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LETTER

Amino acids essential for the interaction between cellular heat shock protein 90 and a Kaposi's sarcoma-associated herpesvirus-encoded protein kinase ORF36

Dear Editor,

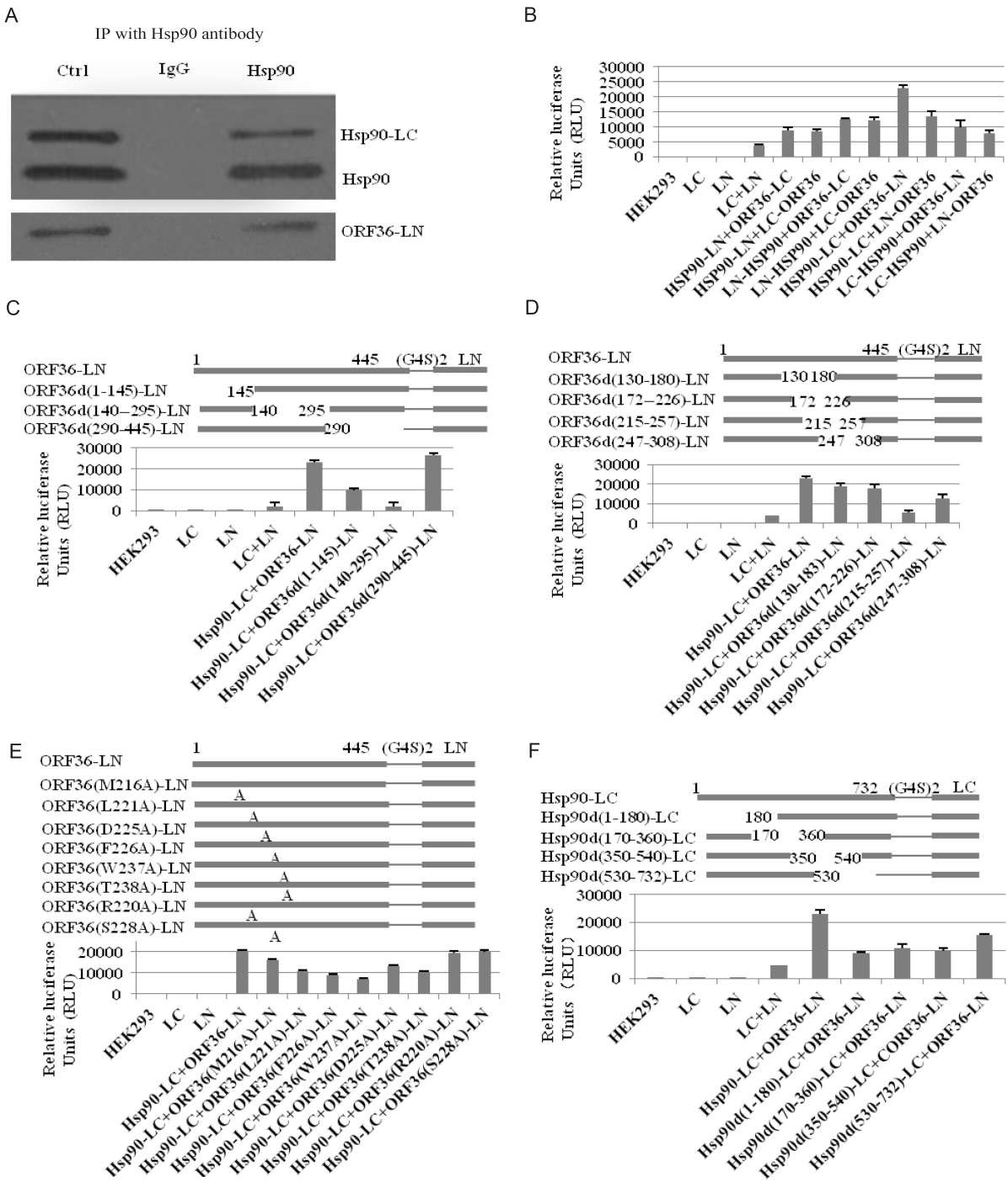
In this study, we use SRLCA to identify the possible Hsp90-ORF36 interaction. Additionally, critical amino acids of ORF36 and Hsp90 are deleted or mutated to evaluate their possible contributions to Hsp90-ORF36 interaction. The results indicated that the aa 215–257 of ORF36 and aa 110–180 region of Hsp90 are essential for the Hsp90-ORF36 interaction. The M216, L221, D225, F226, W237, and T238 in ORF36 and A121, Q133, F134, T149, E158, and G168 in Hsp90 are important for the Hsp90-ORF36 interaction. SRLCA is based on the complementation of the N-terminal domains of *Renilla* luciferase (LN) and C-terminal domains of *Renilla* luciferase (LC) non-functional halves of *Renilla* luciferase fused to possibly interacting proteins and emit luminescence (Deng et al., 2011; Jiang et al., 2010) (Supplementary Figure S1A). The Kaposi's sarcoma-associated herpesvirus open reading frame 36 (ORF36) encodes a serine protein kinase, playing an important role in the development of malignancy (Kim et al., 2013). Heat shock protein 90 (Hsp90) plays crucial roles in post-translational folding and stability of many proteins (Chehab et al., 2015; Pennisi et al., 2015). In this study, we use SRLCA to identify the possible Hsp90-ORF36 interaction. Additionally, critical amino acids of ORF36 and Hsp90 are deleted or mutated to evaluate their possible contributions to Hsp90-ORF36 interaction. The results indicated that the aa 215–257 of ORF36 and aa 110–180 region of Hsp90 are essential for the Hsp90-ORF36 interaction. The M216, L221, D225, F226, W237, and T238 in ORF36 and A121, Q133, F134, T149, E158, and G168 in Hsp90 are important for the Hsp90-ORF36 interaction.

The plasmids pcDNA3.1, PA-LN and PA-LC were obtained from Prof. Feng Li (South Dakota State University, South Dakota State, USA). The first group of full length KSHV open reading frame 36 (ORF36) and heat shock protein 90 (Hsp90) sequences were PCR-amplified using the primers designed with *Xho* I and *Eco*R I (NEB, USA), and subcloned upstream of LN and LC with a linker DNA sequences GGGGSGGGGS ((G4S)2) (Paulmurugan and Gambhir, 2003). ORF36 plasmids were tagged by Flag. The second group of full-length KSHV ORF36 and Hsp90 sequences were PCR-amplified using the primers designed with *Xho* I and *Not* I, and subcloned downstream of LN and LC with a linker (G4S)2 (Supplementary Figure S1B, S1C). All plasmids were effectively expressed in HEK293 cells (Supplementary Figure S2A). All possible combinations of Hsp90-ORF36 pairs were transfected into 293T cells for 48 h and the luciferase activity was detected. As shown in Figure 1B, the pair of Hsp90-LC + ORF36-LN has the highest luciferase activity (~22827 RLU). These results suggest that the pair Hsp90-LC/ORF36-LN has the most powerful interaction. Therefore, the pair of Hsp90-LC/ORF36-LN was selected to study the Hsp90-ORF36 interactions through the SRLCA. Additionally, according to our previous study (Wang et al., 2016), deletion mutants of ORF36 were constructed to confirm the essential amino acids region. Most of the polar amino acids in this region were mutated. Moreover, several non-polar amino acids were randomly selected to mutate in this region. All deletion and point mutation of ORF36 were linked to upstream of LN. The recombinant plasmids ORF36d (1–145)-LN and ORF36d (290–445)-LN were PCR-amplified from ORF36-LN. Primers were designed by Primer5 as follows: ORF36d (1–145) *Xho* I-F/ORF36 *Eco*R I-R, ORF36 *Xho* I-F Flag/ORF36d (290–445)-R. The plasmid ORF36d (140–295)-LN was amplified from ORF36-LN. Primers were designed by Primer5 as follows: ORF36 *Xho* I-F Flag/ORF36d (140–295)-R, ORF36d (140–295)-F/ORF36 *Eco*R I-R Flag. After the first PCR-amplification, the products were mixed and amplified for ten cycles using primers ORF36 *Xho* I-F Flag/ORF36 *Eco*R I-R Flag. The plasmids ORF36d (130–183)-LN, ORF36d (172–226)-LN, ORF36d (215–257)-LN, and ORF36d (247–308)-LN were constructed by the same method. The mutants ORF36 (R220A)-LN, ORF36 (S228A)-LN, ORF36 (M216A)-LN, ORF36 (L221A)-LN, ORF36 (D225A)-LN, ORF36 (F226A)-LN, ORF36 (W237A)-LN, and ORF36 (T238A)-LN were amplified from ORF36-LN. The primers were designed by Primer 5 as follows: ORF36 (R220A)-F/R, ORF36 (S228A)-

F/R, ORF36 (M216A)-F/R, ORF36 (L221A)-F/R, ORF36 (D225A)-F/R, ORF36 (F226A)-F/R, ORF36 (W237A)-F/R, and ORF36 (T238A)-F/R. All deletion and point mutations of Hsp90 were linked to upstream of LC by the same way. All primers used in this study were shown in Supplementary Table S1.

To determine if the expressed fusion proteins (Hsp90-LC and ORF36-LN) can interact with each other, IP was performed to detect Hsp90-ORF36 interaction after transfection. As shown in Figure 1A, both wild-type Hsp90 and fusion protein Hsp90-LC were precipitated by Hsp90 antibody, and ORF36-LN could be detected by Flag antibody. These results suggest that the fusion proteins (Hsp90-LC and ORF36-LN) are able to interact with each other in living cells.

To detect the complementation of Hsp90-LC and ORF36-LN, HEK293 cells were transfected with various constructs as indicated. As shown in Figure 1B, when



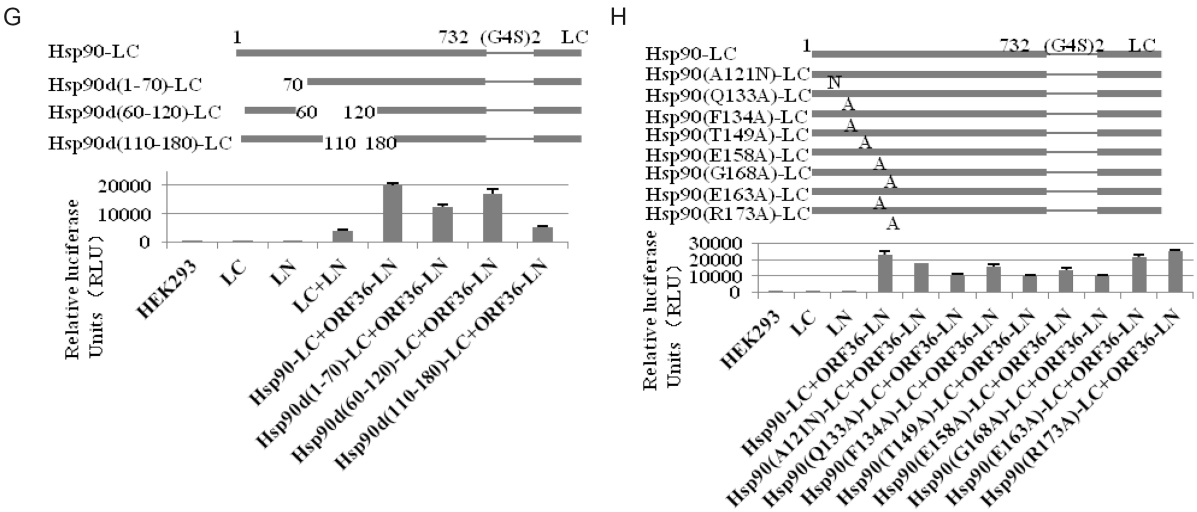


Figure1. PPIs was detected by IP and Luciferase assay. (A) The plasmids Hsp90-LC and ORF36-LN were transiently co-transfected into HEK293 cells. Cell lysates were immunoprecipitated with Hsp90 antibody. IgG was used as a negative control. Cell lysates without immunoprecipitation were used as control. (B) HEK293 cells were transfected with various plasmids. Luciferase assay was performed under experimental procedures. Data are presented as mean ± SD (n = 6). (C, D and E) The recombinant mutants of ORF36 linked to LN via the linker (G4S)2. Luciferase assay of HEK293 cells transfected with these mutants after 48 h. Data are presented as mean ± S.D. (n = 6). (F, G and H) The recombinant mutants of Hsp90 linked to the LC via the linker (G4S)2. Luciferase assay was performed under experimental procedures. Data are presented as mean ± SD (n = 6).

the HEK293 cells were transfected with each fragment alone (LN, LC, LN + LC), the luciferase activity was low. However, when the two plasmids Hsp90-LC and ORF36-LN were co-transfected into HEK293 cells, the luciferase activity was elevated (about 22827 RLU). These results suggest that the fusion proteins (Hsp90-LC and ORF36-LN) are able to interact with each other in cells.

To determine the essential region of ORF36 for the Hsp90-ORF36 interaction, deletion mutants of ORF36 (ORF36d (1–145)-LN, ORF36d (140–295)-LN, ORF36d (290–445)-LN) were constructed. All mutants can be detected in HEK293 cells (Supplementary Figure S2B). As demonstrated in Figure 1C, co-transfection of Hsp90-LC/ORF36d (140–295)-LN only restored 9.05% luciferase activity compared with full length Hsp90-LC/ORF36-LN. However, co-transfection of Hsp90-LC/ORF36d (1–145)-LN and Hsp90-LC/ORF36d (290–445)-LN restored 41.67% and 105.53% luciferase activity compared with full length Hsp90-LC/ORF36-LN. These results suggest that the aa140–295 region of ORF36 is essential for the Hsp90-ORF36 interaction. Therefore, four recombinant plasmids (ORF36d (130–183)-LN, ORF36d (172–226)-LN, ORF36d (215–257)-LN, and ORF36d (247–308)-LN) were constructed from the wild type of ORF36-LN. The results of SRLCA indicated that deletion of the aa 215–257 region in ORF36 only restored 22.90% luciferase activity compared with full-length of Hsp90-LC/ORF36-LN. However, the luciferase activity of Hsp90-LC/ORF36-LN was not decreased significantly by Hsp90-LC/ORF36d (130–183)-LN, Hsp90-LC/ORF36d (172–226)-LN, and Hsp90-LC/ORF36d (247–308)-LN (Figure 1D). These results suggest the aa215–257 region of ORF36 is crucial in the ORF36-Hsp90 interaction.

Since the above results indicated that the aa215–257 region of ORF36 is important for Hsp90-ORF36 interaction, most of the polar amino acids in this region were mutated. Moreover, several non-polar amino acids were randomly selected to mutate in this region. Here we show the most important amino acids M216, L221, D225, F226, W237, and T238 of ORF36. In addition, R220 and S228 were used as the negative controls. All mutants can be detected in HEK293 cells (Supplementary Figure S2D). The result of SRLCA showed that luciferase activity were not changed by Hsp90-LC/ORF36 (R220A)-LN or Hsp90-LC/ORF36 (S228A)-LN. The mutations in ORF36 (M216A, L221A, D225A, F226A, W237A, and T238A) reduced the luciferase activity by 22.55%, 47.97%, 35.10%, 57.19%, 69.44%, and 49.80%, respectively, compared with the full-length Hsp90-LC/ORF36-LN (Figure 1E). The data indicate that M216, L221, D225, F226, W237, and T238 in ORF36 are essential for Hsp90-ORF36 interaction.

To investigate the functional region of Hsp90 for the Hsp90-ORF36 interaction, deletion mutants of Hsp90 (Hsp90d (1–180)-LC, Hsp90d (170–360)-LC, Hsp90d (350–540)-LC, and Hsp90d (530–732)-LC) were constructed. These mutants of Hsp90-LC can be detected by

Hsp90 antibody (Supplementary Figure S2E). The result of SRLCA showed us that Hsp90d (1–180)-LC/ORF36-LN restored about 39.07% luciferase activity compared with full-length Hsp90-LC/ORF36-LN, which was lower than others (Figure 1F). Therefore, three recombinant plasmids (Hsp90d (1–70)-LC, Hsp90d (60–120)-LC, Hsp90d (110–180)-LC) were constructed. These mutants can be detected by Hsp90 antibody (Supplementary Figure S2F). The result of SRLCA showed us that deletion of the aa 110–180 region in Hsp90 reduced Hsp90-ORF36 interaction by 72.73% compared with wild type of Hsp90-LC and ORF36-LN (Figure 1G). The data suggest that the aa 110–180 region of Hsp90 is essential for the Hsp90-ORF36 interaction.

To investigate the important amino acids of Hsp90 for the Hsp90-ORF36 interaction, most of the polar amino acids in this region (110–180) were mutated. Moreover, several non-polar amino acids were randomly selected to mutate in this region. Here we show the most important amino acids A121, Q133, F134, T149, E158, and G168 of Hsp90 were mutated. In addition, E163 and R173 were used as the negative controls. All point mutants of Hsp90-LC can be detected by Hsp90 antibody (Supplementary Figure S2G). The result of SRLCA indicated that the Hsp90-LC/ORF36-LN interaction was not changed by Hsp90 (E163A)-LC/ORF36-LN or Hsp90 (R173A)-LC/ORF36-LN. However, Hsp90 (A121N)-LC/ORF36-LN, Hsp90 (Q133A)-LC/ORF36-LN, Hsp90 (F134A)-LC/ORF36-LN, Hsp90 (T149A)-LC/ORF36-LN, Hsp90 (E158A)-LC/ORF36-LN, and Hsp90 (G168A)-LC/ORF36-LN abolished Hsp90-ORF36 interaction by 75.76%, 46.17%, 68.26%, 42.64%, 50.02%, and 41.25%, respectively, as measured by the restored luciferase activity (Figure 1H). These results suggest that A121, Q133, F134, T149, E158, and G168 in Hsp90 are directly involved in the Hsp90-ORF36 interaction.

PPIs are believed to play complex roles in many cellular process, such as transcription, translation, signal transduction, cell division, and oncogenic transformation (Zhang et al., 2016). The inhibition of Hsp90 has become a new strategy for cancer therapeutics (Peng et al., 2007). The functional Hsp90 requires cochaperones to form the Hsp90 chaperone machinery, which plays a key role in orchestrating the spatial and temporal order of protein interactions (Pennisi et al., 2015). Therefore, investigation of PPIs roles in Hsp90 superchaperone machinery may offer a new therapeutic strategy for virus infections and the associated diseases.

In this study, SRLCA was used to study full-length Hsp90-ORF36 interaction in living cells. Furthermore, we evaluated the contributions of the essential amino acids in the Hsp90-ORF36 interaction. SRLCA can be used alone or associated with IP or other techniques to detect PPIs.

In summary, the Hsp90-ORF36 interaction was tested using SRLCA method, and the interacting motifs were investigated. It is found that the aa 215–257 of ORF36 and aa 110–180 region of Hsp90 are essential for the Hsp90-ORF36 interaction. The M216, L221, D225, F226, W237, and T238 in ORF36 and A121, Q133, F134, T149, E158, and G168 in Hsp90 are important for the Hsp90-ORF36 interaction. These results provide a rationale to develop inhibitors for disruption of the Hsp90-ORF36 interactions.

FOOTNOTES

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Supplementary figures/table are available on the websites of *Virologica Sinica*: www.virosin.org; link.springer.com/journal/12250.

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