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P62 and the Sequestosome, a Novel Mechanism for Protein Metabolism

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In addition to selecting proteins for degradation by the 26S proteasome, ubiqitination appears to serve other regulatory functions, including for endosomal/lysosomal targeting, protein translocation, and enzyme modification. Currently, little is known how multiubiquitin chains are recognized by these cellular mechanisms. Within the 26S proteasome, one subunit (Mcb1/S5a) has been identified that has affinity for multiubiquitin chains and may function as a ubiquitin receptor. We recently found that a non-proteasomal protein p62 also preferentially binds multiubiquitin chains and forms a novel cytoplasmic structure "sequestosome" which serves as a storage place for ubiquitinated proteins. In the present manuscript, the role and regulation of p62 in relation to the sequestosomal function will be reviewed.

Key words : P62, Sequestosome, Ubiqitination, Protein metabolism

INTRODUCTION

Eukaryotic cells have two distinct protein degradation systems, lysosomal and nonlysosomal (Ciechanover, 1994). The best known nonlysosomal protein degradation process is proteasomal proteolysis which is responsible for degradation of proteins in the cytoplasm and the nucleus, while lysosomal proteolysis occurs in the lipid bilayered organelles. Unlikely lysosomal proteolysis, protein degradation in the proteasome requires ubiquitination of substrate proteins (Hershko and Ciechanover, 1992). This process is ATP dependent and occurs at neutral pH. The 26S proteasome complex is composed of two distinct 20S and 19S particles (Ciechanover, 1994; Ichihara, 1995). The 20S particle is a protease complex, while 19S contains several proteins including ATPases and a ubiquitin binding proteins.

Ubiquitination-mediated protein degradation can be divided into three steps: signaling for the ubiquitination of a target protein, ubiquitin conjugation of the target protein, and recognition and degradation by proteasomes (Ciechanover, 1994; Gonda, 1994). In general, ubiquitin conjugation to proteins involves three enzymes, ubiquitin activating enzyme (E1), ubiquitin carrier protein (E2), and ubiquitin ligase (E3) (Ciechanover, 1994). Initially, E1 activates ubiquitin by forming a high energy thioester intermediate with the C-terminal glycine using ATP. The activated ubiquitin is sequentially transferred to E2, then to E3 which catalyzes isopeptide bond formation between the activated C-terminal glycine of ubiquitin and an ε-amino group of a lysine residue of the substrate. Following the linkage of the first ubiquitin chain, additional molecules of ubiquitin are attached to lysine side chains of the previously conjugated moiety to form branched multiubiquitin chains.

The multiubiquitin chain then serves as a recognition signal for the 26S proteasome which degrades the conjugated proteins (Ciechanover, 1994). The 19S regulatory complex contains one or more ubiquitin recognition factors. One recently identified is Mcb1 or S 5a which has affinity for multiubiquitin chains, especially those containing four or more ubiquitins (Deveraux *et al.*, 1994; van Nocker *et al.*, 1996). However, yeast lacking Mcb1 are viable and efficiently degrade most ubiquitin-conjugates (van Nocker *et al.*, 1996). Thus, the 26S proteasome must also contain ubiquitin recognition factor in addition to Mcb1/S5a.

Ubiquitin-protein conjugation also has functions unrelated to proteasomal targeting. For example, multiubiquitination is required for the internalization of several yeast and mammalian cell surface proteins into the endocytic pathway (Hicke, 1996, 1997). In addition, ubiquitination-dependent changes in location of monoamine oxidase B and catalytic activity of IκBα kinase have been described (Zhaung and McCauley, 1989). Thus, specific ubiquitin chain recognition components may also exist for these nonproteasomal roles.

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The cytoplasmic p62 protein was previously identified as a binding protein to the p56lck SH2 domain (Park *et al.*, 1995; Joung *et al.*, 1996). Recently, we found that p62 also binds noncovalently to ubiquitin, suggesting a role in the ubiquitin pathway (Vadlamudi, *et al.*, 1996). Furthermore, p62 has high affinity for multiubiquitin chains, but unlike Mcb1/S5a, it is not physically associated with the 26S proteasome. Rather, p62 forms a novel cytoplasmic compartment, sequestosome, into which excess ubiquitinated proteins are segregated. In the present manuscript, the function and regulation of p62 in relation to such new mechanism in protein metabolism will be discussed.

Interaction of p62 with protein kinases

p62 has been initially identified as a phosphotyrosine-independent ligand for the lck SH2 domain (Park *et al.*, 1995; Joung *et al.*, 1996). Different SH2 domains bind to distinct phosphotyrosine containing peptides by specifically recognizing a few residues immediately C-terminal to the phosphotyrosine residue (Songyang *et al.*, 1993). On the other hand, the interaction between p62 and the lck SH2 domain requires neither phosphorylation of tyrosine in p62 nor invarient arginine residue of the SH2 domain (Park *et al.*, 1995). Furthermore, analysis using a cloned p62 cDNA, composed of 2,078 bp and contained an open reading frame encoding a polypeptide of 440 amino acid residues showed that the N-terminal 110 amino acids, but not four tyrosine residues in this region, is required for the binding of p62 to the SH2 domain (Joung *et al.*, 1996).

In addition to binding with the lck SH2 domain, p 62 also associates with a Ser/Thr kinase (Park *et al.*, 1995; Joung *et al.*, 1996). Furthermore, the p62 message is expressed in all tissues tested. Thus, the functions of p62 are not restricted to lck SH2 domain binding in T cells. Rather p62 may serve as a common signal mediator in various cell types. Recently, two independent groups reported that p62 associates with atypical protein kinase C, ζ and λ (Pulse *et al.*, 1997; Sanchez *et al.*, 1998). Thus, p62 may have an unknown biochemical activity which can be regulated by its binding to a SH2 domain containing tyrosine kinase and/or to a Ser/Thr kinase.

Interaction of p62 with multiubiquitin chains

Except its interaction with protein kinases, due to the absence of homology with any known proteins, the biochemical role of p62 was obscure when its cDNA was first cloned and sequence analyzed. The functional implication was suggested when ubiquitin was characterized as another p62 binding protein by a yeast two hybridization system using p62 cDNA fusion to the Gal4 DNA binding domain (Vadlamudi *et al.*, 1996). Sequencing of the cDNA plasmids isolated from the 46 final positive clones in the two hybrid system revealed that the majority of them (43 of 46) belonged to the ubiquitin gene family including diubiqitin, poly ubiquitin, and a ubiquitin-conjugated ribosomal protein UBA52. 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.

Proteins binding to ubiquitin can be grouped into three different categories based on their binding modes: (i) Proteins forming isopeptide bond with ubiquitin; most proteasomal substrates have conjugated multiubiquitin through isopeptide bonds. (ii) Proteins forming a thioester bond with ubiquitin; only ubiquitination enzymes E1, E2, and E3 are known to form a thioester with ubiquitin. (iii) Proteins binding non-covalently to ubiquitin; all members of C-terminal hydrolases and an S5a/Mcb1 subunit of the 26S proteasome bind noncovalently to ubiquitin (Predergast *et al.*, 1995; Hawley-Nelson *et al.*, 1989).

Interestingly, p62 interacted with ubiquitin conjugated sepharose (Ub-sepharose) in the absence of ATP equally as well as in its presence. Furthermore, p62 bound to Ub-sepharose was eluted completely by SDS under nonreducing conditions (Vadlamudi *et al.*, 1996). Thus, p62 binds noncovalently to ubiquitin, rather than forming a thioester or an isopeptide bond. The S5a/Mcb1 subunit of the 26S proteasome and ubiquitin C-terminal hydrolases are the only known proteins which bind noncovalently to ubiquitin. However, p62 did not show any detectable ubiquitin hydrolase activity.

Recently it was shown that S5a/Mcb1 binds the multimeric form of ubiquitin with higher affinity than monomeric ubiquitin (Deveraux *et al.*, 1994; van Nocker *et al.*, 1996; van Nocker *et al.*, 1996). As the function of the 26S proteasome complex is to recognize and hydrolyze multiubiquitin conjugated proteins, its higher affinity for multimeric ubiquitin chains may be used to recruit appropriate substrates efficiently. Thus, each ubiquitin binding protein may have preference towards ubiquitins in different contexts in relation to its function.

Interestingly, GST fused p62 protein efficiently interacts with multiubiquitin chains containing four or more ubiquitins (Fig. 1 and Ko *et al.*). Furthermore, Immunoprecipitation of p62 in several eukaryotic cell lysates coprecipitated a complex mixture of multiubiquitin conjugates present in the cells. Thus, like Mcb1/S5a, p62 has low affinity for ubiquitin monomers and short ubiquitin chains ($n \leq 3$) but has strong affinity for higher order chains ($n \geq 4$) with the strength of binding increasing as the chain length increases (Ko *et al.*).

The binding domains within the multiubiquitin chains

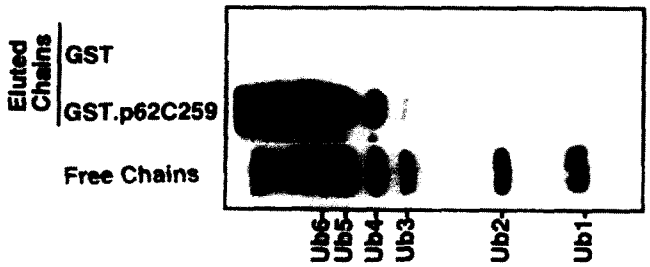

Fig. 1. Preferential interaction of p62 with multiubiquiti chains. GST or GST fused p62 ubiquitin binding region (GST p62C259) was incubated with ^{125}I-labeled multiubiquiti chains, the bound chains were precipitated and eluted; the eluent was separeted by SDS-PAGE and the profile of multiu-biquitin chains analyzed by autoradiography. The mixture o ^{125}I-labeled multiubiquitin chain are included for comparison (free chains).

that interact with Mcb1/S5a involve a hydrophobic patch composed of the side chains of Leu8, Ile44 and Val70 (Cook *et al.*, 1994; Beal *et al.*, 1996). This domain appears to be preferentially exposed on the surface of each ubiquitin unit only in tetra- or longer ubiquitin chains (Cook *et al.*, 1994). This patch presumably interacts with a conserved hydrophobic patch found in the C-terminal half of Mcb1/S5a, which is predicted to form an α-helical structure (Fu *et al.*, 1998; Young *et al.*, 1998). Analysis using several deletion mutants of p62 showed that the C-terminal 80 amino acids are essential for association with ubiquitin chains (Vadlamudi *et al.*, 1996). Although its sequence bears little homology with the binding domain in Mcb1/S5a, this domain is predicted to form three amphipathic α-helix where hydrophobic residues are clustered on one face of each helix (Ko *et al.*). Thus, like Mcb1/S5a, p 62 also may recognize the same hydrophobic motif exposed in the multiubiquitin chains of $n \geq 4$ using the amphipathic helical structure.

p62 forms a novel cytoplasmic "sequestosome" structure

Given its affinity for multiubiquitin chains, it is possible that p62 represents a second chain recognition factor within the 26S proteasome. However, most, if not all, p62 in HeLa cells does not co-purify with the 26S proteasome and thus is not likely to be an integral subunit of the proteolytic complex (Ko *et al.*). Thus, it may be that p62 is loosely associated with the 26S proteasome and dissociates from the complex during purification, or that p62 may be a soluble receptor shuttling ubiquitinated substrates to the 26S proteasome. Alternatively, p62 may form a novel mechanism which modifies the fate of multiubiquitinated proteins.

Immunohistostaining study showed that a large part of p62 is present in the cytoplasmic small punctate structure in normally growing HeLa and Jurkat cells.

Fig. 2. Electron microscopic image of the sequestosome. A electron dense area concentrated with gold labelled anti-p 62 antibody is a typical image of the sequestosome.

However, p62 in this structure is colocalized neither with proteasomal markers nor with late endosomal/lysosomal markers. Thus, the cytoplasmic punctate structure containing p62 is likely the third cellular compartment which regulates the fate of multiubiquitinated proteins. However, relatively small portion of multiubiquitinated proteins were found in the p62 containing punctate structure while most ubiquitin-protein conjugates are diffused in the cytoplasm and nucleus.

Interestingly, treatment of cells with proteasomal inhibitor LLnL or lactacystin induced enlargement of the p62 containing punctate structure with concomitant reduction of the average number. As total cellular p62 level in these cells was only slightly decreased by the same treatment, the enlarged structure is likely caused by fusion of the small punctate structures. Strikingly, the enlarged punctate structure was highly concentrated with cytoplasmic multiubiquitin-protein conjugates. These results indicate that the p62 containing cytoplasmic punctate structure is the compartment into which cytoplasmic multiubiquitinated proteins segregate, and that the degree of the segregation is maximized when the proteasome is malfunctioning. Interestingly, electron microscopic observation shows that p62 localizes in an amorphous structure without membrane confinement (Fig. 2). In order to specify the cytoplasmic punctate structure formed by p62 itself or with ubiquitin-protein conjugates, we propose to use "sequestosome" to describe this unique structure (Fig. 3).

Role of p62 in sequestosome formation

p62 can be devided largely into three domains (Joung *et al.*, 1996; Vadlamudi *et al.*, 1996; Pulse *et al.*, 1997; Sanchez *et al.*, 1998); N-terminal region for interaction with protein kinases, a hinge region with potential modification sites, and C-terminal region

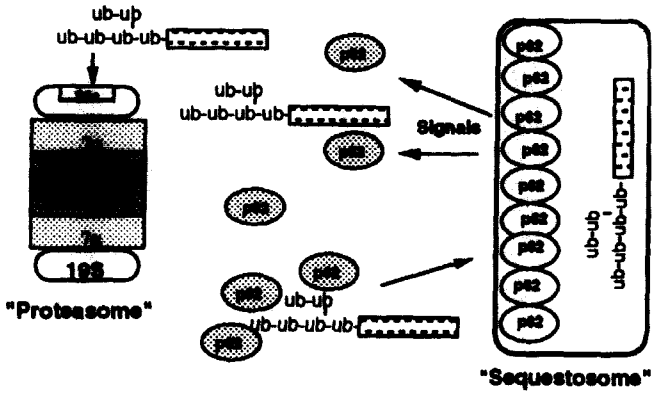

Fig. 3. A hypothetical relationship between the proteasome and the sequestosome. Cytoplasmic ubiquitin-protein conjugates are either degraded in the proteasome or stored in the sequestosome. Proper signals induces release of ubiquitin-protein conjugates from the sequestosome for proteasomal degradation. When proteasomal activity is lowered, most ubiquitin-protein conjugates segregate into the sequestosome.

containing the 80 amino acids long ubiquitin binding domain.Interestingly, p62Δ361, a mutant which lacks the C-terminal ubiquitin binding region, localized in the sequestosome. However, p62Δ361 was not colocalized at all with ubiquitin-protein conjugates. On the other hand, another mutant p62C259, which contains intact ubiquitin binding region but lacks the N-terminal half of p62, diffusely present in the cytoplasm without any sign of sequestosome formation even after proteasomal inhibition. These results indicates that p62 is necessary for the sequestosome formation and the segregation of multiubiquitinated proteins using its N- and C-terminal domains respectively (Ryu *et al.* manuscript in preparation).

Interestingly, a rat p62 homologue translocated from the cytoplasmic punctate structure to the cytosolic diffused form by interaction with PKCζ (Pulse *et al.*, 1997). Thus, p62 may regulate the segregation of cytoplasmic ubiquitinated proteins using its association with protein kinases upon the signals transduced from extracellular environment (Fig. 3).

Transcriptional regulation of the p62 gene

Another factor regulating the sequestosome formation would be expression level of p62. When external stimulus reaches the cell surface, a number of catalytic reactions relay the signals to nucleus for coordinate gene activation (Kieran and Zon, 1996). 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 (Hershman, 1991). Thus far, the best known immediate early response genes are transcriptional regulators such as jun, fos, myc, and egr (Herschman, 1991). Some immediate early genes also encode chemokines, kinases, phosphatases, and ubiquitin hydrolases (Herschman, 1991; Beltman *et al.*, 1996; Zhu *et al.*, 1996; Hedge *et al.*, 1997).

Interestingly, upon a variety signals for proliferation and differentiation, both transcript and protein levels of p62 were rapidly increased and then remained at the same high levels at later time points (Lee *et al.*, submitted). These signals include phorbol 12-myristate 13-acetate and calcium ionomycin for peripheral blood mononu- clear cells, serum or PDGF for the serum-starved NIH3T3 cells, IL-3 for the early G1 arrested pre-B cell line Ba/F3, PMA for a human promyelocytic cell line U937, and an oxidative stress in murine intestinal macrophage (Lee *et al.*, submitted, Ishii *et al.*, 1996). Interestingly, p62 transcription was also enhanced by pretreatment of cells with cycloheximide. Thus, p62 gene activation does not require de novo protein synthesis and can be categorized as an immediate early response gene. Thus, it is expected that the promoter of p62 gene likely has multiple regulatory features in order for responding to such divergent signals in various tissues.

The 5' flanking region of p62 gene lacks both TATA and CAAT boxes (Vadlamudi and Shin). Instead, immediate upstream (within 100 bp 5') of the transcription start site has very G/C rich region which contains three consensus sequences for SP1 transcription factor binding. Further upstream of the 5' flanking region contains a number of binding sites for known transcription factors. These include three TPA-responsive element sites which are known to be high affinity binding sites for heterodimers between Fos- and Jun-related bZip proteins or Jun homodimers (Smith *et al.*, 1993). Another characteristic of the p62 promoter region is the presence of multiple binding sites for Ets-1 family transcription factors, including 2 Ets-1 sites, 6 PEA3 sites, and 2 Pu. 1 sites (Karim *et al.*, 1990). PEA3 is induced by TPA, serum, EGF, v-src, v-raf, or by Ha-ras, thus representing a primary target of mitogenic signal transduction pathways (Karim *et al.*, 1990; D'Orazio *et al.*, 1997). Pu.1 is another Ets-1 family member known to be involved in monocytic differentiation of bone marrow progenitor cells (Rosmarin *et al.*, 1995). Thus, these cis-elements would be responsible, at least partly, for activation of the p62 gene upon signals for proliferation, differentiation, and oxidative stress.

SUMMARY AND CONCLUSION

Proteasomal proteolysis has been known for a long time as a bulk removal system for multiubiquitin conjugated proteins. Physiological significance of this nonlysosomal proteolysis system has been recognized by recent findings of cell cycle dependent and signal dependent proteasomal degradation of cellular regulatory proteins. On the other hand, the sequestosome seems

to regulate the fates of ubiquitinated proteins by segregate and release ubiquitin conjugates. p62, a nonproteasomal multiubiquitin chain binding protein, is likely a key player in the sequestosome formation and regulation. Tight regulation of p62 in response to a variety of extracellular signals strongly suggests that sequestosomal function has essential roles in cell proliferation and differentiation. Further analysis of the role, mechanism, and regulation of p62 in relation to the sequestosomal modification of the fates of ubiquitinated proteins will lead to better understanding of protein metabolism.

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