β-arrestins (βarrs) are two highly homologous proteins that uncouple G protein-coupled receptors from their cognate G proteins, serve as adaptor molecules linking G protein-coupled receptors to clathrin-coat components (AP-2 complex and clathrin), and act as scaffolding proteins for ERK1/2 and JNK3 cascades. A striking difference between the two βarrs (βarr1 and βarr2) is that βarr1 is evenly distributed throughout the cell, whereas βarr2 shows an apparent cytoplasmic localization at steady state. Here, we investigate the molecular determinants underlying this differential distribution. βarr2 is constitutively excluded from the nucleus by a leptomycin B-sensitive pathway because of the presence of a classical leucine-rich nuclear export signal in its C terminus (L395/L397) that is absent in βarr1. In addition, using a nuclear import assay in yeast we showed that βarr2 is actively imported into the nucleus, suggesting that βarr2 undergoes constitutive nucleocytoplasmic shuttling. In cells expressing βarr2, JNK3 is mostly cytosolic. A point mutation of the nuclear export signal (L395A) in βarr2, which was sufficient to redistribute βarr2 from the cytosol to the nucleus, also caused the nuclear relocalization of JNK3. These data indicate that the nucleocytoplasmic shuttling of βarr2 controls the subcellular distribution of JNK3.

Arrestins form a family of homologous and structurally similar proteins involved in the control of G protein-coupled receptor (GPCR) function. Whereas visual arrestin is exclusively expressed in the retina where it turns off the signal mediated by activated rhodopsin, βarrs are almost ubiquitous and play a critical role in desensitization and endocytosis of GPCRs. Phosphorylation of ligand-activated receptors by specialized GPCR kinases promotes the translocation of βarrs from the cytoplasm to activated GPCRs. The binding of βarrs to GPCRs uncouples them from their cognate G proteins, a process referred to as desensitization that terminates receptor signaling (1–3). βarrs have also been shown to interact with the adaptor molecule AP-2 (4) and with clathrin (5), the two major structural components of clathrin-coated pits (CCPs) (6), and have therefore been proposed to target phosphorylated GPCRs for clathrin-mediated endocytosis (2).

In addition to their roles in termination of GPCR signaling, new roles for βarrs have recently been proposed because of the identification of novel βarr-interacting partners. Two isoforms of βarr exist, βarr1 and βarr2 (also known as arrestin 2 and 3, respectively), which were originally described in relation to β2-adrenergic receptor desensitization (7, 8). It has been demonstrated that βarr1 acts as a scaffolding protein for the activation of tyrosine kinases such as Src (9) and Hck (10). In another role as adaptor proteins, βarr2 has been shown to interact with the component kinases of the c-Jun N-terminal kinase 3 (JNK3) cascade, JNK3, and the MAP3K, ASK1, to facilitate JNK3 signaling (11). Likewise, βarrs interact with the component kinases of the ERK1/2 mitogen-activated protein kinase (MAPK) cascade, Raf and ERK2, to facilitate GPCR-mediated ERK activation (12).

Although the βarrs share a high degree of homology (~80%), it is becoming clear that there are differences between the regulation and function of the two isoforms. For example, βarr1 is phosphorylated by ERK, and following receptor activation and its recruitment to the plasma membrane it is dephosphorylated, which promotes Src binding and targets activated GPCRs to CCPs (13). In contrast, βarr2 is phosphorylated by Casein kinase II under basal conditions, and its dephosphorylation upon receptor activation does not appear to alter its endocytic capacity but has instead been proposed to alter binding to an as yet unidentified βarr2-interacting protein(s) (14). In addition, differences in the ability of the βarrs to promote sequestration of the β2-adrenergic receptor in mouse embryo fibroblasts has been observed, and studies using enhanced green fluorescent protein (GFP)-tagged βarrs have highlighted differences in their relative capacities to translocate to activated GPCRs (15).

Another striking difference between βarr1 and βarr2 is their subcellular localization. While at steady state, βarr2 appears to be almost exclusively extranuclear; β-arrestin1 is found throughout the cell, both in the cytoplasm and nucleus (Ref. 16 and “Results and Discussion” section). Since previous struc-
-Arrestin2 Contains a Functional NES

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture media, fetal bovine serum, and Calcium Phosphate transfection kit were from Invitrogen. TRH was from Sigma, and FuGene 6 reagent was from Roche Molecular Biochemicals. Lepptomycin B (LMB) was a kind gift from Minoru Yoshiida (University of Tokyo, Japan) and Barbara Wolff (Novartis). Mouse monoclonal antibodies AP-6 (anti-AP2, Ref. 24), P5D4 (against the YTDIEMNRLGK epitope of the G protein of vesicular stomatitis virus [VSV], Ref. 25) and M2 (anti-flag) were obtained from ATCC, Dr. T. Kreis, and Sigma, respectively. Alexa-fluor-488-conjugated goat anti-mouse immunoglobulin was obtained from Molecular Probes.

**Plasmids**—The construction of \( \beta ar2-GFP \), pHSV-TK-RHR, pFLAG-JNK3, pNIA and pNIA-VirE2 have been described previously (26–28). \( \beta arr2 \) was generated by cloning the full-length \( \beta ar2 \) cDNA downstream and in frame of EGFP in pEGFPN1 (Clontech) using the ApaI and SacI sites of pEGFPN1. To create pGFP-\( \beta ar2 \), pGFP-\( \beta ar2 \)C1, pGFP-\( \beta ar2 \)C2, pGFP-\( \beta ar2 \)C3, pGFP-\( \beta ar2 \)C4, pGFP-\( \beta ar2 \)C5, and pEGFP-\( \beta ar2 \), the corresponding regions of \( \beta ar2 \) CDNA were amplified using PCR; amino acids 317–410, 317–400, 317–390, 317–380, 317–370, and 1–316. These PCR products were subsequently digested SacI (contained on upper primers) and KpnI (contained on lower primers) and introduced downstream and in frame of EGFP in pEGFPNC1 (Clontech) using the same restriction sites. Similarly, DNA primers corresponding to amino acids 317–418 of \( \beta ar2 \) were amplified by PCR using primers containing HindIII (upper primer) and XhoI (lower primer) sites and cloned downstream and in frame of EGFP in pEGFPNC1 using the same restriction sites to create pGFP-\( \beta arr2 \)C1. Mutagenesis was performed using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene Europe, Amsterdam, The Netherlands). \( \beta ar2 \)-L387TA-GFP, \( \beta ar2 \)-L395A-GFP, \( \beta ar2 \)-R396A-GFP, and \( \beta ar2 \)-L397TA-GFP were generated by replacing Ile-387, Leu-395, Arg-396, or Leu-397 of GFP-tagged \( \beta ar2 \) with alanine residues.

**Immunofluorescence**—HeLa cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, penicillin, and streptomycin. Subconfluent HeLa cells were used for transient expression of the different constructs. Transfections were performed using the FuGENE 6 Transfection System or calcium phosphate transfection kits from Invitrogen according to the manufacturer’s instructions. For immunofluorescence studies, HeLa cells were seeded on coverslips in 6-well plates, transfected with 1 μg plasmid on the following day, and used for immunofluorescence 1 day post-transfection. For translocation experiments HeLa cells were transfected with 1 μg VSV-TRHR and 0.2 μg of a \( \beta ar2 \)-GFP construct. When treatment with LMB was performed, the cells were incubated at 37 °C for 60 min in Dulbecco’s modified Eagle’s medium-2% fetal calf serum containing 20 nM LMB. Cells were fixed and processed for fluorescence microscopy as previously described (29). Samples were examined under an epifluorescence microscope (Carl Zeiss) attached to a cooled CCD camera (Spot 2, Diagnostic Instruments).

**Nuclear Import Assay in Yeast**—The L40 yeast reporter strain containing the two LexA-inducible genes, HIS3 and lacZ, was transformed with the pNIA-hybrid expression vectors and plated on selective medium. Transformants were subsequently assayed for \( \beta \)-galactosidase (\( \beta \)-Gal) activity and histidine auxotrophy as previously described (28).

**Internalization of Thyrotropin-releasing Hormone Receptor-1 (TRHR) as Assayed by Radioligand Binding**—Internalization of TRHR was performed essentially as in (26). Briefly, subconfluent COS cells in 10 cm cell culture dishes were transiently transfected with 2.5 μg VSV-TRHR and either 2.5 μg of pCMV-Tag4A, 2.5 μg of \( \beta ar2 \)-FLAG, or 2.5 μg of \( \beta ar2 \)-L395A-FLAG using FuGENE 6. 24 h after transfection, cells were trypsinized and seeded into 12-well plates (one 10-cm dish was split into 16 wells). The day after, DMEM was removed from the cells and replaced with 0.5 ml HEPES-buffered HD medium (serum free DMEM, 20 μM HEPES, pH 7.2) per well, and cells were incubated at 37 °C. 40 min later plates were placed on ice and cooled for 10 min. HD medium was removed and replaced with 200 μl of 50 nM [\(^{3}H\)TRH in HD medium]. Cells were incubated on ice for 40 min. Subsequently, plates were shifted to 37 °C for 60, 30, or 10 min, replaced on ice, and washed three times with ice-cold 0.15 mM NaCl. Immediately after, membrane-bound [\(^{3}H\)TRH was stripped with 0.5 mM of an acid/salt wash (0.2 M acetic acid, 0.5 mM NaCl, pH 2.6) followed by 0.5 ml of 0.15 M NaCl wash. Internal [\(^{3}H\)TRH was determined after cell solubilization with 1% (w/v) SDS/1% (w/v) Triton X-100/10 mM Tris/HC1, pH 8.0. Samples incubated in the presence of 10 μM cold TRH allowed the determination of nonspecific binding. Results are expressed as a percentage of surface receptors at 0 min (cells incubated on ice for the duration of the experiment).

**Measurement of Intracellular Calcium ([Ca\(^{2+}\)]i)**—Changes in cytosolic-free calcium concentration were measured in HEK-293 cells loaded with Fura 2/AM as previously described (30). Briefly, subconfluent

**FIG. 1. \( \beta ar2 \) constitutively shuttles between the cytoplasm and the nucleus.** HeLa cells transiently transfected with \( \beta ar2 \)-C1 and \( \beta arr1 \)-C1 were incubated with either Me2SO (5000 μM) or 20 nM LMB (b and d) for 60 min at 37 °C, then fixed and processed for fluorescence microscopy as described under “Experimental Procedures.” The cells were observed under an epifluorescence microscope attached to a cooled CCD camera. The focus was made on the central region of the cell, identified using phase contrast (not shown) to better observe the nucleus.
HEK-293 cells in 10-cm dishes were transfected with 5 μg of βarr2-FLAG, 5 μg of βarr2-L395A-FLAG, or pCMV-Tag4A empty control vector. 48 h after transfection, adherent cells were loaded at 37°C with 2 μM Fura2/AM in serum-free Dulbecco’s modified Eagle’s medium for 50 min with gentle stirring. Cells were harvested after incubation for 10 min at room temperature in phosphate-buffered saline containing 5 mM EDTA and washed twice by centrifugation in Hank’s balanced salt solution, pH 7.4. Fura 2 fluorescence assays were performed with 2.5 × 10^6 cells in 2 ml of Hank’s balanced salt solution using a Jobin-Yvon three-dimensional fluorometer (Longjumeau, France) equipped with a thermally controlled cuvette holder and a magnetic stirrer. Excitation and emission wavelengths for Fura 2 assays were 340 and 510 nm, respectively. The concentrations of cytosolic-free calcium were calculated using the equation [Ca^{2+}]_i (nM) = 224 × (F-Fmin)/Fmax-F) as previously described (30). To evaluate muscarinic acetylcholine receptor desensitization, cells were incubated with 100 μM carbachol for 20 min, washed, and then rechallenged with 1 mM carbachol.

**RESULTS AND DISCUSSION**

Previous studies, performed with βarrs fused to the GFP or its variant, yellow fluorescent protein, at either their N or C terminus showed an unanticipated difference in the subcellular localization of βarr1 and βarr2 (16). Under steady-state conditions, the βarr2 constructs expressed in HEK-293 cells were almost exclusively cytoplasmic, whereas the βarr1 fusion proteins were evenly distributed throughout the cell including the nucleus. These results prompted us to study whether this phenomenon could be tag- or cell-dependent. The different subcellular localization of the two βarrs was conserved in HeLa cells (Fig. 1, a and c). Furthermore, experiments performed using βarrs tagged with a short, eight-residue long FLAG-epitope gave similar results (data not shown). The distinct subcellular localization of βarr1 and βarr2 is consistent with the presence of a functional NES in βarr2.

**Fig. 2.** Amino acids 390–400 in the C-terminal region of βarr2 are responsible for nuclear export activity. **Top,** a schematic representation of βarr1- and βarr2-derived constructs is depicted with their nuclear distributions. (+) present in the nucleus, (−) excluded from the nucleus. Locations of clathrin and AP-2 binding sites are indicated. **Bottom,** HeLa cells transiently transfected with GFP alone (a), βarr2/C1- (b), βarr2/C2- (c), βarr2/C3- (d), βarr2/N1- (e), or βarr1/C1- (f) GFP constructs were fixed and processed for fluorescence microscopy as described in Fig. 1.
distribution of βarr1 and βarr2 thus appear to be tag- and cell-type independent and cannot be explained by differences in size or conformation, because the two proteins vary by less than 10 amino acids in length and are structurally similar. Therefore, we investigated whether active transport events between the cytosol and nucleus could be involved. LMB is a drug that specifically inhibits the constitutive nuclear export of proteins that contain classical leucine-rich NESs through inactivation of CRM1/exportin1 (31). Incubation with LMB for 1 h caused the nuclear localization of βarr2-GFP (Fig. 1b), whereas no effect was observed on βarr1-GFP (Fig. 1d). These results indicate that βarr2, but not βarr1, undergoes constitutive nucleocytoplasmic shuttling and suggest the presence of a functional NES in βarr2.

Previous studies suggested that some unidentified molecular determinants located in the C-terminal region of βarrs are responsible for their different nuclear localization at steady state (16). Therefore, we postulated that the potential NES

Fig. 3. The activity of βarr2 NES depends on two leucine residues (L395 and L397). Top left, the 386/400 region from mammalian βarr2 is compared with the corresponding 385/399 region from mammalian βarr1. Residues that have been replaced by Ala (A) or Gln (Q) are also indicated. HeLa cells transiently transfected with βarr2- (a), L395A- (b), L397A- (c), I387A- (d) or L395Q- (e) GFP constructs were fixed and processed for fluorescence microscopy as described in Fig. 1.
underlying the nuclear exclusion of βarr2 was contained within this region. A series of GFP-tagged βarr2 C-terminal tail constructs were generated by progressive deletion from the C terminus to test this hypothesis (Fig. 2, top). We predicted that upon expression all of the constructs containing a functional NES would be cytoplasmic because of CRM1-mediated nuclear export. In contrast, truncations lacking NES would be found in both the nucleus and cytosol at steady state, since proteins of about 40 kDa or less are able to undergo passive diffusion through nuclear pores, as observed for the 27-kDa protein GFP (Fig. 2a). The full-length C-terminal tail construct containing the last 94 amino acids of βarr2 (βarr2/C1) was excluded from the nucleus under steady-state conditions (Fig. 2b) in keeping with the presence of a NES within this region. This hypothesis was further confirmed by the fact that βarr2/C1 was localized in the nucleus upon LMB treatment (Fig. 2, top and data not shown). Similarly, the expression pattern of the 10-residue shorter βarr2/C2 construct was cytoplasmic at steady state (Fig. 2c) and was found in the nucleus upon addition of LMB (Fig. 2, top and data not shown). In contrast with the βarr2/C3 construct, when an additional 10 amino acids were removed the nuclear exclusion of the βarr2 C-terminal tail was lost (Fig. 2d), suggesting that a NES lies between amino acids 390 and 400 of βarr2. Similar distribution profiles were observed for βarr2/C4 and βarr2/C5 as with βarr2/C3 (Fig. 2, top and data not shown). The fact that βarr2/N1, a βarr2 construct lacking the last 94 C-terminal amino acids, was mainly nuclear (Fig. 2e) suggests that there is no further NES in the protein. Finally, a construct encoding the last 94 amino acids of βarr1 was found in both the cytosol and nucleus like the full-length protein, further confirming the lack of a NES in βarr1. Taken together these results indicate that βarr2 is constitutively excluded from the nucleus because of the presence of a NES in its C terminus between amino acids 390 and 400.

The 10-residue region of βarr2 that is responsible for its nuclear export is highly conserved between different species and contains two leucine residues (L395/L397; Fig. 3, top) that are characteristic of a typical CRM1-dependent NES. These residues were individually mutated to alanine in full-length βarr2-GFP, and the effect of the mutations on nuclear exclusion of the subsequent chimeras was tested (Fig. 3). Mutation of each leucine residue, at positions 395 or 397, to alanine (L395A and L397A) caused a complete loss of nuclear exclusion (Fig. 3, b and c) resulting in nuclear localization of the constructs similar to that of wild-type βarr2 in the presence of LMB (Fig. 1b). Similar results were obtained using FLAG-tagged βarr2 constructs (data not shown). Mutation of the isoleucine residue at position 387 to alanine had no effect on nuclear exclusion (Fig. 3d), indicating that this residue is not required for NES activity. Interestingly, the region between amino acids 390–400 is also highly conserved between mammalian βarr1 and βarr2, the only difference being the L395 residue in βarr2 that is replaced by a glutamine residue at the corresponding position in βarr1 (Fig. 3, top). To further test if this one residue difference could account for the different nuclear localizations of βarr1 and βarr2, L395 was replaced by a glutamine residue in βarr2-GFP (L395Q). As expected, the resulting construct was found to be mainly nuclear, similar to what was found for the L395A mutant (compare Fig. 3, b and e). Therefore, these results indicate that βarr2 contains a classical leucine-rich NES (L395/L397) responsible for its constitutive nuclear exclusion. They also demonstrate that a single amino acid difference between βarr2 and βarr1 (L395 and Q394, respectively) is responsible for the lack of a functional NES in βarr1.

The aforementioned results clearly indicate that βarr2 is actively exported from the nucleus. In addition, the fact that the βarr2/N1, the NES βarr2 mutants, as well as wild-type βarr2 in the presence of LMB showed a stronger nuclear accumulation than GFP alone or βarr1 constructs suggests that βarr2 is actively imported in the nucleus (compare Figs. 1b, 2e, 3b, c, and e to 1c and 2f). The analysis of the βarr2 primary sequence did not reveal any classical, lysine-rich or M9-type (32) NLS, preventing the precise characterization of its nuclear import. Therefore, we studied βarr2 using an in vivo assay in yeast that was developed to quantify the active nuclear import of exogenous proteins bearing functional nucleocytoplasmic shuttling signals (28). In this assay, proteins to be tested are
fused to a chimeric protein consisting of the GAL4 activation domain fused to a modified LexA DNA binding domain in which its intrinsic NLS is abolished (Fig. 4A). If the tested protein undergoes active nuclear import the resulting fusion protein enters the nucleus and then activates two reporter genes, lacZ and HIS3, inducing both β-gal activity and growth on histidine-deficient medium (28). Such an activation was observed with both the L395A NES mutant of βarr2 and the human immunodeficiency virus integrase, a protein known to be actively imported into the nucleus (33), but not with wild-type βarr2 or a negative control, VirE2, which encodes an agrobacterium protein without any NLS (28) (Fig. 4B). These results are consistent with the respective intracellular localization of βarr2-GFP constructs and confirm that βarr2 is actively imported into the nucleus, although the NES in βarr2 appears to be functionally predominant at steady state.

Taken together our data indicate that βarr2 undergoes constitutive nucleocytoplasmic shuttling involving a yet under- mined import mechanism and the classical CRM1-dependent export pathway via a NES in its C-terminal extremity. Several recent studies have highlighted the important role that the last 40 amino acids (Fig. 5, top) of the βarrs play in their function and regulation. In this region both βarr1 and βarr2 share the same clathrin binding site (34), the IVF regulatory motif, which is involved in the stabilization of βarr in its basal state (35, 36), and the AP-2 binding site (37). Other regulatory sites are isoform specific, such as the βarr2 phosphorylation site for Casein kinase II (14), the ERK phosphorylation site for βarr1 (13), and the βarr2 NES (present study).

Cytosolic βarr2 rapidly translocates to activated GPCRs and mediates their recruitment to pre-existing CCPs by interacting with the adapter protein AP-2. This phenomenon, which can be
visualized by fluorescence microscopy as the colocalization of receptors H9252 arr2 and AP-2 in punctate areas of the plasma membrane after agonist stimulation (26), constitutes the first step of receptor endocytosis. Since the two leucine residues required for NES activity (L395 and L397) are adjacent to the two arginine residues (R394 and R396) that were identified to be important for H9252-arrestin-binding to the AP-2 complex, we further investigated whether these residues were involved in only one specific function or if mutation of one functional motif would affect the other. The fact that the H9252 arr2 R396A mutant, which demonstrates impaired AP-2 binding (37), remained localized within the cytoplasm under steady-state conditions (Fig. 5a) indicated that the NES was still functional. This was confirmed by the observation that the R396A construct was found in the nucleus upon LMB treatment (Fig. 5b). However, in cells expressing the GPCR TRHR recruitment of the R396A barr2 mutant to CCPs was greatly impaired, as evidenced by the lack of colocalization with the CCP marker AP-2 upon agonist stimulation (Fig. 5, c and d). In contrast, both NES mutants L395A and L397A were efficiently targeted to CCPs upon agonist stimulation of the TRHR like wild-type H9252 arr2 (26), as demonstrated by their complete colocalization with AP-2 (Fig. 5, e and f and data not shown for the L397A mutant) and the receptor (Fig. 6A, panels a and b and data not shown for the L397A mutant). In addition, the kinetics of TRHR endocytosis was similar in cells overexpressing either wild-type or NES mutant H9252 arr2, as shown in Fig. 6B. COS cells express low levels of endogenous b-arrestins that limit the amount and rate of GPCR endocytosis (38). COS cells were co-transfected with plasmids encoding the TRHR and plasmids coding for

**FIG. 6.** Nuclear export activity and barr2 functions on receptor endocytosis and desensitization are independent. A, HeLa cells transiently co-transfected with a VSV-tagged TRHR construct and the L395A-GFP construct were incubated at 4°C for 1 h with the anti-VSV antibody P5D4 before being warmed to 37°C, treated with 10 μM TRH for 2 min, and then fixed and processed for fluorescence microscopy using the secondary Alexa 594-labeled goat antimouse immunoglobulin. The focus was made on the planar plasma membrane adherent to the coverslip to show the localization of the constructs in CCPs. a, green fluorescence emitted by GFP. b, red fluorescence emitted by Alexa 594. Insets show higher magnifications of representative areas. The L395A barr2 construct and TRHR colocalized in dots (arrows). B, COS cells were transiently co-transfected with VSV-TRHR and either barr2-FLAG, barr2-L395A-FLAG, or pCMV-Tag4A empty control plasmid. After a 40-min incubation on ice with 50 nM [3H]TRH, cells were shifted at 37°C for different times. Surface and internal [3H]TRH binding was measured as described under Experimental Procedures. Data are expressed as the percentage of remaining surface receptors and represent means of duplicates. The experiment shown is representative of three independent experiments giving similar results. C, HEK-293 cells were transfected with barr2-FLAG, barr2-L395A-FLAG, or pCMV-Tag4A empty control vector. Desensitization of M1Ach receptor was assessed 48 h following transfection by incubating cells with 100 μM carbachol for 20 min followed by rechallenge with carbachol (1 mM). The data are expressed as a percentage of the peak calcium response to carbachol (1 mM) in non-pretreated cells. The data represent the mean ± S.E. of four independent experiments. Asterisks indicate a significant (p < 0.05) difference in the percentage of desensitization between control and barr2 (wild-type or NES mutant) overexpressing cells.
wild-type or NES mutant βarr2 or a control empty vector. TRH endocytosis reached ~25% after 1 h of agonist stimulation in cells transfected with the empty vector. In contrast, in the presence of overexpressed wild-type or L395A βarr2, endocytosis was faster and reached 80% for both constructs (Fig. 6B). These results indicate that although the NES and AP-2 binding sites are in close proximity, the specific residues implicated in nuclear export and AP-2 binding are able to function independently for each process.

For the majority of GPCRs receptor phosphorylation and subsequent binding of β-arrestins terminate coupling to G proteins in the continued presence of agonist, a phenomenon referred to as desensitization. Phosphorylation by specialized kinases, known as G protein receptor kinases, provides docking sites for β-arrestins on the intracellular regions of receptors. Upon binding to the receptor, β-arrestins sterically block further coupling between GPCRs and G proteins. We investigated whether mutation of the NES would hamper this process. HEK-293 cells express endogenous muscarinic receptors, which signal by increasing intracellular calcium (39, 40). Desensitization of muscarinic receptor signaling was tested in HEK-293 cell overexpressing wild-type βarr2 or βarr2-L395A (Fig. 6C). The increase in intracellular calcium concentration promoted by the muscarinic agonist carbachol was compared in cells preincubated or not with the same ligand to induce desensitization. In cells transfected with the empty control plasmid, desensitization involving endogenous β-arrestins resulted in ~50% decrease of the calcium signal. Overexpressed wild-type βarr2 and βarr2-L395A both significantly increased desensitization to comparable levels (~75% decrease of the calcium signal), indicating that the NES mutation does not interfere with regulation of receptor function.

All known biological functions of βarr2 were associated so far with its extra-nuclear localization. The existence of a nucleocytoplasmic shuttling indicates that this protein may have specific functions within the nucleus, which are likely not shared by βarr1 since the latter isoform is devoid of a NES. βarr2, but not βarr1, was reported to serve as a scaffold for the component kinases of the JNK3 cascade and also to retain JNK3 outside the nucleus under basal conditions (11). To investigate whether the nucleocytoplasmic shuttling of βarr2 could interfere with the subcellular distribution of JNK3, wild-type β-arrestins or NES mutant L395A βarr2 fused to GFP were co-transfected with FLAG-tagged JNK3 (Fig. 7). In agreement with previous findings (11), under basal conditions JNK3 distributed in both the cytoplasm and nucleus in cells expressing JNK3 alone or co-expressing JNK3 and βarr1 (data not shown), whereas it was mainly cytoplasmic in cells co-expressing wild-type βarr2 (Fig. 7A). In contrast, in the presence of the L395A NES mutant of βarr2, JNK3 colocalized with βarr2 within the nucleus (Fig. 7B), indicating that under basal conditions the two proteins remain associated in both nuclear and extra-nuclear compartments.

Taken together, these data and previous observations (11) are consistent with a model in which the nucleocytoplasmic shuttling of βarr2 would serve to redirect nuclear JNK3 to the cytoplasm and to relocalize GPCR-activated JNK3 to the extra-nuclear compartment in the vicinity of specific substrates. Such a scenario was recently demonstrated for PEA-15, a protein nuclear compartment in the vicinity of specific substrates. Such cytoplasm and to relocalize GPCR-activated JNK3 to the extranuclear compartments. Such conditions the two proteins remain associated in both nuclear and extra-nuclear compartments.

ACKNOWLEDGMENTS—We thank Dr. Vitaly Citovsky for kindly providing the pNIA and pNIA-VirE2 plasmids and Dr. R. J. Davis for providing Eps15, clathrin assembly lymphoid myeloid leukemia (CALM), α-adaptin, and now βarr2 have been shown to undergo nucleocytoplasmic shuttling, suggesting the possibility that molecules of the endocytic machinery might also play some function in the nucleus. These proteins, which contain multiple protein-protein interaction surfaces and are characterized by self-assembling properties, were proposed to provide scaffold structures participating in the regulation of transcription factors (22). Eps15 and CALM were reported to act as positive modulators of transcription in a GAL4-based transactivation assay (22), whereas epsin was found to interact with the transcription factor promyelocytic leukemia Zn (2+)-finger protein (PLZF) (21). Therefore, it is tempting to speculate that βarr2 might also play the role of a transcription regulator, a potential function to be added to the growing list of biological functions carried out by β-arrestins.

FIG. 7. JNK3 cycles with βarr2 during its nucleocytoplasmic shuttling: HeLa cells transiently co-transfected with either βarr2-GFP and FLAG-JNK3 constructs (A) or with βarr2-L395A-GFP and FLAG-JNK3 constructs (B) were fixed, permeabilized, and processed for fluorescence microscopy. The mouse M2 monoclonal antibody directed against the flag epitope was revealed by a secondary Alexa 594-labeled goat antimouse immunoglobulin. The nuclei were stained using a 4,6-diamidino-2-phenylindole (DAPI) containing mounting medium. βarr2 and L395A: green fluorescence emitted by GFP fused to βarr2 or L395A βarr2, respectively. JNK3: red fluorescence emitted by Alexa 594 labeling of the FLAG-Jnk3. DAPI: blue fluorescence emitted by DAPI staining of the nuclei. Lower right quadrants: overlaid images, colocalization of the three markers appears in white, whereas colocalization of JNK3 and βarr2 appears in orange.
ing the FLAG-JNK3 plasmid. We thank all of the members of the Marullo lab for helpful discussion and V. Poupon for technical assistance.

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