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行政院國家科學委員會補助專題研究計畫成果報告

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※子計畫九:人類疹病毒第八型引發惡性腫瘤之增生機制研究(2/3)　※
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計畫類別：□個別型計畫　　■整合型計畫

計畫編號：NSC 94-2745-B-039 -003 -URD

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執行單位：中國醫藥大學醫學檢驗生物技術學系

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行政院國家科學委員會專題研究計畫成果報告

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Abstract

Kaposin encoded by the unique open reading frame K12 of human herpesvirus 8 (HHV-8) genome has been demonstrated the induction of tumorigenic transformation. In this study, we intend to investigate the protein-protein interactions and biological functions of kaposin with its interacting cellular protein(s) identified from phage displayed human cDNA libraries. Our results demonstrated in vitro binding specificity and in vivo colocalization between HHV-8 kaposin and the C-terminus (residues 274 to 452) of septin-4 using co-immunoprecipitation and confocal microscopy. Coexpression of kaposin and septin-4 indicated the inhibition of caspase-3 activity induced by the C-terminal septin-4. In addition, HHV-8 kaposin showed an antagonistic effect against septin-4 mediated activation of transcriptional factors AP1 and NF-κB. Thus, the interaction of kaposin and septin-4 was suggested to play a role in the development of HHV-8-associated malignancies. This study provides insights into the mechanism of the kapsoin pathology, in which the interactions of kapsoin with cellular proteins might allow to alter the fundamental cellular processes.

Keywords: human herpesvirus 8, kaposin, septin-4, functional interaction

1. Introduction

A novel gammaherpesvirus, human herpesvirus 8 (HHV-8) is first identified in Kaposi's sarcoma tissue, being also known as Kaposi's sarcoma (KS)-associated herpesvirus (KSHV) [1, 2]. HHV-8 does not appear to be involved in the pathogenesis of AIDS-associated, African endemic and classic Kaposi's sarcoma, but is also associated with development of several human malignancies including multicentric Castleman disease (MCD), primary effusion lymphoma (PEL), multiple myeloma (MM) and sarcoidosis [2, 3]. In addition, HHV-8 DNA was detected in salivary gland tumors, noncleaved cell lymphoma, multiple myeloma and sarcoidosis [2, 3]. Like other herpes viruses, HHV-8 that has been identified in brain tissues shows a neuroinvasive and neuropersistent potential [4], being associated with multiple sclerosis [5]. HHV-8 encoding human homologous genes could be involved in oncogenesis, anti-apoptosis, angiogenesis and cytokine action [6]. Therefore, identifying unique interactions between HHV-8 proteins and host cellular

factors would become important issues for understanding molecular mechanisms of the HHV-8 pathology and developing the rationale treatments and prevention of HHV-8-associated diseases.

HHV-8 is related to the gamma-2 herpesviruses, such as herpesvirus saimiri [2, 3]. Of close to 100 open reading frames (ORFs), the HHV-8 genome contains 15 unique ORFs, being numbered separately and given the prefix K (K1 to K15). ORF-K12, one of HHV-8 genes with oncogenic potential, has been found to express in KS [7] and PEL cell lines [8]. The ORF-K12 encoding protein, also known as kaposin is a unique latent protein with highly hydrophobic 60 amino acids [9]. Kaposin has been shown to induce tumorigenic transformation and to activate cellular serine–threonine kinases [10, 11]. Recently, mutational analysis indicated that the LXXLL motif at residues 31-35 of kaposin could be responsible for nuclear receptor coactivators in transforming activity [12].

In this study, we intend to investigate the protein-protein interactions and biological functions of the kaposin protein with human septin-4 protein by identifying with a phage displayed human brain cDNA library. Septin-4 is expressed in human brain, heart, liver, and endothelial cells [13], being associated with cytokinesis, apoptosis, and tumor suppression [14-15]. The specific interaction in vitro and in vivo was confirmed using co-immunoprecipitation and confocal imaging. In addition, a functional effect of kaposin in the septin-4 expressing cells was characterized with caspase-3 and in vitro signaling pathway assays. This study provides insights into molecular pathogenesis of HHV-8 kaposin.

2. Materials and methods

Viruses and cells

The HHV-8 carrying BC-3 cell line that was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) was maintained in RPMI 1640 medium with 20% heat-inactivated fetal bovine serum (FBS). HHV-8 was harvested from the supernatant of cultured media from the BC-3 cells treated with 0.3 mM of butyric acid (Sigma–Aldrich) for 48 h. Human TE-671 medulloblastoma cells were grown in minimum essential medium (MEM) with 2 mM L-glutamine, 1mM sodium pyruvate and 10 % fetal bovine serum (FBS).

Construction and purification of recombinant HHV-8 kaposin protein

The kaposin gene from the HHV-8 genome was amplified by PCR with the primers 5’-TCGCGGATCCATGGATAGAGGCTTAA-3’ and 5’-CGCACTCGAGGTGCGCGCCCGTTGCA-3’. The PCR product was cloned

into the *Bam*H I-*Xho* I site of the bacterial expression vector pET32a (Novagen). The transformed *E. coli* BL21(DE3) cells with pET32a-Kaposin were grown in LB medium containing 100 μg of ampicillin and grown at 37 ℃. Once the cells reached an absorbance at 600nm of 0.6, they were induced by the addition of 0.5mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 20 ℃ for 4 hr. The supernatant of the transformed *E. coli* was purified with the HisTrap Kit and buffer kit (Amersham). The purified recombinant kaposin protein was examined using SDS-PAGE and Western blotting. The samples from fractions of each purification step were then dissolved in 2X SDS-PAGE sample buffer without 2-mercaptoethanol, and boiled for 10 min. Proteins were resolved on 12% SDS-PAGE gels, and then the electrophoretically separated proteins were transferred to nitrocellulose paper. The resultant blots were blocked with 5% skim milk, and then reacted with the appropriately diluted anti-His tag monoclonal antibody (mAb) (Serotec) for 3-h incubation. The blots were then washed with 1％TBST three times and overlaid with a 1/5000 dilution of goat anti-mouse IgG antibodies conjugated with alkaline phosphatase (PerkinElmer Life Sciences,Inc.). Following 1-h incubation at room temperature, the blots were developed with TNBT/BCIP (Gibco).

Identification of kaposin-interacting cellular proteins from phage display cDNA libraries

A human brain cDNA library (Novagen) was used to screen high-affinity kaposin-interacting cDNA clones as described in our previous reporters [17, 18]. Briefly, biopanning of the phage display cDNA library preabsorbed with the thioredoxin partner protein was carried out in recombinant kaposin-coated microplates (5 μg per well). After six rounds, the kaposin-interacting cDNA phage clones were eluted with the soluble kaposin, and were separately amplified in *E. coli*. Direct ELISA was further performed for determining the binding affinity. The nucleotide sequences of human cDNA displayed on the high affinity phages were directly amplified using the T7 Select UP primer 5'-GGAGCTGTCGTATTCCAGTCA-3' and determined using the sequencing with the ABI PRISM 377 DNA Sequencer (Perkin-Elmer, USA). The deduced amino acid sequences of kaposin-interacting cellular proteins were aligned by the MegAlig program of DNAStar, and analyzed using the BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/).

Co-immunoprecipitation assay

The C-terminal region of the septin-4 gene was amplified from the kaposin-interacting phage clone 25 by PCR with primers:

5’-ATCCGAATTCTTCATGCTGGCCCTGC-3’ and 5’-CGCACTCGAGGTGCGCGCCCGTTGCA-3’. The PCR product was cloned into the *EcoR* I/*Xho* I site of the vector pET24a, and expressed as the fusion protein with T7-tag at the N-terminus and His-tag at the C-terminus. The mixture of purified septin-4 protein with the kaposin fusion protein or the thioredoxin protein was first incubated with anti-T7 tag mAb (Novagen) for 4 h at 4 ℃, and then reacted with the protein A-Sepharose beads for the addition of 2-h incubation. After centrifugation, the pellet was washed four times with NET buffer (150 mM NaCl, 0.1 mM EDTA, 30 mM Tris-HCl, pH 7.4). The immunoprecipitate was dissolved in a 2X SDS-PAGE sample buffer without 2-mercaptoethanol and boiled for 10 min. Proteins were resolved on 12% SDS-PAGE gels and electrophoretically transferred to nitrocellulose papers. The resultant blots were blocked with 5% skim milk, and then reacted with the anti-His tag mAb for 1-h incubation. The blots were then washed with TBS three times and overlaid with a 1/1000 dilution of rabbit anti-mouse IgG antibodies conjugated with alkaline phosphatase (KPL). Following another1-h incubation at room temperature, the blots were developed with NBT/BCIP (Invitrogen).

Expression and subcellular localization of kaposin-GFP and septin-4-RFP fusion proteins

HHV-8 kaposin gene was cloned into the in-framed site of the plasmid pEGFP (pcDNA3.1-His based), and expressed as a kaposin-GFP (green fluorescent protein) fusion protein. Septin-4 gene was inserted into the plasmid pDsRed (BD Biosciences Clontech) for generating a septin-4-RFP (red fluorescent protein) fusion protein. TE-671 cells at 60-90% confluency in 6-well plates were co-transfected with different combination of plasmids pEGFP-kaposin, pEGFP, pDsRed-septin-4, and pDsRed using the GenePorter reagent. In addition, cells were co-transfected with pEGFP-kaposin or pEGFP plus pDsRed-Mito (BD Biosciences Clontech) for determining the subcellular localization of kaposin. According to manufacturer’s direction (Gene Therapy Systems, San Diego,CA). After 5-hour incubation, the transfected cells were maintained in 2 ml of MEM containing 20% bovine serum (FBS), then were selected using the DMEM containing 10 % FBS and 200 μg/ml of G418. Confocal image analysis of the cells was performed using Leica TCS SP2 AOBS laser-scanning microscopy (Leica Microsystems, Heidelberg GmbH, Germany).

Fluorimetric assay of caspase-3 activity

Transfected cells were harvested after 5 days of the G418 selection for

measuring the activity of caspase-3 using BD ApoAlert Caspase Fluorescent Assay Kits (BD Biosciences). 2×10^5 cells per well were lysed in 50 μl of lysis buffer and centrifuged at 12,000 g for 5 min at 4 ℃. Supernatant was transferred into the well of the caspase profiling assay plate and incubated at 37 ℃ for 2 hr. Finally, the caspase-3 assay plate was analyzed in the fluorescent plate reader with excitation at 380 nm and emission at 460 nm.

In vivo signaling pathway assay

The *cis*-reporter plasmids pAP1-Luc, pNF-kB-Luc or pISRE-Luc (Stratagene) and an internal control reporter pRluc-C1 (BioSignal Packard) were used for assessing pathway activation of specified enhancer elements. The cells expressing a single or both of kaposin-GFP and septin-4-RFP were transiently co-trasfected with the indicated *cis*-reporter plasmid and the plasmid pRluc-C1 using the GenePorter reagent. After 18-h incubation, the luciferase activity in the indicated cells was measured using the dual Luciferase Reporter Assay System (Promega) and the Luminometer TROPIX TR-717 (Applied Biosystems).

3. Results

Generation of recombinant kaposin fusion protein

To generate *E. coli*-synthesized kaposin protein, HHV-8 kaposin gene cloned into the expression vector pET32a was in-frame fusion to a thioredoxin tag, an S tag and a His tag at the N- terminus. The kaposin fusion protein was further purified using the immobilized-metal affinity chromatography (IMAC). Coomassie blue-stained gel and Western blotting revealed that the major band of 30 kDa was found for recombinant kaposin fusion protein at the eluted fractions with 400 mM imidazole (Fig. 1A and 1B, lanes 5 to 10). Purified kaposin fusion protein with a high purity was subsequently used for the affinity biopanning of the phage-displayed human cDNA library.

Binding interaction of HHV-8 kapsoin with septin 4 identified by phage display technology

To identify potential kaposin-interacting cellular proteins, a human cDNA library preabsorbed with thioredoxin was used for screening high affinity cDNA clones to the kaposin fusion protein. Fifty eluted phage plaques at the sixth round of biopanning were randomly selected for determining relative kaposin-binding affinities using direct binding ELISA assay (data not shown). Of fifty phage clones, seven kaposin-interacting cDNA clones that showed 2-fold higher kaposin-binding affinity than others were amplified using PCR. The PCR products were subsequently

sequenced for determining the nucleotide sequences of kaposin-interacting cellular proteins encoded by the phage display cDNA clones. The BLAST alignment search indicated that the deduced amino acid sequence of the kaposin-interacting cDNA clone No. 25 containing 179 residues was 99% identical to the C-terminus (residues 274 to 452) of septin-4 (Peanut-like protein 2) (Brain protein H5) (Cell division control-related protein 2) (Bradeion beta) (GenBank accession number O43236). Septin-4 encoded by kaposin-interacting cDNA clone No. 25 contains the incomplete GTP-binding domain and the coiled-coil domain, which was suggested to be involved in protein-protein interaction [19].

To test the specific interaction between HHV-8 kapsoin and septin-4, the septin-4 fragment of kaposin-interacting cDNA clone No. 25 was cloned into the pET24a expression vector for generating the septin-4 fusion protein with a N-terminal T7 tag and a C-terminal His tag in *E. coli*. For co-immunoprecipitation assay, IMAC-purified recombinant septin-4 protein with molecular weight of 20 kDa was incubated with the kapsoin fusion protein, and then the protein complex was precipitated by the anti-T7 tag antibody and the protein A-Sepharose beads. Immunoprecipitation followed by SDS-PAGE and immunoblotting with the anti-His tag antibody revealed that the septin-4 protein bound to the kaposin fusion protein (Fig. 2, lane 3), but not to thioredoxin (the partner of the kaposin fusion protein) (Fig. 2, lane 6). Therefore, co-immunoprecipitation demonstrated the binding specificity of HHV-8 kaposin to the C-terminus of spetin-4 identified by screening of phage display cDNA library.

Confocal image analysis of kaposin-GFP and septin 4-RFP fusion proteins in mammalian cells

For investigating colocalization of kaposin and septin-4, localization of kapsoin-GFP (green fluorescence) and septin-4-RFP (red fluorescence) fusion proteins in TE-671 cells was analyzed using confocal microscopy (Fig. 3). Kaposin-GFP (Fig 3A and Fig 3D), RFP (Fig. 3B), and septin-4-RFP (Fig. 3E) were expressed in co-transfected cells, respectively. Superimposition of the corresponding green fluorescence and red fluorescence images indicated that yellow color was found in the transfected cells expressing both of kaposin-GFP and septin-4-RFP (Fig 3F), but not in the transfected cells expressing kaposin-GFP and RFP (Fig. 3C). The result demonstrated a very close colocalization of kaposin and septin 4 in the co-transfected cells. In addition, subcellular localization of HHV-8 kaposin was further investigated. The cells were also co-transfected with pEGFP or pEGFP-kaposin plus the pDsRed-Mito encoding mitochondrially targeted RFP, respectively. Superimposition of confocal images revealed the mitochondrial localization of the

kaposin protein in the cells (Fig 3L). Therefore, the results suggested that HHV-8 kaposin could bind to septin-4 in mitochondria.

Biological activity of the interaction between kaposin and septin 4 in vivo

To investigate the possible function of kaposin-septin-4 interaction, caspase-3 activity in the transfectd cells expressing different combination of GFP, kaposin-GFP, septin-4-RFP and RFP was measured using fluorimetric assay (Fig. 4). Caspase-3 activity in the transfected cells expressing septin-4-RFP was 6-fold higher than those of the transfected cells expressing GFP, RFP, or kaposin-GFP. Interestingly, caspase-3 activity was suppressed by coexpression of septin-4-RFP and kaposin-GFP in the transfected cells. The results indicated that expression of kaposin resulted in the inhibitory effect on activation of caspase-3 induced by the C-terminus of septin-4 in vivo.

To further test the effect of the interaction on transcriptional activities, the cells expressing a single or both of kaposin-GFP and septin-4-RFP were transiently co-transfected with an internal control reporter pRluc-C1 and the cis-reporting plasmids pAP1-Luc, pNF-kB-Luc or pISRE-Luc, respectively. The Dual Luciferase Reporter assay indicated that expression of kaposin-GFP significantly induced 1.8-fold increase of AP1 activation compared to those of cells expressing GFP or septin-4-RFP (Fig. 5A). Moreover, coexpression of kaposin-GFP and septin-4-RFP led to 50% increase in the AP1-Luc activity than that of the septin-4 expressing cells. In contrast, expression of kaposin-GFP caused no changes in the NF-κB-Luc activity, while expression of septin-4 increased about 50% activity of NF-κB-Luc (Fig. 5B). However, coexpression of kaposin-GFP and septin-4-RFP inhibited the NF-κB-Luc activity. In addition, the profile of the ISRE-Luc activity in the indicated cells was similar to the responses of NF-κB-Luc (data not shown). Together with the results of signal transduction pathways, HHV 8 kaposin altered the transcriptional responses to septin-4 in the transfected cells.

4. Discussion

HHV-8 kaposin that was demonstrated on induction of cellular transformation and activation of serine-threonine kinases has been suggested to correlate with KS pathogenesis [10, 11]. However, rare studies were reported in elucidating the mechanism(s) of kaposin with cellular factors. In this study, we demonstrated in vitro binding specificity and in vivo colocalization between HHV-8 kaposin and the C-terminus (residues 274 to 452) of septin-4 using co-immunoprecipitation and confocal microscopy (Figs. 2, and 3F). Septin-4 was a member of GTPases related to the Ras superfamily of signaling GTPases that are

thought to mediate in diverse cellular functions, including cytokinesis, apoptosis, and tumor suppression [14-16]. Septin-4 was reported in interactions with septin-2, septin-5, phosphatidylinositol polyphosphate (PIP2), collapsin response mediator proteins (CRMPs), X-chromosome-linked inhibitor of apoptosis protein (XIAP), and synuclein (Hall et al., 2004). In addition, the C-terminus of septin-4 was demonstrated to be a protein-protein interaction domain [20]. Therefore, the binding interaction of kaposin with septin-4 could be linked to the pathogenesis of HHV-8.

Septin-4, also called Bradeion beta, was identified to express in human colorectal cancer and malignant melanoma, being a potential target for colorectal cancer therapy [21]. ARTS (apoptosis-related protein in the TGF-beta signalling pathway) encoded by a splice variant of septin-4 gene was a mitochondrial pro-apoptotic protein through activation of caspase-3 [22], being as a tumor suppressor in acute lymphoblastic leukemia [23]. In this study, colocalization of kaposin and septin-4 in mitochondria resulted in the inhibition of caspase-3 activity induced by the C-terminal septin-4 (septin-4-RFP) (Figs 3F, 3L and 4). In addition, HHV-8 kaposin showed an antagonistic effect against septin-4 mediated activation of transcriptional factors AP1 and NF-κB (Fig 5). The findings suggested that the interaction of kaposin and septin-4 could participate in more than one biological process, including anti-apoptosis and proliferation.

In conclusion, our results demonstrate the functional interaction of HHV-8 kaposin with septin-4 in vitro and in vivo. Coexpression of kaposin and septin-4 indicates that the binding of kaposin to septin-4 could have an opposed effect on septin mediated signaling in the transfected cells. Thus, the interaction of kaposin and septin-4 is suggested to play a role in the development of HHV-8-associated malignancies. This study provides insights into the mechanism of the kapsoin pathology, in which the interactions of kapsoin with cellular proteins might allow to alter the key cellular processes.

Acknowledgements

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Cell Cycle 3, 1021-1023.

Figure Caption

Fig. 1. SDS-PAGE (A) and Western blotting (B) of the purified kaposin fusion protein in each purified step. Each fraction of washing and elution was analyzed by 12% SDS-PAGE, then electrophorectically transferred onto nitrocellulose paper. The blot was probed with mouse anti-His tag antibodies, and developed with an alkaline phosphatase- conjugated secondary antibody and NBT/BCIP substrates. Lane 1 was the molecular marker. Lanes 2 to 3 indicated the fraction from washing with 100 mM imidazole, whereas lanes 4 to 10 indicated the fraction from elution with 400 mM imidazole, respectively. kDa, kilodaltons.

Fig. 2. Co-immunoprecipitation of kaposin with septin-4. An equal amount of the kaposin-thioredoxin fusion protein (or thioredoxin partner protein) and the septin-4 protein was first incubated with anti-T7 tag mAb at 4 ℃ overnight, followed by incubation with the protein A-Sepharose beads for the addition of 2-h incubation. After centrifugation, the pellet was washed with NET buffer, and then dissolved in 2X SDS-PAGE sample buffer without 2-mercaptoethanol and boiled for 10 min. Following the western blot procedure, the blot was probed with mouse anti-His tag antibodies, and developed with an alkaline phosphatase- conjugated secondary antibody and NBT/BCIP substrates.

Fig. 3. Colocalization and subcellular localization of kaposin and septin-4 using confocal microscopy. Cells were cotransfected with pEGFP-kaposin and pDsRed (column I), pEGFP-kaposin and pDsRed-septin-4 (column II), pEGFP and pDsRed-Mito (column III) or pEGFP-kaposin and pDsRed-Mito (column IV). A, D, G and J: green fluorescence; B, E, H and K: red fluorescence; C, F, I and L: merge.

Fig. 4. Fluorimetric caspase-3 activity in kaposin and septin-4 expressing cells. Cells expressing different combination of GFP, RFP, kaposin-GFP, and septin-4-RFP were lysed in 50 μl of lysis buffer and centrifuged at 12,000 g for 5 min at 4 ℃. Supernatant was transferred into the well of the BD ApoAlert Caspase Fluorescent Assay Kits assay plate. After the incubation at 37 ℃ for 2

h, the caspase profiling assay plate was analyzed in the fluorescent plate reader with excitation at 380 nm and emission at 460 nm. Fluorescent intensity was normalized by the protein concentration of the lysates.

Fig. 5. Effect of in vivo signal transduction pathway by a single and both of kaposin and septin-4 expression on the *cis*-reporting systems of AP1 (A) and NF-κB (B). Cells expressing different combination of GFP, kaposin-GFP, and septin-4-RFP were transiently co-transfected with an internal control reporter pRluc-C1 and the indicated cis-reporting plasmid. Firefly and Renilla luciferase enzymes were measured using the dual Luciferase Reporter Assay System and the Luminometer TROPIX TR-717. The relative firefly luciferase activity was normalized by Renilla luciferase.

Fig. 1A

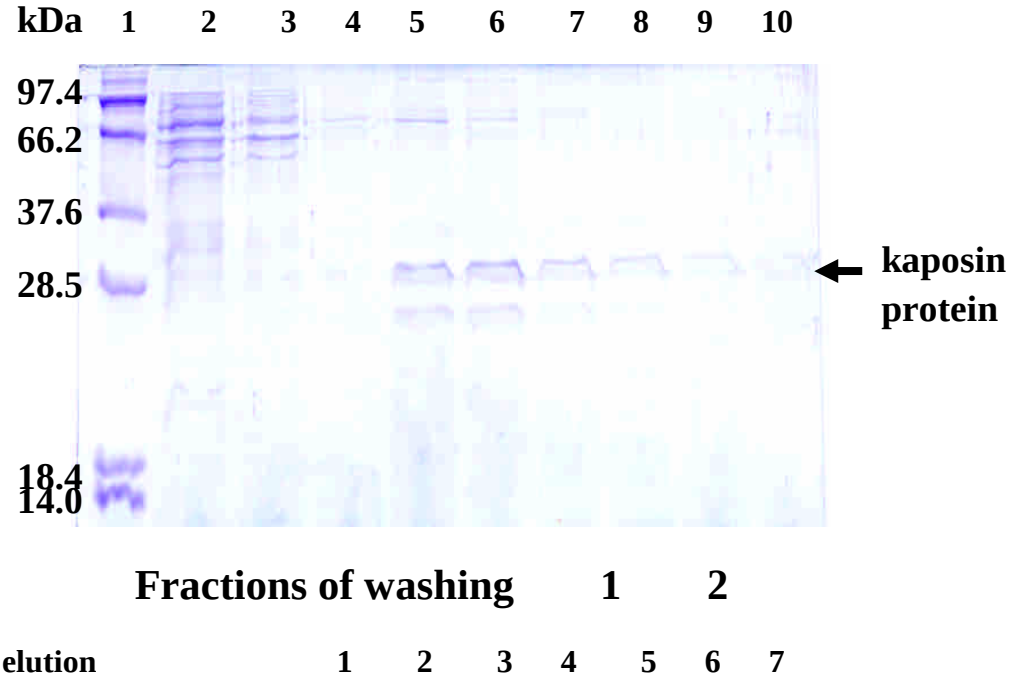
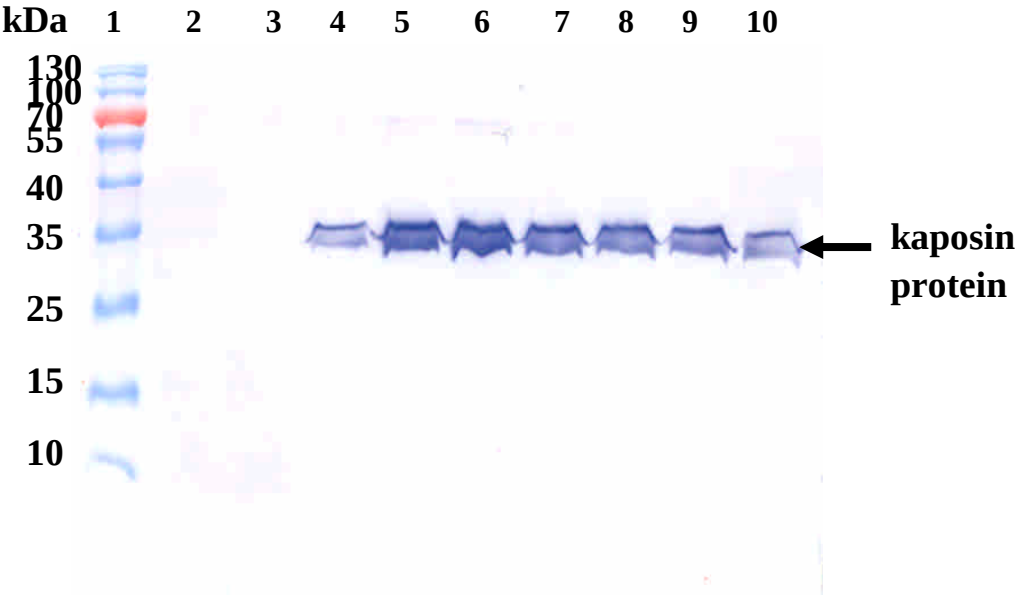

kDa 1 2 3 4 5 6 7 8 9 10
97.4
66.2
37.6
28.5
18.4
14.0
kaposin protein
Fractions of washing 1 2
Fractions of elution 1 2 3 4 5 6 7

Fig. 1B

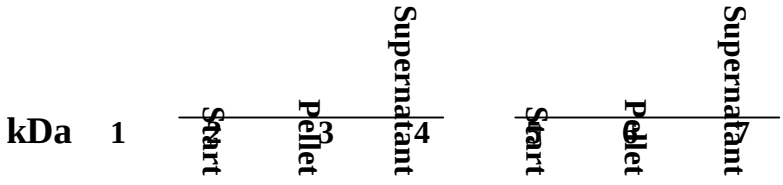


Fractions of washing 1 2

Fractions of elution 1 2 3 4 5 6

7

Fig. 2



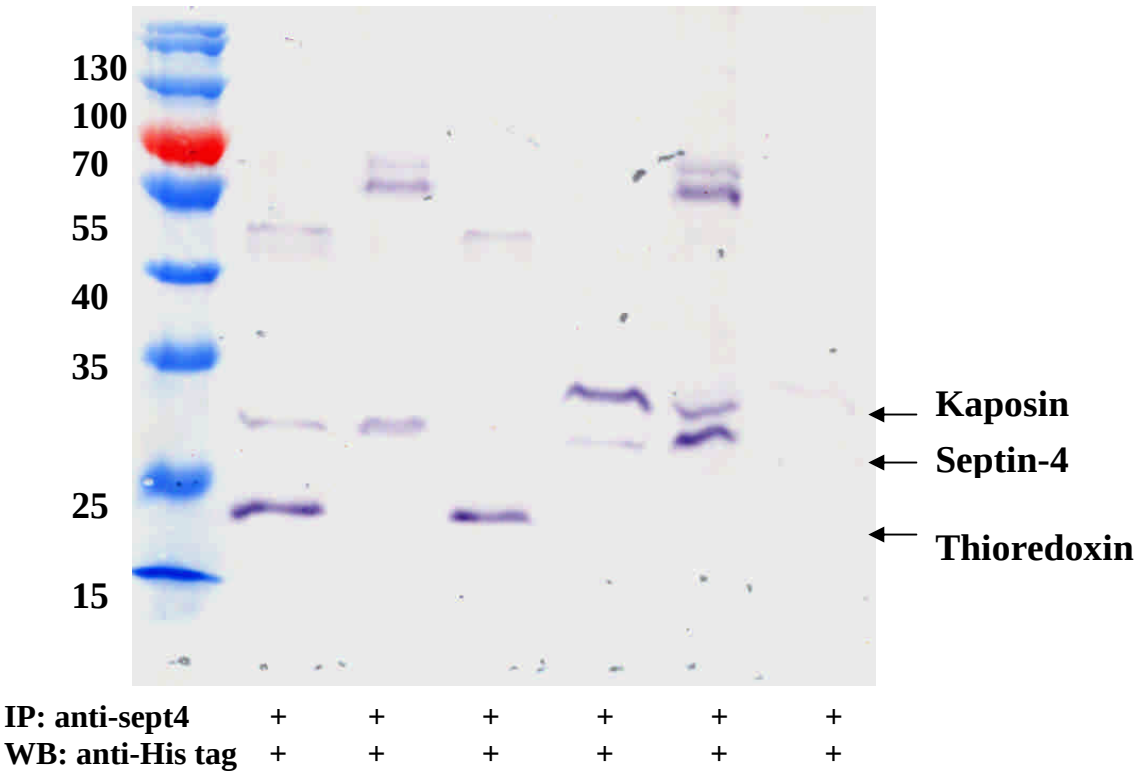

130
100
70
55
40
35
25
15
Kaposin
Septin-4
Thioredoxin
IP: anti-sept4 + + + + + +
WB: anti-His tag + + + + + +

Fig. 3

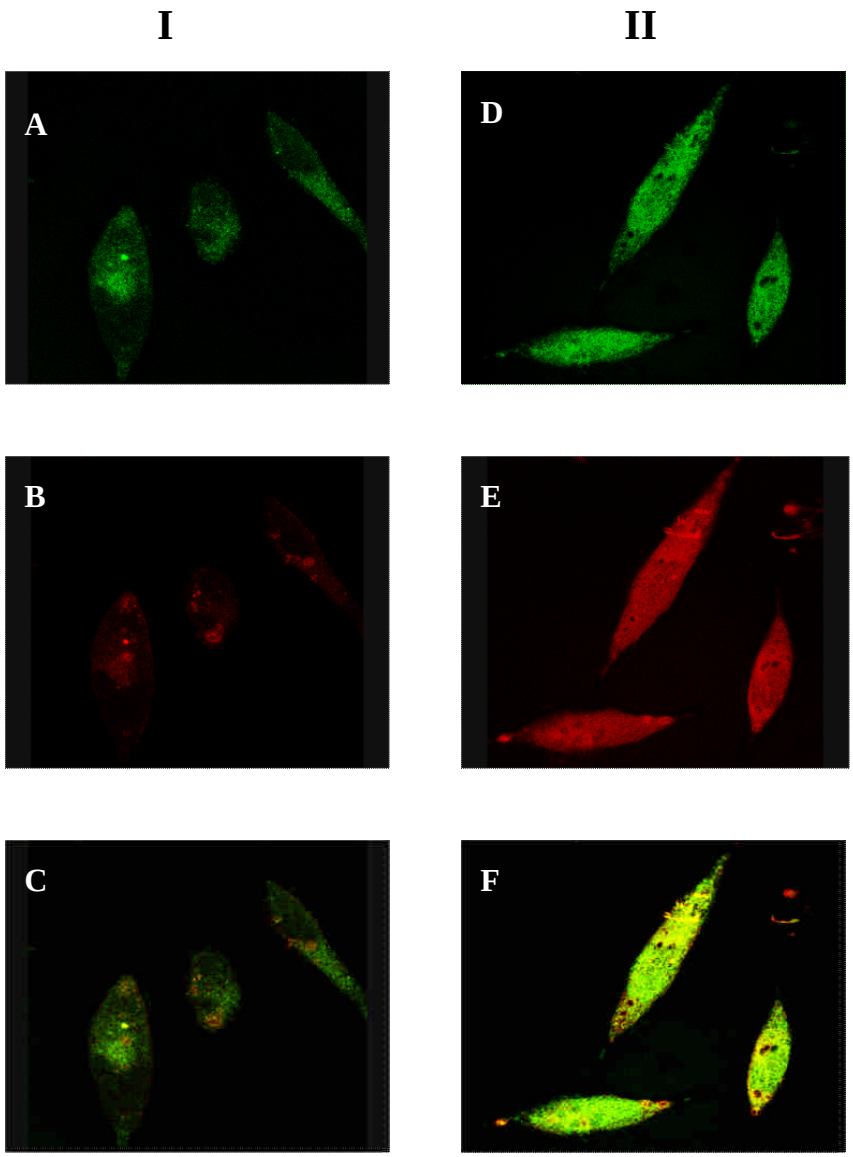
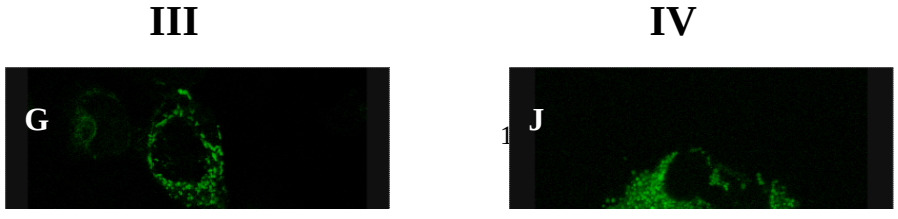
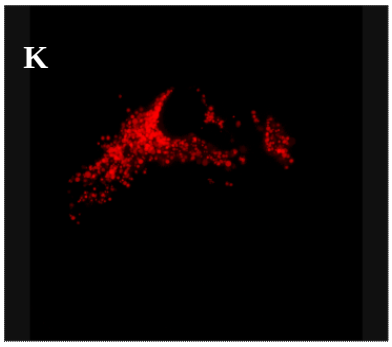


Fig. 3





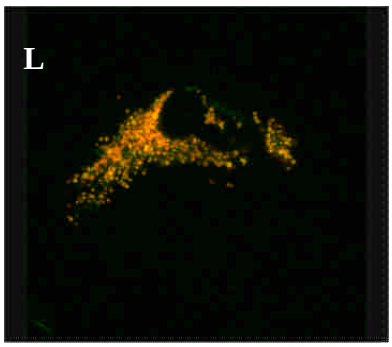


Fig. 4

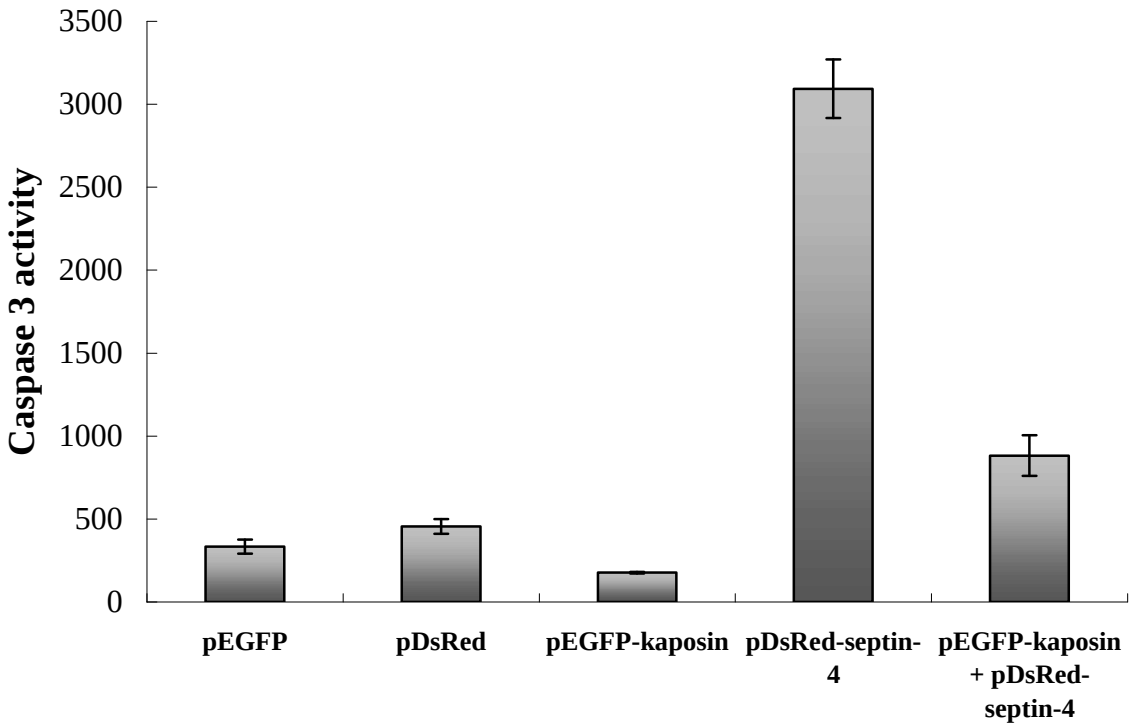

Caspase 3 activity
3500
3000
2500
2000
1500
1000
500
0
pEGFP
pDsRed
pEGFP-kaposin
pDsRed-septin-4
pEGFP-kaposin + pDsRed-septin-4

Fig. 5A

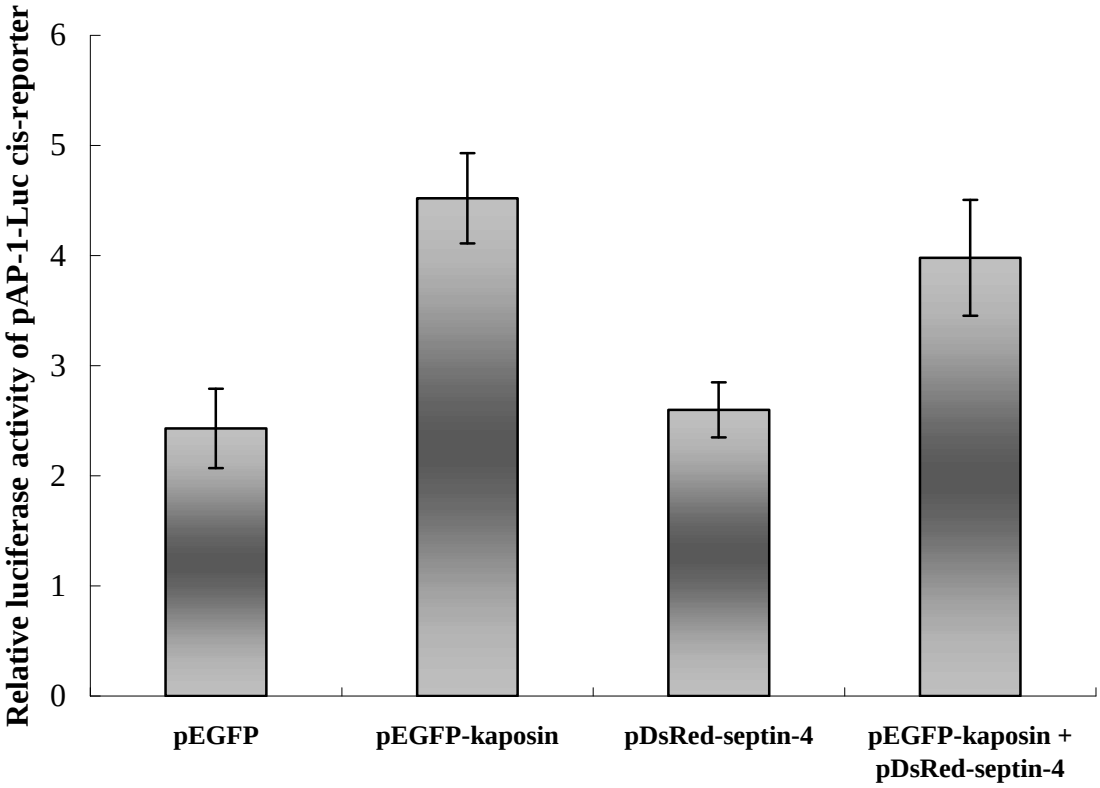
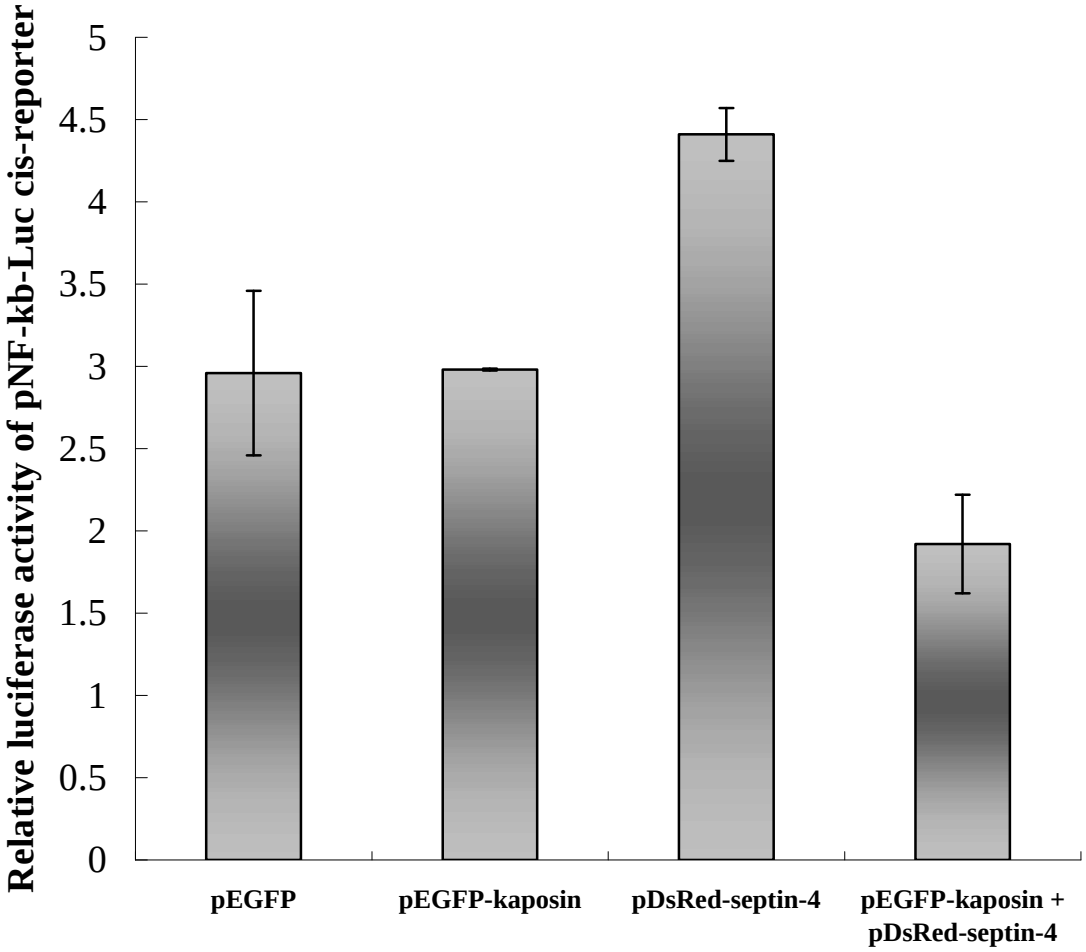

Relative luciferase activity of pAP-1-Luc cis-reporter
0
1
2
3
4
5
6
pEGFP
pEGFP-kaposin
pDsRed-septin-4
pEGFP-kaposin + pDsRed-septin-4

Fig. 5B


Relative luciferase activity of pNF-kb-Luc cis-reporter
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
pEGFP
pEGFP-kaposin
pDsRed-septin-4
pEGFP-kaposin + pDsRed-septin-4