Global phosphorylation analysis of β-arrestin–mediated signaling downstream of a seven transmembrane receptor (7TMR)

Kunhong Xiao[a], Jinpeng Sun[a,1], Jihee Kim[a,1], Sudarshan Rajagopal[a], Bo Zhai[b], Judit Villén[b], Wilhelm Haas[b], Jeffrey J. Kovacs[a], Arun K. Shukla[a], Makoto R. Hara[a], Marylens Hernandez[c], Alexander Lachmann[c], Shan Zhao[c], Yuan Lin[a], Yishan Cheng[a], Kensaku Mizuno[d], Avi Ma'ayan[c], Steven P. Gygi[b,2], and Robert J. Lefkowitz[a,e,f,2]

Departments of [a]Medicine and [e]Biochemistry and [f]Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC 27710; [b]Department of Cell Biology, Harvard University Medical School, Boston, MA 02115; [c]Department of Pharmacology and Systems Therapeutics, Systems Biology Center New York, Mount Sinai School of Medicine, New York, NY 10029; and [d]Department of Biomolecular Sciences, Graduate School of Life Sciences, Tohoku University, Sendai, Miyagi 980-8578, Japan

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β-Arrestin–mediated signaling downstream of seven transmembrane receptors (7TMRs) is a relatively new paradigm for signaling by these receptors. We examined changes in protein phosphorylation occurring when HEK293 cells expressing the angiotensin II type 1A receptor (AT1aR) were stimulated with the β-arrestin–biased ligand Sar^1, Ile^4, Ile^8-angiotensin (SII), a ligand previously found to signal through β-arrestin–dependent, G protein-independent mechanisms. Using a phospho-antibody array containing 46 antibodies against signaling molecules, we found that phosphorylation of 35 proteins increased upon SII stimulation. These SII-mediated phosphorylation events were abrogated after depletion of β-arrestin 2 through siRNA-mediated knockdown. We also performed an MS-based quantitative phosphoproteome analysis after SII stimulation using a strategy of stable isotope labeling of amino acids in cell culture (SILAC). We identified 1,555 phosphoproteins (4,552 unique phosphopeptides), of which 171 proteins (222 phosphopeptides) showed increased phosphorylation, and 53 (66 phosphopeptides) showed decreased phosphorylation upon SII stimulation of the AT1aR. This study identified 38 protein kinases and three phosphatases whose phosphorylation status changed upon SII treatment. Using computational approaches, we performed system-based analyses examining the β-arrestin–mediated phosphoproteome including construction of a kinase-substrate network for β-arrestin–mediated AT1aR signaling. Our analysis demonstrates that β-arrestin–dependent signaling processes are more diverse than previously appreciated. Notably, our analysis identifies an AT1aR-mediated cytoskeletal reorganization network whereby β-arrestin regulates phosphorylation of several key proteins, including cofilin and slingshot. This study provides a system-based view of β-arrestin–mediated phosphorylation events downstream of a 7TMR and opens avenues for research in a rapidly evolving area of 7TMR signaling.

G protein-coupled receptor | GPCR | phosphoproteome

Seven transmembrane receptors (7TMRs), also known as "G protein-coupled receptors," are encoded by nearly 1,000 genes in the human genome and regulate virtually all known physiological processes in humans (1). In the classical paradigm, 7TMR signaling is mediated through the activation of heterotrimeric G proteins, generally referred to as "G-protein–mediated signaling" (1). Dissociation of Gα and Gβγ subunits, each of which signals to downstream effectors, leads to changes in cellular physiology. Following activation, 7TMRs are phosphorylated by G protein-coupled receptor kinases, and subsequently recruit cytosolic β-arrestins. β-Arrestin binding uncouples the receptors from G protein subunits and desensitizes G protein-mediated signaling. More recently, however, it has been shown that, in addition to their desensitizing actions, β-arrestins also serve as multifunctional adaptors and signal transducers, linking the receptors, in an activation-dependent manner, to a growing list of endocytic and signaling molecules [e.g., MAPK, Src, and protein kinase B (AKT)], initiating a newly appreciated method of signaling referred to as "β-arrestin–mediated signaling" (2). Although G protein-dependent signaling downstream of 7TMRs has been studied for many years, a complete understanding of G protein-independent, β-arrestin–mediated signaling is still emerging.

β-Arrestin–mediated 7TMR signaling is an evolving paradigm for 7TMR signaling and function (3). Consequently, developing a global understanding of the signaling events downstream of β-arrestins is of great importance. Recent advances in the techniques and strategies of systems biology and MS-based quantitative proteomics (4–7) offer great opportunities to investigate β-arrestin–mediated signaling on a large scale. To gain insight into the intricacies of β-arrestin–mediated signaling, we previously performed a large-scale MS-based proteomic analysis of β-arrestin interacting partners (the "β-arrestin interactome") with and without angiotensin II (AngII) stimulation of the angiotensin II type 1A receptor (AT1aR) (8). Because many cellular signaling events involve agonist-induced reversible phosphorylation of key targets, in the present study we carried out an MS-based quantitative phosphoproteomics screen downstream of AT1aR upon stimulation by Sar^1, Ile^4, Ile^8-angiotensin (SII). SII is a known β-arrestin "biased ligand" for AT1aR, which is unable to activate G protein-dependent signaling as evidenced by a lack of phosphatidylinositol hydrolysis, calcium mobilization, or diacylglycerol activity (9, 10). However, SII can recruit β-arrestin to the AT1aR and stimulate ERK and several other biochemical effector mechanisms in an entirely β-arrestin–dependent manner (9). Using this β-arrestin–biased ligand, we selectively activated β-arrestin–mediated signaling and identified proteins that are phosphorylated or dephosphorylated downstream of AT1aR in a β-arrestin–dependent fashion. Subsequently, we used multiple bioinformatic and systems biology approaches to construct a kinase network involved in β-arrestin–dependent signaling downstream of the AT1aR.

Results and Discussion

β-Arrestin–Dependent Phosphorylation Events Downstream of AT1aR Are Activated by SII. Because protein phosphorylation is fundamental to many aspects of cell signaling, we set out to interrogate the mechanisms involved in β-arrestin–mediated signaling by exploring SII-induced phosphorylation events. In an initial approach, we used a human phospho-antibody array (Human Phospho-Kinase Array, Proteome Profiler Array Kit, ARY003; R&D Systems) to study a subset of phosphorylation events after

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The authors declare no conflict of interest.

[1]J.S. and J.K. contributed equally to this work.

[2]To whom correspondence may be addressed. E-mail: lefko001@receptor-biol.duke.edu and steven_gygi@hms.harvard.edu.

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SYSTEMS BIOLOGY

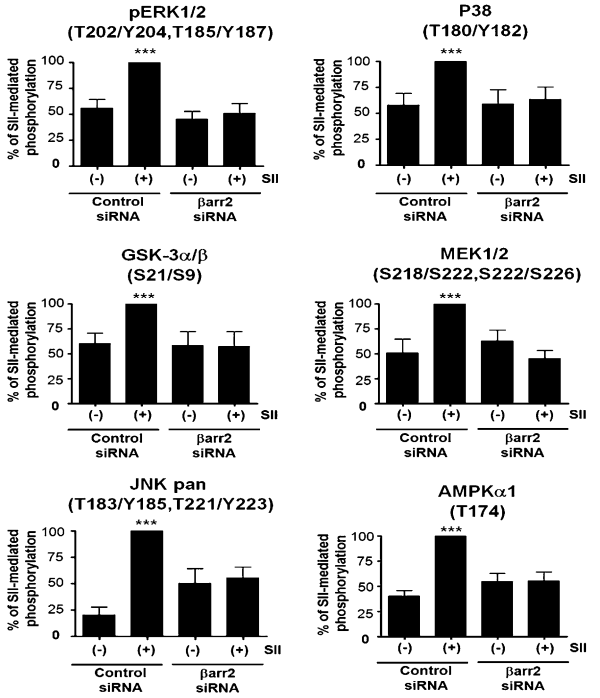
SII stimulation. Furthermore, to assess the degree to which these events depend on β-arrestin, HEK293 cells transiently expressing AT1aR were stimulated with or without SII under conditions in which β-arrestin 2 was present or knocked down using siRNA technology. Among 46 phospho-antibodies (most for proteins known to play roles in cell signaling) spotted on the array, the relative extent of phosphorylation of 35 proteins increased upon SII stimulation relative to nonstimulated samples (Fig. 1 and Fig. S1). This high percentage of proteins observed to undergo SII-stimulated changes in phosphorylation may reflect the fact that the proteins on the array are preselected kinases known to be involved in important signaling pathways. When β-arrestin 2 was depleted by siRNA technology, the SII-induced changes in phosphorylation detected by all 35 antibodies were largely abrogated, consistent with SII-mediated phosphorylation events being β-arrestin dependent.

Quantitative Phosphoproteome Analysis of AT1aR Signaling upon SII Stimulation. Findings with the phospho-antibody array prompted us to investigate protein phosphorylation profiles stimulated by SII activation of AT1aR signaling on a large scale using MS-based quantitative phosphoproteomics technology (11) and a strategy of stable isotope labeling of amino acids in cell culture (SILAC) (Fig. S2 *A–C*) (12). In three independent experiments we identified a total of 14,328 phosphopeptides with 5,046, 5,332, and 3,950 phosphopeptides (in experiments 1, 2, and 3, respectively). Statistical analysis of the data from these three independent experiments showed good correlations between the three data sets (Fig. S3*A*). After removing the redundant peptides, we identified 4,552 unique phosphopeptides (unique phosphorylation events) in 1,555 phosphoproteins (Table S1). To determine phosphorylation sites in these peptides and measure the confidence of assignment, we applied the Ascore algorithm, which computes the probability that a difference in detected site-determining ions in a MS/MS spectrum between two potential site locations occurred by chance (13). We considered sites with Ascore ≥ 13 ($P \leq 0.05$) to be confidently localized. All calculated Ascores are listed in Table S1.

To compare quantitatively the AT1aR phosphoproteome from untreated HEK293 cells with that of cells from stimulated for 5 min with SII, the relative abundances of coeluting "light" (untreated) and "heavy" (SII-treated) phosphopeptide pairs were calculated using a previously described software program (14). Each calculated abundance ratio (Vista ratio) reflects the change in the extent of phosphorylation of a peptide upon SII stimulation. We measured the Vista ratios of phosphopeptides in three independent experiments (Fig. S3*B* and Table S1). We chose a 1.5-fold ratio change (which is equivalent to three times the SD of the $\log_2$ transformation of Vista ratios) as a cutoff and considered an increase of more than 1.5-fold or a decrease of more than 33.3% as a significant increase or decrease in phosphorylation level. We define a "β-arrestin–mediated phosphopeptide" as one whose phosphorylation is increased by at least 1.5-fold or decreased by at least 33.3% in at least two independent experiments. Using this criterion, we have established a "β-arrestin–mediated phosphoproteome" (Table S2) including 288 phosphopeptides ("β-arrestin–mediated phosphopeptides") from 220 phosphoproteins ("β-arrestin–mediated phosphoproteins"). Among these 288 phosphopeptides, the phosphorylation status of 222 peptides (from 171 different phosphoproteins) increased, and the phosphorylation status of 66 peptides (from 53 different phosphoproteins) decreased upon SII stimulation of AT1aR. Table 1 lists some representative β-arrestin–mediated phosphopeptides.

Some phosphorylation sites in this β-arrestin–mediated phosphoproteome, such as T202/Y204 of ERK1 and T184/Y186 of ERK2, have been characterized previously as undergoing β-arrestin–dependent phosphorylation upon SII stimulation of AT1aR (9). The phosphorylation of T202/Y204 of ERK1 and T184/Y186 of ERK2 increased 1.5- to 2.4-fold using this technique. This result indicates that the 1.5-fold change threshold we chose may reflect a physiologically significant phosphorylation change. Furthermore, because most of the β-arrestin–mediated phosphorylation sites we identified have not been observed previously in the context of β-arrestin–mediated 7TMR signaling, we went on to validate five selected sites using commercially available antibodies for Western blotting analysis. All SII-induced phosphorylation changes measured by Western blotting analysis confirmed the phosphoproteome data (Fig. S2*D*).

A



B

ERK (T202/Y204, T185/Y187)	JNK pan (T183/Y185, T221/Y223)
MEK1/2 (S218/S222, S222/S226)	Lck (Y394)
Akt (T308)	Lyn (Y397)
AMPKα1 (T174)	Pyk2 (Y402)
AMPKα2 (T172)	Yes (Y426)
Chk-2 (T68)	MSK1/2 (S376/S360)
PLCγ-1 (Y783)	mTOR (S2448)
FAK (Y397)	p27 (T157)
Fgr (Y412)	P38α (T180/Y182)
Fyn (Y420)	p70S6 (421/422)
GSK-3α/β (S21/S9)	p70S6 (T229)
Hck (Y411)	p70S6 (T389)
HSP27 (S78/S82)	RSK1/2/3 (S380)
RSK1/2 (S221)	STAT5a (Y699)
Src (Y419)	STAT5a/b (Y699)
STAT2 (Y689)	STAT5b (Y699)
STAT3 (Y705)	STAT6 (Y641)
STAT4 (Y693)	

Fig. 1. Human phospho-antibody array analysis reveals that phosphorylation events induced by SII are primarily β-arrestin–dependent. (*A*) Normalized intensities for six representative phosphoproteins from samples applied to the phospho-antibody array. Lysates were from HEK293 cells transfected with either control or β-arrestin 2 (βarr2) siRNA followed by 30 μM SII stimulation for 5 min [SII(+)] or no stimulation [SII(–)]. The normalized intensity for each antibody was calculated as a percentage of the corresponding SII-stimulated sample treated with control siRNA. The experiment was repeated at least three times, and statistical analysis was performed using a one-way ANOVA. ***$P < 0.001$. (*B*) A list of β-arrestin–regulated phosphoproteins revealed by human phospho-antibody array analysis. The residues indicated in parentheses are the phosphorylation sites.

Protein Kinases and Phosphatases Are Overrepresented in the β-Arrestin–Mediated Phosphoproteome. Protein kinases and phosphatases are critical for reversible phosphorylation of important signaling proteins. Most of these enzymes are themselves regulated via reversible phosphorylation. It is notable that among the 220 β-arrestin–mediated phosphoproteins identified in this study, 26 are protein kinases (more than would be expected by chance; $P < 10^{-6}$; Fisher test), and three are phosphatases (Table S3). Together with those identified in the phospho-antibody array analysis, we identified 38 protein kinases that are regulated by SII stimulation (Table S3). Included in this group of 38 are kinases in the MAPK

Table 1. Representative phosphoproteins identified in phosphoproteome analysis

Protein name	Phospho-peptide sequence*	Phosphorylation site	Ascore	Relative abundances† Sii (+)/Sii (–)	
				Fold Increase	% decrease
SH3 and multiple ankyrin repeat domains 3	K**pS**PIAAARLFS**pS**LGELSSISAQR	S1533/S1543	44.3/5.4	↑ 773.8-fold	
Nucleoporin NDC1	KLN**pS**PEETAFQTPK	S406	97.8	↑ 27.6 -fold	
Protein phosphatase 1, catalytic subunit, α	YGQFSGLNPGGRPITPPRN**pS**AK	S336	24.2	↑ 25-fold	
PRKD1	SFRR**pS**VVGTPAYLAPEVLR	S744	8.7	↑ 13.1-fold	
MEKK3	SAD**pS**ENALSVQER	S340	22.9	↑ 4.3-fold	
Jun	AKNSDLLT**pS**PDVGLLKLApSPELER	S63/S73	5.9/74.7	↑ 4.1-fold	
MYPT1	**pS**TQGVTLTDLQEAEK	S695	11.4	↑ 2.7-fold	
Rap guanine nucleotide exchange factor 2	QAEDTISNASSQLS**pS**PP**pT**SPQSSPR	S1116/T1119	18.1/13.1	↑ 2.5-fold	
TP53BP1	STPFIVP**pS**SPTEQEGRQDKPMDTSVLSEEGGEPFQK	S379	14.9	↑ 2.3-fold	
PAK2	GTEAPAVV**pT**EEEDDDEETAPPVIAPRPDHTK	T169	72.4	↑ 2.2-fold	
MAP4K5	RQSSP**pS**CGPVAETSSIGNGDGISK	S436	8.5	↑ 2.1-fold	
PEA15	DIIRQP**pS**EEEIIK	S116	1000	↑ 2.0 -fold	
ERK1	IADPEHDHTGFL*pT*E**pY**VATR	T202/Y204	60.3/65.2	↑ 1.9-fold	
GSK3β	GEPNVS**pY**ICSR	Y216	26.4	↑ 1.9 -fold	
MEK1	RSDAEEVDFAGWLCSTIGLNQPS**pT**PTHAAGV	T385	19.8	↑ 1.8 -fold	
CDK2	TY**pT**HEVVTLWYRAPEILLGCK	T160	16.2	↑ 1.8 -fold	
PRKD2	RLS**pS**T**pS**LASGHSVR	S197/S203	5.9/13.2	↑ 1.7 -fold	
AKT1	SGSPSDN**pS**GAEEMEVSLAKPK	S129	17.6	↑ 1.7 -fold	
ERK2	VADPDHDHTGFL**pT**E**pY**VATRWYR	T185/Y187	31.1/28.4	↑ 1.7-fold	
p90RSK	TPKD**pS**PGIPP**pS**AGAHQLFR	S363/S369	42.1/70.5	↑ 1.7 -fold	
GIT1	HG**pS**GADSDYENTQSGDPLLGLEGKR	S592	44.2	↑ 1.7 -fold	
p38	HTDDEM**pT**GYVATR	T180	43	↑ 1.6-fold	
PTK2	YMEDSTpYpYKASK	Y576/Y577	16.2/33.7	↑ 1.6 -fold	
NIK	KQSSV**pY**KLEAVEK	Y40	57.8	↑ 1.6 -fold	
TAK1	**pS**IQDLTVTGTEPGQVSSR	S412	72.4	↑ 1.5-fold	
GSK3α	ARTSpSFAEPGGGGGGGGGGPGGSASGPGGTGGGK	S21	24.4	↑ 1.5-fold	
Cortactin	AK**pT**QTPPVSPAPQPTEERLPSSPVYEDAASFK	T400	24.2		↓ 37%
Nadrin	SPSPP**pT**QHTGQPPGQPSAPSQLSAPR	T679	28.3		↓ 39%
NUP98	YGLQD**pS**DEEEEEHPSKTSTK	S888	78.7		↓ 45%
Slingshot	SS**pS**SD**pS**IHSVR	S937/S940	16.2/21.3		↓ 47%
PAK4	GAPSPGVLGPHASEPQLAPPACTPAAPAVPGPPGPR**pS**PQREPQR	S291	46.4		↓ 52%
c-SRC	KEPEERPTFEYLQAFLEDYFTSTEPQ**pY**QPGENL	Y530	52.6		↓ 53%
MDC1	SSVK**pT**PEPVVPTAPELQPSTSTDQPVTSEPTYQATR	T975	18.4		↓ 60%
NOM1	QLVGTVS**pS**KILELAR	S640	10.3		↓ 99%

*Phosphorylation sites are indicated in bold.

†Relative abundances of phosphopeptides in samples with [SII(+)] and without [SII (–)] SII treatment. Increases of more than 1.5-fold or decreases of more than 33.3% in relative abundance were chosen as cutoffs for significant increases or decreases in phosphorylation.

signaling cascades, p21 protein (Cdc42/Rac)-activated kinases, ribosomal protein S6 kinases, 5′-AMP–activated protein kinases, and kinases involved in cell-cycle regulation. Nonreceptor tyrosine kinases, such as Src, Yes, Lck, Hck, Lyn, Fyn, proline-rich tyrosine kinase, and focal adhesion kinase, also are present in the β-arrestin–mediated phosphoproteome (Fig. 2). The three phosphatases we identified are the serine/threonine phosphatase protein phosphatase 1, the dual-specificity phosphatase slingshot 1, and the SH2 containing protein tyrosine phosphatase 2 (SHP2) (Table S3).

An advantage of the global proteomic approach used here is that details of β-arrestin–dependent signaling networks can be elucidated simply by examining the phosphorylation status of a molecule and comparing it with previously published observations. For example, dephosphorylation of Src Tyr-530 by protein tyrosine phosphatases has been proposed as a likely mechanism for its activation (15). Interestingly, Src was found to be dephosphorylated at its inhibitory site Tyr-530 in our phosphoproteome, suggesting potential involvement of β-arrestins in activation of Src in response to AT1aR activation. Activation of Src was the first β-arrestin–dependent signaling mechanism identified more than 10 y ago.

Phosphorylation Motif and Kinase Enrichment Analysis Reveal Kinases Crucial for β-Arrestin–Mediated Signaling Downstream of AT1aR. To identify candidate kinases that may play roles in regulating the phosphorylation status of the β-arrestin–mediated phosphoproteome, we used the Motif-X program (16) to identify phosphorylation motifs present in the β-arrestin–mediated phosphopeptides and compared them with known consensus sequences of protein

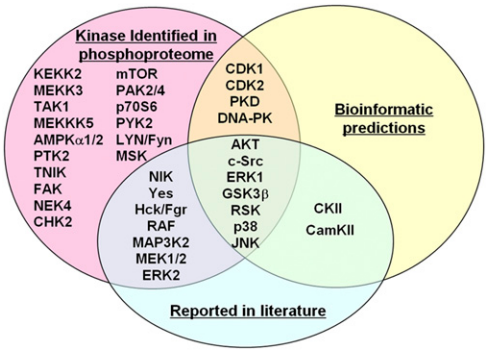


Fig. 2. Protein kinases involved in β-arrestin–mediated cellular signaling downstream of AT1aR. A comparison of experimentally identified, bioinformatically predicted, and previously reported protein kinases involved in β-arrestin–mediated signaling. Bioinformatic predictions were performed using Motif-X analysis and KEA as described in the text.

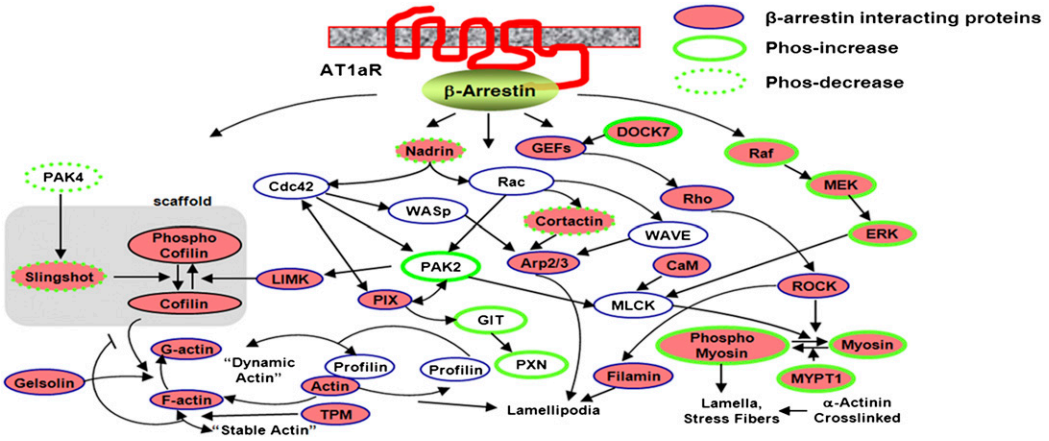


Fig. 3. A β-arrestin–dependent cytoskeletal reorganization network downstream of AT1aR. β-Arrestin–mediated phosphoproteome and β-arrestin interactome revealed that many proteins in cytoskeletal reorganization pathways were regulated downstream of AT1aR/β-arrestin, suggesting a critical role of β-arrestins in cytoskeletal reorganization. The β-arrestin–interacting proteins were identified in the previous β-arrestin interactome study (8) or in biochemical experiments in the current study.

kinases. We found that the consensus sequences for proline-directed kinases [such as MAPKs and cyclin-dependent kinase (CDKs)], AKT, protein kinase D (PKD), calmodulin-dependent protein kinase (CaMKII), and casein kinase II are enriched in the β-arrestin–mediated phosphopeptides (Fig. S4).

In another approach to identifying candidate kinases for the quantitative phosphoproteomics dataset, we used Kinase Enrichment Analysis (KEA) (17). KEA uses a kinase-substrate database of reported mammalian kinase–substrate interactions and protein–protein interactions that involve kinases to compute enrichment for kinase substrates. This analysis identified 10 protein kinases [DNAPK, AKT1, CDK1, CDK2, glycogen synthase kinase 3**β** (GSK3β), Src, and p90RSK3] as well as several kinases in the MAPK pathway (Table S4), consistent with the kinases that were found to be differentially phosphorylated and those identified by Motif-X.

We then compared the kinases identified in this study with those that previously have been reported to be involved in β-arrestin–mediated signaling. As shown in Fig. 2, a number of kinases identified or predicted here have been reported previously to play important roles in β-arrestin–mediated signaling. This comparison demonstrates a good correlation between the phosphoproteomics or bioinformatic data and findings using traditional approaches, although here we have identified a number of previously unreported kinases that appear to be important for β-arrestin–mediated cellular signaling.

Signaling Pathway and Subnetwork Analyses Reveal a β-Arrestin–Dependent Cytoskeletal Reorganization Network Downstream of AT1aR. To analyze the global extent of β-arrestin–mediated AT1aR signaling, we performed a number of bioinformatic analyses that yield complementary information. These analyses include Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) canonical pathway, and Ingenuity subnetwork analyses. These analyses are described in detail in Figs. S5 and S6 *A–H* and Tables S5 and S6. Recent work has shown that β-arrestins play an important role in cytoskeletal reorganization through the regulation of a number of proteins downstream of 7TMRs, including LIM kinase, cofilin, RhoA, and myosin light-chain kinase (18). We found that cytoskeletal reorganization was one of the pathways enriched by both pathway and subnetwork analyses, with a number of key regulators of cytoskeletal reorganization showing changes in phosphorylation status upon SII stimulation of AT1aR. With this phosphoproteomics data, in combination with the previously published β-arrestin interactome (8) and the β-arrestin signaling partners characterized in this study, we identified a β-arrestin–dependent cytoskeletal reorganization subnetwork downstream of AT1aR (Fig. 3).

To test the validity of this putative subnetwork, we examined the β-arrestin–dependent dephosphorylation of slingshot and its effects on cytoskeletal reorganization. Slingshot is a phosphatase that regulates actin filament dynamics via dephosphorylation and activation of the actin depolymerizing protein cofilin. The phosphatase activity of slingshot is known to be inactivated by phosphorylation at Ser-937 and Ser-978 (19). Our phosphoproteome data showed that phosphorylation of slingshot Ser-937 and -940 decreased 2-fold upon SII stimulation, indicating activation of slingshot downstream of AT1aR in a β-arrestin–dependent manner. We did not detect phosphorylation of Ser-978, a well-characterized phosphorylation site of slingshot that is associated with its activation. This site probably was not observed because of the current technical limitations of phosphoproteomic screens that capture only a fraction of

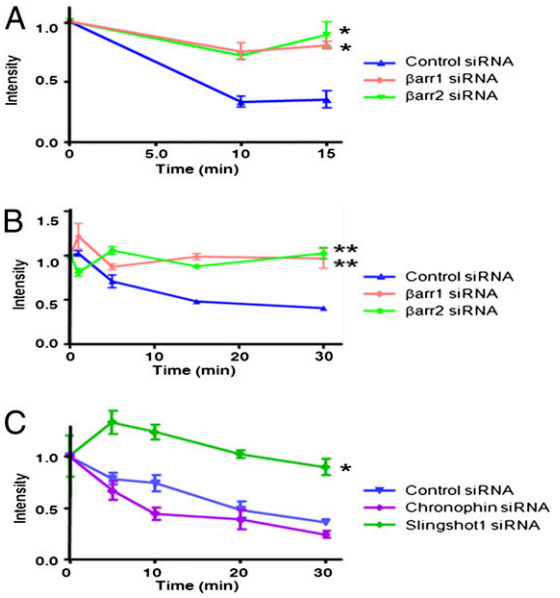


Fig. 4. SII stimulation leads to β-arrestin–mediated dephosphorylation of slingshot and cofilin downstream of AT1aR. HEK293 cells stably overexpressing AT1aR were stimulated with 30 μM SII for the indicated time periods. (*A*) β-Arrestin–dependent dephosphorylation of slingshot. The cell lysates were probed with the slingshot pSer-978 antibody in the presence of control, β-arrestin1 (βarr1), or β-arrestin 2 (βarr2) siRNAs. The ratio of phospho-slingshot/total slingshot was calculated for each data point and plotted as fold decrease from the nonstimulated sample. (*B*) β-Arrestin–dependent dephosphorylation of cofilin. The cell lysates were probed using cofilin pSer-3 antibody. The ratio of phospho-cofilin/total cofilin was calculated for each data point and plotted as fold decrease from the nonstimulated sample. (*C*) Cofilin pSer-3 dephosphorylation downstream of AT1aR is mediated via slingshot and not via chronophin. HEK293 cells stably overexpressing AT1aRs were transfected with control, chronophin, or slingshot1/2 siRNA. Cofilin dephosphorylation was probed with phospho-cofilin Ser-3. The phospho-cofilin/total cofilin ratio was calculated for each data point and plotted as fold decrease from the nonstimulated sample. The data presented here are from at least three independent experiments. Statistical analysis was done using two-way ANOVA. $*P < 0.05$; $**P < 0.01$.

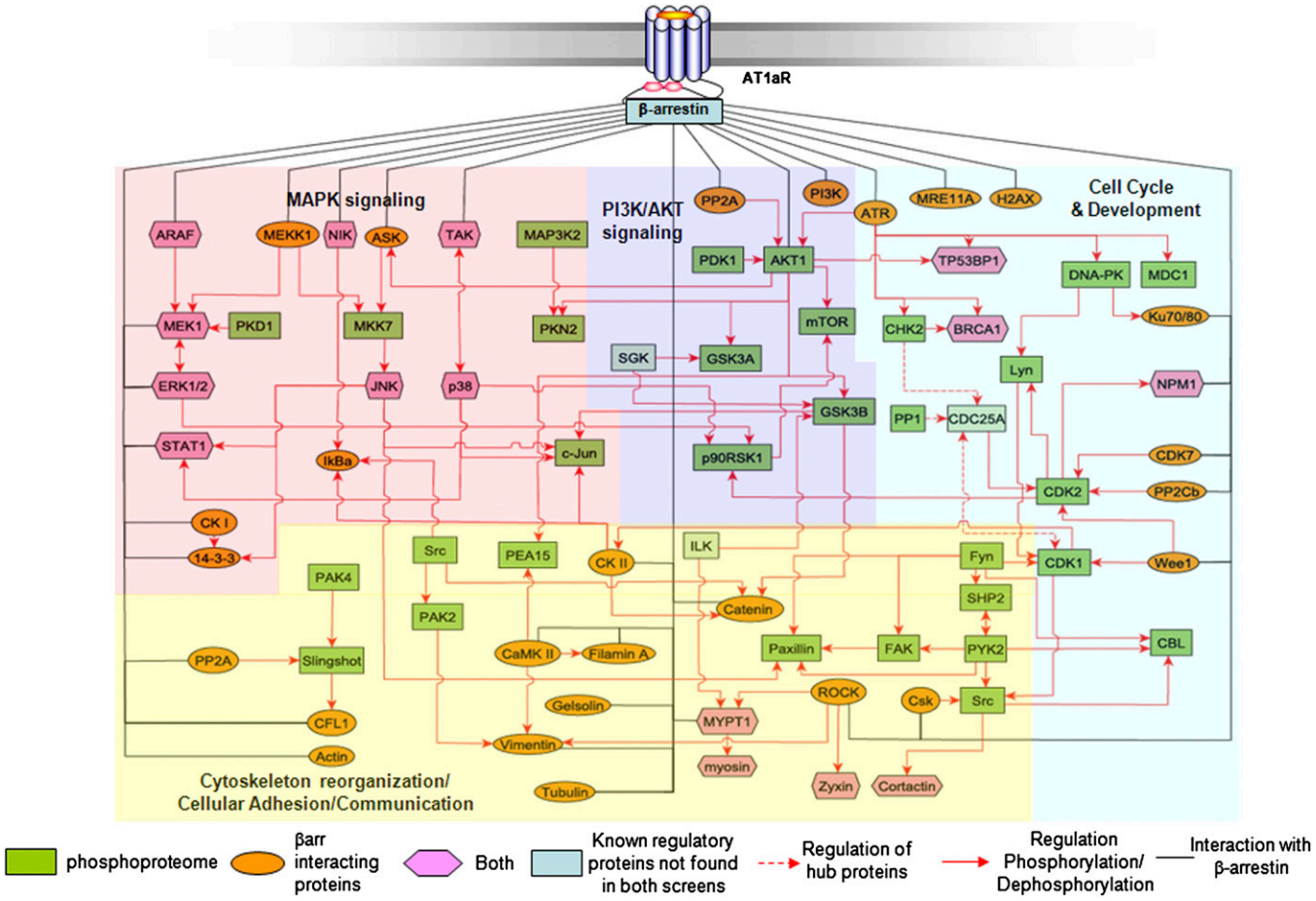


Fig. 5. β-arrestin–dependent kinase network downstream of AT1aR revealed by the β-arrestin–mediated phosphoproteome and β-arrestin interactome. The network was generated by an inference algorithm (24) and literature-based kinome network (17) as described in the text and *SI Text*. This network covers a broad range of cellular functions including MAPK and PI3K/AKT signaling, cytoskeletal reorganization, cellular adhesion, cell-to-cell communication, and cell cycle and development. Green rectangle, identified in β-arrestin–mediated phosphoproteome; orange ellipse, β-arrestin–interacting proteins; pink hexagon, identified in β-arrestin–mediated phosphoproteome and β-arrestin interactome; gray rectangle, "hub proteins" important for the signaling network but not identified in either β-arrestin–mediated phosphoproteome or β-arrestin interactome; red arrow, regulation of phosphorylation/dephosphorylation; black line, interaction with β-arrestin; dashed line, regulation of hub proteins. Several known β-arrestin–interacting proteins (ARAF, MEK1, p38, and JNK) that were not in our β-arrestin interactome are indicated also.

relevant peptides. We then used a slingshot phospho-antibody targeting pSer-978 (the only phospho-slingshot antibody available) to demonstrate a time-dependent activity profile. Upon SII stimulation of AT1aR, the phosphorylation level of slingshot decreased more than twofold at 10 and 15 min (Fig. 4*A* and Fig. S7*A*). In addition, either β-arrestin 1 or 2 siRNA ablated slingshot dephosphorylation and activation, confirming its β-arrestin–mediated activation (Fig. 4*A* and Fig. S7 *A* and *D*).

Activated slingshot dephosphorylates cofilin at Ser-3 (Fig. S8), thereby activating its actin-binding/depolymerizing ability and promoting actin reorganization and lamellipodia formation (20). In the AT1aR system, we used a phospho-antibody against pSer-3 and demonstrated SII stimulation-dependent dephosphorylation of cofilin (Fig. 4*B* and Fig. S7*B*). Knockdown of β-arrestin 1 or 2 significantly blocked SII-mediated cofilin dephosphorylation, indicating β-arrestin–dependent activation of cofilin. Because previous studies on the proteinase-activated receptor 2 suggested that β-arrestin–mediated cofilin dephosphorylation was regulated by the phosphatase chronophin (21), we next studied cofilin dephosphorylation when chronophin or slingshot was individually depleted from cells by siRNA. Knockdown of chronophin had no effect on cofilin dephosphorylation (Fig.4*C* and Fig. S7 *C* and *D*). However, knockdown of slingshot 1 blocked SII stimulation-induced cofilin dephosphorylation. This result suggests that slingshot, and not chronophin, regulates β-arrestin–dependent cofilin dephosphorylation downstream of the AT1aR.

To test the hypothesis that β-arrestins scaffold slingshot to cofilin, we overexpressed FLAG-tagged β-arrestin 1 or 2 in HEK293 cells overexpressing the AT1aR and stimulated the cells for 5 min with SII or vehicle control. Immunoprecipitation with an anti-FLAG antibody demonstrated an association between β-arrestins and slingshot that increased upon SII stimulation (Fig. S7*E*, *Upper*). This interaction is direct, as shown by in vitro GST pull-down experiment (Fig. S7*E*, *Lower*). Cofilin is known to interact with β-arrestin (8), so it is likely that β-arrestin scaffolds a protein complex that contains both slingshot and cofilin, thereby promoting slingshot-dependent cofilin dephosphorylation. These findings verify part of our signaling subnetwork and demonstrate the hypothesis-generating potential of this combined proteomics (8) and phosphoproteomics dataset. Furthermore, these findings, coupled with our previous observation that PP2A and β-arrestin form a complex (8, 22), suggest that β-arrestin may activate slingshot through the recruitment and scaffolding of PP2A which dephosphorylates slingshot to activate its phosphatase activity.

A β-Arrestin–Dependent Kinase Network Downstream of the AT1aR Revealed by the β-Arrestin–Mediated Phosphoproteome. Signals that are transmitted inside cells do not go through linear pathway cascades but rather through highly interconnected networks (23). The identification of the β-arrestin–mediated phosphoproteome offers a system wide view of the phosphorylation signature of the entire β-arrestin signaling network and provides an opportunity to

connect β-arrestin–mediated phosphoproteins to kinases and other proteins that have been reported to regulate them using an inference algorithm (24) and a previously constructed literature-based kinome network (17). Using these approaches (*SI Materials and Methods*), we were able to infer the activation or inhibition of phosphosites detected on kinases in our study (24). These results then were compared with 38 other phosphoproteomics experiments reported for mammalian cells in the literature, validating our predictions for the function, activation, or inhibition of protein kinase phosphosites (further details are given in *SI Materials and Methods* and ref. 24). By combining this kinase-substrate subnetwork and the β-arrestin interactome, along with additional curation, we constructed a cell-signaling network specific for β-arrestin–mediated AT1aR signaling (Fig. 5).

This β-arrestin–mediated AT1aR network provides a global picture of how β-arrestins function to relay messages from AT1aRs at the cell surface, through protein phosphorylation mechanisms, to a wide variety of cellular effectors. This network covers a broad range of cellular functions and processes such as MAPK and PI3K/AKT signaling, cytoskeletal reorganization, cellular adhesion, cell-to-cell communication, and cell cycle and development. For example, this network identifies several key molecules involved in cell-cycle regulation that are subject to β-arrestin–dependent signaling, including CDK1, CDK2, Fyn, SHP2, HSF1, and Lyn. All these proteins are important in keeping the cell cycle from progressing until repairs to damaged DNA have been completed (25) and are linked to a subnetwork for DNA repair in the β-arrestin–mediated kinase network.

A very recent study also used a SILAC-based approach to examine phosphorylation events downstream of stimulated AT1aR in HEK 293 cells (26). Christensen et al. (26) used a TiO_2 phosphopeptide enrichment approach and performed analysis after stimulation with SII or Ang II. However, in their study no attempt was made to relate any of the SII-mediated events to β-arrestin–mediated signaling. As would be expected, there was significant overlap (~30%) in these two studies in the phosphoproteins regulated by SII stimulation of the AT1aR; however, a significant number of proteins are unique to one study or the other, probably because the different phosphopeptide enrichment methods, TiO_2 and immobilized metal ion affinity chromatography (IMAC), detect different, partially overlapping fractions of the phosphoproteome (27). In their study, Christensen et al. identified PKD as an important kinase regulating downstream signaling after SII stimulation, a finding that was confirmed in our analysis. However, although Christensen et al. reported more phosphoproteins that changed because of stimulation, with our more extensive bioinformatic analysis, we have constructed a much broader and more specific β-arrestin–mediated kinase-substrate network that includes additional kinases that, like PKD, regulate many phosphorylation events after AT1aR activation. This β-arrestin–mediated signaling network identifies roles for β-arrestins in a number of cellular pathways, such as cytoskeletal reorganization, DNA damage repair, and MAPK signaling. We also validated the specific role of slingshot in β-arrestin–mediated signaling. Taken together, these complementary studies point the way toward a more global understanding of signaling downstream of 7TMRs.

Materials and Methods

A SILAC strategy was used for quantitative phosphoproteome analysis. Phosphopeptides were prepared by strong cation exchange fractionation followed by IMAC enrichment. Peptide samples were analyzed on a LTQ Orbitrap XL mass spectrometer (Thermo Scientific) or a LTQ-FT mass spectrometer (Thermo Scientific). MS/MS spectra were searched via the SEQUEST algorithm (28) against a composite database containing the human International Protein Index protein database and its reversed complement.

Further details of these and other procedures used in this work are given in *SI Materials and Methods*.

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Correction

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Correction for "Global phosphorylation analysis of β-arrestin–mediated signaling downstream of a seven transmembrane receptor (7TMR)," by Kunhong Xiao, Jinpeng Sun, Jihee Kim, Sudarshan Rajagopal, Bo Zhai, Judit Villén, Wilhelm Haas, Jeffrey J. Kovacs, Arun K. Shukla, Makoto R. Hara, Marylens Hernandez, Alexander Lachmann, Shan Zhao, Yuan Lin, Yishan Cheng, Kensaku Mizuno, Avi Ma'ayan, Steven P. Gygi, and Robert J. Lefkowitz, which appeared in issue 34, August 24, 2010, of *Proc Natl Acad Sci USA* (107:15299–15304; first published August 4, 2010; 10.1073/pnas.1008461107).

The authors note that within the footnotes, the line "This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1008461107/-/DCSupplemental and http://www.lefkolab.org/fileadmin/lefko/pdf/" should instead appear as "This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1008461107/-/DCSupplemental and http://lefko-gpcr-phosphoproteome-data.org/pdf/." The online version has been corrected.

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