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Arkadia complexes with clathrin adaptor AP2 and regulates EGF signalling

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Arkadia is a positive regulator of transforming growth factor (TGF)-β signalling that induces ubiquitin-dependent degradation of several inhibitory proteins of TGF-β signalling through its C-terminal RING domain. We report here that, through yeast-two-hybrid screening for Arkadia-binding proteins, the μ2 subunit of clathrin-adaptor 2 (AP2) complex was identified as an interacting partner of Arkadia. Arkadia was located in both the nucleus and the cytosol in mammalian cells. The C-terminal YXXΦ-binding domain of the μ2 subunit associated with the N-terminal YALL motif of Arkadia. Arkadia ubiquitylated the μ2 subunit at Lys130. In addition, Arkadia interacted with the AP2 complex, and modified endocytosis of epidermal growth factor receptor (EGFR) induced by EGF. Arkadia thus appears to regulate EGF signalling by modulating endocytosis of EGFR through interaction with AP2 complex.

Keywords: AP2/Arkadia/endocytosis/TGF-β/ubiquitylation.

Abbreviations: AP2, clathrin adaptor complex 2; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FBS, foetal bovine serum; HDAC, histone deacetylase; PAGE, polyacrylamide gel-electrophoresis; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PI, propidium iodide; RT, reverse transcription; siRNA, short interfering RNA; TGF-β, transforming growth factor-β.

Arkadia was originally isolated by gene-trap mutagenesis in mice as a factor required for induction of the mammalian node in the extraembryonic lineages, and was also found to induce mesendoderm by enhancing nodal-related signalling in Xenopus embryos (*1*, *2*). Arkadia has a RING domain at the carboxyl terminus and is predominantly located in the nucleus. We previously found that Arkadia is an E3 ubiquitin ligase and binds to Smad7, c-Ski and SnoN in cell-type-dependent fashion (*3*–*5*). Smad7, an inhibitory Smad, is induced by transforming growth factor (TGF)-β and competitively inhibits phosphorylation of Smads through binding to TGF-β type I receptor (TβR-I) at the plasma membrane. In contrast, c-Ski and SnoN are members of the Ski family of oncoproteins, and repress TGF-β signalling through interaction with Smad proteins. Thus, Arkadia is the first example of an E3 ubiquitin ligase that positively regulates TGF-β family signalling. In addition, it has been reported that Arkadia interacts with Axin and forms a ternary complex with Axin and Smad7. Axin sequesters Smad7 in the cytoplasm, where Arkadia facilitates Smad7 polyubiquitylation and degradation (*6*). Arkadia-Axin complexes thus enhance TGF-β signalling.

Endocytosis from the plasma membrane via clathrin-coated pits is the major pathway for intracellular entry of receptors (*7*). Clathrin-adaptor 2 (AP2) complex is a key component of the endocytotic machinery that links cargo membrane proteins to the clathrin lattice (*8*). Heterotetrameric AP2 complex consists of two large subunits, *i.e.* α and β2 (also called adaptins), a medium subunit (μ2) and a small subunit (σ2). The α-subunit of AP2 recruits endocytotic accessory proteins including Epsin, Eps15 and AP180, while the β2 subunit triggers clathrin assembly (*9*). The σ2 subunit forms a heterodimer with α subunit of AP2. The μ2 subunit is comprised of an N-terminal region and a C-terminal region linked by a linker region (*10*). The N-terminus of the μ2 subunit is located at the centre of the AP2 complex and stabilizes the core structure, whereas the C-terminal YXXΦ (X is any amino acid and Φ is a hydrophobic amino acid)-binding domain binds directly to the YXXΦ sorting motif present in the cytoplasmic tail of cargo proteins. AP2 complex is required for endocytosis of cell surface proteins and various types of receptors including EGF receptors (EGFR) (*11*).

In the present study, we employed yeast two-hybrid screening to identify Arkadia-binding proteins, and found that one of the identified clones encoded the μ2 subunit of AP2. We showed that the μ2 subunit in the AP2 complex interacts with Arkadia. In addition, Arkadia modulated EGFR endocytosis. We therefore conclude that Arkadia plays a role in trafficking of AP2-regulated cell surface proteins.

Materials and Methods

Cell culture and transfection

HEK 293T, COS-7 and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 4.5 g/l glucose, 10% foetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Mouse mammary epithelial NMuMG cells (American Type Culture Collection) were cultured in DMEM in the presence of 4.5 g/l glucose, 10 μg/ml insulin, 10% FBS and the same antibiotics. All cells were grown in a 5% CO_2 atmosphere at 37°C. Transient transfection was performed using Fugene 6 Transfection Reagent (Roche Diagnostics) according to the manufacturer's instructions.

Yeast two-hybrid screening

Full-length mouse Arkadia cDNA (*3*) was inserted in-frame into pGBKT7 GAL4 DNA-binding vector and used as a bait. This construct was introduced into the yeast strain AH109 to screen a HeLa cell cDNA library in the pGAD vector (Matchmaker Gal4 Two-Hybrid System 3, Clontech Lab. Inc, Mountain View, CA, USA) on SD-L-W-H-A (synthetic defined medium deficient in leucine, tryptophan, histidine and adenine) with 3 mM of 3-Amino-1,2,4-triazole (3-AT). Positive clones were picked up after 5–13 days and confirmed by retransformation and β-galactosidase activity. Library plasmids were rescued from the yeast and sequenced.

Expression plasmids

The expression plasmids for wild-type (WT), ligase inactive mutant (CA) and deletion mutant (1–936) of mouse Arkadia, and ubiquitin were described previously (*3*). The other mutants of Arkadia and ubiquitin were generated by polymerase chain reaction (PCR) and by point mutagenesis, and confirmed by sequencing. The μ2 subunit (116–433 amino acids) was identified from the yeast two-hybrid system as a protein that binds to Arkadia. Full-length μ2 subunit was obtained by reverse transcription–PCR (RT–PCR) using HeLa cDNAs as a template. The expression plasmids for WT and deletion mutants of the μ2 subunit were 6Myc epitope-tagged at their N-terminus and subcloned into pcDEF3 vector. The expression construct encoding human AP2μ2 was cloned into the pcDNA3 vector. siRNA-resistant mutants of AP2μ2, μ2WT-siRNA-resistant mutant (WT-SR) and μ2K130R-siRNA-resistant mutant (K130R-SR), were generated using a PCR-based mutagenesis method. The sequences of primer for mutagenesis are 5′-CATTGCTCGCACGTCGTTTTTTC ATGTTAAGCGGTCC-3′ and 5′-GGACCGCTTAACATGAAAA AACGACGTGCGAGCAATG-3′.

Reagents and antibodies

Proteasomal inhibitor MG132 was purchased from Peptide Institute (Osaka, Japan). Recombinant human EGF was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Rabbit anti-Arkadia antibody (#62) was previously described (*4*). Mouse monoclonal anti-Flag M2, anti-α-tubulin and anti-AP2 β2 subunit antibodies were from Sigma-Aldrich. Mouse monoclonal anti-c-Myc antibody was from Oncogene Research Products (La Jolla, CA, USA). Mouse anti-HDAC1 and anti-phosphotyrosine 4G10 antibodies were from Millipore (Bedford, MA, USA). Mouse anti-EGFR was from Medical and Biological Laboratories (Nagoya, Japan). Mouse anti-AP2 μ2 subunit (AP50) antibodies was from BD Biosciences (Lexington, KY, USA). Anti-AP2 α-subunit antibody was from Sigma-Aldrich. Anti-EGFR antibody for immunoprecipitation was from Calbiochem (Darmstadt, Germany). Horseradish peroxidase-conjugated secondary antibodies to mouse IgG, rabbit IgG and rat IgG were from Amersham Pharmacia Biotech (Piscataway, NJ, USA) and Cappel Research Products (Columbus, OH, USA), respectively. Normal mouse IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

RNA interference

Short interfering RNAs (siRNAs) were transfected into cells according to the protocol recommended for Lipofectamine RNAi MAX Reagent (Invitrogen). Mouse NMuMG cells and human HeLa cells were transiently transfected with siRNAs against mouse and human Arkadia, respectively (Stealth RNAi, Invitrogen). siRNA against the human μ2 subunit (CCAGCUUCUUCCAUGUUAATT) was obtained from Invitrogen. The final concentrations of the siRNAs were each 10 nM in the present study.

Confocal fluorescence microscopy

HeLa cells were seeded onto 8-well culture slides (BD Biosciences) coated with 0.1% gelatin (Millipore, Bedford). Twenty-four hours after transfection, cells were fixed with 1 : 1 acetone: methanol solution and washed five times with PBS. For detection of Flag-Arkadia WT, cells were incubated with anti-Flag antibody in Blocking One (Nacalai Tesque, Kyoto, Japan) for 1 h at room temperature. Then, the cells were incubated with secondary antibodies and propidium iodide (PI) for 1 h. Fluorescence was examined by confocal laser scanning microscopy.

Immunoprecipitation and immunoblotting

COS-7 and HEK 293T cells transfected with the indicated plasmids were washed with PBS and lysed with cell lysis buffer containing 0.5% Nonidet P-40, 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulphonyl fluoride and 10 μg/ml aprotinin. For detection of endogenous Arkadia, MG132 was added to the cell lysis buffer at a concentration of 10 μM. After clearing with centrifugation, the supernatants were incubated with the indicated antibodies for 1 h and then incubated with Protein G-Sepharose (Amersham) for another 30 min. The beads were washed twice with the cell lysis buffer. Proteins were subjected to SDS polyacrylamide gel-electrophoresis (SDS–PAGE), followed by semidry transfer of them to Fluoro Trans W membrane (Pall, Glen Cove, NY, USA). Non-specific binding of proteins to the membrane was blocked by incubation in TBS-T buffer (50 mM Tris–HCl, pH7.4, 150 mM NaCl and 0.1% Tween-20) containing 5% skim milk. Immunodetection was performed with the ECL blotting system (Amersham) and Luminescent Image Analyzer (LAS4000, Fujifilm, Tokyo, Japan).

Sequential immunoprecipitation

HEK 293T cells were transfected with Myc-μ2, Flag-Arkadia and HA-ubiquitin, and treated with 10 μM MG132 for 4 h prior to harvest. The lysates were subjected to immunoprecipitation with anti-Myc antibody for 1 h. The immune complexes were washed with the cell lysis buffer and boiled for 5 min at 95°C in cell lysis buffer containing 1% SDS. The samples were diluted up to 1 ml with the cell lysis buffer, and secondary immunoprecipitated with anti-Myc antibody followed by immunoblot analysis.

Fractionation of nuclear and cytoplasmic proteins

NMuMG cells were transfected with siRNAs using HiPerFect Reagent (Qiagen). Fractionation of nuclear and cytoplasmic proteins from NMuMG cells was performed with the NE-PER nuclear and cytoplasmic extraction reagents following the manufacturer's instructions (PIERCE, Rockford, USA).

Cell surface biotinylation assay

After stimulation with 20 ng/ml EGF for 15 s on ice, HeLa cells were incubated with 0.3 mg/ml sulphosuccinimidyl 2-(biotinamido) ethyl-dithioproprionate (sulpho–NHS–SS–biotin; PIERCE) for 30 min on ice, followed by washing with sulpho-NHS-SS-biotin blocking buffer (50 mM NH_4Cl in PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$) to quench free sulpho-NHS-SS-biotin. After removal of the blocking buffer, normal media were added to the cells and incubated at 37°C for indicated periods. The cells were then washed twice with a glutathione buffer (60 mM glutathione, 83 mM NaCl, 83 mM NaOH and 1% bovine serum albumin) on ice for 20 min to remove bound biotinyl groups from remaining cell surface-biotinylated proteins, and lysed in radioimmunoprecipitation assay (RIPA) buffer (1% NP-40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris–HCl, pH 8.0 and 5 mM EDTA). The cell lysates were incubated with streptavidin beads (GE Healthcare) to collect biotinylated proteins. Proteins bound were analysed by SDS–PAGE and immunoblotting with anti-EGFR antibody.

Results

Subcellular localization of Arkadia

To examine the subcellular localization of Arkadia, Flag-tagged WT Arkadia (Arkadia WT) was ectopically expressed in HeLa cells and immunostaining was performed using anti-Flag antibody. Arkadia WT was observed in both the nucleus and the cytosol of HeLa cells (Fig. 1A). Next, to confirm the subcellular localization of endogenous Arkadia, we performed fractionation analysis of nuclear and cytosolic proteins, since the anti-Arkadia antibody we had prepared was not of quality sufficient for immunohistochemical analysis. Endogenous Arkadia was detected predominantly in the nucleus of mouse mammary epithelial NMuMG and HeLa cells, and partially in the cytosol (Fig. 1B and data not shown). Interestingly, endogenous Arkadia was detected as double bands on SDS–PAGE, and these bands clearly disappeared after transfection with Arkadia siRNA, indicating that the double bands on SDS–PAGE represent endogenous Arkadia, and that Arkadia is located in both the nucleus and the cytosol in cells.

μ2 subunit of AP2 as a binding protein to Arkadia

It has been reported that Arkadia acts in the nucleus as one of the positive regulators of TGF-β signalling (*4*). To elucidate the functions of Arkadia in the cytosol, we performed yeast two-hybrid screening using a cDNA library from HeLa cells with a full-length Arkadia as bait. Several positive clones were identified as proteins that bound to Arkadia. Among these proteins, the μ2 subunit of AP2 was chosen and further investigated, since AP2 is ubiquitously expressed in the cytosol of almost all cells. μ2 subunit 116–433, lacking the N-terminal 115 amino acids, was isolated from the positive clones, and full-length μ2 subunit (μ2 WT) was thus obtained from cDNAs of HeLa cells by a PCR-based strategy. To examine whether Arkadia binds to the μ2 subunit in cells, we transiently cotransfected HEK 293T cells with expression plasmids of Flag-tagged Arkadia and Myc-tagged μ2, and immunoprecipitated Arkadia with anti-Flag antibody. μ2 WT and μ2 116–433 coimmunoprecipitated with Arkadia (Fig. 2A). Since it is known that μ2 subunit associates with various transmembrane proteins at its YXXΦ-binding domain, we prepared a μ2 deletion mutant consisting only of the YXXΦ-binding domain (166–433). Figure 2A shows that μ2 166–433 mutant also interacted with Arkadia. These findings indicated that the YXXΦ-binding domain of the μ2 subunit is sufficient for binding to Arkadia.

The YXXΦ-binding domain of the μ2 subunit interacts with the YXXΦ motif in various transmembrane proteins. To identify the YXXΦ motif through which Arkadia binds to μ2 subunit, we prepared several deletion mutants of Arkadia and performed co-immunoprecipitation assay. Arkadia WT, a catalytically inactive mutant (CA), a C-terminally truncated mutant (ΔC) and an N-terminal non-catalytic fragment (NT) coimmunoprecipitated with μ2, consistent with the interaction in yeast (Fig. 2B and C and data not shown). Importantly, an N-terminally deleted mutant of Arkadia (ΔN) did not bind to μ2, suggesting the possibility that the YXXΦ motif in NT of Arkadia interacted with μ2 subunit. We found one YXXΦ motif (YALL) at amino acid position 246–249 in the NT region of Arkadia and substituted it to AALL (YA mutant). Figure 2D shows that WT and the NT mutant of Arkadia clearly interacted with μ2, whereas both YA mutants [WT(YA) and NT (YA)] exhibited reduced association with μ2. These findings strongly suggested that the YXXΦ-binding domain of μ2 subunit interacts with the YALL motif of Arkadia.

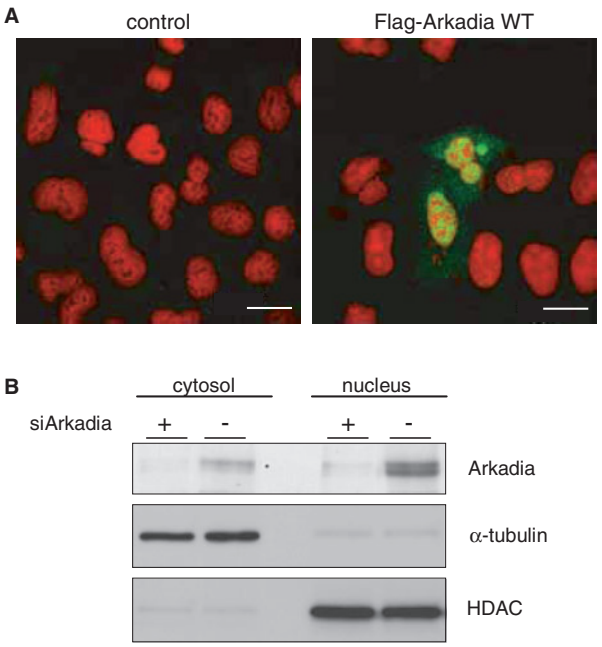

Fig. 1 Subcellular localization of Arkadia. (A) HeLa cells were immunostained with anti-Flag antibody at 24 h after transfection with Flag-Arkadia WT (right) or empty vector control (left). Red, PI; green, anti-Flag antibody. Scale bars indicate 20 μm. (B) Fractionation of cytosolic and nuclear proteins of NMuMG cells was performed as described in 'Materials and Methods' section. Anti-HDAC and α-tubulin antibodies were used as representative markers of nuclear and cytosolic proteins, respectively.

Association of Arkadia with AP2 complex

The AP2 complex consists of four different subunits of proteins including α, β2, μ2 and σ2. We therefore examined whether Arkadia interacts with endogenous AP2 complex. HEK 293T cells were transiently transfected with Flag-Arkadia WT and immunoprecipitated with anti-Flag antibody. Anti-Flag antibody, but not normal mouse serum, specifically coimmunoprecipitated subunits of α, β2 and μ2 with Flag-tagged Arkadia (Fig. 3A). Anti-μ2 antibody could not be used for coimmunoprecipitation assay, since the μ2 subunit has almost the same molecular weight as IgG heavy chain. Therefore, instead of anti-μ2 antibody, anti-β2 subunit antibody was used for immunoprecipitation of AP2 complex (Fig. 3B). Anti-β2 antibody clearly immunoprecipitated endogenous β2 subunit and also coimmunoprecipitated endogenous Arkadia. These findings thus suggest that Arkadia associates with AP2 complex.

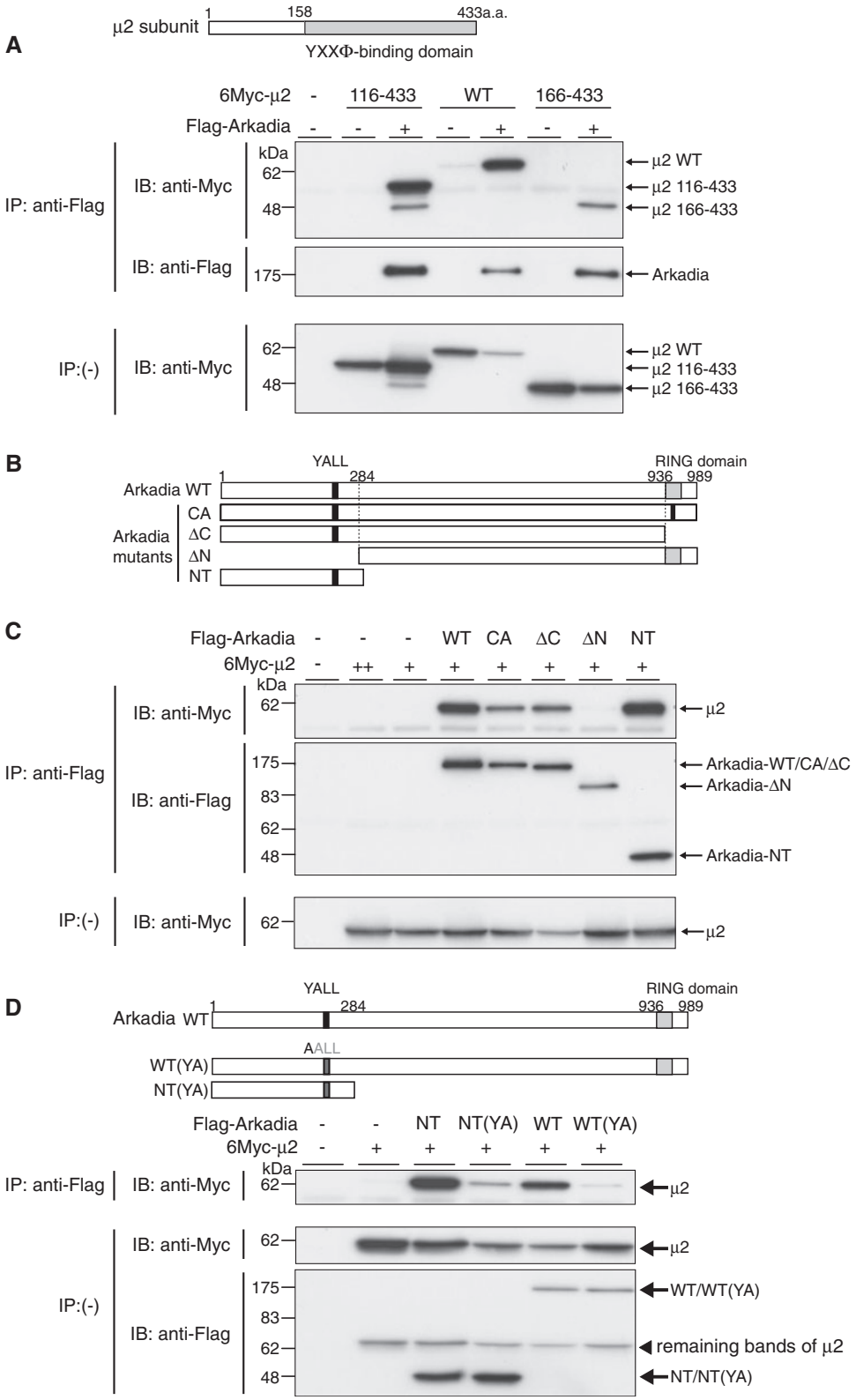

Fig. 2 Interaction of Arkadia with μ2 subunit. (A) Schematic illustration of μ2 subunit is shown in the top panel, and the YXXΦ binding domain in μ2 subunit is shown in grey. After transfection with the indicated plasmids into COS-7 cells, immunoprecipitation (IP) with anti-Flag antibody was performed, followed by immunoblotting (IB) with anti-Myc antibody or anti-Flag antibody. (B) Schematic illustration of Arkadia mutants. The C-terminal RING domain and the YALL sequence are shown in grey and black, respectively. (C) The interaction of 6Myc-μ2 with Flag-Arkadia WT and -Arkadia mutants (CA, ΔC, ΔN and NT) in COS-7 cells were examined by immunoprecipitation (IP) and immunoblot (IB) analyses. (D) Schematic illustration of Arkadia YA mutants is shown in the top panel. The C-terminal RING domain is shown in grey (top). The interaction of Flag-Arkadia WT and -Arkadia mutants with 6Myc-μ2 was determined by immunoprecipitation (IP) and immunoblot (IB) analyses. The bottom panel was reblotted with anti-Flag antibody after detection of 6Myc-μ2 using anti-Myc antibody. Arrowhead indicates the remaining bands of 6Myc-μ2.

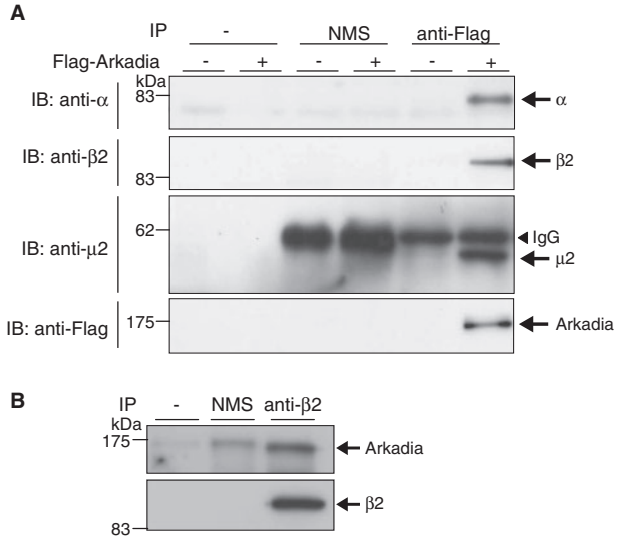

Fig. 3 Association of AP2 complex with Arkadia. (A) Flag-Arkadia was overexpressed in HEK 293T cells. The lysates were immunoprecipitated with anti-Flag and immunoblotted (IB) with anti-α subunit, anti-β2 subunit and anti-μ2 subunit antibodies. Arrowhead indicates IgG heavy chain. (B) Cells were treated with 50 μM MG132 for 5 h. The lysate from HeLa cells was subjected to immunoprecipitation with anti-β2 subunit antibody, followed by immunoblotting with anti-Arkadia antibody. NMS, normal mouse serum.

Ubiquitylation of AP2μ2 by Arkadia

We next examined whether μ2 subunit serves as a substrate of Arkadia. To determine this, Flag-tagged Arkadia, Myc-tagged μ2 and HA-tagged ubiquitin were transfected into HEK 293T cells. Ubiquitylation of μ2 was observed in the presence of Arkadia WT, but not in the presence of Arkadia CA and Arkadia ΔC (Fig. 4A and data not shown). μ2 116–433 mutant was also ubiquitylated by Arkadia WT. However, although μ2 166–433 mutant interacted with Arkadia (Fig. 2A), ubiquitylation of μ2 166–433 by Arkadia was markedly decreased (Fig. 4A), suggesting that amino acids between 116 and 166 of the μ2 subunit are crucial sites for ubiquitylation by Arkadia.

The region from amino acids 116 to 166 in the μ2 subunit contains three lysine residues at amino acid positions 130, 139 and 143. To determine which lysine residues are ubiquitylated by Arkadia, we generated μ2 mutants in which each lysine residue was replaced by an arginine residue. μ2 WT and its derivative mutants (K130R, K139R, K143R and 3KR mutants with alteration of all three lysine residues to arginine residues) were co-transfected with Flag-Arkadia WT and HA-ubiquitin. The K139R and K143R mutants of μ2 were ubiquitylated by Arkadia at levels similar to μ2 WT, whereas K130R and 3KR mutants exhibited reduced ubiquitylation by Arkadia (Fig. 4B). These findings indicated that Lys130 of μ2 is a key site for the ubiquitylation induced by Arkadia. To further confirm the ubiquitylation of μ2 by Arkadia, we performed sequential immunoprecipitation assay. After cotransfection with Myc-μ2, Flag-Arkadia and HA-ubiquitin, Myc-μ2 was immunoprecipitated with anti-Myc antibody, followed by boiling in a SDS-containing buffer. Myc-μ2 was then repeatedly immunoprecipitated with anti-Myc antibody. Figure 4C shows that ubiquitin-bound μ2 was detected only on co-transfection with Arkadia WT, but not with Arkadia ΔC, indicating that Arkadia ubiquitylates the μ2 subunit of AP2.

Addition of ubiquitin chains through Lys27 linkage to the μ2 subunit by Arkadia

Ubiquitin has seven lysine residues at amino acid positions 6, 11, 27, 29, 33, 48 and 63, each of which is able to form an isopeptide bond with the C-terminal glycine and form polyubiquitin chains (*12*). To determine which lysine residues formed polyubiquitin chains by Arkadia, we prepared several ubiquitin mutants, in which one lysine residue was intact and the other six lysine residues were substituted by arginine residues (6K, 11K, 27K, 29K, 33K, 48K and 63K). Among these mutants, only the ubiquitin 27K mutant yielded polyubiquitylation of μ2 by Arkadia, whereas the other ubiquitin mutants and the 7KR mutant, in which all seven lysine residues were substituted by arginine residues, failed to polyubiquitylate (Fig. 5A). We also prepared ubiquitin mutants in which one lysine residue was substituted by an arginine residue (K27R, K29R, K48R and K63R). Ubiquitin K27R mutant, in which Lys27 had been mutated to an arginine residue, markedly decreased polyubiquitylation of μ2 by Arkadia (Fig. 5B). These findings indicated that Arkadia adds ubiquitin chains through Lys27 linkage to the μ2 subunit of AP2.

Regulation of EGF endocytosis by Arkadia

The AP2 complex regulates endocytosis of cell surface proteins. Endocytosis of EGFR is one of the most widely used experimental systems for study of the mechanisms of AP2/clathrin-mediated endocytosis (*11*). We thus examined whether Arkadia regulates endocytosis of EGFR. After stimulation with EGF, HeLa cells were surface-biotinylated on ice, and then moved to 37°C to elicit internalization. After incubation for indicated periods, biotinylated proteins were purified with avidin-beads and determined by immunoblotting with anti-EGFR antibody (Fig. 6A). In control cells, EGF induced endocytosis of EGFR at maximal levels in 10 min. Interestingly, Arkadia siRNA decreased levels of biotinylated EGFR (Fig. 6A), suggesting that Arkadia positively regulates endocytosis of EGFR. In addition, we prepared two μ2 mutants, WT-SR that is resistant to AP2μ2 siRNA and K130R-SR that is resistant to AP2μ2 siRNA and is not ubiquitylated by Arkadia. Revertant HeLa cells were prepared by silencing endogenous AP2μ2 with AP2μ2 siRNA and by transfection with WT-SR or K130R-SR. The revertant HeLa cells with K130R-SR revealed increased levels of tyrosine phosphorylation of EGFR after EGF stimulation (Fig. 6B), and remarkably repressed endocytosis of EGFR (Fig. 6C). These findings suggest that K130R mutation inhibits endocytosis of EGFR and retains EGFR at cell surface. Therefore, Arkadia-mediated K130 ubiquitylation of AP2μ2 may promote endocytosis of EGFR.

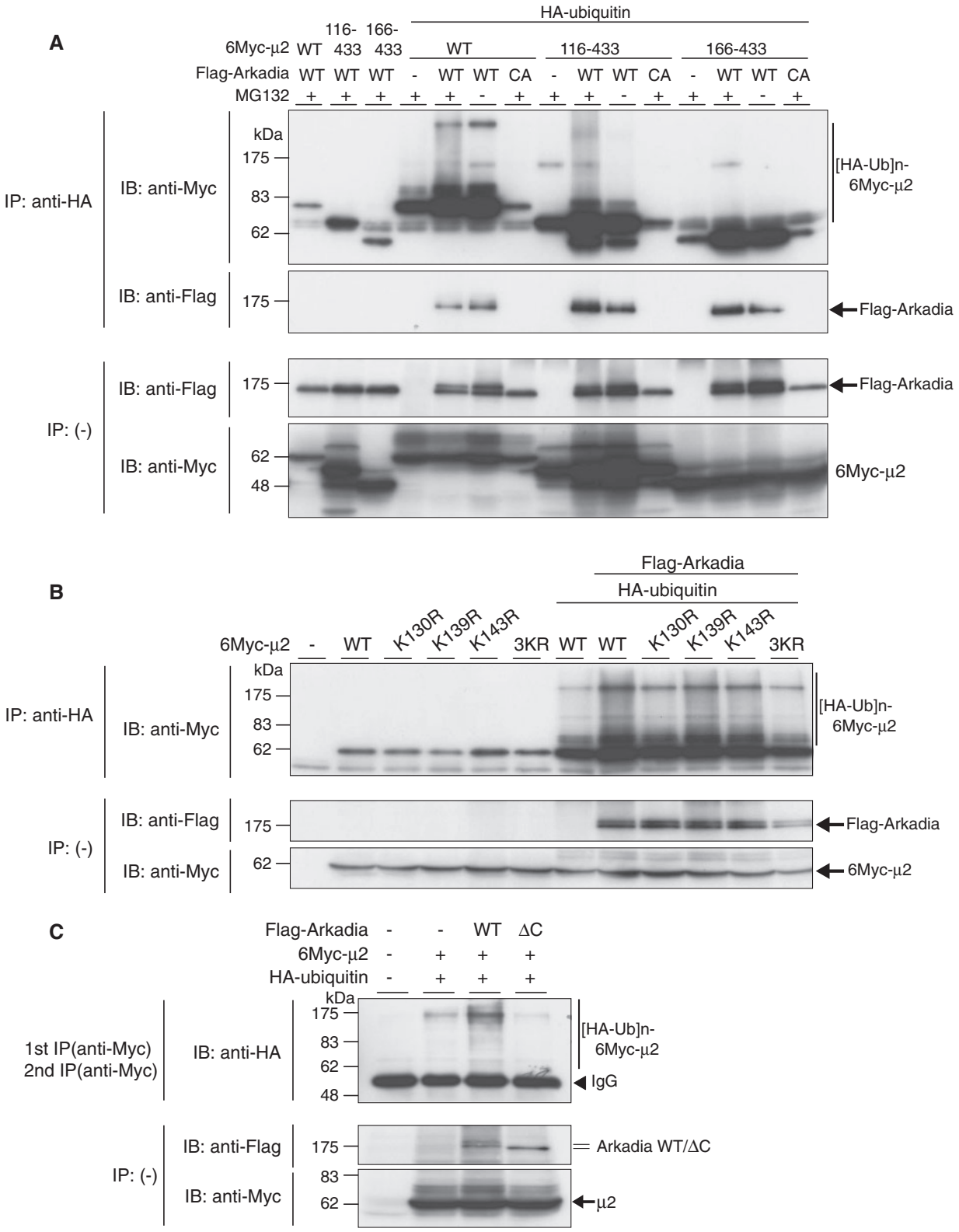

Fig. 4 Ubiquitylation of μ2 subunit of AP2 by Arkadia. (A) HEK 293T cells were transfected with various combinations of HA-ubiquitin, 6Myc-μ2 and Flag-Arkadia. Cells were treated with 10 μM MG132 for 4 h before lysis. The lysates were subjected to immunoprecipitation with anti-HA antibody and then immunoblotted (IB) with anti-Myc antibody. (**B**) HEK 293T cells were transfected with various combinations of HA-ubiquitin, Flag-Arkadia and 6Myc-μ2 or -μ2 mutants (K130R, K139R, K143R and 3KR). Cells were treated with 10 μM MG132 for 4 h. Immunoprecipitation and immunoblot (IB) analyses were performed using the indicated antibodies. (C) 6Myc-μ2, Flag-Arkadia and HA-ubiquitin were overexpressed in HEK 293T cells. Cells were treated with 10 μM MG132 for 4 h. The lysates were subjected to immunoprecipitation with anti-Myc antibody and the immunocomplexes were boiled for 5 min in lysis buffer containing 1% SDS. The samples were diluted up to 1 ml with the lysis buffer, and then secondary immunoprecipitated with anti-Myc antibody followed by immunoblotting (IB) with anti-HA and -Flag antibodies. Arrowhead indicates IgG heavy chain.

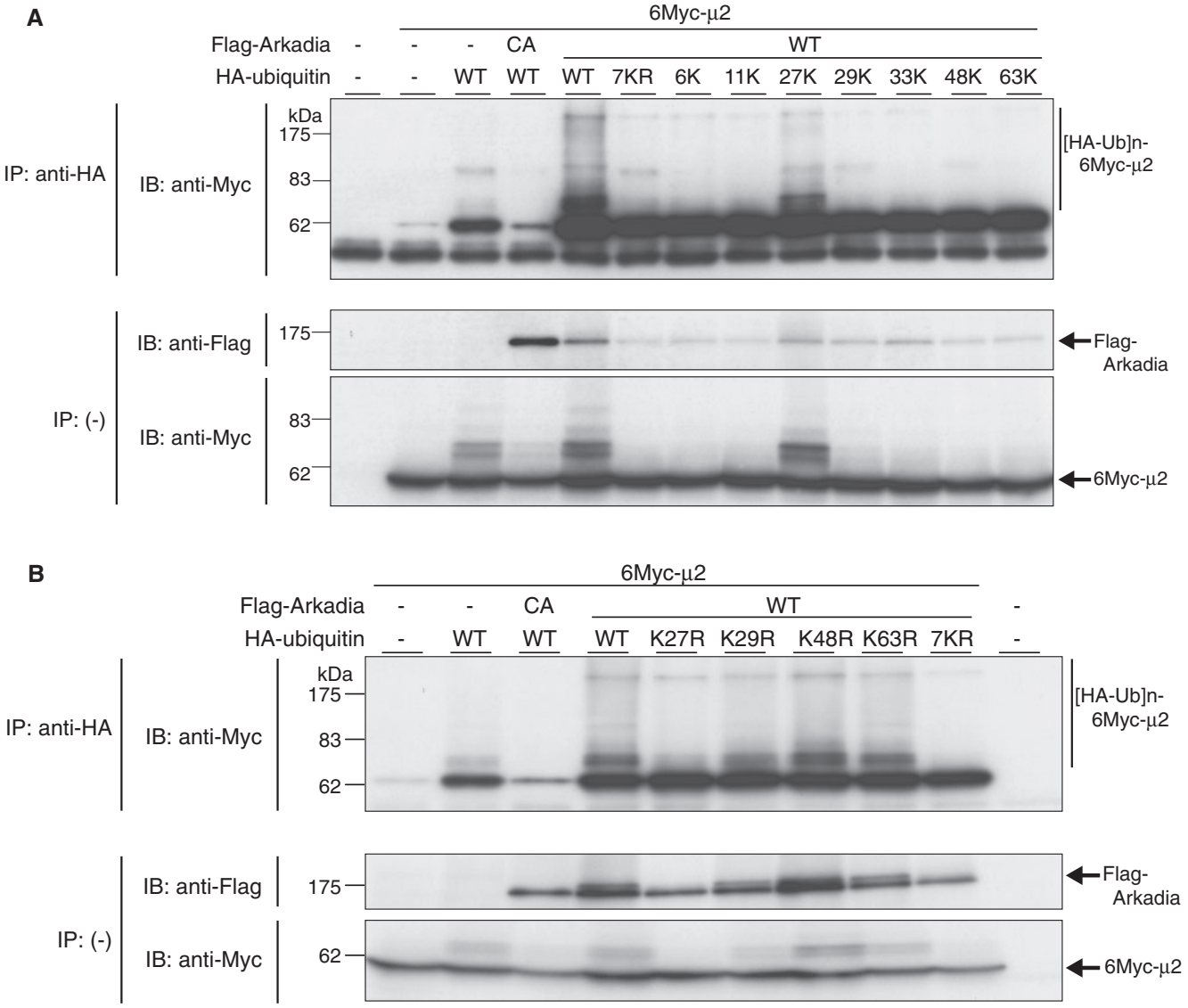

Fig. 5 Addition of ubiquitin chains through Lys27 linkage to the μ2 subunit by Arkadia. (A and B) After the indicated plasmids were transfected into HEK 293T cells, cells were treated with 10 μM of MG132 for 4 h. Lysates were immunoprecipitated (IP) with anti-HA antibody and then immunoblotted (IB) with the indicated antibodies.

Discussion

In the present study, we identified the μ2 subunit of AP2 as a novel protein that binds to Arkadia. The N-terminal YALL motif of Arkadia interacted with the YXXΦ-binding domain of the μ2 subunit, and Arkadia associated with the complex with AP2. In addition, Arkadia modified EGFR internalization through ubiquitylation of AP2μ2. The AP2-bound Arkadia complex thus controls endocytosis of EGFR.

Arkadia has been known to be a positive regulator of TGF-β signalling by targeting Smad7, c-Ski and SnoN in the nucleus, since Arkadia has two putative nuclear localization signals and is predominantly located in the nucleus. It has recently been reported that Axin links interaction of Smad7 with Arkadia in the cytosol and promotes degradation of Smad7 mediated by Arkadia (*6*). In the present study, we found that Arkadia interacted with AP2 complex and regulated localization of AP2μ2 (Supplementary Fig. S1). In addition, Arkadia siRNA modulated endocytosis of EGFR induced by EGF. K130R-SR mutant decreased endocytosis of EGFR, suggesting that EGFR is retained at the cell surface and that tyrosine phosphorylation of EGFR is enhanced after EGF stimulation. These findings suggest that ubiquitylation of AP2μ2 by Arkadia promotes internalization of EGFR. Moreover, pulse-chase assay using cells overexpressing Arkadia or those with silencing of endogenous Arkadia by specific siRNAs revealed that Arkadia did not affect the turnover of endogenous μ2 protein (data not shown), suggesting that EGFR endocytosis mediated by Arkadia may not be induced by decreased levels of AP2μ2 expression. However, other possibilities that μ2-bound Arkadia modulates modifications of EGFR and other clathrin adaptor proteins including AP2α, AP2β2, Epsin1 and Eps15 are not completely excluded yet (*13*,*14*).

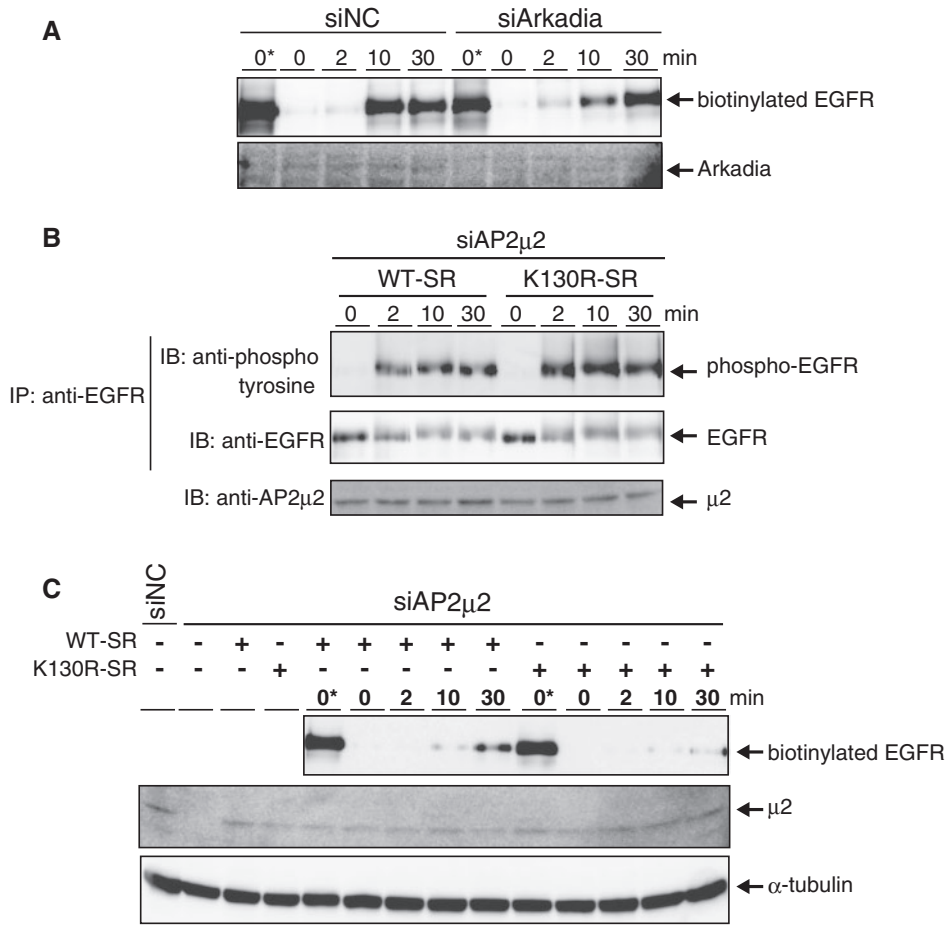

Fig. 6 Regulation of EGFR internalization by Arkadia. (A) HeLa cells transfected with control siRNA (siNC) or siArkadia were treated with EGF and then surface-biotinylated. After purification with avidin-beads, surface-biotinylated and internalized EGFR was detected by immunoblotting with anti-EGFR antibody. 0* indicates 'without washing with a glutathione buffer'. (B) HeLa cells introduced with siAP2μ2 were transfected with AP2 mutants of WT-SR or K130R-SR, and treated with 20 ng/ml of EGF for indicated periods. The lysates were immunoprecipitated with anti-EGFR antibody, followed by immunoblotting with anti-phosphotyrosine or anti-EGFR antibodies. (C) HeLa cells introduced with siRNAs against AP2μ2 or control (siNC) were transfected with WT-SR or K130R-SR. The cells were treated with 20 ng/ml of EGF and surface-biotinylated. Internalized EGFR was detected as in (A). 0* indicates 'without washing with a glutathione buffer'.

It has been reported that β2 of AP2 interacted with the isoleucine–leucine-based (di-leucine) motif of TβR-II as determined by yeast two-hybrid and *in vitro* biochemical studies (*15*). Although TβR-II colocalized with clathrin-coated pits, whether endocytosis of TβR-II and TβR-I by AP2 occurs is unknown. In the present study, we found that Arkadia directly bound to the μ2 subunit and complexed with AP2, raising the possibility that Arkadia mediates endocytosis of receptors for the TGF-β family and regulates TGF-β signalling.

Ubiquitin is a 76-amino acid protein and its C-terminal glycine carboxyl group forms an isopeptide bond with the ε-amino group of lysine residues. There are seven lysine residues in ubiquitin itself, at amino acids 6, 11, 27, 29, 33, 48 and 63 (*12*). Lys48- and Lys63-linked chains have been well characterized. The former is involved in protein proteasomal degradation and the latter in DNA repair, ribosome function and also proteasomal degradation. Lys29-linked chains may be involved in protein degradation. While ubiquitin–ubiquitin conjugation can occur through any of the seven lysine residues, Lys6-, Lys11-, Lys27- and Lys33-linked chains have not been well characterized. Our findings indicate that Arkadia adds ubiquitin chains through Lys27 linkage to the μ2 subunit (Fig. 4). However, it is still unclear whether other substrates of Arkadia are modified by Lys27-linked chains. The function of polyubiquitylation through Lys27 linkage will be elucidated in the near future.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of interest
None declared.

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