Functional specialization of β-arrestin interactions revealed by proteomic analysis

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β-arrestins are cytosolic proteins that form complexes with seven-transmembrane receptors after agonist stimulation and phosphorylation by the G protein-coupled receptor kinases. They play an essential role in receptor desensitization and endocytosis, and they also serve as receptor-regulated signaling scaffolds and adaptors. Moreover, in the past decade, a growing list of protein–protein interactions of β-arrestins pertinent to these functions has been documented. The discovery of several novel functions of β-arrestins stimulated us to perform a global proteomics analysis of β-arrestin-interacting proteins (interactome) as modulated by a model seven-transmembrane receptor, the angiotensin II type 1a receptor, in an attempt to assess the full range of functions of these versatile molecules. As determined by LC tandem MS, 71 proteins interacted with β-arrestin 1, 164 interacted with β-arrestin 2, and 102 interacted with both β-arrestins. Some proteins bound only after agonist stimulation, whereas others dissociated. Bioinformatics analysis of the data indicates that proteins involved in cellular signaling, organization, and nucleic acid binding are the most highly represented in the β-arrestin interactome. Surprisingly, both S-arrestin (visual arrestin) and X-arrestin (cone arrestin) were also found in heteromeric complex with β-arrestins. The β-arrestin interactors distribute not only in the cytoplasm, but also in the nucleus as well as other subcellular compartments. The binding of 16 randomly selected newly identified β-arrestin partners was validated by coimmunoprecipitation assays in HEK293 cells. This study provides a comprehensive analysis of proteins that bind β-arrestin isoforms and underscores their potentially broad regulatory roles in mammalian cellular physiology.

mass spectrometry | seven-transmembrane receptor | angiotensin II type 1a receptor | interactome | signal transduction

Seven-transmembrane receptors (7TMRs), the largest group of plasma membrane receptors, classically signal via activation of heterotrimeric G proteins and generation of second messengers such as cAMP, DAG, and IP3. Their signaling is rapidly quenched by a universal mechanism involving two families of proteins. G protein-coupled receptor kinases phosphorylate activated receptors, thereby promoting the binding of β-arrestin molecules (β-arrestin 1 or 2, aka arrestin 2 and 3) or in the case of rhodopsin, visual arrestin (aka arrestin 1). The arrestin molecules "desensitize" the receptors by sterically inhibiting further G protein activation (1, 2).

Over the past decade, however, a variety of additional functions of β-arrestins have been discovered. These include important roles in clathrin-mediated endocytosis of receptors and as signal transducers for a growing list of effector pathways such as MAP kinases, AKT, and phosphatidylinositol 3-kinase (PI3-kinase). Both the endocytic and signaling roles of β-arrestins rely on their ability to serve as adaptors and scaffolds that engage in regulated interactions with a variety of cellular partners. A number of such partners (approximately two dozen) have been previously identified by a variety of techniques including yeast two-hybrid screens and coimmunoprecipitation studies (2, 3). These include elements of the endocytic machinery such as clathrin and AP-2 and signaling molecules such as ERK, JNK3, and Src (2). Moreover, quite recently β-arrestins have been found to interact with a wide variety of receptors other than 7TMRs such as the tyrosine kinase IGF1 receptor, TGFβ III receptor, nicotinic cholinergic receptor, and Notch (3). Taken together, these findings hint at much broader roles of these multifunctional adaptor molecules than are currently known.

Accordingly, we set out to assess the potential scope of β-arrestin functions by performing a global proteomics analysis to determine, in a mammalian cell, the protein partners of both β-arrestin 1 and 2 under basal and 7TMR [angiotensin II type 1a receptor (AT1aR)] stimulated conditions. Both gel-based and non-gel-based proteomics methods for protein resolution and identification were used, aimed at enhancing the coverage and reliability of the analysis (4). Thus, by a combination of these two methods, we identified 337 nonredundant proteins interacting with β-arrestin 1 and 2. The large and diverse set of proteins obtained provides evidence of significant specialization in the functions of β-arrestins and major clues to previously unsuspected functions.

Results and Discussion

Identification of β-Arrestin-Interacting Proteins by Mass Spectrometry. We used a comprehensive proteomics approach to identify proteins as potential β-arrestin interaction partners (β-arrestin interactome) [Tables 1 and 2, supporting information (SI) Tables 3–6, and SI Fig. 3]. The interacting proteins were identified by mass spectrometry analysis of the β-arrestin complexes immunoprecipitated from HEK293 cells stably overexpressing β-arrestin 1 or 2 with a C-terminal FLAG epitope and the AT1aR (SI Fig. 4). The experiments were performed under both basal and receptor-stimulated conditions. To enhance the coverage and reliability of the identified proteins, each experiment was repeated six to eight times using either the multidimensional protein identification technique (MudPIT) or gel-based LC tandem MS approach. A total of 337 nonredundant proteins were found to interact with β-arrestins (SI Tables 3–6). One hundred seventy-three were found to interact with β-arrestin 1, and 266 were found to interact with β-arrestin 2. Of these, 102 were found to interact with both β-arrestin 1 and 2. For β-arrestin 1, 43 proteins were found only in the nonstimulated sample and 52 were found only in the stimulated sample, with 78 in both samples. For β-arrestin 2, 81 proteins were found only in

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Abbreviations: AT1aR, angiotensin II type 1a receptor; 7TMR, seven-transmembrane receptor; PI3-kinase, phosphatidylinositol 3-kinase; DGK, diacylglycerol kinase.

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Table 1. β-arrestin 1-interacting proteins

Signal Transduction

Adaptor proteins:

β-arrestin 1; β-arrestin 2;S-arrestin;X-arrestin; 14-3-3 β/α; γ; τ; ε; ζ/δ; η; RanBPM

Protein kinases:

STK38; SCY1-like 2; DGK-ζ; DGK-ε; DGK-γ; p160ROCK; c-Yes; Serine/threonine-protein kinase ATR; PI3-K-C2α; PI3-K-p85β; DGK-η; MEKK1; Serine/threonine kinase 38 like

Phosphatases: PP2Cα; PP2Cβ

Trafficking:

Clathrin light chain A; Clathrin heavy chain; AP-3 δ1; Gaf-1; LRP4; LRP-DIT; dynamin-1; Tomosyn-1; Copine-8

G protein- and small GTPase-related:

RGS3S; Rab-1A; RICS; Ras-GTPase-activating protein binding protein 1

Other signaling proteins:

Calmodulin; Annexin II; Patched 2; Syndecan-3; Calgranulin B; Zyxin

Metabolic Enzymes

Fatty acid synthase; PK3; Dehydrogenase/reductase SDR family member 2; ATP synthase, a; β; iPFK-2;Protein disulfide-isomerase precursor; Acetyl-CoA carboxylase 1; Complex I-75kD; Cyclophilin A; Phospholipase A1-α; α-enolase

Cellular Organization

Cytoskeleton Constituent Proteins:

Actin α-1; Tubulin α-1; Tubulin α-2; Tubulin α-6; Tubulin β-5; Vimentin; Tubulin β-2A;

Cytoskeleton Accessory Proteins:

Filamin A;Gelsolin;Eplin;p116Rip; Caldesmon; MAP 1B; Tropomyosin 4; α-fodrin; CKAP4 protein; Tropomodulin-3; Cofilin-1; Cortactin; Titin; Dystrophin; p16-ARC; Kinectin; α-internexin

Motor Proteins:

Myosin-9; Myosin-1; Myosin regulatory light chain; KIF26A; Myosin Ic; Myosin light chain; KIF2C; KIF3A; Dynein, axonemal heavy chain 3

Chaperone and Stress Response

HSC70; HSPCA; HSP70.1; HSPA7

Ion Channels

CNG channel α 3; potassium channel tetramerisation domain containing 3

Miscellaneous

Apoptosis: BIP; APLP; Mortalin 2; BTF

Proteolysis and ubiquitination:

Ubiquitin and ribosomal protein S27a; CEP52; Ubiquitin B; C; Carboxypeptidase A1; Deubiquitinating enzyme 24

Others: β-casein; Spindlin-1; Vigilin

Nucleic Acid Binding

DNA binding, Chromatin Structure and Cell Cycle Regulation:

Histone H1.2; H2B; H4; H3.3; H2A; Ku80; Histone H2A.1; H2A.x; H1x

Transcription Factor and Transcription Regulatory Proteins:

Nopp140; Treacle; JBP1; Nucleophosmin; PAF49; PAF53; RPA40; RPA194;Trap150; ZNF265; Thymopoietin; YB-1; Tho4; RPB25; BACH2

RNA Processing and Ribonucleoprotein:

Nop10; RNA binding motif protein 10; SAP145; Nucleolin; SmD1; SRRM2; Dyskerin; hnRNP M; K; A3; A1; H; U

Protein Biosynthesis:

eIF-4B; eEF1A-2; Ribosomal protein L22; Ribosomal protein S3a; S17; IMP-2; Ribosomal protein L3; L6; L7a; S2; S8; eIF-3β

Unknown

Methylosome protein 50; 11 kDa protein; 46 kDa protein; HDGF2; hypothetical protein LOC345651; FLJ45296; DKFZp686J1593; Similar to POTE2A; similar to FKSG30; similar to cytoskeletal β actin; Similar to Actin, cytoplasmic 2; Similar to SHK1 kinase-binding protein 1; Similar to bA92K2.2; Dpy-30-like protein; Hypothetical protein DKFZp566G0346

Shown is an abbreviated version of the β-arrestin 1-interacting proteins (SI Tables 3 and 4 show the full versions). Protein names are separated from each other by semicolons. Blue, proteins identified only in nonstimulated conditions; red, proteins identified only in stimulated conditions; black, proteins identified in both conditions.

the nonstimulated sample and 53 were found only in the stimulated sample, with 132 in both samples (SI Fig. 3).

We also performed control experiments in which the bait proteins, β-arrestin-FLAGs, were absent. In these experiments, only isoforms of keratins, immunoglobulins, and actins were found (SI Table 7), demonstrating a low background binding associated with our experimental conditions. Keratins are common contaminants in mass spectrometry-based proteomics studies, and immunoglobulins are most likely introduced by the anti-FLAG M2 beads. Both of these proteins were also present in the experimental data and hence were removed. We set up the criteria for a minimum two unique peptides per positive protein identification, and we report only those proteins that were identified in at least two independent experiments. Some proteins such as treacle, Nopp140, and ZNF265 were found in as many as eight independent experiments. Additionally, many of the already known β-arrestin binding partners such as ERK, casein kinase II, c-Yes, PI3-kinase, diacylglycerol kinases (DGKs), PP2A, clathrin, AP-2, PDE4D, filamin A, and cofilin were detected. Moreover, the β-arrestin interactome was cross-validated by coimmunoprecipitation assay of β-arrestins and a set of randomly selected partners.

Confirmation of β-Arrestin-Interacting Proteins by Coimmunoprecipitation and Western Blot Analysis. We selected 16 proteins from the β-arrestin interactome, subcloned them into a FLAG-tagged expression vector, and coexpressed them with β-arrestins in HEK293 cells stably expressing the human AT1aR. The overexpressed FLAG-tagged target proteins were coimmunoprecipitated by anti-FLAG M2 antibodies, and their interactions with β-arrestins were examined by Western blot using anti-β-arrestin antibodies (Fig. 1). In these experiments, 14 of 16 selected proteins were found to interact with β-arrestins (SI Table 8). Angiotensin II stimulation enhanced binding affinities of four proteins (PP2Cα, PP2Cβ, PRP4 kinase, and ZNF265) to β-arrestins. The binding affinities of three proteins (14-3-3τ, HSC70, and pyruvate kinase M2) to β-arrestins decreased upon angiotensin II stimulation. Although agonist effects on some proteins, such as calgranulin B, could not be confirmed in the coimmunoprecipitation assay, on the whole the agonist effects were in good agreement with the data obtained by the mass spectrometry-based proteomics analysis (SI Table 8). A more detailed study of agonist effects requires quantitative analysis using techniques such as stable isotope-based metabolic labeling.

β-Arrestin-Interacting Proteins Are Ubiquitous in Cells and Have Diverse Functions. Our analysis demonstrates that β-arrestin-interacting proteins are widely distributed in the cell. The breakdown of subcellular localizations of these proteins is as follows: the cytoplasm (53%), the nucleus (26%), the nucleolus (5%), the plasma membrane (5%), the mitochondrion (2%), the endoplasmic reticulum and Golgi apparatus (2%), and all other compartments (6%) (Fig. 2*A*). It is noteworthy that the AT1aR, the receptor that is overexpressed in the HEK293 cells, is missing from the β-arrestin interactome. The failure to detect AT1aR and other 7TMRs seems surprising and maybe due to the inaccessibility to proteases of these receptors because of the transmembrane regions. In addition, posttranslational modifications can make the receptor peptides hard to ionize and difficult to detect by mass spectrometry (5).

Table 2. β-arrestin 2-interacting proteins

Signal Transduction

Adaptor proteins:

β-arrestin 2; β-arrestin 1; S-arrestin; 14-3-3 β/α; γ; τ; η; ε; X-arrestin

Protein kinases:

ERK-2; Casein kinase I; Casein kinase II; DGK-ζ; DGK-ε; CaM kinase II δ; STK38; CDC2L5; CDK3; CDK7; SCY1-like 2; GCN2 eIF2α kinase; PAK-7; NIK; MEKK1; Wee1A kinase; CDK4; PRP4 kinase; TAK1; PI3-K-C2α; SRPK2; Phosphorylase kinase alpha L subunit

Phosphatases:

PP2Cα; PP2Cβ; PP2A, subunit A, PR65-α; Myosin phosphatase-targeting subunit 1

Trafficking proteins:

AP-2 α1; AP-2 β1; VPS35; Copine-1; Gaf-1; Clathrin light chain A; Clathrin light chain B; AP-3 δ1; AP-2 α2; AP-3 β2; LRP11 protein

G protein- and small GTPase-related:

α-Pix; Ran GTPase-activating protein 1; Rho GTPase activating protein 10; Ras-GTPase-activating protein-binding protein 2; Nadrin; PDZ-RhoGEF; Leukemia-associated RhoGEF

Other signaling proteins:

Calmodulin; Annexin II; I κ-B kinase α ; TAK1-binding protein 1; Catenin α-1; Catenin δ-1; Syndecan-3; PRMT1; WDR26; PDE4D protein; Afadin; Calgranulin B

Metabolic Enzymes

PK3; GAPDH; iPFK-2; SDH2; ARD1; DDX1;Ribophorin II; Fatty acid synthase; CTP synthase; Protein disulfide-isomerase precursor; Cyclophilin A

Nucleic Acid Binding

DNA binding, Chromatin Structure and Cell Cycle Regulation:

Histone H1.1; H1x; H2A; HDAC2; Ku70; Histone H2B; Ku80; Lamin-B receptor;MCM3; Histone H1.2; H2A.x; RPA1

Transcription Factors and Transcription Regulatory Proteins:

Nopp140; Treacle; JBP1; Nucleophosmin; ERH; TIF 1B; YB-1; LEO1; Tho4; ZNF265; Trap150; Tat-SF1; RPA194; RPA135; MPP4; TWIST neighbor; NAP1; CSDA protein; STAT1; RPA40; RPB25; Myocardin; Mitosin

RNA Processing and Ribonucleoproteins:

Nop10; Nop56; SAP145; SAP155; SRRM2; DDX3; DDX15; SmD1; SmD2; NONO protein; Nucleolin; RNA binding motif protein 10; PSF; hnRNP A1; A2; D0; H; K; M; U; hnRNP A0; C; F; H'; I; R; SAP130; Dyskerin; Fibrillarin; U5-116 kDa; PABP3; PABP4; PAI-RBP1; DDX5

Protein Biosynthesis:

eIF-4B; eEF1A-2; PABP1; IGF-II mRNA-binding protein 1; 40S ribosomal protein S3a; S3; S17; S19; 60S ribosomal protein L7a; L7; L12; L18; L21; L22; L31; P0; P1; P2; 40S ribosomal protein S6; S7; S8; S13; 60S ribosomal protein L3; L6; L14; L15; L28; EF-2; EF1A; EEF1A protein; 40S ribosomal protein S4, X isoform; 60S ribosomal protein L4; L11; L19; L26; L30; L35a; L35; L36

Chaperone and Stress Response

HSP70.1; HSP70-Hom; HSC70; HSP90; T-complex protein 1ζ; HSPA6

Ion Channels

Kv-β-1; TrpV4; Nuclear chloride ion channel 27; Chloride channel, nucleotide sensitive 1A

Cellular Organization

Cytoskeleton Constituent Proteins:

Tubulin α-1; α-2; α-6; β-2A; β-2; β-3; Actin β; Vimentin; Actin γ-2; Tubulin α-3; α-8; β-4; α-cardiac actin

Cytoskeleton Accessory Proteins:

Gelsolin; Filamin A; α-fodrin;Tropomodulin-3; Cofilin-1; Cortactin; Eplin; Drebrin; MAP 1B; β-fodrin; ARC16-2; CapZ α-1; α-2; Thymopoietin; DAL1; ZNF231

Motor Proteins:

Myosin-9; Myosin light chain; Myosin Ic; Dynein heavy chain, cytosolic; Myosin-10

Miscellaneous

Apoptosis: BTF; BIP

Proteolysis and ubiquitination:

Ubiquitin and ribosomal protein S27a; Ubiquitin B; C; Calpain-1 catalytic subunit; CEP52; EDD1; Cullin-5

Others:

Spindlin-1; Spindlin-3; NK-TR protein; Dermcidin; p33; Myosin-reactive immunoglobulin light chain variable region; IGKV1-5 protein; β-casein; Importin 90; TER ATPase

Unknown

HDGF2; Hepatoma-derived growth factor 2; Hypothetical protein FLJ10100; LOC345651; Methylosome protein 50; Hypothetical protein FLJ45296; Similar to cytoskeletal β actin; BolA-like protein 2; Mob4B protein; 11 kDa protein; Hypothetical protein LOC144097; 29 kDa protein; 46 kDa protein; 61 kDa protein; TTP1; Similar to PI-3-kinase-related kinase SMG-1 isoform 1; Similar to FKSG30; nsun2 protein; Similar to Actin, cytoplasmic 2; Hypothetical protein DKFZp566G0346

Shown is an abbreviated version of the β-arrestin 2-interacting proteins (SI Tables 5 and 6 show the full versions). Color codes are same as those in Table 1.

The important roles of β-arrestins in the cytoplasm and at the plasma membrane have been well documented over the past decade (1–3). Both the endocytic and signaling functions of β-arrestins rely on their ability to serve as adaptors/scaffolds that regulate a variety of cytoplasmic proteins such as clathrin, AP-2, ERK, and PDE4 (3). In addition to these known partners, >160 new cytoplasmic proteins have been found to interact with β-arrestins in this study, suggesting that the roles of β-arrestins in the cytoplasm may be much broader than our current understanding. The second largest group of β-arrestin-interacting proteins is localized in the nucleus. More than 68 nuclear proteins, including a number of nucleic acid binding proteins, nuclear kinases, and nuclear signaling proteins, were found to interact with β-arrestins. In addition, many cytoplasmic proteins, like β-arrestins themselves, have nucleocytoplasmic shuttling capabilities. All of these suggest previously unsuspected nuclear functions of β-arrestins, an emerging role for these multifaceted molecules (6). Surprisingly, 16 nucleolar proteins were also identified as β-arrestin interactors. A functional theme for these nucleolar proteins is rRNA production/modification and ribosome assembly. Along these lines, 34 ribosomal proteins were also identified during this study. These varied subcellular distributions of the β-arrestin-interacting proteins reflect a widespread role for β-arrestins in the cell.

To elaborate the functional scope of β-arrestins, the identified β-arrestin-interacting proteins were grouped according to their Gene Ontology biological and molecular functions (Tables 1 and 2). In comparison with the functional distribution of the whole human genome (7) (Fig. 2 *B* and *C*), β-arrestin-interacting proteins are overrepresented in signal transduction (25.1%), cellular organization (15.1%), and nucleic acid binding (35.5%) categories. Other functional categories of the β-arrestin interactome include metabolic enzymatic activity (5.3%), chaperone activity (2.7%), and ion channel activity (1.8%). Having a wide subcellular and functional distribution of proteins in its interactome, β-arrestin is an adaptor/scaffold involved in a wide range of cellular processes in diverse cellular compartments. The functional specialization of the β-arrestin interactome in terms of cellular roles of the interacting proteins is further discussed below.

Cellular Communication and Signal Transduction. It is increasingly appreciated that β-arrestins play essential roles in cellular com-

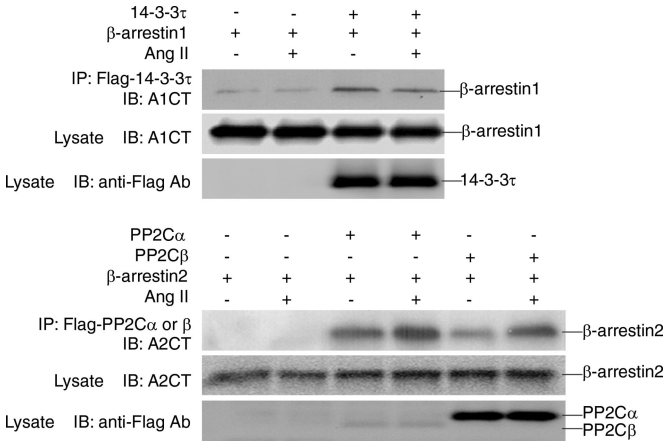

Fig. 1. Confirmation of β-arrestin-interacting proteins by coimmunoprecipitation. Shown are three examples (PP2Cα, PP2Cβ, and 14-3-3τ) of the coimmunoprecipitation assays used to validate the β-arrestin-interacting proteins identified by mass spectrometry. The cDNAs of 16 randomly selected proteins from the β-arrestin interactome were subcloned into a p3xFLAG-CMV-10 vector and transfected together with pcDNA3–β-arrestin 1 or 2 into HEK293 cells stably overexpressing HA-AT1aR. The 3xFLAG-tagged protein complexes were coimmunoprecipitated with anti-FLAG M2 affinity agarose beads and analyzed by Western blot using anti-β-arrestin antibody A1CT or A2CT. These data show representative blots from one of the three independent experiments. The detailed coimmunoprecipitation assay data are summarized in SI Table 8. Ang II, angiotensin II (100 nM).

munication and signal transduction processes in which they function as 7TMR signal transducers/mediators, scaffolding signaling molecules such as kinases and phosphatases and modulating their activities (1–3). Consistent with their roles as key signaling adaptors/scaffolds, β-arrestins were found to interact with numerous proteins involved in cellular communication and signal transduction (Tables 1 and 2). In addition to many known β-arrestin binding partners, such as ERK, casein kinase II, c-Yes, PI3-kinase, PP2A, clathrin, AP-2, DGKs, and PDE4D, a multitude of new signaling proteins were also discovered. These signaling proteins include adaptor proteins, protein kinases, phosphatases, G protein-related and small GTPase-related proteins, and proteins involved in trafficking (Tables 1 and 2).

The two major sets of adaptor proteins found in our proteomics screen are β-arrestins themselves and 14-3-3s. The identification of β-arrestins was expected because they were used as the bait proteins in the study. Interestingly, endogenous β-arrestin 1 bound to β-arrestin 2–FLAG when the latter was used as a bait, and vice versa. This result is consistent with the ability of β-arrestins to form homo- or heterodimers/oligomers (8). However, it is surprising indeed that both S-arrestin (visual arrestin) and X-arrestin (cone arrestin), which were previously thought to be expressed exclusively in the visual system, were also identified. Western blot using antibodies that specifically recognize S- or X-arrestin confirmed the expression of these two arrestin isoforms in the HEK293 cells (SI Fig. 5). It is tempting to speculate that S- and X-arrestin may be involved in the regulation of β-arrestin-mediated cellular signaling via the formation of heterodimers/oligomers with β-arrestins.

Another set of adaptor proteins found to interact with β-arrestins are the multiple isoforms of 14-3-3. The interaction of the 14-3-3τ isoform and β-arrestin was confirmed by coimmunoprecipitation assay. 14-3-3s are universal adaptors whose functional roles have been implicated in the regulation of a wide range of cellular processes via their association with a multitude of functionally dissimilar proteins (9). In the past few years, hundreds of proteins have been identified as 14-3-3 interactors through extensive proteomics studies (10, 11). The functional distribution of the 14-3-3 interactome is quite similar to the β-arrestin interactome (Fig. 2*D*). Given this, one may argue that the proteins found in this study may interact with β-arrestins through their associations with 14-3-3s. This seems unlikely because only 8% of the β-arrestin interactome overlaps with the 14-3-3 interactome (SI Fig. 6). However, because both β-arrestin and 14-3-3 are very general adaptors, future studies of the interaction between these two proteins and their cellular consequences should shed light on the mechanism of many signaling pathways.

Reversible protein phosphorylation by the interplay between kinases and phosphatases is a major regulatory mechanism for 7TMR-mediated signal transduction (1–3). β-Arrestins have been shown to play an essential role in this process by acting as scaffolds/adaptors for functionally diverse protein kinases such as the members of the MAPK family including ERK, p38, and JNK3 and phosphatases such as PP2A and MKP3 (1). With more

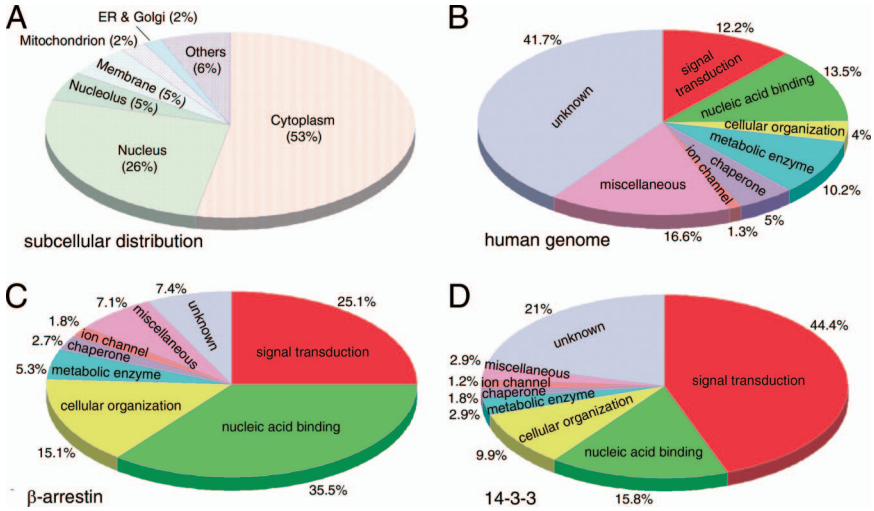

Fig. 2. Subcellular and functional distribution of β-arrestin interactome. (*A*) Subcellular distribution of β-arrestin-interacting proteins. The subcellular distribution information of proteins was obtained from the human International Protein Index database (www.ebi.ac.uk/IPI) and the Human Protein Reference Database (www.hprd.org). (*B–D*) Functional distributions of the whole human genome (*B*), β-arrestin-interacting proteins (*C*), and 14-3-3-interacting proteins (*D*). The functional distribution information of the whole human genome and 14-3-3-interacting proteins was taken from previously published reports (7, 10).

new kinases/phosphatases such as DGKs (12) emerging as novel β-arrestin binding partners, it would seem that understanding of the roles of β-arrestins as signal scaffolds/adaptors is far from complete. Consistent with this speculation, in addition to the known β-arrestin partners such as ERK, casein kinase II, DGKs, and PP2A, this study identified many new kinases and phosphatases that interact with β-arrestins (Tables 1 and 2). These proteins are implicated in a broad range of cellular processes that include signal transduction (p160ROCK, MEKK1, TAK1, and PI3-kinase), cellular organization and movement (p160ROCK, SCY1-like2, and PAK-7), cell cycle regulation and cell proliferation (Wee1A kinase, PAK-7, CDKs, and PP2C), nucleic acid synthesis and processing (NIK, MEKK1, and SRPK2), cell stress (PP2C), and apoptosis (PAK-7 and PI3-kinase). In coimmunoprecipitation assays we were able to confirm the interactions between β-arrestins and all of the kinases/phosphatases (STK38, PRP4 kinase, PP2Cα, and PP2Cβ) we tested (SI Table 8).

It is noteworthy that many proteins (e.g., Nopp140 and treacle) in the β-arrestin interactome are phosphoproteins. Thus, it is quite possible that β-arrestins act as scaffolds for the phosphorylation of these proteins by bringing kinases and their substrates into close proximity. On the other hand, it is clear that β-arrestins prefer to bind phosphorylated proteins, such as the G protein-coupled receptor kinase-phosphorylated 7TMRs. Therefore, it is also possible that β-arrestins are "phosphosensors" that scaffold phosphoproteins and regulate their activities. If so, the identification of specific phosphorylation motifs to which β-arrestins bind would be important to understand β-arrestin-mediated cell signaling.

Other signaling proteins in the β-arrestin interactome include G protein-related and small GTPase-related proteins (e.g., RGS3S and α-Pix), proteins involved in trafficking (e.g., AP-2 and VPS35), and others (e.g., calmodulin and calgranulin B). In the coimmunoprecipitation assay we confirmed the interactions between β-arrestins and two randomly selected proteins among these proteins, calmodulin and calgranulin B (SI Table 8). Although a detailed discussion of these proteins is beyond the scope of this article, the discovery of a variety of signaling molecules in the β-arrestin interactome suggests a widespread involvement of β-arrestins in signal transduction.

Cellular Organization and Movement. In the past few years, accumulating evidence has indicated that β-arrestins may play an essential role in the processes of cytoskeleton reorganization, chemotaxis, and metastasis (13). β-Arrestins are reported to mediate these processes at multiple levels either by scaffolding/activating signaling proteins (p38, ERK, PI3-kinase, or RhoA) to promote localized cellular reorganization or by directly binding cytoskeletal proteins such as filamin and cofilin (13). In the β-arrestin interactome, in addition to many signaling proteins involved in cellular organization mentioned earlier, numerous cytoskeletal proteins were also identified. These cytoskeletal proteins account for 15.1% of the β-arrestin interactome and fall into three subgroups: constituent proteins of cytoskeleton filaments, cytoskeletal accessory proteins, and motor proteins.

The β-arrestin interactome contains the prominent constituent proteins for the three major cytoskeletal filaments: actins for the actin filaments, tubulins for the microtubules, and vimentin for the intermediate filaments. Although these cytoskeletal constituent proteins are abundant in cells and several isoforms of actins were found in the control experiments because of nonspecific binding, accumulating evidence suggests that the interaction between these proteins and β-arrestins is plausible and may have functional implications. For example, a recent study demonstrated that β-arrestins bound to microtubules and regulated their activity by recruiting ERK and Mdm2 to microtubules (14). It is plausible that β-arrestins interact with and regulate the actin and intermediate filaments in a similar manner.

The second group of proteins in the β-arrestin interactome involved in cellular organization are the cytoskeletal accessory proteins. Among these are filamin, which facilitates the bundling of actin filaments and gelsolin, cofilin, and p16-ARC, which work together to regulate actin filament polymerization/depolymerization. Several other cytoskeletal accessory proteins such as p116Rip, cortactin, and CKAP4 protein are also identified. β-Arrestins have been reported to associate with filamin and cofilin and to be involved in the processes of cytoskeleton reorganization and chemotaxis (13). Similarly, β-arrestins may regulate cytoskeleton reorganization and chemotaxis via their interactions with the other cytoskeletal accessory proteins found in this study.

In addition to the cytoskeleton constituents and accessory proteins, several motor proteins, such as myosin, were also found to associate with β-arrestins. Myosin IIA (myosin-9) has been reported recently to bind to β-arrestins and play a role in the endocytosis of CXCR4 (15). Axonemal dynein and kinesins, which are involved in ciliary and flagellar transport, were also present in the β-arrestin interactome. The interaction between β-arrestins and motor proteins may play an important role in cellular processes, such as intracellular transportation and signal transduction. Thus, β-arrestins may serve as adaptors to link various types of cargo to the receptors and the cytoskeleton.

Nucleic Acid and Protein Synthesis and Processing. More than 35% of the β-arrestin interactome is composed of nucleic acid binding proteins generally involved in gene expression (Fig. 2*C*). Although most of these proteins [e.g., histones and heterogeneous ribonucleoproteins (hnRNPs)] are localized primarily in the nucleus, some (e.g., eIF-4B and PABP4) are in the cytoplasm, and some (e.g., treacle and STAT1) shuttle between these two locations. They modulate the concerted processes of gene expression at various steps including nuclear DNA replication/transcription, RNA processing, and cytoplasmic protein synthesis. Although it has been appreciated recently that β-arrestins play important roles in these processes, current knowledge in this context is limited only to the regulation of gene transcription via the interaction of β-arrestin and p300/CREB (6). The present findings suggest that much remains to be discovered about β-arrestins and the regulation of gene expression.

Functionally, the nucleic acid binding proteins in the β-arrestin interactome fall into four groups including proteins involved in DNA binding, transcription, RNA processing, and translation. The proteins in the first group are involved in DNA binding, chromatin structure, and cell cycle. Among those we identified were components of the core histones (H2A, H2B, H3, and H4) as well as members of the histone H1 family. Interestingly, interaction of β-arrestin 2 and histones was recently reported in the nucleus of mature spermatozoa (16). In our study, besides the histones, HDAC2, an enzyme responsible for deacetylating lysine residues on the N-terminal part of the core histones and important for transcription regulation, was also present, thus suggesting a possible role for β-arrestins in the regulation of chromatin remodeling and the cell cycle.

Proteins in the second group are the transcriptional regulators. More than two dozen proteins present in the β-arrestin interactome are known to be involved in the regulation of transcription. These include RNA polymerases I (RPA40, 135, and 194) and II (RPB25), transcription factors such as STAT1, YB-1, and myocardin, and transcription regulatory proteins such as TIF 1B, Tho4, and BACH2. These transcriptional regulators also include several other proteins, such as treacle, Nopp140, ZNF265, and Trap150, which have been found in almost all of the experiments. It is interesting that a recent report revealed a direct nuclear role of β-arrestin in transcription via its associa-

tion with the transcription factor CREB and the histone acetyltransferase p300 (6). The identification of more transcriptional proteins in this proteomics screen raises the strong possibility of a more general involvement of β-arrestins in transcription.

The proteins in the third group are RNA processing proteins, such as splicing factors, splicing coactivators, and ribonucleoproteins. Many hnRNPs, responsible for pre-mRNA processing, were identified. Several small nuclear ribonucleoproteins, the components of spliceosomes for splicing the introns from pre-mRNA, were also found. Interestingly, importin 90, which controls nuclear import of small nuclear ribonucleoproteins, was also identified. These results indicate that β-arrestin may also be involved in RNA splicing.

The fourth group of nucleic acid binding proteins is involved in protein synthesis. Several elongation factors, such as eEF1A and eIF-4B, were identified. As mentioned earlier, many nucleolar proteins were also identified as β-arrestin interactors, including (*i*) several high-frequency identifications such as Nopp140 and treacle, (*ii*) RNA polymerase I, and (*iii*) components of both C/D and H/ACA small nucleolar ribonucleoproteins. These nucleolar proteins, working together, are in charge of rRNA production/modification and ribosome assembly. It is surprising that >34 ribosomal proteins were also identified. Although ribosomal proteins are often observed as contaminants of coimmunoprecipitation, it is tempting to speculate that β-arrestins may associate with ribosomal proteins in the cytoplasm, nucleolus, or ribosomal assembly machinery and regulate their activities. Previously a good correlation between GPCR activation and the translational control of gene expression mediated by G protein-coupled receptor kinase 2 activation and ribosomal protein P2 phosphorylation was demonstrated (17). Thus, it is plausible that β-arrestins maybe involved in such processes. All of these data indicate a potential role of the β-arrestins in protein biosynthesis.

Other β-Arrestin-Interacting Proteins. In addition to those discussed above, proteins with other cellular functions were also identified in this proteomics screen. These proteins include metabolic enzymes [e.g., pyruvate kinase M2 (PK3) and fatty acid synthase], chaperones (e.g., HSP90 and HSC70), ion channels (e.g., Kv-β-1 and TrpV4), and proteins involved in proteolysis, ubiquitination (e.g., ubiquitin B and EDD1), and apoptosis (e.g., APLP, BIP, and mortalin 2). Although the presence of metabolic enzymes in the β-arrestin interactome is surprising, more than a dozen of them were found to interact with β-arrestins. Interestingly, most of the β-arrestin-interacting metabolic enzymes are involved in the glycolysis pathway (e.g., PK3, GAPDH, and enolase) and the energy pathways such as the mitochondrial electron transfer chain (e.g., ATP synthases and SDH2). The interaction of PK3 and β-arrestins was confirmed by coimmunoprecipitation assay (SI Table 8). The functional implications of the interactions between β-arrestin and these enzymes are yet to be established. β-Arrestins may scaffold these glycolytic enzymes and form a complex to facilitate energy production in the microenvironment. Alternatively, novel roles for several glycolytic enzymes, such as GAPDH and enolase (18), in signaling pathways have been demonstrated, and perhaps β-arrestins are involved in some of these functions. The involvement of β-arrestins in regulation of antiapoptotic signaling is just beginning to be appreciated. In this study we identified two proteins (BIP and mortalin 2) that are involved in antiapoptosis, underscoring the possible roles of β-arrestins in this process. The identification of many proteins with other functions, such as chaperone activity and ion channel activity, further expands the horizon of understanding of β-arrestin-dependent cellular processes.

Conclusion

By mass spectrometry-based proteomics approaches, we have identified 337 proteins that interact with β-arrestins. These proteins, ubiquitously distributed in the cell, have numerous functions ranging from receptor desensitization, endocytosis, and signal transduction to regulation of gene expression, protein synthesis, cellular reorganization, chemotaxis, apoptosis, and many more. It is becoming increasingly evident that the biological functions of β-arrestins are much broader than we currently understand. A complete functional understanding of the multifaceted β-arrestins will require further characterization of the interactions between β-arrestins and individual target proteins and defining the cellular consequences of these interactions. However, our current study opens up avenues of research related to novel and previously unsuspected functions of the β-arrestins.

Materials and Methods

The mass spectrometry gel-based proteomics method was conducted by SDS/PAGE separation of proteins, in-gel digestion, and nano-LC tandem MS of gel slices. The non-gel-based method, named multidimensional protein identification technique (MudPIT), was conducted by direct digestion of protein complexes, multidimensional liquid chromatography, and tandem mass spectrometry. Please refer to *SI Methods* for further details of these and other procedures used in this work.

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