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PR65A Regulates The Activity of The Zinc-finger Antiviral Protein*

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Abbreviations: ZAP, zinc finger antiviral protein; ZRE, ZAP responsive element; MLV, murine leukemia virus; EBOV, Ebola virus; MARV, Marburg virus; SINV, Sindbis virus; TAP, tandem-affinity purification; PP2A, protein phosphatase 2A; HEAT, huntingtin/elongation/A- subunit/TOR

Abstract The zinc-finger antiviral protein (ZAP) is a host factor that inhibits the replication of certain viruses, including murine leukemia virus and Sindbis virus by destabilizing viral mRNA in the cytoplasm. ZAP binds to specific viral mRNAs and recruits cellular RNA degradation machinery to degrade the RNA. Identifying ZAP-interacting proteins provides a viable strategy to uncover the mechanism by which ZAP inhibits viral replication. In the present study, we developed a method to search for proteins interacting with ZAP. Lysates of ZAP-expressing cells were subjected to glycerol gradient centrifugation. Fractions co-migrating with ZAP were collected, followed by immunoprecipitation of ZAP. Proteins co-immunoprecipitated with ZAP were identified by mass spectrometric analysis. By this method, PR65A, a structural subunit of PP2A, was identified as a putative ZAP-interacting protein. Coimmunoprecipitation assays confirmed the interaction between PR65A and ZAP in an RNA-independent manner. Downregulation of PR65A reduced the antiviral activity of ZAP. We conclude that PR65A interacts with ZAP and is required for the optimal antiviral activity of ZAP.

Key words zinc-finger antiviral protein (ZAP), PR65A, anti-virus
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The zinc finger antiviral protein (ZAP) was originally recovered from a cDNA library as it conferred Rat2 cells resistance to infection by Moloney murine leukemia virus (MLV) [1]. Several other viruses including Sindbis virus (SINV)[2], Ebola virus (EBOV) and Marburg virus (MARV)[3] were later found to be restricted in ZAP-expressing cells, with SINV exhibiting the strongest sensitivity. ZAP is an interferon stimulated gene (ISG)[4], its expression can be induced by viral infection or stimulation with dsRNA or dsDNA in an IRF3-dependent manner [5]. These observations suggest that ZAP plays an important role in innate immunity to viral infection.

ZAP is a nuclear-cytoplasmic shuttling protein [6] and prevents the accumulation of viral mRNA in the cytoplasm[1]. Through the CCCH-type zinc-finger motifs residing in the N-terminal domain, ZAP directly and specifically binds to viral mRNAs [7]. Mutations of some residues in the zinc-finger motifs abolished ZAP's activity[7-8], highlighting the importance of this region. Furthermore, ZAP recruits cellular mRNA degradation machinery to degrade the target viral RNA [9]. To date, several proteins involved in ZAP-mediated antiviral activity have been identified [9]. Among them, RNA processing exosome [10], RNA helicase p72 [11], and some other factors of RNA degradation machinery [9] are required for optimal function of ZAP.

Identification of ZAP-interacting proteins is a viable strategy to unravel the mechanism by which ZAP restricts viral replication. By means of

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Co-immunoprecipitation and tandem-affinity purification (TAP), a number of candidate ZAP-interacting proteins have been identified. ZAP and its partners including exosome components[10], RNA helicase p72 (Chen unpublished data) displayed similar distribution patterns in the cell lysates fractionated by sucrose or glycerol velocity gradient centrifugation. Thus, we reasoned that collecting ZAP-containing fractions for subsequent immunoprecipitation might provide a novel approach to search for ZAP-interacting proteins.

Serine/Threonine-protein phosphatase 2A (PP2A) is one of the three type 2 protein phosphatases. It plays regulatory roles in a number of signaling pathways[12–15]. Generally, it exists in two forms, a heterodimeric core enzyme and a heterotrimeric holoenzyme. The former is composed of a scaffold subunit of 65 ku (subunit A or PR65 subunit) and a catalytic subunit of 36 ku (C subunit), and the latter consists of the core enzyme and a variable regulatory subunit (B, B', B" and B"') which is related to the substrate specificity[15–17]. The PR65 subunit contains 15 tandem HEAT (Huntingtin/elongation/A- subunit/TOR) repeats, each comprising a pair of antiparallel α helices. The conserved ridge of the four C-terminal HEAT repeats is recognized by the catalytic subunit, and is essential for their interaction[18]. PR65 has two isoforms, with α isoform (also termed PR65A) encoded by *PPP2R1A*, and β isoform (also termed PR65B) by *PPP2R1B*. These two isoforms share a 87% sequence identity[19], and the β isoform is much more abundant than the β isoform[15].

In this report, we identified a list of candidate ZAP-interacting proteins and confirmed the interaction between PR65A and ZAP.

1 Materials and methods

1.1 Plasmid construction

The luciferase reporter plasmids pMLV-luc and pRL-TK have been described previously[1]. pCMV-HA-Flag-PR65A expresses Flag-tagged PR65A. The coding sequence was PCR amplified using forward primer 65A-FP bearing an *Eco*R Ⅰ site and reverse primer 65A-RP bearing a *Sal* Ⅰ site with the plasmid pMIG-Aalpha WT (kindly provided by Dr. Hahn[20]) as the template. After digestion with the restriction enzymes, the PCR product was inserted into expression vector pCMV-HA-Flag. The primer sequences are listed below. 65A-FP, 5′ aagaattcatggcggcggccgacgg 3′; and 65A-RP, 5′ ttgtcgactcaggcgagagacagaac 3′. pSuper-retro65Ai2, pSuper-retro65Ai3 and psuper-retro65Ai4 (for short, PR65Ai2, PR65Ai3 and PR65Ai4) express shRNAs directed against the coding sequence of PR65A at different sites. pSuper-retroScr (for short, Scr) expresses scrambled shRNA and serves as a negative control. To construct the shRNA-expressing plasmids, synthesized oligos were annealed and inserted into pSuper-retro (OligoEngine) digested with *Bgl* Ⅱ and *Hin*d Ⅲ. The sequences of the oligonucleotides are as follows with the target sequences italicized.

PR65Ai2S: 5′ GATCCCC*AACGTCAAGAGTGA-GATCATC*TTCAAGAGAGATGATCTCACTCTTG-AGTTTTTTTA 3′; PR65Ai2A: 5′ AGCTTAAAAA-AACGTCAAGAGTGAGATCATCTCTCTTGAAGA-TGATCTCACTCTTGACGTTGGG 3′; PR65Ai3S: 5′ GATCCCC*AACAGCATCAAGAAGCTGTCC*TTCA-AGAGAGGACAGCTTCTTGATGCTGTTTTTTA 3′; PR65Ai3A: 5′ AGCTTAAAAAAACAGCATCAAG-AAGCTGTCCTCTCTTGAAGGACAGCTTCTTGA-TGCTGTTGGG 3′; PR65Ai4S: 5′ GATCCCC*GT-GGAGTTCTTTGATGAGA* TTCAAGAGATCTCATC-AAAGAACTCCACTTTTTA 3′; PR65Ai4A: 5′ AG-CTTAAAAAGTGGAGTTCTTTGATGAGATCTCT-TGAATCTCATCAAAGAACTCCACGGG 3′; ScrS: 5′ GATCCCC*GCGCGCTTTGTAGGATTCG*TTCAAG-AGACGAATCTACAAAGCGCGCTTTTTA 3′; ScrA: 5′ AGCTTAAAAAGCGCGCTTTGTAGGATTCGT-CTCTTGAACGAATCCTACAAAGCGCGCGGG 3′.

1.2 Velocity sedimentation centrifugation

To prepare a discontinuous glycerol density gradient, glycerol was completely dissolved in 1 × hypotonic buffer (10 mmol/L Tris •HCl (pH 7.6)/1.5 mmol/L $Mg(Ac)_2$/1 mmol/LKAc/10 μmol/L $ZnCl_2$/2 mmol/L DTT) at concentrations of 30%, 25%, 20%, 15% 10%, and 5%. 1.6 ml of the 30% glycerol was first loaded at the bottom of the polyallomer centrifuge tube (Beckman, Fullerton, CA), on top of which was layered 1.6 ml of each of the 25%, 20%, 15%, 10%, and 5% glycerol solutions. To prepare the cytoplasmic extract, 293TRex-ZAP cells were treated with tetracycline (final concentration, 1 mg/L) for 48 h to induce ZAP expression, and then trypsinized, washed twice with ice-cold 1 × PBS, and suspended in 1 × hypotonic buffer. The suspension was homogenized in a Dounce homogenizer by 20 up and down strokes for complete lysis and then treated with RNaseA at the concentration of 100 mg/L. The resultant cell lysates were clarified by centrifugation at 13 000 r/min for 10 min at 4℃ in a microcentrifuge (Sorvall, Bad

Homberg, Germany), and the supernatant was loaded on top of the glycerol gradients. The final centrifugation was performed in a Beckman SW41 rotor at 36 000 r/min for 3 h at 4℃. Twelve fractions were collected from the bottom of the tube with ~1 ml per fraction. 30 μl of each fraction was boiled in 1 × SDS-PAGE sample buffer and subjected to Western blot. The same treatment was performed with 293TRex cells, which do not express ZAP and thus serve as a negative control.

1.3 Immunoprecipitation and coimmunoprecipitation

The distribution of ZAP in the fractions collected after velocity sedimentation was detected by Western blotting. The three fractions with highest amounts of ZAP and the corresponding fractions (with the same glycerol densities) collected from TRex cells were applied for sequential immunoprecipitation in parallel. The cell lysates were incubated with 50 μl protein G plus agarose(Amersham Pharmacia) and 9 μl anti-myc antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) to precipitate ZAP. One hour later, after centrifugation at 4 000 r/min for 5 min at 4℃, the supernatant was removed, and the resultant beads were incubated with the other fractions sequentially in the same way. Finally, the resins were washed with ice cold 1 ×PBS for three times and then boiled in 1 × SDS-PAGE sample buffer. The proteins were resolved on SDS-PAGE and visualized by Coomassie blue staining. Compared with the precipitates of 293TRex control cells, the specific bands were excised and subjected to matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) analysis.

For coimmunoprecipitation, ZAP-expressing cells and control cells were lysed in lysis buffer (30 mmol/L Hepes (pH 7.6), 100 mmol/L NaCl, 0.5% Nonidet P-40 and a cocktail of protease inhibitors) supplemented with or without RNaseA(final concentration 100 mg/L) for 30 min on ice, and the lysates were clarified by centrifugation at 13 000 r/min for 10 min at 4℃. The supernatant was mixed with protein G Sepharose agarose and anti-Flag (Sigma-Aldrich) antibody to precipitate Flag-tagged PR65A or anti-myc (SantaCruz) to precipitate myc-tagged ZAP. After incubation at 4℃ for 2 h, the resins were washed three times with 1 × cold PBS and the bound proteins were detected by Western blotting.

1.4 Cell culture and antiviral activity assay

All the cells were maintained in DMEM supplemented with 10% FBS. Transfection was performed using Lipofectamine™ 2000 (Invitrogen, CA) following the manufacturer's instructions. 293TRex, 293TRex-ZAP cell lines and the luciferase reporter assay have been described previously [7]. To assay the effects of RNAi on the activity of ZAP, the shRNA-expressing plasmids were transfected into 293Trex-ZAP cells together with the reporters pMLV-luc and pRL-TK (Promega) at the ratio of 1∶0.1∶0.01. Immediately after transfection, the cells were mock-treated or treated with tetracycline to induce ZAP expression. At 48 h posttransfection the cells were lysed and luciferase activities were measured. The expression level of pRL-TK, a plasmid expressing Renilla luciferase insensitive to ZAP, was used to normalize the transfection efficiency. Fold inhibition was calculated as the normalized firefly luciferase activity in the mock treated cells divided by the normalized luciferase activity in the tetracycline treated cells.

2 Results

2.1 Identification of PR65A as a putative ZAP-interacting protein

We previously observed that ZAP cosediments with the RNA exosome and some other ZAP-interacting proteins in sucrose or glycerol velocity gradient centrifugation [10]. These proteins displayed similar sedimentation patterns and peaked in high mobility fractions, implying the existence of a complex containing ZAP and its interacting proteins. To optimize our procedure for isolating ZAP-interacting proteins, lysates of ZAP-expressing cells were first subjected to glycerol velocity gradient centrifugation followed by immunoprecipitation of ZAP. To prevent possible nonspecific RNA tethering, the cell lysates were treated with RNaseA before glycerol velocity sedimentation. Due to the dissociation of the proteins that interact with ZAP in an RNA-dependent manner, ZAP peaked in lower mobility fractions (Figure 1a, Fractions *9* & *10*). Fractions *8*, *9* and *10* were immunoprecipitated with anti-myc antibody and resolved on SDS/PAGE. The proteins were visualized by Coomassie blue staining. As shown in Figure 1b, ZAP was efficiently immunoprecipitated (indicated by arrow). Compared with the precipitates of 293TRex control cells, a specific band of ~62 ku (Figure 1b, Lane *3*, indicated by asterisk) was detected in the precipitates of the ZAP-expressing cells. This band

was excised and the proteins were identified by matrix-assisted laser desorption ionization time-of-flight(MALDI-TOF) mass spectrometry(MS). Database search identified protein phosphatase 2A 65 ku regulatory subunit A α isoform (PR65A) and some other proteins as candidates (Table 1). In this study, PR65A was chosen for further study.

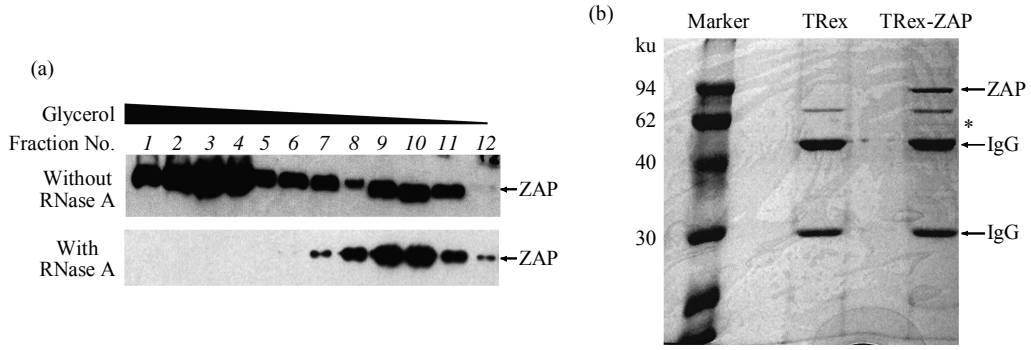

Fig. 1 Identification of ZAP-interacting proteins by immunoprecipitation

(a) The distribution patterns of ZAP in glycerol velocity sedimentation were determined by Western blotting with anti-myc antibody. The cell lysates were treated without (a) or with (b) RNase A prior to sedimentation. (b) After immunoprecipitation with anti-myc antibody, the precipitates were resuspended in SDS-loading buffer and resolved on SDS-PAGE. The proteins were visualized by Coomassie Blue Staining. The arrows indicate the immunoprecipitated ZAP, heavy chain and light chain. The asterisk indicates the specific band that was subsequently excised and trypsinized for mass spectrometry analysis. The result is representative of three independent experiments.

Table 1 Summary of MS analysis

	Reference Scan(s)	Score XC	M_r Sp	Accession RSp	Peptide (Hits) Ions
1	Prolyl 4-hydroxylase, beta subunit	120.29	57 116.9	20070125	12 (12 0 0 0 0)
2	Pyruvate kinase 3 isoform 2	110.32	58 062.8	33286420	11 (11 0 0 0 0)
3	Coactivator-associated arginine methyltransferase 1	110.26	65 854.4	40288288	11 (11 0 0 0 0)
4	α isoform of regulatory subunit A, protein phosphatase 2	80.26	65 309.3	21361399	8 (8 0 0 0 0)
5	Heterogeneous nuclear ribonucleoprotein K isoform a	40.29	51 028.9	14165439	4 (4 0 0 0 0)
6	Leucine rich repeat containing 1	40.25	59 242.4	95113664	4 (4 0 0 0 0)
7	U2 (RNU2) small nuclear RNA auxiliary factor 2 isoform a	30.26	53 501.7	6005926	3 (3 0 0 0 0)
8	60 ku Ro/SSA autoantigen isoform 2	30.18	60 671.4	31377800	3 (3 0 0 0 0)
9	Asparaginyl-tRNA synthetase	28.18	62 943.6	4758762	3 (2 1 0 0 0)

In this table, the candidates with more than 3 peptides (hits) are listed.

2.2 ZAP interacts with PR65A in an RNA-independent manner

To confirm the interaction between ZAP and PR65A, coimmunoprecipitation assay was performed. Flag-tagged PR65A and myc-tagged ZAP were coexpressed in HEK293T cells. Immunoprecipitation of ZAP with anti-myc antibody coprecipitated PR65A (Figure 2a). In the complementary experiment, immunoprecipitation of PR65A coprecipitated ZAP (Figure 2b). The pretreatment with RNase A didn't affect the interaction between these two proteins, suggesting that they interact with each other in an RNA-independent manner. This is consistent with the experimental settings for searching ZAP-interacting proteins (Figure 1a), where the cell lysates were treated with RNase A prior to velocity sedimentation. The results of coimmunoprecipitation established that PR65A is a ZAP-interacting protein.

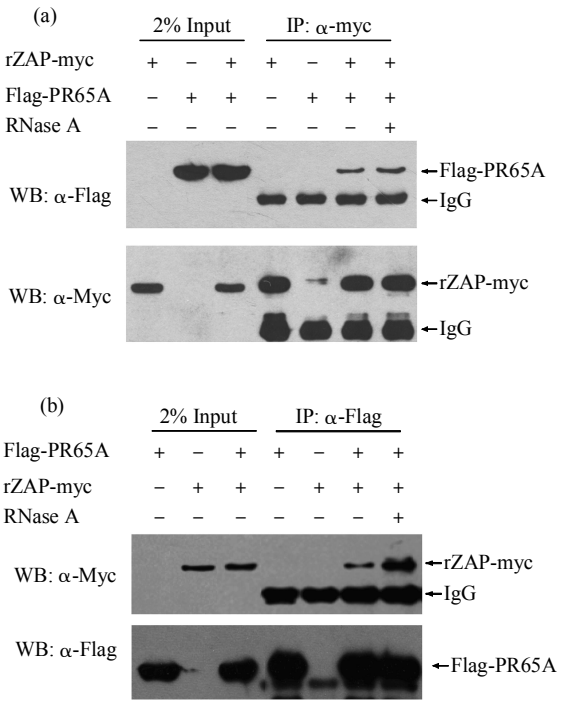

Fig. 2 ZAP interacts with PR65A in an RNA-independent manner

Myc-tagged ZAP and Flag-tagged PR65A were transiently expressed in HEK293T cells. The cell lysates were immunoprecipitated with anti-myc (a) or anti-Flag antibody (b) in the presence (+) or absence (–) of RNaseA and Western blotted with antibodies indicated. The positions of ZAP, PR65A and IgG are indicated.

2.3 Depletion of PR65A decreases the activity of ZAP

To analyze the role of PR65A in ZAP-mediated antiviral activity, PR65A was depleted with shRNAs targeting its coding sequence (CDS). Three shRNAs directed against PR65A (65Ai2, 65Ai3, 65Ai4) were constructed. Their abilities to downregulate the expression of PR65A was tested by cotransfection into HEK293TRex cells with plasmids expressing Flag-tagged PR65A and myc-tagged GFP (GFP-myc, control for transfection efficiency). Compared with the scrambled shRNA (Scr), 65Ai2 and 65Ai4 reduced the expression level of PR65A significantly, while 65Ai3 exhibited little effect (Figure 3a). To test whether PR65A is involved in ZAP's antiviral activity, the shRNA-expressing plasmids were cotransfected with pMLV-luc reporter into 293TRex-ZAP cells. The antiviral activity of ZAP was calculated and presented as fold inhibition. Indeed, compared with the control shRNA (Scr) 65Ai2 and 65Ai4 reduced ZAP's activity by 50% approximately (Figure 3b). Consistent with the result that 65Ai3 failed to downregulate the expression of PR65A (Figure 3a), 65Ai3 had little effect on ZAP, further confirming the specificity of the effects mediated by 65Ai2 and 65Ai4. These results indicated that PR65A is required for optimal activity of ZAP.

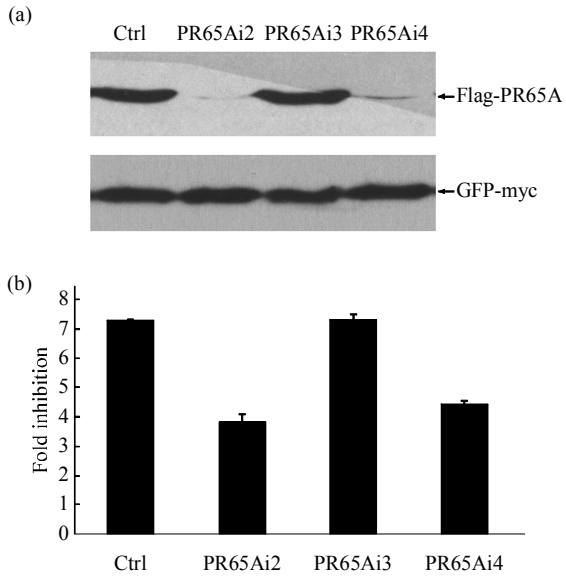

Fig. 3 Depletion of PR65A by RNAi reduced ZAP′s activity

(a) 65Ai2 and 65Ai4 downregulate the expression of PR65A. Scr, scrambled shRNA; 65Ai2, 65Ai3 and 65Ai4, shRNAs targeting the CDS of PR65A; the expression of GFP-myc served as a control for transfection efficiencies. (b) Knocking down of PR65A resulted in reduction of antiviral activity of ZAP. The fold inhibition data were $\bar{x} \pm s$ of three independent experiments.

3 Discussion

ZAP inhibits the replication of certain viruses by binding to the viral RNA and recruiting cellular mRNA degradation machinery to degrade the target RNA. In mammalian cells, mRNA degradation is a highly complex and organized process, involving multiple factors. In addition, as an important effector in innate immunity, the antiviral activity of ZAP is regulated. Identification of ZAP-interacting proteins helps to elucidate the mechanisms by which the RNA degradation process is carried out and how ZAP's activity is regulated.

In this study, we developed a method to search for ZAP-interacting proteins. The lysates of ZAP-expressing cells were first fractionated through the glycerol velocity sedimentation and ZAP-interacting

proteins were recovered by immunoprecipitation of ZAP from the enriched fractions. This search led to the identification of PR65A as one of the ZAP-interacting proteins. Coimmunoprecipitation assays confirmed the interaction between ZAP and PR65A. These results validate the method reported here.

Downregulation of PR65A by RNAi reduced the antiviral activity of ZAP, indicating that PR65A is required for optimal function of ZAP. It remains to be determined how PR65A participates in or regulates ZAP's activity. There exist many Ser/Thr phosphorylation sites in the N-terminal domain of ZAP (Sun & Lü unpublished data). Considering that PR65A is a subunit of Ser/Thr phosphatase 2A, we speculate that PR65A may be involved in regulating the phosphorylation status and thereby the activity of ZAP.

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PR65A 调控抗病毒蛋白 ZAP 的活性 *

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摘要 锌指结构抗病毒蛋白(ZAP)能够特异性地识别病毒 RNA 并促进其特异性降解，从而抑制病毒的复制．ZAP 不能独立地发挥抗病毒的功能，需要招募细胞内很多因子，包括外切酶复合体(exosome)、RNA 解旋酶 p72 等．通过蛋白质组学分析方法，我们找到了与 ZAP 可能存在相互作用的一些蛋白质并证实蛋白磷酸酶 2 调节亚基 A 的 α 亚型(PR65A)与 ZAP 之间存在着直接的相互作用．细胞内 PR65A 的表达水平，降低削弱 ZAP 的活性， 表明 PR65A 是 ZAP 发挥最佳抗病毒活性所必需的．这丰富了我们对 ZAP 抗病毒作用机理的认识，同时也为更好地利用其抗病毒活性提供了重要的指导．

关键词 锌指抗病毒蛋白(ZAP)，PR65A， 抗病毒

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