PART I

ASSESSING IDPs IN THE LIVING CELL

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IDPs AND PROTEIN DEGRADATION IN THE CELL

Yosef Shaul, Peter Tsvetkov, and Nina Reuven
Department of Molecular Genetics, Weizmann Institute of Science, Rehovot, Israel

ABSTRACT

The degradation of the majority of cellular proteins is mediated by the proteasomes. Ubiquitin-dependent proteasomal protein degradation is executed by a number of enzymes that interact to modify the substrates prior to their engagement with the 26S proteasomes. The 26S proteasome is made of two complexes, the 20S and the 19S. The role of 19S is to unfold the proteins to gain entry into the 20S particle, where the protein is cleaved into short peptides. Thus, some of the functions of the 19S complex are expected to be dispensable for degradation of intrinsically disordered proteins, or IDPs. Indeed, in cell-free systems, at least some of the IDPs are digested by 20S particles in the absence of the 19S. In fact, it appears that susceptibility to the 20S proteasome may represent a hallmark of IDPs. Recent evidence suggests that IDPs are susceptible to degradation *in vivo* by the 20S proteasome as well. The process is ubiquitin independent and takes place by default. However, the process of default degradation can be regulated by different strategies. The described process of IDP degradation provokes new predictions and explanations in the field of protein regulation and functionality.

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1.1 INTRODUCTION

The proteins that provide the machinery for growth and maintenance of living cells turn over by complex processes of synthesis and degradation. Thus, the extended polypeptide chain synthesized on the ribosome collapses to a compact molten globule (MG; 80), which subsequently acquires the three-dimensional (3D) structure of the functional protein, with or without the aid of molecular chaperones (49). However, many proteins contain extensive disordered regions, and some are even completely disordered under physiological conditions (42). These natively unfolded (136) or intrinsically disordered proteins (IDPs) are involved in many cellular processes, including transcription regulation and signal transduction (44, 132). Thus, as well formulated by Dyson and Wright (44), functional proteins fall on a structural continuum, ranging from tightly packed proteins, which are almost completely ordered, thus displaying well-defined tertiary structures, through proteins that contain both large-ordered and large-disordered stretches, a good example being the p53 tumor suppressor (15, 52), on to whole proteins such as the ~440-residue tau protein, which appears to be ID from its NH_2-terminus to its COOH-terminus (123). Furthermore, in multidomain proteins, tightly packed and ordered domains may be connected by ID flexible linker sequences, as is the case for a construct that contains the first three zinc fingers of transcription factor-IIIA (24).

Protein degradation plays an important role in almost every basic cellular process. The selective degradation of many short-lived proteins in the cell is mediated via the ubiquitin-26S proteasomal degradation pathway (54, 60). Ubiquitin, a 76-amino acid residue protein, is covalently conjugated in a highly regulated multistep process to the substrate protein, marking it for degradation by the 26S proteasomes. Recent discoveries in the field of protein degradation have revealed that several proteins are also susceptible to ubiquitin-independent degradation that is mediated via the core 20S proteasomes (10). New evidence raises the interesting possibility that IDPs are subjected to this pathway of degradation that does not require prior modification, a process we refer to as “degradation by default.”

In this chapter, the evidence for IDPs degradation by the default mechanism, both *in vitro* and in the cells, is summarized. In addition, the biological outcomes and the unique contributions of IDPs’ susceptibility to degradation by default are addressed. Given the fact that this concept is in its infancy, some of the proposed models call for additional experimental data.

1.2 PROTEASOMAL DEGRADATION

Protein degradation, a pivotal biochemical process, regulates many basic aspects of cellular behavior. Degradation of the majority of cellular proteins is carried out by the proteasomes (section 1.3). In the ubiquitin–proteasome

degradation pathway, substrates are marked by covalent linkage to ubiquitin chains to be recognized and degraded by the 26S proteasome (54, 61, 63). Ubiquitination generally requires three proteins: the ubiquitin-activating enzyme (E1), the ubiquitin-carrier or conjugating enzyme (E2), and the ubiquitin ligase (E3). The E1 enzyme transfers the activated ubiquitin to one of many E2 carrier enzymes, which, in combination with one of many E3 ubiquitin ligases, transfer the ubiquitin monomer to the substrate. The E3 ubiquitin ligase binds to both the substrate and the E2 enzyme, assembling them in the correct position to allow efficient ubiquitin transfer. E3 ubiquitin ligases can either be single proteins or protein complexes. Ubiquitin is conjugated to its target protein in most cases by the formation of an isopeptide bond between its carboxyl group of glycine 76 (the carboxy terminus) and the epsilon amino group of one or more lysine residues or N-terminal amino group of the target protein (34, 61). Additional ubiquitin molecules are added to the lysine 48 residue of the previous ubiquitin in the chain. Specificity of the ubiquitination reaction is determined by the E3 ubiquitin ligase (61, 104).

Many cellular proteins are substrates of ubiquitin-mediated proteasomal degradation. The tumor suppressor p53 is one of the best studied substrates of the ubiquitin proteasome degradation pathway. Wild-type p53 is a short-lived protein that accumulates following various types of stress and induces growth arrest or apoptosis. The level of p53 is tightly regulated by the rate of its proteasomal degradation (138). A major regulator of p53 is Mdm2, an E3 really interesting new gene (RING) finger ubiquitin ligase that binds to the amino terminal transactivation domain of p53 and ubiquitinates p53 (59, 79). p53 is further polyubiquitinated either by p300 or Mdm2, and the polyubiquitinated p53 is eventually degraded by the 26S proteasomes. Mdm2 is a RING domain protein that binds and ubiquitinates p53, directing p53 to 26S proteasomal degradation. Mdm2 is transcriptionally upregulated by p53, thus establishing an autoregulatory negative feedback loop. p53 is also ubiquitinated by several other independent E3 ligases. In addition, it is ubiquitinated following its recruitment into a complex containing the viral E6 protein of the human papilloma viruses (HPVs) and the E6-associated protein, which serves as an E3 HECT domain ubiquitin ligase (117).

While ubiquitin-dependent proteasomal degradation is likely to represent the primary means of proteasomal degradation for most proteins, there are a number of proteins that have been demonstrated to undergo proteasomal degradation that is ubiquitin independent (reviewed recently by Jariel-Encontre et al. [65]). Ornithine decarboxylase (ODC) was the first protein found to undergo ubiquitin-independent degradation by the proteasome, and it is the most well studied. ODC is the first rate-limiting enzyme in polyamine biosynthesis that catalyzes the decarboxylation of ornithine to form putrescine. In its active and stable form, ODC is a homodimer with two enzymatic active sites. Binding of antizyme, a polyamine-induced protein, to ODC disrupts ODC homodimers and exposes the C-terminal region of ODC, inducing ubiquitin-independent 26S proteasomal degradation of ODC (37). The mam-

malian ODC carboxy-terminal 37 amino acids constitute a degron necessary and sufficient to destabilize ODC. This region is a molecular mimic of a polyubiquitin chain (143) and also provides an unstructured or loosely structured region from which degradation can initiate (126).

1.3 THE VARIOUS COMPLEXES OF THE PROTEASOMES

In eukaryotic cells, most proteins are degraded by the 26S proteasome, a huge molecular machine designed for controlled proteolysis. It degrades the majority of cellular proteins in eukaryotes. It controls the levels of various regulatory proteins and prevents the accumulation of misfolded mutant and damaged proteins (61). The 26S proteasome is comprised of a 19S regulatory complex and the 20S protealytic core. The symmetrical core 20S proteasome consists of 28 subunits arranged in a cylindrical form of four rings. Seven different alpha and beta subunits are assembled as four heptameric rings to form the 20S catalytic core particle. The rings are stacked on top of each other to form a hollow cylindrical structure (57). Beta subunits in the middle of the complex provide the proteolytic activity with little specificity for their substrates. Alpha subunits form the external rings of the cylinder to generate the entry gates (56). The 20S proteasome is in a latent state, since entry is gated, and therefore, the substrate entry is the rate-limiting step for hydrolysis.

The 19S regulatory complex is composed of about 18 polypeptides, six of which are ATPases. It associates with the small ends of the 20S core particle at the entrance to the degradation channel. The 19S complex can be dissociated into two subcomplexes, the "lid" and the "base." The lid possesses the subunits for recognition of polyubiquitin chains. The base, containing the ATPase activity, binds and unfolds proteins, the "reverse chaperone" activity, to unfold substrates in preparation for their degradation by the 20S core (150). The base also controls the gating to the degradation channel (124). Together, the lid and the base of the 19S regulatory complex recognize the polyubiquitin chains and catalyze substrate deubiquitination, denaturation, and translocation into the 20S catalytic core for degradation.

Correctly folded globular proteins in their "native" conformation should not be readily susceptible to 20S proteasomal attack unless denatured by the regulatory 19S subunit (68, 140). Interestingly, the proteolysis of tightly folded proteins by the 26S proteasome is greatly accelerated when an unstructured region is attached to the substrate (107). It has been proposed that the unstructured region acts as a site for initiation of unfolding. This region, which may lie at the terminus of the protein or constitute a surface loop, is the first portion of the substrate to enter the proteasome, a process that improves substrate unfolding. Thus, a ubiquitin chain is sufficient for proteasome docking, but unfolding cannot be initiated efficiently in the absence of an unstructured initiation site.

The proteasome 20S core particle in isolation is active in degradation of certain proteins. It has been proposed that unfolded protein substrates are fed into the 20S proteasome via their gates as extended chains and are degraded progressively (93, 139). Liu and coworkers, however, have provided evidence for susceptibility of internal disordered regions to endoproteolytic activity of the proteasome (84), suggesting that a disordered protein can be degraded by the proteasome in a unique manner as discussed below.

There are two additional and smaller protein complexes, PA28α/β (REGα/β) and PA28γ (REGγ), that belong to the REG or 11S family of proteasome activators that have been shown to bind and activate the 20S proteasome based on their ability to promote the degradation of model peptide substrates. The REG complex is an ATP-independent and ubiquitin-independent proteasome activator that strongly enhances the catalytic activity of proteasomes *in vitro* (110). REGs are rings composed of seven subunits that bind the alpha rings (the gates) of the 20S proteasome and activate peptide hydrolysis. Of the three REG family members, REGα and REGβ subunits form a heteroheptameric complex, whereas REGγ forms a homoheptameric complex with a molecular mass of about 200 kDa (111). REGγ has a nuclear-restricted expression pattern and can be found independently or associated with 20S proteasomes. REGγ is found in worms, insects, and higher animals, whereas REGα and REGβ are found only in vertebrates (86). Originally, REGγ had been only characterized in terms of its ability to degrade small peptide model substrates *in vitro*, and it was initially thought that REGγ is not involved in the destruction of intact endogenous proteins. As discussed below, REGγ directs degradation of proteins by the 20S proteasome in an ATP-independent and ubiquitin-independent manner.

1.4 IDPs AND DEGRADATION BY 20S PROTEASOME *IN VITRO*

The architecture of the proteasome suggests that correctly folded globular proteins in their "native" conformation are too big to enter the gates of the 20S proteasome and would not be readily susceptible to 20S proteasomal attack unless denatured by the regulatory 19S subunit. It has been proposed that structured substrates are denatured prior to being fed into the 20S gates as extended chains to progressively undergo degradation (93, 139). However, IDPs override this requirement. For example, p21 and α-synuclein are unstructured proteins and are directly susceptible to uncapped 20S proteasomal degradation (21, 131), possibly by partial opening of the 20S gates.

The differential behavior between IDPs and the globular proteins can be easily demonstrated *in vitro* where only IDPs are susceptible to the 20S proteasome digestion (135). This is also the case with partially disordered proteins, such as p53 (see section 1.7). IDPs are completely degraded to smaller fragments. However, certain motifs may be resistant to degradation as was reported for the polyglutamine chain (137).

Well-defined preparations of proteins in a long-lived MG state, in which the protein is devoid of most of its tertiary structure, but retains its secondary structure, and is still quite compact, are also resistant to the 20S proteasome. The MG species are significantly more flexible than the corresponding native species, based on their capacity to undergo disulfide reshuffling, as well as their being highly susceptible to proteinase K. Nevertheless, they apparently do not present sufficiently extended unstructured sequences to the 20S proteasome to permit their penetration into its cavity so as to undergo peptide bond cleavage (135). Given that folded proteins are resistant to proteinase K and 20S digestion, that IDPs are sensitive to both 20S and to proteinase K digestion, and that proteins in the MG state are sensitive to proteinase K degradation only, the utilization of these two digestion protocols in tandem provides a convenient operational tool for discriminating between the three states (135). The susceptibility to 20S proteasomal degradation is a simple, rapid, and reliable method of operationally defining IDPs and unstructured sequences. Furthermore, utilization of this method could lead to the characterization of motifs that sensitize IDPs to proteasomal degradation.

Certain structured proteins contain one or more disordered regions. The question is whether there is a specific requirement for the position of the disordered region in order to be sensitized to degradation *in vitro* by the 20S proteasome. Location of a disordered region either at the N-terminus or the C-terminus facilitates proteasomal degradation in general (107). Interestingly, however, the susceptibility to 20S proteasomal digestion was maintained even when these IDPs were hooked at both N and C-termini to GFP, a highly structured protein, suggesting that internal disordered regions are susceptible to endoproteolytic activity of the proteasome (84). Thus, the location of the disordered region seems not to follow a simple rule.

The ability of the core 20S proteasomes to degrade primarily unstructured proteins and protein regions can be utilized as an assay for the identification of such protein regions. The protein of interest can be incubated *in vitro* with purified 20S proteasomes, and the degradation products can be analyzed with the expectation that the ID region will be degraded first. Furthermore, in cases when a protein folds upon binding to its biological counterpart, such an assay can be a valuable tool for identifying the interacting target protein or molecule as these are expected to rescue the protein from the 20S proteasomal degradation. More importantly, such a biological assay enables us to examine whether a suspected candidate protein is degraded by the 20S proteasomes and protected upon binding to its biological counterpart.

1.5 *IN VITRO* STABILIZATION OF IDPs

It has been estimated that 36–63% of the proteins in eukaryotic proteomes contain long sequences that are ID *in vitro* (43). This does not imply that these IDPs, or protein sequences, are necessarily disordered *in vivo*. On the con-

trary, in many cases, convincing evidence has been presented that they form functional complexes within the cell, adapting a well-defined conformation upon interaction with their partner(s). Based on this observation, it was predicted that protein–protein interactions can protect IDPs from 20S proteasomal degradation. This may be achieved either by masking the unstructured domain or by folding it. We have recently demonstrated the validity of this principle experimentally (135). It has further been argued that their ID character may enable IDPs to form relatively low-affinity complexes, thus permitting plasticity and rapid turnover, and, consequently, to serve as hub proteins interacting with multiple partners (41). These kinds of interactions may be enough to protect IDPs from 20S proteasomal degradation.

The Cdk inhibitor p21, a naturally unstructured protein, is a critical regulator of cell division and DNA replication (21). In the cells, p21 proteins are rarely found in a "free" form and are almost always detected as a part of a complex, raising the possibility that the "free" p21 is rapidly degraded in the cells. *In vitro* degradation studies with p21 show that this protein is degraded by the 20S proteasomes in a ubiquitin-independent manner. Degradation of p21 is inhibited upon binding of p21 to the proliferating cell nuclear antigen (PCNA) or in the presence of the cyclin E and Cdk2 complex (20, 38, 134). p21 is just one example of an IDP that is stabilized by its binding partners. More examples exist, as in the case of p53 and other proteins discussed below and specified in Table 1.1.

1.6 UBIQUITIN-INDEPENDENT PROTEASOMAL DEGRADATION *IN VIVO*

As discussed in section 1.2, recognition of substrates by the 26S proteasome is typically accomplished through ligation of a polyubiquitin chain to one or more internal lysine residues or to the N-terminal amino group of a target substrate. Once bound to the proteasome, the polyubiquitinated substrates must be unfolded and inserted into the proteasome's catalytic chamber, where proteolysis occurs. As discussed in this chapter, a growing number of IDPs are subjected to proteasomal degradation without prior ubiquitination. Experimental strategies were formulated to demonstrate that proteasomal degradation of a given protein is neither ubiquitinated nor dependent on ubiquitination. The key experiments are:

1. Biochemical analysis ruling out the possibility that ubiquitin moieties are attached to the substrate.
2. Demonstration that polyubiquitinated substrates do not accumulate in cells treated with known chemicals that inhibit proteasomes. However, certain proteins are subjected to both ubiquitin-dependent and ubiquitin-independent pathways; therefore the fact that protein is ubiquitinated does not rule out the possibility that it is subjected to the other

TABLE 1.1 IDPs Suspected to Undergo Degradation by Default

Protein	Ubiquitin-Independent Proteasomal Degradation	26S/20S *In Vitro* Degradation	Binding Stabilizes	Unstructured
α-Catenin	(64)	—	—	FoldIndex† (255 disordered residues, 11 segments)
α-Synuclein	(131)	(84, 131)	—	DisProt (completely)
Calmodulin	(128)	(16, 48, 128)	Ca^{2+} (16, 48, 128)	DisProt (77–81)
Casein	—	(73)	—	DisProt (completely)
C/EBPδ	(149)	—	(149)	FoldIndex (207 disordered residues, 4 segments)
c-Fos	(22)	—	—	DisProt (216–380)
Cholera toxin	—	(98)	CTA2 (98)	FoldIndex (132 residues, 4 segments)
c-Jun	(66)	(66)	—	DisProt (185–235)
DHFR	—	(2)	MTX (2)	DisProt (43 residues, 5 segments)
eIF4F and eIF3	(14)	(14)	—	DisProt (393–490)
Fra-1	(13)	—	—	High homology to c-Fos
GRK2	—	(102)	—	FoldIndex (367 residues, 6 segments)
HIF-1α	(74)	(74)	—	(115)
IκBα	(76, 87, 103)	(1, 78, 87)	p65 (1, 76, 87, 94)	DisProt 1–66, 276–317)
NF-κB (p65)	(91)	(91)	—	FoldIndex (167 residues, 8 segments)
ODC	(114)	(4, 17, 18, 36)	NQO1 (4)	DisProt (1–35, 158–165, 297–311, 412–425)
$p14^{ARF}$	—	(105)	TBP-1 (105)	DisProt $p19^{ARF}$ (1–37)
p16	(30)	(30)	—	(127)
p21	(119) REGγ (30, 82)	(30, 82, 84, 134)	PCNA (134) Cdk/cyclin (82) cyclinE/Cdk2 (30)	DisProt (completely)

Table 1.1 Continued

Protein	Ubiquitin-Independent Proteasomal Degradation	26S/20S *In Vitro* Degradation	Binding Stabilizes	Unstructured
p33ING1b	(53)	(53)	NQO1 (53)	FoldIndex (165 disordered residues, 2 segments)
p53	(7, 27)	(11, 55)	NQO1 (11) NQO2 (55)	(15)
p73	(11)	(11)	NQO1 (11)	DisProt* (549–564)
Pertussis toxin S1	—	(97, 98)	NAD^+(97)	FoldIndex (70 residues, 6 segments)
PrP	—	(129)	—	DisProt (23–120)
Rb	(70, 118)	(118)	—	FoldIndex (319 disordered residues, 12 disordered segments)
RPN4	(69)	—	—	FoldIndex (394 disordered residues, 5 segments)
SRC-3	(83)	(83)	—	FoldIndex (831 residues, 30 segments)
Tau	—	(28, 39, 106, 145)	Hsc70, BAG-1 (45)	DisProt (completely)
Thymidylate synthase	(51)	—	—	(51)
Troponin C	—	(16)	Ca^{2+} (16)	DisProt (103–158)
YB-1	(125)	(125)	RNA (125)	FoldIndex (246 residues, 2 segments)

Note: This table provides a partial list of proteins suspected to undergo degradation by default as defined by parameters in section 1.10. References are provided for: UI degradation *in vivo*, 20S/26S proteasomal degradation *in vitro*, stabilization by binding to a protein or co-factor and intrinsic disorder. In addition to specific citations regarding structure, the DisProt database and the FoldIndex program were used to identify proteins listed here as disordered.

*The Database of Protein Disorder (DisProt) is a curated database that provides information about proteins that lack fixed 3D structure in their putatively native states, either in their entirety or in part. DisProt is a collaborative effort between the Center for Computational Biology and Bioinformatics at Indiana University School of Medicine and the Center for Information Science and Technology at Temple University (122).

†FoldIndex©: a tool to predict whether a given protein sequence is intrinsically unfolded (108).

CTA2, cholera toxin A2 polypeptide; DHFR, dihydrofolate reductase; GRK2, G protein-coupled receptor kinase 2; HIF, hypoxia-inducible factor; MTX, methotrexate; RPN4, Regulatory Particle Non-ATPase.

mode of degradation. Also, since lysine is the site of ubiquitin ligation in most molecules targeted for proteasomal degradation, “lysine-less” substrates, usually with lysine residues replaced by arginine, are used to show that degradation is not compromised. However, the maintenance of normal protein degradation in “lysine-less” mutants does not rule out the possibility that ubiquitin ligation occurs at the N-terminal amino group (20). Therefore, it is important to show, often by MS measurements, that the N-terminal end is blocked by N-acetylmethionine. For these reasons, these methods are insufficient to show ubiquitin-independent degradation for proteins that are susceptible to both pathways.

3. Genetic manipulation offers another method to demonstrate the ubiquitin-independent nature of the proteasomal degradation. A conventional tool is a cell line that bears a temperature-sensitive E1 mutant, such as the A31N-ts20 cell line (33). E1 is the first enzyme in the consecutive steps of protein ubiquitination, and under the restrictive condition (i.e., high temperature), the primary vertebrate E1, encoded by Ube1, is inactivated, and therefore, proteasomal degradation under this condition provides biological evidence for ubiquitin-independent degradation of the substrate. Using these approaches, an increasing number of proteins have been shown to be degraded by the proteasome in a ubiquitin-independent manner, including ODC (4), inhibitory κBα (IκBα) (76), p53 (7), retinoblastoma protein (Rb) (70), T-cell receptor α (142), c-Jun (66), calmodulin (128), and thymidylate synthase (TS) (51, 101). For some of these, degradation is completely independent of ubiquitination, while for others, it occurs through both ubiquitin-dependent and ubiquitin-independent pathways. Recently, an alternate ubiquitin-activating E1, called Ube1L2, Uba6, or E1-L2, has been discovered (32, 67, 100). Although this E1 appears to interact with a different subset of E2s than Ube1, this finding demonstrates the complexity of the ubiquitin-proteasome system and the need to use several experimental systems to prove that a particular protein is being degraded in a ubiquitin-independent manner.

TS is an S-phase enzyme involved in thymidine 5′-monophosphate synthesis. Recently, it was shown that degradation of human TS is carried out by proteasomes in a ubiquitin-independent fashion (51, 101). The N-terminal region of the polypeptide was identified as a primary determinant of this degradation (51). The region is disordered in X-ray crystallographic structures (29). Deletion of as few as six amino acids from the N-terminal end of the molecule elicits marked stabilization of the enzyme, with further deletions resulting in varying degrees of stability (51). Thus, the disordered N-terminal domain seems to mediate recognition by the proteasome and to target the polypeptide for proteolytic destruction. This domain is sufficient to destabilize an evolutionarily distinct TS molecule, indicating that it functions as an independent degradation signal (101).

As exemplified by ODC degradation, a ubiquitin-independent pathway is executed by the 26S proteasome and not necessarily by the 20S proteasome. However, in that case, a second protein, antizyme, is required for targeting of ODC to the 26S particle. However, the ODC case appears to be an exception, since the emerging picture is that the 26S proteasome is not the catalytic particle in many other cases of ubiquitin-independent degradation. As discussed below, other particles, such as 20S either in isolation or in association with REG or other proteins, may provide the catalytic domain in the process of ubiquitin-independent degradation.

1.7 *IN VIVO* DEGRADATION OF IDPs

An important question is whether IDPs are present as such within the living cell. By analogy with the *in vitro* scenario, it might be possible to address this question by the mode of degradation to which IDPs are subjected. A growing number of proteins bearing disordered regions are reported to undergo ubiquitin-independent degradation. However, we are not yet in the position to say that a given protein that undergoes ubiquitin-independent degradation has to contain disordered regions. As discussed below, another angle is susceptibility to the 20S proteasome or related particles that lack the 19S particle with the "reverse chaperone" activity. Susceptibility to the 20S proteasome *in vitro* may be extended in the future to address the issue of the status of IDPs *in vivo*, either by inducing the 20S proteasomal degradation or by selectively blocking 26S proteasome activity, but not that of the uncapped 20S proteasome. Proteins found to undergo proteasomal degradation under such conditions are likely to have retained their ID status *in vivo*. In addition, IDPs are protected from 20S proteasome digestion *in vitro* when they are in association with a partner. This principle of "interact to protect" may be applied for *in vivo* analysis as well to further demonstrate the ID nature of a given protein. Furthermore, in the future, this kind of assay should enable identification of novel binding partners of a given IDP.

An example is the tumor suppressor p53, which is a short-lived protein that accumulates following exposure to different types of stress and induces cell cycle arrest or apoptosis. Regulation of p53 stability plays a central role in the control of proper function of p53. Degradation of p53 has been intensively studied, and indeed, p53 has become a hallmark for ubiquitin-dependent 26S proteasomal degradation. Several specific E3 ubiquitin ligases were reported to bind and polyubiquitinate p53, marking it for degradation by the 26S proteasomes (see references 7–10 in Asher et al. [10]; 40, 59, 79, 81). Both the p53 N-terminal transactivation domain and the C-terminal regulatory domain were identified as unstructured regions (15). These unstructured regions may facilitate and serve as a signal for degradation by the core 20S proteasomes. Interestingly, p53 is also susceptible to degradation in a ubiquitin-independent manner in the cells that are mediated via the core 20S proteasomes (5–9, 11).

The c-Fos proto-oncogene is a component of activator protein-1 (AP-1) transcription factor regulating numerous cellular processes. c-Fos acts as an obligatory heterodimer with members of basic region leucine zipper proteins from the JUN, ATF, and MAF families (31, 62), and binds to specific DNA motifs via a basic domain N-terminally adjacent to the leucine zipper domain. The C-terminal half of c-Fos is ID (26), and *in vitro* c-Fos is susceptible to 20S proteasome digestion (see Jariel-Encontre et al. [65]). This region is involved in ubiquitin-independent degradation of c-Fos (22). Fra-1, a related protein, appears to behave in a similar manner. Indeed, enhanced GFP is destabilized by a 40-amino acid C-terminal fragment of Fra-1 (13). Alternative pathways may contribute to c-Fos degradation in different subcellular compartments, in the nucleus ubiquitin-independent but in the cytoplasm ubiquitin-dependent (116). It will be interesting to investigate whether or not ubiquitin independence in the nucleus may be attributed to the nuclear REGγ-20S complex.

These examples, and the others listed in Table 1.1, suggest that two distinct proteasomal pathways can degrade IDPs *in vivo*. The ubiquitin-dependent pathway is a common feature of many proteins including long-lived structured proteins in addition to IDPs. In contrast, ubiquitin-independent proteasomal degradation, with the exception of ODC, has been mainly shown to degrade IDPs, raising the possibility that the ubiquitin-independent degradation is a specific degradation pathway common to IDPs.

1.8 IDPs AND *IN VIVO* EVIDENCE FOR DEGRADATION BY THE 20S PROTEASOME

IDPs are subjected to degradation by the conventional ubiquitination pathway involving ATP-dependent substrate denaturation by the 19S cap particle. However, as summarized above, a growing number of proteins undergo degradation in a ubiquitin-independent manner. Given the fact that IDPs are unstructured, ATP-dependent substrate denaturation by the 19S particle is dispensable. Therefore, in principle, IDPs may undergo degradation by the 20S proteasome. To date, it is rather difficult to distinguish the activity of the 26S from that of the 20S in the cells. A major problem is that the known chemical inhibitors of the 26S proteasome inhibit 20S as well. This introduces confusion in the field since many reports have demonstrated the involvement of the 26S proteasome in their system by simple utilization of these inhibitors, ignoring the possibility of coinhibition of the "free" and uncapped 20S proteasome as well. Therefore, in this chapter, we prefer to refer to these cases as 26S/20S proteasome. This will include any proteasome containing the 20S core particle regardless of the presence and the nature of the cap particle. One strategy to determine whether the uncapped 20S proteasome is responsible for IDPs degradation is to eliminate subunits from the 19S particle and, therefore, to reduce or eliminate 26S proteasomes in the cells. This can be achieved by knocking down crucial subunits of the 19S regulatory complex that would

prevent the association of the 19S complex to the 20S proteasome. In addition, proteasomal ATPase-associated factor 1 (PAAF1) protein has been shown to associate with the ATPases of the 19S regulatory complex, and overexpression of PAAF1 interferes with the association of the 19S with the 20S proteasomes (99). Thus, PAAF1 can increase the level of 20S proteasomes, and susceptibility to PAAF1 would provide an additional way to determine the involvement of the uncapped 20S proteasome in the process. We anticipate that these strategies will help answer the question as to whether the 20S proteasome degrades IDPs *in vivo*.

Another possible strategy would be to find the proper physiological settings to alter the 20S/26S ratio. For example, starvation to glucose increases the relative amount of the uncapped 20S proteasome in yeast cells and changes proteasomal distribution in mammalian cells (12, 96). Oxidative stress was also shown to alter the activity of the 20S/26S proteasomes (112, 113). However, at the moment, it is not clear what physiological settings elevate the 20S/26S proteasome ratio in mammalian cells.

There are several examples of IDPs that are degraded *in vitro* by 20S proteasomes and *in vivo* in a ubiquitin-independent manner that does not require interaction with E3 ubiquitin ligase or any other tagging such as polyubiquitination. For example, the degradation of p53 and its related family member p73 both exhibit these characteristics (11). Because susceptibility to this degradation is determined by the inherent characteristics of the protein, we refer to it as degradation by default (see section 1.10). By analogy with the *in vitro* system, our model is that *in vivo* degradation by default is carried out by the uncapped 20S proteasomes. The methods delineated above will be useful in definitively establishing that the 20S proteasomes are responsible for default degradation. The present *in vitro* and *in vivo* data at hand suggest that this is the case.

ODC is an interesting example since it undergoes ubiquitin-independent degradation via both 26S and 20S proteasomes. The former is true for the homodimer complex, whereas the latter is true for the ODC monomer (4). Degradation of ODC monomers by the 20S proteasomes does not require tagging (such as polyubiquitination) or protein–protein interaction, suggesting that these monomers are inherently unstable and are degraded by the 20S proteasomes in the cells. In contrast, ODC dimers are resistant to degradation by the 20S proteasomes. Remarkably, antizyme that binds ODC also sensitizes ODC to degradation by the 26S proteasome but inhibits it from degradation by the 20S proteasome (4). This switching mechanism suggests a cross talk between these distinct pathways.

1.9 IDPs AND *IN VIVO* DEGRADATION, THE ROLE OF REGγ

As detailed in section 1.3, in the cells, some of the 20S particles are capped with two smaller protein complexes, PA28α/β (REGα/β) or PA28γ (REGγ),

both members of the 11S family of proteasome activators. The REG complex is an ATP-independent and ubiquitin-independent proteasome activator that strongly enhances the catalytic activity of proteasomes *in vitro* (110). REGγ has been only characterized in terms of its ability to degrade small peptide model substrates *in vitro*, but recently, evidence was provided for REGγ to direct degradation of proteins by the 20S proteasome in an ATP-independent and ubiquitin-independent manner.

REGγ interacts with and enhances the proteolysis of several proteins such as HCV core protein (92), steroid receptor coactivator-3 (SRC-3) protein (83), p21 (30, 82), $p19^{ARF}$, and p16 (30). REGγ enhances 20S proteasome-mediated degradation of these proteins in a ubiquitin-independent and ATP-independent manner. Given the fact that this process does not require the "reverse chaperone" activity of the 19S, it has been speculated that IDPs are the natural substrates of the REGγ-activated 20S proteasome (83). $p19^{ARF}$, p21, and p16 are all IDPs when not associated with specific binding partners (such as cyclins and Cdks, for p21 and p16, and nucleophosmin in the case of $p19^{ARF}$). Moreover, although monomeric p21 is unstructured, the great majority of p21 *in vivo* is highly structured within p21–cyclin–Cdk complexes. Therefore, the susceptibility to degradation by this pathway is likely to be confined to the monomer. Certain truncation mutants of p21, which are predicted to be unstructured like the full-length p21, are not substrates of the REGγ pathway. Therefore, unfolded structure might be necessary but not sufficient.

1.10 IDPs AND DEGRADATION BY DEFAULT

In eukaryotes, the conventional model of ubiquitin-dependent proteasomal degradation argues that the regulatory step is in the hands of the E3 ligase group of enzymes (see above). A given protein is stable unless polyubiquitinated by a specific E3 ligase, a modification that targets the modified protein to the 26S proteasome, the major protein destructive machine. In addition to this conventional model of "modification to degradation," the IDPs also seem to undergo degradation without prior modification. Furthermore, unlike the ODC case, IDPs do not require an antizyme-like entity to target the substrate to the proteasome. Therefore, IDPs appear to be inherently susceptible to uncapped 20S degradation. We regard this mechanism as degradation by default, that is, the protein is digested by the 20S proteasome unless it actively escapes this process, such as through interaction with a partner. To reach the conclusion that a protein is subjected to proteasomal degradation by default, the following observations have to be made:

1. The protein is digested *in vitro* by the 20S proteasome.
2. The *in vitro* degradation is blocked in the presence of an interacting protein.

3. In the cells, the protein is degraded by proteasomes in the absence of a functional ubiquitin system.
4. The half-life of the protein is increased under high expression of an interacting protein.
5. Knocking down the partner protein destabilizes the substrate.

The definition is operational at the moment, and in the future, new strategies are expected to be developed. For example, at least some of the proteins, such as p53, are stabilized by NQO1, an NADH-dependent enzyme that is in association with the 20S proteasomes (see below). We have been able to show that the p53 degradation process meets all the experimental criteria of degradation by default. There are many other proteins that meet at least some of these criteria (as listed in Table 1.1), and additional experiments are needed for their better classification. Degradation by default is a regulated process, and some of the 20S-associated proteins are suspected candidates for accelerating or inhibiting the process. In addition, when our knowledge of the molecular basis of substrate targeting to the uncapped 20S proteasome is improved, we will be in a better position to identify the substrates of the process of degradation by default.

This operational definition can be used to reevaluate some of the published observations. For example, the protein α-catenin undergoes ubiquitin-independent proteasomal degradation, but this process is blocked by the interacting β-catenin (64). Therefore, in accordance with our experimental criteria, α-catenin is likely to undergo degradation by default. To fulfill all the criteria, we have to demonstrate that α-catenin is a substrate of the uncapped 20S proteasome. Although no experiments were performed to meet these criteria, some hints are provided by sequence analysis. Using different algorithms to predict the structure of α-catenin, we found that this protein is expected to contain several unstructured regions and therefore might be a putative substrate of the uncapped 20S proteasomes in the cells. This prediction can be easily challenged by using the *in vitro* assay described above.

1.11 PROTEASOME TARGETING OF IDPs

Proteasome targeting of proteins is a crucial step in degradation. In the ubiquitin-dependent proteasomal degradation pathway, the 19S cap particle binds to the polyubiquitin chains and catalyzes substrate deubiquitination, denaturation, and translocation of the unfolded substrate into the 20S catalytic core for degradation. A major role of polyubiquitination is to target proteins to proteasomes. The actual degradation step requires that ubiquitinated proteins first be denatured and deubiquitinated, to allow them to fit into the narrow channel of the core particle. The polyubiquitin chain is not crucial for proteasomal degradation per se as has been first demonstrated for ODC (18). Thus, the substrates for the actual proteolysis are unfolded and nonubiquitinated.

Therefore, if a protein can be delivered to the proteasome in a denatured or partially unfolded state, ubiquitination might be required for substrate targeting but not for its degradation.

Direct binding of the substrates to the uncapped 20S via its different subunits could provide a possible mechanism for targeting of IDPs for proteasomal degradation. Consistent with this model, it has been shown that p21, a substrate of uncapped 20S proteasome, binds α7, a 20S subunit (134). The authors proposed that this physical interaction is responsible for p21 targeting to the proteasome for degradation. However, although p21 can bind to isolated α7, it has not been possible to detect p21 in complex with the uncapped 20S proteasome (134). More importantly, a p21 (1–82) mutant lacking the binding to α7 is also readily degraded by purified proteasomes (20). Interestingly, the same six amino acids in p21 that are necessary for binding the α7 subunit are required for binding REGγ (30). Although the relationship between the binding of p21 to both α7 and REGγ remains to be defined, it is possible that these reflect concerted steps in the pathway of p21 degradation, namely, targeting and entry to the catalytic chamber.

Interestingly, interaction with the 20S α7 subunit is important for degradation of other proteins as well. The Rb plays a critical role in the development of human malignancies. Based on the available prediction algorithms, Rb is a highly unstructured protein. Mdm2 promotes Rb proteasomal degradation in a ubiquitin-independent manner by promoting Rb interaction with the α7 subunit of the 20S proteasome (118). This model attributes to Mdm2 a role of adaptor protein that acts to target Rb to the 20S proteasome. However, the human cytomegalovirus has developed an alternative mechanism to target Rb to the proteasome (see below). The 20S proteasome α7 subunit seems to be a preferred subunit for substrate targeting. Whether this subunit facilitates degradation of the 20S proteasome client proteins is an important possibility that would improve our understanding of the mechanism of 20S proteasome targeting and activation.

As discussed below, another emerging mechanism of 20S proteasome targeting was recently described. The enzyme NQO1 selectively associates with the uncapped 20S proteasome. Furthermore, NQO1 interacts with a number of IDPs. It is therefore likely that at least certain IDPs are targeted to the 20S via NQO1. As it is clarified below, NQO1 also protects the substrate from the 20S proteasomal digestion (4, 11).

1.12 IDPs STABILIZATION VIA PROTEIN–PROTEIN INTERACTION

An intuitive conclusion from the model that IDPs are prone to degradation by default is that IDPs are, in general, less stable proteins. However, there are mechanisms to protect IDPs from degradation by default. A large body of evidence suggests that IDPs escape degradation by default when they are in

association with a partner, possibly by masking the disordered region. This process might provide a mechanism for supporting the formation of functional complexes and at the same time, for eliminating excess of "free" subunits that might interfere with the function of these complexes. A number of cases are described here, but the literature is loaded with many more cases.

The p21 IDP is rarely found in a "free" form and is often detected as a part of a complex. A likely explanation is derived from the fact that degradation of p21 is inhibited upon binding of p21 to the PCNA or in the presence of the cyclin E and Cdk2 complex pushing the equilibrium toward complex formation (20, 38, 134). How p21 is protected is an important issue. It is very likely that p21 is in a more defined 3D structure while engaging into complexes, but some alternative explanations should also be considered. For example, p21 is targeted to the 20S proteasome via interaction with the α7 subunit. Thus, inhibition of p21 degradation via masking of the α7-binding site may represent an alternative mechanism of p21 stabilization. Consistent with this possibility is the fact that PCNA binds p21 at the region very close to that considered to be critical for α7 binding (134). A similar mechanism was proposed for cyclin D1-dependent p21 stabilization (38). This may also explain how another p21-interacting protein, CCAAT/enhancer binding protein alpha (C/EBPα), stabilizes p21 (130).

Another example is the case of IκBα, a major regulator of the NF-κB transcription factor. IκBα is unstructured at its N and C-termini (summarized in the DisProt database [122]). Following exposure to different types of stimuli, IκBα is phosphorylated and rapidly degraded via the ubiquitin 26S proteasome degradation pathway (76). Independent of its rapid stimulation-induced breakdown, IκBα is inherently unstable and undergoes continuous turnover. Analysis of both the basal degradation and the stimulation-induced breakdown of IκBα revealed that although in both cases the degradation is mediated via the proteasome, these two pathways of degradation are distinct. In contrast to the stimulation-induced breakdown, the basal degradation of IκBα does not require polyubiquitination of IκBα (76). Interestingly, expression of p65, which interacts with IκBα, significantly reduces the basal turnover of IκBα. Several studies have shown that IκBα is degraded by the uncapped 20S proteasomes in a ubiquitin-independent manner and that p65 can protect IκBα from this degradation (1, 78, 87).

In these last two examples, the basal degradation of both p21 and IκBα is ubiquitin independent and mediated via the core 20S proteasomes, whereas ubiquitin-dependent degradation occurs only upon exposure to certain physiological stimuli. These examples further suggest that the degradation of certain proteins through the ubiquitin-independent 20S degradation pathway depends upon the inherent quality of the substrate protein, whereas ubiquitin-dependent degradation is an active process that promotes increased levels of degradation. It is our assumption that assembly of substrate proteins into large protein complexes protects these substrates only from the 20S proteasomal degradation.

The $p14^{ARF}$ tumor suppressor is a key regulator of cellular proliferation, frequently inactivated in human cancer. ARF can activate the p53 tumor surveillance pathway by interacting with and inhibiting the p53 antagonist Mdm2 (120). $p14^{ARF}$ is dynamically disordered in aqueous solution and becomes structured upon binding to Mdm2 (23). $p14^{ARF}$ is degraded *in vitro* by the 20S proteasome in the absence of ubiquitination. $p14^{ARF}$ also binds transactivator of transcription (tat) binding protein 1 (TBP-1). TBP-1 inhibits ubiquitin-independent $p14^{ARF}$ degradation both *in vitro* and *in vivo* (105). By the experimental parameters outlined in this chapter, it is very likely that the IDP $p14^{ARF}$ undergoes degradation by default.

1.13 NQO1 REGULATES IDPS DEGRADATION BY DEFAULT

The process of degradation by default must be blocked under certain conditions to allow protein accumulation on demand. As discussed above, large protein complexes are not susceptible to 20S proteasomal degradation; however, this is not the only means of protecting proteins from degradation by default. A novel mechanism of protection is regulated by NQO1, a ubiquitous enzyme that utilizes NADH to catalyze the reduction of various quinones. NQO1 binds a subset of short-lived proteins including p53 and p73α (11), ODC (4), $p33^{ING1b}$ (53), p21, and Fos (unpublished observations). All these proteins bear ID regions and are subjected to degradation *in vitro* by the 20S proteasome and *in vivo* to degradation by both ubiquitin-dependent and ubiquitin-independent mechanisms. This set of proteins that undergo degradation by default is protected by NQO1, with the exception of p73β, which does not bind NQO1 (11). Binding of NQO1 to at least some of these proteins is augmented in the presence of NADH and inhibited by dicoumarol, an inhibitor of NQO1, which competes with NADH (11). In the case of the tumor suppressor $p33^{ING1b}$, phosphorylation of the protein in response to genotoxic stress increases its association with NQO1 and its half-life (53). Inhibition of NQO1 by dicoumarol or NQO1 knockdown with specific NQO1 siRNA induces ubiquitin-independent degradation of these proteins. *In vitro* degradation assays further confirmed that NQO1 together with NADH selectively protects p53, p73α, and ODC from 20S proteasomal degradation, and dicoumarol reduces the protection. The binding of NQO1 to the 20S proteasomes and the ability of NQO1 to bind and protect a subset of short-lived proteins from uncapped 20S proteasomal degradation suggest that NQO1 is an important regulator of degradation by default of IDPs.

1.14 NQO1 AS A 20S PROTEASOME GATEKEEPER

NQO1 is in association with the 20S core particle where it plays the function of “gatekeeper” by regulating the degradation of certain substrates (11).

Purification of the 20S proteasomes from mice livers (11) and human red blood cells (unpublished data) shows that NQO1 is physically associated with the 20S proteasomes but not with the 26S proteasomes. As described above, NQO1 interacts with certain 20S substrates such as p73α, p53, and ODC. Interestingly, NADH regulates the association of NQO1 with the substrates but not with the 20S proteasome. At high levels of NADH, the substrates are protected and do not enter the 20S catalytic chamber. At low levels of NADH, the substrates are not effectively protected and are degraded by the proteasome. Certain small drugs, like dicoumarol, by competing with NADH, abolish the NQO1–client substrate interaction and, therefore, sensitize these proteins to degradation by the 20S proteasomes. Dicoumarol does not alter the binding of NQO1 to the 20S proteasomes. Indeed, treatment of the 20S–NQO1–p53 ternary complexes with dicoumarol resulted in dissociation only of p53 but not of NQO1 and 20S.

NQO1, the gatekeeper, can be switched to an "on" or "off" position by NADH to allow or to block protein degradation. NADH level in turn is determined by the energy and oxidative state of the cells. The balance between these conditions, and the overall cell metabolism and catabolism processes must be properly maintained. Thus, NQO1 and NADH may provide a novel means of keeping this balance. Consistent with this hypothesis, the interaction of NQO1 with p53, p73, and ODC is modulated under oxidative state and ionizing radiation (4, 11).

The finding that an enzyme regulates the 20S proteasome in the context of degradation of IDPs may be the early bird of more regulators to be discovered. There are a number of additional roles that NQO1 may play in this context. NQO1 may be involved in targeting a subset of ID-containing proteins to the 20S proteasome, a possibility that is discussed below. Also, NQO1 may be functioning in regulating the latent state of the 20S catalytic core particle, with yet unknown mechanism.

1.15 OTHER MECHANISMS OF ESCAPING IDPs DEGRADATION BY DEFAULT

As discussed so far, the ID region, upon interacting with a partner, either acquires a defined structured or is sequestered. In either case, this would protect the IDPs from degradation by the 20S proteasome *in vitro*. By the same rationale, we explained the fact that IDPs in the cells are stabilized in the presence of a binding partner or destabilized when the level of the partner is decreased. For example, IκB proteins bind tightly to NF-κB dimers to ensure their nuclear exclusion. Free IκBα undergoes degradation by default, a process that depends on the C-terminal sequence. NF-κB binding to IκBα masks the C-terminus domain from proteasomal recognition, precluding its degradation by default, and as a result, degradation of free IκBα is about three orders of magnitude slower (94). As discussed above, bound IκBα undergoes

degradation by a second pathway that requires its N-terminal phosphorylation by the IκB kinase.

Protein interaction with other macromolecules may provide another possible mechanism of escaping degradation by default. Y-box binding protein-1 (YB-1) exemplifies a case of protein–RNA interaction. Based on the FoldIndex prediction program, YB-1 is a highly unstructured protein. YB-1 is a nucleocytoplasmic shuttling protein involved in many DNA-dependent and RNA-dependent events. In the nucleus, YB-1 regulates transcription of many genes involved in cell proliferation and differentiation. Recently, it was demonstrated that YB-1 undergoes a specific proteolytic cleavage by the 20S proteasome. Cleavage of YB-1 by the 20S proteasome is ubiquitin independent and is inhibited following association of YB-1 with messenger RNA (125).

Protein modification may provide another mechanism of escaping degradation by default. For example, c-Fos degradation by default (ubiquitin independent) is blocked when c-Fos heterodimerizes with c-Jun. However, c-Fos may escape degradation by default by modification. The C-terminus of c-Fos is an ID region. Under certain conditions, phosphorylation at this region (S362, S374) stabilizes c-Fos (47). Fra-1, a Fos-related protein, behaves in a similar manner in response to phosphorylation at the C-terminus (13).

Another emerging mechanism is interaction with small molecules. Some small molecules that serve as protein substrates or cofactors stabilize IDPs, probably by reducing their disordered conformation. Calmodulin becomes partially resistant to proteasomal degradation upon binding to Ca^{2+} (16, 48, 128), and the same was shown for troponin C (16). Other small molecules are NAD^+ and NADH. These may regulate IDP stability either indirectly via NQO1, as described above, or by direct binding to 20S substrates. For example, the binding of NAD^+ prevents pertussis toxin S1 degradation by the 20S proteasome (97). Another case is p53 that binds NAD^+ and NADH, and undergoes conformational changes (89); however, it is not known whether NAD^+ and NADH alone, in the absence of NQO1, may regulate p53 stability. NADH and NAD(P)H regulate some other protein–protein interactions that may indirectly lead to protein stabilization. These include the increased binding of the transcriptional repressor C-terminal binding protein (CtBP) to the transcription factor E1A in the presence of NADH (50, 144) and the increased binding of the metabolic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to the POU domain of the transcription factor OCT-1 by NAD^+. The NAD^+-dependent binding of GAPDH to OCT-1 does not require the enzymatic activity of GAPDH (148). A CtBP mutant that cannot bind NADH was also shown to be less stable (85). Stabilization of the lens quinone oxidoreductase ζ-crystallin and its binding to the chaperone ζ-crystallin require NADPH, which induces a conformational change in ζ-crystallin (109). Changes in metabolism that result in altered NAD^+/NADH ratio may thus alter the stability of IDPs through the default degradation pathway. This may occur due to NADH-induced interaction of NQO1 with its client proteins, or due

to direct interaction of NAD^+ or NAD(P)H with IDPs, that affects their conformation and susceptibility to default degradation.

1.16 THE BIOLOGICAL MEANING OF IDPs DEGRADATION BY DEFAULT

An important question is how degradation by default of IDPs would serve the cell's needs. An interesting case is activation of NF-κB in response to UV radiation. Inflammatory signaling induces rapid degradation of NF-κB-bound IκBα, but it does not induce the degradation of free IκBα (87, 94). In contrast, UV irradiation causes the depletion of both free and NF-κB-bound pools of IκBα. This is possible because free IκB undergoes degradation by default and because UV-induces IκB translation inhibition. The unstable nature of free IκBα demands its high synthesis rate to accumulate to the level needed to bind NF-κB to saturation. Inhibition of the synthesis of highly unstable free IκB, the inhibitor of NF-κB, rapidly shifts the equilibrium toward active NF-κB (95). Thus, the IκBα degradation by default is essential to obtain NF-κB activation in response to UV radiation.

Because many unstructured regions are folded upon binding to their biological targets in the cells, it seems that nature has developed an elegant way to link protein stability with biological functionality. Such a mechanism is extremely important in cases of functional multiprotein complexes, in which any excess of one of the subunits might interfere with the proper biological function of the complex. A theoretical analysis of gene circuits in bacteria presents a model for how selective degradation of monomers could be advantageous to the cells (25). This study analyzes the nonlinear dependence of degradation rate on the protein concentration. The degradation rate of proteins at lower concentrations is elevated compared with the degradation rate at higher protein concentrations. Since monomers predominate at low protein concentrations and dimerization is favored at higher concentrations, the model proposes that multimeric complexes support protein stability and terms it "cooperative stability." The authors of the "cooperative stability" model suggest that it improves the functioning of genetic networks and provides the means for evolutionary changes. This is based on the premise that genetic networks can be better regulated if the proteins involved are at higher concentrations. For example, if a protein dimer is the active species, then a low protein concentration would require high affinity for the dimer to be formed, and also would demand very stringent control of the protein expression. Higher protein concentrations offer the cell more flexibility in the regulation of expression, allow for lower affinity interactions, and allow for the variability that is essential for evolution. However, high protein concentrations and lower affinity mean that excess protein monomers may interfere with reactions catalyzed by the active dimers. Therefore, there must be a means to selectively degrade the excess monomers. The degradation by default model explains

how excess monomers are selectively degraded without requiring any additional molecular components.

One example of this type of regulation is the degradation of poly(A)-binding protein (PABP). PABP binds to the mRNA 3′ poly(A) tail and stimulates recruitment of the ribosome to the mRNA at the 5′ end. PABP activity is tightly controlled by the PABP-interacting protein 2 (Paip2), which inhibits translation by displacing PABP from the mRNA. The carboxy-terminal third of PABP contains an unstructured region (75). An E3 ubiquitin ligase, EDD (E3 isolated by differential display), targets Paip2 for degradation. Interestingly, EDD-mediated degradation of Paip2 gives rise to the IDP PABP degradation as well possibly by the default mechanism. Upon PABP knockdown, Paip2 interacts with EDD, which leads to Paip2 ubiquitination and degradation. Thus, an IDP protein (PABP) interacts with a regulatory-structured protein (Paip2). The PABP/Paip2 complex resists proteasomal degradation by sequestering the structured protein from interacting with the E3 ligase on the one hand, and protecting the IDP from default degradation on the other hand. This interesting coregulatory mechanism serves as a homeostatic feedback to control the activity of PABP in cells (141).

The nonhomologous DNA end-joining pathway is used in animal cells to repair double-strand DNA breaks. To this aim, the heterodimer Ku70–Ku80 proteins, which form a DNA end-binding complex, play an important role. The central region of the Ku80 C-terminal domain has a well-defined structure with disordered N- and C-termini (146). Ku70 is unstructured in part as well (147). Not much is known about the mechanisms of degradation of these proteins. Interestingly, knockout of one results in dramatic reduction in the level of the other (46, 58). Therefore, the Ku70 and Ku80 proteins appear to play reciprocal roles in stabilizing each other. The simplest explanation is that these proteins are subjected to degradation by default but protected upon their interaction. Having said so, the data on knockout of a gene encoding an IDP should be treated cautiously while attributing to a gene a given function. Based on this principle, the coreduced protein level under a knockout background may provide a simple way to identify the potential interactors.

1.17 DEGRADATION OF IDPs AND VIRUSES

HCV capsid is, by large, a disordered protein based on prediction by FoldIndex. This protein undergoes degradation via the 20S proteasome and the 20S activator REGγ (92). This degradation process is ubiquitin independent and initiated by direct contact between the capsid protein and REGγ. The fact that the monomers of the capsid protein that form the highly structured capsid are partially ID is not unique to HCV and appears to be the case with other viruses such as HBV and human immunodeficiency virus type 1 (HIV) (based on in silico analysis). However, in the case of HCV, the capsid has many regulatory roles as well (88), and therefore, its ID nature may be important to this function.

Tat is a small RNA-binding protein that plays a central role in the regulation of HIV replication and in approaches to treating latently infected cells. The tat amino acid sequence has a low overall hydrophobicity and a high net positive charge, and analyses by several algorithms and experimental data suggest that it is a natively unfolded protein (121). Tat has been shown to interact with a number of 20S subunit including α7 (3). EBNA, an EBV-coded protein, also targets the 20S α7 subunit (133). Whether this subunit facilitates degradation of its client proteins is an important possibility that may explain how substrates are targeted to the uncapped 20S proteasome.

DNA tumor viruses often induce degradation of the major tumor suppressors p53 and Rb, both of which are, in part, unstructured or predicted to be so. Recent findings suggest that this process can take place by both ubiquitin-dependent and ubiquitin-independent pathways. The human papilloma virus (HPV) E6 protein interacts with two regions of p53, namely, the DNA-binding region and the C-terminus unstructured region. The former enhances p53 degradation via the ubiquitin pathway, whereas the latter enhances via a ubiquitin-independent mechanism that is likely to be 20S proteasomal default degradation (27). Consistent with this possibility is the finding that the HPV E6 protein was ineffective in destabilizing p53 under overexpression of NQO1, which blocks degradation by default (6). E7, another regulatory protein encoded by HPV, interacts to destroy Rb. Interestingly, E7 also binds to the 19S subunit of the proteasome and might therefore direct the degradation of the Rb protein (19). However, proteasome binding has not yet been shown to be required for degradation, and the dependence of the degradation on polyubiquitination has not been examined. The pp71 protein of cytomegalovirus interacts with and facilitates the ubiquitin-independent proteasomal degradation of Rb, p107, and p130 (70). The question of whether pp71 targets the 20S proteasome was not addressed.

Viruses, therefore, encode IDPs and exploit the process of IDP degradation to satisfy their needs. These studies are in their infancy, and it is expected that many additional examples will be reported in the future. Nevertheless, already at this initial stage, one can think of future directions of how to manipulate IDPs roles in virus–host interactions in order to block virus infection and the associated diseases.

1.18 IDPs AND DISEASE

Protein degradation plays a role in many cellular processes, such as cell cycle regulation, antigen presentation, and the disposal of denatured, unfolded, or oxidized proteins. Several diseases, including certain types of cancers and neurological disorders, can result from aberrant protein degradation. In many neurodegenerative diseases, the basis for the pathology is the cellular inability to degrade misfolded and damaged proteins, resulting in the formation of cytotoxic aggregates such as seen in AD, Parkinson's Huntington's, and prion

diseases. Parkinson's is caused by the accumulation of aggregated proteins in the neuronal or glial cytoplasm. The inclusions of aggregated and filamentous proteins, called Lewy bodies, are comprised primarily of α-synuclein, a protein of unknown function that is highly expressed in the human brain. Monomeric α-synuclein is a natively unfolded protein without defined structure that undergoes ubiquitin-independent degradation by the 20S proteasomes (131). α-Synuclein spontaneously multimerizes to form highly stable insoluble fibrils that are resistant to degradation. Inhibition of the proteasomes in cells leads to an accumulation of α-synuclein that is phenotypically similar to the inclusions found in diseased tissue. Furthermore, inherited forms of Parkinson's disease include mutations that increase the copy number of α-synuclein, mutations that impair proteasome function, and mutations in α-synuclein that promote oligomerization of the protein and resistance to proteasomal degradation (90). One of the characteristics in Alzheimer's (AD) is the formation of neurofibrillary tangles that are intracellular and rich in tau, a structural protein that is normally associated with the microtubules. In several studies, it has been suggested that in AD, the proteasomal machinery is impaired (71, 72). Furthermore, it has been shown that the disordered tau protein can undergo ubiquitin-independent degradation by the proteasome, whereas the induction of conformational change by sodium dodecyl sulfate (SDS) or the binding to heat shock cognate 70 (Hsc70) or BCL2-associated athanogene (BAG-1) can prevent tau degradation (39, 45). Prion diseases are fatal neurodegenerative disorders whose pathogenesis is associated with a conformational rearrangement of the normal cellular prion protein (PrP^C) to abnormal conformers (PrP^{Sc}). PrP^C can undergo ubiquitin-independent degradation (129), whereas not only is the PrP^{Sc} conformation resistant to proteasomal degradation but it also inhibits the 20S proteasome catalytic activity (77).

These neurodegenerative diseases illustrate the importance of functioning 20S degradation. Interestingly, the proteins that are discussed are IDPs (see Table 1.1), highlighting the importance of degradation by default. Although some have pointed out that the ubiquitin system is important in the pathology of these diseases (as reviewed [35]), this does not contradict the possibility that ubiquitin-independent proteasomal degradation by default might play a significant role in these abnormalities. Monomeric proteins, such as α-synuclein and tau, if not forming their functional complex, need to be degraded to prevent their accumulation and subsequent spontaneous oligomerization that leads to the protein inclusions associated with these diseases. The degradation by default pathway can prevent the accumulation of uncomplexed ID proteins, suggesting that if this pathway is impaired, it could lead to the associated diseases.

1.19 IDPs: ARE THEY DISORDERED OR HIGHER ORDERED?

We do not think that the terms IUPs or IDPs adequately describe the functional significance of this category of proteins. Both terms are inaccurate,

inasmuch as these proteins may become structured/ordered at given points in time and space, both in the presence and absence of partners. Furthermore, the use of the terms IDP and IUP may also give the impression that these proteins are in some way “inferior” to structured proteins. This is completely wrong, given the fact that such proteins are mainly associated with higher organisms and functions, as can be seen, for example, by their prevalence at synapses in the nervous system. We proposed that they be called “4D proteins,” based on the fact that their structures are not fixed, as is generally the case for “3D proteins,” but are rather defined by time and space (135). IDPs, therefore, are more highly ordered than the 3D proteins, and the term 4D proteins would more accurately define this group of proteins.

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