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Homo- and Hetero-oligomerization of β -Arrestins in Living Cells^{*S}

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Arrestins are important proteins, which regulate the function of serpentine heptahelical receptors and contribute to multiple signaling 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 heptahelical G protein-coupled receptors (GPCRs).⁵ 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 β -arrestin 1 (β arr1) and 2 (β arr2), respectively, are ubiquitous and translocate to a large variety of ligand-activated GPCRs. Originally identified as negative regulators of GPCR function, promoting desensitization (2), β arrs were subsequently shown to be adaptor proteins connecting GPCRs to the endocytic machinery (3, 4). β arrs also serve as signaling scaffolds linking receptors to a growing number of effector pathways (5). For example, β arrs act as scaffolds for the activation of ERK and JNK3 (5). In addition,

β arr2 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 β arr2 (6, 7). This signal is absent from β arr1, 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 β arr1 is very similar to that of visual arrestin, but unlike visual arrestin, full-length β arr1 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 β arr1 and β arr2 only exist as monomers in cells (14, 15). However, β arr1 truncated of its COOH-terminal tail was found to form dimers in crystals (15). Therefore, the possibility that β arrs 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- β arr2 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, monoclonal 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 p β arr2-FLAG, p β arr2-YFP, p β arr2-GFP, p β arr2-L395A-GFP, and p β arr2-R396A-GFP have been described previously (6). The plasmid p β arr2-CFP was generated by subcloning the β arr2 coding region into ECFPN1 (Clontech Europe, Erembodegem, Belgium). β arr2 and β arr1 cDNAs were amplified by PCR and subcloned in pCMV-Tag3A (Stratagene) to create pMyc- β arr2 and pMyc- β arr1. To obtain Rluc- β arr1 and β arr2-Rluc, appropriate forms of β arr were subcloned in frame, in the pHluc-C1 or -N3 vector, respectively, encoding the humanized *Renilla* luciferase (BioSig-

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[§] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental methods, Table 1, supplemental Fig. 1, and supplemental Refs. 1–5.

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⁵ The abbreviations used are: GPCR, G protein-coupled receptor; β arr, β -arrestin; BRET, bioluminescence resonance energy transfer; FRET, fluorescence resonance energy transfer; NES, nuclear export signal; YFP, yellow fluorescent protein; TSCSPC, time- and space-correlated single photon counting; FLIM, fluorescence lifetime imaging microscopy; GFP, green fluorescent protein; CFP, cyan fluorescent protein; JNK, c-Jun NH₂-terminal kinase.

nal, Montreal, Canada). YFP- β arr1 and YFP- β arr2 were obtained by subcloning 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 μ g/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 3×10^5 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 μ M Na₃VO₄, 1 mM NaF, supplemented with protease inhibitors) and clarified by centrifugation at $13,000 \times g$ for 20 min at 4 °C. Immunoprecipitations were performed using 20 μ l 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 1×10^5 cells were distributed in 96-well microplates (White Optiplat, PerkinElmer Life Sciences/Packard Biosciences). The luciferase substrate, coelenterazine h (Molecular Probes Europe, Leiden, The Netherlands), was added at a final concentration of 5 μ M, and emitted luminescence and fluorescence were measured simultaneously using the MithrasTM 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 (β arr2 fused to CFP) or both FRET donor and acceptor (β arr2 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 DMIRBE, Leica, France) for wide field illumination. The fluorescence emitted by the sample was imaged with a 100 \times 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.

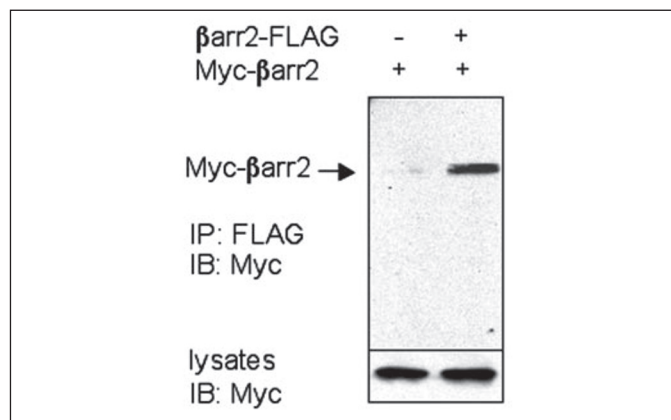


FIGURE 1. Homo-oligomerization of β arr2 identified by co-immunoprecipitation experiments. β arr2-FLAG or control empty vector and Myc- β arr2 constructs were co-transfected in COS-7 cells (1 μ g of each construct for a 10-cm diameter plate). After immunoprecipitation with anti-FLAG antibodies, immunoblots were performed using anti-Myc antibodies. The material loaded on the gel to quantify Myc- β arr2 corresponds to 10% of the input. The amount of Myc- β arr2 that could be immunoprecipitated under these conditions by β arr2-FLAG was $5.5 \pm 1.5\%$ of the total input (average \pm S.D. from three independent experiments).

Appropriate band pass emission filter ($460 \text{ nm} < \lambda_{em} < 500 \text{ nm}$) 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

β arr2 and β arr1 Form Homo-oligomers—Yeast two-hybrid screens were conducted to identify new β arr1 and β arr2 interaction partners (data not shown). Both β arrs were present among the identified preys, consistent with the formation of homo-oligomers. Interestingly, the β arr1 bait also interacted with a β arr2 prey, indicating possible hetero-oligomerization between the different β arr isoforms.

The two-hybrid findings were confirmed by biochemical assays. COS-7 cells, which were reported to express very low levels of endogenous β -arrs (19), were transfected with plasmids encoding differentially epitope-tagged β arr2 for co-immunoprecipitation experiments (Fig. 1). Myc-tagged β arr2 co-immunoprecipitated with FLAG-tagged β arr2, supporting the hypothesis that constitutive β arr2 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 β arr2 recruitment to activated receptors (16, 23). BRET saturation experiments (24) were carried out in COS-7 cells to investigate basal β arr2 oligomerization. A constant amount of β arr2-Luc

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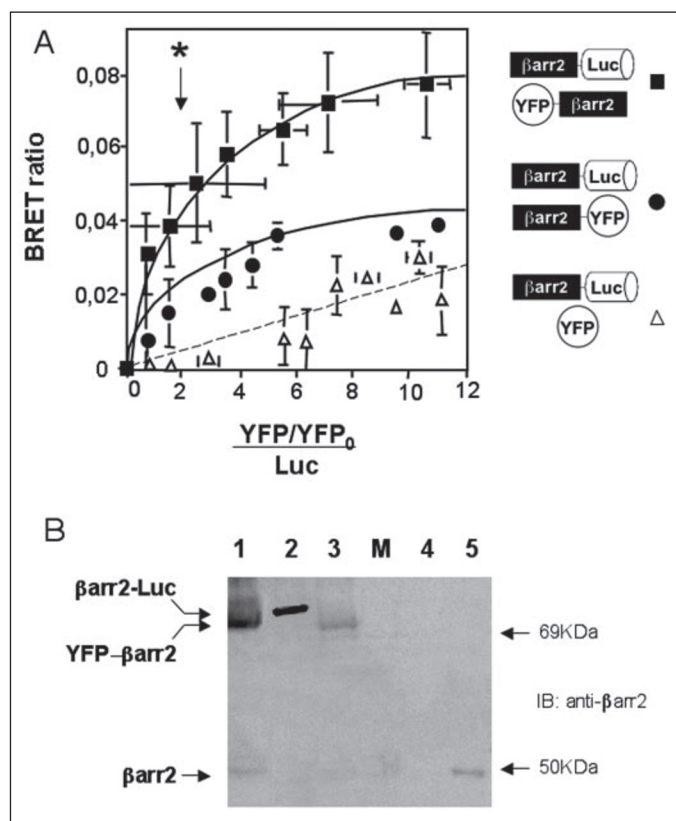


FIGURE 2. BRET study of constitutive β arr2 oligomerization in living cells. *A*, BRET experiments in COS-7 cells. BRET donor (Luc) and acceptor (YFP) were fused upstream or downstream to β arr2 as indicated. YFP was used as nonspecific control. Energy transfer was initiated by the addition of the cell-permeable luciferase substrate, coelenterazine h. Donor saturation curves were obtained by measuring the BRET in the presence of fixed quantities of donor and increasing amounts of acceptor. The relative amounts of BRET acceptor are expressed as the ratio between the fluorescence of the acceptor and the luciferase activity of the donor. YFP₀ corresponds to the fluorescence value in cells expressing the BRET donor alone. Error bars indicate S.D. of mean specific BRET ratio values of individual experiments ($n = 146$ (■); $n = 98$ (●); $n = 66$ (△)) grouped as a function of the amount of BRET acceptor, determined as indicated above. The asterisk indicates the position on the x axis of the sample quantified by immunoblot in the *B*. *B*, a sample of cells transfected with 10 ng of β arr2-Luc and 50 ng YFP- β arr2 (in a 3.5-cm diameter subconfluent plate) for a BRET experiment was lysed, and the fusion proteins were quantified by comparison with β arr2 contained in the same amount of cell lysates from various controls. Lane 1, COS-7 cells overexpressing YFP- β arr2; lane 2, COS-7 cells overexpressing β arr2-Luc; lane 3, cell lysate from cells used in the BRET experiment indicated by the asterisk in *A*; M, marker lane; lane 4, untransfected COS-7; lane 5, untransfected HEK293 cells. In lane 3 the amount of β arr2-Luc was at the limits of immunological detection. Gel quantification revealed that the signal corresponding to the tagged forms of β arr2 in lane 3 was 1.9 times higher than that measured in lane 5. Note that in COS-7 cells overexpressing YFP- β arr2 some free β arr2 is likely released from YFP, presumably by proteolytic cleavage.

cDNA, encoding a fusion protein, in which Luc is linked to the carboxyl-terminal extremity of β arr2, was co-transfected with increasing amounts of cDNAs coding for β arr2 fused either downstream (YFP- β arr2) or upstream (β arr2-YFP) of YFP. YFP alone was used to determine "bystander" non-selective BRET (24) (Fig. 2A). Specific BRET signals were measured with acceptors in either orientation, indicating the presence of β arr2 oligomers. Maximal BRET values were higher in experiments carried out with YFP- β arr2, compared with β arr2-YFP, indicating that BRET signals reflect an oriented oligomerization of β arr2 and not its nonspecific aggregation. Indeed, in the case of non-specific aggregation, BRET signals would have likely been independent of donor and acceptor orientations. The position of the BRET acceptor, relative to β arr2, does not modify the propensity of β arr2 to form constitutive oligomers, since the ratio of acceptor/donor at which half-maximal BRET was obtained (BRET₅₀) is similar for both orientations.

Immunoblot experiments were carried out to quantify the amount of YFP- β arr2 and β arr2-Luc expressed in aliquots of COS-7 cells, in which specific BRET signals were obtained (Fig. 2B). The amount of material immunostained with anti- β arr2 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 β arr2, which was quantified based on luciferase and fluorescence signals, was 30–40% below the amount of tagged β arr2 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 β arr2 (and β arr1) than HEK293 cells (25), demonstrating that constitutive β arr2 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 β arr2-CFP alone or coexpressing β arr2-CFP and β arr2 fused downstream to the FRET acceptor YFP (Fig. 3a). Because CFP fluorescence decay is bi-exponential, mean fluorescence lifetimes (τ_m) 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 τ_m ($\Delta\tau_m = 0.191$ ns), caused by FRET in cells coexpressing β arr2-CFP and YFP- β arr2, compared with cells expressing β arr2-CFP alone, indicates the presence of β arr2 oligomers. No significant FRET was measured in cells expressing β arr2-CFP and free YFP (data not shown). As a positive control, a τ_m 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 τ_m can be visualized pixel by pixel on a color code scale. As shown in Fig. 3b β arr2 oligomers appeared evenly distributed throughout the cytoplasm in unstimulated cells. In agreement with the reported nuclear exclusion of β arr2 at steady state (6), no β arr2 was visible in the nucleus.

Co-immunoprecipitation and BRET experiments were also conducted with β arr1 constructs (Fig. 4). Myc- β arr1 co-immunoprecipitated with FLAG- β arr1 and specific BRET signals were obtained using Luc- β arr1 and YFP- β arr1 as donor and acceptor, respectively. Maximal BRET signals and BRET₅₀ value were comparable with those measured for BRET studies on β arr2 indicating that the two β arr isoforms have a similar propensity for self-association.

β arrs Can Form Hetero-oligomers—As the two-hybrid results indicated that β arr1 and β arr2 hetero-dimers have the capacity to form in yeast, we investigated whether hetero-oligomerization between the two different β arr isoforms occurs in living mammalian cells. A first confirmation of this hypothesis came from experiments with epitope-tagged β arrs showing that, indeed, Myc- β arr1 can be co-immunoprecipitated with β arr2-FLAG (Fig. 5A). BRET studies were also conducted in cells expressing a donor and an acceptor of each β arr species (Fig. 5B). Again, specific BRET signals, comparable in amplitude and BRET₅₀ to those obtained in the experiments above, were measured whatever the β arr species used as BRET donor or acceptor. Together, these results indi-

FIGURE 3. Constitutive β arr2 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 β arr2 as indicated. YFP was used as nonspecific control. *A*, decay comparison of β arr2-CFP alone (blue curve) and β arr2-CFP co-expressed with YFP- β arr2 (green curve). Average τ_m 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 ($\Delta\tau_m = \tau_m(\beta$ arr2-CFP) - $\tau_m(\beta$ arr2-CFP + β arr2-CFP)), which reflects FRET. *B*, pixel-by-pixel representation of τ_m 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.

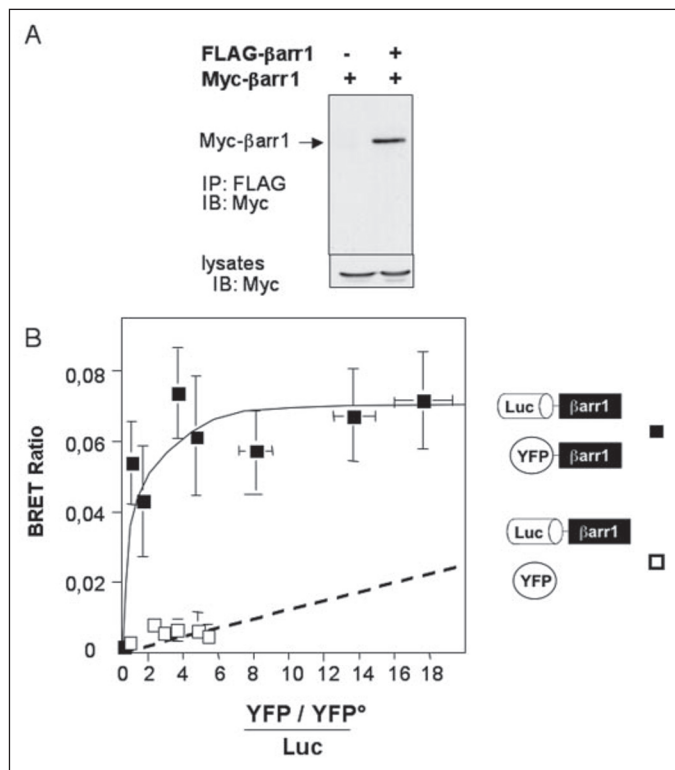
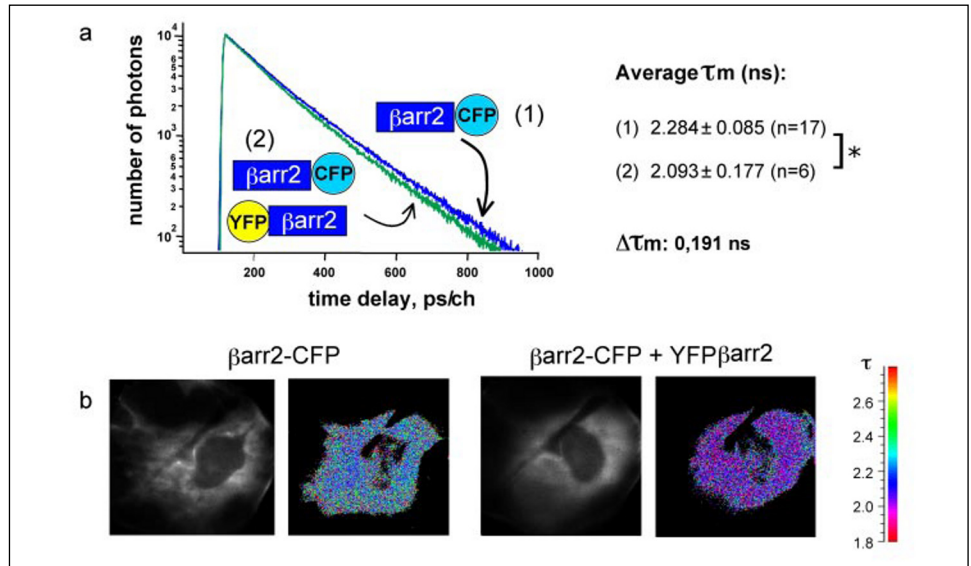


FIGURE 4. Constitutive β arr1 oligomerization. *A*, homo-oligomerization of β arr1 identified by co-immunoprecipitation experiments. FLAG- β arr1 or control empty vector and Myc- β arr1 constructs were co-transfected in COS-7 cells (1 μ g of each construct for a 10-cm diameter plate). After immunoprecipitation with anti-FLAG antibodies, immunoblots were performed using anti-Myc antibodies. 10% of the Myc- β arr1 input was co-immunoprecipitated in the shown experiment. *B*, β arr1 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 ($n = 129$ (■); $n = 15$ (△)).

cate that β arrs have the propensity to self-associate into either homo or hetero-oligomers in living cells.

Subcellular Distribution of β arr Homo- and Hetero-oligomers—To determine whether β arr oligomerization may be correlated with biological effects, we took advantage of a recent report showing that β arr2 permanently shuttles between the cytosol and the nucleus in resting cells and that inhibition of the nuclear export machinery leads to the

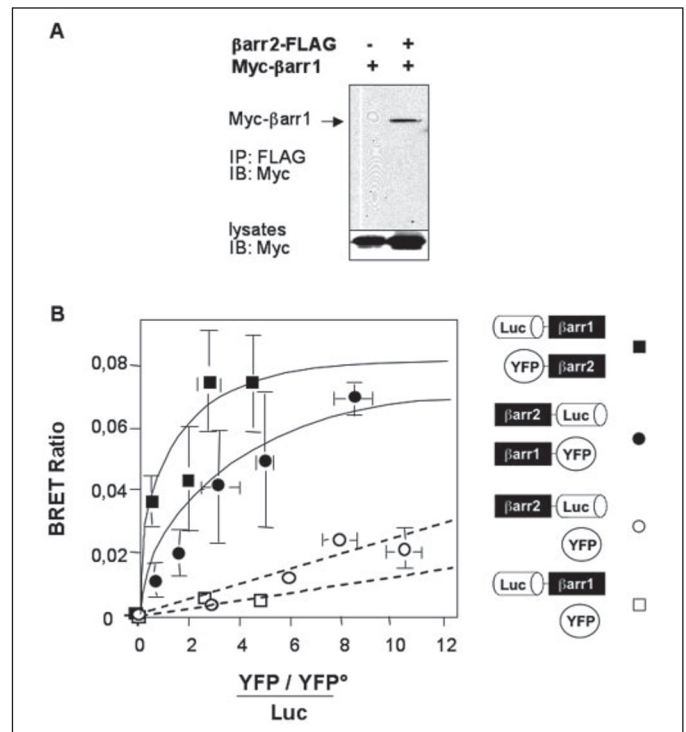


FIGURE 5. Constitutive β arr1- β arr2 hetero-dimerization. *A*, hetero-oligomerization of β arrs identified by co-immunoprecipitation experiments. β arr2-FLAG or control empty vector and Myc- β arr1 constructs were co-transfected in COS-7 cells (1 μ g of each construct for a 10-cm diameter plate). After immunoprecipitation with anti-FLAG antibodies, immunoblots were performed using anti-Myc antibodies. 3% of the Myc- β arr1 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 ($n = 114$ (■); $n = 36$ (■); $n = 15$ (□); $n = 66$ (○)).

nuclear accumulation of β arr2 (6, 7). Accordingly, disruption of the β arr2 NES by a single amino acid residue substitution (β arr2-L395A) caused nuclear accumulation of the resulting mutant (Fig. 6A, upper right panel). Upon co-expression with β arr2-L395A, a significant proportion of wild type β arr2, which is normally undetectable in the nucleus (Fig. 6A, upper left panel), was sequestered into this compartment where it co-localized with β arr2-L395A (Fig. 6A, lower panels). The dominant effect of β arr2-L395A on wild type β arr2 subcellular

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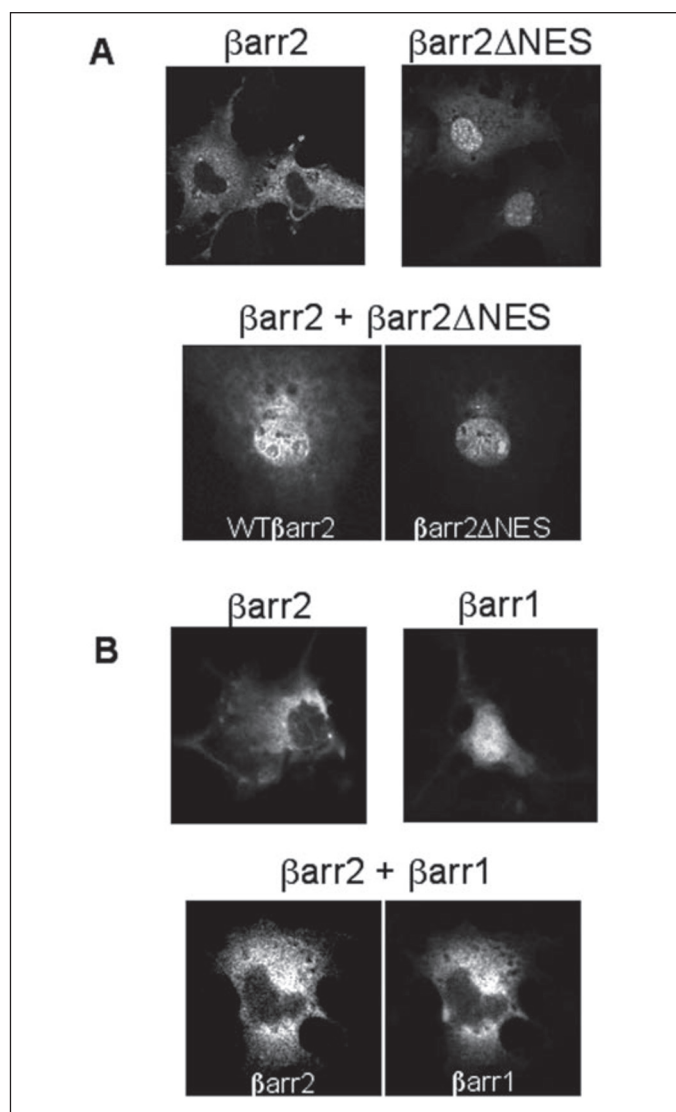


FIGURE 6. Subcellular distribution of β arrestins. *A*, immunofluorescence experiments performed in COS-7 cells expressing β arr2-FLAG and/or the NES mutant of β arr2 (β arr2-L395A-GFP) as indicated. Subconfluent cells were transfected (250 ng each of the β arr2-FLAG and the β arr2-L395A-GFP constructs 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 β arr2-CFP and/or β arr1-YFP. Subconfluent cells were transfected (250 ng of DNA coding for β arr1-YFP and 250 ng of DNA coding for β arr2-CFP), fixed, and analyzed by confocal microscopy.

localization supports the occurrence of hetero-oligomerization between these two species and the nuclear localization of these oligomers. In contrast to β arr2, β arr1 has no NES in its carboxyl-terminal tail and is both nuclear and cytosolic at steady state (Fig. 6*B*, upper panels). In cells co-expressing β arr1 and β arr2, however, β arr1 was excluded from the nucleus, indicative of its cytosolic retention through hetero-oligomerization with β arr2 (Fig. 6*B*, lower panels).

Although oligomerization of β arrestins 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 β arrestins, we studied the effect of forced and controlled β arr oligomerization on β arr activity. We designed a construct in which a FKBP binding motif was fused downstream of the β arr2 carboxyl-terminal tail and upstream of GFP (β arr2-FKBP-GFP).

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 β arr2 fusion proteins was clustered through FKBP dimerization (supplemental Fig. 1*A* and supplemental TABLE ONE). Upon angiotensin AT₁AR stimulation, pre-oligomerized β arr2-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. 1*B* and data not shown). These data indicate that the oligomeric organization of β arr2 molecules *per se* does not prevent their reactivity to receptor activation.

DISCUSSION

We have provided biochemical and biophysical evidence for the existence of constitutive β arr homo- and hetero-oligomers. The ability of β arr2 to self-associate and form hetero-oligomers with β arr1 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 β arrestins. Finally, NES mutant or wild type forms of β arr2 altered the subcellular localization of wild type β arr2 and β arr1, 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 β arrestins 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 β arrestins, which are far below than those of visual arrestins in the retina. In addition, we have shown that artificially oligomerized β arr2 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 β arrestins, an alternative plausible model emerges, in which oligomers might represent an important functional form of β arrestins.

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 β arrestins 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 β arrestins might facilitate their interaction with multiple partners at the same time. Also, consistent with the hypothesis that β arr oligomerization might contribute to MAP kinase activation is a recent study showing that dimerization of MEKK2 (an upstream activator of JNK) 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- β arr 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 β arrestins.

Additional functions of β arr2 have been recently associated with its

capacity of shuttling permanently between the cytosol and the nucleus. In particular β arr2 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 β arr2 also retains β arr1 in the cytosol through hetero-dimerization, nuclear β arr1 availability and functions may vary depending on the specific stoichiometry of the two isoforms. So far, there is limited information on the physiological nuclear effects of β arr1. Still, in the context of the GPCR-mediated anti-inflammatory response, it was reported that β arr1 modulates NF- κ B activation likely through a direct effect in the nucleus (32).

In conclusion, we have shown that the self-association properties of β arrs, initially hypothesized from crystallographic data, result in the presence of oligomers in living cells. In addition, the subcellular distribution of β arrs can be modulated through oligomerization suggesting that the quaternary structure of β arrs may control at least part of their biological functions.

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