The E3 Ubiquitin Ligase Siah-1 Suppresses Avian Reovirus Infection by Targeting p10 for Degradation

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ABSTRACT Avian reovirus (ARV) causes viral arthritis, chronic respiratory diseases, retarded growth, and malabsorption syndrome. The ARV p10 protein, a viroporin responsible for the induction of cell syncytium formation and apoptosis, is rapidly degraded in host cells. Our previous report demonstrated that cellular lysosome-associated membrane protein 1 (LAMP-1) interacted with p10 and was involved in its degradation. However, the molecular mechanism underlying LAMP-1-mediated p10 degradation remains elusive. We report here that the E3 ubiquitin ligase seven in absentia homolog 1 (Siah-1) is critical for p10 ubiquitylation. Our data show that Siah-1 ubiquitylated p10 and targeted it for proteasome degradation. Furthermore, the ubiquitylation of p10 by Siah-1 required the participation of LAMP-1 by forming a multicomponent complex. Thus, LAMP-1 promotes the proteasomal degradation of p10 via interacting with both p10 and the E3 ligase Siah-1. These data establish a novel host defense mechanism where LAMP-1 serves as a scaffold for both Siah-1 and p10 that allows the E3 ligase targeting p10 for ubiquitylation and degradation to suppress ARV infection.

IMPORTANCE Avian reovirus (ARV) is an important poultry pathogen causing viral arthritis, chronic respiratory diseases, retarded growth, and malabsorption syndrome, leading to considerable economic losses to the poultry industry across the globe. The ARV p10 protein is a virulence factor responsible for the induction of cell syncytium formation and apoptosis and is rapidly degraded in host cells. We previously found that cellular lysosome-associated membrane protein 1 (LAMP-1) interacts with p10 and is involved in its degradation. Here we report that the E3 ubiquitin ligase seven in absentia homolog 1 (Siah-1) ubiquitylated p10 and targeted it for proteasomal degradation. Furthermore, the ubiquitylation of p10 by Siah-1 required the participation of LAMP-1 by forming a multicomponent complex. Thus, LAMP-1 serves as an adaptor to allow Siah-1 to target p10 for degradation, thereby suppressing ARV growth in host cells.

KEYWORDS ARV, LAMP-1, Siah-1, complex, degradation, interaction, p10, viral replication, ubiquitination

Avian reovirus (ARV), a member of the *Orthoreovirus* genus in the *Reoviridae* family, is an important pathogen of chickens, causing viral arthritis, chronic respiratory diseases, retarded growth, and malabsorption syndrome and leading to considerable losses to the poultry industry. ARV infection induces apoptosis (1–3), cell-cell fusion, and syncytium formation (4–7). The ability of ARV to induce apoptosis is not restricted to a particular virus strain or to a specific cell type, since different ARV isolates were able to induce apoptosis in several avian and mammalian cell lines (1). ARV is an icosahedral nonenveloped virus with a double-protein capsid shell containing a genome consisting of 10 double-stranded RNA (dsRNA) segments (L1, L2, L3, M1, M2, M3, S1, S2, S3, and

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S4) (1, 8). These segments encode at least 10 different structural proteins, 8 of which (λA , λB , λC , μA , μB , σA , σB , and σC) are primary translation products of their encoding mRNAs, whereas the other two, μBN and μBC , originate by the posttranslational cleavage of their precursor μB (8, 9). In addition to the structural proteins, ARV expresses four nonstructural proteins (μNS , σNS , p10, and p17) (10).

The viral protein p10, encoded by the first open reading frame of the S1 gene (11), contains a central transmembrane domain that separates ecto- and endodomains of approximately equal sizes (9, 12). It was found that p10 plays a key role in the fusogenic phenotype displayed by avian reoviruses because the expression of p10 induces extensive cell-cell fusion in transfected cells (10, 12). p10 is rapidly degraded in host cells, and the rate of p10 degradation was significantly reduced by the proteasome inhibitor MG132, indicating that p10 is degraded via the proteasomal degradation pathway (7, 13).

Seven in absentia homolog 1 (Siah-1) is the mammalian homolog of the *Drosophila* seven in absentia (Sina) protein that it is evolutionarily conserved from plants to mammals and functions primarily as an E3 ubiquitin (Ub) ligase (14, 15). The N terminus of Siah-1 encodes a RING domain that confers its E3 ubiquitin ligase activity, and the C terminus encodes a domain that mediates its binding to substrate proteins (16). Through direct and specific interactions with substrates, Siah-1 targets many proteins for ubiquitylation and proteasome-dependent degradation (17–21).

In our previous study, we found that the degradation of p10 was associated with lysosome-associated membrane protein 1 (LAMP-1) (13). However, the underlying molecular mechanism remains elusive. In the present study, we found that Siah-1 served as an E3 ligase interacting with both p10 and LAMP-1, forming a complex. Importantly, the knockdown of Siah-1 by RNA interference (RNAi) markedly reduced p10 ubiquitylation, allowing the accumulation of p10 in host cells and facilitating ARV infection. Furthermore, the knockdown of LAMP-1 significantly mitigated the interaction of Siah-1 with p10, reducing Siah-1-mediated p10 degradation, suggesting that LAMP-1 works as a scaffold protein for interactions with both Siah-1 and p10 that allows Siah-1-mediated ubiquitylation and degradation of p10 to suppress ARV replication.

RESULTS

Interaction of ARV p10 with the cellular protein Siah-1. Our previous study showed that ARV p10 interacted with the cellular protein LAMP-1 and was degraded via the proteasomal degradation pathway (13). However, the E3 ligase responsible for p10 ubiquitin-dependent degradation remains elusive. It was reported previously that the expression of p10 induces apoptosis in DF-1 cells (13), ARV induces apoptosis through p53 (22), and Siah-1 is a p53-inducible E3 ubiquitin ligase playing a role in p53-mediated apoptosis (23–26). Furthermore, Siah-1 targets several viral proteins for proteasomal degradation, such as Kaposi's sarcoma-associated herpesvirus (KSHV) open reading frame 45 (ORF45) (15), herpes simplex virus (HSV) ICP0 (21), and hepatitis B virus (HBV) X (20). Thus, we examined whether Siah-1 interacted with ARV p10 and served as the candidate E3 ubiquitin ligase for the proteasomal degradation of the ARV protein p10. We transfected DF-1 cells with pRK5-flag-p10 and/or pCMV-Myc-Siah-1 and performed immunoprecipitation assays. As a result, when the lysates from DF-1 cells expressing both Myc-Siah-1 and FLAG-p10 were immunoprecipitated with FLAG antibody, Myc-Siah-1 was detected in the precipitate, indicating that p10 interacted with exogenous Siah-1 in DF-1 cells (Fig. 1A). To further substantiate the binding of Siah-1 to ARV p10, we transfected DF-1 cells with pRK5-flag-Siah-1, infected these cells with ARV, and performed a pulldown assay using anti-FLAG antibodies. Our data show that p10 was readily detectable in the precipitate, indicating that Siah-1 interacted with ARV p10 (Fig. 1B). To further determine whether ARV p10 and Siah-1 could interact under physiological conditions, we infected DF-1 cells and performed a pulldown assay using anti-p10 antibodies. As a result, Siah-1 was detected in the precipitates of the lysates from ARV-infected cells but not from those of mock-infected controls (Fig. 1C). These data clearly show that ARV p10 interacts with Siah-1 in host cells.

FIG 1 Interaction of ARV p10 with Siah-1. (A) Interaction of p10 with exogenous Siah-1. DF-1 cells were transfected with the indicated expression plasmids. Twenty-four hours after transfection, cell lysates were prepared, immunoprecipitated (IP) with anti-FLAG antibody, and immunoblotted with anti-Myc or anti-FLAG antibodies. (B) Interaction of ARV p10 with exogenous Siah-1 in ARV-infected cells. DF-1 cells were transfected with the indicated expression plasmids. Six hours after transfection, cells were infected with ARV at an MOI of 5. Thirty-six hours after infection, cell lysates were prepared, immunoprecipitated with anti-FLAG antibody, and immunoblotted with anti-p10 or anti-FLAG antibodies. (C) Interaction of ARV p10 with endogenous Siah-1 in ARV-infected cells. DF-1 cells were infected with ARV at an MOI of 5. Thirty-six hours after infection, cell lysates were prepared, immunoprecipitated with anti-p10 antibody, and immunoblotted with anti-p10 or anti-Siah-1 antibodies. WB, Western blot.

The ectodomain spanning amino acid residues 1 to 43 of p10 is responsible for interaction with the C-terminal binding domain of Siah-1. To determine the region of p10 that is responsible for the interaction with Siah-1, we constructed a series of truncated p10 mutants fused to a green fluorescent protein (GFP) tag (Fig. 2A) with reference to the information that p10 contains a central transmembrane domain (amino acids [aa] 44 to 62) lying between an ectodomain (aa 1 to 43) and an endodomain (aa 63 to 98) (12). These p10 derivatives were individually coexpressed with FLAG-Siah-1 in DF-1 cells, and the lysates of the cells were immunoprecipitated with FLAG antibody. Our results indicated that with the exception of mutants (p10 Δ 3 and p10 Δ 4) lacking aa 1 to 43, other p10 mutants with aa 1 to 43 (mutants p10 Δ 1 and p10 Δ 2) retained the ability to interact with Siah-1 (Fig. 2B), suggesting that the ectodomain spanning aa 1 to 43 of p10 is a direct and specific region responsible for its interaction with Siah-1 (Fig. 2C).

It was reported previously that the substrate binding domain of Siah-1 is located in the C-terminal portion of the protein (27). Using a similar strategy, we constructed truncated Siah-1 mutants (Fig. 2D). We cotransfected cells with pEGFP-p10 and different FLAG-Siah-1 mutants and found that only the truncated Siah-1 mutant with the C-terminal portion (Siah-1 Δ 2) could bind to GFP-p10 (Fig. 2E and F). These results indicated that the portion of p10 spanning aa 1 to 43 directly and specifically interacted with Siah-1, while the C-terminal portion of Siah-1 served as the binding domain for p10 (Fig. 2C and F).

ARV p10 colocalizes with Siah-1. To determine the subcellular localization of Siah-1 and p10, we performed confocal microscopy assays with DF-1 cells transfected to express DsRed-p10 and GFP-Siah-1. As shown in Fig. 3A and B, both Siah-1 and p10 were localized in the cytoplasm of pEGFP-Siah-1- or pDsRed-p10-transfected cells. When cells were transfected with both plasmids, Siah-1 and p10 were well colocalized in the cytoplasm (Fig. 3C to E), supporting the above-described results that Siah-1

FIG 2 Determination of the binding domains of p10 for Siah-1 and of Siah-1 for p10. (A) Schematics showing the genes encoding full-length p10 and truncated p10 molecules (from $\Delta 1$ to $\Delta 4$). The numbers indicate the amino acid positions in the molecule. WT, wild type. (B) Siah-1 interacts with different truncated p10 mutants. DF-1 cells were transfected with the indicated plasmids. Cell lysates were prepared and immunoprecipitated with anti-FLAG monoclonal antibody. The pellets were examined by Western blotting using anti-GFP and anti-FLAG antibodies. (C) Schematic representing the binding domain (aa 1 to 43) of p10 for Siah-1. (D) Schematics representing the genes encoding full-length Siah-1 and truncated Siah-1 molecules ($\Delta 1$ and $\Delta 2$). The numbers indicate the amino acid positions in the molecule. (E) p10 interacts with different truncated Siah-1 mutants. DF-1 cells were transfected with the indicated plasmids. Cell lysates were prepared and immunoprecipitated with anti-FLAG monoclonal antibody. The pellets were examined by Western blotting using anti-GFP and anti-FLAG antibodies. (F) Schematic representing the binding domain (aa 141 to 282) of Siah-1 for p10.

interacted with p10 in cells. To consolidate these findings, we examined the colocalization of Siah-1 with p10 in ARV-infected DF-1 cells. We transfected DF-1 cells with pEGFP-Siah-1, infected these cells with ARV at a multiplicity of infection (MOI) of 5, and performed an immunofluorescent-antibody assay (IFA) using anti-p10 antibody. As a result, Siah-1 was also colocalized with p10 in the cytoplasm of ARV-infected cells (Fig. 3F to K). These results strongly support that ARV p10 interacts with Siah-1 in cells.

Siah-1 induces p10 degradation via a proteasome pathway. Since p10 can be modified by ubiquitylation and degraded rapidly *in vivo* (7, 13), we examined the effect of Siah-1 on p10 degradation in cells. We transfected DF-1 cells with pRK5-flag-p10 with or without pCMV-Myc-Siah-1 in the presence or absence of MG132, an inhibitor of

FIG 3 Colocalization of p10 with Siah-1 in DF-1 cells. (A to E) Localization of p10 and exogenous Siah-1 in DF-1 cells. DF-1 cells (2×10^5) were seeded onto 24-well plates with coverslips in the wells and cultured overnight. Cells were transfected with pDsRed-p10 (A) or pEGFP-Siah-1 (B) or with both pDsRed-p10 and pEGFP-Siah-1 (C to E). Sixteen hours after transfection, cells were fixed with 4% paraformaldehyde. After washes, the cell nuclei were counterstained with DAPI (blue). The cell samples were observed with a confocal laser scanning microscope. (F to K) Colocalization of ARV p10 with exogenous Siah-1 in mock- or ARV-infected cells. DF-1 cells were transfected with the indicated expression plasmids. Six hours after transfection, cells were mock infected or infected with ARV at an MOI of 5. Sixteen hours after infection, cells were fixed and probed with mouse anti-p10 antibody followed by TRITC-conjugated goat anti-mouse antibodies (red). Nuclei were counterstained with DAPI (blue). The cell samples were observed with a confocal laser scanning microscope.

proteasomal degradation, and examined the effect of Siah-1 overexpression on the p10 content in cells by Western blotting. We found that the overexpression of Siah-1 significantly reduced p10 levels in cells (Fig. 4A and B). Furthermore, the reduction in the p10 level by Siah-1 was markedly inhibited by the proteasome inhibitor MG132. Similar results were also obtained from an experiment with ARV infection of DF-1 cells (Fig. 4C and D). In contrast to MG132, chloroquine (Chlq), a commonly used lysosomal inhibitor, had no inhibitory effect on Siah-1-induced p10 degradation in ARV-infected cells (Fig. 4E and F). These results indicate that ARV p10 was degraded via proteasomal rather than lysosomal degradation pathways.

It was reported previously that the Siah-1-C44S mutant abrogated the E3 ubiquitin ligase activity of Siah-1 (16, 21, 28). To determine the role of the cysteine residue at amino acid 44 of Siah-1 in p10 degradation, we constructed a Siah-1 mutant with a cysteine-to-serine substitution at codon 44 in its N-terminal RING domain (Siah-1-C44S) and examined the effect of the Siah-1 mutant on p10 degradation. As a result, the reduction in the level of the p10 protein by Siah-1 was reversed by its mutant Siah-1-C44S (Fig. 4G and H), indicating that cysteine 44 of Siah-1 is critical for the catalytic activity of Siah-1 in the process of p10 degradation. Moreover, our data show that the knockdown of endogenous Siah-1 by RNAi markedly enhanced p10 levels in pRK5-flag-p10-transfected cells compared to that of the RNAi control (Fig. 5A and B). Consistently, the knockdown of Siah-1 also significantly increased p10 levels in ARV-infected cells (Fig. 5C and D). These data demonstrate that Siah-1 is directly involved in p10 degradation via the proteasomal degradation pathway.

FIG 4 Siah-1 induces p10 protein degradation via the proteasome pathway. (A) DF-1 cells were transfected with the indicated plasmids. Eighteen hours after transfection, cells were treated with 20 μ M MG132 or dimethyl sulfoxide (DMSO) for 6 h, and the levels of proteins were examined by Western blotting. (B) The levels of ARV p10 expression in panel A were quantified by densitometry and normalized to that of actin. The expression level of p10 in the first column was set at 1.0. (C) DF-1 cells were transfected with the indicated plasmids. Six hours after transfection, cells were infected with ARV at an MOI of 5. Eighteen hours after infection, cells were treated with 20 μ M MG132 or DMSO for 6 h, and the levels of proteins were examined by Western blotting using the corresponding antibodies. (D) The levels of p10 expression in panel C were quantified by densitometry and normalized to that of actin. The expression level of p10 in the first column was set at 1.0. (E) Chloroquine does not affect Siah-1-induced p10 degradation. DF-1 cells were pretreated with chloroquine (Chlq) (50 μ M) for 4 h, followed by transfection with the indicated plasmids. Distilled water was used as a control. Six hours after transfection, cells were infected with ARV at an MOI of 5. One hour after virus absorption at 37°C, the cell cultures were changed for fresh medium in the absence or presence of Chlq. Twenty-four hours after infection, the levels of proteins were examined by Western blotting using the corresponding antibodies. (F) The levels of p10 expression in panel E were quantified by densitometry and normalized to that of actin. The expression level of p10 in the first column was set at 1.0. (G) Transfection of cells with pRK5-flag-Siah-1-C44S abolishes Siah-induced degradation of p10. DF-1 cells were transfected with the indicated expression plasmids. Six hours after transfection, cells were infected with ARV at an MOI of 5. The levels of proteins were examined by Western blotting. (H) The levels of ARV p10 expression in panel G were quantified by densitometry and normalized to that of actin. The expression level of p10 in the first column was set at 1.0. Data are presented as means $\pm$ standard deviations ($n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant.

Siah-1 inhibits ARV-induced syncytium formation and suppresses viral growth in host cells. Since Siah-1 is required for p10 degradation in cells and p10 plays a key role in the fusogenic phenotype displayed by ARV (10, 12), it would be logical and intriguing to examine the effects of Siah-1 on ARV-induced syncytium formation. As a result, the knockdown of Siah-1 markedly enhanced ARV-induced syncytium formation (Fig. 6A to G) compared to that of RNAi controls, suggesting that Siah-1 plays an inhibitory role in ARV pathogenesis.

As the knockdown of Siah-1 allows ARV p10 accumulation and ARV p10 acts as a porin that facilitates viral growth, Siah-1 should therefore inhibit ARV growth. To test this hypothesis, we examined viral replication in cells transfected with pEGFP-Siah-1 by measuring viral loads in cultures of ARV-infected cells at different time points postinfection. As a result, cells with Siah-1 overexpression had much lower viral loads in both the supernatant and cell cultures than those of controls (Fig. 7A and B). On the contrary, the knockdown of Siah-1 enhanced ARV growth in both the supernatants and cell cultures compared to that in control DF-1 cells (Fig. 7C and D). These data suggest that Siah-1 is involved in the host response against ARV infection via facilitating the ubiquitin-proteasome degradation of p10, which reduces p10-mediated cell-cell fusion and suppresses ARV growth.

FIG 5 Knockdown of Siah-1 results in the accumulation of p10 in cells. (A) DF-1 cells were treated with Siah-1 RNAi or control RNAi and transfected with pRK5-flag-p10. At 24 h posttransfection, cell lysates were prepared, and the expression levels of p10 were examined by Western blotting. (B) The levels of p10 expression in panel A were quantified by densitometry and normalized to that of actin. The expression level of p10 in normal control DF-1 cells was set at 1.0. (C) DF-1 cells treated with Siah-1 RNAi or control RNAi were infected with ARV at an MOI of 5. At 24 h postinfection, cell lysates were prepared, and the expression of p10 was examined by Western blotting. (D) The levels of ARV p10 expression in panel C were quantified by densitometry and normalized to that of actin. The expression level of p10 in normal control DF-1 cells was set at 1.0. Results are representative of data from three independent experiments. Data are presented as means $\pm$ standard deviations ($n = 3$). **, $P < 0.01$.

Siah-1 facilitates ubiquitylation of p10. The facts that Siah-1 acts as an E3 ligase in the proteasomal degradation of proteins (19, 20), interacts with p10, and is involved in p10 degradation prompted us to investigate whether Siah-1 is involved in the ubiquitylation of p10 in the proteasomal degradation pathway. We transfected plas-

FIG 6 Knockdown of Siah-1 by RNAi enhances ARV-induced syncytium formation in cells. (A to F) Knockdown of Siah-1 enhances ARV-induced syncytium formation. DF-1 cells treated with or without Siah-1 RNAi or control RNAi were mock infected or infected with ARV at an MOI of 10. Twenty-four hours after infection, cells were fixed and stained with Giemsa stain to visualize syncytia. The cell samples were observed under a regular microscope. Syncytia are indicated by arrows. Bar, 50 μ m. The magnification is the same in all panels. (G) Average number of syncytial nuclei per field. The significance of the difference between Siah-1 RNAi and control RNAi treatments in syncytium formation was analyzed by ANOVA. Data are the means $\pm$ standard deviations of results from three independent experiments. ***, $P < 0.001$.

FIG 7 Siah-1 suppresses ARV growth. (A and B) Overexpression of Siah-1 suppresses ARV growth in DF-1 cells. DF-1 cells were transfected with pEGFP-Siah-1 or pEGFP-C1 as a control. Twenty-four hours after transfection, cells were infected with ARV at an MOI of 5. At different time points (12, 24, 48, and 72 h) after ARV infection, the viral titers in the cell cultures (A) and supernatants (B) were determined by TCID₅₀ analysis using 96-well plates. The significance of the differences between pEGFP-Siah-1 and pEGFP-C1 control groups was determined by ANOVA. The graph shows the mean viral titers in DF-1 cells from three independent experiments. (C and D) Knockdown of Siah-1 by RNAi facilitates ARV growth in DF-1 cells. DF-1 cells were treated with Siah-1 RNAi or control RNAi, followed by infection with ARV at an MOI of 5. At different time points (12, 24, 48, and 72 h) after ARV infection, the viral titers in the cell cultures (C) and supernatants (D) were determined by TCID₅₀ analysis using 96-well plates. The significance of the differences between Siah-1 RNAi and control RNAi groups was determined by ANOVA. The graph shows the mean viral titers in DF-1 cells from three independent experiments. The significance of the difference between Siah-1 RNAi and control RNAi treatments in viral growth was determined by ANOVA.

mids encoding hemagglutinin (HA)-ubiquitin, pRK5-flag-p10, and pEGFP-Siah-1 or the pEGFP-C1 control into DF-1 cells and performed immunoprecipitation assays. As a result, the overexpression of Siah-1 significantly enhanced p10 ubiquitylation in cells compared to that of controls (Fig. 8A). On the contrary, the knockdown of Siah-1 by RNAi markedly reduced the ubiquitylation of p10 (Fig. 8B). These results clearly show that Siah-1 mediates the ubiquitylation of p10, leading to p10 degradation via the proteasome.

Siah-1 is required for the ubiquitylation of p10 *in vitro*. Since Siah-1 interacted with p10 and was involved in p10 ubiquitylation in cells, it would be vital to examine the effect of Siah-1, as an E3 ligase, on p10 ubiquitylation *in vitro*. We expressed a recombinant His-p10 protein in an *Escherichia coli* expression system, purified the protein, and performed ubiquitylation assays *in vitro*. As shown in Fig. 9, p10 was remarkably ubiquitylated in the presence of Siah-1 but not its mutant Siah-1-C44S. These data clearly show that Siah-1 is required for the ubiquitylation of p10 and that cysteine 44 of Siah-1 is critical for this process.

LAMP-1, Siah-1, and ARV p10 form a complex. Since our previous report showed that LAMP-1 interacted with p10 and played a critical role in p10 degradation, it was intriguing to determine whether LAMP-1 is also associated with Siah-1 in cells. We transfected DF-1 cells with pEGFP-LAMP-1 and/or pRK-flag-Siah-1 and performed immunoprecipitation assays. When lysates of cells expressing both FLAG-Siah-1 and GFP-LAMP-1 were immunoprecipitated with FLAG antibody, GFP-LAMP-1 was detected in the precipitate, indicating that Siah-1 interacted with LAMP-1 in DF-1 cells (Fig. 10A). To confirm the binding of Siah-1 to LAMP-1, we expressed Siah-1 in DF-1 cells and examined its interaction with endogenous LAMP-1 using a pulldown assay. The

FIG 8 Siah-1 mediates the ubiquitylation of p10. (A) Overexpression of Siah-1 enhances the ubiquitylation of p10 [p10-(Ub)_n]. DF-1 cells were transfected with plasmids encoding HA-ubiquitin, pRK5-flag-p10, and pEGFP-Siah-1 or pEGFP-C1 as a control. Twenty-four hours after transfection, cells were treated with MG132 for 6 h. The cell lysates were immunoprecipitated with anti-FLAG antibodies and immunoblotted with anti-HA or anti-FLAG antibodies. The whole-cell lysates were examined for protein expression (bottom panels) by Western blotting. (B) Knockdown of Siah-1 reduces p10 ubiquitylation. DF-1 cells were treated with Siah-1 RNAi or control RNAi together with HA-ubiquitin. Six hours after transfection, cells were infected with ARV at an MOI of 5. Eighteen hours after infection, cells were treated with 20 μ M MG132 for 6 h. Cell lysates were prepared, immunoprecipitated with anti-p10 antibodies, and immunoblotted with anti-p10 or anti-HA antibodies. The whole-cell lysates were examined for protein expression (bottom panels) by Western blotting.

binding of FLAG-Siah-1 with endogenous LAMP-1 was readily detectable in cells expressing Siah-1 (Fig. 10B). To determine the interaction of LAMP-1 with Siah-1 in ARV-infected cells under physiological conditions, we infected DF-1 cells with ARV and performed a pulldown assay. When lysates of ARV-infected cells were immunoprecipitated with p10 antibodies, both endogenous LAMP-1 and Siah-1 were detected in the precipitate (Fig. 10C), indicating that LAMP-1, Siah-1, and p10 formed a complex in host cells infected with ARV.

LAMP-1 affects Siah-1-mediated p10 ubiquitylation and degradation via acting as a scaffold protein for their interaction. Since LAMP-1 promoted ARV p10 degradation and interacted with both Siah-1 and ARV p10, we assumed that LAMP-1 would

FIG 9 Siah-1 is required for the ubiquitylation of p10 *in vitro*. An *in vitro* p10 ubiquitylation assay was performed with a reaction buffer containing UbE1, UbcH5b, Ub, GST-Siah-1, or the catalytically inactive GST-Siah-1-C44S mutant and His-p10, as indicated. Reaction mixtures were incubated at 37°C for 2 h. The ubiquitylated products were detected by Western blotting using anti-Ub, anti-GST, or anti-His antibodies.

FIG 10 Siah-1, LAMP-1, and ARV p10 form a complex. (A) Interaction of exogenous LAMP-1 with exogenous Siah-1. DF-1 cells were transfected with the indicated expression plasmids. Twenty-four hours after transfection, cell lysates were prepared, immunoprecipitated with anti-FLAG antibodies, and immunoblotted with anti-FLAG or anti-GFP antibodies. (B) Interaction of exogenous Siah-1 with endogenous LAMP-1. DF-1 cells were transfected with the indicated expression plasmids. Twenty-four hours after transfection, cell lysates were prepared, immunoprecipitated with anti-FLAG antibody, and immunoblotted with anti-FLAG or anti-LAMP-1 antibodies. (C) ARV p10 interacts with both endogenous Siah-1 and LAMP-1, forming a complex. DF-1 cells were infected with ARV at an MOI of 5. Thirty-six hours after infection, cell lysates were prepared, immunoprecipitated with anti-p10 antibodies, and immunoblotted with anti-p10, anti-Siah-1, or anti-LAMP-1 antibodies.

induce p10 degradation by interacting with Siah-1. To test this hypothesis, we examined the effect of LAMP-1 on the interaction of Siah-1 with p10 in ARV-infected cells in the presence of MG132. We found that the knockdown of LAMP-1 by RNAi markedly reduced ARV p10 levels in the precipitates from the lysates immunoprecipitated by Siah-1 compared to those of RNAi controls (Fig. 11A and B). In addition, the knockdown of LAMP-1 decreased the colocalization of Siah-1 with p10 (Fig. 11C to H), while the colocalization of LAMP-1 with p10 was not much affected by Siah-1 knockdown compared to that of the RNAi control (Fig. 11I to N), suggesting that LAMP-1 is required for the interaction of Siah-1 with p10. Furthermore, the knockdown of LAMP-1 by RNAi significantly inhibited the reduction of p10 levels by Siah-1 overexpression compared to that of RNAi controls without affecting ARV σ C expression (Fig. 12A and B), indicating that p10 degradation is specifically induced by Siah-1 associated with LAMP-1. Moreover, we examined the effect of LAMP-1 on Siah-1-mediated p10 ubiquitylation *in vitro*. As shown in Fig. 12C, p10 ubiquitylation *in vitro* was markedly enhanced in the presence of His-LAMP-1. These results reveal that LAMP-1, via interactions with both Siah-1 and p10, serves as a scaffold protein that allows the E3 ligase to target p10 for its ubiquitylation and degradation.

Taken together, our data show that LAMP-1 interacted with both p10 and Siah-1, forming a complex, leading to p10 degradation via proteasomal pathways, and suppressing viral growth.

DISCUSSION

ARV causes viral arthritis, chronic respiratory diseases, retarded growth, and malabsorption syndrome, leading to considerable losses to the poultry industry. ARV infection induces apoptosis in cultured cells (1, 2, 29) and in infected chicken tissues (30). It was

FIG 11 Knockdown of LAMP-1 reduces the interaction of Siah-1 with p10. (A) DF-1 cells were treated with LAMP-1 RNAi or control RNAi together with pRK5-flag-Siah-1 or pRK5-flag. Six hours after transfection, cells were infected with ARV at an MOI of 5. Twenty-four hours after infection, cells were treated with 20 μ M MG132 for 6 h. Cell lysates were prepared, immunoprecipitated with anti-FLAG antibodies, and immunoblotted with anti-FLAG, anti-LAMP-1, or anti-p10 antibodies. The whole-cell lysates were examined for protein expression (bottom panels) by Western blotting. (B) The levels of p10 in the precipitates in panel A were quantified by densitometry and normalized to that of actin. Results are representative of data from three independent experiments. Data are presented as means $\pm$ standard deviations ($n = 3$). *, $P < 0.05$. (C to H) Knockdown of LAMP-1 decreases the colocalization of Siah-1 with p10. DF-1 cells were transfected with control RNAi or LAMP-1 RNAi and GFP-C1-Siah-1 plasmids. Six hours after transfection, cells were infected with ARV at an MOI of 5. Twenty-four hours after infection, cells were fixed and probed with mouse anti-p10 antibody, followed by TRITC-conjugated goat anti-mouse antibodies (red). Nuclei were counterstained with DAPI (blue). The cell samples were observed with a confocal laser scanning microscope. (I to N) Knockdown of Siah-1 does not affect the colocalization of LAMP-1 with p10. DF-1 cells were transfected with control RNAi or Siah-1 RNAi. Six hours after transfection, cells were infected with ARV at an MOI of 5. Twenty-four hours after infection, cells were fixed and probed with mouse anti-p10 and rabbit anti-LAMP-1 antibodies, followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (green) and TRITC-conjugated goat anti-mouse antibody (red). Nuclei were counterstained with DAPI (blue). The cell samples were observed with a confocal laser scanning microscope. Bar, 10 μ m. The magnification is the same in all panels.

reported previously that the ARV p10 protein induced syncytium formation and apoptosis in cells (11, 13). As the cell-cell fusion induced by p10 is restricted by the rapid degradation of the majority of newly synthesized p10 (7), it is likely that the degradation of ARV p10 might be one of the essential mechanisms employed by host cells to control ARV.

LAMP-1 is a multifunctional protein. It was reported previously that LAMP-1 was involved in apoptosis (31–33), autophagy (34, 35), bacterial binding (36), and ARV p10 degradation (13). In this study, we show a new function of LAMP-1 in which LAMP-1 serves as a scaffold for both Siah-1 and p10 that allows the E3 ligase targeting p10 for ubiquitylation and degradation to suppress ARV infection.

Our previous report indicates that LAMP-1 plays a critical role in the proteasomal degradation of ARV p10 (13). In the present study, we searched for the E3 ubiquitin ligase responsible for p10 ubiquitin-dependent degradation. We found that the E3 ubiquitin ligase Siah-1 interacted with ARV p10. The overexpression of Siah-1 markedly promoted p10 ubiquitylation and degradation in host cells. On the contrary, the knockdown of Siah-1 considerably suppressed p10 degradation and ubiquitylation and facilitated ARV replication. Importantly, Siah-1 mediated p10 ubiquitylation *in vitro*.

FIG 12 LAMP-1 promotes the ubiquitylation and degradation of ARV p10 by Siah-1. (A) Knockdown of LAMP-1 mitigates p10 degradation induced by Siah-1. Cells were treated with LAMP-1 RNAi or control RNAi together with pRK5-flag-Siah-1 or pRK5-flag. Six hours after transfection, cells were infected with ARV at an MOI of 5. Twenty-four hours after infection, cell lysates were prepared and immunoblotted with anti-FLAG, anti-LAMP-1, anti-σC, or anti-p10 antibodies. (B) The levels of p10 in panel A were quantified by densitometry and normalized to that of actin. Results are representative of data from three independent experiments. Data are presented as means $\pm$ standard deviations ($n = 3$). *, $P < 0.05$. (C) LAMP-1 promotes p10 ubiquitylation *in vitro*. An *in vitro* p10 ubiquitylation assay was performed with a reaction buffer containing UbE1, UbcH5b, Ub, and GST-Siah-1 in the presence or absence of His-LAMP-1, as indicated. Reaction mixtures were incubated at 37°C for 2 h. The ubiquitylated products were detected by Western blotting using anti-Ub, anti-GST, or anti-His antibodies.

Thus, our data clearly established Siah-1 as an E3 ligase for p10 ubiquitylation, the only E3 ligase found so far for the proteasomal degradation of ARV p10. Moreover, we found that LAMP-1 interacted with both p10 and Siah-1 and that the knockdown of LAMP-1 mitigated the reduction of p10 levels by Siah-1, suggesting that LAMP-1 induces p10 degradation via Siah-1, the E3 ligase for p10 ubiquitylation.

It is known that Siah-1 plays different roles in cells, such as transcription regulation, mitosis, apoptosis, and tumor progression (37–41). Our data show that Siah-1, via interactions with both LAMP-1 and ARV p10 and the facilitation of the ubiquitin-proteasome degradation of p10, plays an important role in host defense against ARV infection because the knockdown of Siah-1 enhanced ARV growth. Besides ARV p10, Siah-1 can mediate the ubiquitylation and degradation of several viral proteins, such as the Kaposi's sarcoma-associated herpesvirus ORF45 protein (15), the hepatitis B virus X protein, and the herpes simplex virus immediate early protein ICP0 (19, 20). As the expression levels of these viral proteins are crucial for the pathogenesis of these pathogens, Siah-1 may play an important role in host defense against viral infection by the ubiquitylation of these viral proteins for proteasomal degradation to restrict virus replication.

Many Siah-1 binding proteins contain a consensus PXAXVXP motif, such as SIP (42) and synphilin-1 (43). Some proteins without this motif can also bind to and be degraded by Siah-1, such as CtIP (44), c-Myb (45), and the hepatitis B virus X protein (20). Although ARV p10 lacks a similar motif, our findings reveal that Siah-1 interacted with p10 and that Siah-1 mediated p10 ubiquitylation both *in vivo* and *in vitro*. We also found that the knockdown of LAMP-1 reduced the interaction of Siah-1 with p10 (Fig. 8A and B), indicating that LAMP-1 serves as a scaffold for interactions with Siah-1 and p10 and facilitates Siah-1-mediated p10 ubiquitylation.

We previously reported that the transfection of DF-1 cells with pEGFP-p10 induced

apoptosis (13), and others found that the overexpression of Siah-1 induced apoptosis in 293T cells and pc12 cells (38). As such, those observations raised several questions. For example, is the apoptosis induced by ARV p10 associated with Siah-1? In addition to ARV p10, are there any other ARV proteins that can be targeted by Siah-1 for proteasomal degradation? More efforts will be required to elucidate the mechanism underlying the host defense against ARV infection via p10 degradation.

It was reported previously that STAT3 regulates the β -catenin protein level through interacting with and stabilizing Siah-1 (46). Synphilin-1 recruits Siah-1 to mitochondria, where it promotes mitochondrial protein ubiquitylation (47). Similar to those reports, we also found that LAMP-1 degraded p10 via Siah-1. It seems that Siah-1 can be employed by multiple proteins to affect cellular processes.

Of note, a comparative sequence analysis showed that there is extensive sequence divergence between ARV and other orthoreoviruses (48). It has been found that ARV displays significant polymorphism in the migration patterns of the dsRNA segments among different isolates (49, 50), and the diversity within the S1-encoding gene is neither dependent on the viral serotype nor correlated with the disease state caused by ARV (48). Our data were obtained from experiments using ARV S1133, a strain that is commonly used for studies of ARV (1, 2, 8, 10, 51, 52). More efforts will be required to investigate the diversity of functional domains of p10 among ARV strains.

In summary, our data show that LAMP-1 interacted with both p10 and Siah-1 in ARV-infected cells, forming a multicomponent complex. The knockdown of Siah-1 by RNAi reduced p10 ubiquitylation, allowing p10 accumulation and facilitating ARV growth in cells. Furthermore, the knockdown of LAMP-1 markedly reduced the interaction of p10 with Siah-1 and mitigated p10 degradation by Siah-1. Thus, LAMP-1 promotes the proteasomal degradation of p10 via Siah-1, indicating a novel host defense mechanism in which LAMP-1 serves as a scaffold protein interacting with both the E3 ligase Siah-1 and the viral protein p10 to target p10 for Siah-1-mediated proteasomal degradation to suppress ARV infection.

MATERIALS AND METHODS

Cells and virus. DF-1 (immortal chicken embryo fibroblast) cells were obtained from the ATCC. DF-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) in a 5% CO₂ incubator. ARV S1133 was kindly provided by Jingliang Su (China Agricultural University, Beijing, China).

Reagents. All of the restriction enzymes were purchased from NEB. Endotoxin-free plasmid preparation kits and EasySpin Plus RNA extraction kits were purchased from Aidlab (China). pCMV-Myc, pRK5-flag, pDsRed-monomer-N1, and pEGFP-C1 vectors were obtained from Clontech. Anti- β -actin (catalog number sc-1616-R), anti-HA (Y-11), anti-Siah-1 (N15), anti-c-Myc (catalog number sc-40), and anti-GFP (catalog number sc-9996) antibodies were purchased from Santa Cruz Biotechnology. Rabbit anti-LAMP-1 polyclonal antibody (catalog number ab24170) was purchased from Abcam. Anti-FLAG M2 (catalog number F1804) antibody, MG132, and Chlq (catalog number C6628) were purchased from Sigma. Giemsa stain (catalog number 0679) was purchased from Amresco.

Mouse anti-ARV p10 (catalog number EU-0210) monoclonal antibody and mouse anti- σ C (catalog number EU-0211) monoclonal antibody were purchased from CAEU Biological Company (Beijing, China). Tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG and horseradish peroxidase (HRP)-conjugated goat anti-mouse and anti-rabbit IgG antibodies were purchased from Ding-GuoShengWu (Beijing, China). HRP-labeled donkey anti-goat IgG was purchased from Beyotime Biotechnology (Beijing, China). DMEM, Opti-MEM, and RNAiMAX were obtained from Invitrogen. The jetPRIME transfection reagent was purchased from Polyplus-transfection (France). 4',6-Diamidino-2-phenylindole (DAPI) was purchased from Beyotime (Nanjing, China). Protease inhibitor cocktail C was obtained from YTHX Biotechnology Company (Beijing, China). The recombinant ubiquitin-activating enzyme Ube1, the ubiquitin-conjugating enzyme UbcH5b, and ubiquitin were purchased from Boston Biochem (Cambridge, MA).

Constructs. The Siah-1 gene was cloned from the cDNA of DF-1 cells by using specific primers (sense primer 5'-CGGAATTCGGATGAGCCGTCAGACTGCTAC-3' and antisense primer 5'-ACGCCTCGAGTCAACACATCGATATAGTAA-3' [GeneID number 374076]). pEGFP-C1-Siah-1, pCMV-Myc-Siah-1, and pRK5-flag-Siah-1 expression plasmids were constructed by standard molecular biology techniques. The catalytically inactive Siah-1-C44S mutant was generated by using a TaKaRa mutagenesis kit.

All of the primers were synthesized by Sangon Biotechnology (Beijing, China). pRK5-flag-p10 and pEGFP-LAMP-1 expression plasmids were reserved in our laboratory (13).

Cell culture and transfection. DF-1 cells were seeded onto 6- or 24-well plates. Twenty-four hours later, cells were transfected with plasmids by using the jetPRIME transfection reagent.

Immunoprecipitation and Western blot analyses. For immunoprecipitation, DF-1 cells (6×10^5 cells) were seeded onto 6-well plates and cultured for 24 h before being cotransfected with the indicated plasmids or empty vectors as controls. Twenty-four hours after transfection, cell lysates were prepared by using lysis buffer (1% Triton X-100, 20 mM Tris-Cl, 5 mM EDTA, 137 mM NaCl, 0.02% NaN_3 , and 1% protease inhibitor cocktail C). The cell lysates were incubated with 2 μg of anti-FLAG antibody at 4°C for 2 h and then mixed with 20 μl of a 50% slurry of protein A/G plus-agarose and incubated for another 2 h. Beads were washed three times with lysis buffer and boiled with 2 $\times$ SDS loading buffer for 10 min. The samples were fractionated by electrophoresis on 10% SDS-PAGE gels, and resolved proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking with 5% skim milk, the membranes were incubated with either anti-Myc or anti-FLAG antibodies followed by an appropriate HRP-conjugated secondary antibody. Blots were developed by using an enhanced chemiluminescence (ECL) kit. For endogenous pulldown assays, DF-1 cells were transfected with pRK5-flag-p10. Twenty-four hours after transfection, the cell lysates were subjected to immunoprecipitation with anti-FLAG antibody and immunoblotted with anti-LAMP-1, anti-Siah-1, or anti-p10 antibodies.

Confocal laser scanning microscopy assays. DF-1 cells (1×10^5) were seeded onto coverslips in 24-well plates and cultured overnight before transfection with pEGFP-Siah-1 and pDsRed-p10. Twenty-four hours after transfection, cells were fixed with 4% paraformaldehyde, and the nuclei were stained with DAPI.

For the examination of endogenous Siah-1 in ARV-infected cells, DF-1 cells were transfected with pEGFP-Siah-1 for 6 h before infection with ARV. Sixteen hours after ARV infection, DF-1 cells were fixed with 1% paraformaldehyde, permeabilized with 0.2% Triton X-100 for 15 min, blocked with 1% bovine serum albumin, and then probed with mouse anti-p10 monoclonal antibody followed by TRITC-conjugated goat anti-mouse IgG antibodies. After three washes with phosphate-buffered saline (PBS), the cell nuclei were stained with DAPI. The samples were analyzed by using a confocal laser scanning microscope.

Ubiquitylation assay *in vivo*. DF-1 cells (6×10^5) were seeded onto 6-well plates and cultured for 24 h before being transfected with a plasmid encoding HA-ubiquitin, pRK5-flag-p10, and pEGFP-Siah-1 or the pEGFP-C1 control. Twenty-four hours after transfection, cells were treated with the proteasome inhibitor MG132 (20 μM) for 6 h and then lysed in lysis buffer (1% Triton X-100, 20 mM Tris-Cl, 5 mM EDTA, 137 mM NaCl, 0.02% NaN_3 , and 1% protease inhibitor cocktail C). The cell lysates were incubated with 2 μg of anti-FLAG antibody at 4°C for 2 h and then mixed with 20 μl of a 50% slurry of protein A/G plus-agarose and incubated for another 2 h. Beads were washed three times with lysis buffer and boiled with 2 $\times$ SDS loading buffer for 10 min. The samples were detected by Western blotting using anti-HA or anti-FLAG antibodies.

Ubiquitylation assay *in vitro*. The *in vitro* ubiquitylation reaction was set up with a total volume of 30 μl containing 100 nM UbE1, 392 nM UbcH5b, 39.2 μM ubiquitin, 8.3 ng/ μl glutathione S-transferase (GST)-Siah-1, and ~17 to 170 $\mu\text{g}/\mu\text{l}$ His-p10 in reaction buffer (50 mM NaCl, 2 mM dithiothreitol [DTT], 5 mM MgCl_2 , 50 mM Tris-Cl [pH 7.4], 5 mM ATP). Two hours after incubation at 37°C, 6 μl of 6 $\times$ SDS-PAGE buffer was added to the mixture, and the samples were analyzed by SDS-PAGE and Western blotting.

Knockdown of Siah-1 and LAMP-1 by RNAi. The small interfering RNAs (siRNAs) were designed by Genepharma Company (Shanghai, China) and used to knock down the expression of Siah-1 in DF-1 cells. The siRNA for targeting Siah-1 in DF-1 cells is Siah-1RNAi (sense primer 5'-CCAGCAUUGCACAGCUCUU TT-3' and antisense primer 5'-AAGAGCUGUGCAAUGCUGGTT-3'). The siRNA for targeting LAMP-1 in DF-1 cells is LAMP-1RNAi (sense primer 5'-GCUCGUGUGGUGAAGGUAA-3' and antisense primer 5'-UUACCU UACCCUCUGAGC-3'). The sequences for the negative-control siRNA are 5'-UUCUCCGAACGUGUCACG UTT-3' for the sense primer and 5'-AUCUCUAUCCAGCAUUGATT-3' for the antisense primer. DF-1 cells (2×10^5) were seeded onto 12-well plates and cultured for 24 h before transfection with siRNA using RNAiMAX according to the manufacturer's instructions (Invitrogen, USA). Double transfections were performed at 24-h intervals. Twenty-four hours after the second transfection, cells were harvested for further analysis.

Examination of syncytium formation. DF-1 cells were mock infected or infected with ARV at an MOI of 10. Twenty-four hours after ARV infection, cells were fixed with 4% paraformaldehyde and stained with Giemsa stain to visualize syncytia. Syncytium formation was examined by microscopy. The syncytial nuclei in each microscopic field were counted, and the average number of syncytial nuclei from three fields was calculated.

Measurement of ARV growth in DF-1 cells. Untreated cells or cells receiving Siah-1-specific siRNA or control siRNA were infected with ARV at an MOI of 5. Cell cultures were collected at different time points (12, 24, 48, and 72 h) after infection. The culture samples were freeze-thawed three times and centrifuged at $2,000 \times g$ for 10 min. The virus in the supernatants and cell cultures was titrated by using a TCID₅₀ (50% tissue culture infective dose) assay on DF-1 cells. Briefly, the viral solution was diluted 10-fold in DMEM. A 100- μl aliquot of each diluted sample was added to the wells of 96-well plates, followed by the addition of 100 μl of DF-1 cells at a density of 5×10^5 cells/ml. Cells were cultured for 5 days at 37°C in 5% CO₂. Cell cultures with cytopathic effect (CPE) were considered positive. The titer was calculated by using a previously described method (53).

Statistical analysis. The significance of the differences between pCMV-Myc-Siah-1-transfected cells and controls in the levels of p10 in cells, between Siah-1 RNAi cells and controls in the levels of p10 in cells and in viral growth, and between LAMP-1 RNAi cells and controls in the levels of p10 in cells was determined by a Mann-Whitney test or analysis of variance (ANOVA).

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