Proteasome Interactions with Viral and Cellular Proteins

An interaction map of proteasome subunits

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Abstract
Despite the central role of the 26 S proteasome in eukaryotic cells, many facets of its structural organization and functioning are still poorly understood. To learn more about the interactions between its different subunits, as well as its possible functional partners in cells, we performed, with Marc Vidal’s laboratory (Dana-Farber Cancer Institute, Boston, MA, U.S.A.), a systematic two-hybrid analysis using Caenorhabditis elegans 26 S proteasome subunits as baits [Davy, Bello, Thierry-Mieg, Vaglio, Hitti, Doucette-Stamm, Thierry-Mieg, Reboul, Boulton, Walhout et al. (2001) EMBO Rep. 2, 821–828]. A pair-wise matrix of all subunit combinations allowed us to detect numerous possible intra-complex interactions, among which some had already been reported by others and eight were novel. Interestingly, four new interactions were detected between two ATPases of the 19 S regulatory complex and three α-subunits of the 20 S proteolytic core. Possibly, these interactions participate in the association of these two complexes to form the 26 S proteasome. Proteasome subunit sequences were also used to screen a cDNA library to identify new interactors of the complex. Among the interactors found, most (58) have no clear connection to the proteasome, and could be either substrates or potential cofactors of this complex. Few interactors (7) could be directly or indirectly linked to proteolysis. The others (12) interacted with more than one proteasome subunit, forming ‘interaction clusters’ of potential biological interest.

Introduction
The last 10 years have highlighted the essential role of intracellular proteolysis in cell physiology. Indeed, protein breakdown is required not only for removal of abnormal or ‘aged’ proteins, but also to control most biological pathways through the regulated degradation of key positive or negative factors, and to confer an ‘irreversible’ character to cellular transitions. Cell proliferation and differentiation, as well as activation or extinction of signalling pathways, are well-documented examples of processes that bear intimate relationship with proteolysis, and numerous pathologies, including certain cancers, illustrate the deleterious impact on cells of abnormal protein degradation [1].

In eukaryotic cells, the ubiquitin-proteasome pathway is a major actor in intracellular proteolysis [2]. Present in both the cytoplasm and the nucleus, this pathway usually functions in two main ATP-dependent steps. First, the protein to be degraded is tagged for proteolysis by the covalent addition of a polyubiquitin chain, through the action of an enzymic cascade involving three types of enzyme, called E1, E2 and E3 [2]. In this cascade, the E3 component recruits both the substrate and the ubiquitylation machinery, and thus acts as the specificity factor of the reaction. As a consequence, eukaryotic cells contain a very high number of E3 proteins [3]. Once polyubiquitylated, the substrate is then degraded by the 26 S proteasome, an ATP-dependent 2500 kDa multienzyme degradation machine that usually hydrolyses proteins into small peptides without biological functions [4,5]. However, it should be noted that the proteasome can also act in the processing of certain precursors into active proteins [6], and degrade certain proteins without their prior ubiquitylation [7,8].

The 26 S proteasome is thus an essential piece of the intricate molecular circuits that govern cell physiology. Its purification from different species allowed us to define its subcomponents and to understand its overall structural organization. Briefly, the 26 S proteasome is made by the ATP-dependent association of the 20 S proteasome (also...
called the proteolytic core particle), and the 19S regulatory particle that caps the 20S at both ends. The 20S proteasome, a 720 kDa cylindrical structure formed by four stacked rings, is composed of 14 different subunits that, based on sequence similarity, may be classified into two groups, \( \alpha \) and \( \beta \), each group having distinct structural and functional roles. The seven \( \beta \)-subunits form the inner rings of the 20S proteasome, and define an internal cavity that contains six proteolytic sites in eukaryotes. The seven \( \alpha \)-subunits form the outer rings, which control the entry to the catalytic chamber and mediate interaction of the 20S proteasome with its regulators [4,5]. While the crystal structure and the main enzymic mechanisms of the 20S proteasome complex have been elucidated [9], the overall organization and function of the 19S particle are less well defined. The 19S particle appears to be composed of two structurally distinct modules called the base and the lid [10]. The base contains nine different subunits and interacts with the outer \( \alpha \)-rings of the 20S proteasome. Six are related but nonetheless distinct ATPases of the ‘AAA’ family that have non-redundant functions [11] and are collectively involved in substrate unfolding and 20S proteasome activation [12,13]. The yeast lid module is necessary for degradation of ubiquitylated proteins in vitro. It contains eight subunits and appears to be evolutionarily related to the COP9 signalosome and the translational initiation factor eIF3, although it remains unclear whether they have common functions [5,10].

Despite the great level of information available concerning the 26S proteasome, both structural and functional questions remain unanswered. In addition, it is clear that this complex, as it can be purified by classical biochemistry, is only the core of a more complex structure that most probably functions in vivo [14]. To better understand its structure and define its possible interactors, we performed a systematic two-hybrid analysis in yeast, using Caenorhabditis elegans 26S proteasome subunits [15]. For this purpose, the sequences of 29 subunits of C. elegans 26S proteasome, as well as that of the p27 subunit of the proteasome modulator [16], were PCR-amplified from a C. elegans cDNA library and gateway-cloned into two-hybrid vectors. Two complementar-y two-hybrid experiments were then performed. First, all pair-wise combinations of subunits were tested for interactions in order to better define the internal organization of the 26S proteasome. Second, an interaction map was generated by screening the 30 subunits against a C. elegans cDNA library [15].

### Interactions between subunits of the 26S proteasome

Combining the results obtained in both two-hybrid experiments, a total of 25 interactions were detected between proteasome subunits (Table 1). Within the 19S regulatory complex, six potential interactions between the regulatory particle non-ATPase (RPN) and regulatory particle triple-A protein (RPT) subunits (RPN10–RPT3, RPT4–RPT4, RPN3–RPN7, RPN8–RPN9, RPN9–RPN11 and RPN11–RPN11) were novel, while three (RPN1–RPT2, RPT4–RPT5 and RPN8–RPN11) were reported previously in other studies [17–19]. An interaction was also detected between the modulator subunit p27 and the 19S ATPase RPT5, which correlates well with the co-purification of p27 with the ATPases RPT4 and RPT5 [16].

An important question regarding the assembly of the 26S proteasome is how the 20S proteasome and the 19S regulatory complex interact. The association between these two particles is ATP-dependent, and is controlled by phosphorylation [20,21]. Different arguments strongly suggest that the interface between these complexes is mediated by direct contact of the outer \( \alpha \)-rings of the 20S proteasome and the ATPases of the 19S complex [10,22]. However, little detailed information is available on this matter. We detected four potential interactions between two 19S ATPases and three 20S \( \alpha \)-subunits (\( \alpha 2 \)-RPT5, RPT4–\( \alpha 2 \), RPT4–\( \alpha 7 \), RPT4–RPT4). These interactions scored relatively weakly in our two-hybrid system, but are of great potential interest since they could participate in the association of the 20S proteasome and the 19S complex. However, further work will be necessary to understand their biological relevance.

Although our two-hybrid analyses were performed under stringent conditions to minimize the number of false positives, the question of the validity of the interactions detected between the subunits of the 26S proteasome was of particular concern since proteasome subunits are abundant and, a priori, prone to interact with many proteins. To assess this issue, we took advantage of the fact that the structure of the 20S proteasome is known [9]. Indeed, it is thus possible to ask whether the interactions we detected between subunits of this complex are compatible with the crystal structure. We detected 11 pairs of interactors between 20S proteasome subunits. Among these, five (\( \alpha 1 \)-\( \alpha 7 \), \( \alpha 4 \)-\( \alpha 5 \), \( \alpha 6 \)-\( \alpha 7 \), \( \beta 2 \)-\( \beta 3 \) and \( \beta 2 \)-\( \beta 7 \)) could be expected since the subunits involved are direct neighbours in the complex. In addition, the 20S structure suggests that \( \alpha 2 \) is in contact

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**Table 1 | Interactions found between subunits of the 26S proteasome**

<table>
<thead>
<tr>
<th>Location</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within the lid of the 19S complex</td>
<td>RPN3–RPN7, RPN8–RPN9, RPN8–RPN11, RPN9–RPN11, RPN11–RPN11</td>
</tr>
<tr>
<td>Within the base of the 19S complex</td>
<td>RPN1–RPT2, RPN10–RPT3, RPT4–RPT5, RPT4–RPT5</td>
</tr>
<tr>
<td>Between 20S proteasome and 19S complex</td>
<td>( \alpha 2 )-RPT4, ( \alpha 2 )-RPT5, ( \alpha 4 )-RPT4, ( \alpha 7 )-RPT4</td>
</tr>
<tr>
<td>Within 20S proteasome</td>
<td>( \alpha 1 )-( \alpha 6 ), ( \alpha 1 )-( \alpha 7 ), ( \alpha 2 )-( \alpha 2 ), ( \alpha 2 )-( \alpha 4 ), ( \alpha 2 )-( \alpha 6 ), ( \alpha 2 )-( \beta 5 ), ( \alpha 4 )-( \alpha 5 ), ( \alpha 6 )-( \alpha 7 ), ( \alpha 7 )-( \alpha 7 ), ( \beta 2 )-( \beta 3 ), ( \beta 2 )-( \beta 7 )</td>
</tr>
<tr>
<td>Between p27 and 26S proteasome</td>
<td>p27–RPT5</td>
</tr>
</tbody>
</table>
with β5 and that its N-terminal tail interacts with the N-terminal tail of α4 and with a domain of α6. Accordingly, β5–α2, α4–α2 and α6–α2 interactions are present in our two-hybrid map. Thus >70% (8/11) of the observed interactions between 20 S subunits seem legitimate in view of the crystal structure of the complex. The remaining three interactions are α6–α1, which could be explained by the fact that their N-termini are in contact, and two homodimeric interactions (α2–α2 and α7–α7), which could be due to the presence of bridging partners, as they occur in some two-hybrid interactions. The overall degree of confidence in the interaction map between 20 S proteasome subunits thus appears to be quite high, and probably can be extrapolated to the other interactions found within the 26 S proteasome.

### Proteasome interactors

Our second approach was to screen a cDNA library for proteasome subunit interactors. A total of 138 different interacting sequences were found, and since some reacted with several proteasome subunits they collectively represented 94 different genes (see http://vidal.dfci.harvard.edu/Proteasome/data.htm for details). Among them, 16 corresponded to proteasome subunits, and the majority of the others had no clear characterized functions in proteolysis. We also tried to evaluate the potential biological relevance of the results. The number of interactors identified for each subunit varied between 0 and 22, with an average of 4.6. As this average number is similar to that observed for other proteins to proteasome subunits is biologically meaningful.

The [C. elegans] EFT-3 (EF-1α) protein can interact with both RPN2 and RPT4. This protein is a translation elongation factor, which catalyses the GTP-dependent binding of amino acyl-tRNA to the A-site of the ribosome. Interestingly, several reports have suggested a possible role of EF-1α in protein degradation. First, it has been shown to be necessary for the in vitro degradation of certain proteins [24], although its exact function in this process has not been elucidated. Later, biochemical analyses demonstrated that in yeast EF-1α was associated with the proteasome complex under certain conditions [25]. Our own work now suggests that this interaction is direct, since EF-1α can bind to two individual proteasome subunits by two-hybrid analysis would be able to do so when the subunit is incorporated into the complex. It is thus difficult to make any sensible prediction for most of the non-proteasome subunit interactors (58/78) that we obtained, since they interact with a single proteasome subunit and have no clear function within the ubiquitin-proteasome pathway.

The other interactors have a higher likelihood of biological relevance, and fall into two classes. One (12 members; Table 2) comprises interactors that associate with at least two proteasome subunits (and thus form ‘interaction clusters’) while showing no obvious functional link to the ubiquitin-proteasome pathway. Interestingly, two interactors (T08G5.8, an orthologue of Vps39/Vam6-like protein, and H15N14.1, an orthologue of N-ethylmaleimide-sensitive fusion protein (NSF)) appear to belong to the intracellular vesicle trafficking machinery. Among the other interactors, two (W02G9.2, a protein with a kelch motif, and C48D5.1, predicted to be the nuclear hormone receptor NHR-6) were found surprisingly to bind six different proteasome subunits.

The other class (seven members; Table 3) corresponds to proteins that can be directly or indirectly related to the degradation machinery, suggesting that the interaction of these proteins to proteasome subunits is biologically meaningful.

### Table 2 | Proteins interacting with several subunits of the 26 S proteasome

Note that the protein PAL-1 was repeatedly obtained with unrelated proteins and was removed from this list as a likely false positive. NSF, N-ethylmaleimide-sensitive fusion protein.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Comment</th>
<th>Interacting with . . .</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y42H9AR.1</td>
<td>Strong similarity with human DKFZP434D156</td>
<td>α2, α7</td>
</tr>
<tr>
<td>T08G5.5</td>
<td>Similar to the human Vps39/Vam6-like protein</td>
<td>α3, β5</td>
</tr>
<tr>
<td>Y79H2A.1</td>
<td>No clear orthologue or function</td>
<td>α6, β6</td>
</tr>
<tr>
<td>ZK1098.4</td>
<td>Translation initiation factor elf-2B</td>
<td>α6, β6</td>
</tr>
<tr>
<td>C06A8.1</td>
<td>Probable methylene tetrahydrofate reductase</td>
<td>α6, β6</td>
</tr>
<tr>
<td>F57F5.1</td>
<td>Member of the cathepsin B family</td>
<td>RPT5, RPN10</td>
</tr>
<tr>
<td>Y62E10A.14</td>
<td>No clear orthologue or function</td>
<td>RPN2, RPN7</td>
</tr>
<tr>
<td>H15N14.1</td>
<td>NSF required for vesicle-mediated transport</td>
<td>α2, α4, β2</td>
</tr>
<tr>
<td>T28C6.7</td>
<td>No clear orthologue or function</td>
<td>α7, RPT6, RPN1</td>
</tr>
<tr>
<td>W04D2.1</td>
<td>α-Actinin</td>
<td>α1, β3, β5, RPN11</td>
</tr>
<tr>
<td>W02G9.2</td>
<td>Member of the kelch motif family</td>
<td>α2, α4, α5, α6, α7, β6</td>
</tr>
<tr>
<td>C48D5.1</td>
<td>Nuclear hormone receptor NHR-6</td>
<td>α4, α7, RPT2, RPT6, RPN1, RPN12</td>
</tr>
</tbody>
</table>
proteasomal subunits. A recent article provided evidence that EF-1α could have chaperone-like activity, and could play a role in quality surveillance of newly synthesized proteins [26]. Altogether, these data obtained by different laboratories in different organisms and using different approaches strongly suggest that EF-1α could play an important role in the degradation of abnormal proteins by delivering them to the proteasome to promote their rapid degradation.

F55A11.3 (a potential interactor with RPN11) and the chaperone heat-shock protein HSP-3 (a potential interactor with RPN2) are orthologues of the yeast proteins Hrd1p and Kar2p, respectively. These two yeast proteins are both important for endoplasmic reticulum (ER)-associated protein degradation [27]. In this process, abnormal ER proteins are retro-translocated into the cytoplasm and degraded by the proteasome. Since Hrd1p is an ER-bound ubiquitin ligase (E3) with a RING finger motif exposed on the cytosolic side of the ER [28], it is possible that its interaction with RPN11 allows the recruitment of the proteasome to the ER membrane in order to promote rapid removal of abnormal ER proteins. Interestingly, other E3s have recently been shown to interact with the proteasome [25,29,30], suggesting that E3s could play an important role, more general than suspected previously, in bringing together the substrates and the proteasome.

A central question concerning the functioning of the proteasome is how it recognizes its substrates. The 19S subunit RPN10 might be involved in the recognition of ubiquitylated proteins, but since this subunit is dispensable for yeast viability [31], it is commonly accepted that other proteasome subunits and/or bridging proteins are involved in this process. Among the possible bridging proteins are cytosolic chaperones, which are important for the degradation of certain substrates [32], and proteins with a ubiquitin-like domain which are thought to link the ubiquitylation machinery or the chaperone to the proteasome [33,34]. In our work, we found that the cytosolic chaperone HSP-1 interacts with the 19S RPN1 subunit, and that the ubiquitin-related proteins C16C8.16 and C26F1.4 interact with β3 and RPN2 respectively. Interestingly, we also found that RPN1 interacts with the linear polyubiquitin protein UBQ-1. RPN1 and RPN2 are two large subunits of the base of the 19S complex, which are homologous with each other and possess a large domain with a proposed toroid structure which could participate in the unfolding of substrates [35]. Our findings that these proteins interact in two-hybrid experiments with chaperones and ubiquitin (-like)-domain-containing proteins suggest that they could be a major docking site on the proteasome for the delivering of substrates.

**Conclusion**

Our results shed new light on the organization of the 19S regulatory particle and on its interface with the 20S proteasome when assembled into the 26S complex, and suggest new potential substrates or factors involved in proteasome regulation, function and liaison with the ubiquitin machinery. Due to the intrinsic limitations of the two-hybrid approach, especially when studying multimeric complexes, the interactions revealed throughout this work will require further work to be validated. However, a major advantage of our analyses, together with similar studies with other organisms [19,36], is to directly point to the proteasome subunit involved in a given interaction, thus providing important information for future dissection of the biological role(s) of this interaction.

**References**


Table 3 | Proteasome potential interactors previously linked to proteolysis

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Comment</th>
<th>Interacting with . . .</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16C8.16</td>
<td>Ubiquitin-like domain</td>
<td>β3</td>
</tr>
<tr>
<td>F31E3.5</td>
<td>EFT-3, translation elongation factor EF-1α</td>
<td>RPT4, RPN2</td>
</tr>
<tr>
<td>F25B5.4</td>
<td>UBQ-1, polyubiquitin protein</td>
<td>RPN1</td>
</tr>
<tr>
<td>F26D10.3</td>
<td>HSP-1, member of the heat-shock Hsp70 protein family</td>
<td>RPN1</td>
</tr>
<tr>
<td>C26F1.4</td>
<td>Ubiquitin-like protein</td>
<td>RPN2</td>
</tr>
<tr>
<td>C15H9.6</td>
<td>HSP-3, member of the Hsp70 family, similar to mammalian GRP78/BIP</td>
<td>RPN2</td>
</tr>
<tr>
<td>F55A11.3</td>
<td>Similar to human Hrd1p, involved in ER-associated protein degradation</td>
<td>RPN11</td>
</tr>
</tbody>
</table>

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