# THE UBIQUITIN SYSTEM FOR PROTEIN DEGRADATION AND SOME OF ITS ROLES IN THE CONTROL OF THE CELL DIVISION CYCLE

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by

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## INTRODUCTION

All living cells contain many thousands of different proteins, each of which carries out a specific chemical or physical process. Due to the importance of proteins in basic cellular functions, there has been a great interest in the problem of how proteins are synthesized. In the fifties and sixties of the 20th century, the discovery of the double helical structure of DNA and the cracking of the genetic code focused attention on the mechanisms by which the order of bases in DNA determines the sequence of amino acids in proteins, and on further molecular mechanisms that regulate the expression of specific genes. Because of the intensive research activity on protein synthesis, little attention was paid to at that time to the fact that many proteins are rapidly degraded to amino acids. This dynamic turnover of cellular proteins had been previously known by the pioneering work of Schoenheimer and co-workers, who were among the first to introduce the use of isotopically labeled compounds to biological studies. They administered $^{15}$N-labeled L-leucine to adult rats, and the distribution of the isotope in body tissues and in excreta was examined. It was observed that less than one-third of the isotope was excreted in the urine, and most of it was incorporated into tissue proteins (1). Since the weight of the animals did not change during the experiment, it could be assumed that the mass and composition of body proteins also did not change. It was concluded that newly incorporated amino acids must have replaced those in tissue proteins in a process of dynamic protein turnover (1). Schoenheimer's studies on the dynamic state of proteins and of some other body constituents were published in a small booklet in 1942, soon after his untimely death (ref. 2, see Fig. 1).

In the subsequent decades research on protein degradation was neglected, mainly because of the great interest in the mechanisms of protein synthesis, as described above. However, gradually experimental evidence accumulated which indicated that intracellular protein degradation is extensive, selective and has basically important cellular functions. It was observed that abnormal
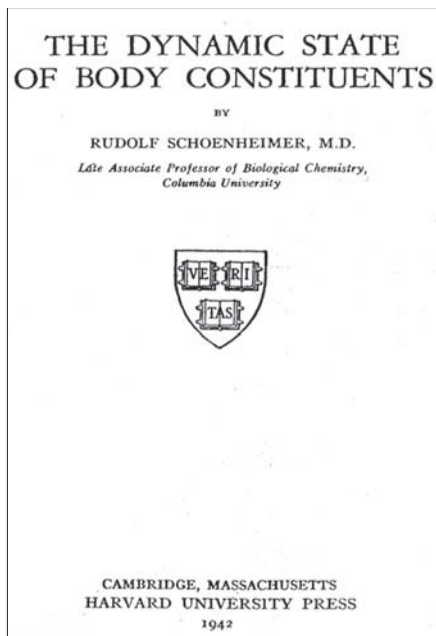
*Figure 1.* Front page of Schoenheimer's collected lectures, edited by his colleagues soon after his death.

proteins produced by the incorporation of some amino acid analogues are selectively recognized and are rapidly degraded in cells (3). However, intracellular protein degradation was not thought to be merely a "garbage disposal" system for the elimination of abnormal or damaged proteins. By the late sixties, it became apparent that normal proteins are also degraded in a highly selective fashion. The half-life times of different proteins ranged from several minutes to many days, and rapidly degraded proteins usually had important regulatory functions. These properties of intracellular protein degradation and the role of this process in the regulation of the levels of specific proteins were summarized in an excellent review by Schimke and Doyle in 1970 (4). Thus, it was known at that time that protein degradation has important functions, but it was not known what is the biochemical system that carries out this process at such a high degree of selectivity and sophistication.

## MY FIRST ENCOUNTER WITH PROTEIN DEGRADATION

I became interested in the problem of how proteins are degraded in cells when I was a post-doctoral fellow in the laboratory of Gordon Tomkins in 1969–71 at the University of California, San Francisco. At that time, Gordon was mainly interested in the mechanisms by which steroid hormones induce the synthesis of specific proteins. His model system for this purpose was the synthesis of the enzyme tyrosine aminotransferase (TAT) in cultured hepato-
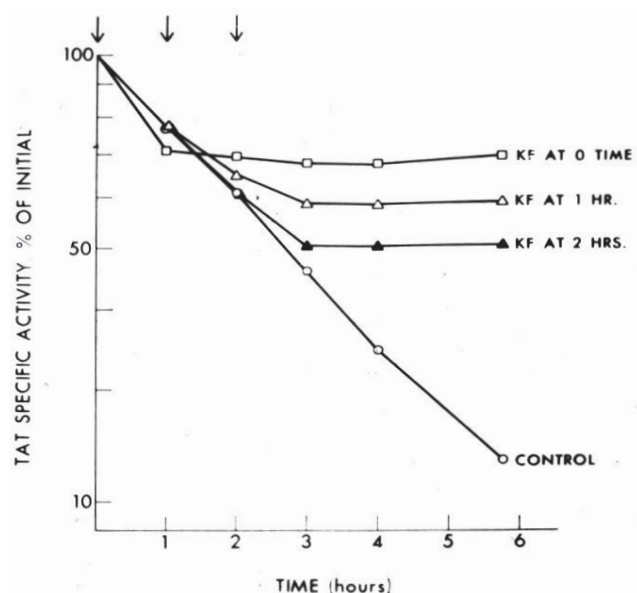
*Figure 2*. Energy-dependence of the degradation of tyrosine aminotransferase. From ref. 5.

ma cells. When I arrived there I saw that it was a large laboratory, with many post-doctoral fellows working on different aspects of the synthesis of TAT. I thought that this was a bit too crowded and I asked Gordon for a different project. He suggested that I should study the degradation of TAT, a process that also regulates the level of this enzyme. This was how I became involved in protein degradation, a problem on which I have been working ever since.

Fig. 2 shows one of the first experiments that I did as a post-doctoral fellow in the Tomkins lab. It was quite easy to follow the degradation of TAT: first, hepatoma cells were incubated with a steroid hormone, which caused a great increase in the level of this protein. Then the hormone was removed by changing the culture medium, and a rapid decline in the level of this protein, due to its degradation, could be observed. As with other regulatory proteins, this protein also had a relatively rapid rate of degradation, with a half-life time of about 2-3 hours. I found then that the degradation of TAT was completely arrested by potassium fluoride, an inhibitor of cellular energy production (ref. 5 and Fig. 2). The effect was not specific to fluoride, because I got similar results with other inhibitors of cellular energy production. These results confirmed and extended earlier findings of Simpson (6) on the energy-dependence of the liberation of amino acids from proteins in liver slices. This observation was later dismissed as being indirect, and that energy is needed to keep the acidic pH inside the lysosomes (described in ref. 7). However, in the case of TAT energy was needed for the selective degradation of a specific enzyme, and it did not

seem reasonable to assume that engulfment into lysosomes can be responsible for the highly selective degradation of specific cellular proteins. Since ATP depletion also prevented the inactivation of the enzymatic activity of TAT, it was concluded that energy is required at an early step in the process of protein degradation (5).

I was very much intrigued by the energy-dependence of intracellular protein degradation. Energy is usually needed to synthesize a chemical bond, and not to break a chemical bond. Thus, the action of extracellular proteinases of the digestive system is an exergonic process, *i. e.*, it actually releases energy. This suggested that within cells a novel, as yet unknown proteolytic system exists, that presumably uses energy to attain the high selectivity of the degradation of cellular proteins.

## DISCOVERY OF THE ROLE OF UBIQUITIN IN PROTEIN DEGRADATION

Parts of the story of the discovery of the ubiquitin system have been described previously (7–9). Following my return to Israel and setting up my own laboratory at the Technion in Haifa, I continued to pursue this problem of how proteins are degraded in cells, and why is energy required for this process. It was clear to me that the only way to find out how a completely novel system works is that of classical biochemistry. This consists of using a cell-free system that faithfully reproduces the process in the test tube, fractionation to separate its different components, purification and characterization of each component and reconstitution of the system from isolated and purified components. A cell-free ATP-dependent proteolytic system from reticulocyte lysates was first established by Etlinger and Goldberg (10). Subsequently, my laboratory subjected this system to biochemical fractionation, with the aim of the isolation of its components and the characterization of their mode of action. A great part of this work was done by Aaron Ciechanover, who was my graduate student at that time (1976–1981). This work has also received a lot of support, great advice and helpful criticism from Irwin Rose, in whose laboratory at Fox Chase Cancer Center I worked in a sabbatical year in 1977–78 and in many summers afterwards.

In the initial experiments, we have resolved reticulocyte lysates on DEAE-cellulose into two crude fractions: Fraction 1, which contained proteins not adsorbed to the resin, and Fraction 2, which contained all proteins adsorbed to the resin and eluted with high salt. The original aim of this fractionation was to get rid of hemoglobin, which was known to be in Fraction 1, while most non-hemoglobin proteins of reticulocytes were known to be in Fraction 2. We found that neither fraction was active by itself, but ATP-dependent protein degradation could be reconstituted by the combination of the two fractions (11). The active component in Fraction 1 was a small, heat stable protein; we have exploited its stability to heat treatment for its purification to near homogeneity. We termed this protein at that time APF-1, for <u>A</u>TP-dependent <u>P</u>roteolysis <u>F</u>actor 1. The identity of APF-1 with ubiquitin was established later by Wilkinson *et al.* (12), subsequent to our discovery of its covalent ligation to
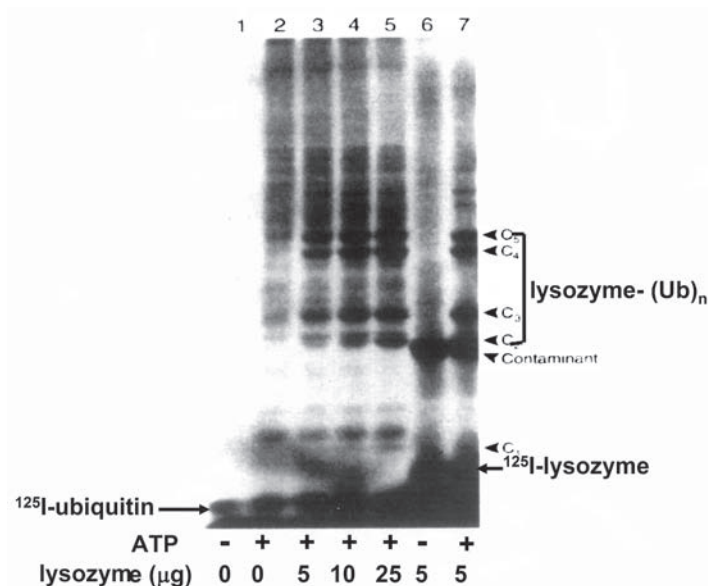
*Figure 3.* Discovery of covalent ligation of ubiquitin to substrate protein. See the text. From ref. 16.

protein substrates, as described below. Ubiquitin was originally isolated by Goldstein and co-workers in a search for hormones of the thymus, but was subsequently found to be present in all tissues and eukaryotic organisms, hence its name (13). The functions of ubiquitin were not known, though it was discovered by Goldknopf and Busch that ubiquitin was conjugated to histone 2A by an isopeptide linkage (14).

The purification of APF-1/ubiquitin from Fraction 1 was the key to the elucidation of the mode of its action in the proteolytic system. It looked smaller than most enzymes, so at first we thought that it might be a regulatory subunit of some enzyme (such as a protein kinase or an ATP-dependent protease) present in Faction 2. To test this notion, we looked for the association of APF-1/ubiquitin with some protein in Fraction 2. For this purpose, purified radiolabeled APF-1/ubiquitin was incubated with Fraction 2 in the presence or absence of ATP, and was subjected to gel filtration chromatography. A marked ATP-dependent association of APF-1/ubiquitin with high molecular weight material was observed (15). It was very surprising to find, however, that ubiquitin was bound by covalent amide linkage, as indicated by the resistance of high molecular weight derivative to alkali, hydroxylamine and boiling with SDS in the presence of mercaptoethanol (15). The analysis of reaction products by SDS-polyacrylamide gel electrophoresis showed that ubiquitin was ligated to a great number of endogenous proteins. Since crude Fraction 2 from reticulo-
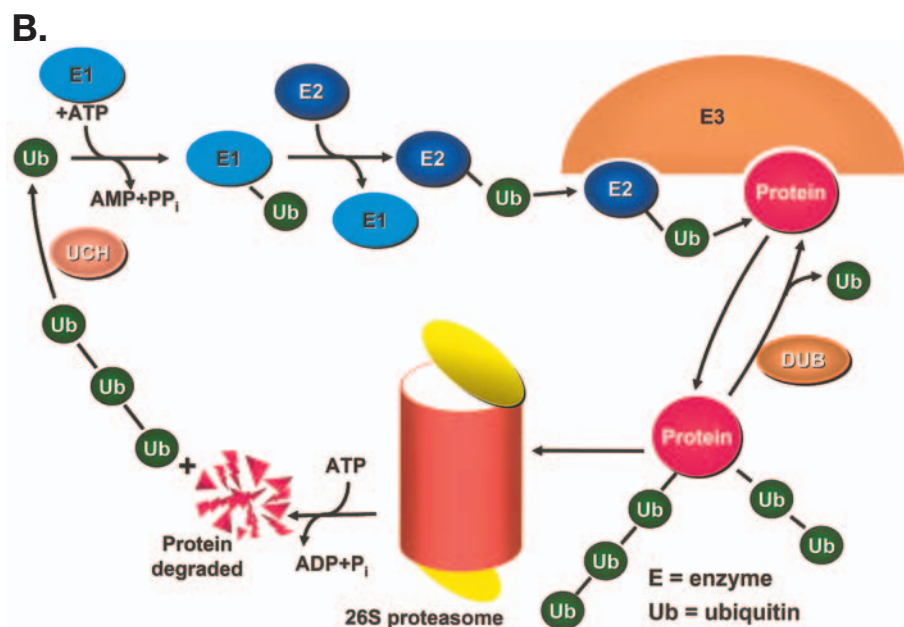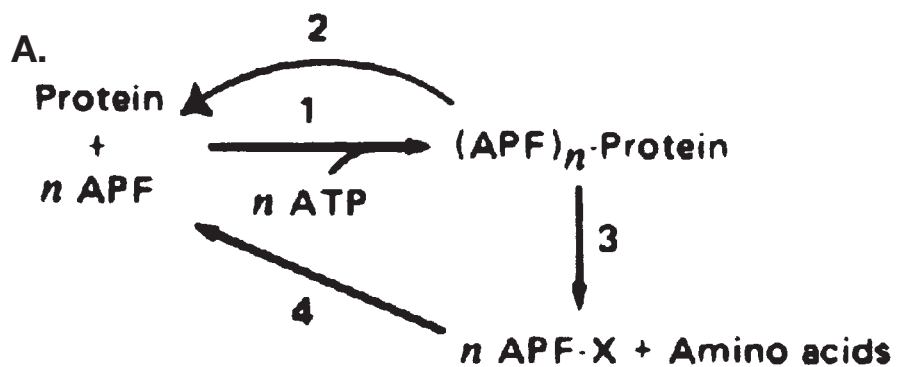
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*Figure 4.* The ubiquitin system then and now. A. Original proposal of the sequence of events in protein degradation. See the text; from ref. 16. B. Our current view of the main enzymatic reactions in ubiquitin-mediated protein degradation. See the text. Ub, ubiquitin; DUB, deubiquitylating enzyme; UCH, ubiquitin carboxyl-terminal hydrolase.

cytes contained not only enzymes, but also endogenous substrates of the proteolytic system, we began to suspect that ubiquitin may be linked to protein substrates, rather than to an enzyme. We indeed found that proteins that are good (though artificial) substrates of the ATP-dependent proteolytic system, such as lysozyme, are conjugated to ubiquitin (16). The original experiment is shown in Fig. 3. We found that similar high-molecular weight derivatives were formed when ^{125}I-labeled ubiquitin was incubated with unlabeled lysozyme

(Fig. 3, lanes 3–5), or when ^{125}I-labeled lysozyme was incubated with unlabeled ubiquitin in the presence of ATP (Fig. 3, lane 7). Based on these observations, we proposed that proteins are targeted for degradation by covalent ligation to APF-1/ubiquitin (16). The original hypothesis from 1980, formulated jointly with Irwin Rose, is shown in Fig. 4A. We proposed that a putative enzyme, which we called "APF-1-protein amide synthetase", ligates multiple molecules of ubiquitin to the protein substrate (Step 1) and then some other enzyme degrades proteins which are linked to several molecules of ubiquitin (Step 3), and finally free and reutilizable ubiquitin is released (Step 4). According to this proposal, ubiquitin is essentially a tag, which when attached to a protein, dooms this protein to be degraded.

## IDENTIFICATION OF ENZYMES OF THE UBIQUITIN-MEDIATED PROTEOLYTIC SYSTEM

In subsequent work, we tried to isolate and characterize enzymes of the ubiquitin pathway, using the same biochemical fractionation-reconstitution approach. The original proposal (Fig. 4A) was found to be essentially correct, but much important detail was added. Our present knowledge of the main enzymatic steps in the ubiquitin-mediated proteolytic pathway is shown in Fig. 4B (reviewed in ref. 17). This scheme summarizes about 10 years of our work (from 1980 to 1990), as well as that of some other researchers. Thus, we found that ubiquitin is ligated to proteins not by one enzyme, but by the sequential action of three enzymes. These are the ubiquitin-activating enzyme E1 (18), a ubiquitin-carrier protein E2 (19) and a ubiquitin-protein ligase E3 (19). E1 carries out the ATP-dependent activation of the carboxy-terminal glycine residue of ubiquitin (20) by the formation of ubiquitin adenylate, followed by the transfer of activated ubiquitin to a thiol site of E1 with the formation of thiolester linkage (18, 21). Activated ubiquitin is transferred to a thiol site of E2 by transacylation, and is then further transferred to amino group of the protein substrate in a reaction that requires E3 (19). We found that the role of E3 is to bind specific protein substrates (22). Based on this observation, it was proposed that the selectivity of ubiquitin-mediated protein degradation is mainly determined by the substrate specificity of different E3 enzymes (23). This notion was verified by subsequent work in many laboratories on the selective action of a large number of different E3 enzymes on their specific protein substrates. Proteins ligated to polyubiquitin chains are degraded by a large 26S proteasome complex (discovered by other investigators) and free ubiquitin is released by the action of ubiquitin-C-terminal hydrolases or isopeptidases (reviewed in 17).

## MECHANISMS OF THE DEGRADATION OF CYCLIN B: DISCOVERY OF THE CYCLOSOME/ANAPHASE-PROMOTING COMPLEX

All our studies on the basic biochemistry of the ubiquitin pathway were carried out in the reticulocyte system, using artificial model protein substrates. Though many gaps remained in our understanding of the basic biochemical

*Figure 5.* The north Atlantic surf clam, *Spisula solidissima.*

processes of the ubiquitin system, at around 1990 I thought that it was important to turn to the question of how is the degradation of specific cellular proteins is carried out by the ubiquitin system in a selective and regulated fashion. This is how I became interested in the roles of the ubiquitin system in the cell division cycle, because the levels of many cell cycle regulatory proteins oscillate in the cell cycle. I first worked on the biochemical mechanisms of the degradation of cyclin B in the early embryonic cell cycle. Cyclin B was discovered by Hunt and co-workers as a protein that is degraded at the end of each mitosis (24). It was subsequently found that it is a positive regulatory subunit of protein kinase Cdc2/Cdk1 (Cyclin-dependent kinase 1) (reviewed in ref. 25). In the early embryonic cell cycles, cyclin B is synthesized during the interphase and then is rapidly degraded in the metaphase-anaphase transition. The active protein kinase Cdk1-cyclin B (also called MPF or M phase-promoting factor) is formed at the beginning of mitosis and promotes entry of cells into mitosis. The inactivation of MPF, caused by the degradation of cyclin B, is required for exit from mitosis. Our question was: what is the system that degrades cyclin B and why does it act only at the end of mitosis?

I have approached this problem again by biochemistry, and here the quest for a cell-free system led me to marine biology and to the surf clam, *Spisula solidissima* (Fig. 5). This is a large clam that produces large numbers of oocytes. Luca and Ruderman (26) established a cell-free system from fertilized clam

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oocytes that faithfully reproduced cell cycle stage-specific degradation of mitotic cyclins. In this work, I was greatly helped first by Robert Palazzo and Leonard Cohen, and then by collaboration with Joan Ruderman. Initial fractionation of the system (27) showed that in addition to E1, two novel components were required for the ligation of cyclin B to ubiquitin: these were a specific E2 called E2-C and an E3-like activity, which in clam extracts, was associated with particulate material. We have solubilized the E3-like activity and found it to be a large (~1,500 kDa) complex that has ubiquitin ligase activity on mitotic cyclins. The activity of this enzyme is regulated in the cell cycle: it is inactive in the interphase and becomes active at the end of mitosis, an event that requires the action of Cdk1/cyclin B (28). We called this ubiquitin ligase complex the cyclosome, to denote its large size and important roles in cell cycle regulation (28). A similar complex was isolated at about the same time from extracts of *Xenopus* eggs by the Kirschner laboratory and was called the Anaphase-Promoting Complex (29). Parallel genetic work in yeast by the Nasmyth group identified several subunits of the Anaphase-Promoting Complex/cyclosome (or APC/C, as it is now called) as products of genes required for exit from mitosis (30). Thus, the discovery of APC/C was due to the convergence of biochemical with genetic work. Subsequent work by other investigators showed that the APC/C is also involved in the degradation of several other important cell cycle regulators, such as securin, an inhibitor of anaphase onset (reviewed in 31, 32). In addition, the APC/C is the target of the spindle assembly checkpoint system, an important surveillance mechanism that allows the separation of sister chromatids only after they are all properly attached to the mitotic spindle (reviewed in 33).

## ROLE OF SCF[SKP2] UBIQUITIN LIGASE IN THE DEGRADATION OF THE CDK INHIBITOR P27[KIP1]

Another problem on which I have been working recently, in collaboration with Michele Pagano, is on the mode of the degradation of the mammalian Cdk inhibitor p27[Kip1]. This inhibitor is present at high levels in G0/G1, preventing the action of Cdk2/cyclin E and Cdk2/cyclin A to drive cells into the S-phase. Following growth stimulation by mitogenic agents p27 is rapidly degraded, allowing the action of these kinases to promote entry into the S-phase (reviewed in 34). It has been shown that p27 is degraded by the ubiquitin system (35). We have tried to identify the ubiquitin ligase system that targets p27 for degradation. It was first found that the process of p27-ubiquitin ligation can be faithfully reproduced *in vitro* in extracts of HeLa cells. Thus, the rate of ligation of p27 to ubiquitin was much greater in extracts from growing cells than in extracts from G1-arrested cells. It was also found that the phosphorylation of p27 on T187 by Cdk2/cyclin E is required for p27-ubiquitin ligation *in vitro* (36), as is the case *in vivo* (37). Having established that the cell-free system accurately reflects the characteristics of p27 ubiquitylation in cells, we then proceeded to utilize this cell-free system to identify the ubiquitin ligase (E3 enzyme) involved in this process. Because of the

requirement for the phosphorylation of the p27 substrate, we suspected that an SCF (<u>S</u>kp1-<u>C</u>ullin1-<u>F</u>-box protein) type ubiquitin ligase might be involved. SCF complexes comprise a large family of ubiquitin-protein ligases, whose variable F-box protein subunits recognize a variety of phosphorylated protein substrates (reviewed in 38). We have identified Skp2 (<u>S</u>-phase <u>k</u>inase-associated <u>p</u>rotein 2) as the specific F-box protein component of an SCF complex that ubiquitylates p27, based on the following biochemical evidence: (a) Immunodepletion of extracts from proliferating cells with an antibody directed against Skp2 abolished p27-ubiquitin ligation activity; (b) Addition of recombinant, purified Skp2 to such immunodepleted extracts completely restored p27-ubiquitin ligation; (c) Specific binding of p27 to Skp2, dependent upon phosphorylation of p27 on T187, could be demonstrated *in vitro*. Combined with further *in vivo* evidence from the Pagano lab, Skp2 was identified as the specific and rate-limiting component of an SCF complex that targets p27 for degradation (39). It is notable that levels of Skp2 also oscillate in the cell cycle, being very low in G1, increasing upon entry of cells into the S-phase and declining again later on (40). These fluctuations in Skp2 levels provide an important mechanism for cell cycle stage-specific regulation of p27 degradation.

We have next tried to reconstitute the SCF[Skp2] system that ligates p27 to ubiquitin from purified components. We found that in addition to the known components (Cullin 1, Skp1, Skp2, Roc1, Cdk2/cyclin E, E1 and the E2 enzyme Cdc34), an additional protein factor is required for this reaction. We have purified the missing factor from extracts of HeLa cells and have identified it as Cks1 (<u>c</u>yclin <u>k</u>inase <u>s</u>ubunit 1), both by mass spectrometry sequencing and by functional reconstitution with recombinant Cks1 protein (41). Cks1 belongs to the highly conserved Suc1/Cks family of proteins, which bind to some cyclin-dependent kinases and to phosphorylated proteins, and are essential for several cell cycle transitions (42). Human Cks1, but not other members of this protein family, reconstituted p27-ubiquitin ligation in a completely purified system. While all members of the Suc1/Cks protein family have Cdk-binding and anion-binding sites, only mammalian Cks1 binds to Skp2 and promotes the association of Skp2 with p27 phosphorylated on T187 (41). Similar results were independently obtained by another research group (43). More recently, we have mapped the Skp2-binding site of Cks1 by site-directed mutagenesis and found that it is located on a region that includes the $\alpha2$ and $\alpha1$ helices, well separated from the other two binding sites of Cks1. All three binding sites of Cks1 are required for its action to promote p27-ubiquitin ligation and for the association of Skp2 with T-187-phosphorylated p27 (44). Based on these and on further observations, a model was proposed according to which Cks1 serves as an adaptor necessary for enzyme-substrate interaction: the Skp2-Cks1 complex binds to phosphorylated p27, a process which requires the anion-binding site of Cks1. The affinity of Skp2 to the substrate is then further strengthened by the association of the Cdk-binding site of Cks1 with Cdk2/cyclin E, to which phosphorylated p27 is tightly bound (44). It is notable that the expression of Cks1 also oscillates in the cell cycle (45, 46), providing an additional mechanism for the regulation of p27 degradation.

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## CONCLUDING REMARKS

The ubiquitin system has come a long way since its humble beginnings described in this paper. Ubiquitin-mediated degradation of positively or negatively acting regulatory proteins is involved in a variety of cellular processes such as the control of cell division, signal transduction, transcriptional regulation, immune and inflammatory responses, embryonic development, apoptosis and circadian clocks, to mention but a few. The involvement of malfunction of ubiquitin-mediated processes in diseases such as certain cancers, and the therapeutic implications of this knowledge, are also beginning to emerge. I am quite certain that we are still seeing only the tip of the iceberg of the multitude of functions of the ubiquitin system in health and disease. The main lesson from the story of the discovery of the ubiquitin system that I would like to convey, mainly to young researchers, is the continued importance of biochemistry in modern biomedical research. In his book *"For the Love of Enzymes"*, Arthur Kornberg divided the history of biomedical research into four main periods. First were the "microbe hunters", the great microbiologists of the 19th century. They were followed by the "vitamin hunters", the discoverers of the vitamins. Next were the "enzyme hunters" – the biochemists, followed by the "gene hunters" – the molecular geneticists. However, the times of enzyme (or protein) hunting are far from being over. With the completion of the human genome project, all genes have been "hunted", but we know the functions of only about one-third of our genes. If we want to know what are the roles of the rest of our genes in health and in disease, we shall have to continue to use biochemistry, in combination with functional genetics, well into the future. Our story shows that the ubiquitin system could not have been discovered without biochemical approaches. We could not have a clue to the ubiquitin tagging mechanisms by genetics alone, or by the sequence of genes of the ubiquitin system. On the other hand, once the basic biochemistry was known, molecular genetic approaches were essential for the discovery of the multitude of functions of the ubiquitin proteolytic pathway. So my advice to young investigators in biomedical sciences is: if you have a problem that cannot be solved by molecular genetics alone, do not be afraid to use biochemistry, do not hesitate to enter the cold room, and do not be wary of approaching the FPLC machine!

*Figure 6*. At the end of summer of 1979 in Fox Chase Cancer Center, Philadelphia. Seated left to right: Avram Hershko, Sandy Goldman, Jessie Warms, Hanna Heller. Standing left to right: Zelda Rose, Arthur Haas, Aaron Ciechanover, Mary Williamson, Irwin Rose, Keith Wilkinson and Leonard Cohen (last three people standing on the right side not identified).

more recently, on some roles of this system in cell cycle control. Out of my several friends-collaborators, mentioned in this paper, Irwin Rose had a very special role. My association with Ernie started with a sabbatical year in his laboratory in Fox Chase Cancer Center, Philadelphia, in 1977–78 (see also accompanying biography). During this year, I continued to work on the initial fractionation of the reticulocyte system and the purification of ubiquitin, which we started in Haifa. In the following summer of 1979, Ernie invited me back to his laboratory, together with my graduate student Aaron Ciechanover and research assistant Hanna Heller. When we got there we already knew, from work done in the Haifa lab, that ubiquitin becomes bound to proteins in an ATP-dependent process. However, the discovery that a covalent amide bond is formed between ubiquitin and the substrate protein was made together with Ernie Rose in that summer in Philadelphia. A group picture, taken at the end of this memorable summer of 1979 at Fox Chase Center, included the people involved (Fig. 6). The results of this summer's work were reported in ref. 16.

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Portrait photo of Professor Hershko by photographer Dan Porges.