Identification of NSF as a β-Arrestin1-binding Protein

IMPLICATIONS FOR β2-ADRENERGIC RECEPTOR REGULATION*

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Previously studies have demonstrated that β-arrestin1 serves to target G protein-coupled receptors for internalization via clathrin-coated pits and that its endocytic function is regulated by dephosphorylation at the plasma membrane. Using the yeast two-hybrid system, we have identified a novel β-arrestin1-binding protein, NSF (N-ethylmaleimide-sensitive fusion protein), an ATPase essential for many intracellular transport reactions. We demonstrate that purified recombinant β-arrestin1 and NSF interact in vitro and that these proteins can be coimmunoprecipitated from cells. β-Arrestin1-NSF complex formation exhibits a conformational dependence with β-arrestin1 preferentially interacting with the ATP bound form of NSF. In contrast to the β-arrestin1-clathrin interaction, however, the phosphorylation state of β-arrestin1 does not affect NSF binding. Functionally, overexpression of NSF in HEK 293 cells significantly enhances agonist-mediated β2-adrenergic receptor (β2-AR) internalization. Furthermore, when coexpressed with a β-arrestin1 mutant (βarr1S412D) that mimics a constitutively phosphorylated form of β-arrestin1 and that acts as a dominant negative with regards to β2-AR internalization, NSF rescues the βarr1S412D-mediated inhibition of β2-AR internalization. The demonstration of β-arrestin1-NSF complex formation and the functional consequences of NSF overexpression suggest a hitherto unappreciated role for NSF in facilitating clathrin coat-mediated G protein-coupled receptor internalization.

Many G protein-coupled receptors (GPCRs),1 such as the β2-adrenergic receptor (β2-AR), utilize classical clathrin-coated vesicle pathways for internalization. Several lines of evidence indicate that phosphorylation of the receptor by a G-protein-coupled receptor kinase (GRK) followed by β-arrestin binding are crucial steps in this process (reviewed in Refs. 1–3). The role of GRK-mediated receptor phosphorylation in the internalization of receptors is to facilitate the binding of β-arrestins. β-Arrestins serve a dual role in the regulation of GPCRs. First, they mediate rapid desensitization by binding to GRK-phosphorylated receptors, sterically interdicting signaling to the G protein (4). Second, they target receptors for internalization via clathrin-coated pits, allowing subsequent dephosphorylation and resensitization of the receptors (5). Thus, a mutated form of the β2-AR (Y326A) that is a poor substrate for the GRKs fails to undergo agonist-dependent internalization. However, receptor internalization can be rescued by overexpressing GRKs (6). Similarly, overexpressing β-arrestin1 with the Y326A mutant also rescues receptor internalization (7). Further confirming the essential role of β-arrestins in the internalization process is the finding that dominant negative forms of β-arrestin1, namely V53D (8) and βarr1S412D (5), dramatically impair agonist-stimulated receptor sequestration.

It has been previously demonstrated that β-arrestin1 and 2 bind directly and stoichiometrically to clathrin (9) and that the function of β-arrestin1 in GPCR internalization is regulated by phosphorylation/dephosphorylation of the β-arrestin1 molecule (5). Cytoplasmic β-arrestin1 is constitutively phosphorylated on a carboxyl-terminal serine (Ser-412) and is recruited to the plasma membrane upon agonist stimulation of the receptor, where it becomes rapidly dephosphorylated. Dephosphorylation of β-arrestin1 is required for clathrin binding and the subsequent targeting of receptors to clathrin-coated pits but not for receptor binding and receptor desensitization. It is interesting to note that other members of the arrestin family (visual arrestins, β-arrestin2, and splice variants) do not possess the Ser-412 residue and are therefore not subject to the same regulation as β-arrestin1. Whether the ability of β-arrestin1 to promote GPCR internalization arises solely as a consequence of its ability to bind clathrin or whether additional β-arrestin1-binding proteins are involved in this process remains to be determined. In an attempt to further define the role of β-arrestin1 in the regulation of GPCR function, we used the yeast two-hybrid system to screen for novel β-arrestin1-binding proteins.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen—The full cDNA of rat β-arrestin1 was subcloned into the yeast expression vector pGADT9 using EcoRI and BamHI. Following amplification in Escherichia coli, the pGADT9 β-arrestin1 plasmid was transformed into yeast strain HF7c, and a yeast two-hybrid screen was carried out essentially according to the CLONTECH MATCHMAKER protocol using a GAL4 activation domain fusion library in pGAD10 (MATCHMAKER rat whole brain cDNA library, CLONTECH). Interacting proteins were isolated by growth selection on Trp, His, and Leu dropout plates and subsequently by filter β-galactosidase assays. Isolated library clones were analyzed by DNA sequencing using an ABI automated DNA sequencer (Howard Hughes Nucleic Acid Facility, Duke University).

Cell Culture and Transfection—The plasmids of interest were transfected into either HEK 293 cells (for sequestration assays) or COS cells (cellular coimmunoprecipitations) using LipofectAMINE according to the manufacturer’s instructions (Life Technologies, Inc.). To determine the association of β-arrestin1 and NSF, COS cells were cotransfected with either Flag-tagged β-arrestin1 (or Flag-tagged βarr1S412A and βarr1S412D) and/or His6-NSF-c-Myc, pcDNA3 empty vector was used.
as a control. Cells were harvested in lysis buffer (LB; 50 mM HEPES, pH 7.4, 0.5% Nonidet P-40, 250 mM NaCl, 10% glycerol, 2 mM EDTA, 0.5 mM ATP, 1 mM dithiothreitol, 1 μM phenylmethylsulfonyl fluoride 25 μg/ml aprotinin, and 1 μM leupeptin). The β-arrestin1s were immunoprecipitated using a polyclonal M2 antibody specific to the Flag epitope. The proteins were resolved by SDS-PAGE. Western blot analysis was performed using a monoclonal antibody specific for NSF (6E6). NSF was visualized using either ECL or quantitated using alkaline phosphatase-conjugated secondary antibody (Amersham) followed by phosphimager analysis. The nitrocellulose was stripped of immunoglobulin, and NSF was immunoprecipitated using an antibody specific for β-arrestin1 (19) to confirm equal expression of the β-arrestins.

**Determination of Nucleotide Dependence of the β-Arrestin1-NSF Interaction**—Cellular immunoprecipitations were carried out as described above. COS-1 cells were cotransfected with plasmids encoding either empty vector, Flag-β-arrestin1, NSF, or Flag-β-arrestin1 plus NSF. Flag-β-arrestin1 was immunoprecipitated using a M2 monoclonal antibody specific for the Flag epitope. Immunoprecipitates were washed three times with LB buffer, then equally divided, and transferred to fresh tubes. One-half of each immunoprecipitate was given a final wash with LB buffer containing 0.5 mM ATP and 2 mM EDTA (ATP/EDTA), and the other half was washed with LB buffer containing 2 mM EDTA plus 10 mM MgCl2 (ATP/Mg2+2) to allow ATP hydrolysis on the immunoprecipitated NSF. Immunoprecipitates were resolved by SDS-PAGE and transferred to nitrocellulose membranes. NSF was visualized using the 6E6 monoclonal antibody and quantitated by phosphimager analysis. Immuneblots were stripped of IgG and reprobed for β-arrestin1 using an antibody specific to β-arrestin1, such that the amount of NSF immunoprecipitated under nonhydrolyzable versus hydrolyzable conditions could be normalized to the amount of β-arrestin1 immunoprecipitated.

**β-AR Sequestration as Determined by Flow Cytometry**—Agonist-induced β-AR internalization was determined by immunofluorescence flow cytometry analysis as has been described previously (31). HEK 293 cells were transiently transfected with the plasmid encoding a Flag-tagged β-AR and either a pcDNA3 control vector, a β-arrestin1 expression plasmid (β-arrestin1 or barr1S412D), an NSF expression vector, or NSF with barr1S412D. Receptor fluorescence was defined as signal above the fluorescence of untransfected cells. Sequestration is defined as the fraction of total cell surface receptors that are removed from the plasma membrane (and thus are not accessible to antibodies added to the cells) following agonist treatment.

**RESULTS AND DISCUSSION**

**The Yeast Two-hybrid System Identifies NSF as a β-Arrestin1-binding Protein**—To identify novel proteins that interact with β-arrestin1 we screened a rat brain cDNA library using a full-length β-arrestin1 cDNA as bait in the yeast two-hybrid system screen (10). Several clones positive for His+ cell growth and β-galactosidase expression were isolated including a cDNA clone encoding a portion of the amino-terminal region of NSF (YN-ethylmaleimide-sensitive fusion protein (NSF) (Fig. 1). This clone was found to require the Gal4 DNA-binding domain-β-arrestin1 fusion for activity. NSF is an ATPase whose hydrolytic activity has previously been demonstrated to play an essential role in intracellular membrane trafficking (11).
suggests that NSF could potentially interact with proteins in vitro, this suggests that the β-arrestin1-NSF interaction may be of physiological relevance.

It has previously been shown that the phosphorylation state of β-arrestin1 regulates its endocytic function; the dephosphorylated form of β-arrestin1 preferentially binds to clathrin and promotes GPCR internalization (5). Therefore, we investigated the effect of β-arrestin1 phosphorylation on NSF binding. Two β-arrestin1 phosphorylation mutants, where Ser-412 has been replaced with either alanine (βarr1S412A) or aspartate (βarr1S412D) have been described previously (5). The βarr1S412A mutant has been shown to simulate the dephosphorylated form of β-arrestin1, whereas the βarr1S412D mutant has been shown to mimic its phosphorylated form. The association of NSF with wild type β-arrestin1, βarr1S412A, or βarr1S412D was comparable (Fig. 2B). The observation that β-arrestin1-NSF complex formation is not dependent on the phosphorylation state of β-arrestin1 suggests that NSF could potentially interact with β-arrestin1 either in the cytosol (phosphorylated β-arrestin1) and/or at the plasma membrane (dephosphorylated β-arrestin1). β-Arrestin1 has been shown to translocate from the cytosol to the plasma membrane and colocalize with clathrin-coated vesicles following agonist activation of the β2-AR (8). Thus, interaction of β-arrestin1 with NSF may serve to target NSF to the clathrin-coated vesicles following GPCR activation.

**β-Arrestin1-NSF Complex Formation Is ATP-dependent**—The association of NSF with its previously identified binding partners (SNAPs, SNAREs, and more recently the GluR2 subunit of the AMPA receptor) (17, 18) exhibits an ATP dependence. NSF has been shown to bind stably to SNARE complexes and does not dissociate from them when locked in the ATP state (13). To determine whether β-arrestin1 binding to NSF is also modulated by the nucleotide status of NSF, immunoprecipitations from cells were performed either in the presence of ATP/EDTA or ATP/Mg2⁺ (ATP nonhydrolyzable and hydrolyzable conditions, respectively). As seen in Fig. 3, NSF preferentially coimmunoprecipitated with β-arrestin1 in the presence of ATP/EDTA. The binding of NSF and β-arrestin1 was normalized to the amount of NSF bound to the amount of β-arrestin1 immunoprecipitated. Results represent the means ± S.E. for seven independent experiments.

β-arrestin1 was significantly impaired under conditions in which ATP is hydrolyzed (ATP/Mg2⁺). Notably, a mutant NSF capable of binding but not hydrolyzing ATP (E329Q; described in Ref. 15) showed no significant difference in β-arrestin1 binding in the presence of ATP/EDTA or ATP/Mg2⁺ (data not shown).

**Overexpression of NSF Potentiates β2-AR Internalization**—β-Arrestin1 has been shown to promote internalization (also referred to as sequestration) of agonist-occupied β2-AR (7). If the β-arrestin1-NSF interaction is important in regulating GPCR function, then given the well characterized role of NSF in vesicle trafficking, overexpression of NSF might also regulate β2-AR internalization. Thus, HEK 293 cells were transfected with Flag-tagged β2-AR alone or with wild type β-arrestin1, βarr1S412D, NSF, or NSF and βarr1S412D. Consistent with previously published results (20), overexpression of β2-AR with β-arrestin1 in HEK 293 cells results in a modest enhancement of β2-AR sequestration, and overexpression of the βarr1S412D (5) mutant results in a reduction of β2-AR sequestration compared with cells transfected with β2-AR only (Fig. 4). Dramatically, NSF overexpression enhances β2-AR sequestration approximately 2-fold. Furthermore, coexpression of NSF and the βarr1S412D mutant in HEK 293 cells appears to rescue the dominant negative effect of βarr1S412D, such that sequestration was found to be comparable with that in cells expressing β2-AR only (Fig. 4). Coexpression of wild type β-arrestin1 with NSF did not further enhance NSF-stimulated β2-AR sequestration (data not shown).

NSF exists in both cytosolic and membrane-associated forms and was originally purified on the basis of its ability to complement in vitro intra-Golgi transport reactions that had been blocked by treatment with N-ethylmaleimide (21). NSF has a general role in vesicular transport and functions at most if not
all vesicular transport events by virtue of its action on SNARE proteins. SNARE proteins have been classified into two families known as v-SNAREs (vesicle membrane receptor) and t-SNAREs (target membrane receptor) (12). The cognate pairing of a v-SNARE with a t-SNARE leads to the formation of a docking complex for membrane fusion to occur. The formation of this complex, termed the 20 S particle, is ATP-dependent. ATP hydrolysis by NSF disassembles the 20 S complex releasing the v-SNARE and t-SNAREs, thereby recycling these components for future fusion events (reviewed in Ref. 11). The observation that the neuronal SNAREs, syntaxin (v-SNARE) and synaptobrevin (t-SNARE), no longer interact with NSF following one round of ATP hydrolysis indicates that some structural modification occurs in one or both of these NSF-binding proteins (22). Taken together, these observations suggest that NSF promotes conformational changes in proteins with which it interacts in an ATP-dependent manner.

That the function of NSF is not restricted to vesicular transport is suggested by the observation that NSF binds to the AMPA receptor (17, 18, 23). AMPA receptors are a class of ionotropic glutamate receptors that mediate fast synaptic transmission and are composed of subunits GluR1-GluR4. Notably, NSF can bind directly to the GluR2 subunit of the AMPA receptor, providing the first example of an NSF cell surface receptor interaction (17, 18, 23). NSF and GluR2 exist in a complex in brain extracts not only with each other but also with SNAPs (18, 23). The GluR2-SNAP-NSF complex resembles the SNARE-SNAP-NSF complex in being disassembled by ATP hydrolysis (18). However, in contrast to the classical 20 S particle, NSF can bind directly to GluR2 independently of SNAPs. The binding site for NSF maps to a short region of the cytoplasmic tail of GluR2 distinct from the very carboxyl-terminal domain that interacts with the PDZ protein, GRIP (24). GRIP is believed to serve as a molecular scaffold on which a large complex of proteins is constructed, including AMPA receptors. It has been suggested that ATP hydrolysis by NSF may disassemble AMPA receptor interaction with GRIP leading to mobilization, insertion or internalization of the receptor (reviewed in Ref. 25).

Identification of an interaction between β-arrestin1 and NSF brings together two proteins previously found to be associated with clathrin-coated vesicles. β-Arrestin1 has been shown to interact with clathrin and target receptors to clathrin-coated pits for internalization (9). NSF has been shown to be associated with membranes derived from clathrin-coated vesicles (26). Purified clathrin-coated vesicles not only contain significant amounts of NSF but are enriched in the ability to support formation of 20 S fusion complexes. The current model for β2-AR internalization requires agonist-activated receptor to recruit β-arrestin to the plasma membrane, where it binds receptor. Following receptor binding, β-arrestin1 targets the receptor to clathrin and promotes receptor internalization.

By analogy with the many proposed roles of NSF in vesicular transport, one can envisage several possible functions of NSF binding to β-arrestin1 that might involve the NSF-driven association/disassociation of β-arrestin1 with/from receptor and/or other components of the clathrin-coated vesicle. One potential role for the interaction of NSF with β-arrestin1 may be to alter the conformation of β-arrestin1, thereby regulating the interaction of this protein with other components of the endocytic pathway. One β-arrestin1-binding partner whose interaction with β-arrestin1 could potentially be facilitated by NSF is clathrin. This may provide a potential explanation for the observation that overexpression of NSF is able to rescue the dominant negative effect of the βarr1S1412D mutant. βarr1S1412D is believed to behave as a dominant negative with regards to β2-AR internalization due to a reduced ability to bind clathrin (5). Because NSF binds equally well to βarr1S1412D and wild type β-arrestin1, NSF could potentially induce conformational changes in βarr1S1412D, increasing its affinity for clathrin, thus allowing internalization to proceed. Whether or not NSF has effects on regulating the interaction of β-arrestin with clathrin (and potentially other coat components) remains to be determined. A possible role of NSF in β2-AR internalization by mediating vesicle fusion during endocytosis is presently unclear, because any potential SNAREs for this process have yet to be identified.

An alternative role for the NSF-β-arrestin1 interaction, by analogy to the proposed role of NSF in the AMPA-GRIP interaction, is that NSF binding to β-arrestin1 might involve the NSF-driven dissociation of the β2-AR-β-arrestin1 complex from elements of the cytoskeleton. NSF ATP hydrolysis might possibly release the receptor from some constraining influence, leading to mobilization and internalization. A detailed investigation of the function of β-arrestin1-NSF complex formation may reveal hitherto unsuspected roles for NSF in clathrin-mediated endocytosis.

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