INTRACELLULAR PROTEIN DEGRADATION: FROM A VAGUE IDEA THRU THE LYSOSOME AND THE UBIQUITIN-PROTEASOME SYSTEM AND ONTO HUMAN DISEASES AND DRUG TARGETING

Nobel Lecture, December 8, 2004

by

Aaron Ciechanover

Cancer and Vascular Biology Research Center, Faculty of Medicine, Technion – Israel Institute of Technology, Haifa, Israel.

ABSTRACT

Between the 1950s and 1980s, scientists were focusing mostly on how the genetic code is transcribed to RNA and translated to proteins, but how proteins are degraded has remained a neglected research area. With the discovery of the lysosome by Christian de Duve it was assumed that cellular proteins are degraded within this organelle. Yet, several independent lines of experimental evidence strongly suggested that intracellular proteolysis is largely non-lysosomal, but the mechanisms involved had remained obscure. The discovery of the ubiquitin-proteasome system resolved this enigma. We now recognize that ubiquitin- and proteasome-mediated degradation of intracellular proteins is involved in regulation of a broad array of cellular processes, such as cell cycle and division, regulation of transcription factors, and assurance of the cellular quality control. Not surprisingly, aberrations in the system have been implicated in the pathogenesis of many human diseases, malignancies and neurodegenerative disorders among them, which led subsequently to an increasing effort to develop mechanism-based drugs, one is already in use.

INTRODUCTION

The concept of protein turnover is hardly 60 years old. Beforehand, body proteins were viewed as essentially stable constituents that were subject to only minor 'wear and tear': dietary proteins were believed to function primarily as energy-providing fuel, traversing metabolic pathways which were completely

Abbreviations used: ODC, ornitihine decarboxylase; G6PD, glucose-6-phosphate dehydrogenase; PEPCK, phosphoenol-pyruvate carboxykinase; TAT, tyrosine aminotransferase; APF-1, ATP-dependent Proteolysis Factor 1 (ubiquitin); UBIP, ubiquitous immunopoietic polypeptide (ubiquitin); MCP, multicatalytic proteinase complex (26S proteasome); CP, 20S core particle (of the 26S proteasome); RP, 19S regulatory particle (of the 26Sproteasome).

distinct from those of the structural and functional proteins of the body. The problem was hard to approach experimentally, as research tools were not available. Important research reagents that were lacking at that time were stable isotopes. While radioactive isotopes were developed earlier by George de Hevesy (de Hevesy G., Chemistry 1943. In: *Nobel Lectures in Chemistry 1942–1962*. World Scientific 1999. pp. 5–41), they were mostly unstable and could not be used to follow metabolic pathways. The concept that the body structural proteins are static and the dietary proteins are used only as a fuel was challenged by Rudolf Schoenheimer from Columbia University in New York city. Schoenheimer escaped from Nazi Germany and joined the Department of Biochemistry in Columbia University founded by Hans T. Clarke (1–3). There he met Harold Urey who worked in the Department of Chemistry and discovered deuterium, the heavy isotope of hydrogen, a discovery that enabled him to prepare heavy water, D_2O. David Rittenberg who had recently received his Ph.D. in Urey's laboratory, joined Schoenheimer, and together they entertained the idea of *"employing a stable isotope as a label in organic compounds, destined for experiments in intermediary metabolism, which should be biochemically indistinguishable from their natural analog"* (1). Urey later succeeded in enriching nitrogen with ^{15}N, which provided Schoenheimer and Rittenberg with a "tag" for amino acids, resulting in a series of studies on protein dynamics. They discovered that following administration of ^{15}N-labled tyrosine to rat, only ~50% was recovered in the urine, *"while most of the remainder is deposited in tissue proteins. An equivalent of protein nitrogen is excreted"* (4). They further discovered that from the half that was incorporated into body proteins *"only a fraction was attached to the original carbon chain, namely to tyrosine, while the bulk was distributed over other nitrogenous groups of the proteins"* (4), mostly as an αNH_2 group in other amino acids. These experiments demonstrated unequivocally that the body structural proteins are in a dynamic state of synthesis and degradation, and that even individual amino acids are in a state of dynamic interconversion. Similar results were obtained using ^{15}N-labeled leucine (5). This series of findings shattered the paradigm in the field at that time that: (i) ingested proteins are completely metabolized and the products are excreted, and (ii) that body structural proteins are stable and static. Schoenheimer was invited to deliver the prestigious Harvey Lecture (1937) and Edward K. Dunham Lecture (1941; at Harvard University) where he presented his revolutionary findings. After his untimely tragic death in 1941, his lecture notes were edited by Hans Clarke, David Rittenberg and Sarah Ratner, and were published in a small book by Harvard University Press. The editors called the book "The Dynamic State of Body Constituents" (6), adopting the title of Schoenheimer's presentation. In the book, the new hypothesis is clearly presented: *"The simile of the combustion engine pictured the steady state flow of fuel into a fixed system, and the conversion of this fuel into waste products. The new results imply that not only the fuel, but the structural materials are in a steady state of flux. The classical picture must thus be replaced by one which takes account of the dynamic state of body structure"*. However, the idea that body proteins are turning over was not accepted easily

and was challenged as late as the mid-1950s. For example, Hogness and colleagues studied the kinetics of β-galactosidase in *E. coli* and summarized their findings (7): *"To sum up: there seems to be no conclusive evidence that the protein molecules within the cells of mammalian tissues are in a dynamic state. Moreover, our experiments have shown that the proteins of growing E. coli are static. Therefore it seems necessary to conclude that the synthesis and maintenance of proteins within growing cells is not necessarily or inherently associated with a "dynamic state"*. While the experimental study involved the bacterial β-galactosidase, the conclusions were broader, including also the authors' hypothesis on mammalian proteins. The use of the term *'dynamic state'* was not incidental, as they challenged directly Schoenheimer's studies, using his own term. It should be noted, however, that Schoenheimer's result related, as we know now, to the entire population of body proteins, extracellular and intracellular alike. It is now clear that these two classes of proteins are targeted by two different mechanisms (see below). The complex catabolic pathways, along with the lack of recognition of the importance of the process, made progress in the field slow.

Now, after more then six decades of research in the field and with the discovery of the lysosome and later the complex ubiquitin-proteasome system with its numerous tributaries, it is clear that the area has been revolutionized. While the lysosome is involved mostly (but not solely) in targeting extracellular proteins, the ubiiquitin-proteasome system degrades intracellular proteins. We now realize that intracellular proteins are turning over extensively, that the process is specific, and that the stability of many proteins is regulated individually and can vary under different pathophyiological conditions. From a scavenger, unregulated and non-specific end process, it has become clear that proteolysis of cellular proteins is a highly complex, temporally controlled and tightly regulated process that plays major roles in a broad array of basic pathways. Among these processes are cell cycle, development, differentiation, regulation of transcription, antigen presentation, signal transduction, receptor-mediated endocytosis, quality control, and modulation of diverse metabolic pathways. Subsequently, this development has changed the paradigm that regulation of cellular processes occurs mostly at the transcriptional and translational levels, and has set regulated protein degradation in an equally important position. With the multitude of substrates targeted and processes involved, it is not surprising that aberrations in the pathway have been implicated in the pathogenesis of many diseases, among them certain malignancies, neurodegeneration, and disorders of the immune and inflammatory system. As a result, the system has become a platform for drug targeting, and mechanism-based drugs are currently developed, one of them is already on the market.

THE LYSOSOME AND INTRACELLULAR PROTEIN DEGRADATION: FACTS

In the mid-1950s, Christian de Duve discovered the lysosome (see, for example, Refs. 8 and 9 and Figure 1). The lysosome was first recognized biochemically

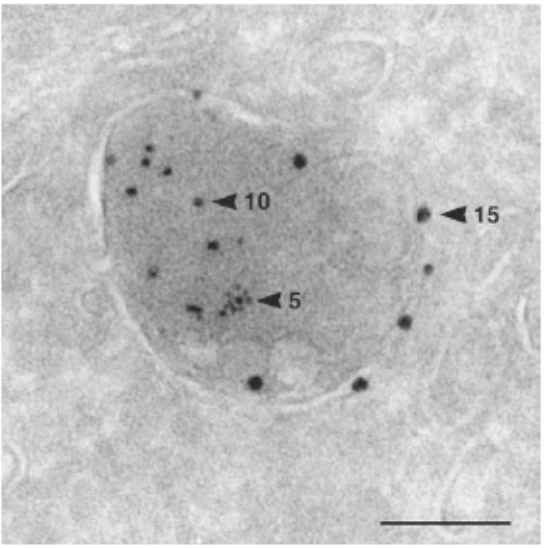

Figure 1: The lysosome: Ultrathin cryosection of a rat PC12 cell that had been loaded for 1 hour with bovine serum albumin (BSA)-gold (5 nm particles) and immunolabeled for the lysosomal enzyme cathepsin B (10-nm particles) and the lysosomal membrane protein LAMP1 (15 nm particles). Lysosomes are recognized also by their typical dense content and multiple internal membranes. Bar, 100 nm. Courtesy of Viola Oorschot and Judith Klumperman, Department of Cell Biology, University Medical Center, Utrecht, The Netherlands.

in the rat liver as a vacuolar structure that contains various hydrolytic enzymes which function optimally at an acidic pH. It is surrounded by a membrane that endows the contained enzymes with latency that is required to protect the cellular contents from their action (see below). The definition of the lysosome has been broadened over the years. This is because it has been recognized that the digestive process is dynamic and involves numerous stages of lysosomal maturation together with the digestion of both exogenous proteins (which are targeted to the lysosome through receptor-mediated endocytosis and pinocytosis) and exogenous particles (which are targeted via phagocytosis; the two processes are known as heterophagy), as well as digestion of endogenous proteins and cellular organelles (which are targeted via micro- and macro-autophagy, in many cases, under stress; see Figure 2). The lysosomal/-vacuolar system as we currently recognize it is a discontinuous and heterogeneous digestive system that also includes structures that are devoid of hydrolases – for example, early endosomes which contain endocytosed receptor-ligand complexes and pinocytosed/phagocytosed extracellular contents. On the other extreme it includes the residual bodies – the end products of the completed digestive processes of heterophagy and autophagy. In between these extremes one can observe: primary/nascent lysosomes that have not been engaged yet in any proteolytic process; early autophagic vacuoles that might contain intracellular organelles; intermediate/late endosomes and phagocytic

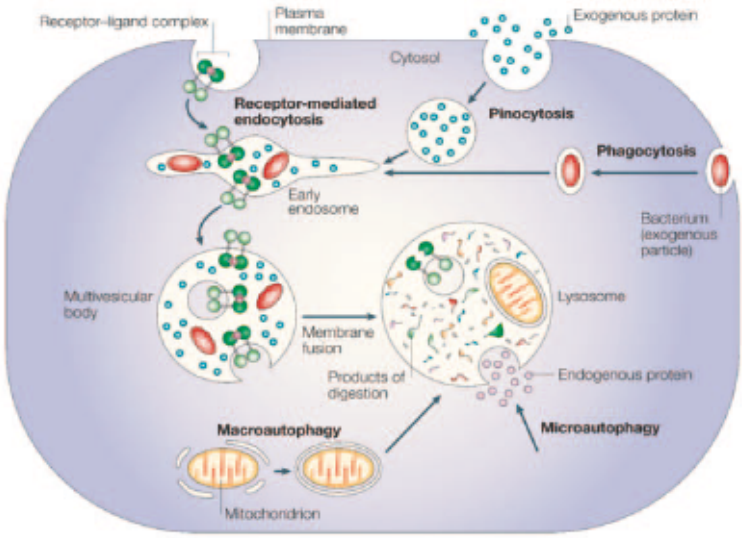

Figure 2: The four digestive processes mediated by the lysosome: (i) specific receptor-mediated endocytosis, (ii) pinocytosis (non-specific engulfment of droplets containing extracellular fluid), (iii) phagocytosis (of extracellular particles), and (iv) autophagy (micro- and macro-; of intracellular proteins and organelles) (with permission from Nature Publishing Group. Published originally in Ref. 83).

vacuoles (heterophagic vacuoles) that contain extracellular contents/particles; and multivesicular bodies (MVBs) which are the transition vacuoles between endosomes/phagocytic vacuoles and the digestive lysosomes.

The discovery of the lysosome along with independent experiments that were carried out at the same time and that have further strengthened the notion that cellular proteins are indeed in a constant state of synthesis and degradation (see, for example, Ref. 10), led scientists to feel, for the first time, that they have at hand an organelle that can potentially mediate degradation of intracellular proteins. The separation of the proteases from their substrates by a membrane provided an explanation for controlled degradation, and the only problem left to be explained (at the time) was how the substrates are translocated into the lysosomal lumen where they are exposed to the activity of the lysosomal proteases and degraded. An important discovery in this respect was the unraveling of the basic mechanism of action of the lysosome – autophagy (reviewed in Ref. 11). Under basal metabolic conditions, portions of the cytoplasm which contain a fraction of the entire population of cellular (cytosolic) proteins, are segregated within a membrane-bound compartment, and are then fused to a primary nascent lysosome, and their contents digested. This process was denoted microautophagy. Under more extreme conditions, starvation for example, mitochondria, endoplasmic reticulum membranes, glycogen bodies and other cytoplasmic entities, can also be engulfed by a

process called macroautophagy (see, for example, Ref. 12; the different modes of action of the lysosome in digesting extra- and intracellular proteins particles and organelles, are shown in Fig. 2).

THE LYSOSOME AND INTRACELLULAR PROTEIN DEGRADATION

However, over a period of more than two decades, between the mid-1950s and the late 1970s, it has become gradually more and more difficult to explain several aspects of intracellular protein degradation based on the known mechanisms of lysosomal activity: accumulating lines of independent experimental evidence indicated that the degradation of at least certain classes of cellular proteins must be non-lysosomal. Yet, in the absence of any 'alternative', researchers came with different explanations, some more substantiated and others less, to defend the 'lysosomal' hypothesis.

First was the gradual discovery, coming from several laboratories, that different proteins vary in their stability, and their half-life times can span a range of three orders of magnitude, from a few minutes to many days. Thus, the $t_{1/2}$ of ornitihine decarboxylase (ODC) is ~10 min, while that of glucose-6-phosphate dehydrogenase (G6PD) is 15 hours (for review articles, see, for example, Refs. 13,14). Also, rates of degradation of many proteins was shown to change under different physiological conditions, such as availability of nutrients or hormones. It was conceptually difficult to reconcile the findings of distinct half-lives of different proteins with the mechanism of action of the lysosome, where the microautophagic vesicle contains the entire cohort of cellular (cytosolic) proteins that are therefore expected to be degraded at the same rate. Similarly, changing pathophysiological conditions, such as starvation or re-supplementation of nutrients, were expected to affect the stability of all cellular proteins to the same extent. Clearly, this was not the case.

Another source of concern about the lysosome as the organelle in which intracellular proteins are degraded were the findings that both specific and general inhibitors of lysosomal proteases have different effects on different classes of proteins, making it clear that distinct groups of proteins are targeted by different proteolytic machineries. Thus, the degradation of endocytosed/-pinocytosed extracellular proteins was significantly inhibited by lysosomal inhibitors, a partial effect was observed on the degradation of long-lived cellular proteins, and almost no effect was observed on the degradation of short-lived and abnormal/mutated proteins.

Finally, the thermodynamically paradoxical observation that the degradation of cellular proteins requires metabolic energy, and more importantly, the emerging evidence that the proteolytic machinery uses the energy directly, were in contrast with the known mode of action of lysosomal proteases that under the appropriate acidic conditions, and similar to all known proteases, degrade proteins in an exergonic manner.

The assumption that the degradation of intracellular proteins is mediated by the lysosome was nevertheless logical. Proteolysis results from direct interaction between the target substrates and proteases, and therefore it was clear

that active proteases cannot co-exist free and active in the cytosol along with their substrates, which would have resulted in destruction of the cell. Thus, it was recognized that any suggested proteolytic machinery that mediates degradation of intracellular proteins must also be equipped with a mechanism that separates – physically or virtually – between the proteases and their substrates, and enables them to associate only when needed. The lysosomal membrane provided this separating/fencing mechanism. Obviously, nobody could have predicted that a new mode of post-translational modification – ubiquitination – could function as a chemical fence and as a proteolysis signal, and that untagged proteins will remain protected. Thus, while the structure of the lysosome as a distinc organelle could explain the separation necessary between the proteases and their substrates, and autophagy could explain the mechanism of entry of cytosolic proteins into the lysosomal lumen, major problems have remained unsolved. As noted, important among them were: (i) the various half lives of cellular proteins, (ii) the energy requirement for intracellular proteolysis, and (iii) the distinct response of different populations of proteins to lysosomal inhibitors. Thus, according to one model, it was proposed that different proteins have different sensitivities to lysosomal proteases, since their half-lives *in vivo* were in correlation with their sensitivity to the action of lysosomal proteases *in vitro* (15). To explain an extremely long half-life for a protein that was nevertheless sensitive to lysosomal proteases, or alterations in the stability of a single protein under various physiological states, it was suggested that although all cellular proteins are engulfed into the lysosome, only the short-lived proteins are degraded, whereas the long-lived proteins exit back into the cytosol: *"To account for differences in half-life among cell components or of a single component in various physiological states, it was necessary to include in the model the possibility of an exit of native components back to the extralysosomal compartment"* (16). According to a different model, selectivity is determined by the binding affinity of the different proteins for the lysosomal membrane which controls their entry rates into the lysosome, and subsequently their degradation rates (17). For a selected group of proteins, such as the gluconeogenetic enzymes phosphoenol-pyruvate carboxykinase (PEPCK) and fructose-1,6-biphosphatase, it was suggested, though not firmly substantiated, that their degradation in the yeast vacuole is regulated by glucose via a mechanism called 'catabolite inactivation' that possibly involves their phosphorylation. However this regulated mechanism for vacuolar degradation was limited only to a small and specific group of proteins (see for example Ref. 18; reviewed in Ref. 19). More recent studies have shown that at least for stress-induced macroautophagy, a general sequence of amino acids, KFFERQ, that was identified in numerous cellular proteins, directs, via binding to a specific 'receptor' and in cooperation with cytosolic and lysosomal chaperones, the regulated entry of many cytosolic proteins into the lysosomal lumen. While further corroboration of this hypothesis is still required, it can explain the mass, stress-induced, entry into the lysosome of a large population of proteins that contain a homologous sequence, but not the targeting for degradation of a specific protein under defined conditions (reviewed in Refs. 20,21). The energy requirement for

protein degradation was described as indirect, and necessary, for example, for protein transport across the lysosomal membrane (22) and/or for the activity of the H^+ pump and the maintenance of the low acidic intralysosomal pH that is necessary for optimal activity of the proteases (23). We now know that both mechanisms require energy. Thus, despite the strained explanations for different stabilities and energy requirement, the lysosome still appeared as the most logical organelle/system that mediates degradation of intracellular proteins. Christian de Duve's view on the subject as summarized in a review article he published in the mid-1960s, saying: *"Just as extracellular digestion is successfully carried out by the concerted action of enzymes with limited individual capacities, so, we believe, is intracellular digestion"* (24) still appeared to be valid even towards the mid 1970s. The problem of different sensitivities of distinct protein groups to lysosomal inhibitors has remained unsolved, and may have served as an important trigger in the future quest for a non-lysosomal proteolytic system that may be involved in at least certain aspects of intracellular protein degradation.

Progress in identifying the elusive, non-lysosomal proteolytic system(s) was hampered by the lack of a cell-free preparation that could faithfully replicate the cellular proteolytic events – degrading proteins in a specific and energy-requiring mode and at neutral pH. An important breakthrough was made by Rabinovitz and Fisher who found that rabbit reticulocytes degrade abnormal, amino acid analogue-containing hemoglobin (25). Their experiments modeled known disease states, the hemoglobinopathies. In these diseases, abnormal mutated hemoglobin chains (such as sickle cell hemoglobin) or excess of unassembled normal hemoglobin chains (generated in thalassemias, diseases in which the pairing chain is not synthesized at all or is mutated and rapidly degraded. Consequently, the bi-heterodimeric hemoglobin complex is not assembled) are rapidly degraded in the reticulocyte (26,27). Reticulocytes are terminally differentiating red blood cells that do not contain lysosomes. Therefore, it was postulated that the degradation of hemoglobin in these cells is mediated by a non-lysosomal machinery. Etlinger and Goldberg (28) were the first to isolate and characterize a cell-free proteolytic preparation from reticulocytes. The crude extract selectively degraded abnormal hemoglobin, required ATP hydrolysis, and acted optimally at a neutral pH, which further corroborated the assumption that the proteolytic activity was non-lysosomal. A similar system was isolated and characterized later by our group (29). Additional studies by the group led subsequently to resolution, characterization, and purification of the major enzymatic components from this extracts, and to the discovery of the ubiquitin signalling system (see below).

THE LYSOSOME HYPOTHESIS IS CHALLENGED

As mentioned above, the unraveled mechanism(s) of action of the lysosome could explain only partially, and at times not satisfactorily, several key emerging characteristics of intracellular protein degradation. Among them were: (i) the

heterogeneous stability of individual proteins (ii), the effect of nutrients and hormones on their degradation, and (iii) the dependence of intracellular proteolysis on metabolic energy. The differential effect of selective inhibitors on the degradation of different classes of cellular proteins could not be explained at all.

The development of methods to monitor protein dynamics in cells together with the discovery of specific and general lysosomal inhibitors have resulted in the identification of different classes of cellular proteins, long- and short-lived, and the findings of the differential effects of the inhibitors on these groups (see, for example, Refs. 30,31). An elegant experiment in this respect was carried out by Brian Poole and his colleagues in the Rockefeller University. Poole was studying the effect of lysosomotropic agents – weak bases such as ammonium chloride and chloroquine – that accumulate in the lysosome and dissipate its low acidic pH by neutralizing the H^+ ions: the lysosomal proteases are inactive in neutral pH. It was assumed that this mechanism also underlies the anti-malarial activity of chloroquine and similar drugs that inhibit the activity parasite's lysosome, 'paralyzing' its ability to digest the host's hemoglobin during the intra-erythrocytic stage of its life cycle. Poole and his colleagues labelled metabolically endogenous proteins in living macrophages with ^3H-leucine and 'fed' them with broken macrophages that had been labelled metabolically with ^{14}C-leucine prior to their disruption. They assumed, apparently correctly, that the dead macrophages debris and proteins will be phagocytosed by the live macrophages and targeted to the lysosome for degradation. They monitored the effect of lysosomotropic agents on the degradation of these two protein populations, the endogenous and the exogenous. In particular, they studied the effect of the weak bases chloroquine and ammonium chloride, and the acid ionophore X537A which dissipates the H^+ gradient across the lysosomal membrane. They found that these drugs inhibited specifically the degradation of extracellular proteins, but not of intracellular proteins (32). Poole summarized beautifully (I would say also poetically) these experiments and explicitly predicted the existence of a non-lysosomal proteolytic system that degrades intracellular proteins: *"Some of the macrophages labeled with tritium were permitted to endocytise the dead macrophages labeled with ^{14}C. The cells were then washed and replaced in fresh medium. In this way we were able to measure in the same cells the digestion of macrophage proteins from two sources. The exogenous proteins will be broken down in the lysosomes, while the endogenous proteins will be broken down wherever it is that endogenous proteins are broken down during protein turnover"* (33).

The requirement for metabolic energy for the degradation of both prokaryotic (34) and eukaryotic (10,35) proteins was difficult to interpret. Proteolysis is an exergonic process and the thermodynamically paradoxical energy requirement for intracellular proteolysis made researchers believe that energy cannot be consumed directly by proteases or the proteolytic process per se, and is used indirectly. As Simpson summarized his findings (10): *"The data can also be interpreted by postulating that the release of amino acids from protein is itself directly dependent on energy supply. A somewhat similar hypothesis, based on*

studies on autolysis in tissue minces, has recently been advanced, but the supporting data are very difficult to interpret. However, the fact that protein hydrolysis as catalyzed by the familiar proteases and peptidases occurs exergonically, together with the consideration that autolysis in excised organs or tissue minces continues for weeks, long after phosphorylation or oxidation ceased, renders improbable the hypothesis of the direct energy dependence of the reactions leading to protein breakdown". Being cautious however, and probably unsure about a single interpretation and conclusion, Simpson still left a narrow slit opened for a proteolytic process that requires energy in a direct manner: *"However, the results do not exclude the existence of two (or more) mechanisms of protein breakdown, one hydrolytic, the other energy-requiring"*. Since any proteolytic process must be at one point or another hydrolytic, the statement that makes a distinction between an hydrolytic process and an energy-requiring (yet non-hydrolytic one), is not clear. Judging the statement from a historical perspective and knowing the mechanism of action of the ubiquitin system, where energy is required also in the pre-hydrolytic step (ubiquitin conjugation), Simpson may have thought of a two step mechanism: an initial conditioning step that is energy-requiring yet not hydrolytic, followed by a second hydrolytic step that is energy-independent. However, he did not provide us with a clear explanation. Clearly, he could not have imagined that even the second step will be energy-requiring (as we know is proteasomal activity). At the end of this clearly understandable and apparently difficult deliberation, he left us with a vague explanation linking protein degradation to protein synthesis, a process that was known already at the time to require metabolic energy: *"The fact that a supply of energy seems to be necessary for both the incorporation and the release of amino acids from protein might well mean that the two processes are interrelated. Additional data suggestive of such a view are available from other types of experiments. Early investigations on nitrogen balance by Benedict, Folin, Gamble, Smith, and others point to the fact that the rate of protein catabolism varies with the dietary protein level. Since the protein level of the diet would be expected to exert a direct influence on synthesis rather than breakdown, the altered catabolic rate could well be caused by a change in the rate of synthesis"* (10). With the discovery of lysosomes in eukaryotic cells it could be argued that energy is required for the transport of substrates into the lysosome or for maintenance of the low intralysosomal pH, for example (see above). The observation by Hershko and Tomkins that the activity of tyrosine aminotransferase (TAT) was stabilized following depletion of ATP (36) indicated that energy may be required at an early stage of the proteolytic process, most probably before proteolysis occurs. Yet, it did not provide a clue as for the mechanism involved: the energy could be used, for example, for specific modification of TAT, e.g. phosphorylation, that would sensitize it to degradation by the lysosome or by a yet unknown proteolytic mechanism, or for a modification that activates its putative protease. It could also be used for a more general lysosomal mechanism, one that involves transport of TAT as well as other proteins into the lysosome or dissipation of the intralysosomal acidic pH, for example. The energy inhibitors used by Hershko and Tomkins abolished almost completely degradation of the entire

population of cell proteins, confirming previous studies (e.g. 10) and suggesting a general role for energy in protein catabolism. In that respect energy inhibitors had an effect that was distinct from that of protein synthesis inhibitors (e.g. cycloheximide) which affected only enhanced degradation of TAT (induced by steroid hormone depletion), but not basal degradation (36). This finding ruled out, at least partially, a tight linkage between protein synthesis and degradation. In bacteria, that lack lysosomes, an argument involving energy requirement for lysosomal degradation could not have been proposed, but other indirect effects of ATP hydrolysis could have affected proteolysis in *E. coli*, such as phosphorylation of substrates and/or proteolytic enzymes, or maintenance of the 'energized membrane state'. According to this model, proteins could become susceptible to proteolysis by changing their conformation, for example, following association with the cell membrane that maintains a local, energy-dependent gradient of a certain ion. However, such an effect was ruled out (37), and since there was no evidence for a phosphorylation-mediated mechanism (although, similar to the state of affairs in eukaryotes, the proteolytic machinery in prokaryotes had not been identified at that time), it seemed that at least in bacteria, energy is required directly for the proteolytic process. In any event, the requirement for metabolic energy for protein degradation in both prokaryotes and eukaryotes, a process that is exergonic thermodynamically, strongly indicated that proteolysis is a highly regulated process, and that a similar principle/mechanism has been preserved along evolution of the two kingdoms. Implying from the possible direct requirement for ATP in degradation of proteins in bacteria, it was not too unlikely to assume a similar direct mechanism in the degradation of cellular proteins in eukaryotes. Supporting this notion was the description of the cell-free proteolytic system in reticulocytes (28,29), a cell that lacks lysosomes, which suggested that energy is probably required directly for the proteolytic process, although here too, the underlying mechanisms had remained enigmatic at the time. The description of the cell-free system paved however the road for the later detailed dissection of the underlying mechanisms involved and to the discovery of the ubiquitin system.

THE UBIQUITIN-PROTEASOME SYSTEM

The cell-free proteolytic system from reticulocytes (28,29) turned out to be an important and rich source for the purification and characterization of the enzymes that are involved in the ubiquitin-proteasome system. Initial fractionation of the crude reticulocyte cell extract on the anion-exchange resin diethylaminoethyl (DEAE) cellulose yielded two fractions which were both required to reconstitute the energy-dependent proteolytic activity found in the crude extract: the unadsorbed, flow through material was denoted Fraction I, and the high salt eluate of the adsorbed proteins was denoted Fraction II (38).

Table 1: Resolution of the ATP-dependent proteolytic activity from crude reticulocyte extract into two essentially required complementing activities (adapted from Ref. 38; with permission from Elsevier/Biochem. Biophys. Res. Commun.).

Fraction	Degradation of [^{3}H]globin (%)	
	-ATP	+ATP
Lysate	1.5	10
Fraction I	0.0	0.0
Fraction II	1.5	2.7
Fraction I and Fraction II	1.6	10.6

This finding was an important observation and a methodolgical lesson for future dissection of the system. For one, it suggested that the system is not composed of a single 'classical' protease that has evolved evolutionarily to acquire energy dependence [although energy-dependent multi component/-complex proteases, such as the mammalian 26S proteasome (see below) and the prokaryotic Clp family of proteases have been described later], but that it is made of at least two components. This finding of a two component, energy-dependent protease that had no precedent, left us with no paradigm to follow. In an attempt to explain this finding, we suggested, for example, that the two fractions could represent an inhibited protease and its activator. Second, learning from this reconstitution experiment and the essential dependence between the two active components, we continued to reconstitute activity from resolved fractions whenever we encountered a loss of activity along further purification steps. This biochemical 'complementation' approach resulted in the discovery of additional enzymes of the system, all required to be present in the reaction mixture in order to catalyze the multi-step proteolysis of the target substrate. We chose first to purify the active component from Fraction I (see accompanying Biography). It was found to be a small, ~8.5 kDa heat-stable protein that was designated ATP-dependent Proteolysis Factor 1, APF-1. APF-1 was later identified as ubiquitin (see below; I am using the term APF-1 to the point in which it was identified as ubiquitin and then change the terminology accordingly). In retrospect, the decision to start the purification efforts with Fraction I turned out to be strategically important, as Fraction I contained only one single protein – APF-1 – that was necessary to stimulate proteolysis of the model substrate we used at the time, while Fraction II, as we learnt later contained many more components. Starting the purification efforts with Fraction II would have resulted in a much more complicated route. Later studies showed that Fraction I contains additional components necessary for the degradation of other substrates, but these were not necessary for the reconstitution of the system at that time. This choice enabled us not only to purify APF-1, but also to quickly decipher its mechanism of action. A critically important finding that paved the road for future developments in the field was that multiple moieties of APF-1 are covalently conjugated to the target substrate when incubated in the presence of Fraction II, and that the modification requires ATP (39,40; Figures 3 and 4). It was also found that the modifica-

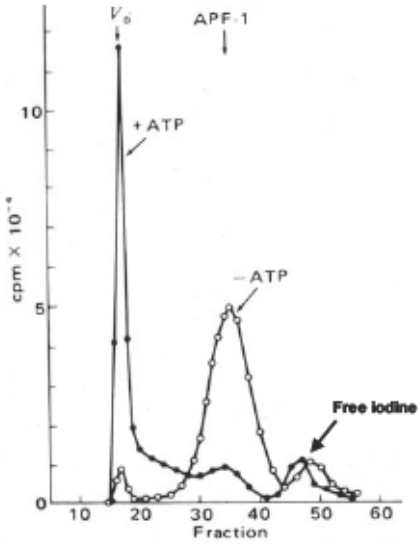

Figure 3: APF-1 is shifted to a high molecular mass "compound(s)" following incubation in ATP-containing crude reticulocyte cell extract. ^{125}I-labelled APF-1 was incubated with reticulocyte crude Fraction II in the absence (open circles) or presence (closed circles) of ATP, and the reaction mixtures were resolved via gel filtration chromatography. Shown is the radioactivity measured in each fraction. As can be seen, following addition of ATP, APF-1 becomes associated with some component(s) in Fraction II, which could be an enzyme(s) or a substrate(s) of the system (with permission from Proceedings of the National Academy of the USA; published originally in Ref. 39).

tion is reversible, and APF-1 can be removed from the substrate or its degradation products and reutilized (40).

The discovery that APF-1 is covalently conjugated to protein substrates and stimulates their proteolysis in the presence of ATP and crude Fraction II, led in 1980 to the proposal of a model according to which protein substrate modification by multiple moieties of APF-1 targets it for degradation by a downstream, at that time an yet unidentified, protease that cannot recognize the unmodified substrate; following degradation, reusable APF-1 is released (40). It was also shown that APF-1 can be released from the intact substrate (40). This can occur, for example, if the substrate was marked eironeously. It was further predicted that the release is mediated by specific protease(s) and not via reversal of the conjugation reaction. Amino-acid analysis of APF-1, along with its known molecular mass and other general characteristics raised the suspicion that APF-1 is ubiquitin (41), a known protein of previously unknown function. Contributing to this suspicion was the previously described conjugate between ubiquitin and histone H2A that raised the possibility that a similar, though obviously not an identical, conjugate is generated between APF-1 and its target substrate (see below). Indeed, in a series of biochemical and functional experiments, Wilkinson and colleagues confirmed unequivo-

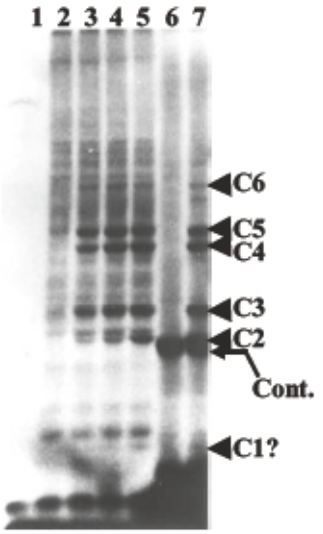

Figure 4: Multiple molecules of APF-1/ubiquitin are conjugated to the proteolytic substrate, probably signalling it for degradation. To interpret the data described in the experiment depicted in Figure 3 and to test the hypothesis that APF-1 is conjugated to the target proteolytic substrate, ^{125}I-APF-1 was incubated along with crude Fraction II (Figure 3 and text) in the absence (lane 1) or presence (lanes 2–5) of ATP and in the absence (lanes 1,2) or presence (lanes 3–5) of increasing concentrations of unlabeled lysozyme. Reaction mixtures resolved in lanes 6 and 7 were incubated in the absence (lane 6) or presence (lane 7) of ATP, and included unlabeled APF-1 and ^{125}I-labeled lysozyme. C1–C6 denote specific APF-1-lysozyme adducts in which the number of APF-1 moieties bound to a the lysozyme moiety of the adduct is probably increasing, from 1 to 6. Reactions mixtures were resolved via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized following exposure to an X-ray film (autoradiography) (with permission from Proceedings of the National Academy of the USA; published originally in Ref. 40).

cally that APF-1 is ubiquitin (42). Ubiquitin is a small, heat-stable and highly evolutionarily conserved protein of 76 residues. It was first purified during the isolation of thymopoietins (43) and was subsequently found to be ubiquitously expressed in all kingdoms of living cells, including prokaryotes (44). Interestingly, it was initially found to induce lymphocyte differentiation, a characteristic that was attributed to the stimulation of adenylate cyclase (44,45). Accordingly, it was named UBIP for *ubi*quitous *i*mmunopoietic *p*olypeptide (44). However, later studies showed that ubiquitin is not involved in the immune response (46), and that it was a contaminating endotoxin in the preparation that was responsible for the adenylate cyclase and the T-cell differentiating activities. Furthermore, the unraveling of the sequence of several eubacteria and archaebacteria genomes, as well as earlier biochemical analyses in these organisms (unpublished) showed that ubiquitin is restricted only to eukaryotes. The finding of ubiquitin in bacteria (44) was probably due to contamination of the bacterial extract with yeast ubiquitin derived from the

yeast extract in which the bacteria were grown. While in retrospect the name ubiquitin is a misnomer as it is restricted to eukaryotes and is not ubiquitous as was previously thought, from historical reasons it has still remained the name of the protein. Accordingly, and in order to avoid confusion, we suggest that names of other novel enzymes and components of the ubiquitin system, but of other systems as well, should remain as were first coined by their discoverers.

An important development in the ubiquitin research field was the discovery that a single ubiquitin moiety can be covalently conjugated to histones (see above), and particularly to histones H2A and H2B. While the function of these adducts has remained elusive until recently, their structure was unraveled in the mid 1970s. The structure of the ubiquitin conjugate with H2A (uH2A; was also designated protein A24) was deciphered by Goldknopf and Busch (47,48) and by Hunt and Dayhoff (49), who found that the two proteins are linked through a fork-like, branched isopeptide bond between the carboxy-terminal glycine of ubiquitin (Gly^{76}) and the ε-NH_2 group of 119 internal lysine (Lys^{119}) of the histone molecule. It was suggested that the isopeptide bond found in the histone-ubiquitin adduct is identical to the bond that was found between ubiquitin and the target proteolytic substrate (50), and between the ubiquitin moieties in the polyubiquitin chain (51,52) that is synthesized on the substrate and that functions, most probably, as a proteolysis recognition signal for the downstream 26S proteasome. In this particular polyubiquitin chain the linkage is between Gly^{76} of one ubiquitin moiety and internal Lys^{48} of the previously conjugated ubiquitin moiety. It appears that only Lys^{48}-based polyubiquitin chain is recognized by the 26S proteasome and serves as a proteolytic signal. In recent years it has been shown that the first ubiquitin moiety can also be attached in a linear mode to the N-terminal residue of the proteolytic target substrate (53). However, even in this case, the subsequent ubiquitin moieties are generating a Lys^{48}-based polyubiquitin chain on the first linearly fused moiety. N-terminal ubiquitination is clearly required for targeting naturally occurring lysine-less proteins for degradation. Yet, several lysine-containing proteins have also been described that traverse this pathway, the muscle-specific transcription factor MyoD, for example. In these proteins the internal lysine residues are probably not accessible to their cognate ligases. Other types of polyubiquitin chains have also been described that are not involved in targeting the conjugated substrates for proteolysis. Thus, a Lys^{63}-based polyubiquitin chain has been described that is probably necessary to activate transcription factors (reviewed recently in Ref. 54). Interestingly, the role of monoubiquitination of histones has also been identified recently. It is involved in regulation of transcription, probably via modulation of the structure of the nucleosomes (for recent reviews, see, for example, Refs. 55,56).

The identification of APF-1 as ubiquitin, and the discovery that a high-energy isopeptide bond, similar to the one that links ubiquitin to histone H2A, links it also to the target proteolytic substrate, resolved at that time the enigma of the energy requirement for intracellular proteolysis (see however below) and paved the road to the untangling of the complex mechanism of isopeptide bond formation. This process turned out to be similar to that of

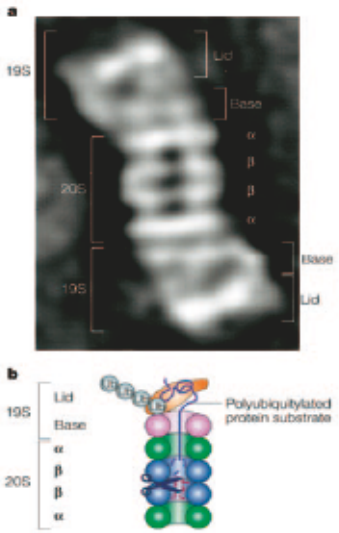

Figure 5: The Proteasome. The proteasome is a large, 26S, multicatalytic protease that degrades polyubiquitinated proteins to small peptides. It is composed of two sub-complexes: a 20S core particle (CP) that carries the catalytic activity, and a regulatory 19S regulatory particle (RP). The 20S CP is a barrel-shaped structure composed of four stacked rings, two identical outer α rings and two identical inner β rings. The eukaryotic α and β rings are composed each of seven distinct subunits, giving the 20S complex the general structure of $\alpha_{1\text{-}7}\beta_{1\text{-}7}\beta_{1\text{-}7}\alpha_{1\text{-}7}$. The catalytic sites are localized to some of the β subunits. Each extremity of the 20S barrel can be capped by a 19S RP, each composed of 17 distinct subunits, 9 in a "base" sub-complex, and 8 in a "lid" sub-complex. One important function of the 19S RP is to recognize ubiquitinated proteins and other potential substrates of the proteasome. Several ubiquitin-binding subunits of the 19S RP have been identified, however, their biological roles and mode of action have not been discerned. A second function of the 19S RP is to open an orifice in the α ring that will allow entry of the substrate into the proteolytic chamber. Also, since a folded protein would not be able to fit through the narrow proteasomal channel, it is assumed that the 19S particle unfolds substrates and inserts them into the 20S CP. Both the channel opening function and the unfolding of the substrate require metabolic energy, and indeed, the 19S RP "base" contains six different ATPase subunits. Following degradation of the substrate, short peptides derived from the substrate are released, as well as reusable ubiquitin (with permission from Nature Publishing Group. Published originally in Ref. 83).
a. Electron microscopy image of the 26S proteasome from the yeast *S. cerevisiae*.
b. Schematic representation of the structure and function of the 26S proteasome.

peptide bond formation that is catalysed by tRNA synthetase or during non-ribosomal synthesis of short peptides (57). In these processes, that occurs during protein synthesis a single amino acid is activated, whereas during activation of ubiquitin, the c-terminal Gly^{76} residue of the protein is activated, but otherwise the three processes appear to be almost identical. Using the unravelled mechanism of ubiquitin activation and immobilized ubiquitin as a 'covalent' affinity bait, the three enzymes that are involved in the cascade reaction of ubiquitin conjugation were purified by Ciechanover, Hershko, and their colleagues. These enzymes are: (i) E1, the ubiquitin-activating enzyme, (ii) E2, the ubiquitin-carrier protein, and (iii) E3, the ubiquitin-protein

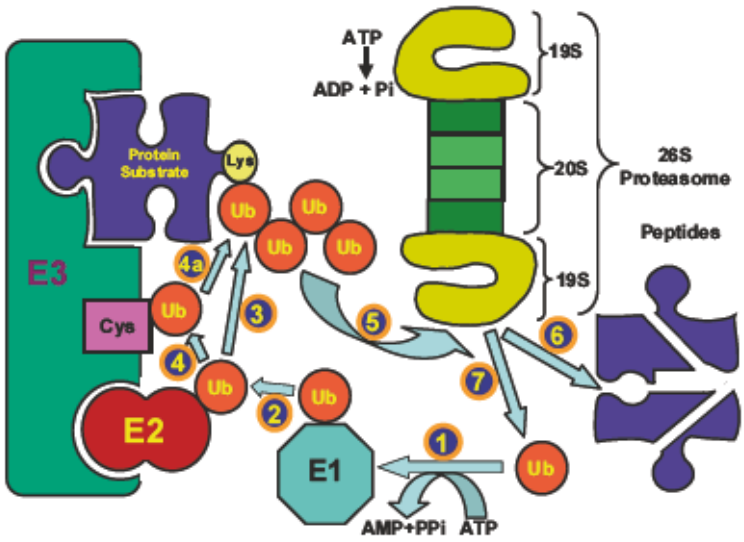

Figure 6: The ubiquitin-proteasome proteolytic system. Ubiquitin is activated by the ubiquitin-activating enzyme, E1 (1) followed by its transfer to a ubiquitin-carrier protein (ubiquitin-conjugating enzyme, UBC), E2 (2). E2 transfers the activated ubiquitin moieties to the protein substrate that is bound specifically to a unique ubiquitin ligase E3. The transfer is either direct [(3) in the case of RING finger ligases and possibly some other smaller groups of E3s such as U-Box ligases], or via an additional thiol-ester intermediate on the ligase [(4, 4a) in the case of HECT domain ligases]. Successive conjugation of ubiquitin moieties to one another generates a polyubiquitin chain that serves as the binding (5) and degradation signal for the downstream 26S proteasome. The substrate is degraded to short peptides (6), and free and reusable ubiquitin is released by de-ubiquitinating enzymes (DUBs) (7).

ligase (58,59). The discovery of the E3 component which is the specific substrate-binding component of the system, pointed to a possible solution to the problem of the heterogeneous stabilities of different proteins – they might be specifically recognized and targeted by different ligases.

Within a short period, the ubiquitin tagging hypothesis received substantial support. For example, Chin and colleagues injected into HeLa cells labeled ubiquitin and hemoglobin and denatured the injected hemoglobin by oxidizing it with phenylhydrazine. They found that ubiquitin conjugation to globin is markedly enhanced by denaturation of hemoglobin, and the level of globin-ubiquitin conjugates was proportional to the rate of hemoglobin degradation (60). Hershko and colleagues observed a similar correlation for abnormal, amino acid analogue-containing short-lived proteins (61). A previously isolated cell cycle arrest mutant that loses the ubiquitin-histone H2A adduct at the permissive temperature (62), was found by Finley, Ciechanover and Varshavsky to harbor a thermolabile E1 (63). Following heat inactivation, the cells fail to degrade normal short-lived proteins (64). Although the cells did not provide direct evidence for substrate ubiquitination as a destruction signal, they still provided a strong and direct linkage between ubiquitin conjugation and

intracellular protein degradation.

At this point, the only missing link was the identification of the downstream protease that would specifically recognize ubiquitinated substrates. Tanaka and colleagues identified a second ATP-requiring step in the reticulocyte proteolytic system which was independent of ubiquitination (65), and Hershko and colleagues demonstrated that energy is required for conjugate degradation (66). An important advance in the field was a discovery by Hough and colleagues who partially purified and characterized a high-molecular mass alkaline protease that degraded, in an ATP-dependent mode, ubiquitin adducts of lysozyme but not untagged lysozyme (67). This protease, which was later called the 26S proteasome (see below), provided all the necessary criteria for being the specific proteolytic arm of the ubiquitin system. This finding was confirmed, and the protease was further characterized by Waxman and colleagues who found that it is an unusually large, ~ 1,5 MDa enzyme, unlike any other known protease (68). A further advance in the field was the discovery (69) that a smaller, neutral multi-subunit 20S protease complex that was discovered together with the larger 26S complex, is similar to a "multicatalytic proteinase complex" (MCP) that was described earlier by Wilk and Orlowski in bovine pituitary gland (70). This 20S protease is ATP-independent and has various catalytic activities, cleaving on the carboxy-terminal side of hydrophobic, basic and acidic residues. Hough and colleagues raised the possibility – although they did not show it experimentally – that this 20S protease can be a part of the larger 26S protease that degrades ubiquitin adducts (69). Later studies showed that indeed, the 20S complex is the core catalytic particle of the larger 26S complex (71,72). However, a strong evidence that the active 'mushroom'-shaped 26S protease is generated through the assembly of two distinct sub-complexes – the catalytic 20S cylinder-like MCP and an additional 19S ball-shaped sub-complex (that was predicted to have a regulatory role) – was provided only in the early 1990s by Hoffman and colleagues (73): These researchers mixed the two purified particles and generated the active 26S enzyme.

The proteasome is a large, 26S, multicatalytic protease that degrades poly-ubiquitinated proteins to small peptides. It is composed of two sub-complexes: a 20S core particle (CP) that carries the catalytic activity, and a regulatory 19S regulatory particle (RP) (for the structure of the 26S proteasome, see Figure 5; for a scheme describing the ubiquitin system, see Figure 6).

CONCLUDING REMARKS

The evolvement of proteolysis as a centrally important regulatory mechanism is a remarkable example for the development of a novel biological concept and the accompanying battles to change paradigms. The five decades journey between the early 1940s and early 1990s began with fierce discussions on whether cellular proteins are static as had been thought for a long time, or are in a "dynamic state" of synthesis and degradation. The discovery of protein dynamics was followed by the discovery of the lysosome, that was believed – between the mid-1950s and mid-1970s – to be the organelle within which in-

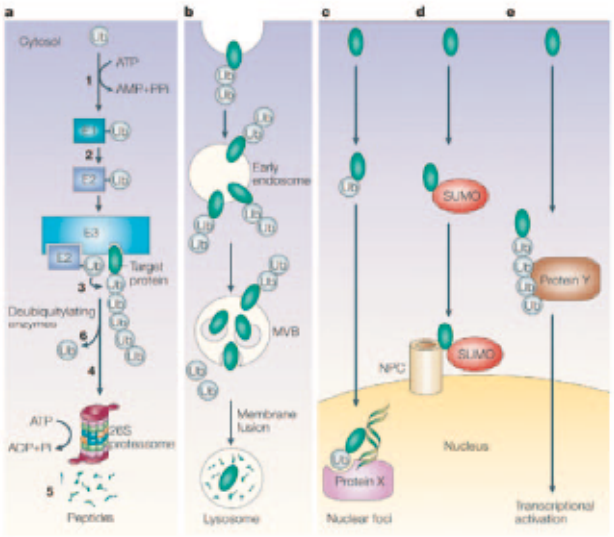

Figure 7: Some of the different functions of modification by ubiquitin and ubiquitin-like proteins. a. Proteasomal-dependent degradation of cellular proteins (see Figure 6). b. Mono- or oligoubiquitination targets membrane proteins to degradation in the lysosome/vacuole. c. Monoubiquitination, or d. a single modification by a ubiquitin-like (UBL) protein, SUMO for example, can target proteins to different subcellular destinations such as nuclear foci or the nuclear pore complex (NPC). Modification by UBLs can serve also other, non-proteolytic functions, such as protecting proteins from ubiquitination or activation of E3 complexes (not shown). e. Generation of a Lys^{63}-based polyubiquitin chain can activate transcriptional regulators, directly or indirectly via recruitment of other proteins (protein Y, for example; shown), or activation of upstream components such as kinases. Ub denotes ubiquitin. (With permission from Nature Publishing Group. Published originally in Ref. 83).

tracellular proteins are destroyed. Independent lines of experimental evidence gradually eroded the lysosomal hypothesis and resulted in the evolvement of new concept, that the bulk of intracellular proteins are degraded – under basal metabolic conditions – via a non-lysosomal machinery. This resulted in the discovery of the ubiquitin system in the late 1970s and early 1980s.

With the identification of the reactions and enzymes that are involved in the ubiquitin-proteasome cascade, a new era in the protein degradation field began at the late 1980s and early 1990s. Studies that showed that the system is involved in targeting of key regulatory proteins – such as light-regulated proteins in plants, transcriptional factors, cell cycle regulators and tumor suppressors and promoters – started to emerge (see for example Refs. 74–78). These studies were accompanied by functional analysis of the system in the yeast *Saccharomyces Cerevisial* (carried out initially mostly by Varshavsky and colleagues), taking advantage of the power of genetics. They were then followed by numerous studies on the underlying mechanisms involved in the degradation of specific proteins, each with its own unique mode of recognition and regulation. The unraveling of the human genome revealed the

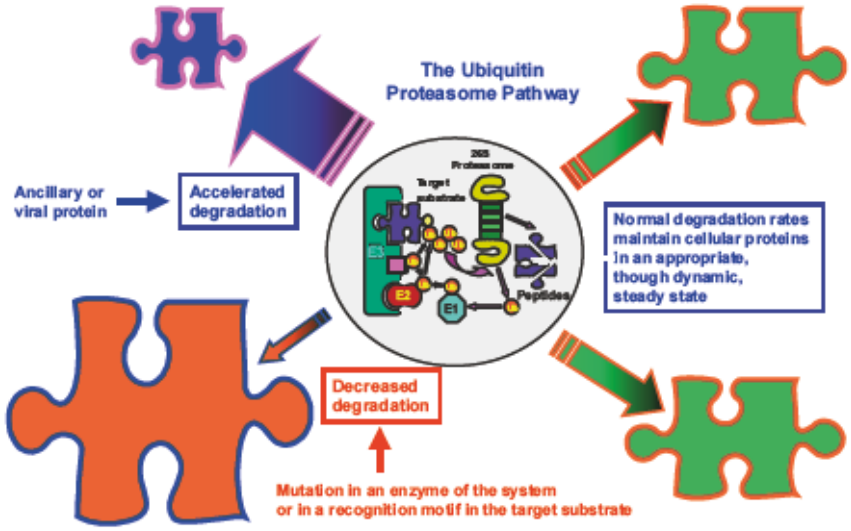

Figure 8: Aberrations in the ubiquitin-proteasome system and pathogenesis of human diseases. Normal degradation of cellular proteins maintains them in a steady-state level, though, this level may change under various physiological conditions (upper and lower right side). When degradation is accelerated due to an increase in the level or activity of an E3 (Skp2 in the case of p27, for example), or overexpression of an ancillary protein that generates a complex with the protein substrate and targets it for degradation (the Human Papillomavirus E6 oncoprotein that associates with p53 and targets it for degradation by the E6-AP ligase, or the cytomegalovirus-encoded ER proteins US2 and US11 that target MHC class I molecules for Endoplasmic Reticulum-Associated Degradation, ERAD), the steady state level of the protein decreases (upper left side). A mutation in a ubiquitin ligase or in a substrate's scaffold/binding protein [such as occurs in *Adenomatous Polyposis Coli* – APC, or in E6-AP (Angelmans' Syndrome)] or in the substrate's recognition motif (such as occurs in β-catenin or in ENaC) will result in decreased degradation and accumulation of the target substrate (lower left side).

existence of hundreds of distinct E3s, attesting to the complexity and the high specificity and selectivity of the system. Two important advances in the field were the discovery of the non-proteolytic functions of ubiquitin such as activation of transcription and routing of proteins to the lysosome/vacuole, and the discovery of modification by ubiquitin-like proteins (UBLs), that is involved in numerous non-proteolytic functions such as directing proteins to their subcellular destination, protecting proteins from ubiquitination, and controlling entire processes such as autophagy (see for example Ref. 79) (for some of the different roles of modifications by ubiquitin and UBLs, see Figure 7). All these studies have led to the emerging realization that this novel mode of post-translational modification/covalent conjugation plays key roles in regulating a broad array of cellular processes – among them cell cycle and division, growth and differentiation, activation and silencing of transcription, apoptosis, the immune and inflammatory response, signal transduction, receptor-mediated endocytosis, various metabolic pathways, and the cell quality control – all

that through proteolytic and non-proteolytic mechanisms. The discovery that ubiquitin modification plays a role in routing proteins to the lysosome/vacuole, and that modification by specific and unique ubiquitin-like proteins and modification system controls autophagy closed an exciting historical cycle, since it demonstrated that the two apparently distinct proteolytic systems – the ubiquitin/proteasome and the lysosome – communicate with one another. With the many processes and innumerable substrates targeted by the ubiquitin pathway, it is not surprising to find that aberrations in the system underlie, directly or indirectly, the pathogenesis of many diseases. While inactivation of a major enzyme such as E1 is obviously lethal, mutations in enzymes or in recognition motifs in substrates that do not affect vital pathways or that affect the involved process only partially, may result in a broad array of phenotypes. Likewise, acquired changes in the activity of the system can also evolve into certain pathologies. The pathological states associated with the ubiquitin system can be classified into two groups: **(a)** those that result from loss of function – mutation in a ubiquitin system enzyme or in the recognition motif in the target substrate that result in stabilization of certain proteins, and **(b)** those that result from gain of function – abnormal or accelerated degradation of the protein target (for a general scheme describing aberrations in the ubiquitin system that result in disease states, see Figure 8). Studies that employ targeted inactivation of genes coding for specific ubiquitin system enzymes and substrates in animals can provide a more systematic view into the broad spectrum of pathologies that may result from aberrations in ubiquitination, ubiquitin-mediated proteolysis, and modification by UBLs. Better understanding of the processes and identification of the components involved in the degradation of key regulators will lead to the development of mechanism-based drugs that will target specifically only the involved proteins. While the first drug, a specific proteasome inhibitor is already on the market (80), it appears that one important hallmark of the new era we are entering now will be the discovery of novel drugs based on targeting of specific processes such as inhibiting aberrant Mdm2- or E6-AP-mediated accelerated targeting of the tumor suppressor p53 which will result in regaining its lost function.

Many review articles have been published on different aspects of the ubiquitin system. The purpose of this article was to bring to the reader several milestones along the historical pathway of the evolvement of the ubiquitin system. For additional reading on the ubiquitin system the reader is referred to the many reviews written on the system, among them, for example, are Refs. 81,82. Some parts of this review, including several Figures, are based on another recently published review article (Ref. 83).

ACKNOWLEDGEMENT

Research in the laboratory of Aaron Ciechanover has been supported along the years by grants from the US-Israel Binational Science Foundation (BSF), the Israel Science Foundation (ISF) founded by the Israeli National Academy of Humanities, Arts and Sciences, the German- Israeli Foundation (GIF) for

Scientific Research and Development, the Israel Cancer Research Fund (ICRF) USA, the Deutsche-Israeli Cooperation Program (DIP), the European Commission (EC), the Israel Cancer Society (ICS), the Prostate Cancer Foundation (PCF) – Israel, the Foundation for Promotion of Research in the Technion and various research grants administered by the Vice President of the Technion for Research. Infrastructural equipment for the laboratory of A.C. and for the Cancer and Vascular Biology Research Center has been purchased with the support of the Wolfson Charitable Fund – Center of Excellence for Studies on Turnover of Cellular Proteins and its Implications to Human Diseases.

REFERENCES

1. Clarke, H.T. (1958). Impressions of an organic chemist in biochemistry. *Annu. Rev. Biochem.* **27**, 1–14.
2. Kennedy., E.P. (2001). Hitler's gift and the era of biosynthesis. *J. Biol. Chem.* **276**, 42619–42631.
3. Simoni, R.D., Hill, R.L., and Vaughan, M. (2002). The use of isotope tracers to study intermediary metabolism: Rudolf Schoenheimer. *J. Biol. Chem.* **277** (issue 43), e1-e3 (available on-line at: http:/www.jbc.org).
4. Schoenheimer, R., Ratner, S., and Rittenberg, D. (1939). Studies in protein metabolism. VII: The metabolism of tyrosine. *J. Biol. Chem.* **127**, 333–344.
5. Ratner, S., Rittenberg, D., Keston, A.S., and Schoenheimer, R. (1940). Studies in protein metabolism. XIV: The chemical interaction of dietary glycine and body proteins in rats. *J. Biol. Chem.* **134**, 665–676.
6. Schoenheimer, R. The Dynamic State of Body Constituents (1942). Harvard University Press, Cambridge, Massachusetts, USA.
7. Hogness, D.S., Cohn, M., and Monod, J. (1955). Studies on the induced synthesis of, β-galactosidase in *Escherichia coli:* The kinetics and mechanism of sulfur incorporation. *Biochim. Biophys. Acta* **16**, 99–116.
8. de Duve, C., Gianetto, R., Appelmans, F., and Wattiaux, R. (1953). Enzymic content of the mitochondria fraction. *Nature (London)* **172**, 1143–1144.
9. Gianetto, R., and de Duve, C. Tissue fractionation studies 4. (1955). Comparative study of the binding of acid phosphatase, β-glucoronidase and cathepsin by rat liver particles. *Biochem. J.* **59**, 433–438.
10. Simpson, M.V. The release of labeled amino acids from proteins in liver slices. (1953). *J. Biol. Chem.* **201**, 143–154.
11. Mortimore, G.E., and Poso, A.R. (1987). Intracellular protein catabolism and its control during nutrient deprivation and supply. *Annu. Rev. Nutr.* **7**, 539–564.
12. Ashford, T.P., and Porter, K.R. (1962). Cytoplasmic components in hepatic cell lysosomes. *J. Cell Biol.* **12**, 198–202.
13. Schimke, R.T., and Doyle, D. (1970). Control of enzyme levels in animal tissues. *Annual Rev. Biochem.* **39**, 929–976.
14. Goldberg, A.L., and St. John, A.C. (1976). Intracellular protein degradation in mammalian and bacterial cells: Part 2. *Annu. Rev. Biochem.* **45**, 747–803.
15. Segal, H.L., Winkler, J.R., and Miyagi, M.P. (1974). Relationship between degradation rates of proteins *in vivo* and their susceptibility to lysosomal proteases. *J. Biol. Chem.* **249**, 6364–6365.
16. Haider, M., and Segal, H.L. (1972). Some characteristics of the alanine-aminotransferase and arginase-inactivating system of lysosomes. *Arch. Biochem. Biophys.* **148**, 228–237.
17. Dean, R.T. Lysosomes and protein degradation. (1977). *Acta Biol. Med. Ger.* **36**, 1815–1820.
18. Müller, M., Müller, H, and Holzer, H. (1981). Immunochemical studies on catabolite inactivation of phosphoenolpyruvate carboxykinase in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **256**, 723–727.

19. Holzer, H. (1989). Proteolytic catabolite inactivation in *Saccharomyces cerevisiae*. *Revis. Biol. Celular* **21**, 305–319.
20. Majeski, A.E., and Dice, J.F. (2004). Mechanisms of chaperone-mediated autophagy. *Intl. J. Biochem. Cell Biol.* **36**, 2435–2444.
21. Cuervo, A.M., and Dice, J.F. (1998). Lysosomes, a meeting point of proteins, chaperones, and proteases. *J. Mol. Med.* **76**, 6–12.
22. Hayashi, M., Hiroi, Y., and Natori, Y. (1973). Effect of ATP on protein degradation in rat liver lysosomes. *Nature New Biol.* **242**, 163–166.
23. Schneider, D.L. (1981). ATP-dependent acidification of intact and disrupted lysosomes: Evidence for an ATP-driven proton pump. *J. Biol. Chem.* **256**, 3858–3864.
24. de Duve, C., and Wattiaux, R. Functions of lysosomes. (1966). *Annu. Rev. Physiol.* **28**, 435–492.
25. Rabinovitz, M., and Fisher, J.M. (1964). Characteristics of the inhibition of hemoglobin synthesis in rabbit reticulocytes by threo-α-amino-β-chlorobutyric acid. *Biochim. Biophys. Acta.* **91**, 313–322.
26. Carrell, R.W., and Lehmann, H. (1969). The unstable hemoglobin hemolytic anaemias. *Semin. Hematol.* **6**, 116–132.
27. Huehns, E.R., and Bellingham, A.J. (1969). Diseases of function and stability of hemoglobin. *Br. J. Hematol.* **17**, 1–10.
28. Etlinger, J.D., and Goldberg, A.L. (1977). A soluble ATP-dependent proteolytic system responsible for the degradation of abnormal proteins in reticulocytes. *Proc. Natl. Acad. Sci. USA* **74**, 54–58.
29. Hershko, A., Heller, H., Ganoth, D., and Ciechanover, A. (1978). Mode of degradation of abnormal globin chains in rabbit reticulocytes. In: Protein Turnover and Lysosome Function (H.L. Segal & D.J. Doyle, eds.). Academic Press, New York. pp. 149–169.
30. Knowles, S.E., and Ballard, F.J. (1976). Selective control of the degradation of normal and aberrant proteins in Reuber H35 hepatoma cells. *Biochem J.* **156**, 609–617.
31. Neff, N.T., DeMartino, G.N., and Goldberg, A.L. (1979). The effect of protease inhibitors and decreased temperature on the degradation of different classes of proteins in cultured hepatocytes. *J. Cell Physiol.* **101**, 439–457.
32. Poole, B., Ohkuma, S., and Warburton, M.J. (1977). The accumulation of weakly basic substances in lysosomes and the inhibition of intracellular protein degradation. *Acta Biol. Med. Germ.* **36**, 1777–1788.
33. Poole, B., Ohkuma, S. & Warburton, M.J. (1978). Some aspects of the intracellular breakdown of exogenous and endogenous proteins. In: Protein Turnover and Lysosome Function (H.L. Segal and D.J. Doyle, eds.). Academic Press, New York. pp. 43–58.
34. Mandelstam, J. (1958). Turnover of proteins in growing and non-growing populations of *Escherichia coli*. *Biochem. J.* **69**, 110–119.
35. Steinberg, D., and Vaughan, M. (1956). Observations on intracellular protein catabolism studied *in vitro*. *Arch. Biochem. Biophys.* **65**, 93–105.
36. Hershko, A., and Tomkins, G.M. (1971). Studies on the degradation of tyrosine aminotransferase in hepatoma cells in culture: Influence of the composition of the medium and adenosine triphosphate dependence. *J. Biol. Chem.* **246**, 710–714.
37. Goldberg, A.L., Kowit, J.D., and Etlinger, J.D. (1976). Studies on the selectivity and mechanisms of intracellular protein degradation. In: Proteolysis and Physiological Regulation (D.W. Ribbons and K. Brew, eds.). Academic Press, New York. pp. 313–337.
38. Ciechanover A., Hod, Y., and Hershko, A. (1978). A heat-stable polypeptide component of an ATP-dependent proteolytic system from reticulocytes. *Biochem. Biophys. Res. Commun.* **81**, 1100–1105.
39. Ciechanover, A., Heller, H., Elias, S., Haas, A.L., and Hershko, A. (1980). ATP-dependent conjugation of reticulocyte proteins with the polypeptide required for protein degradation. *Proc. Natl. Acad. Sci. USA.* **77**, 1365–1368.
40. Hershko, A., Ciechanover, A., Heller, H., Haas, A.L., and Rose, I.A. (1980). Proposed role of ATP in protein breakdown: Conjugation of proteins with multiple chains of the polypeptide of ATP-dependent proteolysis. *Proc. Natl. Acad. Sci. USA* **77**, 1783–1786.

41. Ciechanover, A., Elias, S., Heller, H., Ferber, S. and Hershko, A. (1980). Characterization of the heat-stable polypeptide of the ATP-dependent proteolytic system from reticulocytes. *J. Biol. Chem.* **255**, 7525–7528.
42. Wilkinson, K.D., Urban, M.K., and Haas, A.L. (1980). Ubiquitin is the ATP-dependent Proteolysis Factor I of rabbit reticulocytes. *J. Biol. Chem.* **255**, 7529–7532.
43. Goldstein, G. (1974). Isolation of bovine thymin, a polypeptide hormone of the thymus. *Nature (London)* **247**, 11–14.
44. Goldstein, G., Scheid, M., Hammerling, U., Schlesinger, D.H., Niall, H.D., and Boyse, E.A. (1975). Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells. *Proc. Natl. Acad. Sci. USA* **72**, 11–15.
45. Schlessinger, D.H., Goldstein, G., and Niall, H.D. (1975). The complete amino acid sequence of ubiquitin, an adenylate cyclase stimulating polypeptide probably universal in living cells. *Biochemistry* **14**, 2214–2218.
46. Low, T.L.K., and Goldstein, A.L. (1979). The chemistry and biology of thymosin: Amino acid analysis of thymosin α1 and polypeptide β1. *J. Biol. Chem.* **254**, 987–995.
47. Goldknopf, I.L., and Busch, H. (1975). Remarkable similarities of peptide fingerprints of histone 2A and non-histone chromosomal protein A24. *Biochem. Biophys. Res. Commun.* **65**, 951–955.
48. Goldknopf, I.L., and Busch, H. (1977). Isopeptide linkage between non-histone and histone 2A polypeptides of chromosome conjugate-protein A24. *Proc. Natl. Acad. Sci. USA* **74**, 864–868.
49. Hunt, L.T., and Dayhoff, M.O. (1977). Amino-terminal sequence identity of ubiquitin and the non-histone component of nuclear protein A24. *Biochim. Biophys. Res. Commun.* **74**, 650–655.
50. Hershko, A., Ciechanover, A., and Rose, I.A. (1981). Identification of the active amino acid residue of the polypeptide of ATP-dependent protein breakdown. *J. Biol. Chem.* **256**, 1525–1528.
51. Hershko, A., and Heller, H. (1985). Occurrence of a polyubiquitin structure in ubiquitin-protein conjugates. *Biochem. Biophys. Res. Commun.* **128**, 1079–1086.
52. Chau, V., Tobias, J. W., Bachmair, A., Mariott, D., Ecker, D., Gonda, D. K., and Varshavsky, A. (1989). A multiubiquitin chain is confined to specific lysine in a targeted short lived protein. *Science* **243**, 1576–1583.
53. Ciechanover, A., and Ben-Saadon R. (2004). N-terminal ubiquitination: More protein substrates join in. *Trends Cell Biol.* **14**, 103–106.
54. Muratani, M., and Tansey, W.P. (2003). How the ubiquitin-proteasome system controls transcription. *Nat. Rev. Mol. Cell Biol.* **4**, 192–201.
55. Zhang, Y. (2003). Transcriptional regulation by histone ubiquitination and deubiquitination. *Genes & Dev.* **17**, 2733–2740.
56. Osley, M.A. (2004). H2B ubiquitylation: The end is in sight. *Biochim. Biophys. Acta.* **1677**, 74–78.
57. Lipman, F. (1971). Attempts to map a process evolution of peptide biosynthesis. *Science* **173**, 875–884.
58. Ciechanover, A., Elias, S., Heller, H. & Hershko, A. (1982). "Covalent affinity" purification of ubiquitin-activating enzyme. *J. Biol. Chem.* **257**, 2537–2542.
59. Hershko, A., Heller, H., Elias, S., and Ciechanover, A. (1983). Components of ubiquitin-protein ligase system: Resolution, affinity purification and role in protein breakdown. *J. Biol. Chem.* **258**, 8206–8214.
60. Chin, D.T., Kuehl, L., and Rechsteiner, M. (1982). Conjugation of ubiquitin to denatured hemoglobin is proportional to the rate of hemoglobin degradation in HeLa cells. *Proc. Natl. Acad. Sci. USA* **79**, 5857–5861.
61. Hershko, A., Eytan, E., Ciechanover, A. and Haas, A.L. (1982). Immunochemical Analysis of the turnover of ubiquitin-protein conjugates in intact cells: Relationship to the breakdown of abnormal proteins. *J. Biol. Chem.* **257**, 13964–13970.
62. Matsumoto, Y., Yasuda, H., Marunouchi, T., and Yamada, M. (1983). Decrease in uH2A (protein A24) of a mouse temperature-sensitive mutant. *FEBS Lett.* **151**, 139–142.

63. Finley, D., Ciechanover, A., and Varshavsky, A. (1984). Thermolability of ubiquitin-activating enzyme from the mammalian cell cycle mutant ts85. *Cell* **37**, 43–55.
64. Ciechanover, A., Finley D., and Varshavsky, A. (1984). Ubiquitin dependence of selective protein degradation demonstrated in the mammalian cell cycle mutant ts85. *Cell* **37**, 57–66.
65. Tanaka, K., Waxman, L., and Goldberg, A.L. (1983). ATP serves two distinct roles in protein degradation in reticulocytes, one requiring and one independent of ubiquitin. *J. Cell Biol.* **96**, 1580–1585 (1983).
66. Hershko, A., Leshinsky, E., Ganoth, D., and Heller, H. (1984). ATP-dependent degradation of ubiquitin-protein conjugates. *Proc. Natl. Acad. Sci. USA* **81**, 1619–1623.
67. Hough, R., Pratt, G., and Rechsteiner, M. (1986). Ubiquitin-lysozyme conjugates: Identification and characterization of an ATP-dependent protease from rabbit reticulocyte lysates. *J. Biol. Chem.* **261**, 2400–2408.
68. Waxman, L., Fagan, J., and Goldberg, A.L. (1987). Demonstration of two distinct high molecular weight proteases in rabbit reticulocytes, one of which degrades ubiquitin conjugates. *J. Biol. Chem.* **262**, 2451–2457.
69. Hough, R., Pratt, G., and Rechsteiner M. (1987). Purification of two high molecular weight proteases from rabbit reticulocyte lysate. *J. Biol. Chem.* **262**, 8303–8313.
70. Wilk, S., and Orlowski, M. (1980). Cation-sensitive neutral endopeptidase: Isolation and specificity of the bovine pituitary enzyme. *J. Neurochem.* **35**, 1172–1182.
71. Eytan, E., Ganoth, D., Armon, T., and Hershko, A. (1989). ATP-dependent incorporation of 20S protease into the 26S complex that degrades proteins conjugated to ubiquitin. *Proc. Natl. Acad. Sci. USA.* **86**, 7751–7755.
72. Driscoll, J., and Goldberg, A.L. (1990). The proteasome (multicatalytic protease) is a component of the 1500-kDa proteolytic complex which degrades ubiquitin-conjugated proteins. *J. Biol. Chem.* **265**, 4789–4792.
73. Hoffman, L., Pratt, G., and Rechsteiner, M. (1992). Multiple forms of the 20S multicatalytic and the 26S ubiquitin/ATP-dependent proteases from rabbit reticulocyte lysate. *J. Biol. Chem.* **267**, 22362–22368.
74. Shanklin, J., Jaben, M., and Vierstra, R.D. (1987). Red light-induced formation of ubiquitin-phytochrome conjugates: Identification of possible intermediates of phytochrome degradation. *Proc. Natl. Acad. Sci. USA* **84**, 359–363.
75. Hochstrasser, M., and Varshavsky, A. (1990). *In vivo* degradation of a transcriptional regulator: the yeast α2 repressor. *Cell* **61**, 697 708.
76. Scheffner, M., Werness, B.A., Huibregtse, J.M., Levine, A.J., and Howley, P.M. (1990). The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* **63**, 1129–1136.
77. Glotzer, M., Murray, A.W., and Kirschner, M.W. (1991). Cyclin is degraded by the ubiquitin pathway. *Nature* **349**, 132–138.
78. Ciechanover, A., DiGiuseppe, J.A., Bercovich, B., Orian, A., Richter, J.D., Schwartz, A.L., and Brodeur, G.M. (1991). Degradation of nuclear oncoproteins by the ubiquitin system *in vitro*. *Proc. Natl. Acad. Sci. USA* **88**, 139–143.
79. Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., George, M.D., Klionsky, D.J., Ohsumi, M., and Ohsumi, Y. (1998). A protein conjugation system essential for autophagy. *Nature* **395**, 395–398.
80. Adams J. (2003). Potential for proteasome inhibition in the treatment of cancer. *Drug Discov. Today.* **8**, 307–315.
81. Glickman, M.H., and Ciechanover, A. (2002). The ubiquitin-proteasome pathway: Destruction for the sake of construction. *Physiological Reviews* **82**, 373–428.
82. Pickart, C.M., and Cohen, R.E. (2004). Proteasomes and their kin: Proteases in the machine age. *Nature Rev. Mol. Cell Biol.* **5**, 177–187.
83. Ciechanover, A. (2005). From the lysosome to ubiquitin and the proteasome. *Nature Rev. Mol. Cell Biol.* **6**, 79–86.

Portrait photo of Professor Ciechanover by photographer Dan Porges.