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β-Arrestin-1 Competitively Inhibits Insulin-Induced Ubiquitination and Degradation of Insulin Receptor Substrate 1

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β-Arrestin-1 is an adaptor protein that mediates agonist-dependent internalization and desensitization of G-protein-coupled receptors (GPCRs) and also participates in the process of heterologous desensitization between receptor tyrosine kinases and GPCR signaling. In the present study, we determined whether β-arrestin-1 is involved in insulin-induced insulin receptor substrate 1 (IRS-1) degradation. Overexpression of wild-type (WT) β-arrestin-1 attenuated insulin-induced degradation of IRS-1, leading to increased insulin signaling downstream of IRS-1. When endogenous β-arrestin-1 was knocked down by transfection of β-arrestin-1 small interfering RNA, insulin-induced IRS-1 degradation was enhanced. Insulin stimulated the association of IRS-1 and Mdm2, an E3 ubiquitin ligase, and this association was inhibited to overexpression of WT β-arrestin-1, which led by decreased ubiquitin content of IRS-1, suggesting that both β-arrestin-1 and IRS-1 competitively bind to Mdm2. In summary, we have found the following: (i) β-arrestin-1 can alter insulin signaling by inhibiting insulin-induced proteasomal degradation of IRS-1; (ii) β-arrestin-1 decreases the rate of ubiquitination of IRS-1 by competitively binding to endogenous Mdm2, an E3 ligase that can ubiquitinate IRS-1; (iii) dephosphorylation of S412 on β-arrestin and the amino terminus of β-arrestin-1 are required for this effect of β-arrestin on IRS-1 degradation; and (iv) inhibition of β-arrestin-1 leads to enhanced IRS-1 degradation and accentuated cellular insulin resistance.

β-Arrestins are versatile adaptor proteins that form complexes with most seven-transmembrane receptors (7TMR) following agonist binding and receptor phosphorylation by G-protein-coupled receptor kinases. Binding of β-arrestin to the 7TMR cytoplasmic domain interrupts further heterotrimeric G-protein interaction with the receptor, causing signal termination. β-arrestin also mediates endocytosis and receptor sequestration, further enhancing desensitization of receptor signaling (1, 17, 19–21, 27). β-Arrestin can also play a role as a 7TMR signal transducer by recruiting activated Src to the receptor complex, leading to mitogen-activated protein kinase activation (6, 7, 18, 19). We have also reported that β-arrestin can play an important role in the process of heterologous desensitization of receptor tyrosine kinases (RTKs), as well as 7TMRs. Thus, insulin treatment leads to β-arrestin-1 Ser412 phosphorylation, ubiquitination, and degradation, all of which impair mitogen-activated protein kinase phosphorylation mediated by Gα i-coupled receptors, such as the lysophosphatidic acid (LPA) receptor, β_2-adrenergic receptor (β_2-AR), and the insulin-like growth factor I (IGF-I) receptor (4).

Since β-arrestin is involved in homologous G-protein-coupled receptor (GPCR) desensitization, as well as insulin-induced heterologous desensitization of Gα i-coupled receptor signaling, we wondered whether β-arrestin could also function in the process of insulin-induced homologous desensitization. It is well known that chronic insulin treatment leads to subsequent desensitization of insulin signaling at several steps and that an important component of this involves insulin-induced ubiquitination and degradation of one of its major substrates, insulin receptor substrate (IRS) (9, 23–26, 28–30, 33). While β-arrestin-1 does not appear to function in the normal process of insulin signaling, since insulin treatment causes β-arrestin phosphorylation as well as β-arrestin degradation, we sought to determine whether β-arrestin might play a role in the normal desensitization process of insulin signaling induced by chronic insulin treatment.

The present studies show that β-arrestin-1 can regulate the process of insulin-mediated IRS-1 ubiquitination and degradation by competing with IRS-1 for Mdm2, an E3 ubiquitin ligase. As such, these studies identify a novel role for β-arrestin-1 as a regulation locus modulating the overall state of cellular insulin sensitivity.

MATERIALS AND METHODS

Materials. Rabbit polyclonal anti-IRS-1, anti-IRS-2, and anti-Shc antibodies and protein A agarose were purchased from Upstate Biotechnology, Inc. (Lake Placid, N.Y.). Mouse monoclonal antiphosphotyrosine (PY20), anti-ERK1, and β-arrestin-1 antibodies were from Transduction Laboratories (Lexington, Ky.). Rabbit polyclonal anti-insulin receptor, anti-Gα q/11, antiubiquitin, anti-Mdm2 (C-18), anti-Mdm2 (N-20), and horseradish peroxidase-linked anti-rabbit and-mouse antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.). Mouse monoclonal anti-Mdm2 antibody (Ab-1) was from Oncogene (San Diego, Calif.). Rabbit polyclonal anti-Akt, anti-phospho-Akt (Thr 308), and anti-phospho-ERK1/2 antibodies were from Cell Signaling Technology (Beverly, Mass.). Lactacystin and wortmannin were from Calbiochem (San Diego, Calif.). SuperFECT, was purchased from QIAGEN (Valencia, Calif.). Oligofectamine was from Invitrogen (Carlsbad, Calif.). Dulbecco's modified Eagle's medium and

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fetal bovine serum were purchased from Life Technologies (Grand Island, N.Y.). [γ-^{32}P]ATP was from ICN (Costa Mesa, Calif.). Mouse monoclonal anti-FLAG antibody and all other reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.). Plasmid expression vectors encoding wild-type and mutant β-arrestin-1 and Mdm2 were kindly provided by R. J. Lefkowitz (Duke University, Durham, N.C.).

Cell culture. Rat-1 fibroblasts overexpressing human insulin receptors (HIRc-B cells) were cultured in DME low glucose medium with 10% heat-inactivated serum and 0.5% methotrexate in a 5% CO_2 environment at 37°C as described previously (5). Cultures were never allowed to be completely confluent.

Transfection of plasmid vectors and small interfering RNAs (siRNAs). Transient transfection of plasmid vectors was performed with SuperFECT (QIAGEN) in accordance with the manufacturer's instructions as described previously (4). Cells were reseeded in complete culture medium and incubated for 16 h, when the confluency of the cells was nearly 50 to 60%. Transfection reagent and vectors were removed 3 h after transfection. For each assay, cells were cultured in complete culture medium for 36 h and then serum starved for 16 h prior to use. Custom-made siRNA of β-arrestin-1 (sense, AGC CUU CUG UGC UGA GAA C dTdT; antisense, G UUC UCA GCA CAG AAG GCU dTdT), and control siRNA were purchased from Dharmacon (Lafayette, Colo.). Transfection of siRNA was performed with Oligofectamine (Invitrogen) in accordance with the manufacturer's instructions. Cells were reseeded in complete culture medium and incubated for 16 h, when the confluency of the cells was nearly 50 to 60%. Cells were cultured in serum-free medium with transfection reagent and siRNAs for 4 h. Transfection medium was then replaced with complete medium as described above.

Western blotting. Serum-starved HIRc-B cells were stimulated with 17 nM insulin at 37°C for various time periods as indicated in each experiment. The cells were lysed in solubilizing buffer containing 20 mM Tris, 1 mM EDTA, 140 mM NaCl, 1% NP-40, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 10 mM NaF, pH 7.5, for 15 min at 4°C. The cell lysates were centrifuged to remove insoluble materials. Immunoprecipitation was performed as described previously (13). For Western blot analysis, whole-cell lysates (20 to 50 μg of protein) or immunoprecipitates were denatured by boiling in Laemmli sample buffer containing 100 mM dithiothreitol and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gels were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, Mass.), using a Transblot apparatus (Bio-Rad, Hercules, Calif.). For immunoblotting, membranes were blocked with 5% bovine serum albumin (only for phospho-tyrosine blot or ubiquitin blot) or 5% nonfat dried milk in TBS-T and probed with specific antibodies. Blotting was then performed as described previously (14).

PI3-kinase assay. Phosphatidylinositol 3-kinase (PI3-kinase) activity was measured as described previously (31). Briefly, HIRc-B cells were starved for 16 h and stimulated with insulin (17 nM) for 10 min, washed once with ice-cold phosphate-buffered saline (PBS), lysed, and subjected to immunoprecipitation (300 to 500 μg of total protein) with anti-IRS-1 antibody for 4 h at 4°C. Immunocomplexes were precipitated with protein A-plus agarose. The immunoprecipitates were washed twice with each of the following buffers: (i) PBS, containing 1% NP-40 and 100 μM sodium orthovanadate, pH 7.4; (ii) 100 mM Tris, 0.5 M LiCl, 100 μM sodium orthovanadate, pH 7.4; and (iii) 10 mM Tris, 100 mM NaCl, and 100 μM sodium orthovanadate, pH 7.4. The washed immunocomplexes were incubated with phosphatidylinositol for 5 min and then with [γ-^{32}P]ATP (3,000 Ci/mmol) for 5 min at room temperature. Reactions were stopped with 20 μl of 8 N HCl, mixed with 160 μl of $CHCl_3$-methanol (1:1). Samples were centrifuged, and the lower organic phase was applied to a silica gel thin-layer chromatography (TLC) plate which had been coated with 1% potassium oxalate. TLC plates were developed in $CHCl_3$-CH_3OH-H_2O-NH_4OH (60:47:11.3:2), dried, and exposed to an X-ray film. PI3-kinase activity was quantitated by scanning the film using NIH Image.

RESULTS

Overexpression of wild-type β-arrestin-1 attenuates insulin-induced degradation of IRS-1. We have recently reported that long-term treatment of cells with insulin leads to ubiquitination and degradation of β-arrestin (4, 12). To investigate whether the degradation of β-arrestin-1 could affect the process of insulin-mediated homologous desensitization, we examined the expression level of insulin signaling molecules, including the insulin receptor, IRS-1, IRS-2, Shc, and Gαq/11, with or without overexpression of wild-type (WT) β-arrestin-1 after insulin treatment for up to 16 h. As seen in Fig. 1, insulin treatment had no appreciable effect on the expression level of insulin receptor (IR), Shc, Gα q/11, or Akt proteins, whereas IRS protein levels were dramatically altered. Insulin led to a marked decrease in IRS-1 levels, which was apparent by 1 h and progressed to ~85% downregulation by 16 h. Overexpression of wild type β-arrestin-1 strikingly decreased insulin-induced degradation of IRS-1 at all time points, and this effect is quantitated in Fig. 1B. Insulin also causes an electrophoretic mobility shift of IRS-1 as a result of Ser/Thr phosphorylation, and this is no longer apparent at longer time points (e.g., 6- and 16-h time points in Fig. 1A), indicating that phosphorylated IRS-1 is preferentially degraded. In β-arrestin-1-overexpressing cells, the electrophoretic mobility shift was maintained for up to 16 h, indicating that phospho-IRS-1 degradation was attenuated. These results demonstrate that expression of β-arrestin-1 does not affect insulin-induced serine/threonine phosphorylation of IRS-1 but inhibits its subsequent degradation. Expression of IRS-2 was increased by insulin stimulation for up to 6 h, thereafter returning to baseline levels. This increased expression of IRS-2 by insulin may reflect a compensatory response to degradation of IRS-1. β-Arrestin overexpression largely blocked the fall of IRS-2 levels to baseline at 6 and 16 h, consistent with decreased IRS-2 degradation over this time frame.

Overexpression of β-arrestin-1 rescues insulin-induced desensitization of insulin signaling. To determine whether insulin signaling downstream of IRS-1 was affected by β-arrestin-1 overexpression, HIRc-B cells were pretreated with insulin for 6 h, incubated in insulin-free medium for 2 h, and then restimulated with insulin for 5 or 10 min, followed by measurement of IRS-1 tyrosine phosphorylation, PI3-kinase activation, and Akt stimulation (Fig. 2A to C). As expected, pretreatment with insulin for 6 h inhibited subsequent insulin-stimulated tyrosine phosphorylation of IRS-1 (Fig. 2A), IRS-1-associated PI3 kinase activity (Fig. 2B), and phosphorylation of Akt (Fig. 2C). Importantly, all of these desensitizing effects were rescued by β-arrestin-1 overexpression. Overexpression of β-arrestin-1 resulted in only a partial recovery of IRS-1 tyrosine phosphorylation and PI3-kinase activity (~60% of control), whereas Akt phosphorylation was returned to normal. To assess this further, we examined the effect of wortmannin, a PI3-kinase inhibitor, on PI3-kinase activity and Akt phosphorylation in β-arrestin-1-overexpressing cells (Fig. 2B and C). The enhanced activity of PI3-kinase and Akt in β-arrestin-expressing cells was completely abolished by 100 nM wortmannin. Thus, insulin-induced Akt phosphorylation is preserved in chronically insulin-treated β-arrestin-expressing cells through IRS-1-associated PI3-kinase activity, rather than through some alternate input into Akt phosphorylation. These results suggest that IRS-1 degradation is a major mechanism for insulin-induced insulin resistance and that β-arrestin-1 plays a role in this process.

Silencing of endogenous β-arrestin-1 enhances IRS-1 degradation. To show that endogenous levels of β-arrestin-1 affect IRS-1 degradation, we silenced β-arrestin-1 by treating cells with a β-arrestin-1 siRNA. Two days after transfection of β-arrestin-1 siRNA, β-arrestin-1 protein expression was reduced by >90% (Fig. 3A). Insulin treatment led to a decrease in IRS-1 levels, and this effect was greatly enhanced when endogenous β-arrestin-1 protein was decreased by the use of siRNA (Fig.

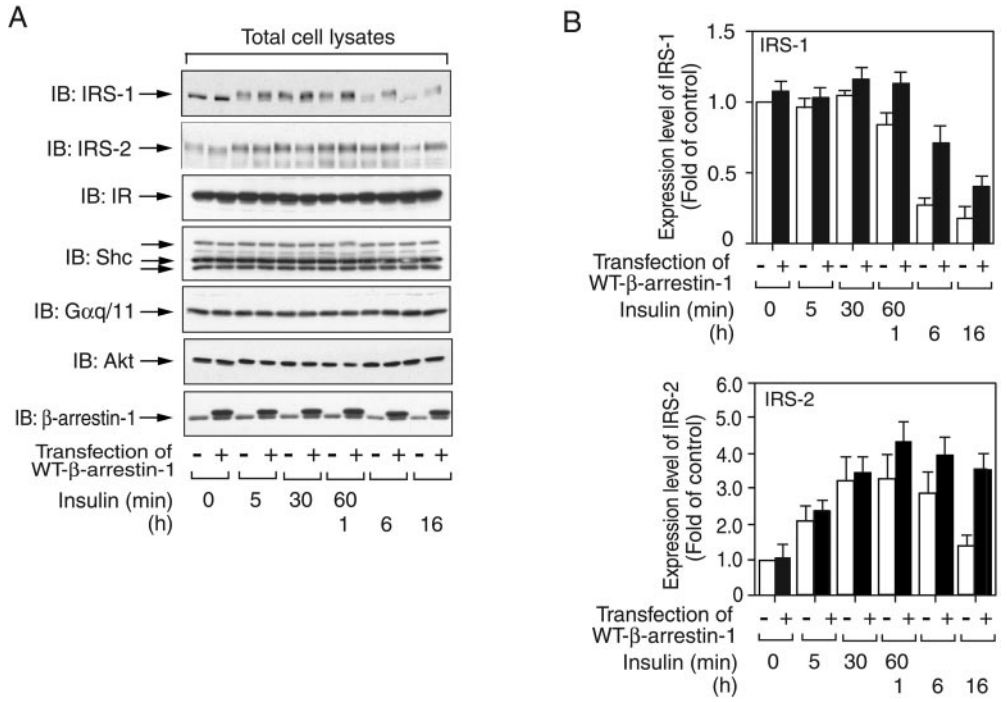

FIG. 1. Effects of wild-type β-arrestin-1 overexpression on insulin-induced mobility shift and degradation of IRS-1 and IRS-2. (A) HIRc-B cells were transfected with wild-type β-arrestin-1 (+) or control vector (−). Forty-eight hours after transfection, cells were serum starved for 16 h, stimulated with insulin for the indicated time periods, and lysed. Total cell lysates were analyzed by Western blotting, using the indicated antibodies, as described in Materials and Methods. Representative results from three independent experiments are shown. (B) The amounts of IRS-1 and IRS-2 were quantitated using NIH Image. Data represent the means ± standard errors of the means from three independent experiments.

3A and B). These results clearly show that β-arrestin-1 is an endogenous inhibitor of insulin-induced IRS-1 degradation.

β-Arrestin-1 overexpression inhibits proteasomal degradation of IRS-1. The above-described results raise the possibility that β-arrestin-1 expression inhibits proteasome-mediated degradation of IRS proteins. To explore this further, we compared the effects of the proteasomal inhibitor lactacystin with β-arrestin-1 overexpression. As seen in Fig. 3C, treatment of cells with a maximally effective dose of lactacystin (10 μM) essentially completely blocks the effects of insulin (6 h) to cause decreased IRS-1 protein expression, whereas β-arrestin-1 overexpression has a smaller effect than lactacystin alone. Importantly, when lactacystin treatment and β-arrestin-1 overexpression were used together, the effects were not additive. Thus, when proteasomal function was completely inhibited, β-arrestin-1 overexpression was now without effect, indicating that β-arrestin-1 expression works at this mechanism. These results also indicate that the effect of β-arrestin-1 on IRS-1 protein content is not a result of increased IRS-1 production. Similar experiments were conducted with a submaximal dose of lactacystin (1 μM) (Fig. 3D). In these studies the effect of submaximal lactacystin and β-arrestin-1 overexpression alone were comparable, and the combination of these two treatments was additive, again consistent with the concept that β-arrestin-1 inhibits proteasome-mediated IRS-1 degradation.

β-Arrestin-1 decreases ubiquitination but does not affect serine phosphorylation of IRS-1. Ubiquitination is a common mechanism targeting proteins for proteasome degradation (3, 10, 15, 16), and recent studies have shown that insulin treatment leads to IRS serine phosphorylation, followed by ubiquitination and degradation (9, 25, 30). To assess the mechanism by which β-arrestin-1 expression inhibits proteasomal degradation of IRS proteins, we examined serine phosphorylation and ubiquitination of IRS-1 (Fig. 4). Insulin treatment for 6 h had a modest effect of increasing ubiquitin content of IRS-1, while pretreatment with lactacystin markedly enhanced the accumulation of ubiquitinated IRS-1, most likely by inhibiting rapid degradation of ubiquitinated IRS-1 by the 26S proteasome as reported previously (9, 26, 30, 34). Expression of WT β-arrestin-1 decreased the ubiquitin content of IRS-1, both before and after insulin stimulation, either with or without lactacystin treatment (Fig. 4, upper panel). The total amount of IRS-1 was increased in β-arrestin-expressing cells in the absence of lactacystin, as also seen in Fig. 3. Insulin-stimulated serine phosphorylation causes an upward mobility shift of IRS-1 which is clearly seen with lactacystin treatment, or β-arrestin expression, and is not inhibited when lactacystin and β-arrestin expression are used together. These results indicate that expression of β-arrestin-1 does not affect serine phosphorylation of IRS-1 but rather decreases the rate of phosphorylated IRS-1 ubiquitination, resulting in decreased proteasomal degradation of this protein.

The β-arrestin-1 Ser412 phosphorylation state regulates degradation of IRS-1. β-Arrestin-1 is phosphorylated at Ser412 in the basal state, and dephosphorylation of Ser412 is required for many β-arrestin functions, such as binding to Src or endo-

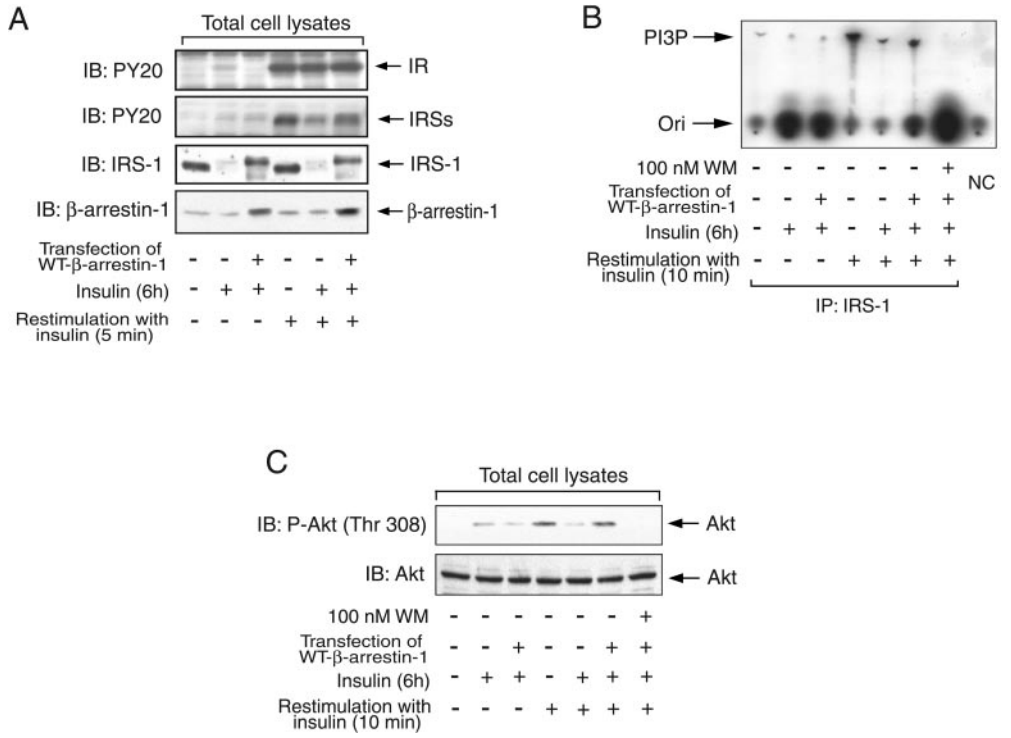

FIG. 2. Effects of wild-type β-arrestin-1 overexpression on the activation of insulin signaling after 6 h of insulin treatment. HIRc-B cells were transfected with wild-type β-arrestin-1 (+) or control vector (−). Forty-eight hours after transfection, cells were serum starved for 16 h, stimulated with insulin for 6 h, and then serum starved again for 2 h. (A) HIRc-B cells were restimulated with insulin for 5 min. Total cell lysates were analyzed by Western blotting, using the indicated antibodies, as described in Materials and Methods. (B) HIRc-B cells were treated with 100 nM wortmannin (+) or 0.1% dimethyl sulfoxide (−) for 30 min and restimulated with insulin for 10 min. Total cell lysates were immunoprecipitated with anti-IRS-1 antibody, and PI3-kinase activity was measured as described in Materials and Methods. Representative results from three independent experiments are shown. (C) HIRc-B cells were treated with 100 nM wortmannin (+) or 0.1% DMSO (−) for 30 min and restimulated with insulin for 10 min. Total cell lysates were analyzed by Western blotting, using the indicated antibodies, as described in Materials and Methods. Representative results from three independent experiments are shown.

cytosis of the β 2-AR. Therefore, we examined the role of Ser412 phosphorylation in the inhibition of insulin-induced degradation of IRS-1, using β-arrestin mutants. Wild type-β-arrestin-1, S412A (which mimics the dephosphorylated state), or S412D (which mimics the phosphorylated state) β-arrestin was expressed in HIRc-B cells, and IRS-1 degradation was measured after insulin stimulation (Fig. 5A and B). The expression levels of the three β-arrestin-1 constructs were comparable (Fig. 5A, lower panel). Both wild-type and S412A β-arrestin inhibited the degradation of IRS-1, whereas the inhibitory effect of S412D β-arrestin was much weaker (Fig. 5B), indicating that the Ser412-dephosphorylated form of β-arrestin-1 functions in the IRS degradation process.

The N terminus of β-arrestin-1 is necessary for inhibition of insulin-induced IRS degradation. The N terminus of β-arrestin contains important functional determinants (18, 19), and therefore, we examined the role of the β-arrestin-1 N terminus on the degradation of IRS-1 and IRS-2 (Fig. 5C). We expressed wild-type β-arrestin-1 (shown as 1-418) or mutants of β-arrestin-1 which lack N-terminal amino acids 1 to 23 (shown as 24-418) or amino acids 1 to 185 (shown as 186-418). The expression levels of all three forms of β-arrestin were comparable (data not shown). Both wild-type and 24-418 β-arrestin inhibited insulin-induced degradation of IRS-1 and IRS-2, whereas the 186-418 mutant was without effect, and these data are quantitated in Fig. 5D.

Both β-arrestin-1 and IRS-1 competitively bind to Mdm2. Recently, it has been reported that an E3 ubiquitin ligase, Mdm2, directly binds to β-arrestin-1 and catalyzes its ubiquitination (27). To determine if the ubiquitination machinery catalyzed by Mdm2 might be used for the degradation of both IRSs and β-arrestin-1, we first assessed the association of Mdm2 with IRS-1. Coimmunoprecipitation experiments demonstrated that the association of Mdm2 and IRS-1 was minimal in the basal state, but insulin stimulation enhanced this interaction (Fig. 6A). The expression level of Mdm2 was not altered by insulin treatment. Next, we compared the association of IRS-1 and Mdm2 before and after β-arrestin-1 overexpression. As seen in Fig. 6B, overexpression of β-arrestin-1 inhibited insulin-induced association of IRS-1 and Mdm2, suggesting that both β-arrestin-1 and IRS-1 competitively bind to Mdm2 and that the overexpressed β-arrestin-1 titrates Mdm2 away from endogenous IRS-1, preventing its ubiquitination and degradation.

Structural features of Mdm2 necessary for insulin-induced IRS-1 degradation. We examined the roles of various Mdm2 domains on IRS-1 degradation by using three different Mdm2 deletion mutants, as seen in Fig. 7A. Wild-type Mdm2 consists

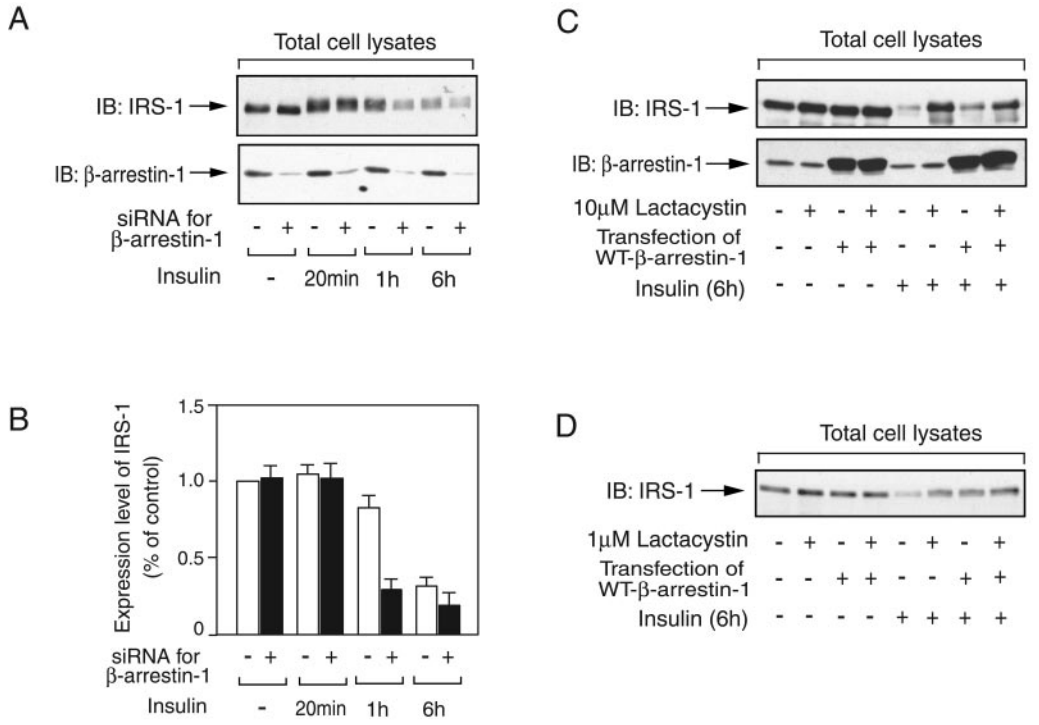

FIG. 3. The effects of β-arrestin-1 siRNA and overexpression of wild-type β-arrestin-1 on insulin-induced IRS-1 degradation. (A) HIRc-B cells were transfected with siRNA of β-arrestin-1 (+) or control siRNA (−). Forty-eight hours after transfection, cells were serum starved for 16 h and stimulated with insulin for the indicated time periods. Total cell lysates were analyzed by Western blotting, using the indicated antibodies, as described in Materials and Methods. Representative results from three independent experiments are shown. (B) The amounts of IRS-1 and IRS-2 were quantitated using NIH Image. Data represent the means ± standard errors of the means from three independent experiments. Open bar, control siRNA; closed bar, β-arrestin-1 siRNA transfection. (C and D) HIRc-B cells were transfected with wild-type β-arrestin-1 (+) or control vector (−). Forty-eight hours after transfection, cells were serum starved for 16 h, pretreated with 10 μM (C) or 1 μM (D) lactacystin (+) or 0.1% dimethyl sulfoxide (−) for 1 h and stimulated with insulin for 6 h. Total cell lysates were analyzed by Western blotting using the indicated antibodies as described in Materials and Methods. Representative results are shown from three independent experiments.

of 491 amino acids. The three Mdm2 deletion mutants used were Mdm2(1-400), lacking only the RING domain (401-491), which is the E3 ubiquitin ligase catalytic domain; Mdm2(1-161), which lacks the β-arrestin-1 binding domain; and Mdm2(1-321), which retains the β-arrestin binding region (27). We transfected wild-type and mutant Mdm2 into HIRc-B cells, and binding of the expressed Mdm2 proteins to IRS-1 was examined. Comparable amounts of these proteins were expressed in these experiments as shown in Fig. 7A. WT Mdm2, Mdm2(1-400), and Mdm2(1-321) were all readily detected in IRS-1 immunoprecipitates, whereas Mdm2(1-161) was not (Fig. 7B). These results suggest that IRS-1 binds to the 162-321 amino acid region of Mdm2, comparable to what has been reported for β-arrestin binding (27). This raises the possibility that Mdm2(1-321) and Mdm2(1-400), both of which can bind to IRS-1 but lack ubiquitin ligase catalytic activity, could behave as dominant-negative ubiquitin ligases for IRS-1. To assess this, we examined the effect of these Mdm2 constructs on insulin-induced IRS-1 degradation. Consistent with a dominant-negative function, Mdm2(1-321) and Mdm2(1-400) inhibited insulin-induced IRS-1 degradation. On the other hand, WT Mdm2 and Mdm2(1-161C) (which does not bind to IRS-1) had no effect on IRS-1 degradation. These results indicate that direct binding of Mdm2 to IRS-1 as well as the catalytic activity of the RING domain are necessary for insulin-induced IRS-1 degradation.

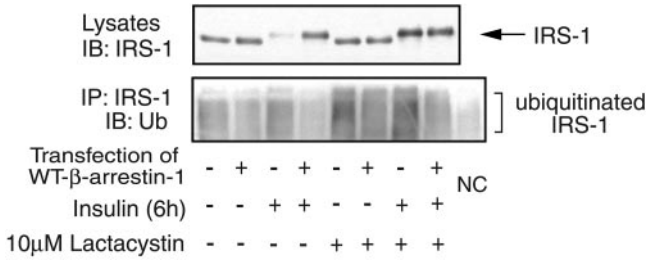

FIG. 4. Effects of wild-type β-arrestin-1 overexpression on insulin-stimulated ubiquitination of IRS-1. HIRc-B cells were transfected with wild-type β-arrestin-1 (+) or control vector (−). Forty-eight hours after transfection, cells were serum starved for 16 h, pretreated with 10 μM lactacystin (+) or 0.1% dimethyl sulfoxide (−) for 1 h and stimulated with insulin for 6 h. Total cell lysates or immunoprecipitates with or without (negative control [NC]) anti-IRS-1 antibody were analyzed by Western blotting, using anti-IRS-1 or antiubiquitin antibody, as described in Materials and Methods. Representative results are shown from three independent experiments.

DISCUSSION

β-Arrestin-1 is a versatile protein which functions as an adapter molecule and signal transducer for many GPCR path-

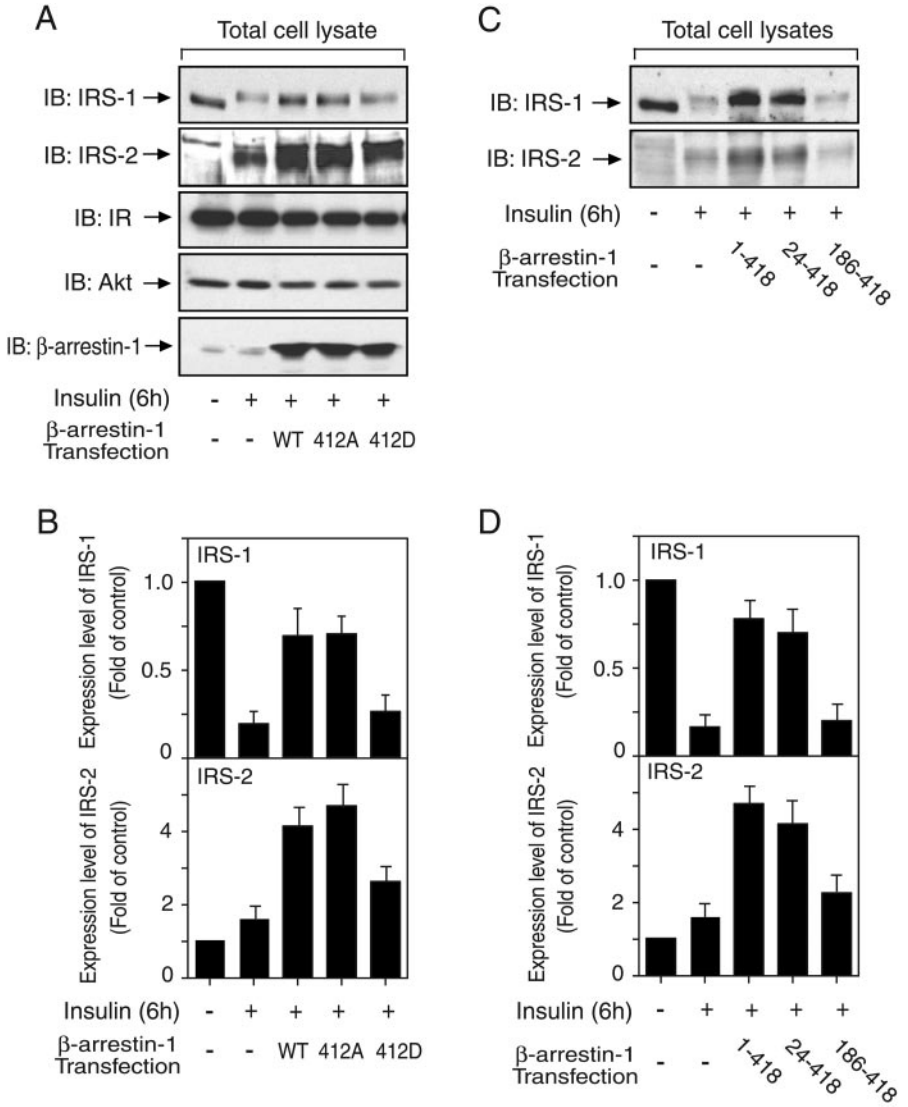

FIG. 5. Role of β-arrestin-1 at serine 412 and amino terminus of β-arrestin-1 for insulin-induced degradation of IRS-1 and IRS-2. HIRc-B cells were transfected with wild-type β-arrestin-1 (WT in panel A or 1-418 in panel C), mutant β-arrestin-1 (412A, 412D, 24-418, or 186-418), or control vector (−). Forty-eight hours after transfection, cells were serum starved for 16 h and stimulated with insulin for 6 h. Total cell lysates were analyzed by Western blotting, using the indicated antibodies, as described in Materials and Methods. Representative results are shown in panels A and C. The amounts of IRS-1 and IRS-2 were quantitated using NIH Image and shown in panels B and D. Data represent the means ± standard errors of the mean from three or four independent experiments.

ways. One of its major properties is mediation of homologous desensitization of GPCR action. β-Arrestin accomplishes this by binding to liganded receptors, interrupting further heterotrimeric G-protein association, and by linking the GPCR to the endocytotic machinery (1, 17, 19–21, 27). We have also shown that β-arrestin can function as a nodal point for heterologous desensitization and cross talk between RTK and GPCR signaling pathways (4). Thus, insulin treatment leads to β-arrestin Ser412 phosphorylation, ubiquitination, and degradation, which desensitizes GPCR and RTK signaling events dependent on β-arrestin, such as β-AR-, LPA-, and IGF-I-stimulated mitogenesis. Insulin can also down regulate its own signaling pathways (homologous desensitization), and since liganded IRs associate with β-arrestin-1 (5), we speculated that β-arrestin could also function in insulin-mediated desensitization of insulin signaling (i.e., insulin resistance). These studies provide strong support for this idea, showing that β-arrestin-1 competes with IRS proteins for ubiquitination and degradation, such that β-arrestin-1 deficiency accelerates insulin-induced IRS-1 degradation, whereas overexpression of β-arrestin retards this process. In this manner, insulin-mediated insulin resistance is regulated by β-arrestin-1 function.

E3 ligases recognize specific ubiquitination signal sequences on target proteins and promote the transfer of activated ubiquitin molecules from a ubiquitin-carrying enzyme (E2) to lysine residues on these proteins, as well as onto the growing ubiquitin chain (3, 10, 15, 16). For many cell membrane receptors, ubiquitination targets the receptor to lysosomes, where

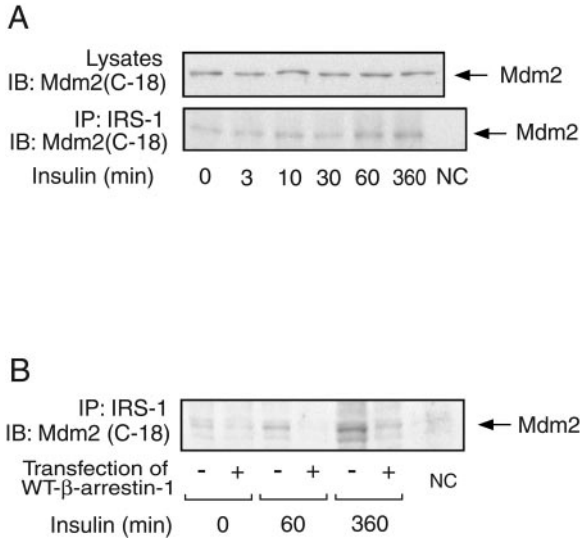

FIG. 6. Direct binding of Mdm2 to IRS-1. (A) HIRc-B cells were serum starved for 16 h and stimulated with insulin for the indicated time periods. Total cell lysates or immunoprecipitates with or without (negative control [NC]) anti-IRS-1 antibody were analyzed by Western blotting using anti-Mdm2 antibody as described in Materials and Methods. (B) Hirc-B cells were transfected with wild-type β-arrestin-1 (+) or control vector (−). Forty-eight hours after transfection, cells were serum starved for 16 h and stimulated with insulin for the indicated time periods. Immunoprecipitates with or without (negative control [NC]) anti-IRS-1 antibody were analyzed by Western blotting using anti-Mdm2 antibody as described in Materials and Methods. Representative results from three independent experiments are shown.

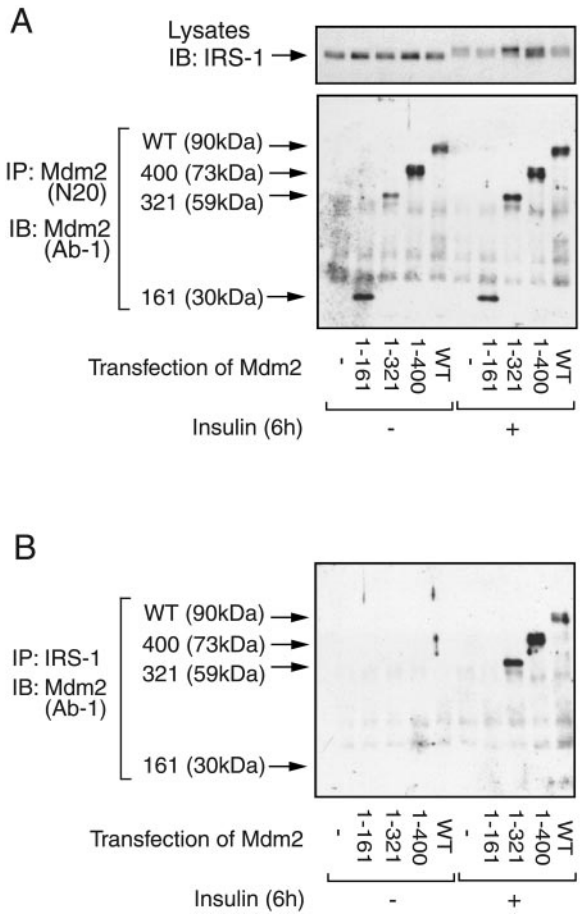

FIG. 7. Identification of Mdm2 domains necessary to induce IRS-1 degradation. HIRc-B cells were transfected with the wild type or the indicated Mdm2 deletion mutant. Forty-eight hours after transfection, cells were serum starved for 16 h and stimulated with insulin for 6 h. Total cell lysates or immunoprecipitates with or without (negative control [NC]) anti-Mdm2 (N20) antibody (A) or anti-IRS-1 antibody (B) were analyzed by Western blotting, using the indicated antibodies, as described in Materials and Methods. Representative results from three independent experiments are shown.

they can be degraded or deubiquitinated and recycled to the membrane (2). Mdm2 has been described as a RING E3 ubiquitin ligase that constitutively targets p53 to proteasomes (8, 11, 22). Mdm2 recognition and ubiquitination of p53 depend on a sequence in the transactivation domain at the N terminus of p53. Serine phosphorylation in this sequence, as occurs with DNA damage, prevents Mdm2 from recognizing p53, and thus, p53 levels are increased.

The interaction between β-arrestin and Mdm2 has been recently reported using yeast two-hybrid and coimmunoprecipitation approaches (27, 32). Isoproterenol-mediated ubiquitination of β-arrestin-2, which is required for internalization of the β 2-AR, requires Mdm2, indicating that Mdm2 is the E3 ligase for β-arrestin. Interestingly, β-arrestin can regulate the ubiquitination of p53 by binding to Mdm2 (32). Overexpression of β-arrestin-2 reduces ubiquitination of p53, leading to enhanced p53-mediated apoptosis. This effect of β-arrestin was lost if cells were transfected with a mutant form of β-arrestin-2 lacking the region required for Mdm2 binding. In this way, p53 ubiquitination and degradation are modulated by the cellular content of β-arrestin-2, analogous to our present results showing that β-arrestin-1 can regulate Mdm2-mediated ubiquitination of IRS-1.

Ubiquitination is a well-described cellular process targeting proteins for 26S proteasome-mediated degradation (3, 10, 15, 16). In this way, targeted proteins are directed to the degradative pathway by covalent ligation of ubiquitin, a 76-amino-acid protein to ε amino groups on lysine residues within the substrate protein (10).

These studies elucidate some of the mechanisms underlying the effects of β-arrestin and Mdm2 on IRS-1 degradation. Members of our group (9) and others (25, 29, 33) have shown that insulin-induced serine phosphorylation of IRS-1 occurs prior to ubiquitination and degradation. Using the IRS-I mobility shift as an approximation of Ser/Thr phosphorylation, our data show that insulin-induced IRS-1 Ser/Thr phosphorylation proceeds normally when β-arrestin-1 is either overexpressed or silenced. Specifically, when β-arrestin is overexpressed, it is the Ser/Thr-phosphorylated form of IRS-1 which accumulates, because the subsequent step, i.e., ubiquitination, is inhibited. The point of interaction of β-arrestin with the IRS-1 degradation process is not with IRS-1 directly but with the ubiquitin ligase Mdm2, since β-arrestin coprecipitates with Mdm2 but not with IRS-1. In the basal situation, β-arrestin exists mostly in a Ser412-phosphorylated form. Upon recruitment to membrane receptors, β-arrestin-1 becomes dephos-

phorylated, and it is the dephosphorylated species which can functionally interact with downstream effector molecules such as Src and components of the endocytic machinery (17, 19). In keeping with this, we find that dephosphorylated β-arrestin is necessary for inhibition of IRS-1 degradation, since β-arrestin 412D, which constitutively mimics the phosphorylated state, does not cause this effect. This is also fully consistent with earlier results (4, 12) showing that insulin stimulation leads to β-arrestin-1 Ser412 phosphorylation and degradation. This provides two coordinate mechanisms (inactivation [412 phosphorylation] and degradation) whereby chronic insulin treatment modulates the β-arrestin system to promote IRS-1 downregulation. Thus, for insulin-induced homologous desensitization to proceed efficiently, insulin stimulation of β-arrestin phosphorylation, ubiquitination, and degradation is a necessary step. Viewed in this way, β-arrestin-1 can function as a new regulator of cellular insulin sensitivity.

Mdm2 is an E3 ubiquitin ligase which ubiquitinates β-arrestin-1 (27). The present results show that Mdm2 can also ubiquitinate IRS-1 and that dephosphorylated β-arrestin-1 competes with IRS-1 for Mdm2-mediated ubiquitination. Recently, White et al. (25) reported that elongin, another E3 ligase, can also promote IRS-1 ubiquitination, and thus, the relative contribution of these two ligases to this process is unclear. Mdm2 clearly associates with IRS-1, and the structural features of Mdm2 required for this interaction were partially identified. Amino acids 162 to 321 are required for binding of Mdm2 to β-arrestin (27), and by using deletion mutants, our studies show that this same domain is necessary for Mdm2–IRS-1 association, consistent with competition between IRS-1 and β-arrestin-1 for Mdm2. Ligase-defective Mdm2 does not mediate IRS-1 degradation, and mutant Mdm2 constructs which bind to, but cannot ubiquitinate, IRS-1 behave as dominant-negative inhibitors of IRS-1 degradation. The latter finding further supports a role for Mdm2 as a biologically relevant IRS-1 ubiquitinase. Mdm2 participates in ubiquitination of a number of cellular proteins besides β-arrestin-1 and IRS-1. For example, ubiquitination by Mdm2 of p53 has been described. It is possible that overexpression of β-arrestin-1 influences ubiquitination and degradation of proteins in addition to IRS-1, although our studies have shown that overexpression of β-arrestin-1 has no effect on p53 levels (data not shown).

We also explored some of the structural features of β-arrestin-1 which are required for inhibition of IRS-1 degradation. Using a deletion mutant strategy, we found that wild-type, as well as β-arrestin-1(124-418), effectively inhibited IRS-1 degradation, whereas β-arrestin-1(186-418) does not. This indicates that residues 24 to 186 of β-arrestin-1 contain the domain necessary to allow β-arrestin-1 to compete with IRS-1 for ubiquitination. Most likely, this domain of β-arrestin-1 is important for the functional interaction with Mdm2, consistent with previous results. Taken together, residues 161 to 321 of Mdm2 and 24 to 186 of β-arrestin-1 represent the interaction domains of these two proteins in this system.

Interestingly, in previous studies we have shown that insulin or IGF-I binding leads to recruitment of β-arrestin-1 to the cognate receptor(s) (5). Since β-arrestin-1 binds to the E3 ligase Mdm2, and since chronic insulin and IGF-I treatment leads to downregulation of their respective receptors, it is tempting to speculate that β-arrestin-1 serves an adaptor function, allowing ubiquitination and degradation of the insulin receptor and the IGF-I receptors after chronic ligand stimulation.

In summary, these studies identify a novel role for β-arrestin-1 in the regulation of cellular IRS-1 expression. Thus, insulin treatment leads to homologous desensitization, in part by inducing IRS-1 ubiquitination and degradation. The present data show that β-arrestin-1 can interrupt this process by competitively binding to endogenous Mdm2, an E3 ligase that ubiquitinates IRS-1 under the influence of insulin. Only the serine 412-dephosphorylated form of β-arrestin performs this function, and the amino terminus of β-arrestin is required for this effect. In this way, β-arrestin-1 can modulate insulin sensitivity by inhibiting insulin-induced proteasomal degradation of IRS-1. Conversely, inhibition of β-arrestin-1 accelerates the rate of IRS-1 degradation. Importantly, insulin treatment leads to β-arrestin-1 Ser412 phosphorylation, as well as β-arrestin-1 degradation, and both of these effects enhance insulin-mediated IRS-1 downregulation. In this way, the two effects of insulin on β-arrestin-1 function are coordinated with the effects of chronic insulin treatment to cause IRS-1 degradation and decreased insulin sensitivity. Not only do these studies show a new role for β-arrestin as a modulator of insulin sensitivity, but they also demonstrate a novel general mechanism for regulation of hormonal insulin sensitivity provided by a ligand-controlled ubiquitin-mediated proteasomal degradative process.

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