

The Ubiquitin System and Some of Its Roles in Cell Cycle Control

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The selective degradation of many short-lived proteins in eukaryotic cells is carried out by the ubiquitin-mediated proteolytic system. In this pathway, proteins are targeted for degradation by covalent ligation to ubiquitin, a highly conserved small protein. The ligation of ubiquitin to protein involves the successive action of three types of enzymes: the ubiquitin-activating enzyme E1, a ubiquitin-carrier protein E2 and a ubiquitin-protein ligase, E3. The selectivity and the regulation of the degradation of a specific protein are usually determined by the properties of its specific ubiquitin ligase (E3) enzyme. Recently we have been studying two ubiquitin ligase complexes that have important roles in different aspects of cell cycle regulation. One is the cyclosome, or Anaphase-Promoting Complex (APC), which acts on mitotic cyclins and some other regulators in exit from mitosis. The cyclosome is activated at the end of mitosis by phosphorylation, a process that allows its further activation by the ancillary protein Cdc20. A different complex, which belongs to the SCF (Skp1-Cullin-F-box protein) family of ubiquitin ligases, is involved in the degradation of p27, a mammalian G1 cyclin-dependent kinase (Cdk) inhibitor, following mitogenic stimulation. Its action is triggered by Cdk2-dependent phosphorylation of p27, as well as by the increase in levels of a specific F-box protein Skp2 and of the cell cycle regulatory protein Cks1, that all take place in the G1 to S-phase transition.

For many years, I have been interested in the problem of how proteins are degraded in cells. The dynamic state of cellular proteins (Schoenheimer, 1942) and the important roles of protein degradation in the control of cellular enzyme levels (Schimke & Doyle, 1970) have been recognized for a long time, but the underlying molecular mechanisms remained unknown. A clue to an unusual mechanism was provided by observations indicating that the degradation of cellular proteins requires metabolic energy (Simpson, 1953; Hershko & Tomkins, 1971). In 1978, biochemical fractionation in my laboratory of an ATP-dependent cell-free proteolytic system from reticulocytes showed that a small, heat-stable protein was required for its activity (Ciechanover *et al.*, 1978). In 1980, we have found that this protein (subsequently identified as ubiquitin) is covalently ligated to protein substrates, and proposed that ubiquitin ligation targets proteins for degradation (Hershko *et al.*, 1980). Our further fractionation-reconstitution work has shown (Hershko *et al.*, 1983) that ubiquitin-protein ligation involves the sequential action of three enzymes. First, a ubiquitin-activating enzyme, E1, uses the energy of ATP hydrolysis to form a thiolester bond with ubiquitin. Next, activated ubiquitin is transferred to a ubiquitin-carrier protein, E2. Finally, ubiquitin is transferred to the protein substrate by the action of a ubiquitin-protein ligase, E3 (Hershko *et al.*, 1983). Further work by several laboratories has shown that there is a single E1, but there are multiple species of E2s and E3s, involved in the ligation to ubiquitin of different proteins. The selectivity of protein degradation is mainly determined by the specificity of the binding of a certain class of cellular proteins to a specific E3 enzyme. Proteins ligated to polyubiquitin chains are degraded by the 26S proteasome complex and free ubiquitin is released by the action of ubiquitin-C-terminal hydrolases or isopeptidases (reviewed in Hershko & Ciechanover, 1998).

These studies on the basic biochemistry of the ubiquitin pathway were all carried out in the reticulocyte system, using artificial protein substrates, such as extracellular proteins or denatured proteins. Though many gaps remained in our understanding of the basic biochemistry of the ubiquitin system, about ten years ago I thought that it was important at this stage to turn to the question of how the degradation of some 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 important cell cycle regulatory proteins rise and then fall in the cell cycle (reviewed in Hershko, 1977). Here I describe briefly our more recent work on the mode of the degradation of two different cell cycle regulators: cyclin B, the major mitotic cyclin, and p27, an inhibitor of cyclin-dependent kinases in animal cells.

Cyclin B is the positive regulatory subunit of protein kinase Cdk1. It was the first cyclin discovered, as a protein that is destroyed at the end of each cell cycle in early embryos of marine invertebrates, such as sea urchins and clams (Evans *et al.*, 1983). In 1991, independent work by Glotzer *et al.* (1991) and from our laboratory (Hershko *et al.*, 1991) has indicated that cyclin B is degraded by the ubiquitin system. Both laboratories have employed biochemical approaches, using cell-free systems from early embryonic cells that faithfully reproduce the selectivity and regulation of cyclin B degradation *in vitro*. With a cell-free system from *Xenopus* eggs, Glotzer *et al.* (1991) have shown that cyclin B is degraded and is ubiquitinated only in mitosis, but not in the interphase. We found that in extracts of fertilized clam oocytes, the degradation of both cyclin A and cyclin B was inhibited by methylated ubiquitin, a derivative of ubiquitin that blocks the formation of polyubiquitin chains (Hershko *et al.*, 1991).

The above-described studies have suggested that the degradation of mitotic cyclins is carried out by the ubiquitin pathway, but they have not identified the components responsible for the specificity and regulation of cyclin degradation. For this purpose, the fractionation of extracts and the isolation of the active components was required. Initial fractionation was achieved by our laboratory (Hershko *et al.*, 1994). Fractionation of extracts of clam oocytes showed that in addition to E1, two novel components were required to reconstitute cyclin-ubiquitin ligation *in vitro*. These were a specific E2, called E2-C, and an activity associated with particulate material. Though E2-C specifically acts on this system, and homologues of E2-C are present in many (though not all) eukaryotic organisms (reviewed in Hershko, 1997; Townsley & Ruderman, 1998), its activity is not regulated in the cell cycle. By contrast, the activity of the component associated with particulate material was cell cycle regulated: it was inactive in the interphase, and became active in mitosis (Hershko *et al.*, 1994).

This slow start has paved the way to rapid progress in our knowledge of this system that took place in 1995. This progress was due to convergence of information from biochemical work with genetic analysis in yeasts. In a work done in collaboration with Joan Ruderman, we have dissociated the cell cycle-regulated component from the particulate fraction oocytes and have partially purified and characterized it (Sudakin *et al.*, 1995). It was found to be a large (~1,500 -kDa) complex that has cyclin-ubiquitin ligase activity. The activity of the isolated complex remained cell cycle regulated. Furthermore, the inactive complex from interphase cells could be converted *in vitro* to the active form by incubation with protein kinase Cdk1/cyclin B. We have called this complex the cyclosome, to denote its large size and important roles in cell cycle regulation (Sudakin *et al.*, 1995). A similar biochemical approach of fractionation of *Xenopus* egg extracts by King *et al.* (1995) has identified a similar complex that was termed Anaphase-Promoting Complex, or APC. The identification of the subunits of the cyclosome/APC was made possible by the work of Irniger *et al.* (1995), who have used an elegant screen to isolate yeast mutants defective in cyclin B proteolysis. Vertebrate homologues of the products of some of these genes were shown to be subunits of the *Xenopus* cyclosome/APC (King *et al.*, 1995). Further work has identified 10-12 subunits of the cyclosome/APC in yeast and in higher eukaryotes (reviewed in Zachariae & Nasmyth, 1999). It thus seems that the cyclosome/APC is highly conserved in evolution, from yeast to clams to humans.

The activity of the cyclosome/APC is tightly regulated in the cell cycle. In early embryonic cells, a basal regulatory mechanism is the reversible phosphorylation of the cyclosome/APC. We found that the active, mitotic form of the clam cyclosome is inactivated by incubation with an okadaic acid-sensitive phosphatase. Addition of protein kinase Cdk1/cyclin B to phosphatase-treated cyclosome preparation restored activity (Lahav-Baratz *et al.*, 1995). More recent genetic and biochemical work in other laboratories has identified two WD-40 repeat containing proteins, called Cdc20 and Cdh1, that are required for the activity of the cyclosome/APC in the M and G1 phases of the cell cycle, respectively (reviewed in Zachariae and Nasmyth, 1999). We found that the phosphorylation of the cyclosome is required for its activation *in vitro* by Cdc20 (Shteinberg *et al.*, 1999), thus explaining the M-phase specific action of this ancillary factor. Similar

results were obtained in a recent genetic study in yeast (Rudner & Murray, 2000). Thus, both *in vitro* and *in vivo* evidence indicate an inter-relationship between protein phosphorylation and protein degradation in the control of exit from mitosis: the protein kinase Cdk1/cyclin B activates the ubiquitin ligase cyclosome/APC by its phosphorylation, and the cyclosome/APC subsequently inactivates the protein kinase by the degradation of its cyclin B subunit. Other substrates, functions and regulatory mechanisms of the cyclosome/APC are discussed in an excellent review of Zachariae and Nasmyth (1999).

The second project on which I have been working recently, in collaboration with Michele Pagano, is the mode of the degradation of p27^{Kip1}. p27 is an inhibitor of mammalian G1 cyclin-dependent kinases such as Cdk2/cyclin E, which is responsible for driving cells from G1 to the S-phase of the cell cycle (reviewed in Sherr & Roberts, 1999). Levels of p27 are high in quiescent cells, thus inhibiting the action of Cdk2. Following stimulation of cells to grow p27 is rapidly degraded, coincident with a rise in levels of cyclin E. The degradation of p27 is thus essential to allow the action of Cdk2/cyclin E to drive cells into the S-phase. It has been previously shown that p27 is degraded by the ubiquitin system (Pagano *et al.*, 1995). We asked the question which ubiquitin ligase targets p27 for degradation and how is its action regulated in the G1 to S-phase transition. Here again, I have used a cell-free system that faithfully reproduces cell cycle stage-specific ubiquitinylation of p27 in the test tube, this time a cell-free system from cultured HeLa cells (Montagnoli *et al.*, 1999). Again we have used biochemical fractionation, except that in this case we could try to guess the nature of the ubiquitin ligase involved, based on some previous bits of information. It was known that the phosphorylation of p27 on Thr187 by Cdk2 is required for its degradation *in vivo* (Sheaff *et al.*, 1997; Vlach *et al.*, 1997), and for its ubiquitinylation *in vitro* (Montagnoli *et al.*, 1999). In many cases, substrate phosphorylation is required for its recognition by an SCF (Skp1-Cullin-F-box protein) type of ubiquitin ligase (reviewed in Deshaies, 1999). These ubiquitin ligase complexes contain several constant components and a variable subunit, called an F-box protein, that recruits specific protein substrates for ubiquitinylation. Because of the requirement of p27 ubiquitinylation on its phosphorylation, we suspected that an SCF-type ubiquitin ligase may be involved. We have therefore subjected extracts

of HeLa cells to immunodepletion with antibodies directed against mammalian F-box proteins and found that immunodepletion of the F-box protein Skp2 (S-phase kinase-associated protein 2) completely abolished p27-ubiquitin ligation activity. Activity could be completely restored by the supplementation of purified recombinant Skp2. These *in vitro* findings, together with *in vivo* results of the Pagano laboratory, established that p27 is targeted for degradation by an SCF complex which contains Skp2 as its specific F-box protein (Carrano *et al.*, 1999). Similar conclusions were reported by other investigators (Sutterluty *et al.*, 1999; Tsvetkov *et al.*, 1999). However, an important piece of the puzzle was missing, since we could not reconstitute p27-ubiquitin ligation with known purified components of the SCF^{Skp2} complex. Using biochemical fractionation and purification, we have recently identified the missing component as Cks1 (cyclin kinase subunit 1), a protein which belongs to the Suc1/Cks family of cell cycle regulatory proteins (reviewed in Pines, 1996). Cks1 reconstitutes p27-ubiquitin ligation in a completely purified system, binds to Skp2 and greatly increases the binding of T187-phosphorylated p27 to Skp2 (Ganoth *et al.*, 2001). Similar conclusions were reached in an independent study of Spruck *et al.* (2001), using Cks1 knockout mice. These findings indicate that an accessory protein is required for the activity of an SCF complex and for its binding to a phosphorylated substrate. The results also show that the degradation of p27 in the G1 to S transition is subject to several levels of regulation. It has been shown that levels of Skp2 are very low in G0/G1, and increase greatly before entry into the S-phase and the expression of Cks1 is similarly regulated in the cell cycle (see Ganoth *et al.*, 2001 and references therein). Thus, in the G1 to S-phase transition, p27 is first phosphorylated by the increase of Cdk2/cyclin E levels, and is then targeted for degradation by a ubiquitin ligase complex that assembles at this stage from newly synthesized Skp2 and Cks1 components. Such multiple levels of regulation allow a tight control of this important cell cycle transition. For further information on the mechanisms and regulation of p27 degradation, the reader is referred to the chapter by Bloom and Pagano.

As I have pointed out previously (Hershko, 1996; Hershko *et al.*, 2000), the main lesson from my story is the continued importance of biochemistry in current biomedical research. The biochemical fractionation of the cell-free ATP-dependent proteolytic system from reticulocytes allowed the

discovery of the role of ubiquitin ligation in protein degradation and the identification of the main enzymatic components of this pathway. More recently, extracts from clam oocytes and from *Xenopus* eggs, that faithfully recapitulate cell cycle-related events in the test tube, were instrumental in the identification of the cyclosome/APC, an important player in cell cycle control. Most recently, extracts of HeLa cells were used to define the components of the SCF/Skp2/Cks1 system that targets p27 for degradation, an event necessary for entry of mammalian cells into the S phase. I would also like to point out that biochemistry is most useful when combined with molecular genetics. In the ubiquitin story, the use of molecular genetics was essential in uncovering the widespread roles of this system in basic cellular processes such as cell cycle control, signal transduction, development and the immune and inflammatory responses.

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References

- Carrano, A. C., Eytan, E., Hershko, A. and Pagano, M. (1999). Skp2 is required for ubiquitin-mediated degradation of the Cdk inhibitor p27. *Nature Cell Biol.* 1, 193–199.
- 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.
- Deshaies, R. J. (1999). SCF and Cullin/RING2-based ubiquitin ligases. *Annu. Rev. Cell Biol.* 15, 435–467.
- Evans, T., Rosenthal, E.T., Youngblom, J., Distel, D. and Hunt, T. (1983). Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell* 33, 389–396.
- Ganoth, D., Bornstein, G., Ko. T. K., Larsen, B., Tyers, M., Pagano, M. and Hershko, A. (2001). The cell-cycle regulatory protein Cks1 is required for SCFSkp2-mediated ubiquitylation of p27. *Nature Cell Biol.* 3, 321–324.

- Glotzer, M., Murray, A.W. and Kirschner, M.W. (1991). Cyclin is degraded by the ubiquitin pathway. *Nature* 349, 132–138.
- 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.
- 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.
- 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.
- Hershko, A., Ganoth, D., Pehrson, J., Palazzo, R.E. and Cohen, L.H. (1991). Methylated ubiquitin inhibits cyclin degradation in clam oocyte extracts. *J. Biol. Chem.* 266, 16376–16379.
- Hershko, A., Ganoth, D., Sudakin, V., Dahan, A., Cohen, L.H., Luca, F.C., Ruderman, J.V. and Eytan, E. (1994). Components of a system that ligates cyclin to ubiquitin and their regulation by protein kinase cdc2. *J. Biol. Chem.* 269, 4940–4946.
- Hershko, A. (1996). Lessons from the discovery of the ubiquitin system. *Trends Biochem. Sci.* 21, 445–449.
- Hershko, A. (1997). Roles of ubiquitin-mediated protein degradation in cell cycle control. *Curr. Op. Cell Biol.* 9, 788–799.
- Hershko, A. and Ciechanover, A. (1998). The ubiquitin system. *Annu. Rev. Biochem.* 67, 425–479.
- Hershko, A., Ciechanover, A. and Varshavsky, A. (2000). The ubiquitin system. *Nature Med.* 6, 1073–1081.
- Irniger, S., Piatti, S., Michaelis, C. and Nasmyth, K. (1995). Genes involved in sister chromatid separation are needed for B-type cyclin proteolysis in budding yeast. *Cell* 81, 269–277.
- King, R.W., Peters, J.-M., Tugendreich, S., Rolfe, M., Hieter, P. and Kirschner, M.W. (1995). A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. *Cell* 81, 279–288.
- Lahav-Baratz, S., Sudakin, V., Ruderman, J.V. and Hershko, A. (1995). Reversible phosphorylation controls the activity of cyclosome-associated cyclin-ubiquitin ligase. *Proc. Natl. Acad. Sci. USA* 92, 9303–9307.
- Montagnoli, A., Fiore, F., Eytan, E., Carrano, A. C., Draetta, G. F., Hershko, A. and Pagano, M. (1999). Ubiquitination of p27 is regulated by Cdk-dependent

- phosphorylation and trimeric complex formation. *Genes & Dev.* 13, 1181–1189.
- Pagano, M., Tam, S.W., Theodoras, A. M., Beer-Romero, P., Del Sal, G., Chau, V., Yew, P.R., Draetta, G.F. and Rolfe, M. (1995). Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. *Science* 269, 682–685.
- Pines, J. (1996). Reaching for a role for the Cks proteins. *Curr. Biol.* 6, 1399–1402.
- Rudner, A. D. and Murray, A. W. (2000). Phosphorylation by Cdc28 activates the Cdc20-dependent activity of the anaphase-promoting complex. *J. Cell Biol.* 149, 1377–1390.
- Schimke, R.T. and Doyle, D. (1970). Control of enzyme levels in animal tissues. *Annu. Rev. Biochem.* 39, 929–976.
- Sheaff, R., Groudine, M., Gordon, M., Roberts, J. and Clurman, B. (1997). Cyclin E/Cdk2 is a regulator of p27. *Genes & Dev.* 11, 1464–1478.
- Sherr, C. J. and Roberts, J. M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes & Dev.* 13, 1501–1512.
- Schoenheimer, R. (1942). *The Dynamic State of Body Constituents*. Harvard University Press, Cambridge, Mass.
- Simpson, M.V. (1953). The release of labeled amino acids from the proteins of rat liver slices. *J. Biol. Chem.* 201, 143–154.
- Shteinberg, M., Protopopov, Y., Listovsky, T., Brandeis, M. and Hershko, A. (1999). Phosphorylation of the cyclosome is required for its stimulation by Fizzy/Cdc20. *Biochem. Biophys. Res. Commun.* 260, 193–198.
- Spruck, C., Strohmaier, H., Watson, M., Smith, A. P. L., Ryan, A., Krek, W. and Reed, S. I. (2001). A Cdk-independent function of mammalian Cks1: targeting of SCFSkp2 to the Cdk inhibitor p27Kip1. *Mol. Cell.* 7, 639–650.
- Sudakin, V., Ganoh, D., Dahan, A., Heller, H., Hershko, J., Luca, F.C., Ruderman, J.V. and Hershko, A. (1995). The cyclosome, a large complex containing cyclin-selective ubiquitin ligase activity, targets cyclins for destruction at the end of mitosis. *Mol. Biol. Cell* 6, 185–198.
- Sutterluty H., Chatelain, E., Marti, A., Wirbelauer, C., Seufter, M., Muller, U. and Krek, W. (1999). p45Skp2 promotes p27Kip1 degradation and induces S phase in quiescent cells. *Nature Cell Biol.* 1, 207–214.
- Townsend, F.M. and Ruderman, J.V. (1998). Proteolytic ratchets that control progression through mitosis. *Trends Cell Biol.* 8, 238–234.
- Tsvetkov, L. M., Yeh, K. H., Lee, S. J., Sun, H. and Zhang, H. (1999). p27(Kip1) ubiquitination and degradation is regulated by the SCF(Skp2) complex through phosphorylation of Thr187 in p27. *Curr. Biol.* 9, 661–664.

- Vlach, J., Hennecke, S. and Amati, B. (1997). Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27Kip1. *EMBO J.* 15, 5987–5997.
- Zachariae, W. and Nasmyth, K. (1999). Whose end destruction: cell division and the anaphase-promoting complex. *Genes & Dev.* 13, 2039–2058.