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Potential Involvement of p62, a Phosphotyrosine-independent Ligand of SH2 Domain of p56^{lck}, on UV-induced Apoptosis in Jurkat T-cell Line

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p56^{lck} SH2 domain 결합 단백질 p62가 Jurkat T-세포주의 세포예정사에 미치는 영향

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ABSTRACT : p62 is a novel cytoplsmic protein that binds to SH2 domain of p56^{lck}, lymphocyte-specific protein tyrosine kinase, and the expression of p62 was observed in most tissues. In addition p62 interacts with various proteins including ubiquitin and atypical PKC isoform, indicating its diverse biological role in different tissues. However, little is known about functional connection between p62 and its binding proteins. In the present study, a novel cellular protein, p62 has been shown to bind to 14-3-3 τ isoform that is specific for T cells. Moreover, overexpression of p62 in T cells caused to delay onset of UV-induced apoptosis characterized by DNA fragmentation and breakdown of poly (ADP-ribose) polymerase (PARP). Lately, 14-3-3 proteins have been shown to mediate survival signal via interacting proapoptotic Bad protein in the lymphocyte. These results suggested the presence of p62-mediated regulatory mechanism during apoptosis in T cells, in which activation-induced apoptotic signal could be interfered by p62 and 14-3-3 protein.

Key words: p62, 14-3-3 protein, p56^{lck}, T-cell, Apoptosis, Yeast two-hybrid screen.

요 약 : p62는 임파구에 특이적으로 발현하는 단백질 티로신 키나제인 p56^{lck}의 SH2 doamin과 결합하는 세포질 단백질로서 두 단백질의 결합에는 지금까지 알려진 바와 다르게 인산화된 티로신이 필요없다. p62는 기능이 다른 여러 조직에서 공통적으로 발현되며 유비퀴틴, 단백질 키나제 C 이성질체 등 다양한 단백질과 결합하는 것이 알려져 있다. 이와 같은 현상으로 p62가 다양한 생물학적 기능을 수행할 수 있음을 예측할 수 있으나 그 자세한 기작은 잘 알려져 있지 않다. 본 연구에서는 p62가 T-세포에 특이적으로 발현하는 14-3-3 τ 이성질체와 결합하는 것을 확인하였으며, p62를 인위적으로 T-세포에 다량으로 발현시키면 세포예정사 (apoptosis)의 시작이 지연되는 현상을 조사하였다. 이때 세포사멸과정에서 전형적으로 나타나는 DNA 절단현상 (DNA fragmentaion)과 poly (ADP-ribose) polymerase의 분해가 지연됨을 알 수 있었다. 최근 14-3-3 단백질이 임파구에서 세포예정사를 촉진시키는 기능을 가진 Bad와 결합함으로써 세포의 생존 신호 전달에 중요한 역할을 한다는 것이 보고된 바 있다. 따라서 본 연구의 결과는 T-세포의 활성으로 일어나는 사멸예정사 과정 중에 p62와 14-3-3 단백질에 의해 수행되는 조절 기작이 있음을 시사하고 있다.

INTRODUCTION

p56^{lck}, a member of src family protein tyrosine kinase (PTK), is preferentially expressed in thymocyte and peripheral T lymphocytes. Like other src family PTK, p56^{lck} has distinct structural motifs composed of unique N-terminal domain, src-homology 2 (SH2) domain, src homology 3 (SH3) domain, and a kinase domain. It associates specifically with the cytoplasmic domains of both CD4 and CD8 T-cell surface glycoproteins and interacts with the beta-chain of the interleukin-2 receptor, which implicates Lck activity in signal transduction during T-cell development and activation of mature T cells (for reviews see, Ravichandran et al., 1996; Weil & Veillette 1996). Mice lacking Lck showed a pronounced thymic atrophy, with a dramatic reduction in immature thymocyte population, demonstrating its importance in T cell maturation (Molina et al., 1992). Moreover, there are implications that p56^{lck} participates in activation induced or Fas mediated apop-

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tosis in thymocyte and mature cycling T cells (Garcia-Welsh et al., 1994; Di Somma et al., 1995; Gonzalez-Garcia et al., 1997). Upon activation through TCR $p56^{lck}$ is rapidly phosphorylated on tyrosine residues, and T cells become anergic or dead without costimulation or secondary signals (Kabelitz et al., 1995).

While interacting via its unique N-terminal domain with CD4 and CD8 $p56^{lck}$ also interacts via its SH2 and SH3 domains with other intracellular signaling proteins, subsequently transmits signals inside the cell (Ravichandran et al., 1996). Lately, a novel 62-kDa cellular protein has been identified as a phosphotyrosine independent binding protein of $p56^{lck}$ SH2 domain (Joung et al., 1996). p62 also interacts with ubiquitin (Vadlamudi et al., 1996) and atypical protein kinase C isoforms (Pulse et al., 1997; Sanchez et al., 1998), suggesting its diverse functions in signal transduction pathway. However, functional connection between p62 and its binding proteins is not clearly understood. In the present study, a novel cellular protein, p62 has been shown to bind to 14-3-3 τ isoform that is specific for T cells. In addition, overexpression of p62 in T cells inhibited apoptosis characterized by DNA fragmentation and breakdown of poly (ADP-ribose) polymerase (PARP). 14-3-3 proteins have been shown to mediate survival signal via interacting proapoptotic Bad protein in the lymphocyte (Zha et al., 1996). These results suggested the presence of p62-mediated regulatory mechanism during apoptosis in T cells, in which the activation induced apoptotic signal could be inhibited by p62 and 14-3-3 protein.

MATERIALS AND METHODS

1. Yeast two-hybrid screen

N-terminal part of p62 containing amino acids 1 to 254 fusion to Gal4 binding domain (Gal4B-p62N) was derived from Gal4B-p62 in GBT9 vector (Vadlamudi et al., 1996) by deleting the C-terminal domain (amino acid residues 255-440) of p62 using internal restriction sites, EcoRV. A HeLa cDNA library fused to the Gal4 activation domain (Gal4A) in pGADGH vector (Clontech) was introduced into Gal4B-p62N transformed yeast strain HF7c. Approximately 10^6 transformants were plated on *his*$^-$ plates, and *his*$^+$ colonies were isolated and tested for β-galactosidase activity using the filter assay, as described below. Plasmids were isolated from *his*$^+$ *Lacz*$^+$ colonies and transformed into an *E.coli Leu*$^-$ strain of HB101 in order to rescue the plasmids of library origin. Specific interaction of N-terminal and full length of p62 with the isolated plasmids was confirmed in a different yeast strain SFY526 in which LacZ is under control of the Gal1 promoter which is different from the promoter used in HF7c strain. β-galactosidase assay was performed both in SFY526 and HF7c strains after cotransformation of the isolated plasmid with either Gal4B-p62N or Gal4B-p62. cDNA inserts of the positive clones were characterized by sequencing using a Sequenase Kit S. Biochemical Corp.).

2. β-galactosidase filter assay

Yeast colonies were patched to synthetic medium plates lacking leucine and tryptophan, incubated for 2 days, and then transferred to nitrocellulose filter. The filter was immersed in the liquid nitrogen for 1 to 2 seconds. Filter was allowed to warm to room temperature and then placed on top of Whatman no. 1 paper that have been prewet in Z buffer containing 0.75 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactoside. The filters were incubated for 3 h at 30℃. Blue coloration is indicative of β-galactosidase assay.

3. Cell and Reagents

Jurkat leukemic T cells were maintained in RPMI 1640 medium supplemented with 100 units/ml penicillin/streptomycin and containing 10% fetal calf serum. Polyclonal p62 antibody was raised against a synthetic peptide consisting of C-terminus (amino acids 401-424) of human p62 protein. Monoclonal anti-PARP antibody was from Oncogene Research Product, and anti-T7 epitope antibody was from Novagen.

4. Generation of stable Jurkat cell line expressing p62

T7-epitope tagged p62 in pCDNA1 was excised and cloned into mammalian expression vector pLXSN (Miller & Rosman, 1989) using EcoRI and XhoI sites for the generation of stable cell lines. This construct was transfected into Jurkat leukemic T cells by electroporation using a Gene

Pulse apparatus (Bio-Rad). 20 μg of DNA was introduced into 10^7 cells suspended in RPMI by a 250V/960 μF electrical pulse at room temperature. Transfected cells were plated out 48 hr later at limited dilution in media containing 500μ g/ml G418 in 96-well microtiter plates. Single cell originated clones were selected after 2~3 weeks. Empty pLXSN vector was also transfected into Jurkat cells and G-418 resistant cells were isolated and used for the control experiment.

5. Western Blot Analysis

Cells were lysed in 20 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1% Triton containing 5 mM NaF, 20 mM leupeptin, and 1 mM phenymethylsulfonyl fluoride. Lysates were separated by SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was first blocked with 5% milk powder for 1 hr, followed by incubation with primary and secondary antibodies, and finally developed with enhanced chemiluminescence (Amersham).

6. Cell Stimulation and Immunoprecipitation

Cells were left unstimulated or incubated with 20 ng/ml of PMA (Phorbol-12-myristate-13-acetate) and 1 μM of Ionomycin in RPMI 1640 medium containing 10% FCS for 9 hrs at 37℃, 5% CO_2. Cells were lysed in 137 mM NaCl, 20 mM Tris (pH8.0), 1.5 mM $MgCl_2$, 1 mM EDTA, 5 mM NaF, 0.3% NP-40 containing 20 mM leupeptin, and 1 mM PMSF. Lysates were cleared with protein A-Sepharose for 1 hr, followed by incubation with anti-14-3-3 or anti-T7 antibodies overnight at 4℃. Next day, the Ab complexes were captured with protein A-Sepharose beads for 1 hr. Precipitates were dissolved by SDS-PAGE, and transferred to nitrocellulose membrane for further immunoblot analysis.

7. DNA Fragmentation Analysis

Cells were treated with UV-C (40J/m^2) using a UV-cross linker (Bio-Rad) and incubated in RPMI 1640 medium containing 10% FCS for indicated times and low molecular weight chromosomal DNA was purified. Briefly, 6×10^5 cells were washed phosphate-buffered saline and lysed in 500 μl of lysis buffer (5 mM Tris-HCl, pH 7.4, 20 mM EDTA, and 0.5% Triton X-100). Samples were incubated on ice for 5 min, and insoluble materials were removed by centrifugation. The supernatant was transferred to a new tube, and the nucleic acid fraction was purified by extraction with phenol/chloroform twice followed by ethanol precipitation. The precipitates was dissolved in 15 μl of TE (10 mM Tris-HCl, pH8.0 and 1 mM EDTA) containing 2 μ g/ml RNase A and incubated for 30 min at 37℃. Each sample was separated by 1.8% agarose gel electrophoresis, and DNA was visualized by ethidium bromide staining.

RESULTS AND DISCUSSION

1. Two-hybrid Screen

Previous study showed that C-terminal 182-amino acid deletion of p62 completely abolished the binding of p62 to ubiquitin (Vadlamudi et al., 1996). In order to isolate associating protein of p62 other than ubiquitin, N-terminus of p62 (amino acids 1 to 245) fused to the Gal4 DNA binding domain (Gal4B-p62N) was used for screening a HeLa cDNA library. cDNA librabry was fused to the Gal4 activation domain (Gal4A) using two reporter genes (*his* and *lacZ*) under the control of Gal4 upstream activating sequence. In a yeast strain HF7c, interaction of Gal4B-p62N with any protein fused to Gal4 activation domain will activated transcription of reporter genes. Initial screening of 1×10^6 transformants for activation of *his* and *lacZ* genes yielded 89 positive clones. 37 of positive clones were sequenced and searched for matching sequences in GenBank database. The deduced sequences from two isolates (#20 and #90) shared high homology with 14-3-3 protein family (Fig. 1). It showed highest homology with the τ and θ isoforms of 14-3-3 gene family that are, among other functions, implicated in T-cell signal transduction and brain development, respectively (Watanabe et al. 1994; Bonnefoy-Bérard et al. 1995). Plasmids of these clones were examined for its specific interaction with p62 in another yeast strain SFY526 that carries either Gal4B-p62 or Gal4B-p62N. Coexpression of Gal4B fused to either full-length (Gal4B-p62) or N-terminus of p62 (Gal4B-p62N) and Gal4A-14-3-3 in yeast specifically induced activation of β-galactosidase that results in blue colony formation (Table 1). This result indicated that 14-3-3 protein interacted specifically with full length as well as N-terminus of p62.

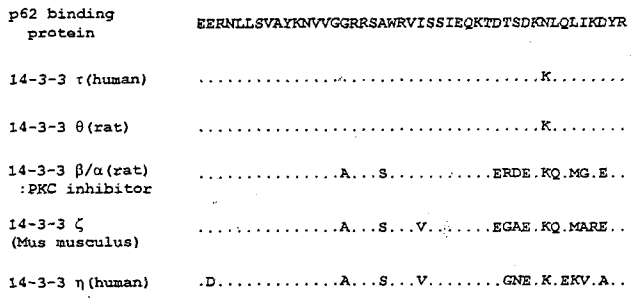

Fig. 1. Amino Acids sequence alignment between p62 binding protein and 14-3-3 gene family.
Amino acid sequences of isolates that bind to p62 in yeast two hybrid system are shown along with their alignments to different isoforms of 14-3-3 gene family. Periods denote amino acids identical to those of p62 binding protein shown on the top.

Table 1. Specific interaction of p62 with 14-3-3 in Yeast activates β-galactosidase reporter gene

	Ga 14 A	Ga14A-14-3-3
Ga14B	W	W
Ga14B-p62	W	B
Ga14B-p62N	W	B
Ga14B-Lamin	W	W

Specificity of the interaction of 14-3-3 with full length and N-terminus of p62 in Yeast was assayed using the 5-bromo-4-chloro-3-indolyl β-galactoside filter method as described under the "Materials and Methods". B, blue color colony; W, white color colony, p62; full length p62 peptide, p62N; N-terminus of p62 peptide (amino acids 1 to 254).

2. Association of p62 with 14-3-3 protein *in vivo*

Gal4A-fused with 14-3-3 τ cDNA isolated from #20 and #90 clones, when coexpressed with Gal4B-p62 in HF7c yeast strain conferred the *His*$^+$ phenotype (Fig. 2A). A control bait Gal4B-Lamin, however, when coexpressed with either of 14-3-3 τ fusion genes, failed to induce the *His*$^+$ phenotype. This result ascertained the specific interaction between 14-3-3 τ and p62 *in vivo*.

Interaction of p62 with 14-3-3 protein was further confirmed in stably transfected Jurkat T-cells, expressing T7 epitope tagged p62 (p62-T7). Western analysis of 14-3-3 immunoprecipitates with T7 antibody revealed coprecipitation of 14-3-3 protein with p62-T7 (Fig. 2B). The association of p62-T7 with 14-3-3 protein in T cells was markedly increased when cells were stimulated with PMA and Ionomycin (lanes 3 and 4). This result does not appear to be due to the higher expression of p62-T7 because increase in the amount of immunoprecipitates with T7 antibody was not pronounced in stimulated cells (lanes 1 and 2). It is likely that p62/14-3-3 interaction is mediated through phosphoserine. It is known that 14-3-3 protein binds to signaling proteins via phosphoserine containing motif (Muslin et al., 1996). p62 is phosphorylated on serine/threonine residues (Joung et al., 1996), and its phosphorylation is induced by various signals including activation by PMA and ionophore (Joung, I; unpublished result). Therefore it is possible that p62 becomes phosphorylated upon T-cell activation and interacts with 14-3-3 proteins.

3. Overexpressed p62 interferes UV-induced apoptosis in Jurkat T-cells

The physiological role and functional consequences of the association between 14-3-3 proteins and different signaling molecules are far from clear, however, the p62/14-3-3 τ interaction suggested several possible functional roles. p62 was first identified as a binding protein of Lck SH2 domain. Both p56^{lck} and 14-3-3 proteins have been implicated in the regulation of development and apoptosis (Garcia-Welsh et al., 1994; Gonzalez-Garcia et al., 1997; Kockel et al., 1997). I accessed whether p62 is involved in the regulation of apoptosis in T-cells. Upon treatment of UV, control Jurkat cells underwent rapid apoptosis characterized by DNA fragmentation within 1 hr, whereas same degree of chromosomal DNA degradation was observed 2 hrs after the treatment of UV in p62-T7 expressing cells (Fig. 3A).

The potential inhibitory effect of p62 on apoptosis was further confirmed by down-regulation of caspase activity. One of the later events observed in cells undergo apoptosis is activation of caspases, resulting in the proteolytic degradation of cellular substrates such as poly (ADP-ribose) polymerase (PARP) (Cohen, 1997). PARP degradation was examined in Jurkat cells by Western blotting analysis using anti-PARP antibody (Fig. 3B; α-PARP). In vector transfected cells treatment of UV yielded 89-kDa apoptotic fragment detected as fast-moving bands on the gel, and most of original 110-kDa protein had disappeared in 30 min. in response to UV exposure. By contrast, in p62-ex-

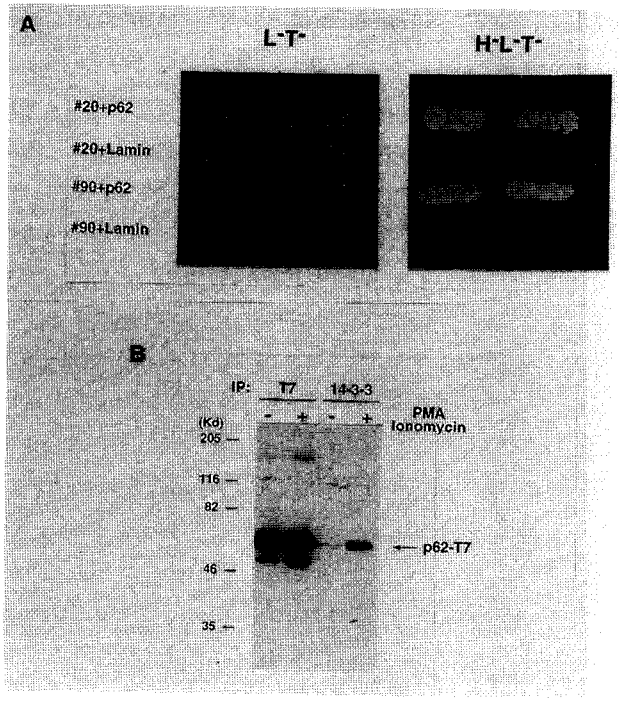

Fig. 2. Association of p62 with 14-3-3 *in vivo*.
(A) Specific interaction of p62 with 14-3-3 protein in the yeast two hybrid system. Yeast strain HF7c was transformed with plasmids carrying Gal4B-p62 or Gal4B-Lamin with each of two positive isolates (#20 and #90) fused to Gal4A. Colonies containing different combinations of plasmids were streaked on *leu⁻trp⁻* (L^-T^-) or *his⁻leu⁻trp⁻* ($H^-L^-T^-$) plates and incubated for 2 days at 30℃. For each combination, two different colonies were picked.
(B) Coimmunoprecipitation of p62 and 14-3-3 in T cells. Western blot of p62-T7 and 14-3-3 immunoprecipitates (anti-T7 and anti 14-3-3) from p62-T7 expressing Jurkat cells developed with anti-T7 antibody. Cells were unstimulated(−) or treated with PMA and Ionomycine (+) as described under "Materials and Methods". Arrow indicated the position of p62-T7.

pressing cells 110-kDa protein was still detected 1 hr after treatment of UV, indicating that onset of PARP breakdown was delayed. These results suggested that over-producing p62 in T-cells down-regulated caspase activity and following DNA fragmentation during apoptosis. Similar inhibitory effect was observed in p62 over-producing cells when apotosis was induced by Fas-antibody ligation (data not shown). Interestingly, Western blot analysis of same lysates using anti-p62 antibody showed p62 rapidly disappeared like PARP as apoptosis was progressed (Fig. 3B; α-p62), implicating that p62 itself is subjected to the regulation of apoptosis.

Programmed cell death or apoptosis is a characteristic feature of normal developmental process as well as a response of cells to stress or other environmental insults. Regulation of cell death is crucial for the normal physiology of multicellular organisms. Perturbation of normal survival or death mechanisms that leads to either excessive cell death or survival may play a role in the pathogenesis of many diseases. T lymphocytes are notoriously prone to undergo apoptosis in the development and homeostatic regulation in immune system, including elimination of reactive thymocytes, development of memmory T cells and killing of target cells by activated cytotoxic T lymphocyets (Smith et al., 1995; Rowan & Fisher, 1997). Resistance to apoptosis may be involved in the pathogenesis of autoimmune diseases, by contrast, an enhanced apoptosis in peripheral T lymphocytes resulted in functional deficiency of T lymphocyte as seen in HIV infection (Kroemer et al., 1995; Rowan & Fisher, 1997).

It has been reported that $P56^{lck}$, a lymphocyte-specific PTK, participates in apoptosis mediated through T-cell receptor or Fas, in which the activation of Lck kinase activity was required in the process (Di Somma et al., 1995; Gonzalez-Garcia, et al., 1997). It is well known that Lck is activated by phosphorylation on its tyrosine residue and signals are further transmitted through interaction between phosphotyrosine containing molecules and SH2 domains (Weiss & Littman, 1994).

In this study I showed p62, a phosphotyrosine-independent binding protein of Lck SH2 domain down-regulated T-cell apoptosis. This inhibitory effect may be carried by interaction of p62 to 14-3-3 protein in T-cells. 14-3-3 family members are acidic, abundantly expressed, highly conserved proteins that have been implicated in a variety of signal transduction process, including those involved in cell cycle regulation and cell survival. In addition many evidences have suggested that 14-3-3 proteins are involved in differentiation and development processes in yeast as well as verterbrates (McConnell et al., 1995; Roberts et al., 1997). However, the specific role for individual 14-3-3 proteins remains elusive, although the function of 14-3-3 binding is known in a few cases. 14-3-3 binding to proapoptotic protein Bad interfere the interaction of Bad with Bcl-2, resulted in the cell survival (Zha et al., 1996). The interaction be-

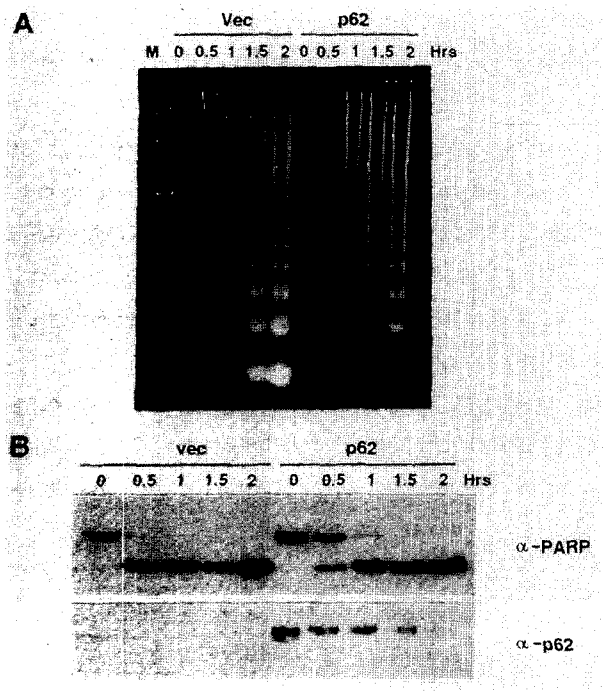

Fig. 3. Down-regulation of apoptosis in p62-expressing T cells.
(A) Inhibition of DNA fragmentation in p62-expressing T cells. Vector transfected cells (vec) or p62-T7 expressing cells (p62) were treated with UV (40J /m2) and incubated for the indicated times shown above the gel, and low molecular weight chromosomal DNA was isolated as described in "Materials and Methods". M indicates the DNA marker.
(B) Prevention of PARP breakdown in p62-expressing T cells. UV- treated control(vec) and p62-T7 Jurkat cells (p62) were lysed, and cell lysates were subjected to Western blot analysis as described in "Materials and Methods". Upper gel indicated as α-PARP showed degradation of the endogenous apoptotic substrate, PARP. Anti-PARP antibody used recognizes both the full-length PARP protein (110 kDa), and fast migrating apoptotic fragment (89 kDa). p62 protein was also shown in lower gel indicated as α-p62. Incubating times after UV-treatment was shown above the gel.

tween 14-3-3 and other signaling molecules are mediated via phosphoserine containing recognition motif (Muslin et al., 1996). It is likely that p62/14-3-3 interaction is also mediated through phosphoserine because p62 is phosphorylated on serine/threonine residue (Joung et al., 1996), and its phophorylation may be induced by several signals including activation. It should be noted that Lck is phosphorylated on tyrosine residues upon T cell activation, however, the interaction between Lck/p62 is not dependent on phosphotyrosine. Therefore, it is possible that p62 may sequester Lck, upon activation, to 14-3-3 proteins, consequently interrupt the activation-induced apoptotic signal. Interestingly, the induction of expression of p62 in macrophage by oxidative stress was reported in mice (Ishii et al, 1996). Taken together, these results suggest that p62 acts as a "keeper" for lymphocytes against stress or activation induced cell death. By doing it, p62 may prevent unnecessary cell death during T-cell activation and development.

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