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Ubiquitin System: Selectivity and Timing of Protein Destruction

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A growing number of cellular functions have been shown to be regulated through protein degradation. The selective degradation of many short-lived proteins in eukaryotic cells is mediated by the ubiquitin system, by which proteins covalently ligated to ubiquitin are targeted for degradation. The selectivity of the destruction is ensured by the substrate specificity in the ubiquitination steps composed of a series of enzymatic reactions. Ubiquitin-ligase (E3), in conjunction with ubiquitin-conjugating enzyme (E2), has been implicated as playing an essential role in the substrate recognition. The substantial character, however, of the ligase was not clear until several recent studies demonstrated ligases that exert key roles in irreversible steps of the cell-cycle control. In this review, attention is focused on the molecular basis of target recognition of ubiquitination, particularly as exemplified in the ubiquitin-ligases in the cell-cycle control mechanisms.

Key words: cell cycle control, proteolysis, ubiquitin, ubiquitin-conjugating enzyme, ubiquitin-ligase.

Selective protein degradation in eukaryotic cells is mainly carried out by the ubiquitin system. Ubiquitin (Ub) is a highly conserved 76-residue protein which is distributed throughout all eukaryotic cells, and linked to a vast range of proteins. The ubiquitin-tagged proteins are mainly targeted for proteolysis by proteasome, although Ub has also been implicated in functions other than proteolysis. Thus, the many short-lived proteins are subjected to be substrates of ubiquitination. Ultimately causing the destruction of various regulatory proteins, the ubiquitin system plays important roles in many cellular functions, including cell-cycle control, signal transduction, transcriptional regulation, the nuclear transport process, receptor control by endocytosis, the processing of antigens in the immune system, and so on. Some pathological alterations are known to be linked to abnormalities in the ubiquitin system. Programmed cell death is also thought to be associated with ubiquitin-mediated proteolysis (reviewed in Refs. *1* and *2*).

Since a vast range of information has accumulated on this subject, it would be impossible to provide a full survey of all the advances. In this review, I summarize the ubiquitin system and then focus on the ubiquitin pathways involved in the cell cycle control, in which the molecular basis of selective protein degradation is demonstrated as a molecular recognition among a variety of components of the ubiquitin system and cell cycle regulators.

Ubiquitin pathway and enzymes

Conjugation of Ub to proteins is a multiple enzymatic process. Ub, activated with ATP by ubiquitin-activating enzyme (E1), is attached to a specific Cys residue of the same enzyme by a high-energy bond, and then transferred to a Cys residue of the second enzyme, ubiquitin-conjugating enzyme (E2 or Ubc). While, in many cases, Ub can be directly transferred from the E2 and covalently attached to a substrate protein, ubiquitination of some proteins requires the additional activity of ubiquitin ligase (E3). Finally, the Ub is covalently linked *via* an isopeptide bond to Lys residues of the target protein. The Ub attached to the protein is further ubiquitinated by another Ub molecule, forming a branch of multiubiquitin chains, which is recognized by 26S proteasome, a huge complex of proteases, and leading to degradation. Thus, the selectivity of protein degradation is assured by the substrate specificity of the ubiquitination. These enzymatic reactions were summarized in Fig. 1.

Usually the E1 enzyme exists as a single species, and the loss of its function is lethal to the cells, indicating that many cellular functions essential for viability are regulated by ubiquitin-mediated proteolysis. In mammalian cell lines, E1 mutants have been predominantly isolated, probably because E1 is encoded on the X chromosome (*3*). These mutants exhibited pleiotropic phenotype, since the ubiquitin pathway is responsible for a vast range of cellular functions. One of the prominent phenotypes of E1 mutants is cell-cycle arrest at the G2 phase, suggesting that the ubiquitin system is tightly associated with cell-cycle control, although the molecular basis of the G2-arrest has not yet been clarified (*4*). The primary structure of E1 is highly conserved among eukaryotic species. Another intriguing point is that sequences very similar to E1 have been identified, but none that are relevant to Ub activation. However, some of these were very recently shown to be necessary for activation of Ub-like proteins (see later section) (*5, 6*).

In contrast to E1, E2, and E3 are known to comprise many species (*7*). In *Saccharomyces cerevisiae*, 13 E2s (Ubc1-Ubc13) have been identified by virtue of a shared structural feature called the UBC domain, the list of which

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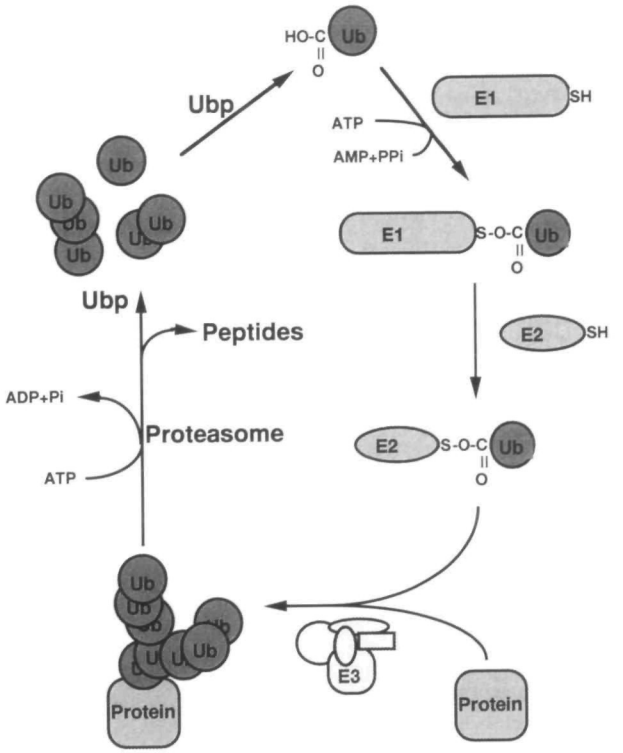

Fig. 1. **Ubiquitin and proteasome degradation pathway.** Proteins covalently tagged with poly-ubiquitin chains by enzymatic reactions involving E1, E2, and E3, are degraded by 26S proteasome. See details in text.

is frequently referred to for purpose of clarifying E2 families. It is reasonable to suppose that there are still more E2s in higher eukaryotic cells. Some E2s have distinct sequences in addition to the core UBC domain, and others do not. In spite of the structural similarity, the E2 species seem to be functionally differentiated, as indicated by the phenotypes of the mutants. Mutations in each E2 display different phenotypes. Some E2s have redundant functions, while others play very specific role. Simultaneously, some E2s play roles that are non-essential for cell viability, while the functions of others are essential. These facts suggest that E2 is responsible, not fully but in part, for selecting the target protein(s) to be ubiquitinated. Interestingly, some E2s have been proved recently not to conjugate Ub, but to conjugate Ub-like small proteins, SUMO-1 or Rub1, to other proteins, although their structural feature is indistinguishable from E2s for Ub (see later section) (*5, 8*).

The E3s play the most crucial role in determining the specificity of the ubiquitination, but the least well understood. It is only very recently that a few classes of E3 have been uncovered. This is because, in contrast to E2s, different E3 families share little or no homology, and also because E3s are in low abundance in the cells. Some E3s are composed of large numbers of subunits, but in many cases the basic function of each component is largely unknown. The major types of E3 will be described in the next section in connection with their selective recognition of substrates.

Last enzyme associated with the ubiquitin system is ubiquitin peptidase (Ubp) which degrades poly-ubiquitin, or releases Ub from Ub-fused or conjugated oligopeptides. In *S. cerevisiae*, 15 Ubps have been identified and classified into two types of families (*2*). Since most of the Ubps are non-essential for cell viability and their mutants do not exhibit specific phenotypes, their biological functions are not clear. I will not describe in greater detail on this enzyme.

Signals and a class of E3s for the selective proteolysis

1) Signals for ubiquitination. The N-end rule system, in which the rate of ubiquitination and therefore degradation of proteins is determined by the N-terminal amino acid residue, was initially demonstrated by a clear observation of the stability of the artificially modified substrates in the cells (*9*). However, few of the natural substrates of this system are known. This pathway in *S. cerevisiae* requires Rad6 (Ubc2) as E2 and Ubr1 as E3. Rad6 is one of the predominant E2s conferring multiple functions, including DNA repair, the process of sporulation and germination, and the N-end rule pathway. *UBR1* has been genetically identified as N-end rule E3, and presumably corresponds to E3α that was enzymatically identified in reticulocyte lysate. Neither *RAD6* nor *UBR1* is an essential gene, and the biological meaning of this pathway is not clear. It may be possible that this pathway is involved in degradation of mis-processed peptides or intermediates of degradation.

Many short-lived proteins have been shown to contain a PEST sequence that, while not a unique sequence, is rich in Pro, Glu, Ser, and Thr residues (*10*). Consequently, the PEST region is expected to be enriched in S/TP sequences, which are minimum consensus signals of phosphorylation by protein kinases, including CDKs. It has been pointed out that phosphorylation and degradation of proteins are tightly associated in many cases. The yeast G1 cyclins, Cln2 and Cln3, are phosphorylated at multiple sites in the PEST region prior to its degradation. Phosphorylation of Sic1, a CDK inhibitor in yeast, is a trigger of ubiquitination and degradation. Mammalian G1 cyclin, cyclin E and D1, are also phosphorylated and degraded.

The search for degradation signals in mitotic cyclins has resulted in the identification of a partially conserved motif. Comparison of A- and B-type mitotic cyclins from various sources has resulted in generalization of 9 amino acid sequence, R(A/T)(A)L(G)X(I/V)(G/T)(N), which is called the "destruction box" (D-box) and is located near the N-terminus of cyclins. The bracketed amino acids are the preferential ones at the sites. Cyclins deleted or mutated in this region are stabilized *in vitro* and *in vivo* (*3, 11*). Reporter proteins fused to this signal are degraded at the cell cycle stages when the cyclin is destructed. Similar destruction box motifs are found in cell-cycle regulator proteins other than cyclin, and the proteins with these motifs are also degraded during mitosis. APC/cyclosome, a cyclin-specific E3 complex (see later section), recognizes these destruction box.

2) hect-E3. The oncogenic human papillomavirus (HPV) types 16 and 18 encode two oncoproteins, E6 and E7. Both E6 and E7 bind to cellular regulatory proteins, E6 to tumor suppressor protein p53, and E7 to RB protein. Mutations in p53 are among the most prominent genetic alterations found in a variety of human cancers. Such mutations may lead to loss of the tumor-suppressor activity. In cell lines transformed with HPVs, the E6 binds to normal p53 with the assistance of another cellular protein termed E6-AP (E6 associated protein), which target p53 to rapid ubi-

quitination and degradation (*12*). The human E2 enzyme involved in this ubiquitination has been identified as UbcH5 belonging to subfamily that includes yeast Ubc4 and Ubc5 species (*13*). The E6/E6-AP complex exerts E3 activity in the p53 ubiquitination, in which a novel mode of reaction has been revealed. In this reaction, activated Ub is transferred from the E2 to form a thiolester with a specific Cys residue near the C-terminus of E6-AP protein (*14*). This thiolester is known to function as the donor of Ub, based on the fact that substitution of this Cys residue abolishes its activity of p53 ubiquitination.

A variety of proteins which contain a C-terminal region homologous to that of E6-AP have been identified in many eukaryotic cells. Proteins in the so-called hect-domain family (homologous to E6-AP carboxy terminus) contain a conserved Cys residue as the active site in approximately the 300 amino acid hect region (*15*). In contrast to the conserved homologies in the hect-domain, the N-terminal region of different hect-proteins are highly variable, suggesting that the N-terminal region confers specific substrate recognition. Thus, the hect-domain was expected to be a common feature in at least some subfamilies of the ubiquitin ligase, and it was assumed that most of the hect-proteins might have an activity of E3 or a component of E3 complex. Though not yet proved in all cases, this is the case in some hect-proteins.

Five hect-proteins have been identified in budding yeast. Rsp5 was found to bind the largest subunit of RNA polymerase II (*16*). The N-terminal domain confers the binding activity to the polymerase, but the C-terminal hect-domain does not. When expression of Rsp5 is inhibited *in vivo*, the level of the RNA polymerase subunit increases. The RNA polymerase is a long-lived protein and the biological importance of its ubiquitination for rapid destruction is not clear. Under the restricted condition like various stress, the RNA polymerases might be targeted to degradation. In fact, it has been reported that the large subunit of RNA polymerase is ubiquitinated in response to DNA damage by UV irradiation. In fission yeast, a hect-protein Pub1 has been shown to be involved in the degradation of a key cell-cycle protein, the Cdc25 phosphatase (*17*). The Cdc25 phosphatase activates Cdc2 kinase by removing a inhibitory phosphate group from a tyrosine residue in Cdc2, resulting in the activation of Cdc2/Cdc13 as a mitosis promoting factor. The levels of Cdc25 oscillate during the cell cycle. In the cells disrupted in *pub1*, the level of Cdc25 is increased. *pub1* shows genetic interaction with genes that control Cdc25 phosphatase activity. Finally, it has been observed that polyubiquitinated Cdc25 accumulates in *pub1*$^+$ but not in *pub1*$^-$ cells.

3) Cyclosome/APC. Progression of the eukaryotic cell cycle is driven mainly by repeated activation and inactivation of cyclin-dependent kinases (Cdks). Different Cdks function at various stages of cell cycle to promote different cell-cycle transitions. Although Cdk activity is regulated through the level of phosphorylation or dephosphorylation of the catalytic subunit, or inactivation of Cdk inhibitors (Ckis), it is controlled at the most basic level by periodic synthesis and degradation of the regulatory subunits, cyclins. The most prominent CDK complex, cyclin B/Cdc2, plays a major role in progression through mitosis and is designated as MPF (mitosis promoting factor). The accumulation of the mitotic cyclin activates MPF, triggering the transition from G2 through mitosis. In late mitosis, MPF activates degradation pathway for anaphase inhibitors through their ubiquitination and 26S proteasome-mediated destruction, resulting in the timely onset of anaphase. At the exit from mitosis, the same ubiquitination machinery targets the mitotic cyclin itself to degradation, leading to the irreversible inactivation of the MPF activity (Fig. 2).

In the past few years the E2 and E3 components required for ubiquitination of the mitotic cyclins have been identified genetically in yeast and biochemically in cell-free systems of clam and *Xenopus* egg extract. The cyclin-specific E2 was

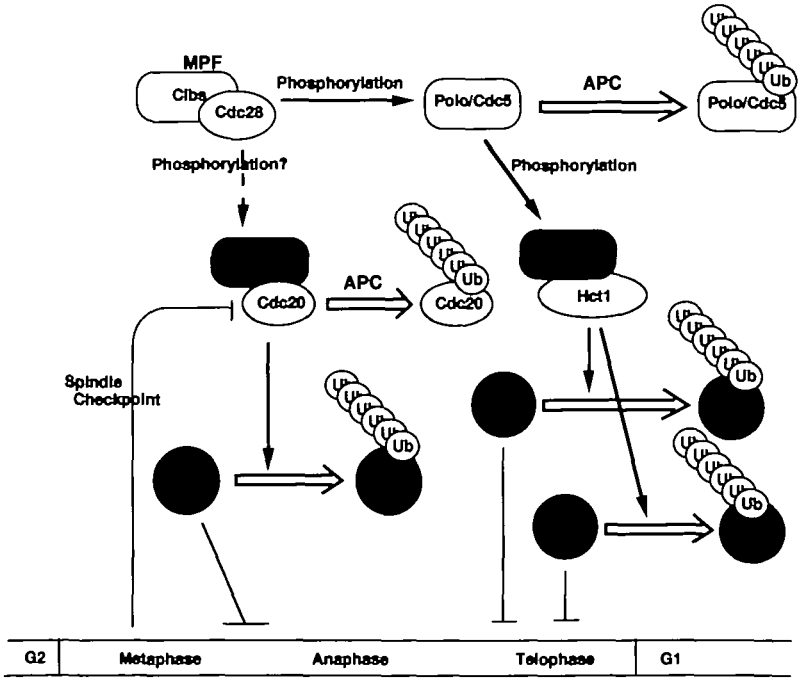

Fig. 2. **APC circuit in budding yeast.** In mitotic phase, Cdc20 activates APC to ubiquitinate Pds1, the destruction of which triggers anaphase by initiating separation of chromatids. Spindle checkpoint works through Cdc20 by inhibiting this initiation step. After initiation of anaphase, APC forms complex with another Cdc20 family, Hct1. Polo-like kinase, Cdc5, which is activated by MPF, phosphorylates this 2nd complex to be active to direct destruction of spindle protein, Ase1, and mitotic cyclin, Clb2. Cdc5 and Cdc20 are destructed through ubiquitination mediated by APC. This mechanism is basically conserved in higher eukaryotic cells as indicated in text.

first identified as E2-c in clam extract (*18, 19*), and then UBCx (*Xenopus*) (*20*), UbcH10 (humans) and UbcP4 (*S. pombe*) (*21*) were found to be homologous. A distinct 30 amino acid sequences in N-terminus has been found in all these E2-c homologues. In *Xenopus* extract, Ubc4 homologue could also support *in vitro* ubiquitination of cyclin B, suggesting that it might function in a manner redundant to the function of UBCx. Curiously, mitotic cyclin-specific E2 has not yet been identified in *S. cerevisiae*. Ubc9 has been implicated as responsible for the cyclin ubiquitination, based on the observation that *ubc9* mutant cells arrest in late G2/early mitosis with accumulation of mitotic cyclins. The direct evidences, however, has not been found; rather, Ubc9 was shown to be involved in conjugation of small Ub-like modifier, SUMO-1, and to be not relevant to Ub. Ubc11 is structurally most homologous to E2-c, although it lacks the N-terminal extension sequence characteristic to E2-c. Ubc11 is not essential for cell viability, and disruption of *ubc11* alone or in combination with *ubc4* and *ubc5* does not affect mitosis.

The E3 activity for cyclin B ubiquitination was found in a huge complex in clam (*19*) and *Xenopus* egg extracts (*22*), and also in yeasts (*23*). This complex is required for metaphase/anaphase transition in mitosis as well as cyclin destruction at the end of mitosis, and hence has been termed as Anaphase Promoting Complex (APC), or cyclosome (Fig. 2). While E2-c is constitutively expressed, the activity of APC is cell-cycle regulated. It is inactive in the interphase, and activated by phosphorylation during mitosis. Thus, the mitotic cyclin destruction seems to be mainly regulated by modulating the APC ubiquitin-ligase activity. Genetic studies in yeast, comparable with biochemical analysis in embryonic cell extracts, have shown that the APC is composed of 8-12 subunits, including homologues of *S. cerevisiae* Cdc16, Cdc23, and Cdc27 (*24*), and *A. nidulans* BIME (*25*). All subunits of human and yeast APC were recently determined (*26, 27*). The APC2 subunit is a novel protein that has a homologous region to a motif of the cullin protein family. One member of the cullin family is Cdc53, which is a component of the SCF complex, another E3 complex required for G1/S transition in yeast (see next section). There is no evidence that any subunit of APC forms high-energy bonds with Ub, like hect-proteins. Thus, it is unlikely that APC is a composition of the thioester cascade. Instead, APC presumably bridges E2 and substrates to transfer Ub between them.

Mutations in subunits of APC arrest cells at metaphase. Expression of the dominant negative mutant of E2-c in human cells (*28*), or UbcP4 mutant of *S. pombe* causes metaphase arrest (*21*). These findings indicate that the APC pathway is necessary for the segregation of sister chromatids during anaphase (Fig. 2). The metaphase arrest is not a consequence of a defect of the mitotic cyclin degradation, since nondegradable cyclin B lacking a D-box prevents the completion of mitosis, but sustains anaphase progression (*29-31*). Furthermore, expression of the cyclin B fragment containing the D-box delays onset of anaphase as well as cyclin degradation, suggesting that proteins other than cyclin B are required to be degraded for chromosome segregation. Candidates for these targets have been identified in yeast. Cut2 in *S. pombe* is essential for sister chromatid segregation, and degraded at the onset of anaphase *via* the APC pathway (*32*). This destruction requires two D-boxes located in the N-terminal region of Cut2. Overexpression of the two D-boxes prevents anaphase and Cdc13 (mitotic cyclin) destruction (*33*). In *S. cerevisiae*, Pds1 is necessary to maintain sister chromatid cohesion under anaphase inhibition (Fig. 2). Pds1 also contains the D-box sequence, which is essential for its APC-mediated degradation (*34*). No significant sequence homologies have been found between Pds1 and Cut2, and *cut2* is essential for viability, while *PDS1* is not. Thus, it is not clear whether Pds1 and Cut2 are functionally homologous, but it is clear that their destruction is essential for anaphase onset.

APC is inactive during interphase and activated by phosphorylation during mitosis, the mechanisms of which are not well understood. In extracts of clam and *Xenopus* egg, addition of Cdc2/cyclin B complex promotes activation of APC, resulting in phosphorylation of a number of the subunits (*19*). However, there is a lag between the addition of the kinase and the APC activation, suggesting that Cdc2/cyclin B activates APC indirectly. Polo-like kinases that were originally identified as Cdc25-regulatory kinases functioning in an MPF amplification loop are known to be conserved among eukaryotes, such as Plk1 (human), Plx (*Xenopus*), and Plo1 (*S. pombe*). They are required in both entry to and exit from mitosis, except in the case of *S. cerevisiae* (Cdc5), in which Cdc5 affects only the exit from mitosis (Fig. 2). Recently, Cdc2/cyclin B-activated polo-like kinase in human cells was shown to phosphorylate APC subunits, activating APC to ubiquitinate cyclin B (*34*). Interestingly, polo-like kinases are targeted for degradation *via* the APC-mediated pathway, providing a feedback mechanism by which the APC destroys its activator at the onset of the next cell cycle (Fig. 2). In *S. pombe*, genetic evidence suggests the involvement of cAMP-dependent protein kinase A in the negative regulation of APC (*35*). Actually, human PKA was shown to phosphorylate APC subunits, thereby suppressing the E3 activity (*34*).

The APC seems to be activated grossly in M phase through phosphorylation. Different substrates, however, such as anaphase inhibitors and mitotic cyclins, appear to be proteolyzed at different times during mitosis, suggesting that a simple switching on and off of APC is not the sole regulatory mechanism. Recent genetic analysis suggests that targeting different substrates with different timing to APC are controlled by Fizzy/Cdc20 family proteins. *fizzy* mutants of *Drosophila* arrest in metaphase with defect of cyclin A degradation. A homologue of *fizzy* has been identified in *S. cerevisiae* (*CDC20*), *S. pombe* (*slp1*), and human (p55CDC). A fizzy-related gene, *fzr*, of *Drosophila* and its *S. cerevisiae* homologue, *HCT1*, were also found. In *S. cerevisiae*, Cdc20 is required to activate APC-mediated Pds1 destruction in metaphase, while the related protein, Hct1, is required for destruction of the mitotic cyclin, Clbs, in G1 phase (Fig. 2). Simultaneously, it has been suggested that Cdc20 or its homologues is a target of the spindle checkpoint that delays the onset of anaphase until all chromosomes are attached to the spindle (*36, 37*). Finally, the cdc20 protein itself is degraded *via* the APC-mediated ubiquitination. In *Drosophila*, Fizzy and Fzr seem to target the same substrates, such as mitotic cyclins and anaphase inhibitor for APC-dependent destruction, but at different points, mitotic and G1 phase, respectively. It is still uncertain how these accessory proteins are involved in the activation of APC and/or the recruiting of substrates to APC.

4) SCF complex. Ubc3 in *S. cerevisiae* is a unique ubiquitin-conjugating enzyme in that its gene has been identified as a cell cycle mutant, *cdc34*, regulating G1/S transition. Analysis of several *cdc* mutants, the phenotypes of which are linked with that of *cdc34*, revealed a subfamily of ubiquitin-ligase complex. Loss of either Cdc34, Cdc4, or Cdc53 leads to cell-cycle arrest at G1 phase, just before the onset of DNA synthesis. This is attributed to a failure of the degradation of Sic1, a potent inhibitor of the Clb/Cdc28 kinases required for activation of DNA synthesis (*38*). Cdc34 was found to form a complex with Cdc53 and Cdc4, in which the ubiquitin-ligase activity was implied (*39*). This activity was demonstrated by the identification of another component, called Skp1, for this pathway (*40*), since the complex composed of the four proteins is sufficient to support *in vitro* ubiquitination of Sic1 under the presence of E1 and Ub (*41*) (Fig. 3). The G1 cyclins, Cln1 and Cln2, are also degraded *via* the Cdc34-dependent ubiquitin pathway, which requires Cdc53, Skp1 but not Cdc4 (*42*). Instead of Cdc4, another protein, Grr1, was found to be involved in this process (*43, 44*). Grr1 is involved phenotypically with glucose repression and Cln turnover, and these, although not readily understood in early studies, was eventually explicated in terms of the alignments of the Skp1 interacting proteins (see next section).

The yeast Skp1 was isolated as a high copy suppressor of the *cdc4* mutant, and also as a part of the CBF kinetocore complex, and was found to be homologous to human Skp1 that was found together with Skp2, as a part of the cyclin A/CDK2 complex, and subsequently as a cyclin F interacting protein. Skp1 binds Cdc53 with its N-terminal domain. In addition to Cdc53, Skp1 was shown to bind a series of proteins, including Cdc4, human Skp2, and cyclin F.

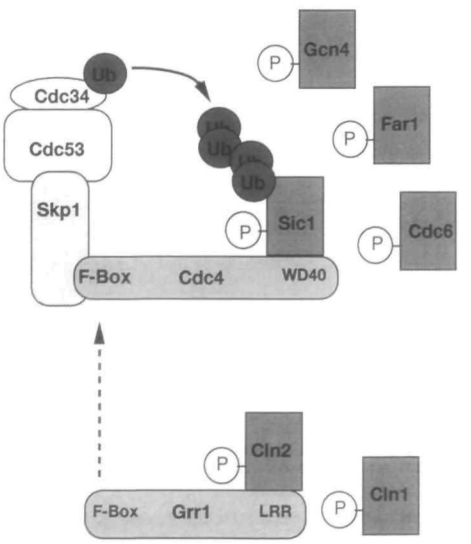

Fig. 3. **F-box proteins as substrate adapters in SCF complex.** Cdc34(E2), Cdc53, and Skp1 form E2-E3 complex together with one of the F-box proteins, and ubiquitinates a variety of proteins. Substrate recognition is accomplished *via* specific interaction between an F-box protein and the target protein. Protein-protein interaction motif like LRR or WD40 in F-box proteins serve for this interaction. An F-box protein binds Skp1 through another motif, F-box domain, which is common to all F-box proteins. In most cases, the target proteins are phosphorylated prior to the ubiquitination. See text for details.

Alignments of these Skp1 interacting proteins deduced an amino acid sequence motif called F-box after cyclin F (*40*). Further investigation of the F-box motif showed that it is contained in a series of proteins, including Grr1. Consequently, the F-box is shown to be required for the interaction of the F-box proteins with Skp1. Disruption of the F-box in Cdc4 and Grr1 abolishes the interaction of Cdc4 and Grr1 with Skp1. Every F-box proteins contains another motif that is necessary for protein-protein interaction. Cdc4 has WD-repeats and Grr1 has leucine-rich region (LRR), disruption of which impairs their interaction with Sic1 and Cln2, respectively. Thus, the Sic1 and Clns degradation pathways require the same core complex composed of Cdc34, Cdc53, and Skp1, but different F-box protein as a tethering molecule for substrates (Fig. 3). The complex including, for example, Cdc4, is now designated as SCF^{Cdc4} (Skp1, Cdc53, and Cdc4 as F-box protein), in which Cdc53 is a scaffold protein bridging Cdc34 and Skp1 (*45*). Skp1 provides multiple ubiquitination pathways by assembling with different F-box proteins which recruit specific substrates to ubiquitination (Fig. 3). Now evidences have accumulated to indicate that SCF^{Cdc4} mediates ubiquitination of several other target proteins, such as another CDK inhibitor, Far1, which inhibits Cln-Cdc28 kinases in arrested cells by mating pheromone, and the replication activator Cdc6. The control of the DNA replication by SCF E3 seems to be conserved in *S. pombe*. Two Cdc4-like proteins, Pop1 and Pop2, regulate DNA replication through degradation of Rum1 and Cdc18, which have functions analogous to those of Sic1 and Cdc6, respectively (*46*). Repression of this degradation pathway, or overexpression of the target substrates of this pathway, results in DNA re-replication without mitosis. The *S. pombe* homolog of Skp1 and Cdc53 is also present. This seems to also be the case in vertebrates, since Cdc34 homolog regulates DNA synthesis in *Xenopus* extracts.

SCF complex seems to be expressed continuously. In contrast to APC, the distinctive mechanisms regulating the activity of SCF complex are not known. SCF^{Cdc4} does not recognize non-phosphorylated but highly phosphorylated forms of Sic1, and similarly SCF^{Grr1} only phosphorylated Clns. Thus, it seems that phosphorylation of the target proteins triggers their recognition *via* cognate F-box proteins. Very recently, Cdc53 was found to be conjugated with Ub-like modifier, Rub1 (*5, 47, 48*). Rub1 is a small protein partially homologous to Ub and distributed among eukaryotic cells. Rub1 is activated by its activating enzyme, the sequence of which is similar to that of E1 but split into two subunits, and then conjugated to Cdc53 using Ubc13 as a conjugating enzyme. The sequence of Ubc13 is very similar to that of other Ubc relevant to Ub and hence has been designated as one of the Ubc. Ubc13, however, reacts with Rub1 but not with Ub, just as Ubc9 reacts with SUMO-1 but not Ub (*6*). The biological significance or implication of the Cdc53 modification by Rub1 is not clear.

Concluding remarks

In the past few years, much headway has been made in our understanding of the roles of selective ubiquitin-mediated proteolysis in a vast range of biological functions, and further progress can be expected in terms of elucidating the involvement of the ubiquitin system in the wider field of biological processes. This is because rapid selective

proteolysis ensures the irreversibility necessary for numerous biological regulations. In spite of accumulated information on the ubiquitin system, our knowledge on the nature of ubiquitin-ligases (E3), which are the main determinants of the timing and specificity of the ubiquitin-mediated protein degradation, has remained obscure until only quite recently. A limited case of ubiquitin-ligases identified casually in the analysis of cell-cycle control indicates that E3 ligases constitute a diverse range of classes that seemingly do not share any common structural features. This is in contrast to E2s which share a common domain and grossly determine the target specificity. The E3s, most strictly specifying the target proteins, function as an interface between the ubiquitin system as a general degradation mechanism and a control network for other biological processes which include regulatory proteins to be degraded *via* the ubiquitin system. Thus, E3 may be rather relevant to its substrate in its function and structure, and it is expected that an increasing number of E3 species will be identified. The diversity of E3 structures suggests the variety of signals to be recognized by the E3s. Our knowledge of the destruction signals is also very limited, and it is reasonable to assume more types of signals different from those discussed above. More studies to identify additional individual E3s and their recognition signals will therefore be required.

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