p62, a Phosphotyrosine-independent Ligand of the SH2 Domain of p56

†Belongs to a New Class of Ubiquitin-binding

Proteins* (Received for publication, May 21, 1996)

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p62 is a novel cellular protein which was initially identified as a phosphotyrosine-independent ligand of the SH2 domain of p56

Recent studies have shown that this interaction involves at least partly through ubiquitination-mediated protein degradation.

The lymphoid-specific Src family tyrosine kinase p56

†has a Src homology 2 (SH2) domain and a Src homology 3 (SH3) domain that mediate protein-protein interactions between signaling proteins through direct binding to regions containing phosphotyrosine and proline-rich sequences, respectively (1, 2). In addition to the well-established protein phosphotyrosine-dependent SH2 domain binding, interaction of SH2 domains with non-phosphotyrosyl proteins are also recognized (3, 4). Recently, a specific phosphotyrosine-independent interaction of the Lck SH2 domain with a novel cytosolic 62-kDa protein (p62) has been identified (5) and its cDNA cloned (6). p62 expression in all human tissues tested (6) suggests that the enzymatic and/or regulatory functions of p62 are not restricted to the lymphoid-specific p56

†SH2 domain binding. Rather, p62 may serve as a common signal transducer in cells and may use the Lck SH2 domain binding for T cell-specific signal transduction.

Recently, ubiquitination of cellular proteins has emerged as a crucial feature in regulation of signal transduction and cell cycle progression through ubiquitination-dependent proteosomal degradation of important cellular proteins, including cell surface receptors such as platelet-derived growth factor receptor and T cell receptor γ chain, mitotic cyclins, Cdk inhibitor p27

†, oncoproteins, transcriptional regulators such as IκB, NF-κB, c-J un, and c-Fos, and the tumor suppressor p53 (reviewed in Ref. 7).

Proteins binding to ubiquitin can be grouped into three different categories based on their binding modes. (i) Proteins forming isopeptide bonds with ubiquitin: most proteasomal substrates are conjugated to multiubiquitins through isopeptide bond formation between the C-terminal glycine of ubiquitin and an ε-amino group of a lysine residue of the substrate (8). (ii) Proteins forming a thioester bond with ubiquitin: only the ubiquitination enzymes E1, E2, and E3 are known to form a thioester with ubiquitin (9). (iii) Proteins binding noncovalently to ubiquitin: all C-terminal hydrolases (deubiquitinating enzymes) and an S5 subunit of the 26 S proteasome bind noncovalently to ubiquitin (10–12). Some studies suggest that E2 and E3 may also bind to ubiquitin noncovalently in addition to the thioester bond formation (13).

In the present study, a novel cellular protein, p62, has been shown to bind to ubiquitin noncovalently, in addition to its association with p56

†(5, 6). However, p62 has no homology to any known ubiquitin-binding protein. Thus, p62 may represent a new ubiquitin-binding protein that regulates signal-mediated ubiquitination and/or proteasomal degradation of cellular proteins.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid System—p62 fusion to the Gal4 binding domain (Gal4B-p62) was constructed using EcoRI and Xhol sites of pGBT9 vector (Clontech) and internal restriction sites (Stul-Xhol) of the p62 cDNA in which the EcoRI and Stul sites were fused by blunt end ligation. A HeLa cDNA library fused to the Gal4 activation domain (Gal4A) in pGADGH vector (Clontech) was introduced into Gal4B-p62 transformed yeast strain HF7c (MATa, his3 3-200, trp 1-901, leu 2-3, 112, URA3::Gal1-lacZ) and an S5 subunit of the 26 S proteasome complex, the only proteins known to bind to ubiquitin noncovalently. These results suggest that p62 belongs to a new class of ubiquitin-binding proteins and that p62 affects signal transduction at least partly through ubiquitination-mediated protein degradation.
Noncovalent Binding of p62 to Ubiquitin

RESULTS AND DISCUSSION

The yeast two-hybrid system was used to identify proteins that interact with p62. p62 cDNA fusion to the Gal4 DNA binding domain (Gal4B-p62) was used to screen a yeast cDNA library fused to the GAL4 activation domain (Gal4A) using two reporter genes (leu, trp) or leu, trp, his plates and incubated for 3 days. Lanes 1, 2, and 3 represent Gal4A-dubiquitin (dUb), Gal4A-polyubiquitin (pUb), and Gal4A-UBA52 (rUb) cDNA fusions, respectively, that were used for transformation. The yeast two-hybrid system was used to identify proteins that interact with p62. p62 cDNA fusion to the Gal4 DNA binding domain (Gal4B-p62) was used to screen a yeast cDNA library fused to the GAL4 activation domain (Gal4A) using two reporter genes (leu, trp) or leu, trp, his plates and incubated for 3 days. Lanes 1, 2, and 3 represent Gal4A-dubiquitin (dUb), Gal4A-polyubiquitin (pUb), and Gal4A-UBA52 (rUb) cDNA fusions, respectively, that were used for transformation.

Figure 1. Interaction of p62 with ubiquitin in the yeast two-hybrid system. Yeast strain HFC7c was transformed with plasmids carrying Gal4B-p62, Gal4B-Lamin, or one of the Gal4A-ubiquitins either alone or in combination. Colonies containing different combinations of plasmids were streaked on leu, trp, or leu, trp, his plates and incubated for 3 days. Lanes 1, 2, and 3 represent Gal4A-dubiquitin (dUb), Gal4A-polyubiquitin (pUb), and Gal4A-UBA52 (rUb) cDNA fusions, respectively, that were used for transformation.

| Beads | Ub-Sph | p62 binding | ATP | DTT |
|-------|--------|-------------|-----|-----|
| Sph   | Ub     | 1           | +   | -   |
| Sph   | Ub     | 2           | +   | -   |
| Sph   | Ub     | 3           | +   | -   |
| Sph   | Ub     | 4           | +   | -   |
| Sph   | Ub     | 5           | +   | -   |
| KCl   | Buffer | 6           |     |     |
| KCl   | Buffer | 7           |     |     |
| KCl   | Buffer | 8           |     |     |

Figure 2. Interaction and binding mode of p62 with ubiquitin in vitro. A, Fraction 2 of HeLa cell lysate was prepared as described under "Experimental Procedures" and incubated with Sepharose (Sph) or Ub-Sepharose beads (Ub-Sph). Proteins bound to the beads were analyzed by immunoblotting with anti-p62 antisera. For competition assays, Fraction 2 was incubated with Ub-Sepharose beads in the presence of 0, 25, and 100 μM soluble ubiquitin, respectively. B, Fraction 2 prepared from HeLa lysates was incubated with Sepharose (lane 1) or Ub-Sepharose beads (lanes 2–9), either in the presence (lanes 2 and 3) or absence of ATP (lanes 4 and 5). Bound proteins were eluted either with SDS sample buffer containing 100 mM DTT (lanes 1–3) or SDS sample buffer without DTT (lanes 4 and 5). The presence of p62 in the complex was analyzed by immunoblotting using anti-p62 antibody. Bound p62 was also eluted either with SDS sample buffer (lane 6) or with 1 M KCl buffer, pH 9.0 (lane 7). Ub-Sepharose beads after KCl elution in lane 7 were further washed with the KCl buffer and eluted with SDS sample buffer containing DTT (lane 8) in order to measure the efficiency of KCl elution.

Lamin, however, when coexpressed with any of those ubiquitin fusion genes, failed to induce the His<sup>+</sup> phenotype. Furthermore, compared to various controls, coexpression of Gal4A-p62 and Gal4A-UBA52 in yeast specifically induced activation of 

\[ \beta \text{-galactosidase} \]

in the yeast two-hybrid screen. These results suggest that p62 interacted specifically with ubiquitin in any context, either in a monomeric or in an oligomeric form.

Interaction of p62 with ubiquitin was further confirmed by in vitro binding studies using ubiquitin-conjugated Sepharose beads (Ub-Sepharose). Proteins bound to Ub-Sepharose were precipitated and analyzed by Western blotting using anti-p62 antibody. Ub-Sepharose but not Sepharose beads alone precipitated p62 from HeLa cell lysates (Fig. 2A). Furthermore, more than a half of p62 binding to Ub-Sepharose, which had been coupled in 10 M°M ubiquitin solution, was inhibited by the presence of 25 M°M soluble ubiquitin in the reaction mixture. These results show that p62 interacts with ubiquitin in vitro as well as in vivo in the yeast two-hybrid screen.

Binding mode of p62 to ubiquitin was then analyzed. p62 interacted with Ub-Sepharose in the absence of ATP equally as well as in its presence (Fig. 2B, lanes 2–5). Furthermore, p62 bound to Ub-Sepharose was eluted completely either by SDS sample buffer or by 1 M KCl in Tris buffer, pH 9.0, both under nonreducing conditions (Fig. 2B, lanes 6–8). These results suggest that p62 binds noncovalently to ubiquitin, rather than forming a thioester or an isopeptide bond.

However, p62 has no homology with C-terminal ubiquitin hydrolases, with the E2 and E3 enzymes, or with the 55a proteins were eluted either with SDS buffer or with other buffers as indicated in figure legends. Presence of p62 in the eluates was analyzed by immunoblotting using a polyclonal anti-p62 antiserum and horse-radish peroxidase-conjugated goat anti-rabbit IgG.

Table 1. Interaction of p62 with ubiquitin in yeast activates 

| Protein     | Gal4B | Gal4B-p62 | Gal4B-Lamin |
|-------------|-------|-----------|-------------|
| Gal4A       | W     | W         | W           |
| Gal4A-dUb   | W     | B         | B           |
| Gal4A-pUb   | W     | B         | B           |
| Gal4A-rUb   | W     | B         | B           |

Table 1 Interaction of p62 with ubiquitin in yeast activates \( \beta \text{-galactosidase} \) reporter gene

Specificity of the interaction of p62 with ubiquitin in yeast was assayed using the 5-bromo-4-chloro-3-indolyl \( \beta \text{-galactosidase} \) filter method as described under "Experimental Procedures." B, blue color colony; W, white color colony; dUb, ubiquitin dimer; pUb, ubiquitin polymer; rUb, ubiquitin ribosomal fusion.

Gall4A-dUb and Lamin, however, when coexpressed with any of those ubiquitin fusion genes, failed to induce the His<sup>+</sup> phenotype. Furthermore, compared to various controls, coexpression of Gal4A-p62 and Gal4A-UBA52 in yeast specifically induced activation of \( \beta \text{-galactosidase} \) that results in blue colony formation (Table 1).

These results suggest that p62 interacted specifically with ubiquitin in any context, either in a monomeric or in an oligomeric form.

Interaction of p62 with ubiquitin was further confirmed by in vitro binding studies using ubiquitin-conjugated Sepharose beads (Ub-Sepharose). Proteins bound to Ub-Sepharose were precipitated and analyzed by Western blotting using anti-p62 antibody. Ub-Sepharose but not Sepharose beads alone precipitated p62 from HeLa cell lysates (Fig. 2A). Furthermore, more than a half of p62 binding to Ub-Sepharose, which had been coupled in 10 M\( \mu \)M ubiquitin solution, was inhibited by the presence of 25 M\( \mu \)M soluble ubiquitin in the reaction mixture. These results show that p62 interacts with ubiquitin in vitro as well as in vivo in the yeast two-hybrid screen.

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| Protein   | Gal4B | Gal4B-p62 | Gal4B-Lamin |
|-----------|-------|-----------|-------------|
| Gal4A     | W     | W         | W           |
| Gal4A-dUb | W     | B         | B           |
| Gal4A-pUb | W     | B         | B           |
| Gal4A-rUb | W     | B         | B           |
subunit of the 26 S proteasome. Furthermore, p62 did not have ubiquitin C-terminal hydrolase activity (data not shown). Thus, p62 belongs to a novel class of ubiquitin-binding proteins. Its function may be exerted through binding to ubiquitin, but it may not be directly involved as an enzyme in the ubiquitination and/or deubiquitination process.

In order to delineate the region of p62 which interacts with ubiquitin, a series of p62 deletion mutants containing C-terminal T7 epitope tag (p62-T7) have been constructed, expressed in HeLa cells, and examined for their ability to bind to Ub-Sepharose. The C-terminal 182-amino acid deletion from Asp^{258} to Leu^{440} (d258–440) completely abolished the binding of p62 to ubiquitin, while deletions from Met^{1} to Lys^{187} (d1–187) and from Glu^{32} to Pro^{322} (d32–322) had little or no effect on binding (Fig. 3A). Further analysis showed that the C-terminal 80 amino acids (Ser^{361} to Leu^{440}) are essential for p62 to associate with ubiquitin (Fig. 3B). Interestingly, partial deletions in this 80-amino acid region, from Asp^{368} to Asp^{391} (d368–391) and from Asp^{391} to Leu^{440} (d391–440), had partial inhibitory effects on the p62-ubiquitin interaction. This result suggests either that two regions of p62 may interact with one ubiquitin molecule to provide maximal binding affinity or that more than one ubiquitin binding site is present.

As all known ubiquitin-binding proteins are involved in ubiquitination-dependent proteasomal proteolysis, the biological function of p62 may also be related to this protein modification/degradation pathway. Since, the N-terminal 50 amino acids of p62 are critical for interaction with the SH2 domain of p56^{lck} (6), p62 has at least two separate domains, one for binding to an SH2 domain and the other for binding to ubiquitin. Furthermore, p62 either has or is associated with a Ser/Thr kinase activity and also has a cysteine-rich zinc-finger-like region and a region homologous to Cdc24, both of which are potential protein-protein interaction sites (6). Thus, a physiological role for p62 may involve connection of mitogenic signals to the ubiquitination-mediated specific proteolytic pathway. Alternatively, p62 may have a still uncharacterized enzymatic activity relating to the ubiquitination-mediated proteasomal degradation pathway that is regulated by binding to such signaling molecules.

Acknowledgment—We thank Dr. Xin Zhang for helpful advice in the yeast two-hybrid screening.

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