International Journal of Applied Biotechnology and Biochemistry.
ISSN 2248-9886 Volume 4, Number 1 (2014) pp. 25-44

http://www.ripublication.com

Oligomerization of the Eukaryotic Initiation Factor 2-Associated Glycoprotein p67 Requires N-terminal 1-107 Amino Acid Residues

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Abbreviations used

p67, eukaryotic initiation factor 2-associated 67-kDa glycoprotein; eIF2α, α-subunit of eukaryotic initiation factor 2 (eIF2); p67-DG, p67-deglycosylase; POEP, protection of eIF2α phosphorylation; EGFP, enhanced green fluorescent protein; GST, glutathione-S-transferase Human p67, also known as MetAP2; and yeast MAP1, also known as MetAP1.

SUMMARY:

The rate of protein synthesis in mammals is regulated by the phosphorylation of the smallest α-subunit of eukaryotic initiation factor 2 (eIF2). The cellular glycoprotein p67 protects eIF2α from phosphorylation by its kinases. To understand the molecular details of this activity, we studied p67-p67 oligomerization by yeast two-hybrid, Far-Western assays, cross-linking experiments, and gel-filtration experiments. In yeast two-hybrid assays and *in vivo* cross-linking experiments, we detected weak interactions between p67 molecules, whereas some of its mutants showed strong interactions with the wild type. To verify these interactions *in vitro,* we performed Far-Western experiments, and found that the full-length molecule (wild type or mutants of p67) did not show strong oligomerization, although its cleavage products did. This interaction was inhibited when either of the lysine-rich domains along with the 1-35 N-terminal amino acid segment was deleted. In addition, alanine substitution at specific conserved amino acid residues in the 166-480 amino acid segment of p67 inhibited oligomerization *in vitro*. Further Far-Western experiments revealed that rat p67 interacts with rabbit and human p67 as well. Taken together, these data suggest

that both the lysine-rich domains and the N-terminal 1-35 amino acid segment of p67 are necessary for its oligomerization, and that the wild type molecule interacts strongly with some of its specific mutant forms.

INTRODUCTION

Phosphorylation of the smallest α-subunit of eukaryotic initiation factor 2 (eIF2α) plays a central role in regulating the overall rate of protein synthesis in eukaryotes [1-2]. Among several mechanisms for the regulation of the eIF2α phosphorylation during normal growth of mammalian cells, the most important is the cellular glycoprotein p67's protection of eIF2α from phosphorylation by its kinases [3-9]. The 480 amino acid residues of mouse, rat, and human p67 are encoded by a recombinant ~1.4 kb cDNA segment [8,10-11]. At the N-terminus 1-165 amino acid segment of p67, there are two lysine-rich domains (I & II) separated by an acidic residue-rich domain. These unique domains are highly conserved among mammals [8-9]. At the 166-480 amino acid segment, five conserved amino acid residues are present at the consensus $^{251}D(X)_{10}{}^{262}D(X)_{68}{}^{331}H(X)_{32}{}^{364}E(X)_{94}{}^{459}E^{460}H$ sequence [8 and references therein, 12]. Our previous studies showed that the N-terminus 1-97 amino acid segment of rat recombinant p67 can increase POEP activity in mammalian cells, and the lysine-rich domain I present at the N-terminus is required for its activity [9]. Although lysine-rich domain II seemed redundant in that study, at a specific time interval (4 hrs) during serum-restoration, this domain can complement lysine-rich domain I. In addition, specific amino acid substitutions at both of these domains inhibited POEP activity [9], indicating that both domains participate in this activity. The most important question is by what mechanism constitutive expression of a p67 mutant inhibits the function of an endogenous molecule. Several lines of evidence indicate that if a mutant protein makes a heterodimer with a wild type molecule and inhibits its function, it will act as a dominant negative mutant [13-17]. To test whether p67 can form oligomer, we have studied p67-p67 interactions by applying several methods routinely used to study protein-protein interaction.

Protein–protein interaction has been shown to be the most powerful mode of communication between upstream and downstream targets of effector molecules, as well as a mean to relay cellular signals within the cells [18-19]. Several methods have been used to identify such protein–protein interactions both *in vivo* and *in vitro*. Among these methods, yeast two-hybrid assay [20-23], Far Western assay [24], and protein cross-linking [25] are routinely used for this purpose.

In this study, we have used several *in vitro* and *in vivo* methods to detect p67-p67 oligomerization. Our study shows that exposure of the lysine-rich domains of p67 is essential for its homodimeric interactions. In addition to the lysine-rich domains, our yeast two-hybrid and Far-Western assays showed that the N-terminal 1-35 amino acid segment is needed for oligomerization. We also found that rat p67 can oligomerize with rat, rabbit, and human p67. Taken together, our data suggest that both the lysine-rich domains and the N-terminal 1-35 amino acid segment of p67 are necessary for its oligomerization, and that the wild type molecule interacts strongly with its specific mutant forms.

MATERIALS AND METHODS

All chemicals used in this study were obtained from Sigma Chemicals (St. Louis, MO), Merck (Darmstadt, Germany), ICN Biomedicals, Inc. (Aurora, Ohio), Fisher Chemicals (New Jersey), or GIBCO-BRL (Rockville, MD). All enzymes used in this study were purchased from New England Biolabs (Beverly, MA). Molecular mass markers were purchased from BioRad, and [^{35}S]methionine was purchased from Amersham.

Generation of p67 mutants

The generation of p67 block mutants, double mutants, and point mutants was previously described [9]. In brief, a ~1.4 kb cDNA insert encoding the entire p67 coding region was produced by PCR using appropriate forward and reverse primers, and mutations at specific sites at the coding region of p67 were introduced following the procedures as described [26]. To obtain various p67 deletion mutants, we first generated the 1-35, 1-76, 108-480, and 88-480 amino acid segments of p67 by PCR using appropriate primers. These fragments were then ligated in different combinations into the pcDNA3 vector (Invitrogen) to make the pcDNA3-P136, pcDNA3-P114, pcDNA3-P134, and pcDNA3-P116 plasmids. These plasmids were subsequently used as templates, and polymerase chain reactions were performed to obtain various fragments. These DNA fragments were then subcloned into pGEX-1 (Gutathione Corporation Ltd) at the *Xma*I site to get in-frame fusion with GST. For generating CΔ143 of P116 and P16, the latter plasmids were digested with *EcoR*I, and self ligated. The oligonucleotides used in this study were purchased from either GIBCO/BRL or Sigma-Genesis. DNA sequencing was performed by sequenase kit version 2.0 (US Biochemicals).

Preparation of glutathione-S-transferase (GST) and its fusion derivatives

The cDNA inserts for p67 with specific mutations at the coding region were isolated from the RF form of M13mp18 and digested with *Xma*I, and the DNA inserts were ligated at the *Xma*I site of the pGEX-1 vector (Glutagene, Amrad Corp. Ltd.) The GST and GST-fusion proteins were purified from JM101 *E. coli* cells using Glutathione-S-Agarose beads (Sigma) following the procedures as described [9].

Cell culture, chemical cross-linking, cell lysate preparation, and Western blot analysis

Rat tumor hepatoma (KRC-7) cells were grown in small petri dishes to near 60-70% confluency, formaldehyde (final concentration 1%) was added to the culture, and the culture was transferred to the 37 °C incubator. As a control, sterile water was added to the cells. At appropriate time interval, cells from the formaldehyde treated and untreated plates were removed and harvested. Cell lysates were made, and Western blot experiments were performed following the procedures as described [9].

Antibodies

Preparation and characterization of polyclonal antibody against p67 has been described [3].

Preparation of specific p67 mutants

The description of p67 mutants used in this study is given in Table 1. Three series of p67 mutants –- block, point, and double mutants -- were generated (Table 1). The block mutants were generated by substitutions of amino acid residues at the lysine-rich domains (I & II) and the acidic residue-rich domain of p67. We also made several double and triple mutants at these domains where one or both lysine-rich amino acid sequences were substituted along with the sequence at the acidic residue-rich domain. To generate point mutants, the conserved amino acid residues D251, D262, H331, E364, and E459 were individually substituted with alanine. These point mutants were then combined with a block mutant (D6/2) to obtain double mutants (Table 1). The expressions of all these mutant forms of p67 were tested individually in L40 yeast cells, and almost equal levels of fusion proteins were detected (data not shown.)

Subcloning p67 mutants into the pGEM vector

At the coding region of p67 cDNA, there are two unique restriction sites: an *EcoR*I site and a *BamH*1 site. To obtain pGEM derivatives of p67 mutants, the *EcoR*I-*BamH*I DNA fragments from the p67 mutant cDNAs were excised and ligated at the same site of a plasmid pGEM-p67 that was previously digested with *EcoR*I and *BamH*I, and the vector containing the partial p67 sequence was gel purified.

Subcloning wild type p67 or its mutants into yeast expression vector

A ~1.4 kb cDNA insert encoding the entire p67 coding region of wild type p67 or its mutants was obtained by PCR using appropriate forward and reverse primers (5' TCGGGCAACCCCGGGGATGGCGGGCGTG 3' and 5' TCCCCCGGGAAGTTTTAATAGTCATCTCCTC 3' respectively) with pGEM-p67 for wild type and EGFP-p67 mutants as templates [9-10]. The resulting DNA fragment was gel purified, digested with *Xma*I, and ligated in pBTM116 [24] at the *Xma*I site. Likewise for pVP16-p67, a 1.4kb cDNA insert encoding the entire p67 coding region was obtained by PCR using appropriate forward and reverse primers (5' AAGGAAAAAAAAGCGGCCGCATGGCGGGCGTGGAAG 3' and 5' AAGGAAAAAAGCGGCCGCAGTTTTAATAGTCATC 3') and pGEM-p67 as a template [10]. The resulting DNA fragment was gel purified, digested with BamH1, and ligated in pVP16 vector [24] at the *BamH*I site. To verify in-frame fusion of wild type p67 or its mutants into the LexA DNA binding domain or the VP16 activation domain, corresponding plasmids were subjected to DNA sequencing by sequenase kit version 2.0 (US Biochemicals). For further verification, these plasmids were expressed in yeast cells, and fusion proteins were detected on Western blots using either LexA or VP16 polyclonal antibodies. The proteins were visualized with the ECL system (Amersham). All procedures for manipulation of recombinant DNA were either published earlier [9] or followed from molecular cloning, a laboratory manual [26], and current protocols in molecular biology [27].

Purification of p67 from rabbit and human

Purification of p67 from rabbit reticulocytes was previously described [3]. Human p67 was tagged with six histidine molecules was a gift of Dr. J. Wang (Abbot

Laboratory). His-tagged human p67 was expressed in bacculovirus cells, and affinity purified on a Ni^{2+}-column following the procedures described by the manufacturer [28]. A sample of yeast MAP1 was a gift of Dr. R. Bradshaw (University of California).

Chromatographic separation of proteins with different molecular weights

A HiPrep Sephacryl S-300 gel filtration column (10-1500 kDa separation range and 120 ml bed volume) was used to determine the molecular weight of native p67. An FPLC purified homogenous preparation of p67 (approx. 2 mg in 1.5 ml) was dialyzed in Buffer A at 4 °C for 15 h and then loaded into the column previously equilibrated with the same buffer. The optical density of the eluted fractions was measured at 280 nm. Peak fractions were then analyzed by SDS-PAGE. The apparent molecular weight of the proteins in the peak fractions was determined using a calibration curve (log M_r vs. K_{av}) generated by running several molecular weight markers (Thyroglobulin , M_r 669,000; Ferritin , M_r 440,000; Catalase, M_r 232,000; Aldolase, M_r 158,000; Bovine Serum Albumin, M_r 67,000, and Cytochrome C, M_r 12,000) through the same column.

Yeast cell growth

For the two hybrid assay, yeast strain L40 (MATa his3Δ200 trp1-901 leu2-3, 112 ade2 LYS2 : : $(lexAop)_4$ – HIS3 URA3 : : $(lexAop)_8$ – lac Z GAL4 gal80) was used. The L40 cells were grown in YPAD media overnight before co-transformation with the indicated plasmids.

Co-transformation of two hybrid plasmids into yeast and amino acid selection

Yeast strain L40 was co-transformed with different combinations of the two-hybrid plasmids. Competent yeast cells were prepared by the lithium acetate method following the procedures previously described [24]. The transformation mixtures (100 µl) were plated on synthetic media lacking tryptophan, leucine, lysine, uracil, and histidine. The cells were grown for 3-5 days, and were then assayed for β-galactosidase activity.

β-galactosidase assay

All transformants were screened for β-galactosidase activity by the filter assay following the procedure as described [24]. In each case, four independent colonies were assayed for β-galactosidase activity by pair-wise interaction between proteins, one of which was fused to the LexA DNA binding domain and the other to the VP16 transactivation domain.

***In vitro* synthesis of [^{35}S]methionine-labeled wild type p67 and its mutants**

The [^{35}S]methionine labeled probes were generated in a rabbit reticulocyte TNT quick coupled transcription/translation system, following the protocols as described by the manufacturer (Promega). In brief, the probe was prepared in a 50 µl reaction mixture containing 40 µl rabbit reticulocyte, 2 µg pGEM-p67 plasmid or pGEM derivatives of

p67 mutants, 6 µl of nuclease free water, and 2 µl (10 µCi/µl) [^{35}S]methionine (specific activity 1175 Ci/mmol, Amersham Corp). The mixture was incubated at 30 ^{o}C for 60–90 min.

Far-Western assays

Protein extracts (~20 µg) were resolved with 15% SDS-PAGE then transferred into nitrocellulose membrane. The protein blot was denatured and then renatured by treating the blot with sequential washings in 0.1 M CZ solution (20 mM HEPES pH 7.9, 17% glycerol, 0.1M KCl, 5 mM $MgCl_2$, 0.1mM $ZnCl_2$, 0.1mM EDTA and 2 mM dithothretol) containing 0.5 mg/ml BSA, 0.02% polyvinyl pyrrolidone, and 6 M guanidine-HCl for 30 min one time; solution CZ 0.1 M plus 0.5mg/ml BSA, 0.02% polyvinyl pyrrolidone and 0.1 M guanidine-HCl for 1 h two times; solution CZ 0.1 M plus 0.5 mg/ml BSA, 0.02% polyvinyl pyrrolidone for 2 h three times, and finally 10 mM HEPES (pH 7.9) plus 5% BSA for 1 h. All washings were performed at room temperature. The blot was then incubated with 10 ml of 0.5X CZ (0.1 M) solution containing [^{35}S]methionine-labeled wild type p67 or its mutant forms for 12–14 h at room temperature. The blot was then washed twice with 0.5 x CZ (0.1M) solution and autoradiographed at –70 ^{o}C.

RESULTS AND DISCUSSION

To determine whether p67 can form oligomer, we used yeast two-hybrid assays. The entire coding region of rat p67 was subcloned into the pBTM116 yeast expression vector to obtain the LexA fusion, and into the pVP16 expression vector to generate the VP16 fusion of p67. These fusion chimeras were separately co-transformed in L40 yeast cells, and selected in medium lacking tryptophan, histidine, uracil, leucine, and lysine (THULL medium). Six representative colonies were grown in liquid culture, and from each colony cell extracts were made, protein concentration was measured, and the expressions of the fusion proteins were tested on Western blots using polyclonal antibodies against LexA, VP16, and p67 separately. Both fusion chimeras were expressed almost equally in this yeast strain (data not shown). Several colonies were then used to test β-galactosidase activity in a filter assay. Our results showed that the interaction between wild type p67 molecules is very weak (Table 2). However, this interaction is prominent when specific p67 mutants are used to study interactions in L40 cells. The β-galactosidase activity in colonies with individual fusion protein (wild type or mutants) was measured, and we found no detectable activity (Table 2, and data not shown). The mutants were then co-transformed in L40 cells and selected in THULL medium. The β-galactosidase activity in the selected colonies was measured by filter assays, and the results are shown in Table 2. Although the interaction between wild type p67 molecules is weak, this interaction increased significantly by replacing amino acid sequences at either of the lysine-rich domains. When both the lysine-rich domains were mutated, their interaction with wild type p67 was not detected. A similar study with the acidic residue-rich domain showed a weak interaction with wild type p67, but double replacement both at the acidic residue-rich domain and lysine-rich domain II resulted in much higher levels of

interaction with wild type p67, indicating that lysine-rich domain I may have a strong interaction with p67 compared to lysine-rich domain II. Surprisingly, however we also noticed that the wild type p67 could interact with a block mutant of p67 (D6K1K2) where the amino acid sequences at all three unique domains (lysine-rich domains I & II, and the acidic residue-rich domain) were mutated, indicating that a second domain apart from the lysine-rich domains of p67 is also involved in oligomerization.

The above finding was further strengthened when we noticed a strong interaction between D6K1K2 mutants in a yeast two-hybrid assay. All the point mutants except D262A did not show any interaction with p67. This lack of interaction was not due to the low levels of the LexA-fusion proteins as confirmed by Western blotting experiments where the levels of these fusion proteins were found to be almost equal (data not shown). Although, we detected a very weak interaction between wild type p67 and a block mutant (D6/2), its second-site mutants D251A, D262A, E364A, and E459A showed stronger interactions with p67 but no interaction was detectable between the H331A double mutant of D6/2 and wild type p67. It is interesting to note that all these interacting double mutants were involved with the acidic amino acid residues, and the basic amino acid residue H331 showed no involvement in interaction with p67. Taken together, these results suggest that the lysine-rich domains are required for oligomerization, and the acidic residue-rich domain and conserved acidic amino acid residues may have an inhibitory effect during oligomerization.

To further verify the interaction of p67 mutants with its wild type form *in vitro*, we generated glutathione-S-transferase (GST) fusions of wild type and the various p67 mutants shown in Table 1. The GST and various GST-fusion proteins were each affinity purified by glutathione-Agarose beads, and the fusion proteins were analyzed with SDS-PAGE. The protein samples were then transferred to nitrocellulose membrane, and subjected to either Western blots with polyclonal antibodies against p67 or monoclonal antibody against GST, or Far-Western assays. In the latter assay, [^{35}S]methionine-labeled probes of wild type p67 or its mutant forms were used to detect protein-protein interaction *in vitro*. As a first step towards these experiments we used four deletion mutants: one containing lysine-rich domain I (P136), another containing lysine-rich domain II (P114), and the third containing both lysine-rich domains I & II (Fig. 1A). The block mutant K1K2, where the lysine-rich domains are substituted with amino acid sequences different from basic lysine residues but the acidic residue-rich domain remains unchanged, was also used to test the protein-protein interaction in p67 [9]. The interaction between radiolabeled wild type p67 and the GST-fusion of each of these mutants was tested with a Far-Western assay, and compared with GST and GST fusion of wild type p67 as controls (Fig. 1B). The P136 mutant showed a stronger interaction and the P114 mutant had a weaker interaction, but the P134 mutant showed a much stronger interaction with p67 than the p67-p67 interactions (compare lanes 3-4 with lane 2, Fig. 1B). In contrast, the interaction between p67 and the K1K2 mutant was undetectable (lane 5, Fig. 1B). The radiolabeled p67 probe did not show any interaction with the GST used as a control (lane 1, Fig. 1B). The weak interaction between the wild type p67 and the P114

mutant, and the lack of interaction with the K1K2 block mutant were not due to the lower protein content in the gel because the Western blot results with p67 antibody (Fig. 1C) and GST antibody (Fig.1D) did show sufficient proteins in their respective lanes. In all cases we have detected the interaction a doublet migrating near 50 kDa and 43 kDa on SDS-PAGE. These fragments originated from the full-length GST-fusion of the proteins. Although we have detected quite a good interaction with the full-length version of the proteins, the interactions with the protein fragments seem to be much stronger than those with the full-length proteins. These results suggest that the presence of lysine-rich domain I produces protein fragments that interact more strongly than fragments made by either mutants containing the lysine-rich domain II or the wild type p67. When both the lysine-rich domains are present in p67, more protein fragments are present in the samples, which bind more strongly with p67. In other words, the presence of lysine-rich domains in p67 is needed for having appropriate peptide fragments to be accessible for binding to p67 in this assay. We have repeated similar binding assays with several-radiolabeled wild type and various p67 mutant probes. The results are shown in Table 3. As expected, the wild type p67 probe interacted poorly with its GST-fusion, but strongly with deletion mutants P136 and P134 (both containing lysine-rich domain I). However, its interaction with the P114 mutant, which has only lysine-rich unique domain II, was very weak. These results indicate that either of the lysine-rich domains could be involved in p67-p67 interaction. Consistent with the findings from a series of related experiments, in all our Far-Western experiments we only detected strong binding with two major peptide fragments migrating near 50 kDa and 43 kDa. These fragments originated from the full-length GST-fusion of the individual wild type or mutant p67 molecules. The full-length GST-fusion showed weak interaction, and its population in the sample was also low. Studies of the interaction between the wild type p67 probe and its several block mutants revealed that the K1/2/3 mutant had a strong interaction, the K2/9/1 mutant had a slightly weaker interaction, and the K1K2 mutant completely inhibited interaction (Table 1, block mutants). Consistent with this, the block mutants D6/2, D6K1/7, and D6K2/5 interacted with wild type p67 probe strongly but the D6K1K2 mutant did not show any such interaction. Although all the point mutants at the conserved amino acid residues D251, D262, H331, E364, and E459 interacted with the wild type p67 probe, some of its second-site mutants, like the acidic residue-rich domain mutant (D6/2) plus D251A, D262A, or E459A did not show any interaction, but other second-site mutants, H331A and E363A, did show interactions. The E364A second-site mutant form of p67 even showed a much stronger interaction than the other second-site mutants tested in this experiment. These interactions were consistent with the presence of the peptide fragments mentioned earlier. This interaction with the peptide fragments is also noticed even when we used radiolabeled probes of the D6/2, K1K2, and D6K1K2 mutant forms of p67. In the D6/2 mutant the amino acid sequences at both the lysine-rich domains are unchanged, whereas in the K1K2 mutant the amino acid sequence at these domains is changed. In the D6K1K2 mutant, the amino acid sequences at all three unique domains are changed (Table 1). We detected interactions between the full-length radiolabeled probes generated from the D6/2, K1K2, and D6K1K2 mutants and deletion mutants P136, P114, and P134;

block mutants D6/2, D6K1/7, and D6K2/5; all point mutants except E459A; and double mutants D6/2+H331A and D6/2+E364A. These results indicate that the lysine-rich domains are necessary to generate peptide fragments that interact with either the N-terminal or the C-terminal portion of the p67 molecule. The mutants that did not show peptide fragments on SDS-PAGE also did not show any interaction with the full-length p67 molecule. These results indicate that the lysine-rich domains and some of the conserved amino acid residues may be involved in the cleavage of p67. These cleavage products may have originated from the p67 molecule due to its endopeptidase activity. We are at present investigating detailed molecular mechanism of endopeptidase activity of p67 *in vitro* and *in vivo*.

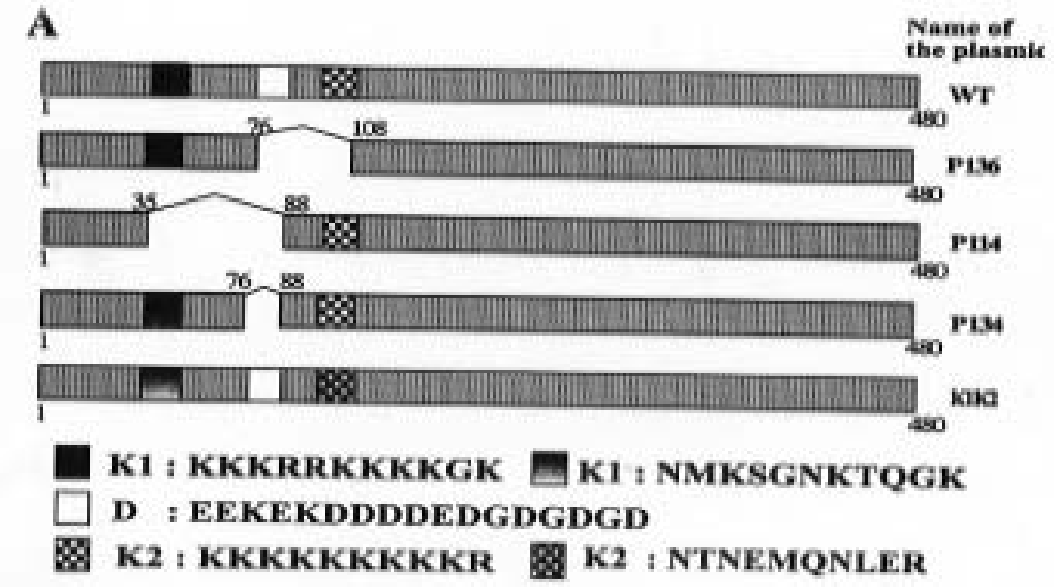
The interactions between wild type p67 and either the D6K1K2 mutant or itself indicate that some domain apart from lysine-rich domains is also involved in oligomerization. To identify such domain(s), we tested the interaction between p67 and each of the P116, P116Δ, P16, and P16Δ mutants (Fig. 2A) in a Far-Western assay (Fig. 2B). In addition to the strong interaction between wild type p67 and full-length GST-P116 (lane 3), a weaker interaction with P116(CΔ143) (lane 4) was also detected. Two additional faster migrating peptides generated from P116 and P116(CΔ143) mutants also interacted with wild type p67 (lane 3-4). This interaction, however, could not be detected with either the P16 or the P16(CΔ143) mutant (lanes 5-6). The difference between P116 and P16 is the N-terminal 1-35 amino acid segment (Fig. 2A). These results therefore suggest that the N-terminal 1-35 amino acid segment is involved in binding with wild type p67. Taken together, these results suggest that the lysine-rich domains along with the N-terminal 1-35 amino acid segment of p67 are involved in oligomerization.

From two different lines of experiments – yeast two-hybrid assays (Table 2) and Far Western (Table 3), we noticed that the p67-p67 interaction was very weak whereas that the interaction between specific mutants of p67 and the wild type was much stronger. This interaction was detected only with two peptide fragments of p67 indicating the possibility that the mutant form of p67 can form oligomer. In our *in vivo* cross-linking experiment we also detected such oligomerization (Fig. 3). In this experiment, KRC-7 cells were grown, treated with 1% formaldehyde or an equal volume of water, transferred to a 37 °C incubator, and kept there for 5 min. Cells were then harvested, and the total protein in the sample was resolved on SDS-PAGE followed by Western blot with p67 polyclonal antibody (Fig. 3). The control cells (lane 1) and the untreated cells (lane 3) did not show any slower migrating protein whereas the formaldehyde treated cells (lane 2) showed at least three slower migrating proteins that were detected by p67 antibody, suggesting that these peptides may be cross-linked oligomers of p67. The amount of cross-linked product was less than 1% of the amount of uncross-linked p67 in the cell lysate, indicating that *in vivo*, a small portion of p67 molecules may exist as a denatured form that can interact with the native molecule. Consistent with these results, we also found that a small fraction of purified rabbit p67 was present as a dimer detectable by gel-filtration assay (Fig. 4). This low level of *in vivo* cross-linking within wild type p67 is possibly due to its major interaction with subunits of eIF2. In agreement with the existence of p67-p67 oligomerization *in vivo*, we also detected a p67-p67 interaction in purified p67

molecules from rat, rabbit, and human (Fig. 5). The yeast MAP1, which has been shown to be homologous to mammalian p67 (~22% sequence similarity, ref. 29) and shares five conserved amino acid residues (D251, D262, H331, E364, and E459), did not show any interaction with rat p67 in a Far-Western assay (lane 4, Fig. 5). These results further confirm that the p67-p67 interaction exists, and this interaction can be detected in heterogonous species that include mammals.

For detecting oligomerization in p67 molecules, we used both a yeast two-hybrid assay and a Far-Western assay. Although the first assay is done in a heterologous system, it represents a more *in vivo* scenario than the Far-Western assay, which is *in vitro*. In yeast cells, we detected a very weak interaction within wild type p67 molecules and strong interactions between wild type and some mutant molecules (Table 2). Among these mutants, D6/2, in which both basic lysine-rich domains remain unchanged (Table 1) and which shows higher levels of POEP activity [9], did not show oligomerization with wild type p67 efficiently (Table 2). This suggests that oligomerization of p67 is indeed inhibitory to its POEP activity. Substitutions of the acidic amino acid residues D251, D262, E364, and E459 with alanine in the D6/2 mutant showed strong interaction with wild type, indicating that these conserved amino acid residues are possibly involved in charge-charge interaction with the lysine-rich domains that are not accessible for oligomerization. This is consistent with the observation that mutants with deletion or amino acid substitutions at either of the lysine-rich domains showed strong interactions with wild type p67, and mutants with changes at both the lysine-rich domains lost the ability to oligomerize. Our Far-Western results detected interactions only with the two major fragments that originated from the GST-fusion proteins, and presence of the lysine-rich domains was essential for such interactions. Although the role of the N-terminal 1-35 amino acid segment in p67-p67 interaction is not clear, it is possible that serine/threonine clusters present at this segment may contribute to oligomerization. The amino acid sequences of p67 from various species are conserved; this was also confirmed by the oligomeric interaction within p67 molecules from different species (Fig. 5). The GST-p67 purified from *E. coli* degraded extensively whereas purified rabbit native p67 or his-tagged recombinant human p67 purified from bacculovirus did not degrade (Fig. 5). These observations indicate that lysine amino acid residues in the lysine-rich domains could be modified or these molecules are glycosylated, thus preventing their degradation. Previously, we noticed that changing the amino acid sequence at the N-terminal lysine-rich domain of rat recombinant p67 from $_{36}$KKKRRKKKK$_{44}$ to $_{36}$NMKSGNKTQ$_{44}$ inhibited its POEP activity in mammalian cells [9]. The level of expression of this mutant was, however, low compared to that of endogenous p67. The mechanism by which the lysine-rich domain mutants K1/2/3 inhibited the function of the endogenous p67 was therefore intriguing. Several lines of evidence suggest that if a mutant form of a protein oligomerizes with its wild type, the mutant might have a “dominant negative” effect. This dominant effect is due to the sequestration of the wild type molecule by its mutant form, or by the mutant form converting the wild type molecule into an inactive form such as a prion [17]. In the latter case, a very low level of the mutant form can inactivate a large excess of endogenous wild type molecules. For example, certain mutant forms of the tumor

suppressor p53 act as 'dominant negative mutants," binding to wild type molecules and driving them into a mutant conformation [30-31]. Likewise, the double-stranded RNA-activated protein kinase PKR controls several cellular activities, including cell growth and proliferation, differentiation, apoptosis, and viral infection [13-14,32]; mutation at the kinase domain K296R makes it non-functional [33]. This mutant acts as a trans-dominant mutant, inhibiting the function of wild-type PKR, and thus it has been considered as a tumor suppressor [34]. The oncoprotein v-ErbA a member of the zinc finger transcription factor superfamily, is a "dominant negative" repressor in avian and mammalian cells, preferably functioning through heterodimerization with retinoid x receptor α [35]. Thyroid hormone receptors (TRs) show a dominant negative effect due to their homodimerization with their selective mutants including R316H and R338W [16]. Breast cancer resistance protein (BCRP) is a half-molecule ABC transporter that makes homodimer, a change from Leu to Pro at residue 554 in the fifth transmembrane domain of BCRP converts it into a mutant form that makes a heterodimer with a wild-type molecule and shows a new strategy to circumvent drug resistance [15]. Furthermore, naturally occurring truncated trkB receptors exert dominant inhibitory effects on BDNF signaling by forming non-functional heterodimer with the full-length receptors [36]. Similarly, co-expression of mutagenized and wild type hUGT1A1 in COS-7 cells shows that the mutant form markedly suppresses the catalytic activity of wild-type hUGT1A1. Homodimerization of hUGT1A1 may explain this dominant negative effect [37]. In all these cases, the mutant form binds to endogenous wild type molecule and inhibits its function. This type of mechanism seems to be also valid in case of p67.



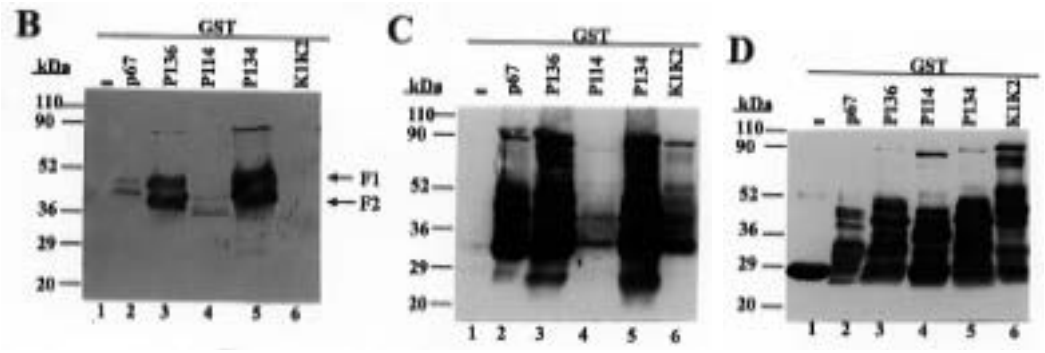


Fig. 1 Far-Western assay to detect p67-p67 interaction. (A) A schematic representation of the various deletion mutants and the K1K2 block mutant of rat p67. The solid bar represents 1-480 amino acid residues of rat p67. At the N-terminus, there are three rectangular boxes representing lysine-rich domain-I (filled box), the acidic residue-rich domain (open box), and lysine rich domain II (partially filled box). In the deletion mutants P136, P114, and P134, lysine-rich domain II, lysine-rich domain I, and the acidic residue-rich domain are deleted, respectively. A schematic diagram of the block mutant K1K2, in which both lysine-rich domains are substituted and the acidic residue-rich domain remains unchanged, is also shown. (B) Far-Western assay. The glutathione-S-transferase fusion proteins of wild type p67 or its deletion mutants and block mutant were resolved with 15% SDS-PAGE, and protein-protein interactions were analyzed by Far-Western assay. (C) Detection of the GST-fusions of wild type p67 and its various mutants. A similar experiment was performed with GST fusion proteins as mentioned in (B), except the nitrocellulose filter was subjected to Western blot with polyclonal antibodies against p67 (C). The antibody from the nitrocellulose membrane was then stripped off and the filter was re-probed with monoclonal antibody against GST (D). Lane 1, GST; lane 2, GST-p67; lanes 3-5, GST-fusions of deletion mutants P136, P114, and P134 respectively. The GST-fusion of the block mutant K1K2 (lane 6) was also analyzed in this experiment. Two prominent peptides migrating near 50 kDa and 43 kDa showed interaction with radiolabeled probes; these are marked as F1 and F2. Molecular mass markers are shown on the left.

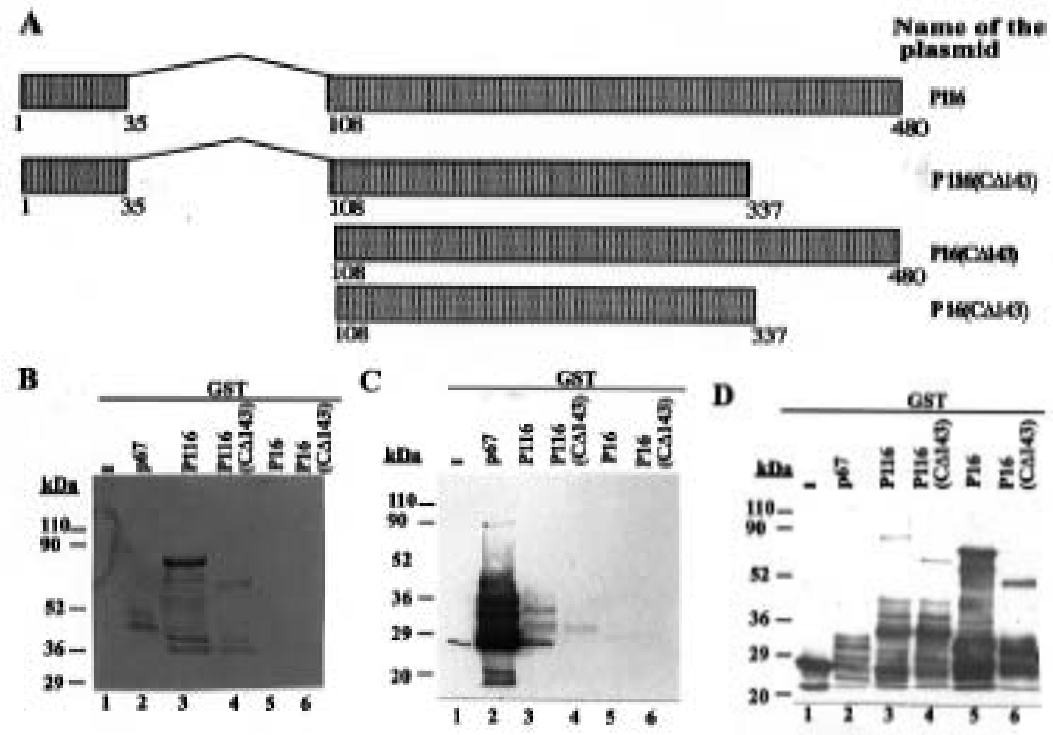


Fig. 2 The N-terminal 1-35 amino acid segment is necessary for p67-p67 interaction. (A) A schematic representation of the various deletion mutants p67. In the P116 mutant, the N-terminal 1-35 amino acid segment and the downstream 108-480 amino acid segment were linked together. The C-terminal 338-480 amino acid segment was removed from P116 to create the P116(CΔ143) mutant. In the P16 mutant, the N-terminal 1-107 amino acid segment was removed. From this mutant, the C-terminal 143 amino acid segment was further deleted to generate the P16(CΔ143) mutant. (B) Far-Western assay. The glutathione-S-transferase fusion proteins of wild type p67 or its deletion mutants were resolved with 15% SDS-PAGE, and protein-protein interactions were analyzed by Far-Western assay. (C) Detection of the GST-fusions of wild type p67 and its various mutants. A similar experiment was performed with GST fusion proteins as mentioned in (B), except the nitrocellulose filter was subjected to Western blot with polyclonal antibodies against p67 (C). The antibody from the nitrocellulose membrane was then stripped off and the filter was re-probed with monoclonal antibody against GST (D). Lane 1, GST; lane 2, GST-p67; lanes 3-5, GST-fusions of deletion mutants P116, P116(CΔ143), P16, and P16(CΔ143) respectively. Molecular mass markers are shown on the left. The polyclonal antibody against p67 interacts strongly with the amino acid sequence present at the N-terminal 1-107 segment. The C-terminal 378-480 amino acid segment is also involved in this antibody binding, indicating the generation of the conformational epitope(s) between C-terminus and N-terminus of the molecule.

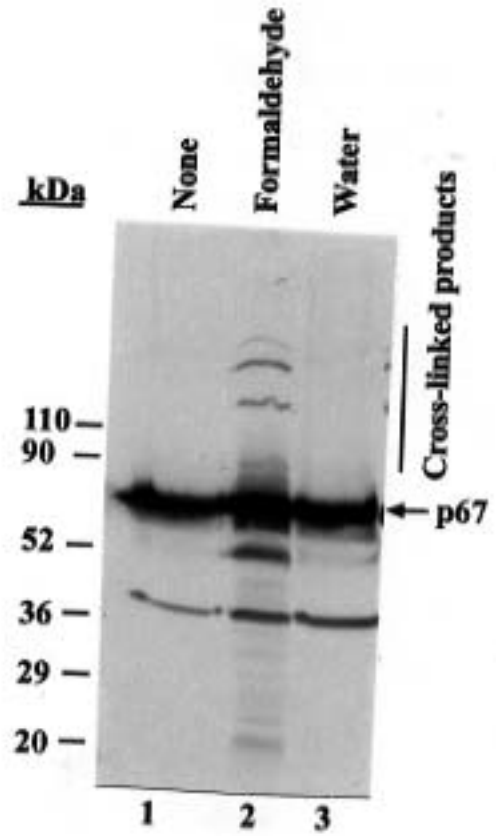


Fig. 3 p67-p67 oligomerization *in vivo*. Rat tumor hepatoma (KRC-7) cells were grown in normal growth medium and cell lysates were prepared at 5 and 10 minutes time intervals after addition of 1% formaldehyde as a cross linking agent. In other sets of the same experiment, sterile distilled water was used as a negative control, and cells grown under normal condition were taken as a control. 100 µg of total protein samples were analyzed with 15% SDS-PAGE and transferred to nitrocellulose membrane followed by immunoblotting with polyclonal antibody against p67.

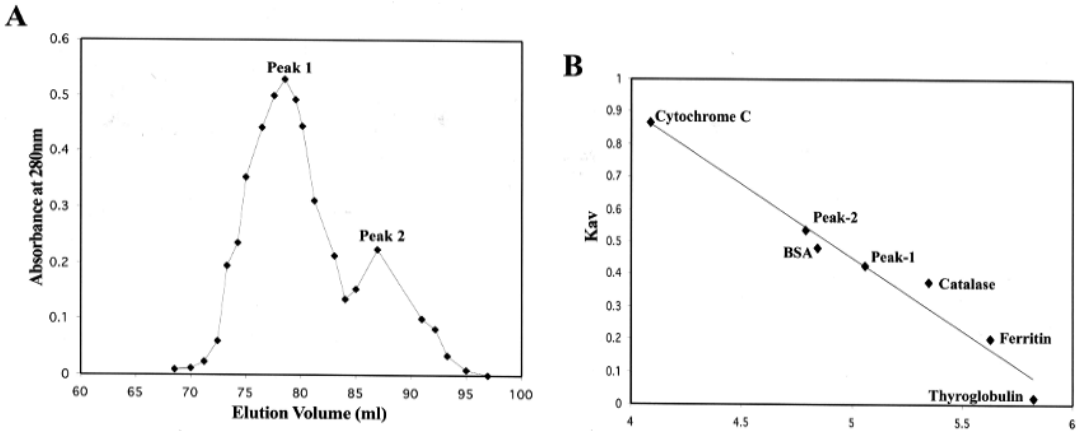


Fig. 4 Gel filtration chromatography of p67. The homogeneous preparation of p67 isolated from rabbit reticulocyte was subjected to Sephacryl S-300 gel filtration chromatography. An FPLC purified homogeneous preparation of p67 (2mg in 1.5ml)

was dialyzed in Buffer A at 4 °C for 15 h and then loaded onto the column previously equilibrated with the same buffer. The absorbance of eluted fractions was measured at 280 nm (A). A calibration curve (log MW vs. K_{av}) was generated by running several molecular weight markers (thyroglobulin, MW 669,000; ferritin, MW 440,000; catalase, MW 232,000; aldolase, MW 158,000; bovine serum albumin, MW 67,000; and cytochrome C, MW 12,000) through the same column (B).

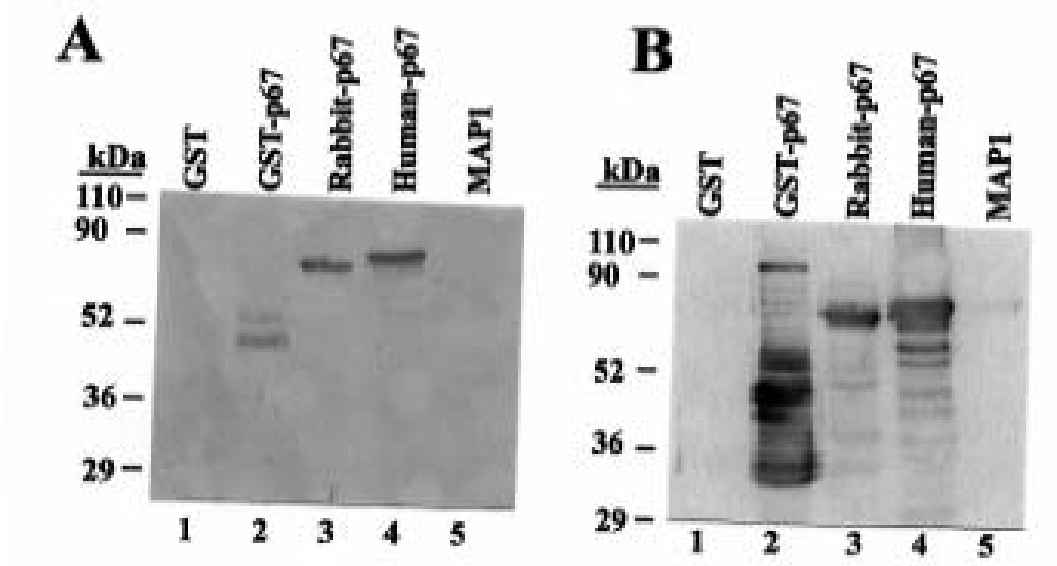


Fig. 5 Analysis of p67-p67 hetero dimerization from different species. (A) Samples of purified p67 from various species were prepared and 2 μg protein samples were analyzed with 15% SDS-PAGE followed by Far-Western analysis with ^{35}S methionine- labeled wild type p67. Lane 1, GST alone; Lane 2, GST-p67; Lane 3, rabbit p67; Lane 4, human p67 or MetAP2; Lane 5, yeast MAP1 or MetAP1. (B) For the analysis of the levels of p67, the same Far-Western blot was washed twice with TTBS and used for Western blotting with polyclonal antibody against p67.

Table 1 Description of different plasmids and various p67 mutants. The LexA DNA binding domain and VP16 transactivation domain are present in yeast expression vectors pBTM116 and pVP16 respectively. The entire coding region of rat p67 cDNA was subcloned into either the pBTM116 vector or the pVP16 vector, to generate the pBTM116-p67 or the pVP16-p67 plasmid respectively. Several p67 mutants (block, point and double) were also subcloned into the pBTM116 vector. The change in amino acid sequences at the block, point, or double mutants were shown as "mutant sequence" from "wild type sequence."

Plasmids	**Wild type sequence**	**Mutant sequence**
pBTM116 (vector)	-	-
pVP16 (vector)	-	-
pBTM116-p67	Full length p67	-
pVP16-p67	Full length p67	-
Block Mutants		
K1/2/3	$_{36}$KKKRRKKKK$_{44}$	$_{36}$NMKSGNKTQ$_{44}$
K2/9/1	$_{98}$KKKKKKKKK$_{106}$	$_{98}$NTNEMQNLE$_{106}$
K1K2/17	$_{36}$KKKRRKKKK$_{44}$, $_{98}$KKKKKKKKK$_{106}$	$_{36}$NMKSGNKTQ$_{44}$, $_{92}$NTNEMQNLE$_{106}$
D6/2	$_{77}$EEKEKDDDDEDGDGD$_{91}$	$_{77}$QNIQKALEPEAGDGA$_{91}$
D6K1/7	$_{77}$EEKEKDDDDEDGDGD$_{91}$, $_{36}$KKKRRKKKK$_{44}$	$_{77}$QNIQKALEPEAGDGA$_{91,}$ $_{36}$NMKSGNKTQ$_{44}$
D6K2/5	$_{77}$EEKEKDDDDEDGDGD$_{91}$, $_{98}$KKKKKKKKK$_{106}$	$_{77}$QNIQKALEPEAGDGA$_{91}$, $_{98}$NTNEMQNLE$_{106}$
D6K1K2/5	$_{77}$EEKEKDDDDEDGDGD$_{91}$, $_{36}$KKKRRKKKK$_{44}$, $_{98}$KKKKKKKKK$_{106}$	$_{77}$QNIQKALEPEAGDGA$_{91}$, $_{36}$NMKSGNKTQ$_{44}$, $_{98}$NTNEMQNLE$_{106}$
Point Mutants		
2/1	D251	A251
3/3	D262	A262
4/3	H331	A331
5/2	E364	A364
6/11	H459	A459
Double Mutants		
D6/2-4/5	$_{77}$EEKEKDDDDEDGDGD$_{91,}$ D251	$_{77}$QNIQKALEPEAGDGA$_{91,}$ A251
D6/2-2/13	$_{77}$EEKEKDDDDEDGDGD$_{91,}$ D262	$_{77}$QNIQKALEPEAGDGA$_{91,}$ A262
D6/2-3/12	$_{77}$EEKEKDDDDEDGDGD$_{91,}$ H331	$_{77}$QNIQKALEPEAGDGA$_{91,}$ A331
D6/2-1/16	$_{77}$EEKEKDDDDEDGDGD$_{91,}$ E364	$_{77}$QNIQKALEPEAGDGA$_{91,}$ A364
D6/2-6/16	$_{77}$EEKEKDDDDEDGDGD$_{91,}$ E459	$_{77}$QNIQKALEPEAGDGA$_{91,}$ A459

Table 2 Summary of the yeast two-hybrid data reflecting pair-wise interactions between proteins fused to the LexA DNA binding domain and VP16 transactivation domain. The β-galactosidase activity was assayed by the filter method using the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), and interactions were identified by the appearance of an indigo blue color on independent yeast colonies grown on selective media. "++++" indicates a very strong interaction, "+++" a strong interaction, "++" a weak interaction, "+" or "+/-" a very weak interaction, and "-" indicates no interaction.

LexA DNA Binding Domain (Bait)	**VP16 Activation Domain** (Prey)	**β-Galactosidase assay**
pBTM116 (vector)	None	-
None	pVP16 (vector)	-
pBTM116(vector)	pVP16(vector)	-
pBTM116–p67	None	-
None	pVP16–p67	-
pBTM116–Lamin	None	-
pBTM116-Lamin	pVP16-p67	-
pBTM116–p67	pVP16–p67	+/-
Block Mutants		
pBTM116–K1/2/3	pVP16–p67	+ + +
pBTM116–K2/9/1	pVP16–p67	+ + +
pBTM116–K1K2/17	pVP16–p67	-
pBTM116–D6/2	pVP16–p67	+
pBTM116–D6K1/7	pVP16–p67	-
pBTM116–D6K2/5	pVP16–p67	+ + + +
pBTM116–D6K1K2/5	pVP16–p67	+ + +
pBTM116-p67	pVP16-D6K1K2/5	+++
pBTM116-D6K1K2/5	pVP16-D6K1K2/5	+++
Point Mutants		
pBTM116–2/1	pVP16–p67	-
pBTM116–3/3	pVP16–p67	-
pBTM116–4/4	pVP16–p67	-
pBTM116–5/2	pVP16–p67	+
pBTM116–6/11	pVP16–p67	-
Double Mutants		
pBTM116–D6/2-4/5	pVP16–p67	+ +
pBTM116–D6/2-2/13	pVP16–p67	+ +
pBTM116–D6/2-3/12	pVP16–p67	-
pBTM116–D6/2-1/16	pVP16–p67	+ + +
pBTM116–D6/2-6/16	pVP16–p67	+ + + +

Table 3 Summary of Far-Western assays for studying p67-p67 interaction. The glutathione-S-transferase (GST) fusion proteins were purified, and appropriate amounts of fusion protein fractions were resolved on 15% SDS-PAGE, and transferred to nitrocellulose membrane. The membranes were then probed with [^{35}S]methionine-labeled wild type p67, D6/2, K1K2 or D6K1K2. "+" indicates interaction with probes; and +/- indicates a very weak interaction, and "-" indicates no interaction.

Plasmid expressing	**Far-Western assays showing interactions with**			
	P67	**D6/2**	**K1K2**	**D6K1K2**
GST	-	-	-	-
Wild type p67	+	+	+	+
Deletion mutants				
P136	+	+	+	+
P114	+	+	+	+
P134	+	+	+	+
Block mutants				
K1/2/3	+	-	-	-
K2/9/1	+	-	-	-
K1K2	-	-	-	-
D6/2	+	+	+	+/-
D6K1/7	+	+	+	+
D6K2/5	+	+	+	+
D6K1K2	-	-	-	-
Point mutants				
2/1	+	+	+	+
3/3	+	+	+	+
4/4	+	+	+	+
5/2	+	+	+	+
6/11	+	-	-	-
Double mutants				
D6/2-4/5	-	-	-	-
D6/2-2/13	-	-	-	-
D6/2-3/12	+	+	+	-
D6/2-1/16	+	+	+	+
D6/2-6/16	-	-	-	-

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