β-Arrestin1, which regulates many aspects of seven transmembrane receptor (7TMR) biology, has also been shown to serve as an adaptor, which brings Mdm2, an E3 ubiquitin ligase to the insulin-like growth factor-1 receptor (IGF-1R), leading to its proteasome-dependent destruction. Here we demonstrate that IGF-1R stimulation also leads to ubiquitination of β-arrestin1, which regulates vesicular trafficking and activation of ERK1/2. This β-arrestin1-dependent ERK activity can occur even when the classical tyrosine kinase signaling is impaired. siRNA-mediated suppression of β-arrestin1 in human melanoma cells ablates IGF-1-stimulated ERK and prolongs the G1 phase of the cell cycle. These data suggest that β-arrestin-dependent ERK signaling by the IGF-1R regulates cell cycle progression and may thus be an important regulator of the growth of normal and malignant cells.

Insulin-like growth factor-1 receptor (IGF-1R) plays crucial physiologic roles in embryonic development, differentiation, and postnatal growth. The IGF-1 pathway is also involved in aging (1) and is crucial for transformation and growth of malignant cells (2–7). Recently, we demonstrated that Mdm2, an E3 ubiquitin ligase, whose most prominent known function is to target and degrade p53, associates with and ubiquitinates the IGF-1R (8). Under conditions where expression of p53 was inhibited, Mdm2 was redistributed and caused ubiquitination and proteasome-dependent degradation of the IGF-1R. However, the Mdm2 E3 ligase cannot interact directly with IGF-1R but requires β-arrestin as an adaptor (9). β-Arrestin was required for Mdm2-mediated IGF-1R ubiquitination under both basal and IGF-1-stimulated conditions. By comparing the efficiencies of the two isoforms β-arrestin 1 and 2 in this context, β-arrestin1 was found to be superior in facilitating ubiquitination in IGF-1-stimulated cells (9, 10).

The role of β-arrestin in IGF-1R ubiquitination was unexpected because its functions have generally been associated with G protein-coupled receptors (GPCRs), also called seven transmembrane receptors (7TMRs) (11, 12). β-Arrestin1 and 2 were originally identified as proteins that desensitize second messenger signaling elicited by 7TMRs (13, 14). Further work has shown that β-arrestin not only acts as an endocytic adaptor for various 7TMRs, but also functions as a scaffolding molecule for activation of MAPKs as well as other signaling pathways (11). β-Arrestin can mediate ERK signaling in the absence of any detectable second messenger activity (15). Moreover, for some receptors such as the angiotensin II type 1a receptor, β-arrestins compartmentalize the signaling complexes in cytoplasmic endosomes. Upon recruitment to agonist-occupied 7TMRs on the cell membrane, β-arrestin is ubiquitinated by Mdm2 and then brings the receptors into endosomal vesicles (16, 17). Additionally, β-arrestin ubiquitination has been shown to regulate both trafficking and ERK activation kinetics (18). In the context of these observations and our previous finding that β-arrestin associates with the IGF-1R, we now sought to investigate whether β-arrestin and its ubiquitination play roles in IGF-1-induced signaling, with a focus on activation of ERKs and control of the cell cycle.

EXPERIMENTAL PROCEDURES

Reagents—Polyclonal IGF-1R antibodies (C-20 and H-60), a monoclonal antibody to phosphotyrosine (PY99), were from Santa Cruz Biotechnology Inc., Santa Cruz, CA. Antibodies against pAkt (S473), Akt, pERK1/2, and ERK1/2 were from Cell Signaling Technology, Danvers, MA. Rabbit polyclonal antibodies against β-arrestin1/2 were generated in the Lefkowitz laboratory. A mouse monoclonal antibody against the human IGF-1R, a mouse monoclonal antibody to Mdm2, were from Calbiochem, Darmstadt, Germany. All other reagents unless stated otherwise were from Sigma.

Cell Culture—The human melanoma cell line BE has been described elsewhere (9). The R−, R+, 46, 56, and 96 mouse cell lines were from Dr. Renato Baserga (Thomas Jefferson University, Philadelphia, PA). The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf
serum. R−, R+, R-46, R-56, and R-96 cell lines were cultured in the presence of G-418 (Promega). Wild type, Mdm2 KO, and β-arrestin mouse embryonic fibroblast (MEFs) were cultured as described elsewhere (19).

Small Interfering RNAs (siRNAs)—Chemically synthesized, double-strand siRNAs, with 19-nt duplex RNA and 2-nt 3′ dTdT overhangs were purchased from Xeragon (Germantown, MD) in deprotected and desalted form. The siRNA sequences targeting human β-arrestin1 and β-arrestin2 have been reported previously (20) and were used to deplete endogenous β-arrestin levels in BE cell line. The siRNA sequences targeting mouse β-arrestin1 and 2 are 5′-AAAGCCCUUCUGUAGUA-ACG-3′ and 5′-AAGACGGAAAGUGUUCGUG-3′. A non-silencing RNA duplex (5′-AAUUCUCAGCAGUUCGUCGU-ACGU-3′), as the manufacturer indicated, was used as a control for both human and mouse cells.

Transfections—40–50% confluent cells in 25-cm² flasks, split 24 h before transfection, were transfected with siRNA (see above) using the Lipofectamine 2000 (Invitrogen) according to the modified manufacturer’s instructions. Briefly, 10 μl of transfection reagent was added to 300 μl of serum-free media, while RNA mixtures containing 12 μl of 20 μM (3.5 μg) RNA, and 188 μl of media were prepared. Both solutions were allowed to stand 5–10 min at room temperature and mixed by inversion. After a 10–20-min incubation at room temperature, the entire transfection mixture was added to cells in a flask containing 3–4 ml of fresh, serum-free media. After cells were incubated for 24 h at 37 °C, the media was replaced with normal (serum-containing) growth media. After additional incubation for 24 h, cells were divided into two flasks or 6-well plates for further experiments. All assays were performed at least 2 days after siRNA transfection. Transfections with pcDNA3 β-arrestin1 and 2 and Mdm mutants were performed as described elsewhere (17).

Immunoprecipitation—The isolated cells were lysed as described previously (21). 15 μl of protein G plus-A/G agarose and 1 μg of antibody were added to 1 mg of protein material. After overnight incubation at 4 °C on a rocker platform, the immunoprecipitates were collected by centrifugation in a microcentrifuge at 2,500 rpm for 2 min. The supernatant was discarded, whereupon the pellet was washed and then dissolved in a sample buffer for SDS-PAGE.

SDS-PAGE and Western Blotting—Protein samples were dissolved in a sample buffer containing 0.0625 M Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, bromphenol blue, and dithiothreitol. Samples corresponding to 5–50 μg of cell protein were analyzed by SDS-PAGE with a 7.5 or 10% separation gel. Molecular weight markers (Bio-Rad) were run simultaneously. Following SDS-PAGE, the proteins were transferred overnight to nitrocellulose membranes (Amersham Biosciences, Uppsala, Sweden) and then blocked for 1 h at room temperature in a solution of 5% (w/v) skimmed milk powder and 0.02% (w/v) Tween 20 in phosphate-buffered saline, pH 7.5. Incubation with appropriate primary antibodies was performed for 1 h at room temperature. This was followed by washes with phosphate-buffered saline and incubation with either a horseradish peroxidase-labeled or a biotinylated secondary antibody (Amersham Biosciences) for 1 h. Following the biotinylated secondary antibody, incubation with streptavidin-labeled horseradish peroxidase was performed. The detection was made with either ECL, Amersham Biosciences or by Supersignal West Pico reagents (Pierce). The films were scanned and quantified by Fluor-S imager (Bio-Rad).

Immunofluorescence Confocal Microscopy—BE cells were plated on collagen-coated 35-mm glass bottom plates. After experimental conditions, cells were fixed with 5% formaldehyde diluted in phosphate-buffered saline containing calcium and magnesium before confocal analyses. For immunostaining endogenously expressed β-arrestins, polyclonal β-arrestin1/2 antibody, and anti-rabbit ALEXA 594 (Molecular Probes) were used as primary and secondary antibodies, respectively.

Cell Cycle and Proliferation Analysis—Cell cycle analysis was performed with a FACS Calibur machine from Becton Dickinson. Following indicated experimental conditions cells were detached with Non-enzymatic Cell dissociation solution from Sigma, and centrifuged for 4 min at 4 °C at 1000 rpm. After two washes with phosphate-buffered saline, cells were incubated for 30 min with 70% ice-cold ethanol on ice. After another two washes, cells were centrifuged and pellet-stained with propidium iodide (50 μg/ml, Sigma) with added RNase A (20 μg/ml, Sigma) and analyzed by flow cytometry using the CellQuest® Program.

RESULTS

Role of β-Arrestin1 in IGF-1-induced ERK Phosphorylation—We first investigated the requirement of β-arrestin1 in IGF-1-activated ERK1/2 phosphorylation. We performed kinetic experiments (0–60 min) in wild-type and β-arrestin1 knockout (KO) MEFs and analyzed the activation of ERK in whole cell lysates. As shown in Fig. 1A, ERK1/2 in wild-type MEFs is phosphorylated after IGF-1 stimulation with a peak at 5–10 min, after which pERK declined substantially. In β-arrestin1 KO MEFs, on the other hand, ERK1/2 remained essentially inactivated upon IGF-1 stimulation (Fig. 1A). However, when the β-arrestin1 KO MEFs were transfected with β-arrestin1, ERK was strongly phosphorylated (Fig. 1, A and B). Although activity peaked at 5 min, it was quite persistent even after 60 min of IGF-1 stimulation (Fig. 1, A and B). This suggests that β-arrestin1 is not only necessary for IGF-1-induced ERK phosphorylation, but also that it prolongs the time course of ERK activation.

The effects of β-arrestin depletion (Fig. 1C) obtained by siRNA on IGF-1 signaling in the human melanoma cell line BE were also examined. Cells transfected with control siRNA responded to IGF-1 with ERK phosphorylation (Fig. 1C). In BE cells transfected with β-arrestin1 siRNA no pERK signals were detected (Fig. 1, C and D). These data provide further evidence that β-arrestin1 is necessary for IGF-1-induced ERK activation. We also investigated whether β-arrestin2 played a similar role. However, no significant reduction in IGF-1-stimulated pERK was observed in BE cells transfected with β-arrestin2 siRNA (Fig. 1, C and D). In contrast, down-regulation of β-arrestin2 levels led to an increase in both basal and IGF-1-stimulated ERK activity that was sustained up to 60 min (Fig. 1, C and D). This denotes that β-arrestin1 but not β-arrestin2 plays an obligatory role in this ERK pathway.
Trafficking and Ubiquitination of H9252-Arrestin1 following IGF-1 Stimulation—The persistent H9252-arrestin1-dependent ERK activity promoted by the IGF-1Rs emulates the situation observed with 7 transmembrane receptors such as the AT1aR that recruit H9252-arrestin2 to endosomal vesicles (22, 23). Therefore, we next tested whether IGF-1 stimulation could cause such a redistribution of β-arrestin. As shown in Fig. 2, under unstimulated conditions, endogenous β-arrestins are diffusely distributed in the cytoplasm as determined by immunostaining with an antibody that detects both β-arrestin isoforms. BE cells treated with control siRNA or siRNA to β-arrestin1 were serum-starved and then either stimulated or not with IGF-1 for 30 min. In the cells treated with control siRNA, ligand stimulation resulted in distinct β-arrestin-containing vesicles (Fig. 2). However, these vesicles did not appear in cells transfected with β-arrestin1 siRNA (Fig. 2), confirming that β-arrestin1 was specifically recruited to these endosomal compartments. Quite surprisingly, when the BE cells were depleted of β-arrestin2 by siRNA transfection, we observed constitutive endosomal distribution of β-arrestin1. This β-arrestin1 recruitment became

**FIGURE 1.Requirement of β-arrestin1 for IGF-1-induced activation of ERK.** A, WT MEFs, β-arrestin1 KO MEFs (mock-transfected), and β-arrestin1 KO MEFs transfected with a β-arrestin1 construct were serum-depleted for 24 h and then stimulated with IGF-1 (50 ng/ml) for 0, 2, 5, 10, 30, or 60 min. The lysates were analyzed for pERK1/2 versus ERK1/2 by Western blotting. B, the pERK signals were quantified by densitometry, and data (means and S.E.) from three different experiments are shown. The expression status of β-arrestin1 and 2 was determined in the transfected stocks before the start of experiments (bottom panels). C, human melanoma cell line BE was transfected with siRNA control, β-arrestin1, or β-arrestin2 siRNA for 72 h. The expression of β-arrestin1 and 2 was verified in the transfected stocks (top panel). The cells were serum-depleted overnight and stimulated with IGF-1 (50 ng/ml) for 0, 2, 5, 10, 30, or 60 min. Cell lysates were analyzed for pERK1/2 versus ERK1/2 (bottom panels). D, the pERK signals were quantified by densitometry, and data (means and S.E.) from three different experiments are shown.
more robust upon stimulation of the IGF-1R (bottom panels, Fig. 2), consistent with the observed pattern of ERK activation (Fig. 1).

Previous studies on agonist-stimulated GPCRs have demonstrated that β-arrestin has to remain stably ubiquitinated to localize on endosomes (18). Similarly, we find that both β-arrestin1 and 2 are ubiquitinated in IGF-1R-stimulated WT MEF cells. The specificity of detection of ubiquitinated β-arrestin is shown in Fig. 3A. In the ubiquitin immunoprecipitates β-arrestins were detected as discrete single bands, rather than smears, suggesting that β-arrestins are monoubiquitinated or oligoubiquitinated under these conditions. Ubiquitinated β-arrestin1 was not observed in β-arrestin1 KO and β-arrestin 1 + 2 KO MEFs, but was detectable in β-arrestin2 KO cells. As also shown, the amount of ubiquitinated β-arrestin2 was much less than that of β-arrestin1 in response to IGF-1 stimulation (Fig. 3A).

β-Arrestins have also been shown to be ubiquitinated by Mdm2 upon ligand activation of GPCRs, and this ubiquitination influences their biological roles (17). To determine whether Mdm2 is involved in IGF-1-induced ubiquitination of β-arrestin1, we stimulated Mdm2 KO MEFs with IGF-1 and examined β-arrestin ubiquitination as indicated above. Fig. 3B shows that β-arrestin1 was not ubiquitinated in these cells unless they were transfected with WT Mdm2. This finding suggests that Mdm2 E3 ligase is responsible for ubiquitination of β-arrestin1 in response to IGF-1 stimulation. In addition, Mdm2 could also enhance the endosomal recruitment of β-arrestin under both basal and IGF-1 stimulated conditions (Fig. 3C, compare vector-transfected cells with Mdm2-transfected cells). In fact, unstimulated Mdm2-overexpressing cells also exhibited some clearly visible β-arrestin-positive endosomal vesicles (compare with control, upper panels of Figs. 3 and 2). Thus, IGF-1 stimulation results in recruitment of β-arrestin into intracellular vesicles, which is enhanced by Mdm2, perhaps by regulating β-arrestin ubiquitination status.

Role of Mdm2 in IGF-1-induced ERK Phosphorylation—Because Mdm2 enhanced IGF-1-stimulated β-arrestin recruitment, we sought to determine if Mdm2 overexpression had any effect on IGF-1-stimulated β-arrestin-dependent ERK activa-
tion. Mdm2 KO MEFs were transfected with either empty vector (mock) or Mdm2L491 (WT Mdm2) (17). The transfectants were serum-depleted overnight and stimulated with IGF-1 or serum and then assessed for phosphorylation of ERK1/2. The expression level of transfected Mdm2 is confirmed in the top panel of Fig. 4A. Phosphorylation of ERK1/2 in mock-transfected Mdm2 KO MEF cells increased only slightly with IGF-1 stimulation (Fig. 4A, left bottom panel). However, after introduction of the WT Mdm2 into these cells, a strong and rapid (5 min.) phosphorylation of both ERK1p42 and ERK2p44 occurred. Upon stimulation with serum instead of IGF-1, phosphorylation of ERKs increased strongly in both mock and Mdm2-transfected Mdm2 KO cells (Fig. 4A, right panel) but was stabilized by Mdm2. This suggests that serum-induced ERK activation mediated by other growth factor receptor pathways is not dependent on Mdm2. Data from three experiments are presented in Fig. 4B, which suggest that Mdm2 is important for IGF-1-induced activation of ERK1/2.

To gain insight about the cooperative roles of Mdm2 and β-arrestin in ERK activity in BE cells, we compared the effects of two different Mdm2 constructs. In addition to WT Mdm2, we made use of Mdm2H400, which like WT Mdm2 binds β-arrestin but lacks the ligase domain. Mdm2H400 (DN Mdm2) was recently shown to exert a dominant-negative effect in the sense that it blocked Mdm2-dependent ubiquitination of IGF-1R (9). BE cells were transfected with empty vector, DN Mdm2, or WT Mdm2, serum-starved, and treated with IGF-1 for 2–60 min. The expression of the Mdm2 constructs is confirmed in the top panel of Fig. 4C. Fig. 4C also shows that stimulation with IGF-1 for 5–10 min induced ERK1/2 phosphorylation in control cells (mock-transfected). DN Mdm2 completely blocked IGF-1-stimulated ERK activation, whereas ectopic expression of WT Mdm2 in BE cells resulted in increased ERK phosphorylation (compare with mock), which also occurred in the unstimulated cells (Fig. 4C). Quantification of the signals is illustrated graphically in Fig. 4D and demonstrates the persistence of the IGF-stimulated ERK activity (Fig. 4D).

**IGF-1-induced ERK Activation Can Occur with Impaired IGF-1R Tyrosine Kinase Signaling (TKS)—** We next analyzed whether IGF-1R tyrosine kinase activity was required for the IGF-1-stimulated ERK pathway. For this purpose we utilized R− cells (IGF-1R KO) and R− (R− cells stably transfected with WT IGF-1R) as negative and positive controls, respectively. In addition, we used “46 cells” that are R− cells stably transfected with an IGF-1R construct possessing a mutation in the substate binding site (SBS) (Y950F), which does not recruit and activate Shc and IRS1, the two major transducers of IGF-1R signaling. This cell line has been shown to be deficient in IGF-1R tyrosine kinase signaling (24). We also tested “56 cells,” which are R− cells stably transfected with IGF-1R with a truncated C-terminal domain and “96 cells,” which are R− cells expressing IGF-1R with both SBS mutation (Y950F) and truncated C terminus. Fig. 5A (right panels) verifies ligand-induced phosphorylation of IGF-1R in all cell lines except R−, R+ cells were responsive to IGF-1 in terms of ERK and Akt phosphorylation with an activity peak at 5 min. Surprisingly, ERK1/2 is phosphorylated in IGF-1-stimulated 46 cells whereas Akt is not. Compared with R+ cells, ERK phosphorylation in 46 cells is weaker and exhibits a later activity peak (30 min) (Fig. 5B). In 56 cells, IGF-1 induces phosphorylation of Akt but has only a weak and transient ERK1/2 activation (Fig. 5, A and B), suggesting that the C-terminal domain of IGF-1R is important for ERK activation. In 96 cells, with combined mutations, neither ERK1/2 nor Akt phosphorylation was stimulated by IGF-1. These data suggest that the recruitment of Shc and IRS proteins is critical only for the Akt pathway but not for ERK activation upon IGF-1R stimulation.

**β-Arrestin Recruitment, Ubiquitination, and Signaling to ERK via IGF-1R Mutant Defective in TKS—** To investigate possible involvement of β-arrestin1 in TKS-independent ERK activation, we first analyzed the association of β-arrestin1 with the different IGF-1R constructs. β-Arrestin1 has previously been shown to associate with WT IGF-1R (9), which is confirmed by co-immunoprecipitation of IGF-1R from R+ and BE cells (Fig. 6A). β-Arrestin1 was also found to be associated with the 46 receptor (mutation at SBS) but not with receptors lacking the C-terminal domain (56 and 96). This indicates that β-arrestin1 is physically bound to the C-terminal domain of the receptor. We also investigated intracellular redistribution of β-arrestin in 46 cells. Fig. 6B shows that IGF-1 stimulation causes endosomal redistribution of β-arrestin in 46. Additionally no redistribution of β-arrestin was observed in 56, 96, and R− cells (Fig. 6B). These results are in agreement with our findings presented in the coimmunoprecipitation experiment in Fig. 6A.

We next analyzed ligand-activated ubiquitination of β-arrestins in BE cells and MEFs with various IGF-1R constructs. The two upper panels of Fig. 7A show that IGF-1 clearly induces ubiquitination of β-arrestin1 in BE and R+ cells, while that of β-arrestin2 is only slightly affected. The maximal effect occurred at 5 and 10 min after addition of IGF-1 to the serum-starved cell cultures. The 46 cells (with mutation of SBS) also responded to IGF-1 with β-arrestin1 ubiquitination that reached a maximal level at 5 min, while cells containing IGF-1Rs with truncated C-terminal (56 and 96), which do not recruit β-arrestin, were not responsive. R− cells (lacking the IGF-1R) did not show any response either (Fig. 7A).

To directly investigate involvement of β-arrestin in TKS-independent ERK phosphorylation, β-arrestin1 expression was inhibited by siRNA in 46 cells (confirmed in the upper panel of Fig. 7B) and cells analyzed for ERK1/2 activation. As shown in the bottom panel of Fig. 7B, the IGF-1-induced ERK activation seen in 46 cells with control siRNA was almost completely eliminated in cells treated with β-arrestin1 siRNA.

**Role of Mdm2 and β-Arrestin in Cell Cycle Progression—** Because the MAPK/ERK pathway is involved in cell cycle progression (25), we investigated the potential role of Mdm2 and β-arrestin in relation to IGF-1R in this respect. Exponentially growing cells, cultured under basal conditions (in medium containing 10% serum), were analyzed for DNA content by cell sorting (FACS) after staining with propidium iodide. Using this method the progression of cells through the different cell cycle phases (G1, S, and G2/M) can be assessed. First, we analyzed the effects of the MEK1 inhibitor PD98059. In Fig. 8A it is shown that BE cells treated with PD98059 accumulated in the G1 phase. We next evaluated the distribution of cells in the different phases of cell cycle under conditions of Mdm2 activity (WT
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A

MEF MDM2 KO

|                | C   | Mock | WT MDM2 |
|----------------|-----|------|---------|
| MDM2           |     |      |         |
| Actin          |     |      |         |

Mock | WT MDM2

| pERK 1/2 |      |          |
| ERK 1/2   |      |          |

Time (min) 0 5 10 20 +IGF-1

Mock | WT MDM2

Time (min) 0 5 10 20 +serum

B

pERK (% of maximum stimulation)

Mock | WT MDM2

IGF-1 (min) 0 5 10 20

Serum (min) 0 5 10 20

C

BE cells

|                |       | WT MDM2 | DN MDM2 |
|----------------|-------|---------|---------|
| MDM2           |       |         |         |
| Actin          |       |         |         |

Mock | WT | DN

| pERK 1/2 |      |          |
| ERK 1/2   |      |          |

Time (min) 0 2 5 10 30 60 +IGF-1

D

pERK (% of maximum stimulation)

Mock | DN MDM2 | WT MDM2

IGF-1 (min) 0 10 20 30 40 50 60
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The expression (pERK/ERK) or inactivity (DN expression) and analyzed the correlation with the presence (BE and R+ cells) and absence (R−) of IGF-1R expression. All these experiments were performed under basal conditions. Fig. 8B (top row) demonstrates that DN Mdm2 caused G1 accumulation in BE and R+ cells similar to the MAPK/ERK inhibitor but did not affect the IGF-1R negative cells (R−). Overexpression of WT Mdm2, on the other hand, accelerated the cell cycle progression in BE and R+ cells, illustrated by a decreased number of cells in the G1 phase (Fig. 8B, bottom row). However, even in this case, R− cells were not affected. These data suggest that Mdm2 is important for adequate G1-S progression in IGF-1R-expressing cells. Reduction of β-arrestin1 causes G1 accumulation in IGF-1R-positive cells (BE and R+) compared with control cells, which were incubated in the presence of transfection reagents and control siRNA (data not shown). Fig. 8C summarizes cell cycle data, showing the effects of WT and DN Mdm2. Also included are bar representations showing the effects of β-arrestin1 depletion under these various conditions. These data confirm that inhibition of Mdm2 E3 ligase and β-arrestin1 increases the number of cells in G1 phase in IGF-1R-expressing cells (p < 0.005–0.0005), whereas elevated Mdm2 accelerates G1 progression, shown as a decrease in the number of cells in the G1 phase, in those cells (p < 0.005–0.0005). We conclude these effects to be specific for the IGF-1R because similar manipulation of Mdm2 and β-arrestin1 did not affect cell cycle progression of the IGF-1R-null R− cells (Fig. 8, B and C).

DISCUSSION

β-Arrestin plays many important roles in 7TMR signaling. Originally, it was discovered that β-arrestin contributes to attenuation or desensitization of the receptors after persistent agonist-induced stimulation (14). Later, it was shown to be involved in endocytosis of 7TMRs, and more recently β-arrestins were found to play important roles in transduction of 7TMR signaling (11, 12, 26, 27). Ubiquitination of β-arrestin has been found to play a role in several of these functions. For example, in the case of agonist-induced activation of β2-adrenergic receptors (β2AR), Mdm2 was demonstrated to serve as the E3 ligase responsible for ubiquitination of β-arrestin. Abrogation of this modification, either by receptor expression in Mdm2 KO cells or by dominant-negative Mdm2 lacking E3 ligase activity, strongly inhibited receptor endocytosis (17).
Currently, it is well established that β-arrestin is involved in the transduction of several signaling pathways stimulated by GPCRs (11). For example, β-arrestin has been shown to transduce activation of ERK as well as c-Src and other nonreceptor tyrosine kinases (28, 29) by a number of different 7TMRs. Moreover, some previous studies have suggested involvement of β-arrestin in MAPK signaling of the IGF-1R (30, 31). We have previously shown that β-arrestins down-regulate IGF-1R by facilitating Mdm2-dependent ubiquitination and degradation of the IGF-1R (9). We now demonstrate that β-arrestin1 is involved in IGF-1-induced signaling to ERK1/2. Thus, for the IGF-1R, β-arrestin appears to mediate both an inhibitory or desensitizing effect (through receptor degradation) and a stimulatory action (through ERK activation).

To transduce IGF-1-stimulated signaling, β-arrestin1 has to bind to the C terminus of the IGF-1R and become ubiquitinated by the Mdm2 E3 ligase. Actually, this response could be obtained in receptors lacking binding sites (designated SBS) for Shc and IRS-1, which are the two docking proteins for IGF-1R tyrosine kinase signaling (32). Even though maximal ERK phosphorylation appears to require an intact receptor, our data indicate that a component of ERK activation by IGF-1R can occur under conditions of impaired tyrosine kinase signaling. By considering the kinetics of ERK activation of two different IGF-1R mutants, one lacking the SBS and the other one being C-terminally truncated (Fig. 5B), it appears that tyrosine kinase signaling is required for rapid induction of ERK phosphorylation (peak at 5 min) while β-arrestin1 is important at a later stage (peak at 30 min). Our data thus suggest that β-arrestin1 may stabilize pERK leading to prolonged activity after ligand stimulation. This kinetic pattern and the stabilizing effect of β-arrestin on ERK phosphorylation has also been observed for agonist-stimulated GPCRs (11) where, of course, the rapid component of ERK activation is G protein-mediated.

β-Arrestin polyubiquitination induced by 7TMRs has been shown to be crucial for receptor internalization (17), and the kinetics of ubiquitination dictates the trafficking and targeting of receptor-signalosomes (16, 18, 33). In the case of IGF-1R,
β-arrestin1 appears to be monoubiquitinated, and this is mediated by Mdm2 (Fig. 3, A and B). Combined with our previous findings, Mdm2 thus plays a dual role in the regulation of IGF-1R signaling, by mediating polyubiquitination of the IGF-1R and monoubiquitination, or oligoubiquitination, of β-arrestin1. Notably, Mdm2 has been previously shown to lead to mono- and polyubiquitination of substrate proteins (34). Monoubiquitination is suggested to be important for regulating the intracellular trafficking of proteins (35, 36) as well as for persistent activity of some signaling kinases (37). Although the exact mechanism is not known, our data indicate that ubiquitination of β-arrestin1 is induced upon IGF-1R stimulation and this is important for trafficking as well as the prolonged downstream ERK activation.

Phosphorylation of ERK1/2 is known to be required for the G1-S transition and inhibition of this reaction causes cells to remain at the G1 phase (25, 38, 39). We could also confirm this cell cycle response using a MEK1 inhibitor, which induced accumulation of cells in the G1 phase of the cell cycle. Through its regulation of ERK activity the expression status of β-arrestin1 affects cell cycle progression of IGF-1R expressing cells. A decrease in G1-S transition is caused by depletion of endogenous β-arrestin1, as well as by dominant-negative Mdm2, under basal culture conditions. This provides evidence for a role of these molecules in cell growth induced by IGF-1R activation. Because β-arrestin1, after becoming ubiquitinated by Mdm2, is needed for IGF-1-induced ERK, this effect on the cell cycle seems appropriate. On the other hand, overexpression of Mdm2 accelerates the G1-S transition leading to considerably fewer cells in the G1 phase (Fig. 8). This effect correlates with an increased recruitment of β-arrestin1 into intracellular vesicles and enhanced ERK phosphorylation in IGF-1-stimulated cells with overexpressed Mdm2. The involvement of Mdm2 and β-arrestin1 in cell cycle progression is mediated through the IGF-1R since β-arrestin depletion and dominant-negative Mdm2, as well as overexpression of wild-type Mdm2, did not affect the cell cycle of IGF-1R KO cells.

β-Arrestin1 and Mdm2 are not only important for transduction of ERK activation, but an increased expression of β-arrestin1 and Mdm2 sustained IGF-1-induced ERK activation (see Figs. 1B and 4, B and D). Activation of the ERK signaling pathway in cell cycle progression from G1 to S phase is linked to

**FIGURE 8. Requirement of pERK, Mdm2, and β-arrestin1 for G1-S transition.** A, BE cells, growing in full medium, remained either untreated or were treated with the MAPK/ERK (MEK1) inhibitor PD98059 (PD) for 6 h. The cells were harvested, stained with propidium iodide, and analyzed for DNA distribution by FACS. B, BE, R+, and R− cells, growing in full medium, were transfected with empty vector (control), DN Mdm2 (upper row) or WT Mdm2 (middle row). Thirty-six hours later, the cells were harvested and analyzed for DNA distribution by FACS. C, summarization of cell cycle data. The experiments described in B were repeated 3–4 times. The percentages of cells in the G1 phase were calculated, after normalization for dead cells. The data are presented as Relative G1 (%) by relating to appropriate controls (set to 100%). Means, S.D., and statistical levels are shown. *, p < 0.005; **, p < 0.0005; and NS, not significant.
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cyclin D induction and consequent retinoblastoma protein phosphorylation (40). To induce cyclin D the activity of ERK must be sustained before the onset of S phase (41). Sustained, but not transient, ERK activation induces cyclin D expression several hours after growth factor stimulation (40, 42). Thus, the duration of ERK activity is critical for ensuring G1-S phase progression. In this context, β-arrestin1 and Mdm2 may play an important role by increasing the duration of ERK activation.

Considered together with our previous study (4, 8, 9), our current data strongly point to a dual role of β-arrestin1 in regulating IGF-1R functions. First, it acts as an adaptor protein in Mdm2-mediated ubiquitination and degradation of the receptor. Second, it serves as a scaffold protein for transduction of IGF-1-induced MAPK signaling. Both these roles of β-arrestin may be important for IGF-1R function in normal and malignant cell growth.

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