Immediate early response of the p62 gene encoding a non-proteasomal multiubiquitin chain binding protein

Young Han Lee[a,b], Jesang Ko[a], Insil Joung[1,a], Jung-Hye Kim[b], Jaekyoon Shin[a,*]

[a] *Division of Tumor Virology, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115, USA*
[b] *Department of Biochemistry, College of Medicine, Yeungnam University, Daegu, South Korea*

Received 5 August 1998; received in revised form 6 October 1998

Abstract p62 is a cytoplasmic ubiquitin chain binding protein. Upon a variety of extracellular signals, both transcript and protein levels of p62 were rapidly increased. These signals include phorbol 12-myristate 13-acetate (PMA) and calcium ionomycin for peripheral blood mononuclear cells, serum or PDGF for serum-starved NIH3T3 cells, IL-3 for the G1 arrested pre-B cell line Ba/F3, and PMA for a human promyelocyte line U937. The elevation of p62 transcript level is due to temporal stabilization of mRNA and rapid activation of the p62 gene. Cycloheximide-induced enhanced transcription suggests the immediate early response of the p62 gene. The rapid induction of p62 indicates the presence of a novel ubiquitination-mediated regulatory mechanism during cell proliferation and differentiation.

Key words: p62; Ubiquitin; Immediate early response

1. Introduction

When an external stimulus reaches the cell surface, a number of catalytic reactions relay the signals to the nucleus for coordinate gene activation [1,2]. Upon stimulation, cells rapidly activate the very first set of genes, known as 'primary' or 'immediate early response' genes, which do not require de novo protein synthesis for their activation [3,4]. Thus far, the best known immediate early response genes are transcriptional regulators such as jun, fos, myc, and egr [3,4]. Some immediate early genes also encode chemokines, kinases, phosphatases, and ubiquitin hydrolases [3,5–9]. Immediate early induction of ubiquitin hydrolases indicates that ubiquitination-dependent proteasomal proteolysis is a target for transcriptional regulation during early changes in the cellular environment.

Proteasomal recognition of ubiquitinated substrates is likely initiated by a proteasomal multiubiquitin chain binding subunit Mcb1/S5a [10,11]. Recently, a cytoplasmic 62 kDa protein (p62) has been characterized as another ubiquitin chain binding protein (Ko and Shin, unpublished observation; and [12]). As p62 also interacts with signaling molecules such as $p56^{lck}$ and PKCζ [13,14], p62 likely regulates fates of ubiquitinated proteins in a signal-dependent manner. The present study shows that p62 is an immediate early response gene product for a variety of signals. Thus, by analogy with other immediate early response genes, the p62-mediated regulatory pathway for multiubiquitinated proteins may have an essential function in cell proliferation and differentiation.

2. Materials and methods

2.1. Cell culture

Human peripheral blood mononuclear cells (PBMC) were isolated from leukophoresis packs using Ficoll gradient [15]. Cells were cultured in 10% fetal calf serum (FCS) supplemented DMEM for NIH3T3, and RPMI 1640 for PBMC, Ba/F3, and U937 cells. For Ba/F3 cells, WEHI-3B conditioned medium was also supplemented as a source of IL-3. For cell cycle arrest, NIH3T3 and Ba/F3 cells were incubated either in DMEM containing 0.5% FCS for 48 h or without supplementation of WEHI-3B conditioned medium for 16 h. Cells were stimulated by adding the following reagents into the culture media: 15% FCS for NIH3T3, 200 U/ml recombinant human IL-3 for Ba/F3, 20 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 μM ionomycin for PBMC, and 20 ng/ml PMA for U937.

2.2. Northern and Western analysis

Total RNA preparation and Northern analysis were performed as described [16]. Briefly, total RNA (10 μg) was electrophoresed on 1% agarose gel containing 6% formaldehyde, transferred onto Hybond-N nylon membrane (Amersham), and cross-linked by UV irradiation. The filter was hybridized overnight at 42°C with [α-^{32}P]dCTP-labeled probes: the 750 bp long *Eco*RI-*Bam*HI fragment of human p62 cDNA [13], the 0.9 kb *Nco*I/*Sal*I fragment of the human c-*fos* gene purified from the pc-fos-1 plasmid [17], and the 0.85-kb *Xba*I-*Bam*HI insert from the pRGAPDH-13 plasmids [18]. After washing with 2×SSC and 0.1% SDS at 25°C, the signal was visualized by autoradiography. For quantitative analysis of p62 mRNA stability, cells were either untreated or treated with PMA/ionomycin for 2, 4, or 19 h before the addition of actinomycin D (10 μg/ml). To measure the stability of p62 mRNA in PBMC, total RNA was isolated from the cells at the indicated times after addition of actinomycin D (10 μg/ml), and the p62 transcript level was analyzed by Northern blot. Western blot analysis was performed as described [13] using rabbit polyclonal anti-human p62 antiserum raised against a C-terminal peptide (Ser^{407} to Asp^{423}). The immunogen site is well conserved (100% identical) in murine p62 [14]. In all experiments, immunoblots were subsequently stripped in a buffer containing 62.5 mM Tris, pH 6.7, 100 mM 2-mercaptoethanol and 2% SDS for 30 min at 55°C, and then re-probed with rabbit anti-MAPK antibody (UBI).

2.3. Nuclear run-on assay

Nuclear run-on was performed following published procedures [19]. PBMC were treated with PMA/ionomycin for the indicated times, and then nuclei were isolated from 5×10^6 cells. After in vitro transcription in the presence of [α-^{32}P]UTP, radiolabeled RNA transcripts were purified and used for hybridization. pBluescript plasmids containing full length cDNAs for human p62 and c-fos, or empty plasmid were linearized, denatured, and immobilized on the Hybond-N nylon membrane. The membrane was hybridized for 48 h at 42°C with the radiolabeled transcripts, washed twice in 2×SSC and 0.1% SDS at room temperature and once each in 0.2×SSC and 0.5% SDS at 55°C for 1 h, and then autoradiographed.

*Corresponding author. Fax: (1) (617) 632-2662.
E-mail: shin@mbcrr.harvard.edu

[1]Present address: Department of Biology, Hanseo University, Chungnam, South Korea.

0014-5793/98/$19.00
PII: S0014-5793(98)01323-4

3. Results and discussion

3.1. Immediate early response of the p62 gene in PBMC

Most T cells in PBMC maintain their quiescence until proper antigenic stimulation that leads to clonal expansion. Simultaneous treatment with PMA and ionomycin has been widely used to mimic the antigenic stimulation [19]. Time-dependent changes in message and protein levels of p62 were examined in PBMC after PMA/ionomycin treatment. Upon stimulation, a rapid increase of both p62 transcript and protein was observed within 2 h, and the elevated levels were maintained for up to 19 h (Fig. 1A,B). During this stimulation period, the levels of 28S rRNA and MAP kinase (MAPK) protein were relatively constant. Under the same stimulation condition, cells progress into the S phase only after 24 h post-stimulation (data not shown). Thus, p62 gene activation and the following p62 protein accumulation occur very early in the entry into the cell cycle, possibly during G0 exit.

The transcriptional inhibitor actinomycin D was used to measure half-lives of p62 mRNA in PBMC (Fig. 1C). The half-life of p62 mRNA was about 2.5 h in resting PBMC. Stimulation of PBMC with PMA/ionomycin for 2 h significantly stabilized p62 mRNA ($t_{1/2} = 5$ h). The increased mRNA stability then gradually decreased back to the basal stability within 19 h. However, the increased mRNA stability alone cannot account for the dramatic elevation of p62 transcript level.

Nuclear run-on assay showed that transcriptional activity of the p62 gene is induced within 30 min after PMA/ionomycin treatment (Fig. 1D). Interestingly, the induced transcriptional activity of the p62 gene persisted during the 6 h observation period, while that of the c-*fos* gene, which is also activated within 30 min, returns to basal level within 1 h (Fig. 1D). These results suggest that the observed elevation of p62 transcript level is due to a combination of transient mRNA stabilization and continuous activation of transcription.

A possibility of immediate early response of the p62 gene was then examined. As expected, mRNA level of c-*fos* in PBMC was rapidly induced and the induction was largely

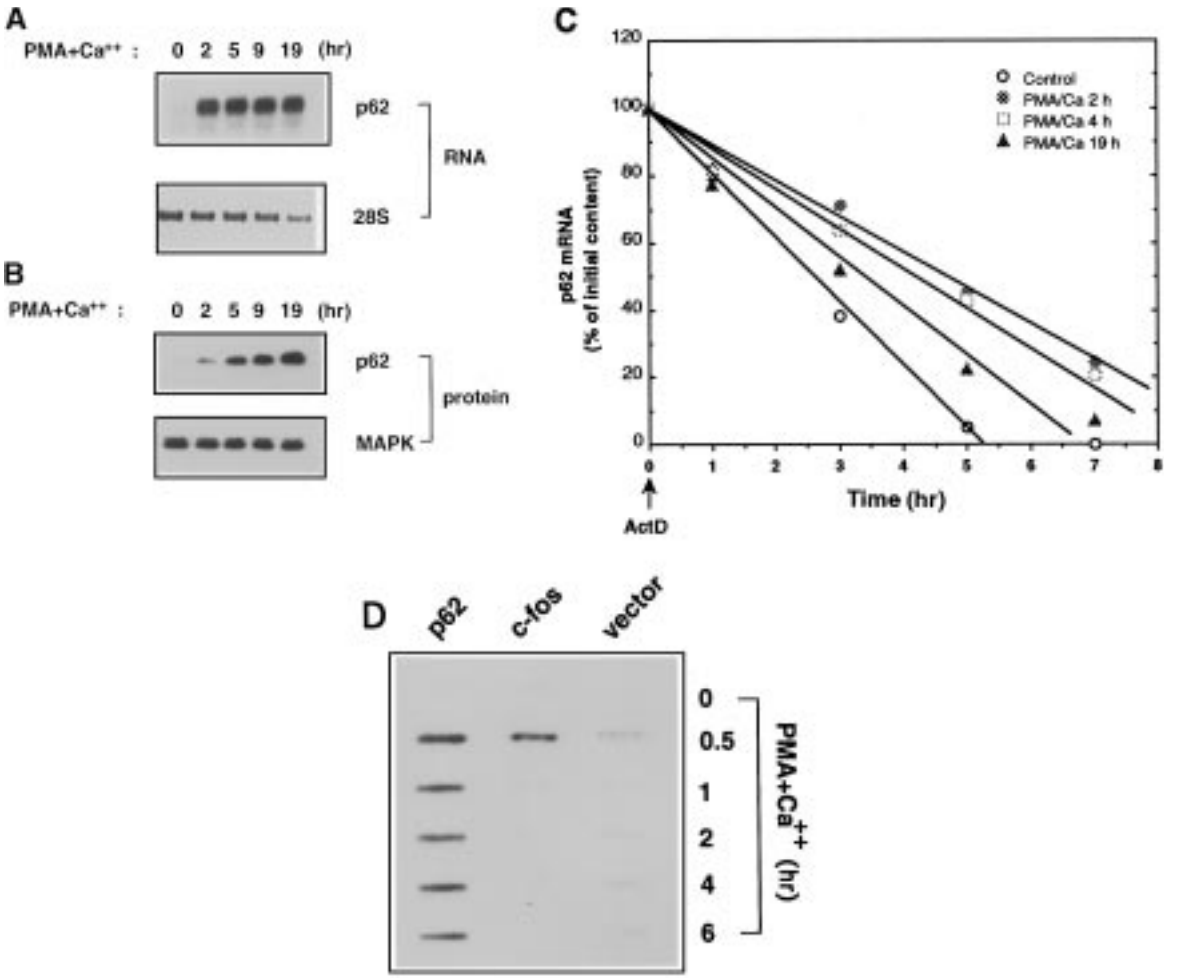

Fig. 1. Effects of PMA/ionomycin on p62 expression in PBMC. A: PBMC was stimulated with PMA/ionomycin for the indicated times, and then 10 μg of cytoplasmic RNA was isolated and analyzed for p62 transcripts (2.1 kb) by Northern blot. Equivalent loading of samples was confirmed by staining the 28S rRNA on the same filter. B: 15 μg of total lysates from the stimulated PBMC were electrophoresed, transferred, and analyzed for p62 and MAPK proteins by immunoblot using anti-p62 and anti-MAPK polyclonal antisera. Typical immunoreactive bands for p62 (62 kDa) and MAPK (42 kDa and 44 kDa) are presented separately. C: PBMC were stimulated with PMA/ionomycin for 0, 2, 4, or 19 h, and then transcription was inhibited by actinomycin D. Total RNA was isolated from the cells at the indicated times after actinomycin D treatment, and analyzed for p62 transcript by Northern blot. The data are expressed as percent of the initial p62 transcript ($t = 0$ for actinomycin D treatment). D: Nuclei were isolated at the indicated times after stimulation. Nuclear run-on assay was performed as described in Section 2 using 10 μg each of denatured pBluescript containing cDNAs inserts for p62 and c-fos, or without insert (vector).

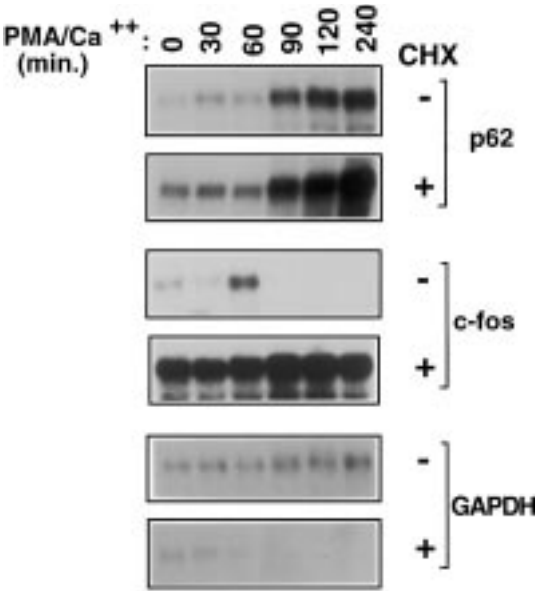

Fig. 2. Effect of cycloheximide on p62 transcription. Cells were pretreated with or without 10 μg/ml cycloheximide (CHX) for 1 h, and then stimulated with PMA/ionomycin for the indicated times. 10 μg each of total RNA was analyzed for p62 transcript by Northern blot. The same filter was stripped and reanalyzed for transcripts of c-fos or glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

enhanced by pretreatment of cells with the translational inhibitor cycloheximide, confirming the immediate early response of the c-*fos* gene (Fig. 2). Similarly, induction of p62 transcription was detected as early as 0.5 h post stimulation and reached maximum level by 1.5 h (Fig. 2). Furthermore, p62 transcription was also enhanced by pretreatment of cells with cycloheximide. Thus, the p62 gene does not require de novo protein synthesis for activation and can be categorized as an immediate early response gene.

3.2. p62 gene activation in other cell types

Changes of p62 level by mitogenic and differentiation signaling have been examined in NIH3T3 fibroblast, IL-3-dependent murine pro-B lymphocytes Ba/F3, and human promyelocytes U937. To this end, NIH3T3 and Ba/F3 cell lines growth arrested by serum and IL-3 starvation were stimulated with 15% serum and 200 U/ml human IL-3 respectively [7]. For monocytic differentiation of U937, cells were treated with 20 ng/ml PMA [20]. Protein levels of p62 in the stimulated NIH3T3 and Ba/F3 cells were markedly elevated within 2 h after stimulation (Fig. 3A,B). A similar elevation of p62 transcript levels in these stimulated cells was also observed (data not shown). Thus, it seems likely that p62 is an early responder to various mitogenic stimuli in divergent cell types.

U937 can be differentiated to monocyte/macrophage upon stimulation with PMA, vitamin D3, or GM-CSF [20,21]. As growth arrest at G1 precedes monocytic differentiation of U937, if p62 function is solely coupled with cell proliferation, p62 level in the differentiating U937 cells would decline. However, when U937 cells were induced to differentiate by either PMA (Fig. 3C) or vitamin D3 (data not shown), both the p62 protein (Fig. 3C) and the transcript (Fig. 3D) levels also rapidly increased and were maintained for a 72 h observation period. Thus, the enhanced function of p62 is not restricted to cell proliferation, but is also associated with physiological changes during cell differentiation.

The genomic structure and promoter region of the p62 gene have been characterized [22]. The 1.8 kb long 5′-flanking region of the p62 gene contains multiple binding sites for transcriptional regulatory factors including SP-1, AP-1, NF-κB, and Ets-1 family. Furthermore, a reporter gene linked to the 5′-flanking region was rapidly activated by serum and PMA in NIH3T3 and U937 cells respectively. Thus, the elevation of p62 levels upon signals for proliferation and differentiation observed in the present study (Figs. 1 and 3) is indeed due to p62 gene activation. Nevertheless, sustained expression of p62 suggests that p62 may function to 'keep' rather than to 'initiate' the newly established cellular environments.

Recently, ubiquitin hydrolases DUB-1 and DUB-2 have been characterized as immediate early response gene products [7,8]. DUB-1 activity is important for the sustained proliferation of IL-3, GM-CSF, or IL-5 responsive cells by removing

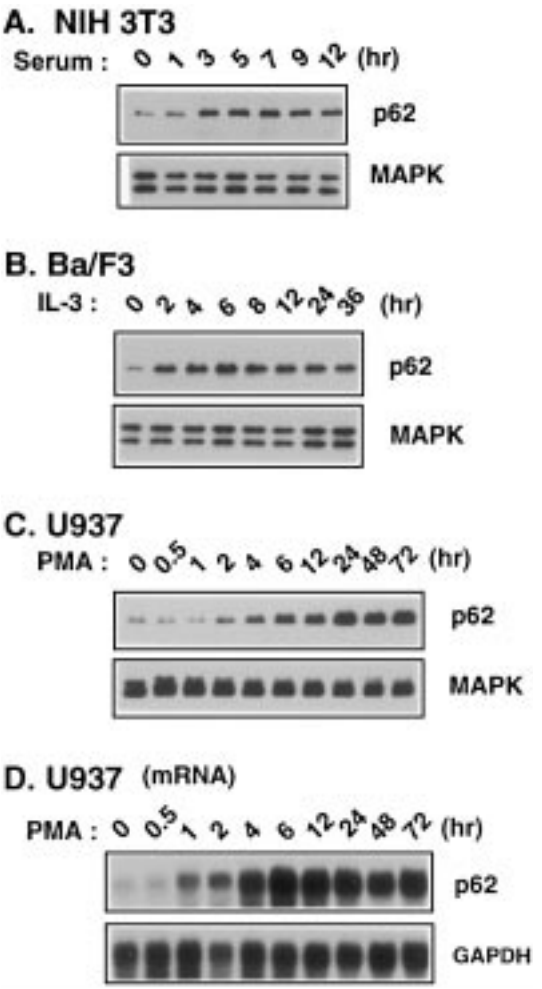

Fig. 3. Induction of p62 under distinct environments. Total lysates were prepared from (A) NIH3T3 cells quiesced in 0.2% serum for 48 h and then stimulated with 15% serum for the indicated times, (B) Ba/F3 cells growth arrested in the IL-3 depleted RPMI medium for 16 h and then stimulated with IL-3 (200 U/ml) for the indicated times, and (C) U937 cells treated with PMA (20 ng/ml) for the indicated times. Lysates (10 μg protein) were analyzed for p62 and MAPK by Western blot. D: 10 μg each of total RNA isolated from U937 cells treated with PMA (20 ng/ml) for the indicated times was probed on Northern blot with a specific cDNA probe for p62 or GAPDH.

ubiquitin chains from target regulatory proteins [7]. Another immediate early gene product, *Aplysia* ubiquitin hydrolase, is essential for long-term facilitation of the synapses through enhancing the proteasomal proteolytic activity [9]. Thus, the regulation of the fate of ubiquitinated proteins appears to be the essential biochemical changes in responding to extracellular stimuli, and is supported by an immediate early transcriptional activation mechanism.

Proteasomal recognition of substrates is initiated by a ubiquitin chain binding protein Mcb1/S5a [10,11]. Interestingly, p62 turns out to be another multiubiquitin chain binding protein (Ko and Shin, unpublished observation). Now we show immediate early activation of the p62 gene upon signals for cell proliferation and differentiation. Thus, p62 likely carries an essential function in cell regulation by modifying the fate of ubiquitinated proteins.

Acknowledgements: We thank Drs. H. Choi and J.L. Strominger and Ms. T. Ogren for their helpful advice and support. This work was supported by National Institute of Health Research Grant GM48961 and American Cancer Society PRG-97-018-01CN.

References

[1] Kieran, M.W. and Zon, L.I. (1996) Curr. Opin. Hematol. 3, 27–34.
[2] King, R.W., Jackson, P.K. and Kirschner, M.W. (1994) Cell 79, 563–571.
[3] Herschman, H. (1991) Annu. Rev. Biochem. 60, 281–319.
[4] McMahon, S.B. and Monroe, J.G. (1992) FASEB J. 6, 2707–2715.
[5] Webster, M.K., Goya, L. and Firestone, G.L. (1993) J. Biol. Chem. 268, 11482–11485.
[6] Beltman, J., McCormick, F. and Cook, S.J. (1996) J. Biol. Chem. 271, 27018–27024.
[7] Zhu, Y., Carroll, M., Papa, F.R., Hochstrasser, M. and D'Andrea, A.D. (1996) Proc. Natl. Acad. Sci. USA 93, 3275–3279.
[8] Zhu, Y., Lambert, K., Corless, C., Copeland, N.G., Gilbert, D.J., Jenkins, N.A. and D'Andrea, A.D. (1997) J. Biol. Chem. 272, 51–57.
[9] Hedge, A.N., Inokuchi, K., Pei, W., Casadio, A., Ghirardi, M., Chain, D.G., Martin, K.C., Kandel, E.R. and Schwartz, J.H. (1997) Cell 89, 115–126.
[10] Deveraux, Q., Ustrell, V., Pickart, C. and Rechsteiner, M. (1994) J. Biol. Chem. 269, 7059–7061.
[11] Van Nocker, S., Deveraux, Q., Rechsteiner, M. and Vierstra, R.D. (1996) Proc. Natl. Acad. Sci. USA 93, 856–860.
[12] Vadlamudi, R.K., Joung, I., Strominger, J.L. and Shin, J. (1996) J. Biol. Chem. 271, 20235–20237.
[13] Joung, I., Strominger, J.L. and Shin, J. (1996) Proc. Natl. Acad. Sci. USA 93, 5991–5995.
[14] Pulse, A., Schmidt, S., Grawe, F. and Stabel, S. (1997) Proc. Natl. Acad. Sci. USA 94, 6191–6196.
[15] Cheung, R.K., Grinstein, S. and Gelfant, E.W. (1982) J. Clin. Invest. 70, 632–638.
[16] Sambrook, J., Frisch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[17] Curran, T., MacConnell, W., vanStraaten, F. and Verma, I. (1983) Mol. Cell Biol. 3, 914–921.
[18] Fort, P., Marty, L., Piechaczyk, M., El Sabrouty, S., Dani, C., Jeanteur, P. and Blanchard, J.M. (1985) Nucleic Acids Res. 13, 1431–1442.
[19] Isakov, N. and Altman, A. (1985) J. Immunol. 135, 3674–3680.
[20] Harris, P. and Ralph, P. (1985) J. Leukocyte Biol. 37, 407–422.
[21] Liu, M., Lee, M.H., Cohen, M., Bommakanti, M. and Freedman, L.P. (1996) Genes Dev. 10, 142–153.
[22] Vadlamudi, R.K. and Shin, J. (1998) FEBS Lett. 435, 138–142.