrs were subcloned in frame, in the phiRluc-C1 or -N3 vector, respectively, encoding the humanized Renilla luciferase (BioSig-

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nal, Montreal, Canada). YFP-ßarr1 and YFP-ßarr2 were obtained by  
succloning corresponding cDNAs in the pEYFP-C1 vector (Clontech). 
All constructs were verified by nucleotide sequencing.

**Cell Culture and Transfection**—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (all from Invitrogen, Cergy Pontoise, France), at 37 °C in an atmosphere of 5% CO₂. Cells were seeded at a density of 3 × 10⁵ cells in the 35-mm diameter wells of 6-well plates. Transient transfections were performed the following day using FuGENE (Roche, Meylan, France), according to the manufacturer’s protocol. Cells were harvested 24 h after transfection and either used for BRET experiments or grown on coverslips for FRET or immunofluorescence experiments. About 50–70% cells were fluorescent at the time of the experiments.

**Co-immunoprecipitation Experiments**—48 h post-transfection cells were lysed in 1 ml of cold glycerol lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA 1% Triton X-100, 10% glycerol, 100 μM Na3VO4, 1 mM NaF, supplemented with protease inhibitors) and clarified by centrifugation at 13, 000 × g for 20 min at 4 °C. Immunoprecipitations were performed using 20 μl of a 50% slurry of monoclonal M2 anti-FLAG-affinity agarose, with constant agitation overnight at 4 °C. Following incubation, immune complexes were washed four times with lysis buffer. Immunoprecipitated proteins were subjected to SDS-PAGE and Western blot analysis was performed using a polyclonal anti-Myc antibody. The chemiluminescence reaction was performed using the ECL reagent (Amersham Biosciences).

**BRET Assay**—COS cells were transfected with 10 ng/well of the DNA construct coding for BRET donor and increasing (10–250 ng/well) amounts of the construct coding for BRET acceptor (or control YFP). 24 h after transfection, cells were detached with phosphate-buffered saline/EDTA and washed in phosphate-buffered saline. Aliquots of 1 × 10⁶ cells were distributed in 96-well microplates (White Optiplate, PerkinElmer Life Sciences/Packard Biosciences). The luciferase substrate, coelenterazine h (Molecular Probes Europe, Leiden, The Netherlands), was added at a final concentration of 5 μM, and emitted luminescence and fluorescence were measured simultaneously using the Mithras™ fluorescence-luminescence detector (Berthold, Germany). Cells expressing BRET donors alone were used to determine background. Filter sets were 485 ± 10 nm for luciferase emission and 530 ± 12.5 nm for YFP emission. BRET ratios were calculated as described (16).

**Fluorescence Lifetime Imaging Microscopy**—Fluorescence decays were measured in cells expressing the FRET donor alone (ßarr2 fused to CFP) or both FRET donor and acceptor (ßarr2 fused to YFP). The microscopy system, based on time- and space-correlated single photon counting (TSCSPC), has been described elsewhere (17). Briefly, a mode-locked titanium sapphire laser (Millennia 5W/Tsunami 3960-M3BB-UPG kit, Spectra-Physics, France) delivering picosecond pulses was tuned at 880 nm to obtain a 440 nm excitation wavelength after frequency doubling. The repetition rate was 4 MHz after pulse-picker (Spectra-physics 3980-35). The laser beam was expanded and directed into an inverted epifluorescence microscope (Leica DMIRE, Leica, France) for wide field illumination. The fluorescence emitted by the sample was imaged with a 100× objective (NA = 1.3) directly at the entrance of the quadrant-anode TSCSPC detector (QA, Europhoton GmbH, Germany). Fluorescence decay imaging was established by counting for 10 min and sampling single emitted photons according to:

- (i) the time delay between their arrival and the laser pulse (picosecond time scale, 4096 channels),
- (ii) their xy coordinate (256 × 256 pixels image), and
- (iii) their absolute time. The count rate was up to 50 kHz.

![FIGURE 1. Homo-oligomerization of ßarr2 identified by co-immunoprecipitation experiments. ßarr2-FLAG or control empty vector and Myc-ßarr2 constructs were co-transfected in COS-7 cells (1 μg of each construct for a 10-cm diameter plate). After immunoprecipitation with anti-FLAG antibodies, immunoblots were performed using anti-Myc antibodies. The material loaded on the gel to quantify Myc-ßarr2 corresponds to 10% of the input. The amount of Myc-ßarr2 that could be immunoprecipitated under these conditions by ßarr2-FLAG was 5.5 ± 1.5% of the total input (average ± S.D. from three independent experiments).](image)

Appropriate band pass emission filter (460 nm < λem < 500 nm) was chosen to select the donor fluorescence and to reject the acceptor fluorescence. Fluorescence decays were determined from different regions of interest in cells expressing the fluorescence donor alone or both donor and acceptor. Decays were fitted with a Marquardt nonlinear least square algorithm ( Globals Unlimited Software, University of Illinois at Urbana, Champaign, IL) by using two lifetimes as theoretical model. Images were obtained by analyzing pixel-by-pixel fluorescence decays with a mean lifetime.

**Immunofluorescence**—COS-7 cells were seeded on coverslips in 6-well plates, transfected with appropriate plasmids and used for immunofluorescence 1 day later. Cells were fixed and processed for fluorescence microscopy as described previously (18). Samples were examined under a confocal microscope (Bio-Rad 1024 MRC). Images were processed using Bio-Rad Lasersharp 2000 software and optimized for contrast using Adobe Photoshop.

**RESULTS**

ßarr2 and ßarr1 Form Homo-oligomers—Yeast two-hybrid screens were conducted to identify new ßarr1 and ßarr2 interaction partners (data not shown). Both ßarrs were present among the identified preys, consistent with the formation of homo-oligomers. Interestingly, the ßarr1 bait also interacted with a ßarr2 prey, indicating possible hetero-oligomerization between the different ßarr isoforms.

The two-hybrid findings were confirmed by biochemical assays. COS-7 cells, which were reported to express very low levels of endogenous ß-arrs (19), were transfected with plasmids encoding differentially epitope-tagged ßarr2 for co-immunoprecipitation experiments (Fig. 1). Myc-tagged ßarr2 co-immunoprecipitated with FLAG-tagged ßarr2, supporting the hypothesis that constitutive ßarr2 homo-oligomers exist in cells.

Energy transfer-based approaches can detect protein-protein interactions occurring in living cells at physiological expression levels (20). In particular, BRET between Renilla luciferase (Luc, the BRET-donor) and a yellow variant of the green fluorescence protein (YFP, the BRET acceptor) has been extensively used to monitor GPCR oligomerization (21, 22) and ßarr2 recruitment to activated receptors (16, 23). BRET saturation experiments (24) were carried out in COS-7 cells to investigate basal ßarr2 oligomerization. A constant amount of ßarr2-Luc
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Immunoblot experiments were carried out to quantify the amount of YFP-βarr2 and βarr2-Luc expressed in aliquots of COS-7 cells, in which specific BRET signals were obtained (Fig. 2B). The amount of material immunostained with anti-βarr2 antibodies was quantified by gel scanning and was found to be 1.9 times higher than the endogenous level detected in untransfected HEK293 cells. In several experiments of the BRET saturation curve, the total amount of exogenous βarr2, which was quantified based on luciferase and fluorescence signals, was 30–40% below the amount of tagged βarr2 quantified in the Western blot of Fig. 2B. In addition, several other cell lines have been reported to express higher (up to 2-fold) endogenous βarr2 (and βarr1) than HEK293 cells (25), demonstrating that constitutive βarr2 oligomers may form in living cells expressing physiological concentrations of this protein.

The BRET approach cannot identify the subcellular localization in which the interaction between the donor and the acceptor occurs. Such information can be obtained by using FRET imaging. Among the various available FRET-based approaches, picosecond fluorescence lifetime imaging microscopy (picosecond FLIM) was reported to be particularly well adapted to in vivo studies because it requires low excitation intensity (avoiding photobleaching) and low levels of fluorescent proteins to be detected (26, 27). FLIM is based on the principle that the decay of the donor fluorescence is accelerated when FRET occurs between the donor and an acceptor. Fluorescence lifetime (τ) of the FRET donor, the cyan variant of the GFP (CFP), was measured at steady state in COS-7 cells either expressing βarr2-CFP alone or coexpressing βarr2-CFP and βarr2 fused downstream to the FRET acceptor YFP (Fig. 3a). Because CFP fluorescence decay is bi-exponential, mean fluorescence lifetimes (τm) were calculated from each component. The curves presented in Fig. 3a represent fluorescence decays averaged for 10 min over the entire surface of single cells. The significant decrease of average τm (Δτm = 0.191 ns), caused by FRET in cells coexpressing βarr2-CFP and YFP-βarr2, compared with cells expressing βarr2-CFP alone, indicates the presence of βarr2 oligomers. No significant FRET was measured in cells expressing βarr2-CFP and free YFP (data not shown). As a positive control, a τm difference of 0.250 ns was measured between COS-7 cells expressing a CFP-YFP fusion protein (for which strong FRET is expected) and COS cells expressing CFP alone (data not shown). The average τm can be visualized pixel by pixel on a color code scale. As shown in Fig. 3b, βarr2 oligomers appeared evenly distributed throughout the cytoplasm in unstimulated cells. In agreement with the reported nuclear exclusion of βarr2 at steady state (6), no βarr2 was visible in the nucleus.

Co-immunoprecipitation and BRET experiments were also conducted with βarr1 constructs (Fig. 4). Myc-βarr1 co-immunoprecipitated with FLAG-βarr1 and specific BRET signals were obtained using Luc-βarr1 and YFP-βarr1 as donor and acceptor, respectively. Maximal BRET signals and BRETm0 value were comparable with those measured for BRET studies on βarr2 indicating that the two βarr isoforms have a similar propensity for self-association.

βarrs Can Form Hetero-oligomers—As the two-hybrid results indicated that βarr1 and βarr2 hetero-dimers have the capacity to form in yeast, we investigated whether hetero-oligomerization between the two different βarr isoforms occurs in living mammalian cells. A first confirmation of this hypothesis came from experiments with epitope-tagged βarrs showing that, indeed, Myc-βarr1 can be co-immunoprecipitated with βarr2-FLAG (Fig. 5A). BRET studies were also conducted in cells expressing a donor and an acceptor of each βarr species (Fig. 5B). Again, specific BRET signals, comparable in amplitude and BRETm0 to those obtained in the experiments above, were measured whatever the βarr species used as BRET donor or acceptor. Together, these results indi-
cate that β-arrestins have the propensity to self-associate into either homo- or hetero-oligomers in living cells.

Subcellular Distribution of β-Arrestin Homo- and Hetero-oligomers—To determine whether β-arrestin oligomerization may be correlated with biological effects, we took advantage of a recent report showing that β-arrestin2 permanently shuttles between the cytosol and the nucleus in resting cells and that inhibition of the nuclear export machinery leads to the nuclear accumulation of βarr2 (6, 7). Accordingly, disruption of the βarr2 NES by a single amino acid residue substitution (βarr2-L395A) caused nuclear accumulation of the resulting mutant (Fig. 6A, upper right panel). Upon co-expression with βarr2-L395A, a significant proportion of wild type βarr2, which is normally undetectable in the nucleus (Fig. 6A, upper left panel), was sequestered into this compartment where it co-localized with βarr2-L395A (Fig. 6A, lower panels). The dominant effect of βarr2-L395A on wild type βarr2 subcellular

FIGURE 3. Constitutive βarr2 oligomerization examined by FLIM. COS-7 cells plated in 3.5-cm diameter dishes were transfected with plasmids coding for FRET donor (CFP, 100 ng of plasmid) alone or donor and acceptor (YFP, 300 ng of plasmid) fused upstream or downstream to βarr2 as indicated. YFP was used as nonspecific control. A, decay comparison of βarr2-CFP alone (blue curve) and βarr2-CFP co-expressed with YFP-βarr2 (green curve). Average τm values (see “Results” for definitions) were calculated from the cytoplasmic area of multiple individual cells (n indicated in the figure) and the mean of these values was used to calculate the variation of the average decay (Δτm = τm(βarr2-CFP) − μ(βarr2-CFP + βarr2-CYP)), which reflects FRET. B, pixel-by-pixel representation of τm values using color code. Left panels, steady state fluorescence intensity images taken with the quadran-anode TSCSPC detector, to delineate cell contours and location of the nucleus.

FIGURE 4. Constitutive βarr1 oligomerization. A, homo-oligomerization of βarr1 identified by co-immunoprecipitation experiments. FLAG-βarr1 or control empty vector and Myc-βarr1 constructs were co-transfected in COS-7 cells (1 μg of each construct for a 10-cm diameter plate). After immunoprecipitation with anti-FLAG antibodies, immunoblots were performed using anti-Myc antibodies. 10% of the Myc-βarr1 input was co-immunoprecipitated in the shown experiment. B, βarr1 homo-oligomerization examined by BRET. BRET experiments, conducted with the indicated constructs, were performed as in Fig. 2; error bars indicate S.D. of mean specific BRET ratio values of individual experiments (n = 114 (f); n = 36 (f); n = 15 (E); n = 66 (E)).

FIGURE 5. Constitutive βarr1-βarr2 hetero-dimerization. A, hetero-oligomerization of βarrs identified by co-immuno-precipitation experiments. βarr2-FLAG or control empty vector and Myc-βarr1 constructs were co-transfected in COS-7 cells (1 μg of each construct for a 10-cm diameter plate). After immunoprecipitation with anti-FLAG antibodies, immunoblots were performed using anti-Myc antibodies. 3% of the Myc-βarr1 input was co-immunoprecipitated in the shown experiment. B, BRET experiments, conducted with the indicated constructs, were performed as in Fig. 2. Error bars indicate S.D. of mean specific BRET ratio values of individual experiments (n = 114 (f); n = 36 (f); n = 15 (E); n = 66 (E)).
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Using an anisotropy-based living cell imaging system (see supplemental material) we verified that, upon addition of the FKBP-dimerizing small molecule AP20187, the totality of βarr2 fusion proteins was clustered through FKBP dimerization (supplemental Fig. 1A and supplemental TABLE ONE). Upon angiotensin AT1,AR stimulation, pre-oligomerized βarr2-FKBP-GFP translocated to the activated receptors and accumulated in clathrin-coated pits and endosomes with the same kinetics as non-dimerized controls (supplemental Fig. 1B and data not shown). These data indicate that the oligomeric organization of βarr2 molecules per se does not prevent their reactivity to receptor activation.

DISCUSSION

We have provided biochemical and biophysical evidence for the existence of constitutive βarr homo- and hetero-oligomers. The ability of βarr2 to self-associate and form hetero-oligomers with βarr1 was first documented by co-immunoprecipitation experiments. Resonance energy transfer-based approaches demonstrated the presence of homo- and hetero-oligomers in intact cells at physiological concentrations of βarrs. Finally, NES mutant or wild type forms of βarr2 altered the subcellular localization of wild type βarr2 and βarr1, respectively.

The approaches we used cannot discriminate, however, between a simple dimerization and more complex organizations of monomers, such as tetramers composed of 2 dimers, reported for visual arrestin (9). The actual proportion of monomeric (if they exist in vivo) versus oligomeric βarrs remains to be established, as saturation BRET experiments can measure the propensity of two partners to interact but do not detect free monomers. It has been proposed that because of the high concentration of visual arrestin in the retina, oligomerization would limit the availability of active monomeric species (13), which might be harmful for the retina if present in excess (28). Data reported here indicate that oligomerization may actually occur in living cells at physiological concentrations of βarrs, which are far below than those of visual arrestins in the retina. In addition, we have shown that artificially oligomerized βarr2 fully retains its capacity to translocate to activated GPCRs. Although our data are not sufficient to disprove the model of monomers constituting the active form of βarrs, an alternative plausible model emerges, in which oligomers might represent an important functional form of βarrs.

Recent studies mapping the arrestin-receptor interface, identified arrestin elements implicated in receptor binding on the concave sides of the two globular domains (29). Phosphate binding residues (contributing to the phosphate sensor, which recognizes phosphorylated GPCRs) are localized to the NH2-terminal globular domain, whereas other residues, which are involved in the binding to cytoplasmic unphosphorylated elements of the receptor (which sense the activation and determine the specificity), are distributed in both domains. It is well established that βarrs are capable of interacting with multiple proteins of MAP kinase cascades via the same globular domains while still binding to the activated GPCRs (30). Therefore, the oligomeric organization of βarrs might facilitate their interaction with multiple partners at the same time. Also, consistent with the hypothesis that βarr oligomerization might contribute to MAP kinase activation is a recent study showing that dimerization of MEKK2 (an upstream activator of NK) through its catalytic domain is essential to propagate the activation cascade (31). Whether the interaction with activated GPCRs physiologically involves arrestin monomers or oligomers, as receptor-βarr complexes rapidly accumulate in clathrin-coated pits, (18), it is reasonable to speculate that oligomers, if not preformed, are enriched in these compartments because of the much higher local concentration of βarrs.

Additional functions of βarr2 have been recently associated with its localization supports the occurrence of hetero-oligomerization between these two species and the nuclear localization of these oligomers. In contrast to βarr2, βarr1 has no NES in its carboxyl-terminal tail and is both nuclear and cytosolic at steady state (Fig. 6B, upper panels). In cells co-expressing βarr1 and βarr2, however, βarr1 was excluded from the nucleus, indicative of its cytosolic retention through hetero-oligomerization with βarr2 (Fig. 6B, lower panels).

Although oligomerization of βarrs has not been documented so far, previous studies on visual arrestin in vitro have proposed a model in which oligomerization would prevent the inappropriate activation of arrestin and subsequent deleterious cellular effects. To verify whether this hypothesis could be valid for βarrs, we studied the effect of forced and controlled βarr oligomerization on βarr activity. We designed a construct in which a FKBP binding motif was fused downstream of the βarr2 carboxyl-terminal tail and upstream of GFP (βarr2-FKBP-GFP).

FIGURE 6. Subcellular distribution of βarrs. A, immunofluorescence experiments performed in COS-7 cells expressing βarr2-FLAG and/or the NES mutant of βarr2 (βarr2-L395A-GFP) as indicated. Subconfluent cells were transfected (250 ng each of the βarr2-FLAG and the βarr2-L395A-GFP constructs per 3.5-cm diameter dish), fixed, permeabilized, and incubated with anti-FLAG polyclonal antibody, followed by incubation with Alexa-594-conjugated goat anti-rabbit antibody. Cells were subsequently analyzed by confocal microscopy. B, immunofluorescence experiments performed in COS-7 cells expressing βarr2-CFP and/or βarr1-YFP. Subconfluent cells were transfected (250 ng of DNA coding for βarr1-YFP and 250 ng of DNA coding for βarr2-CFP), fixed, and analyzed by confocal microscopy.
capacity of shuttling permanently between the cytosol and the nucleus. In particular βarr2 titrates JNK3 out of the nucleus (6) and increases the activity of p53 by relocalizing the E3 ligase Mdm2 from the nucleus to the cytosol (8). Since we reported here that βarr2 also retains βarr1 in the cytosol through hetero-dimerization, nuclear βarr1 availability and functions may vary depending on the specific stoichiometry of the two isoforms. So far, there is limited information on the physiological functions of shuttling permanently between the cytosol and the nucleus. In addition, the subcellular distribution of βarrows can be modulated through oligomerization suggesting that the quaternary structure of βarrows may control at least part of their biological functions.

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