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Interaction of the Transcription Factor Sp1 With the Nuclear Pore Protein p62 Requires the C-Terminal Domain of p62

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Abstract The transcription factor Sp1 plays an important role in the expression of many cellular genes. In studies of proteins that associate with Sp1, a 62-kDa glycoprotein was found in immunoprecipitates of Sp1. This protein was detected in these immunoprecipitates by the monoclonal antibody, RL2, which was originally raised against nuclear pore proteins but was subsequently found to recognize an epitope that contains O-linked N-acetylglucosamine (O-GlcNAc). The association of this protein with Sp1 could be blocked by SDS denaturation of the protein complex. Western blot analysis of the Sp1 immunoprecipitate using antibodies to p62 nucleoporin indicated that this nuclear pore protein associates with Sp1. Furthermore, immunoprecipitation of p62 nucleoporin resulted in the coprecipitation of Sp1. Recombinant p62, expressed as a GST-fusion protein using a vaccinia virus system, also interacted with both recombinant and native Sp1. This interaction between p62 and Sp1 required the C-terminus of p62 and the C-terminus was able to bind Sp1, albeit less efficiently than native p62. A mammalian two-hybrid interaction assay was devised in which p62 was fused to the Gal4 DNA-binding domain. This system also indicated that p62, through its C-terminus, interacts with Sp1 in the living cell. We propose that this interaction of a nuclear pore protein with Sp1 may reflect the nuclear organization required to bring transcribable DNA in contact with the transcription factors. J. Cell. Biochem. 68:50–61, 1998.

Key words: Sp1; p62; interaction assay

The transcription factor Sp1 is ubiquitously expressed and plays a major role in the expression of most cellular genes, including both constitutive housekeeping and inducible genes [Courey and Tjian, 1993]. Sp1 binds to a GC-rich promoter element and stimulates transcription from the promoters containing this consensus element [Kadonaga et al., 1986]. Sp1, like other transcription factors, has a modular structure with domains that confer DNA binding and the protein–protein interactions involved in multimerization and transcriptional activation [Courey and Tjian, 1988; Pascal and Tjian, 1991]. It is well documented that Sp1 forms heterodimeric complexes such as with the TATA-binding protein-associated factor, TAF110, which functions as a coactivator in Sp1-dependent transcription [Hoey et al., 1993]. Sp1 is modified by both phosphorylation and O-glycosylaton; however, the role of these posttranslational modifications in Sp1-dependent transcription is unclear [Jackson et al., 1990; Jackson and Tjian, 1988].

In studies of the role of the O-glycosylation of Sp1 [Jackson and Tjian, 1989] we made use of the monoclonal antibody, RL2 [Snow et al., 1987]. This antibody was originally raised against nuclear pore proteins [Snow et al., 1987] but was subsequently found to recognize an epitope containing the monosaccharide, N-acetylglucosamine (O-GlcNAc) when O-linked to glycoproteins [Roos et al., 1996; Sayeski and Kudlow, 1996]. In immunoprecipitates of Sp1, we consistently noted a 62-kDa protein that co-immunoprecipitated with the Sp1. This protein could be recognized, like Sp1, by RL2 on Western blots, suggesting that it too was modified by O-GlcNAc. The size of this coprecipitat-

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ing protein, the fact that it might be modified by O-GlcNAc, and its presence in nuclear extracts were consistent with the possibility that this protein might be p62 nucleoporin, an abundant nuclear pore protein. This paper shows that this 62-kDa glycoprotein is indeed nucleoporin and that the interaction between Sp1 and nucleoporin can occur in vitro and in vivo.

Nucleoporin p62 was first recognized [Davis and Blobel, 1986] as one of a family of GlcNAc-containing proteins [Snow et al., 1987] in the nuclear pore complex (NPC). The cDNA encoding this abundant member of the NPC was cloned [D'Onofrio et al., 1988; Starr et al., 1990]. The sequence revealed that p62 nucleoporin consists of three distinctive domains. The N-terminal domain of p62 contains several repeats of the sequence XFXFG, a motif conserved in a class of yeast and mammalian nuclear pore proteins [Davis and Fink, 1990; Starr et al., 1990]. The central region of p62 is highly serine- and threonine-rich and contains the major site(s) of O-GlcNAc modification. The C-terminus contains heptad hydrophobic repeats that are characteristic of the coiled-coil regions of filament-forming proteins, such as myosin, tropomyosin, and keratin [Starr et al., 1990]. Here, we report that it is this latter region of p62 that is necessary and partially sufficient for the interaction with the transcription factor, Sp1. This association of Sp1 with a nuclear pore protein may reflect the interactions between transcription factors and the nuclear pore that occur either during translocation of these factors from the cytoplasm to the nucleus or during DNA transcription, at which time there is a requirement for the colocalization of transcriptionally active DNA with the cognate transcription factors.

MATERIALS AND METHODS

Cell Culture

BSC40 cells and MDA468 cells were grown as monolayers in Dulbecco modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (Gibco/BRL, Grand Island, NY), 100 µg/ml penicillin (Sigma Chemical Co., St. Louis, MO) and 50 µg/ml gentamicin (Sigma). Cells were grown at 37°C in a humidified incubator with 7.5% CO_2.

Transfection and Assay for Luciferase

In this study, 7×10^6 NRK cells were used for each transfection. Cells were trypsinized, washed two times with cold phosphate-buffered saline (PBS), resuspended in DMEM + 10% serum; 10 µg of reporter luciferase plasmid and 5 µg of expression vector DNA were added. Cells were electroporated at 250 V and 500 µF in a Gene Pulser (BioRad, Richmond, CA) and plated at a density of 1×10^6 cells per well using 6-well plates (Fisher Scientific, Atlanta, GA). Cells were harvested and assayed for luciferase activity at 24-h post-transfection. Also, a CMV promoter/β-galactosidase reporter was used as a control for transfection efficiency. Luciferase activity was measured in cell extracts in the presence of ATP and luciferin as previously described [Raja et al., 1991] using a Monolight 2010 luminometer with light integration over a 10-s period. Light output was expressed as relative light unit (RLU). Background activity was less than 4% of all readings. β-Galactosidase activity was determined as described by Sambrook et al. [1989]. All transfections were done in triplicate.

Vaccinia Virus Expression of Sp1 and GST-Fusion Proteins

The full-length human Sp1 cDNA (kindly provided by Dr. James Kadonaga) was cloned in appropriate frame into the pTM3 vector and recombinant vaccinia virus was generated using selection methods previously described [Moss, 1991; Moss and Earl, 1995]. After co-infection of BSC-40 cells with the Sp1 gene-containing recombinant virus and vTF7-3 virus, the expressed Sp1 protein was purified as described using sequential wheat germ agglutinin [Jackson and Tjian, 1989] and DNA affinity chromatography [Briggs et al., 1986; Kadonaga and Tjian, 1986]. The cDNA encoding the full-length N-terminal (amino acids 1–298) and C-terminal (amino acids 298–525) segments of human nucleoporin p62 were cloned downstream of the cDNA encoding glutathione-S-transferase (GST). The GST-p62 cassettes were then cloned into the pTM3 vector and recombinant vaccinia viruses were generated as above. Virus co-infected cells were harvested 24 hours post infection, washed with PBS and broken by two cycles of freezing and thawing. The fusion proteins in the cell lysate were affinity purified on glutathione–Sepharose beads (Pharmacia, Piscataway, NJ) as described previously [Smith and Johnson, 1988]. The concentration of the purified proteins was measured using the Bio-

Rad D_c Protein Assay (Richmond, CA) with bovine serum albumin (BSA) as a standard.

Antibodies

The polyclonal antiserum to Sp1 (3517) was raised against the C-terminal portion of Sp1 that had been expressed in *Escherichia coli*, as previously described [Shin et al., 1992]. The Sp1 specific polyclonal antibody (4627) was raised in a rabbit by injecting full-length Sp1 expressed and purified from recombinant vaccinia virus-infected HeLa-S cells. The full-length p62 nuclear pore protein expressed in *E. coli* from the pGEMp62 plasmid (generous gift from Dr. G. Hart, University of Alabama, Birmingham, AL) was used as an antigen to raise p62 specific polyclonal antibodies (688, 689, 690). The p62 was found in inclusion bodies and partially purified according to method described by Sambrook et al. [1989]. This partially purified protein was subject to 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane. After visualizing the protein on the membrane using 0.2% Ponceau-S (Fisher Scientific, Fairlawn, NJ), the portion of the membrane containing the p62 protein was excised. The membrane was completely dried by lyophilization, solubilized in dimethylsulfoxide (DMSO) (Eastman Kodak, Rochester, NY), then injected into rabbits every 2 weeks (500 µg of protein for each injection). Sera obtained 8 weeks after the initial injection were screened by immunoblotting and immunoprecipitation. The mouse monoclonal antibody, 414, directed against the nuclear pore complex but recognizing p62 nucleoporin [Laura and Blobel, 1986; Davis and Blobel, 1987] was purchased from Babco (Berkeley Antibody Co., Richmond, CA). Monoclonal antibody, RL2, specific for GlcNAc containing proteins, was a gift from Dr. L. Gerace (Scripps Clinic, La Jolla, CA).

Immunoprecipitation and Immunoblot Analysis

Approximately 80% confluent MDA468 cells were scraped, pelleted, and washed twice with cold PBS. The cell pellets were resuspended at 4°C in a buffer containing 20 mM Hepes (pH 7.9), 500 mM NaCl, 20% glycerol, 1 mM dithiothreitol (DTT), and 1 mM phenylmethane sulfonyl fluoride (PMSF). Cells were disrupted through two cycles of freezing and thawing. Cellular debris was pelleted for 30 min at 135,000*g* at 4°C in a TL100.3 rotor (Beckman, Palo Alto, CA). The resulting high salt extracts were dialyzed against binding buffer (20 mM Hepes–KOH at pH 7.9, 0.25 M KCl, 2 mM $MgCl_2$, 10 µM $ZnSO_4$, 1 mM DTT, 10% glycerol, 0.5% Nonidet P-40, and 1 mM PMSF). The protein concentration was determined using the BioRad D_c Protein Assay (BioRad Laboratories, Hercules, CA). For each immunoprecipitation reaction, cell extract containing approximately 500 µg of protein was incubated with 5 µg of affinity-purified anti-Sp1 antibodies or anti-p62 antibodies in binding buffer at 4°C for 2 h. Protein A–Sepharose 4B (Pharmacia) was then added, and the mixture was incubated at 4°C on a rotating wheel for 2–4 h. The antibody–protein A complexes were pelleted by centrifugation (2,000 rpm) and washed four times with binding buffer. The pellets were either resuspended in SDS–PAGE sample buffer and loaded directly onto polyacrylamide gels or were subjected to reimmunoprecipitation after elution from beads. For reimmunoprecipitation, the immune complexes on the protein A beads were eluted from beads with 1% SDS in binding buffer. The SDS was diluted 10-fold with binding buffer and immunoprecipitation with either anti-Sp1 or antinuclear pore (414) antibodies was performed. The immunoblots were developed using a 1:1,000 dilution of affinity-purified antibodies and the signals were detected using the Enhanced Chemiluminescence System (Amersham, Arlington Heights, IL).

In Vitro Binding Assay

The GST-p62, GST-p62N, and GST-p62C fusion proteins and GST were expressed using the vaccinia virus expression system in BSC 40 cells, and then affinity purified with glutathione –Sepharose 4B beads as described above. Binding assays were performed by incubating the Sepharose linked GST or GST fusion proteins (approximately 10 µg for each incubation) with the purified Sp1 (55 µg) or with HeLa cell nuclear extract in binding buffer (above) for 1 h at 4°C. The beads were washed three times in cold binding buffer and three times with binding buffer containing 1.5% NP-40 prior to analysis by SDS–PAGE and Western blotting.

RESULTS

Sp1 Immunoprecipitate Contains Nuclear Pore Protein p62

Sp1 is known to interact with many cellular proteins [Lee et al., 1993; Li et al., 1991; Seto et

al., 1993; Sif et al., 1993]. Since many nuclear proteins, including transcription factors, contain the O-GlcNAc modification, we used Western blot analysis with the monoclonal antibody, RL2, to determine whether any proteins containing O-GlcNAc, coprecipitate with Sp1. Following immunoprecipitation with an affinity purified Sp1 antibody, proteins in the Sp1 immunoprecipitate were analyzed by Western blot. When the blot was probed with the Sp1 antibody, the major proteins observed were Sp1 and a smaller, approximately 80-kDa protein (Fig. 1). However, when the blot was probed with RL2, an additional glycoprotein was detected with a mobility corresponding to a molecular weight of 62 kDa. The intensity of the p62 band was considerably weaker than the Sp1 band when detected with RL2. Assuming that both proteins are detected equally well by RL2, this finding suggests that a considerable fraction of Sp1 is not complexed with p62. The failure of the Sp1 antibody to detect this 62-kDa band suggests that the Sp1 antibody does not cross-react with this protein. Based on the molecular size of the coprecipitated band, we probed the blot with a characterized monoclonal antibody (414) that recognizes the p62 nucleoporin [Laura and Blobel, 1986; Davis and Blobel, 1987]. This antibody recognized a protein of indistinguishable mobility with the p62 glycoprotein recognized by RL2. Thus, the 62-kDa protein that immunoprecipitates with Sp1 is immunologically indistinguishable from nucleoporin p62.

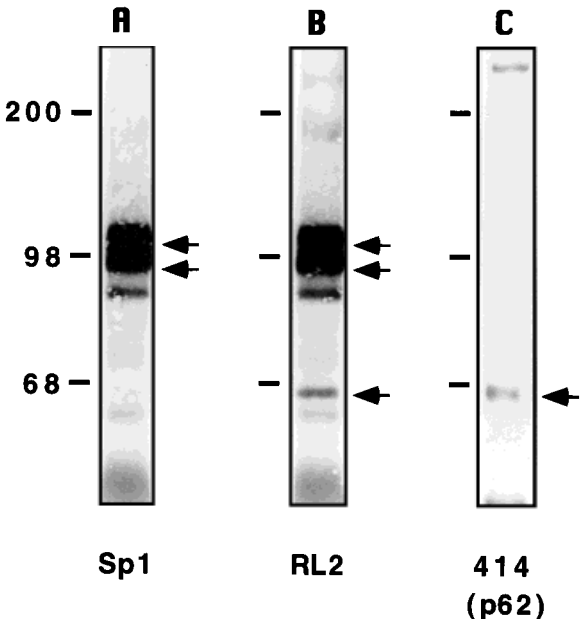

Fig. 1. Immunoprecipitates of Sp1 contain nuclear pore protein p62. MDA468 total cell lysates (500 µg of protein for each reaction) were incubated with 6 µg of affinity-purified anti-Sp1 rabbit polyclonal antisera (4627). Protein A–Sepharose beads were then added to precipitate the antibody. The protein complex retained on the beads was analyzed by SDS–PAGE (7.5% polyacrylamide gel) and subjected to Western blot analysis using Sp1 polyclonal antibody (4627) **(A),** RL2 monoclonal antibody **(B),** or monoclonal antinuclear pore antibody (414) **(C).** Positions of the molecular-weight markers are indicated. Arrows, positions of either Sp1 or p62 nucleoporin.

SDS Denaturation of Sp1 and p62 Prevents the Protein–Protein Interaction

The ability of the Sp1 antibody to precipitate p62 could either be a result of cross-reactivity between the Sp1 antibody with p62 or as a result of protein–protein interaction between Sp1 and p62. Assuming that this putative protein–protein interaction depends upon the proteins being in their native state, a double immunoprecipitation experiment was carried out with both anti-Sp1 and anti-p62 antibodies. Proteins bound to the anti-Sp1 antibody were denatured with 1% SDS and reprecipitated with either an Sp1 or p62 antibody following dilution of the SDS. While this Sp1 antibody could coprecipitate p62 prior to denaturation of the proteins with SDS (Fig. 2, lane 1), this antibody could only precipitate Sp1 following denaturation (Fig. 2, lane 2), suggesting that the Sp1 antibody does not simply recognize a common epitope shared between p62 and Sp1. That the coprecipitated 62-kDa protein is a nucleoporin is indicated by the observation that the p62 protein can be precipitated from the denatured Sp1 immunoprecipitate with the antinuclear pore monoclonal antibody, 414 (Fig. 2, lane 3). The results of this experiment are most compatible with the notion that the interaction of Sp1 and nucleoporin p62 depends on at least one of these proteins being in its nondenatured state.

p62 Immunoprecipitates Contain Transcription Factor Sp1

As a further test of the interaction between Sp1 and p62, we carried out the reciprocal experiment to determine whether p62 antibodies can coprecipitate Sp1 protein from MDA468 cell extracts. Four different rabbit polyclonal p62 nucleoporin antibodies, raised against bacterially expressed (nonglycosylated) full-length p62 protein, were used to immunoprecipitate p62 from MDA468 cell extracts. The immunoprecipitates were analyzed by Western blotting

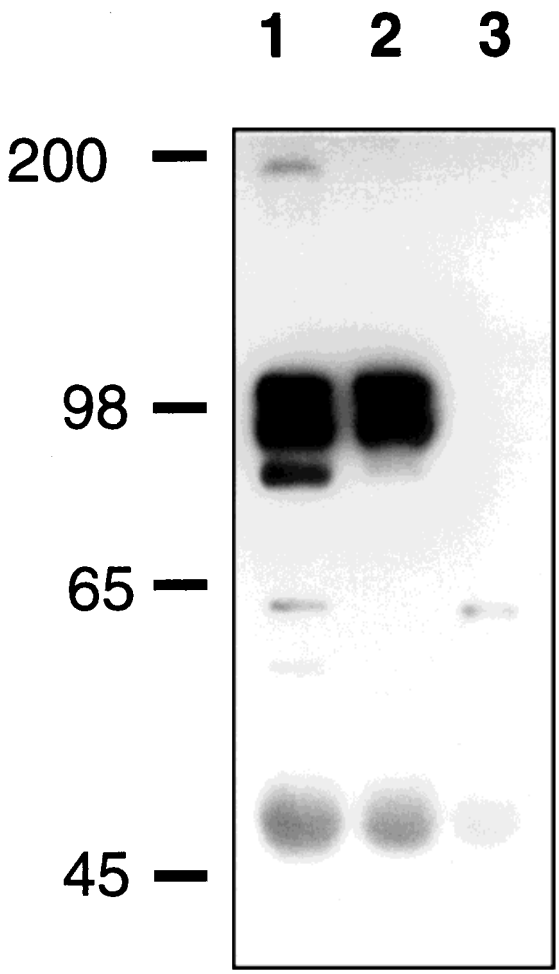

Fig. 2. The Sp1 antibody does not coprecipitate denatured p62 protein. MDA468 total cell extracts (600 µg of protein) were immunoprecipitated with polyclonal anti-Sp1 antibody (6 µg) (4627) **(lane 1).** The immunoprecipitates were eluted from the protein G beads with 1% SDS in binding buffer and subjected to reimmunoprecipitation with either anti-Sp1 (4627) **(lane 2)** or antinuclear pore (414) **(lane 3)** antibody following dilution of the SDS content to 0.1%. The proteins in Sp1 or p62 immunoprecipitates were separated by SDS–PAGE (7.5% polyacrylamide gel) and detected with the RL2 monoclonal antibody. Left, positions of the molecular-weight markers.

using a p62 or Sp1 antibody. The three antibodies raised in our laboratory against rat p62 recognized human p62 in the immunoprecipitates from the MDA468 cells (Fig. 3A). In each case, the p62 immunoprecipitates also contained immunoreactive Sp1 (Fig. 3B). Preimmune serum from each of the rabbits failed to precipitate p62 (data not shown) or Sp1 (Fig. 3C, preimmune rabbit 688 is shown). Furthermore, a p62 antibody, AS474, raised against a p62 synthetic peptide by Dr. Hanover, also coprecipitated Sp1 (Fig. 3C). None of the p62 antibodies recognized Sp1 on Western blots (e.g., Fig. 3C, using Ab 688) again suggesting that the coimmunoprecipitation resulted from interaction between Sp1 and p62, rather than from cross-reactivity of the antibodies in these polyclonal antisera.

C-Terminal Domain of p62 Involved in Protein–Protein Interaction With Sp1

To identify the region of p62 that is important for the interaction with Sp1, GST fusion proteins were generated (Fig. 4), and an immobilized protein interaction assay was performed. The GST chimeric proteins containing the full-length p62 (residues 1–525, GST-p62), the N-terminal domain of p62 (residues 1–298; GST-p62N) or C-terminal domain of p62 (residues 298–525; GST-p62C) were expressed in BSC40 cells using a vaccinia virus expression system. Milligram quantities of the expressed proteins were purified on glutathione—Sepharose beads and analyzed by SDS–PAGE stained with Coomassie blue (Fig. 5A). These preparations were greater than 90% pure. The major impurity was unfused GST. Of note, the purified GST-p62 and GST-p62C showed little or no contamination with other proteins, suggesting that the C-terminal segment of p62 with its predicted coiled coil structure, does not bind nonspecifically to cellular proteins derived from the vaccinia virus-infected BSC-40 cells.

These GST-fusion proteins were immobilized on glutathione—Sepharose 4B beads and incubated with purified, recombinant Sp1 that had

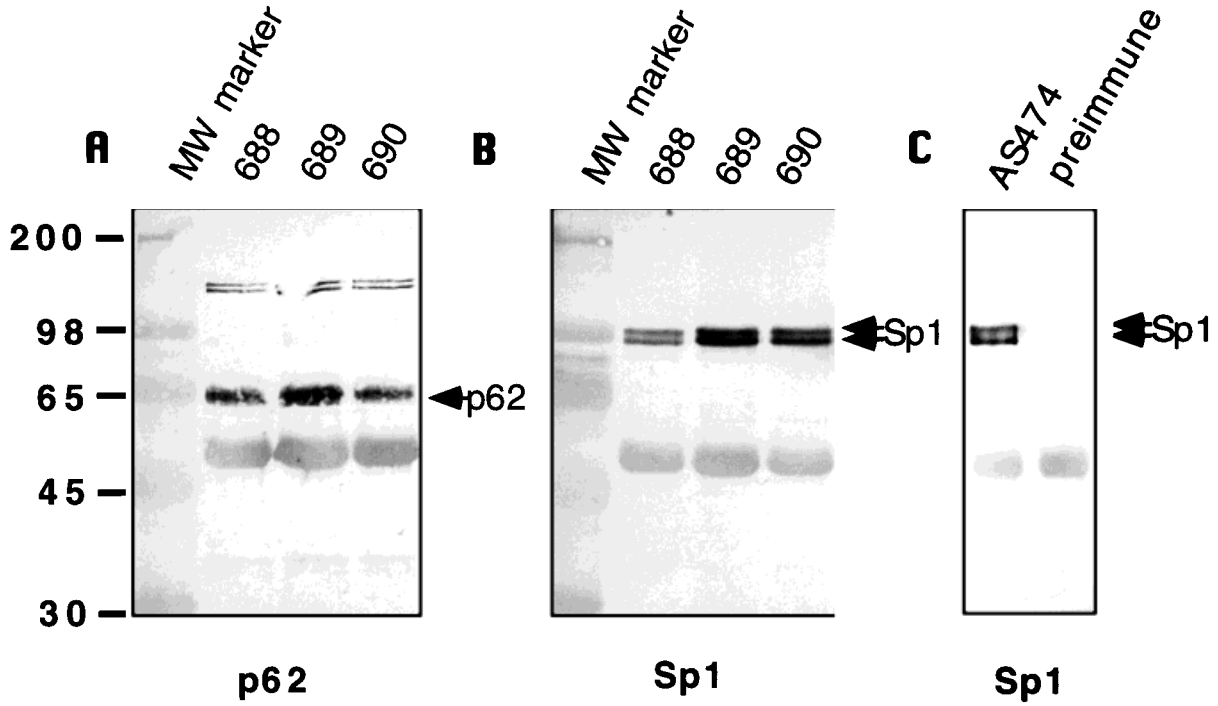

Fig. 3. p62 antibodies coprecipitate cellular Sp1. MDA468 total cell extracts (500 µg for each reaction) were immunoprecipitated with affinity-purified polyclonal p62 antibodies 688, 689, 690, **A, B;** AS474, **C;** or prebleed serum from rabbit 688 (C). The immunoprecipitates were eluted with SDS sample buffer and separated by SDS–PAGE (7.5% polyacrylamide gel). The separated proteins were analyzed by the immunoblot using p62 polyclonal (688) (A) or Sp1 polyclonal (4627) (B,C) antibodies. Left, positions to which the indicated molecular-weight standards migrated.

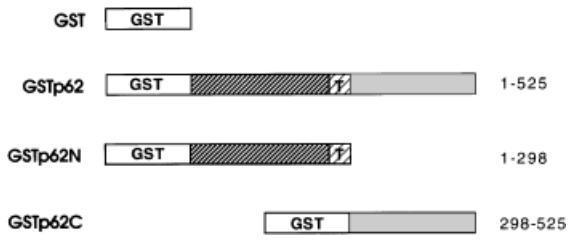

Fig. 4. Schematic diagrams of GST, GST-p62, GST-p62N and GST-p62C constructs.

been expressed using the vaccinia virus system. The incubation and wash conditions were similar to those used for immunoprecipitation. Recombinant Sp1 was retained by GST-p62 and GST-p62C, but not by GST alone or GST-p62N (Fig. 5B). The retained Sp1 could be detected on Western blots with both the Sp1 antibody (Fig. 5B) and RL2 (Fig. 5C). A similar experiment was performed using recombinant retinoblastoma (Rb) protein. We could not detect the binding of Rb protein to the GST-p62 fusion proteins (data not shown), suggesting that the Sp1–p62 interaction was relatively specific. Only full-length and N-terminal p62 could be detected by RL2 (Fig. 5C), confirming the earlier reports that it is the N-terminal domain of p62 that bears the O-GlcNAc modification [Starr et al., 1990]. Based on the intensities of the Western blot signals with RL2 antibody, full-length GST-p62 appeared to interact with an amount of Sp1 roughly equal to the amount of GST-p62; that is, assuming that both Sp1 and p62 are equally detectable with the RL 2 antibody. This result differs from the immunoprecipitation result shown in Figure 1. In that case, the RL2 signal attributable to Sp1 was significantly greater than the signal for p62, implying that the amount of p62 compared to Sp1 in the immunoprecipitate was considerably less than the ratio of these components in the interaction assay. This further suggests that a relatively low fraction of Sp1 in the cell is associated with p62. While GST-p62C also reacted with Sp1, it appeared less efficient than the full-length protein. The GST-p62 fusion proteins were also exposed to crude HeLa cell extracts to determine if p62 could interact with native Sp1. Again, GST-p62 and GST-p62C retained Sp1 whereas GST-p62N did not retain detectable amounts of Sp1 (Fig. 6). As before,

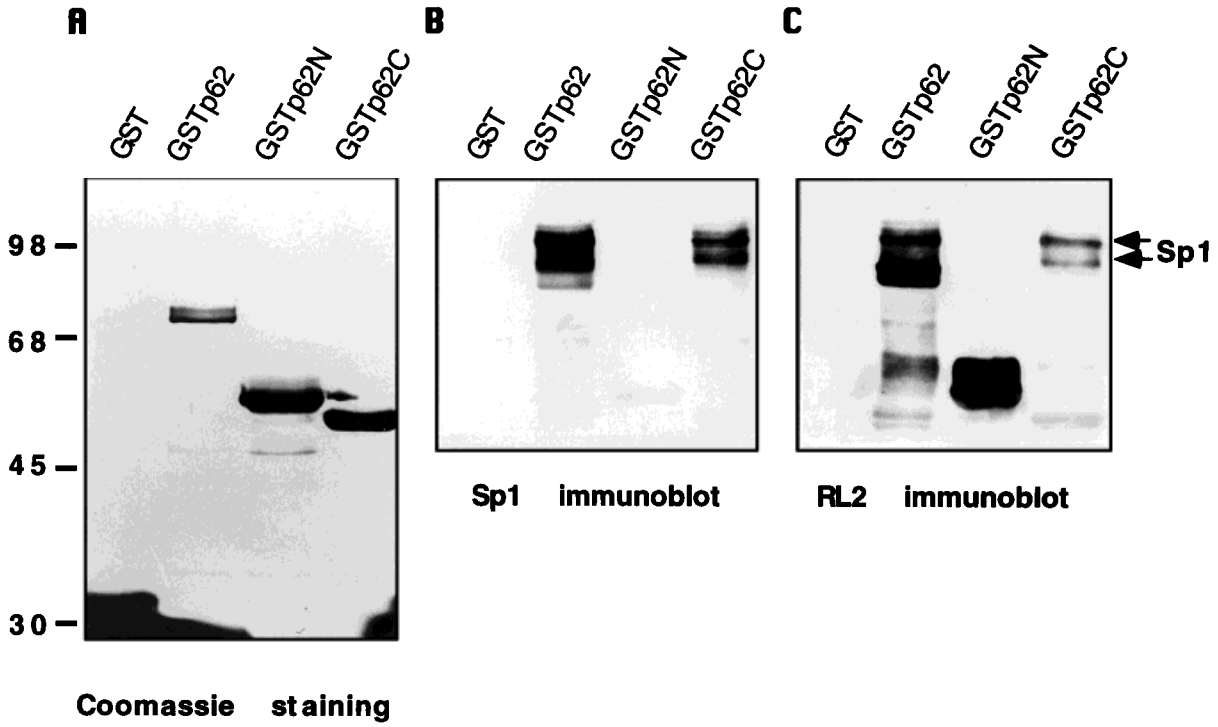

Fig. 5. In vitro binding assay reveals that human Sp1 interacts with the C-terminal domain of p62. Chimeric GST-p62, GST-p62N, and GST-p62C proteins and GST protein were expressed in vaccinia virus and purified on glutathione–Sepharose beads. The affinity-purified proteins were eluted with SDS sample buffer and separated by 7.5% SDS–PAGE (5 µg of purified protein each) and visualized by Coomassie blue staining **(A).** Left, positions to which the indicated molecular-weight standards migrated. The purified proteins were immobilized to glutathione beads (10 µg each) and incubated with purified recombinant Sp1 (55 µg each) in buffer containing 250 mM NaCl. After washing three times with binding buffer and three times with binding buffer containing 1.5% NP-40, the protein remaining bound to the beads was analyzed by Western blot analysis with an anti-Sp1 polyclonal antibody **(B)** or monoclonal RL2 antibody **(C).** The position of Sp1 protein is indicated.

GST-p62 was considerably more potent than GST-p62C at retaining Sp1. Taken together, these results indicate that it is the C-terminal region of p62 that interacts with Sp1 in vitro in a relatively specific manner. The ability of the purified recombinant proteins to interact suggests that the interaction is direct and not mediated by an intervening molecule. That full-length p62 interacts more efficiently than the C-terminal domain, p62C, suggests that the conformation of the intact protein might contribute to the interaction. This finding is in agreement with the data shown in Figure 2, indicating that denatured p62 and Sp1 do not interact in immunoprecipitates.

Sp1 Interacts With C-Terminal Domain of p62 In Vivo

To determine whether Sp1 and p62 can interact in the intact mammalian cell, a two-hybrid system was devised for sensitive detection of such interactions. As indicated in Figure 7, cDNA encoding full-length, N-terminal, and C-terminal domains of p62 were coupled to the cDNA encoding the first 94 amino acids of Gal4. This portion of Gal4 contains the DNA-binding domain of this transcription factor and can direct the fusion partner to the DNA-binding site recognized by Gal4. These fusion constructs were placed downstream of the SV40 promoter to produce the plasmids pG94-p62, pG94-p62N, and pG94-p62C, respectively. The cDNA encoding residues 81–621 of Sp1 (no Sp1 DNA-binding domain) was fused to the cDNA encoding the acidic activation domain of VP16 (amino acids 414–490) and placed downstream of the SV40 promoter. Finally, a reporter plasmid was constructed that contains the Gal4 binding sites placed upstream of a TATA box and the luciferase reporter gene. This reporter plasmid was cotransfected into NRK cells with the expression plasmids encoding the Sp1-VP16 and Gal4-p62 fusion proteins (Fig. 8). The reporter plasmid was transcribed very weakly

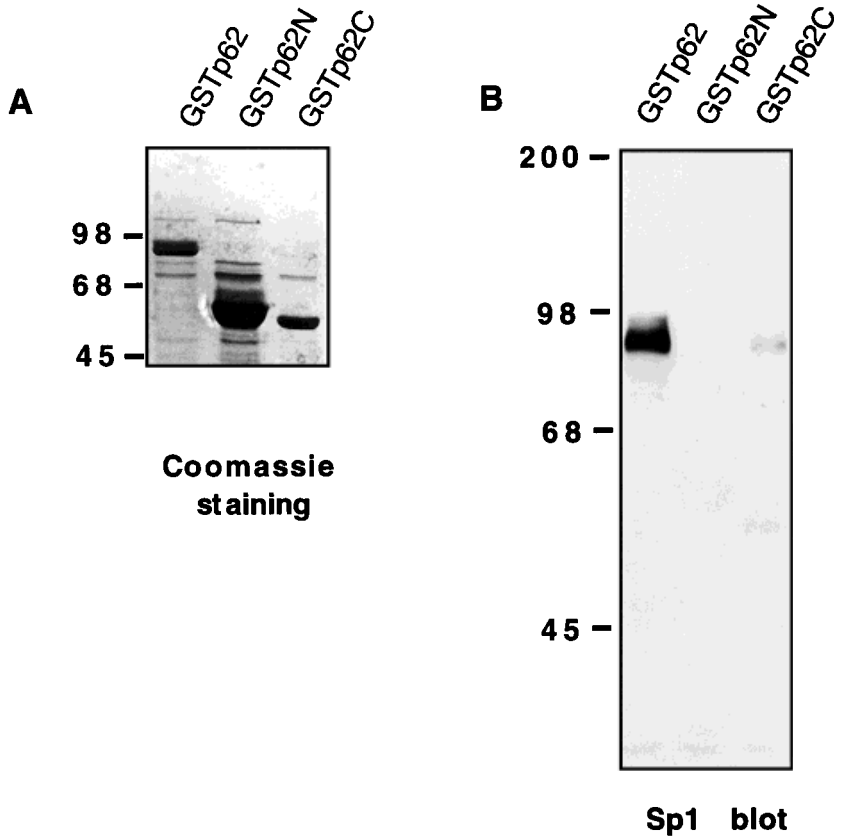

Fig. 6. The C-terminal domain of p62 bind directly to HeLa cell Sp1. Chimeric GST-p62, GST-p62N, and GST-p62C proteins and GST protein were expressed in vaccinia virus and purified on glutathione–Sepharose beads. Purified proteins on the beads were eluted with SDS sample buffer and separated by 7.5% SDS–PAGE and visualized by Coomassie blue staining **(A).** The purified proteins were immobilized on glutathione beads (7 μg each) and incubated with total cell extracts of HeLa cells. After washing three times with binding buffer and three times with binding buffer containing 2% NP-40, the protein remaining bound to the beads was analyzed by Western blot analysis with an anti-Sp1 polyclonal antibody **(B).**

when transfected into cells with unfused Gal4 or with the addition of the VP16-Sp1 activator (Fig. 8, CONT). However, cotransfection of this reporter with either pG94-p62 or pG94-p62C resulted in significant transcriptional activity from the reporter, indicating that p62 and its C-terminus contain a domain capable of transcriptional activation. Coexpression of full-length and C-terminal p62-Gal4 fusion proteins with the VP16-Sp1 activator resulted in a 7-fold activation of transcription from the Gal4 reporter. The N-terminal domain of p62 did not activate transcription either alone or in conjunction with the VP16-Sp1 activator. These results strongly indicate that Sp1 and the C-terminus of p62 can interact in vivo. In this assay, the C-terminal domain of p62 appeared as potent as the full-length molecule, however, quantitative conclusions cannot be drawn from these data, since the stringency of such interactions cannot be controlled. This interaction does not appear to require either the DNA-binding domain of Sp1 or the N-terminal 80 amino acids of Sp1.

DISCUSSION

This study was prompted by our consistent observation of a 62-kDa glycoprotein in immunoprecipitates of the ubiquitous transcription factor Sp1. Using several independently raised antibodies, we were able to identify this protein as the nuclear pore protein, p62. Using several approaches, we have now shown that Sp1 interacts with this nuclear pore protein. While this observation was first made in immunoprecipitates using an Sp1 antibody, we subsequently showed that antibodies directed at p62 could coprecipitate Sp1. Interestingly, another laboratory, studying cell cycle-dependent changes in O-GlcNAc, and using a different Sp1 antise-

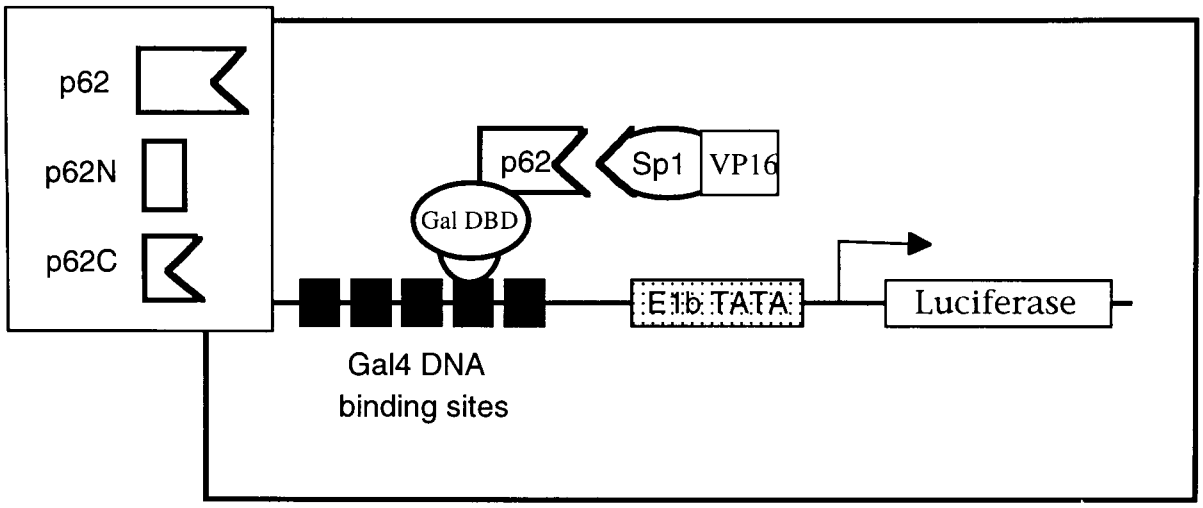

Fig. 7. Schematic representation of the two-hybrid system using Gal4-p62 and VP16-Sp1 mammalian expression vectors. The cDNAs corresponding to full-length p62 (residues 1–525), p62N (residues 1–298), and p62C (residues 298–525) were inserted downstream of the cDNA that encodes the DNA-binding domain of Gal4 (residues 1–94) in the plasmid pGAL. The cDNA encoding residues 81–621 of Sp1 was cloned into pGAD downstream of the cDNA encoding the herpes simplex virus VP-16 protein acidic transcriptional activation domain (residues 414–490). The reporter plasmid, pSgGalLuc, has five Gal4-binding sites placed upstream of the minimal adenovirus E1b promoter and the luciferase coding sequence.

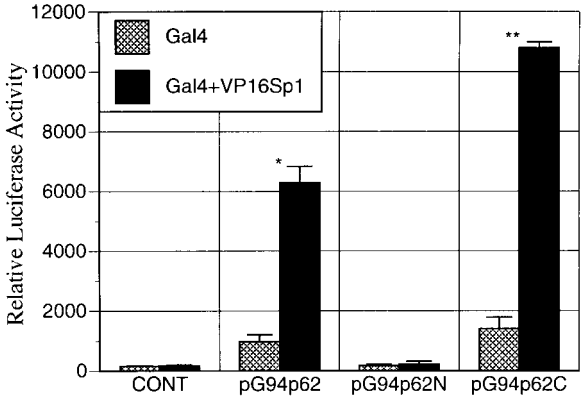

Fig. 8. Luciferase activity induced by the chimeric vectors. NRK cells were transfected with the indicated combinations of expression vectors (5 μg DNA/0.5 × 10^6 NRK cells) together with 10 μg of pSgGalLuc reporter vector and the pCMV-βGal. For the control (CONT), the cells were transfected with the pGAL expression vector containing no p62 sequence. Otherwise the cells were transfected with the expression vectors that encode the indicated Gal4-p62 chimeric proteins. Extracts containing equal amounts of β-galactosidase (transfection efficiency control) were prepared and luciferase activity determined 24-h posttransfection. The activity of the promoter is indicated by the relative light units. *$P < 0.0025$; **$P < 0.001$ (Student's t distribution).

rum, also coprecipitated a 62-kDa glycoprotein with Sp1 [Haltiwanger and Philipsberg, 1997], that we presume to be the same nuclear pore protein. The coimmunoprecipitation that we observed appears to depend on a structural determinant in the native proteins, because prior denaturation of Sp1 and p62 abrogates the interaction of these proteins in the immunoprecipitates. This denaturation experiment also demonstrates that the coprecipitation does not result from a shared epitope in p62 and Sp1 that is recognized by the five separately raised antisera used in these experiments. In addition to the immunoprecipitation data, we were able to show that recombinant p62, immobilized through GST to an affinity column, could also interact with both recombinant and native Sp1. This interaction could be observed between the purified proteins, suggesting that a direct interaction between Sp1 and p62 can occur. Based on the relative intensity of the Western blot signal with the RL2 antibody (Fig. 5) which recognizes the O-GlcNAc residues on both p62 and Sp1, it would appear that this direct interaction is sufficiently efficient to allow p62 to bind approximately equal quantities of Sp1. Finally, using a mammalian two-hybrid system to assay such interactions in vivo, we demonstrated that these proteins interact. This interaction is of sufficient high affinity and duration to allow a sevenfold induction of reporter activity several hours following transfection. While this two-hybrid system is not a test of the specificity of the interaction between p62 and Sp1, the interaction was domain-specific in a manner agreeing with the in vitro interactions. The in vitro experiments did suggest some de-

gree of specificity. No interactions between p62 and the retinoblastoma protein could be detected, suggesting that p62 and its C-terminus were not simply binding all nuclear proteins. Furthermore, the GST-p62 and GST-p62C proteins were easily purified through a single step on a glutathione affinity column, suggesting that this protein is not unusually interactive with proteins in general and thereby suggesting some specificity to the interaction that were observed between p62 and Sp1. Prior studies have indicated that the C-terminus of p62 does interact with other O-glycosylated nuclear proteins, p54 and p58 [Buss and Stewart, 1995; Hu et al., 1996], which reside both on the cytoplasmic and nuclear side of the nuclear membrane. It remains possible that the Sp1 interaction with p62 involved these other proteins. Such an indirect interaction would allow the observed co-immunoprecipitations with anti-Sp1 and anti-p62 sera. However, the experiments using recombinant p62 indicate that these proteins are not necessary for the interaction with Sp1. That is, the vaccinia system directs the expression of at least 1,000-fold more p62 or Sp1 than is normally found in the cell. It is unlikely that the purified recombinant proteins could be contaminated with a sufficient quantity of the naturally expressed nuclear proteins to allow a detectable indirect interaction to occur that would require these nuclear proteins. Furthermore, the affinity purified GST-p62 showed no evidence of contamination with p54 or p58 on protein stained gels (Fig. 5).

The interaction of Sp1 and p62 nucleoporin occurs through the C-terminus of p62. While the interaction with the C-terminus is not as robust as with the entire protein, no interaction was observed with the N-terminus, suggesting that the entire structure of p62 is required for the interaction but the major site of contact probably occurs in the C-terminus. The C-terminal segment of p62 does not contain any O-GlcNAc modifications, implying that the primary mode of interaction between Sp1 and p62 is not dependent on this modification in p62. However, our experiments cannot rule out the possibility that the O-GlcNAc modications on Sp1 or the N-terminal segment of p62 contribute in some way to the interaction. Indeed, Sp1 multimerization is regulated by the O-GlcNAc modification [Roos et al., unpublished observation]. With regard to the nuclear pore proteins, modification of the O-GlcNAc groups on these proteins using galactosyltransferase did not interfere with the in vitro assembly or transport functions of reconstituted nuclear pores, suggesting that the O-GlcNAc residues on these proteins do not play a role in the protein interactions required for the assembly or function of these structures [Miller and Hanover, 1994].

The C-terminal segment of p62 contains numerous heptad hydrophobic repeats that are characteristic of the coiled-coil regions of filament-forming proteins. This potentially filamentous structure is shared with the nuclear lamins that form the fibrous lamina that is closely opposed to the inner membrane of the nuclear envelope [Miller et al., 1991]. It is this fibrous lamina that is part of the larger nuclear matrix. Overall, this fibrous network is believed to impart a three dimensional structure to the nucleus. In addition, the nuclear matrix has been postulated to play an important role in the control and organization of gene transcription [e.g., Davie, 1996]. The structural similarities of the p62 C-terminal domain with nuclear matrix proteins could suggest a functional similarity as well.

The physiological role that might correspond to this biochemical demonstration of the p62–Sp1 interaction has not been addressed by the experiments described in this paper. Attempts were made to express a chimeric form of p62 that would direct its insertion to the cytoplasmic face of the endoplasmic reticulum or plasma membrane. We then planned to determine whether Sp1 could be directed to these ectopic sites. However, no viable transgenic cells could be generated that contained such a construct (data not shown). In the absence of such experimental data, we speculate that the nuclear pore may play a role in nuclear function in addition to the translocation of macromolecules. It remains possible that the interaction we observed between Sp1 and p62 may reflect this transport function. For example, the interaction of the TATA-binding protein with the nuclear pore has been demonstrated using patch clamp and atomic force microscopy techniques [Bustamante et al., 1995], and this interaction was believed to represent a transient interaction related to the transport function of the nuclear pore. However, the interaction between Sp1 and p62 may reflect another function of the nuclear pore. There is some evidence that mRNA synthesis and splicing is concentrated at the nuclear periphery [Singer and Ward,

1982]. This observation has been the basis of the more controversial concept, that the nuclear pore might be more directly involved in transcriptional regulation. Blobel [1985] proposed that the nuclear pore complex contains a distinct DNA-binding subunit that could recognize transcribable genes, thereby directing transcription to the nuclear periphery. Indeed, a nuclear pore protein was subsequently identified and its cDNA cloned [Sukegawa and Blobel, 1993], that binds DNA in a zinc-dependent manner. The cDNA encodes four putative zinc finger motifs of the type found in DNA-binding proteins. This protein also contains the signature XFXFG motif found in p62 and other nucleoporins. That transcribable DNA might be concentrated near or at the nuclear pore would imply that the transcription factors involved in the transcription of this DNA would also be required at this site in the nucleus. While the affinity of Sp1 for its binding sites on the DNA and the concentration of Sp1 in the nucleus [Courey et al., 1989; Kadonaga et al., 1986] may be sufficient to target it to the transcribable DNA by direct binding to the GC-box, the observed ability of Sp1 to interact with p62 might provide a basis for facilitating the localization of transcription factors like Sp1 to the nuclear periphery where transcription might be concentrated. This biochemical study showing that Sp1 interacts with a nuclear pore protein is compatible with the concept that the nuclear pore plays a role in conjunction with the nuclear matrix in the spatial organization of gene transcription.

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