Structural and Functional Characterization of the Phosphoprotein Central Domain of Spring Viremia of Carp Virus

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ABSTRACT Spring viremia of carp virus (SVCV) is a highly pathogenic *Vesiculovirus* in the common carp. The phosphoprotein (P protein) of SVCV is a multifunctional protein that acts as a polymerase cofactor and an antagonist of cellular interferon (IFN) response. Here, we report the 1.5-Å-resolution crystal structure of the P protein central domain (P_{CD}) of SVCV ($SVCV_{P_{CD}}$). The P_{CD} monomer consists of two β sheets, an α helix, and another two β sheets. Two P_{CD} monomers pack together through their hydrophobic surfaces to form a dimer. The mutations of residues on the hydrophobic surfaces of P_{CD} disrupt the dimer formation to different degrees and affect the expression of host IFN consistently. Therefore, the oligomeric state formation of the P protein of SVCV is an important mechanism to negatively regulate host IFN response.

IMPORTANCE SVCV can cause spring viremia of carp with up to 90% lethality, and it is the homologous virus of the notorious vesicular stomatitis virus (VSV). There are currently no drugs that effectively cure this disease. P proteins of negative-strand RNA viruses (NSVs) play an essential role in many steps during the replication cycle and an additional role in immunosuppression as a cofactor. All P proteins of NSVs are oligomeric, but the studies on the role of this oligomerization mainly focus on the process of virus transcription or replication, and there are few studies on the role of P_{CD} in immunosuppression. Here, we present the crystal structure of $SVCV_{P_{CD}}$. A new mechanism of immune evasion is clarified by exploring the relationship between $SVCV_{P_{CD}}$ and host IFN response from a structural biology point of view. These findings may provide more accurate target sites for drug design against SVCV and provide new insights into the function of $NSV_{P_{CD}}$.

KEYWORDS central domain, crystal structure, interferon, negative regulator, P protein, SVCV

Spring viremia of carp virus (SVCV), the causative agent of spring viremia of carp (SVC), causes significant mortality in common carp (*Cyprinus carpio*). It is a negative-sense single-stranded RNA virus belonging to the family *Rhabdoviridae* in the order *Vesiculovirus*. The SVCV genome contains 11,019 nucleotides encoding five proteins in the following order: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glyco-

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protein (G), and large protein (L) (1, 2). The P protein, as a cofactor, plays an essential role in many steps during the replication cycle and an additional role in immunosuppression (3, 4).

SVCV and vesicular stomatitis virus (VSV) are negative-strand RNA viruses (NSVs). NSVs have a single-stranded RNA genome that is encapsidated by the N protein, also called the nucleocapsid protein (5, 6). The viral transcription template used by the viral RNA-dependent RNA polymerase (RdRp; L protein) is the nucleocapsid, but not the free form of the viral RNA genome. The NSV P proteins play an essential role in the recognition of the nucleocapsid and access to the genomic RNA (7). The structures of the VSV and measles virus (MV) N⁰-P complex explained how P protein chaperones N⁰ (RNA-free N) prevent its binding to RNA and self-assembly of N⁰ and supply N⁰ protein in the process of replication (8, 9). The crystal structure showed that the P protein of VSV engaged both the nucleocapsid and L protein as an adaptor and showed that the C-terminal domain of this protein bound to nucleocapsid-like particles, which allowed the polymerase complex to recognize the nucleocapsid as the template for viral synthesis (10, 11).

In addition to playing an important role in the process of replication, the P protein is also a potent inhibitor of the innate immune response, although the evasion mechanisms vary across NSVs. Host immune pattern recognition receptors detect double-stranded RNA (dsRNA), which is a unique product of virus infection, activate signaling cascades, and then lead to the production of interferons (IFNs). For Ebola virus, VP35 (polymerase cofactor, orthologous of the P proteins from other NSVs) bound dsRNA and inhibited IFN- α/β production induced by RIG-I signaling (12). The P protein of rabies virus (RV) inhibited IFN- α - and IFN- γ -induced transcriptional responses, impairing the IFN-induced antiviral state (13). The SVCV P protein could be phosphorylated by cellular TANK-binding kinase 1 (TBK1), which decreased IFN regulatory factor 3 (IRF3) phosphorylation and thus suppressed IFN production (14).

The P protein central domain (P_{CD}) is mainly responsible for its oligomerization. Several structures of P_{CD} from different viruses have been resolved so far, such as VSV, RV, human metapneumovirus (HMPV), and Sendai virus (SeV) (15–18). Furthermore, previous studies on the function of P_{CD} have shown that the dimerization domain of Nipah virus P is important for the initiation of mRNA transcription and/or genome replication (19) and that the homo-oligomers of Marburg virus (MARV) VP35 are essential for the recruitment of polymerase to its nucleocapsid (20). These studies mainly focused on the relationship between P_{CD} and the process of virus replication, whereas there are few on the role of P_{CD} in immunosuppression.

In this study, by solving the structure of SVCV P_{CD} (SVCV_{P_{CD}}) and comparing it to that of VSV P_{CD} (VSV_{P_{CD}}), we illustrate the difference between them, although both of these viruses belong to the genus *Vesiculovirus*. In addition, we investigate the impact of P protein dimerization on the expression of host IFN ϕ 1 and IFN ϕ 3. This study allows us to gain insights into the function of SVCV_{P_{CD}} and provides precise sites for drug design to cure SVC.

RESULTS

Structure of SVCV_{P_{CD}}. The P protein of SVCV can be divided into three domains: the N-terminal domain, the central domain (SVCV_{P_{CD}}; comprised of amino acid residues 101 to 187), and the C-terminal domain (Fig. 1A). To characterize the structure of SVCV_{P_{CD}}, we carried out protein crystallization experiments. The crystal structure of SVCV_{P_{CD}} was solved at 1.5-Å resolution, and we found that it contains two chains in an asymmetric unit (Table 1). The secondary-structure elements of the SVCV_{P_{CD}} monomer include β 1 (residues 121 to 125), β 2 (residues 128 to 133), and the α helix (residues 141 to 161), followed by β 3 (residues 167 to 170) and β 4 (residues 176 to 181) (Fig. 1B). Two P_{CD} monomers form a dimer in which the α helices from each molecule interact with each other in a parallel orientation and the β 1/ β 2/ β 3/ β 4 from each molecule are located beside the α helix. Figure 1C shows two views of the dimer.

FIG 1 Structure of SVCV_{PCD} (residues 101 to 187). (A) Domain organization of SVCV P protein. (B) Topology diagram of SVCV_{PCD}. (C) Ribbon drawing of SVCV_{PCD}.

Similarities and differences between SVCV_{PCD} and VSV_{PCD} in structure. Although both SVCV and VSV belong to the genus *Vesiculovirus*, there are many structural differences between SVCV_{PCD} and VSV_{PCD}. The monomer of VSV_{PCD} also consists of a two-stranded β sheet, an α helix, and a one-turn 3_{10} helix (residues 154 to 156), followed by another two-stranded β sheet, which is the same as SVCV_{PCD} in second-structure composition but different in the orientation of the two-stranded β sheets. The one-turn 3_{10} helix of VSV_{PCD} changes and fixes the orientation of the following two-stranded β sheet, while there is a four-residue loop in SVCV_{PCD} in the corresponding position, which results in the C-terminal sheet of the latter being more inclined to interact with the N-terminal sheet of the same subunit to form a four-stranded sheet (Fig. 2A).

Both the dimers of SVCV_{PCD} and VSV_{PCD} form a charged outside surface and a hydrophobic inside surface, and their two α helices are the main contributors of the flat hydrophobic surface (Fig. 2B). The hydrophobic interactions are formed by four residues (147I, 151M, 154L, and 158I) in SVCV_{PCD} and another four residues (130L, 138W, 142I, and 145V) in VSV_{PCD} (Fig. 2C and D). Hydrogen bonds between the monomers provide additional force to stabilize the dimer. However, the significant difference is that VSV_{PCD} has more ($n = 26$) hydrogen bonds than SVCV_{PCD} ($n = 10$), and it deserves to be mentioned that there are two salt bridges (123R-164E and 164E-135K) between the two monomers in VSV_{PCD} (Fig. 2D). Therefore, the stability of VSV_{PCD} is higher than that of SVCV_{PCD}.

Effects of SVCV_{PCD} mutants on dimerization. Since the hydrophobic interactions of SVCV_{PCD} are the main force maintaining dimerization, four amino acids (147I, 151M, 154L, and 158I) in the hydrophobic interface were mutated to arginine to break the

TABLE 1 X-ray crystallography data collection and refinement statistics

Data set	SeMet-SVCV-P-101-188
Data collection	
Beamline	BL-17U1, SSRF
Wavelength (Å)	0.9791
Space group	P2 ₁ 2 ₁ 2 ₁
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	37.90, 54.11, 89.89
α , β , γ (°)	90, 90, 90
Resolution range (Å) ^a	34.92–1.50 (1.54–1.50)
Completeness (%)	99.9 (100)
<i>I</i> / σ (<i>I</i>)	13.7 (2.2)
<i>R</i> _{merge}	0.095 (1.047)
Multiplicity	12.7 (13.5)
CC half	0.998 (0.673)
Anomalous completeness (%)	99.9 (100)
Anomalous multiplicity	6.7 (7.0)
Refinement	
Resolution (Å)	1.5
No. of reflections	30,305
<i>R</i> _{work} / <i>R</i> _{free} (%)	19.74/21.59
No. of atoms	
Protein	1,300
Solvent	62
B-factors (Å ²)	
Protein	31.0
Solvent	32.1
RMSD ^b	
Bond length (Å)	0.0063
Bond angle (°)	0.88
Ramachandran plot (%)	
Favored region	97.58
Allowed region	0.61
Outliers region	1.82

^aData for the highest-resolution shell are shown in parentheses.^bRMSD, root mean square deviation.

dimerization. Effects on the dimerization were measured by coimmunoprecipitation (Co-IP) experiments. To increase the transfection efficiency, HEK293T cells, but not epithelioma papulosum cyprinid (EPC) cells, were chosen for Co-IP experiments.

As shown in Fig. 3A and B, cotransfection with the plasmids of Myc-P and HA-P, anti-HA antibody (Ab) immunoprecipitated protein complex was recognized by anti-Myc Ab, suggesting that the dimerization existed. When one or two sites of the four amino acids were mutated, the dimerization still existed. The weakest degree of dimerization interruption occurred with the mutation of P4 (I158R). However, in the assay of the interactions between mutants of Myc-P (P-I147R+M151R+I158R, P-I147R+L154R+I158R, and P-M151R+L154R+I158R) and mutants of HA-P (P-I147R+M151R+I158R, P-I147R+L154R+I158R, and P-M151R+L154R+I158R), the dimer of SVCV_{PCD} could not form except with the mutation of P-I147R+M151R+L154R (Fig. 3C). These data indicated that at least three mutations of the four amino acids (147I, 151M, 154L, and 158I) could interrupt the dimerization.

Role of SVCV_{PCD} dimerization in immune escape. Our previous studies showed that the SVCV P protein could dampen the cellular IFN response and facilitate viral replication (14). To further investigate the function of SVCV_{PCD} dimerization in immune escape, its effect on host IFN response was monitored in EPC cells. Both SVCV and MAVS induced the expression of IFNs, but SVCV induced a much lower expression level of IFNs than MAVS (14). Therefore, MAVS was chosen to induce the expression of IFNs in this study. As shown in Fig. 4, overexpression of MAVS upregulated the activation of IFN ϕ 1pro and IFN ϕ 3pro, and this activation was inhibited by cotransfection with the plasmid encoding the wild type of P protein but recovered to some extent by mutation of the P protein. The induction of IFN ϕ 1pro and IFN ϕ 3pro by the single-site mutation

FIG 2 Similarities and differences between SVCV_{PCD} and VSV_{PCD} in structure. (A) Ribbon diagrams of SVCV_{PCD} (left) and VSV_{PCD} (right) monomers colored as rainbow from blue at the N terminus to red at the C terminus. (B) Electrostatic surface representation of one monomer of SVCV_{PCD} (left) and VSV_{PCD} (right) dimer and ribbon representation of another monomer. (C and D) Detailed hydrogen bonds or salt bridges (left) and hydrophobic interactions (right) between two chains in SVCV_{PCD} (C) and VSV_{PCD} (D). Residues are labeled with numbers.

of the P protein had significant recovery except P-I158R (Fig. 4A and B), whereas that by three-site mutation had extremely significant recovery, a finding consistent with the results of the Co-IP experiments (Fig. 4E and F). The induction of IFN ϕ 1pro and IFN ϕ 3pro by two-site mutation of the P protein showed different degrees of recovery, among which P-I147R+L154R and P-I147R+I158R showed no significant difference from the wild type (Fig. 4C and D). Collectively, these data indicated that the lack of dimerization of the P protein weakened the P-induced inhibition activity of IFN ϕ 1pro and IFN ϕ 3pro.

DISCUSSION

The P_{CD} of SVCV is a dimer, consistent with VSV and RV, which belong to the *Rhabdovirus* family (21). In contrast, those of HMPV and SeV are crystallized as tetramers and that of MARV forms an elongated trimer (15, 18, 22). Although all of the structures of NSV P_{CD} differ in oligomeric state, oligomerization is their common characteristic. So far, it is widely accepted that the oligomerization of the P protein is essential during the transcription and replication of the viral genome as a cofactor (23–25). However, recent studies have also shown that P_{CD} is not necessary for the viral transcriptional activity of the polymerase or virus replication in cell cultures. For example, Gérard et al. demonstrated that VSV_{P_{CD}} is dispensable for viral gene expression and for virus growth in cell cultures by using two reverse genetics approaches (26). Therefore, the function of P_{CD} in the viral life cycle is still unclear and needs further study.

The viral P protein plays an important role not only in viral transcription and replication but also in the process of escaping host immune defense (27–29). For example, the 10 C-terminal residues of the RV P protein are required for counteracting JAK-STAT signaling (30, 31). Our data demonstrate that the disruption of SVCV_{PCD} dimerization weakens the inhibition of IFN φ 1 and IFN φ 3 production. Since the SVCV P protein competes with IRF3 to be phosphorylated by TBK1, we hypothesize that the

FIG 3 Effect of hydrophobic surface residue mutation on oligomerization of SVCV P protein. (A) One-site mutation of P protein could not break up the dimer. One-site mutants of P protein contained P1 (I147R), P2 (M151R), P3 (L154R), or P4 (I158R). HEK293T cells were transfected with 5 μ g of I147R-Myc, M151R-Myc, L154R-Myc, and I158R-Myc and 5 μ g of I147R-HA, M151R-HA, L154R-HA, and I158R-HA, respectively. At 24 h posttransfection, the cells were harvested for immunoblotting (IB) with anti-Myc and anti-HA Abs. (B) Two-site mutation of P protein could not break up the dimer. Two-site mutants of P protein contained P12 (I147R+M151R), P13 (I147R+L154R), P14 (I147R+I158R), P23 (M151R+L154R), P24 (M151R+I158R), and P34 (L154R+I158R). The method of transfection and detection was the same as that used in panel A. (C) Three-site mutation of P protein could break up the dimer. Three-site mutants of P protein contained P123 (I147R+M151R+L154R), P124 (I147R+M151R+I158R), P134 (I147R+L154R+I158R), and P234 (M151R+L154R+I158R). The method of transfection and detection was the same as that used in panel A.

FIG 4 Inhibition of IFN̑1/3 activation by overexpression of mutants of SVCV P protein. (A and B) One-site mutation of P protein inhibited MAVS-mediated activation of IFN̑1 and IFN̑3. EPC cells were seeded in 24-well plates overnight and cotransfected with MAVS-expressing plasmids and one-site mutants of P protein plus IFN̑1pro-Luc (A) or IFN̑3pro-Luc (B) at a 1:1:1 ratio. One-site mutants of P protein contained P1 (I147R), P2 (M151R), P3 (L154R), and P4 (I158R). At 48 h posttransfection, the cells were collected for detection of luciferase activities. Error bars represent the standard deviations obtained by measuring each sample in triplicate. Asterisks indicate significant differences from control (*, $P < 0.05$). (C and D) Two-site mutation of P protein inhibited MAVS-mediated activation of IFN̑1 and IFN̑3. Two-site mutants of P protein contained P12 (I147+M151R), P13 (I147R+L154R), P14 (I147R+I158R), P23 (M151R+L154R), P24 (M151R+I158R), and P34 (L154R+I158R). The method of transfection and detection was the same as that used in panels A and B. (E and F) Three-site mutation of P protein inhibited MAVS-mediated activation of IFN̑1 and IFN̑3. Three-site mutation of P protein contained P123 (I147R+M151R+L154R), P124 (I147R+M151R+I158R), P134 (I147R+L154R+I158R), and P234 (M151R+L154R+I158R). The method of transfection and detection was the same as that used in panels A and B.

dimerization of the P protein is necessary for its phosphorylation and the disruption of P_{CD} dimerization results in the loss of its ability to be phosphorylated. In previous studies, the relationship between multimerization and phosphorylation was controversial. For example, in VSV and human respiratory syncytial virus, P protein phosphorylation was essential for P protein oligomerization (32–34), while in SeV and MV, oligomerization was not controlled by phosphorylation (24, 35, 36).

In conclusion, the present study reveals the precise sites for the dimerization of the SVCV P protein. The transcription of host *ifn̑1* and *ifn̑3* is recovered with the disassembly of the SVCV P protein. These data may also provide precise drug design

sites for the treatment of SCV. Further studies will focus on the relationship between the oligomerization and phosphorylation of the P protein to elucidate the immune escape mechanism of SVCV and will provide more strategies for virus control.

MATERIALS AND METHODS

Recombinant protein expression and purification. The gene segment (GenBank accession no. [DQ916053.1](#)) encoding the SVCV_{PCD} (residues 101 to 188) was amplified by PCR and cloned into pET28-SUMO with an N-terminal SUMO tag, followed by a ubiquitin-like-specific protease 1 (ULP1) cleavage site. The His-SUMO-ULP1-P101-188 construct was expressed in the *Escherichia coli* Rosseta strain. Bacteria were cultured in Luria-Bertani broth at 37°C until the optical density at 600 nm (OD₆₀₀) reached 0.6. Expression of the recombinant proteins was then induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 18 h at 18°C. Cells were harvested by centrifugation (4°C, 15 min, 4,000 × g). The deposited cells were suspended in binding buffer (25 mM Tris-HCl [pH 8.0], 100 mM NaCl, 20 mM imidazole) and were mechanically disrupted using a high-pressure homogenizer (ATS AH-1500). The cell debris was removed by centrifugation for 30 min at 17,000 × g at 4°C. The supernatants were loaded onto a column containing 2 ml of preequilibrated Ni-NTA agarose (Qiagen). The loaded column was washed with a 20 column volume of binding buffer. The His-SUMO tag was removed by addition of ULP1 protease at 4°C for 12 h on the column, and then the cleaved product was washed in binding buffer. Proteins were further purified by size exclusion chromatography (Sephacryl S-75; GE Healthcare) in size exclusion buffer containing 25 mM HEPES (pH 8.0) and 100 mM NaCl. Fractions containing the proteins were pooled and concentrated to 15 mg/ml. Purified protein was flash-frozen in liquid N₂ and stored at −80°C.

To produce the selenomethionyl (SeMet) variant of the SVCV_{PCD}, 50-ml cultures were grown in Luria-Bertani broth, and cells were harvested and added to SelenoMet medium base supplemented with SelenoMet nutrient mix (Athena Enzyme Systems) and 50 mg/liter of D,L-SeMet. Cultures were grown to an OD₆₀₀ of 0.8 at 37°C and induced with 0.4 mM IPTG for 18 h at 18°C. SeMet SVCV_{PCD} was purified identically to the wild type.

Crystallization and structure determination. Crystallization of SVCV_{PCD} was performed at 16°C by hanging drop. Then, 1 μl of protein solution was mixed with 1 μl of reservoir solution, followed by equilibration against 600 μl of reservoir solution. The protein was crystallized in Index HT-F5 (0.1 M ammonium acetate, 0.1 M Bis-Tris [pH 5.5], 17% PEG 10,000; Hampton Research). Crystals were cryoprotected in a cryoprotectant containing the reservoir solution supplemented with 15% glycerol, harvested into loops, and flash-cooled by plunging into liquid N₂.

Data collection and structure determinant. Crystals were brought to the Shanghai Synchrotron Radiation Facility for data collection. The data were collected using beamline BL17U (with X-rays at a wavelength of 0.97941 Å) and were processed and scaled with the HKL2000 suite (37). The crystal structures were solved by single-wavelength anomalous dispersion (SAD) method with the program AutoSol in PHENIX (38). AutoBuild in PHENIX was carried out in COOT with manual model revision (39). Multiple rounds of refinement were performed using the program PHENIX. All structure figures were prepared using PyMOL (<http://pymol.org>). Data collection and refinement statistics are listed in Table 1.

Cells and viruses. EPC cells were maintained at 28°C and 5.0% CO₂ in medium 199 (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and antibiotics. HEK293T cells were maintained at 37°C and 5.0% CO₂ in Dulbecco modified Eagle medium (Invitrogen) supplemented with 10% FBS and antibiotics. SVCV (strain 741) was propagated in EPC cells at 28°C until a cytopathic effect was completed, and then the cell culture medium was harvested and stored at −80°C.

Plasmid construction. For prokaryotic expression, the gene (GenBank [DQ916055.1](#)) encoding SVCV_{PCD} was cloned into the BamHI and XhoI sites of the pET28-SUMO vector. For promoter activity analysis, the gene promoters of IFNφ1 (IFNφ1pro-Luc; [NM_207640.1](#)) and IFNφ3pro-Luc ([NM_001111083.1](#)) were respectively cloned and inserted into the KpnI and XhoI sites of the pGL3-basic luciferase (LUC) reporter vector (Invitrogen) as described previously (40). The open reading frame of MAVS ([NM_001080584.2](#)) was amplified by PCR from ZF4 cells and cloned into the pcDNA(+) vectors. The cDNA segments of SVCV_{PCD} and its mutants were amplified by PCR from SVCV and cloned into pcDNA3.1-HA and pCMV-Myc vectors (BD Clontech). All plasmid constructs were verified by sequencing analysis. All of the primers used for plasmid construction are listed in Table S1 in the supplemental material.

Luciferase activity assay. EPC cells were seeded in 24-well plates and cotransfected 24 h later with various plasmids at a ratio of 10:10:1 (expression vectors of P or its mutants: IFNφ1pro-Luc or IFNφ3pro-Luc: pRL-TK). The empty vector pcDNA3.1(+) was used to ensure equivalent amounts of total DNA in each well. Infection with SVCV then occurred 24 h later. At 72 h posttransfection, the cells were washed with phosphate-buffered saline (PBS) and lysed for measuring luciferase activity by using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. LUC activities were measured by using a Junior LB9509 luminometer (Berthold) and normalized to the amount of *Renilla* LUC activities. The results are representative of more than three independent experiments, each performed in triplicate.

Transient transfection and virus infection. EPC cells were seeded in 24-well plates before transfection. The cell culture medium was replaced with fresh medium supplemented with 10% FBS, and then the cells were transfected with the plasmids using X-tremeGENE HP DNA transfection reagent (Roche) according to the manufacturer's instructions.

Coimmunoprecipitation assay. HEK293T cells seeded in 10-cm² dishes overnight were transfected with a total of 10 μ g of the indicated plasmids. At 24 h posttransfection, the medium was removed carefully, and the cell monolayer was washed twice with 10 ml of ice-cold PBS. The cells were then lysed in 1 ml of radioimmunoprecipitation lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM NaF, 1 mM sodium orthovanadate [Na₂VO₄], 1 mM phenylmethylsulfonyl fluoride, 0.25% sodium deoxycholate [C₂₄H₃₉O₄Na]) containing protease inhibitor mixture (Sigma-Aldrich) at 4°C for 60 min on a rocker platform. The cellular debris was removed by centrifugation at 12,000 $\times$ g for 15 min at 4°C. The supernatant was transferred to a fresh tube and incubated with 30 μ l of anti-Myc- or anti-HA-agarose beads (Sigma-Aldrich) overnight at 4°C with constant agitation. These samples were further analyzed by immunoblotting (IB). Immunoprecipitated proteins were collected by centrifugation at 5,000 $\times$ g for 1 min at 4°C, washed three times with lysis buffer, and resuspended in 30 μ l of 2 $\times$ SDS sample buffer. The immunoprecipitates and whole-cell lysates were analyzed by IB with appropriate Abs.

Immunoblotting analysis. Immunoprecipitates or whole-cell extracts were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Bio-Rad). The membranes were blocked for 1 h at room temperature in TBST buffer (25 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20 [pH 7.5]) containing 5% nonfat dry milk, probed with anti-Myc or anti-HA monoclonal antibody at an appropriate dilution overnight at 4°C, washed three times with TBST, and then incubated with secondary Abs for 1 h at room temperature. After three additional washes with TBST, the membranes were stained with the Immobilon Western chemiluminescent horseradish peroxidase (HRP) substrate (Millipore) and detected by using an ImageQuant LAS 4000 system (GE Healthcare). Abs were diluted as follows: anti- β -actin (Cell Signaling Technology) at 1:1,000, anti-Flag/HA (Sigma-Aldrich) at 1:3,000, anti-Myc (Santa Cruz Biotechnology) at 1:2,000, and HRP-conjugated anti-rabbit IgG or anti-mouse IgG (Thermo Scientific) at 1:5,000. The results are representative of three independent experiments.

Statistics analysis. The *P* values were calculated by one-way analysis of variance with Dunnett's *post hoc* test (SPSS Statistics, v19; IBM). A *P* value of <0.05 was considered statistically significant. Data are expressed as means $\pm$ the standard deviations of at least three independent experiments (*n* $\geq$ 3).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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