The p56\textsuperscript{lck} -interacting Protein p62 Stimulates Transcription via the SV40 Enhancer*

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The p56\textsuperscript{lck} is a recently identified ubiquitin-binding, cytosolic phosphoprotein that interacts with several signal transduction molecules including the tyrosine kinase p56\textsuperscript{lck} and the protein kinase C\textsubscript{ζ} (1). p62 is therefore suggested to serve an important role in signal transduction in the cell, although the physiological function of p62 remains undefined. Here we demonstrate by transient transfection assays that p62 stimulates the transcription of reporter genes linked to the simian virus 40 (SV40) enhancer. A putative p62-responsive element was localized to the B domain of the distal 72-base pair repeat of the SV40 enhancer. p62 was unable to bind this element in vitro, nor was it able to activate transcription when directly tethered to a promoter, suggesting that p62 stimulates transcription via an indirect mechanism. Stimulation of transcription mediated by p62 was dependent on its amino-terminal region, which is also necessary for interaction with cell surface signaling molecules. These findings indicate that p62 may link extracellular signals directly to transcriptional responses, and identify the SV40 enhancer as a downstream target for signal transduction pathways in which p62 participates.

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Epitope Tagging of p62—The stop codon of p62 was changed to a codon for glutamine followed by a BglII site by site-directed mutagenesis using the oligonucleotide 5'-GCATCCCCCGCTGGATGeacT- TGGCCACCTCTCTG and pORCA/SG5 single-stranded DNA. A fragment of p62 was amplified from this mutated plasmid with two of the primer pairs 5'-ATTCTCGAGCTGGGGAGCCTGG (forward) and 5'-ATTAGATCTCAAACCCCGCCCAGCG (reverse) to generate pGC/TK-500. 

Northern Blot Analysis—Total RNA was isolated from transfected COS-1 cells by guanidine isothiocyanate extraction using a commercial kit (Trizol, Molecular Research Center, Cincinnati, OH). RNA was quantified by spectrophotometry. Northern blot analysis was carried out according to established methods (18). 10 µg of RNA was loaded per lane. Specific mRNA levels were quantified by densitometry with an Ultrascan XL laser densitometer (LKB Instruments, Bromma, Sweden).

Antibodies and Immunoblot Analysis—Antibodies to full-length mouse PPARα and human RXRs were raised in rabbits by injection of affinity purified maltose-binding protein fusions expressed in Esche- richia coli. The 12C5 monoclonal antibody, which recognizes the 9-amino acid HA epitope, was purchased from the Berkeley Antibody Co. (Richmond, CA). Protein extracts from transfected cells were prepared in 50 mM Tris-HCl (pH 8.0), 0.1% (w/v) Nonidet P-40. Protein concentration was determined with a commercially available kit (Bio-Rad, Mississauga, Ontario, Canada) using bovine serum albumin as a standard. 50 µg of each protein extract was subjected to electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide gel, as described (18). Proteins were transferred to nitrocellulose for immunoblot analysis. Anti-agen-antibody complexes were detected by enhanced chemiluminescence (Amersham Life Sciences, Oakville, Ontario, Canada).

Electrophoretic Mobility Shift Assays (EMSA)—Nuclear extracts were prepared from COS-1 cells transfected with pORCA/SG5 or with control vector, and EMSA was performed as described previously (16). All reactions were normalized for protein content. The entire SV40 enhancer plus 21-bp repeats was amplified by PCR using 5'-ATTGATGACCCGAAATGTGTCAGTTAGC (forward) and 5'-ATTGATGACCCGAAATGTGTCAGTTAGC (reverse primer). The resulting 200-bp product was digested with BamHI and end-labeled with [α-32P]dATP and the Klenow fragment of DNA polymerase I. Binding reactions were analyzed by electrophoresis at 4°C on pre-run 3.5% polyacrylamide gels (30:1, acrylamide/bis-acrylamide weight ratio) with 22 mM Tris base, 22 mM boric acid, 1 mM EDTA acid as running buffer. Reconstituted p62 was produced in E. coli as a fusion to glutathione S-transferase (a kind gift of Christopher Winrow, Uni- versity of Alberta). The fusion protein was purified from bacterial lysates on glutathione-Sepharose 4B resin (Pharmacia, Baie d'Urfe, Quebec, Canada), and the glutathione S-transferase moiety was cleaved with bovine thrombin according to the manufacturer's instructions.

RESULTS

p62 Activates the Transcription of Genes Linked to the SV40 Enhancer/Early Promoter—We originally cloned p62/ORCA using the yeast two-hybrid system with the nuclear hormone receptor COUP-TFII as bait (3). In our investigations of the function of p62, we noted that the activity of reporter genes linked to the SV40 enhancer/early promoter was stimulated in transient transfection assays in the presence of an expression vector for p62. Our results suggested that p62 might be involved in transcription activation.

To explore this initial observation further, we transfected BSC40 cells with the luciferase reporter plasmid pSV2lac, which contains the SV40 enhancer/early promoter, in the absence or presence of the p62 expression plasmid, pORCA/SG5. As shown in Fig. 1, co-transfection of pORCA/SG5 resulted in a 6-fold stimulation of luciferase activity as compared with control transfections carried out with empty expression vector. This increase in activity was specific for the SV40 enhancer/ early promoter, as co-transfection of pORCA/SG5 with a luci- ferase reporter construct containing the cytomegalovirus en- hancer/promoter (pCMVL) or the minimal SV40 promoter (pGL2-promoter) did not result in stimulation of luciferase activity (Fig. 1). Similarly, the activity of a reporter plasmid under control of the Rous sarcoma virus promoter was unaf-
SV40 Transcriptional Activation by p62

p62 activates the transcription of an SV40 enhancer-linked reporter gene. The indicated luciferase reporter plasmids were co-transfected into BSC40 cells in the presence, or absence, of a p62 expression vector, as described under “Experimental Procedures.” Values report luciferase activity in light units, and are the averages of at least two independent transfections carried out in duplicate and normalized to the value obtained with the pGL2-promoter vector in the absence of the p62 expression plasmid, which was taken as 1. Values from individual transfections did not vary by more than 15%.

To determine if p62-mediated stimulation of reporter gene activity was due to an overall increase in transcription, we carried out Northern blot and immunoblot analyses of transfected cells. For this purpose, we used an expression plasmid containing the SV40 enhancer/early promoter (pSG5) linked to cDNAs for different nuclear hormone receptors. The various plasmids were co-transfected into COS-1 cells in the presence, or absence, of pORCA-HA/SG5, which encodes p62 tagged at its carboxyl terminus with the HA epitope to permit monitoring of the expression of p62 in transfected cells. Cells were harvested 48 h post-transfection and divided into two aliquots. Total RNA was isolated from one aliquot and subjected to Northern blot analysis with appropriate probes. Protein extracts were prepared from the other aliquot and subjected to immunoblot analysis with specific antibodies. As shown in Fig. 2, mRNA levels for the nuclear hormone receptors RXRα and PPARα were increased in the presence of p62-HA (2.9- and 1.8-fold, respectively), as compared with control transfections carried out with empty vector (Fig. 2, top panels, compare lane 2 to lane 3 for RXRα and lane 5 to lane 6 for PPARα). The increase in mRNA correlated with an increase in the levels of the corresponding RXRα and PPARα proteins (7.3- and 2.4-fold, respectively) (Fig. 2, bottom panels, compare lane 2 to lane 3 for RXRα and lane 5 to lane 6 for PPARα). The presence of full-length p62-HA in cells transfected with pORCA-HA/SG5 was confirmed by immunoblotting with anti-HA antibodies (Fig. 2, bottom panels, compare lane 7 to lane 8). The above findings indicate that p62-mediated stimulation of reporter gene activity correlates with increased levels of transcription.

The B Site of the SV40 Enhancer Is Required for Responsiveness to p62—The SV40 early promoter consists of three copies of a 21-bp repeat, followed by a TATA box (Fig. 3) (14, 19–23). Each repeat has two copies of a GC-hexanucleotide motif that has been shown to bind the transcription factor Sp1. The prototype SV40 enhancer, derived from SV40 strain 776, contains two tandem copies of a 72-bp repeat. The “minimal” enhancer has been localized to the distal 72-bp repeat and 5′-flanking region (14).

To characterize the putative p62-responsive cis-acting elements of the SV40 enhancer/early promoter, we made several constructs based on the plasmid pGL2-promoter, which contains a luciferase reporter gene linked to the minimal SV40 early promoter (Fig. 4). Co-transfection of this basic plasmid in the presence of p62 expression plasmid resulted in little change (less than 2-fold) in luciferase activity. In contrast, appending the natural SV40 enhancer (two copies of the 72-bp repeat) in the forward (pENH.FOR/GL2) or reverse (pENH.REV/GL2) orientation resulted in a 7–10-fold p62-dependent increase in luciferase activity. A reporter plasmid that contained only the distal 72-bp repeat and 5′-flanking region (pENH.MIN/GL2) also showed increased luciferase activity (4-fold) in the presence of p62, although the absolute level of induction was reduced vis-à-vis the natural SV40 enhancer.

To examine whether the SV40 early promoter, and in particular the GC-rich 21-bp repeats, participate in p62-mediated stimulation, we used a luciferase reporter construct (TKluc) linked to the minimal TK promoter. Co-transfection of either TKluc (contains the TK TATA box) or pGC/TKluc, (contains the 3 GC-rich repeats of the SV40 early promoter placed upstream of the TK TATA box), with p62 expression plasmid did not result in a significant increase in luciferase activity over background levels observed with the reporter genes alone (Fig. 4). However, linking the natural SV40 enhancer directly to TKluc (pENH/TKluc) resulted in a greater than 5-fold p62-dependent increase in luciferase activity. Our results indicate that the distal 72-bp repeat of the SV40 enhancer is sufficient to mediate p62 responsiveness and that induction is independent of the GC-rich 21-bp repeats or the SV40 TATA element.

Full function of the SV40 enhancer has been shown to depend on the cooperation of multiple sequence motifs (GT-I, GT-IIC, SphI, Sph-II, TC-II, octamer, and P), which bind a variety of ubiquitous and cell-specific factors. (20, 21, 23). The minimal SV40 enhancer encompasses ~100 bp, including the
distal 72-bp repeat and its 5' flanking region (14), and is composed of at least two distinct subdomains, A and B. The A and B domains possess very little enhancing activity individually but can cooperate to synergistically activate transcription. Moreover, activity can be potentiated by multimerization of either domain. To determine the contribution of the A and B domains to the transactivating activity of p62 on the SV40 enhancer, we made luciferase reporter gene constructs containing single or multiple copies of either the A or B domain linked to the minimal SV40 early promoter. Transcriptional activation by p62 via a single B domain (pB(x1)/GL2) was modest (3-fold) but was increased (6-fold) when the B domain was duplicated (Fig. 5). In contrast, the activity of a reporter gene containing a single copy (pA(x1)/GL2) or 3 copies (pA(x3)/GL2) of the A domain was unaffected or only marginally stimulated, respectively, by coexpressed p62. Therefore, the B domain of the distal 72-bp repeat is necessary and sufficient to confer responsiveness to p62.

Purified p62 Does Not Bind to the SV40 Enhancer and Does Not Affect Protein/DNA Interactions on this Element—To begin to explore the mechanisms by which p62 might potentiate transcription via the SV40 enhancer, we performed EMSA using a labeled DNA fragment containing the natural SV40 enhancer and 21-bp repeats to determine if p62 binds to this element in vitro. The DNA probe was incubated with extracts prepared from COS-1 cells transfected with pORCA/SG5 or the corresponding empty vector, pSG5. The pattern and/or mobilities of the protein-DNA complexes formed on this element with COS-1 cell extracts did not change in the presence of coexpressed p62 (Fig. 6A, compare lane b to lane c). Purified, bacterially expressed, p62 did not bind to the DNA probe under our assay conditions (Fig. 6B, compare lane a to lane c). Moreover, protein-DNA complexes formed on this element were not altered quantitatively or qualitatively when purified p62 was incubated with increasing amounts of untransfected COS-1 cell extract (Fig. 6B, compare lanes b-d to lanes f-h, respectively). Therefore, within the limits of these assay systems, p62 does not appear to be capable of binding directly or indirectly to the SV40 enhancer, or of altering the interaction of cellular factors with this element.
p62 Does Not Have Activating Potential When Directly Tethered to a Promoter—

To determine if p62 has intrinsic transcription activating potential, we made the plasmid pSG-GAL4-p62 linking the cDNA for p62 to the DNA-binding domain of the yeast transactivator GAL4 and carried out transient transfection assays together with a CAT reporter gene having 5 upstream copies of the GAL4 DNA-binding site (p(GAL4)5E1bCAT). As shown in Fig. 7, the activity of this reporter gene was strongly stimulated by the potent transactivator GAL4-VP16, as expected. However, when directly bound to DNA, GAL4-p62 was unable to stimulate transcription over background levels of the reporter gene alone. Therefore, p62 does not possess an intrinsic activation capacity, and this finding, together with results from the DNA binding assays, suggests that p62 stimulates transcription through an indirect means.

Part of the SH2-binding Domain of p62 Is Essential for Transactivation of the SV40 Enhancer—

p62 contains a small region near its amino terminus (residues 66–82) that shares homology to cdc24 and harbors a putative protein binding motif; a cysteine-rich zinc finger-like motif (residues 128–163) that appears to be involved in homodimerization; a Ser-rich/PEST domain, which includes several potential kinase phosphorylation sites, and an ubiquitin-binding region near its carboxyl terminus. The amino-terminal domain has been shown to be required for interaction with p56\(^{\text{lck}}\), PKC-\(\zeta\), and COUP-TFII.

To identify regions of p62 that are important for the observed transcriptional stimulation of the SV40 enhancer, we generated a series of mutant derivatives of p62 and tested these for activity in transient transfection experiments (Fig. 8). A derivative of p62 truncated at amino acid 257 retained the ability to stimulate transcription of pSV2\(^{\text{luc}}\), albeit with lower efficiency (3-versus 7-fold, respectively). Therefore, the ubiquitin-binding region and the Ser-rich/PEST domain, which includes several potential kinase phosphorylation sites, are not essential for p62-mediated stimulation of transcription of the SV40 enhancer. Similarly, a derivative missing the putative zinc finger/protein interaction region (residues 128–163) retained activity. However, derivatives lacking the first 187 or first 85 residues were inactive.
Fig. 8. The amino-terminal, SH2-binding domain of p62 is essential for transactivation of the SV40 enhancer. The luciferase reporter plasmid pSV2luc was transfected into BSC40 cells in the presence, or absence, of expression vectors for wild-type p62 or mutant forms of p62 lacking the indicated amino acid residues. Values shown represent the fold induction of luciferase activity by a particular p62 expression construct relative to the luciferase activity obtained by cotransfection with the empty expression vector, pSG5, and are the averages of at least 2 independent transfections done in duplicate. Values from independent transfections did not vary by more than 15%. A schematic of p62, highlighting some of its functional domains, is shown at the top. Numbering is according to Ref. 1.

\( \Delta 1-85 \) is missing the cdc24 homology region (residues 66–82). To further demarcate this region, derivatives of p62 were constructed missing residues 1–29 or residues 29–50. As shown in Fig. 8, \( \Delta 1-29 \) activated transcription of pSV2luc and did so as efficiently as the wild-type protein. In contrast, \( \Delta 29-50 \) was inactive. Thus, residues spanning amino acids 29–50 are essential for activity. The cdc24 homology region may also be important; however, in the absence of amino-terminal residues, this region itself is insufficient for activity. Previous work has shown that first 50 residues of p62 are necessary for interaction with the p65\(^{N} \) SH2 domain (12). Thus, our results indicate that at least part of the SH2-binding domain of p62 is also essential for transactivation of the SV40 enhancer.

**DISCUSSION**

p62 has been shown to bind to a variety of cellular factors including signaling molecules such as p66\(^{N^{C}} \), PKC-\( \zeta \), and cytokine receptors; the transcription factor COUP-TFII; and ubiquitin. These findings, coupled with recent observations that p62 is widely expressed in most cell types and is rapidly induced by cell proliferation and differentiation signals, suggest that p62 is a multifunctional protein that serves a general role in signal transduction in the cell, perhaps linked to the regulation of ubiquitin-mediated protein degradation. However, the cellular action of p62 remains unclear, since a direct role for p62 in signal transduction events and/or regulation of protein degradation has not been demonstrated. The findings reported here, demonstrating that p62 also stimulates transcription via the SV40 enhancer, add a further functional dimension to this protein and suggest that p62 provides a link between cell surface signaling and specific gene transcription.

Transcriptional induction mediated by p62 appears to be specific for the SV40 enhancer, since the activity of other promoter/regulatory regions were unaffected by the presence of p62. The natural SV40 enhancer (two copies of the 72-bp repeat) was the most robust at conferring responsiveness to p62 and could do so when appended to the SV40 early promoter or to a heterologous promoter. By mutational analysis, we localized a putative p62-responsive element to the B domain of the 72-bp repeat of the SV40 enhancer. A single copy of the B domain was sufficient to mediate responsiveness to p62; however, the level of induction was significantly increased when the B domain was duplicated, as is the case with the natural enhancer. These results suggest that cooperative interactions of multiple factors participate in mediating full p62 responsiveness.

The mechanism by which p62 potentiates SV40 enhancer-mediated transcription is unknown. p62 does not bind to the SV40 enhancer either on its own or in conjunction with cellular factors, nor does it appear to alter the protein/DNA interactions of cellular factors that interact with this element. Moreover, p62 does not activate transcription when tethered directly to a promoter. Therefore, p62 probably functions indirectly in modulating transcription, perhaps as a transcription intermediary cofactor. Interestingly, p62 shares a small region of homology with the transcriptional coactivator CBP (residues 136–154 of p62 and residues 1715–1722 of CBP), suggesting possible similarities in their mechanisms of action. This region is part of a domain in CBP (residues 1680–1812) that has been shown to bind TFIIB (24). However, this region of CBP homology is not essential for transactivation of the SV40 enhancer by p62 (Fig. 3). Moreover, there is no evidence of p62 being found in the nucleus, as one may expect if it played a direct role in transcription activation. It remains possible that the subcellular distribution of p62 may be altered in the presence of interacting proteins. Indeed, the subcellular localization of ZIP, the rat homolog of p62, is altered in the presence of overexpressed PKC-\( \zeta \) (4).

An alternative possibility is that p62 modulates the function of cellular transcription factors that target the SV40 enhancer. p62 has been shown to have a tightly associated, or intrinsic, Ser/Thr protein kinase activity (1, 2). Therefore, p62 may activate one or more SV40 enhancer-binding transcription factors directly or indirectly by phosphorylation. Since part of the SH2- and PKC-\( \zeta \)-binding domain of p62 is also required for transactivation, p62 may link cell surface signaling pathways to transcription factor phosphorylation. The SV40 enhancer contains multiple binding sites for a number of ubiquitous and cell-specific transcription factors, including transcription factor enhancer factor 1 (23), transcription enhancer factor 2 (GT-IC) (25), AP1 (26), octamer binding transcription factors (27), TCIIA/NF-\( \kappa \)-B and TC-II/B/KBFB1 (28, 29). There is evidence that AP1, TCIIA/NF-\( \kappa \)-B, and TCII/B/KBFB1 may mediate response of the SV40 enhancer to phorbol esters (26, 38–30), and several other transcription factors that bind to this element are subject to modulation by extracellular signals. What role these factors play, if any, in p62-mediated transcriptional activation is unknown, and awaits definition through future mutational, protein binding, and transfection studies. It should be noted that many of the transcription factors that interact with the SV40 enhancer are not specific to this element and play broad and important roles in normal cellular gene regulation (21, 31, 32). Therefore, it is likely that p62 has a role as a more general regulator of gene transcription.

p62 could also act to alter the availability, stability, and/or subcellular distribution of transcription factors. Such actions would be consistent with p62 being a nonproteosomal, ubiquitin-binding protein that may be involved in the regulation of ubiquitin-mediated protein degradation (10). It is possible, for instance, that p62 may target transcriptional repressors that bind to the SV40 enhancer for degradation. There is a growing number of examples of transcription factors whose activity is regulated by ubiquitin-dependent degradation (33). However, such a scenario appears an unlikely explanation for our find-
ings, since the region of p62 that is required for ubiquitin binding is in fact dispensable for its transactivating ability.

In summary, we have demonstrated that p62, a protein known to interact with distinct components of the cell surface signal transduction pathways, is able to activate transcription. We have identified a subdomain of the SV40 enhancer as one of its downstream targets. Our observation that a region of p62 that is necessary for its interaction with cellular signaling molecules is also required for its ability to stimulate transcription, implicates p62 in linking cell surface signal transduction pathways to transcriptional responses.

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