Association of human CUL-1 and ubiquitin-conjugating enzyme CDC34 with the F-box protein p45^{SKP2}: evidence for evolutionary conservation in the subunit composition of the CDC34–SCF pathway

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In normal and transformed cells, the F-box protein p45^{SKP2} is required for S phase and forms stable complexes with p19^{SKP1} and cyclin A–cyclin-dependent kinase (CDK)2. Here we identify human CUL-1, a member of the cullin family, and the ubiquitin-conjugating enzyme CDC34 as additional partners of p45^{SKP2} *in vivo*. CUL-1 also associates with cyclin A and p19^{SKP1} *in vivo* and, with p45^{SKP2}, they assemble into a large multiprotein complex. In *Saccharomyces cerevisiae*, a complex of similar molecular composition (an F-box protein, a member of the cullin family and a homolog of p19^{SKP1}) forms a functional E3 ubiquitin protein ligase complex, designated SCF^{CDC4}, that facilitates ubiquitination of a CDK inhibitor by CDC34. The data presented here imply that the p45^{SKP2}–CUL-1–p19^{SKP1} complex may be a human representative of an SCF-type E3 ubiquitin protein ligase. We propose that all eukaryotic cells may use a common ubiquitin conjugation apparatus to promote S phase. Finally, we show that multiprotein complex formation involving p45^{SKP2}–CUL-1 and p19^{SKP1} is governed, in part, by periodic, S phase-specific accumulation of the p45^{SKP2} subunit and by the p45^{SKP2}-bound cyclin A–CDK2. The dependency of p45^{SKP2}–p19^{SKP1} complex formation on cyclin A–CDK2 may ensure tight coordination of the activities of the cell cycle clock with those of a potential ubiquitin conjugation pathway.

Keywords: CDC34/cell cycle/cyclin-CDK/F-box/ubiquitination

Introduction

Passage through the eukaryotic cell cycle is controlled by the sequential activation of a class of enzymes known as the cyclin-dependent protein kinases (CDKs) (Sherr, 1993; Nigg, 1995). Activation of CDKs is regulated by the availability of their cognate cyclin subunit, positive and negative regulatory phosphorylation and association with CDK inhibitors (CKIs) (Morgan, 1995). Once activated, distinct cyclin–CDK complexes promote cell cycle advance by phosphorylating a set of largely unknown substrates.

In mammalian cells, cyclin A, in complex with its catalytic partner CDK2, has been established as a key promoter for progression through the S phase of the cell cycle (for review, see Heichmann and Roberts, 1994). Interference with cyclin A function in cultured cells by microinjection of antibodies against cyclin A inhibits DNA replication (Girard *et al.*, 1991; Pagano *et al.*, 1992; Zindy *et al.*, 1992), while ectopic expression of cyclin A is sufficient to allow adhesion-independent DNA synthesis in rat kidney cells (Guadagno *et al.*, 1993). In addition, cyclin A has been shown to co-localize with sites of ongoing DNA synthesis (Cardoso *et al.*, 1993; Sobczak-Thepot *et al.*, 1993), which indicates that this kinase may play a direct role in the regulation of one or more aspects of DNA replication.

The S phase-promoting activity of cyclin A–CDK2 is mediated, in part, through its ability to modulate the behavior of stably associated effector proteins. Currently, the known cyclin A–CDK2-associated proteins include CKIs of the Kip/Cip family, namely p21^{WAF1} and p27^{KIP1} (Harper *et al.*, 1993; Zhang *et al.*, 1993; Toyoshima and Hunter, 1994; Polyak *et al.*, 1994; Russo *et al.*, 1996), the DNA replication and repair factor proliferating cell nuclear antigen (PCNA) (Zhang *et al.*, 1993), the growth-suppressing element p107 (Ewen *et al.*, 1992; Faha *et al.*, 1992), p9^{CSK1/2} (Richardson *et al.*, 1990; Zhang *et al.*, 1995) and the transcription factor E2F-1 (Dymlacht *et al.*, 1994; Krek *et al.*, 1994, 1995; Xu *et al.*, 1994). p19^{SKP1} and p45^{SKP2} (for S phase kinase-associated protein 1 and 2) (Zhang *et al.*, 1995) recently have joined the list of proteins stably associated with cyclin A–CDK2. Comparatively little is known about the significance of each of these interactions for cell cycle control.

In normal cells, cyclin A–CDK2–p45^{SKP2}–p19^{SKP1} complexes are quite low in abundance. In contrast, this complex is highly abundant in transformed cells, due, in part, to high levels of p45^{SKP2} present in certain transformed cells (Zhang *et al.*, 1995). Interestingly, cyclin A–CDK2–p21^{WAF1} complexes are absent in such cells (Xiong *et al.*, 1993). The mechanism underlying this effect is unknown. Addition of p45^{SKP2} and/or p19^{SKP1} to active cyclin A–CDK2 did not affect kinase activity *in vitro*, suggesting that these proteins do not function in a manner analogous to CKIs (Zhang *et al.*, 1995). More importantly, microinjection of antibodies to p45^{SKP2}, or introduction of antisense p45^{SKP2} oligonucleotides into transformed or normal cells, inhibited DNA replication, suggesting an essential role for p45^{SKP2} in S phase promotion (Zhang *et al.*, 1995).

Database searches revealed that p45^{SKP2} is a member of the F-box protein family (Bai *et al.*, 1996). The F-box is an evolutionarily conserved domain found in many unrelated proteins (Bai *et al.*, 1996). Recent genetic and

biochemical analyses in *Saccharomyces cerevisiae* suggest a critical role for F-box proteins in ubiquitin-mediated protein degradation (for reviews, see King *et al.*, 1996; Krek, 1998). In general, degradation of proteins by the ubiquitin-proteasome pathway involves the activation of ubiquitin by an ubiquitin-activating enzyme (E1), which forms a thioester bond with ubiquitin, and then the transfer of ubiquitin to a member of a family of ubiquitin-conjugating enzymes (E2s or UBCs), also via a thioester intermediate. From the E2, ubiquitin is then transferred to target proteins either directly, or in combination with an E3 ubiquitin protein ligase. The resulting polyubiquitinated substrates are recognized by the 26S proteasome and degraded (for review, see Ciechanover, 1994). E3 ligases are the components of the ubiquitin conjugation system that are generally thought to be the most directly involved in substrate recognition (Scheffner *et al.*, 1993; Hochstrasser, 1996).

The budding yeast F-box protein CDC4, a WD-40 repeat-containing protein, forms complexes with two other proteins, SKP1 and CDC53, and this multiprotein complex functions as an E3 ubiquitin ligase, which catalyzes multi-ubiquitination of the CDK inhibitor SIC1 in combination with the ubiquitin-conjugating enzyme CDC34 (Goebel *et al.*, 1988) and an E1 (Feldman *et al.*, 1997; Skowrya *et al.*, 1997). Ubiquitin-mediated proteolysis of SIC1 in late G₁ is an essential step in the activation of S phase CDKs and the initiation of DNA replication (Schwob *et al.*, 1994; Bai *et al.*, 1996; Mathias *et al.*, 1996; Schneider *et al.*, 1996; Tyers, 1996; Verma *et al.*, 1997). The reproduction of SIC1 ubiquitination *in vitro* using recombinant proteins revealed that the F-box protein subunit CDC4 also appears to function as the receptor subunit which selectively binds the ubiquitination substrate SIC1 and recruits it for multiubiquitination by CDC34. Moreover, SKP1, which binds through the F-box to CDC4, promotes CDC4-substrate interaction, while CDC53 links the CDC4-SKP1 complex to the E2 CDC34. This novel type of E3 ligase complex has been designated SCF^{CDC4} for SKP1/CDC53(cullin)/F-box protein complex (Feldman *et al.*, 1997; Skowrya *et al.*, 1997). The SCF^{CDC4} appears also to participate in the ubiquitination and degradation of CDC6, a protein required for the formation of pre-replicative complexes (Piatti *et al.*, 1996; Drury *et al.*, 1997), and of the CKI FAR1, which inhibits G₁ CDKs and is required for the cell cycle arrest caused by mating pheromones (Henchoz *et al.*, 1997). Interestingly, ubiquitin-mediated proteolysis of yeast G₁ cyclins CLN1 and CLN2 in late G₁ requires, in addition to CDC34, SKP1 and CDC53, another F-box protein GRR1, which contains a set of leucine-rich repeats (Barral *et al.*, 1995; Deshaies *et al.*, 1995; Yaglom *et al.*, 1995; Bai *et al.*, 1996; Lanker *et al.*, 1996; Willems *et al.*, 1996). Indeed, the SCF^{GRR1} displays binding specificity towards G₁ cyclins and not CKIs (Skowrya *et al.*, 1997). Thus, the identity of the F-box protein subunit determines the repertoire of substrates targeted by these complexes. A close relative of budding yeast CDC4, the fission yeast Pop1 gene product, has been shown to be required for ubiquitin-proteasome-mediated degradation of the CKI Rum1 and the S phase regulator Cdc18 (Kominami and Toda, 1997). Taken together, these examples suggest a critical function of

F-box proteins in the regulation of the cell cycle in these yeasts.

Thus far, analogous complexes have not been described in mammalian cells, although vertebrate cells express structural and functional homologs of yeast CDC34 ubiquitin conjugation pathway components (Plon *et al.*, 1993; Bai *et al.*, 1996; Kipreos *et al.*, 1996; Mathias *et al.*, 1996). A human homolog of CDC34 has been cloned and is capable of complementing the yeast defect (Plon *et al.*, 1993). Moreover, a dominant-negative mutant of human CDC34, when added as recombinant protein to *Xenopus* interphase egg extracts, inhibits DNA replication (Yew and Kirschner, 1997). CDC53 is itself a member of a large evolutionarily conserved multigene family, referred to as the cullins (Kipreos *et al.*, 1996; Mathias *et al.*, 1996). Finally, human p19^{SKP1}, previously isolated as a cyclin A-CDK2-associated protein, is a homolog of SKP1 (Bai *et al.*, 1996).

Here we report that human CUL-1, a member of the cullin family, specifically associates with cyclin A, p45^{SKP2} and p19^{SKP1} *in vivo*. p45^{SKP2} also associates with the human ubiquitin-conjugating enzyme CDC34. These complexes form in an S phase-specific manner and require, at a minimum, an intact F-box motif and the p45^{SKP2}-bound cyclin A-CDK2. We hypothesize that a p19^{SKP1}-CUL-1-p45^{SKP2} complex may be a human representative of an S phase-specific SCF-type E3 ubiquitin ligase complex.

Results

Characterization of antibodies

Polyclonal rabbit antisera were raised against p45^{SKP2} expressed in *Escherichia coli* as a GST fusion protein, against synthetic 20mer and 19mer peptides corresponding, respectively, to the predicted C-termini of p19^{SKP1} (Zhang *et al.*, 1995) and the CDC53-like protein CUL-1 (Kipreos *et al.*, 1996), and against human cyclin A expressed in *E. coli* as a full-length protein (Pines and Hunter, 1990).

The specificities of antibodies for the respective gene products are illustrated in Figure 1. Affinity-purified anti-full-length (fl)p45^{SKP2} antibodies recognized a single protein of the expected molecular weight on immunoblotting of whole cell lysates from human U2-OS cells (Figure 1, lane 4). Importantly, the abundance of the endogenously detected protein was significantly increased in whole cell lysates prepared from U2-OS cells previously transfected with a mammalian expression plasmid encoding a p45^{SKP2} cDNA that utilizes methionine +13 of the reported amino acid sequence of p45^{SKP2} (Figure 1, lane 5) (Zhang *et al.*, 1995), referred to hereafter as untagged p45^{SKP2}. In comparison, the product of a p45^{SKP2} cDNA initiating at methionine +1 (referred to as NT-p45^{SKP2}) migrated on SDS gels as a larger protein (Figure 1, compare lanes 2 and 3, lower panel). Thus, the migration of endogenous p45^{SKP2} on SDS gels is consistent with the view that p45^{SKP2} translation is initiated at methionine +13 *in vivo*. The same migration behavior of endogenous p45^{SKP2} was seen in multiple different cell lines, including T98G, U2-OS and WI-38 cells, as well as 293 cells, squamous cell carcinoma cell lines and Saos-2 cells (see Figure 5C; E.Chatelain and W.Krek, unpublished).

To support this observation further, mammalian expression plasmids were generated encoding NT-p45^{SKP2} and a

Fig. 1. Specificity of antibodies against p45^{SKP2}, p19^{SKP1}, CUL-1 and cyclin A. Human U2-OS cells, either transfected with 5 µg of empty pcDNA3 vector (lanes 4, 6 and 8) or transfected with pcDNA3 mammalian expression plasmids encoding MT-p45^{SKP2} (lane 1), NT-p45^{SKP2} (lane 2), untagged p45^{SKP2} (lanes 3 and 5), untagged p19^{SKP1} (lane 7) or untagged CUL-1 (lane 9) were lysed, equalized for protein content, protein mixtures separated by SDS-PAGE [10% (lanes 4, 5 and 10–15); 12% (lanes 6 and 7); 8% (lanes 8 and 9)] and processed for Western blotting with anti-myc mAb 9E10 (lanes 1–3, upper panel), affinity-purified anti-NT antibodies (lanes 1–3, middle panel), anti-(fl)p45^{SKP2} antibodies (lanes 1–3, lower panel and lanes 4 and 5), anti-(C)p19^{SKP1} antibodies (lanes 6 and 7) or anti-(C)CUL-1 antibodies (lanes 8 and 9). Whole cell extracts were prepared from U2-OS (lane 10), MRC-5 (lane 11) or NIH-3T3 cells (lane 12), as well as extracts of Sf9 cells infected with vector (lane 14) or recombinant baculovirus, encoding cyclin E (lane 13) or cyclin A (lane 15), and processed for immunoblotting with anti-cyclin A rabbit serum. Note that the anti-human cyclin A serum does not recognize mouse cyclin A (lane 12). The positions of the respective p45^{SKP2}, p19^{SKP1}, CUL-1 and cyclin A endogenous proteins are indicated by open arrowheads. Dashes indicate molecular weight markers [from top to bottom in kDa: 78, 46.7, 34.5, 28.8 (lanes 4–7 and 10–15); 109, 78, 46.7, 34.5 (lanes 8 and 9)].

derivative of it carrying a myc epitope, referred to as MT-p45^{SKP2}. By immunoblotting on whole cell lysates of U2-OS cells transfected with the aforementioned mammalian expression plasmids, monoclonal anti-myc antibodies recognized exclusively MT-p45^{SKP2}, but failed to recognize NT-p45^{SKP2} or untagged p45^{SKP2} (Figure 1, compare lanes 1–3, upper panel). Affinity-purified anti-(fl)p45^{SKP2} antibodies, however, detected all three p45^{SKP2} species (Figure 1, lanes 1–3, lower panel). Interestingly, the untagged p45^{SKP2} species co-migrated precisely with endogenous p45^{SKP2} (see Figure 1, lanes 4 and 5), supporting the above observation that endogenous p45^{SKP2} is translated as a 422 amino acid protein. As predicted from these results, peptide antibodies raised against the N-terminal 18 amino acids of NT-p45^{SKP2} recognized MT-p45^{SKP2} as well as NT-p45^{SKP2} (the 435 amino acid version of p45^{SKP2}), but failed to detect transiently expressed, untagged p45^{SKP2} (Figure 1, lanes 1–3, middle panel). Taken together, these results suggest first that anti-(fl)p45^{SKP2} antibodies are specific and recognize endogenous p45^{SKP2} and, second, that the p45^{SKP2} N-terminal peptide antibody, anti-NT, does not recognize endogenous p45^{SKP2}. Hence, this antibody specifically detects NT-p45^{SKP2} and MT-p45^{SKP2}.

Total cell lysates of either untransfected human U2-OS cells or U2-OS cells transfected with mammalian expression plasmids harboring either untagged p19^{SKP1} or CUL-1 were immunoblotted with affinity-purified anti-(C)p19^{SKP1} and anti-(C)CUL-1 antibodies. The respective antibodies recognized single proteins of the expected molecular weights, the abundances of which were significantly enhanced in the corresponding lysates derived from transfected cells (Figure 1, lanes 6–9). Finally, the

specificity of the anti-cyclin A antiserum was assessed. The antibody recognized a single protein of the expected molecular weight in U2-OS and MRC-5 cell extracts (Figure 1, lanes 10 and 11, respectively) which precisely co-migrated with human cyclin A produced in Sf9 cells using recombinant baculovirus (Figure 1, lane 15). Therefore, all antibodies used in this study recognize specifically the relevant protein.

Biochemical evidence for an *in vivo* p45^{SKP2}–CUL-1 interaction in transiently transfected human cells

The cyclin A–CDK2-associated protein p45^{SKP2} shares two recognizable sequence motifs with GRR1, the F-box and the leucine-rich repeat (LRR) (Figure 2A; Bai *et al.*, 1996). This similarity prompted us to ask whether p45^{SKP2} forms specific complexes with cloned human cullins (Pause *et al.*, 1997). To this end, human CUL-1 and CUL-2 were produced in insect cells as bipartite hexahistidine (His₆)-hemagglutinin (HA) epitope-tagged proteins (Figure 2B, lanes 3 and 5, respectively) and the corresponding lysates were mixed individually with baculovirus-produced His₆-NT-p45^{SKP2} (Figure 2B, lane 2). Reaction mixtures were subjected to immunoprecipitation with anti-NT antibodies. His₆-CUL-1-HA, but not His₆-CUL-2-HA, was recovered efficiently in anti-NT immunoprecipitates (Figure 2, compare lanes 4 and 6), suggesting that stable complexes of His₆-NT-p45^{SKP2} and His₆-CUL-1-HA had formed. His₆-CUL-1-HA was not present in anti-NT immunoprecipitates when His₆-NT-p45^{SKP2} was omitted (Figure 2B, lane 3). Thus, p45^{SKP2} can form stable complexes with CUL-1, but not CUL-2, *in vitro*.

Next, we tested whether p45^{SKP2} would complex with epitope-tagged ectopic CUL-1 *in vivo*. Figure 2C shows

that after transient transfection of HA-tagged CUL-1 into U2-OS cells, CUL-1-HA was detected in an anti-(fl)p45^{SKP2} immunoprecipitate (Figure 2C, lane 5). No such band precipitated either with anti-(fl)p45^{SKP2} antibodies from vector-transfected cells (Figure 2C, lane 3) or when the corresponding pre-immune serum was used (Figure 2C, lanes 4 and 6). Hence, p45^{SKP2} and CUL-1 can also interact *in vivo*.

In an effort to confirm further the existence of a physical interaction between p45^{SKP2} and CUL-1 *in vivo*, mammalian expression plasmids carrying MT-p45^{SKP2} and CUL-1-HA were co-transfected either alone or in combination into U2-OS cells. CUL-1-HA was detected specific-

ally in anti-NT immunoprecipitates when present together with MT-p45^{SKP2} (Figure 2D, lane 4), while no such HA-reactive band was seen in the anti-NT immunoprecipitates derived from extracts of either vector-, MT-p45^{SKP2}- or CUL-1-HA-transfected cells (Figure 2D, lanes 1–3, respectively). Western blotting of the relevant whole cell extracts revealed that all proteins were present where expected (Figure 2C, lanes 1–4, lower panels). In a reciprocal experiment, anti-HA immunoprecipitation of an MT-p45^{SKP2}/CUL-1-HA-transfected U2-OS cell extract precipitated MT-p45^{SKP2} (Figure 2E, lane 4). Thus, further evidence of specific complex formation between p45^{SKP2} and CUL-1 was obtained in transiently transfected cells.

Next, we assessed whether these two proteins co-localize within cells. Mammalian expression vectors encoding green fluorescent protein (GFP)–CUL-1 and MT-p45^{SKP2} were transfected individually or in combination into human U2-OS cells. When present alone, GFP–CUL-1 showed nuclear and cytoplasmic localization (Figure 2F, upper panel, left). Interestingly, however, when MT-p45^{SKP2} was expressed together with GFP–CUL-1 in the same cell, the latter localized exclusively to the nucleus (Figure 2F, lower panel, left). This result suggests that p45^{SKP2} is capable of relocating CUL-1 to the nucleus, consistent with the proposal that p45^{SKP2}/CUL-1 form complexes *in vivo*.

Human CUL-1 forms complexes with cyclin A, p45^{SKP2} and p19^{SKP1} *in vivo*

To test whether CUL-1 is an additional partner of the previously described cyclin A–CDK2–p45^{SKP2}–p19^{SKP1} complex (Zhang *et al.*, 1995), immunoprecipitates of p45^{SKP2}, p19^{SKP1} and cyclin A were generated from extracts of untransfected U2-OS cells and immunoblotted with affinity-purified anti-(C)CUL-1 antibodies. As a control, the respective pre-immune sera were used. Human CUL-1 was readily detected in anti-cyclin A (Figure 3A, lane 1), anti-(fl)p45^{SKP2} (Figure 3A, lane 3) or anti-(C)p19^{SKP1} (Figure 3A, lane 5) immunoprecipitates, but not in the respective pre-immune control precipitates (Figure 3A, lanes 2, 4 and 6) or anti-cyclin B1 immunoprecipitate (Figure 3A, lane 7). Thus, CUL-1 interacts with cyclin A, p45^{SKP2} and p19^{SKP1} under physiological conditions. It should be noted that although these CUL-1 antibodies work well on Western blots, they are unable to immunoprecipitate CUL-1, precluding reciprocal immunoprecipitation experiments.

To establish formally whether these proteins assemble into a large protein complex, containing at a minimum p45^{SKP1}, p19^{SKP1} and CUL-1, U2-OS cells were transfected with expression plasmids encoding MT-p45^{SKP2} and CUL-1-HA, with or without flag epitope-tagged (FT)-p19^{SKP1}. Immunoblotting of U2-OS whole cell lysates previously transfected with the plasmids under investigation revealed that each protein was present where expected (Figure 3B, lanes 5 and 6). Extracts of transfected cells were immunoprecipitated with anti-flag antibody, and immune

complexes treated with flag peptide to dissolve antibody–antigen interactions. FT-p19^{SKP1}-containing complexes, released into the supernatant, were then subjected to reprecipitation using anti-NT antibodies, followed by immunoblotting with anti-HA antibodies. CUL-1-HA was only detected when sequential anti-flag and anti-NT immunoprecipitations were performed from extracts derived from FT-p19^{SKP1}/MT-p45^{SKP2}/CUL-1-HA triple-transfected cells (Figure 3B, lane 2), but not from extracts of transfected cells lacking FT-p19^{SKP1} (Figure 3B, lane 3). Hence, FT-p19^{SKP1}, MT-p45^{SKP2} and CUL-1-HA co-exist in a single complex.

p45^{SKP2} associates with a human homolog of budding yeast ubiquitin-conjugating enzyme CDC34

The association of p45^{SKP2} with CUL-1 *in vivo* and the known presence of p19^{SKP1} in this complex suggested that this protein complex might, in analogy to budding yeast, function in a ubiquitin conjugation pathway together with a human homolog of ubiquitin-conjugating enzyme CDC34.

To search for biochemical evidence of an *in vivo* p45^{SKP2}–CDC34 interaction, human CDC34 was equipped with an HA epitope tag at its N-terminus. Mammalian expression plasmids encoding MT-p45^{SKP2} and HA–CDC34 were transfected alone or together into human U2-OS cells. Immunoprecipitation of MT-p45^{SKP2} using anti-NT antibodies led to co-immunoprecipitation of HA–CDC34 when both were co-synthesized (Figure 4A, lane 5). HA–CDC34 was not detected in anti-NT immunoprecipitates derived from extracts of cells transfected with either vector, MT-p45^{SKP2} or HA–CDC34, alone (Figure 4A, lanes 1–3). In addition, transiently produced HA-tagged human UBC9 failed to co-immunoprecipitate with MT-p45^{SKP2}, although HA–UBC9 was expressed at equivalent levels to HA–CDC34 (Figure 4A, lanes 4 and 5, compare lower panels). These results suggest that the cyclin A–CDK2-associated protein p45^{SKP2} interacts with a human homolog of ubiquitin-conjugating enzyme CDC34 *in vivo* and that the interaction is specific. As determined by indirect immunofluorescence microscopy, ectopically synthesized MT-p45^{SKP2} and HA–CDC34 localize predominantly in the nucleus (Figure 4B, upper and lower panels, left). Also in untransfected U2-OS cells, p45^{SKP2}

Fig. 2. p45^{SKP2} associates with human CUL-1 and relocates CUL-1 to the nucleus. (A) Schematic representation of budding yeast GRR1 and human p45^{SKP2}. Two recognizable structural motifs are shared between these two proteins, namely an F-box (filled box) and a leucine-rich repeat (LRR) domain. (B) Aliquots of lysates from baculovirus-infected Sf9 cells expressing His₆-NT-p45^{SKP2}, His₆-CUL-1-HA or His₆-CUL-2-HA were either subjected to immunoprecipitation with anti-NT antibodies (upper panel, lanes 1–6) following mixing of His₆-NT-p45^{SKP2} with His₆-CUL-1-HA (lane 4) or with His₆-CUL-2-HA (lane 6), as indicated by the plus and minus signs on top of the figure, or processed for immunoblotting using anti-HA mAb 12CA5 (middle panel, lanes 1–6) or anti-NT antibodies (lower panel, lanes 1–6). Note that only His₆-CUL-1-HA, but not His₆-CUL-2-HA, co-immunoprecipitated with His₆-NT-p45^{SKP2}. The bracket identifies the IgG band. (C) U2-OS cells were transfected with the indicated mammalian expression plasmids and cell extracts were prepared. Aliquots of lysates were either processed directly for Western blotting using anti-HA mAb 12CA5 (lanes 1 and 2) or subjected to immunoprecipitation using anti-(fl)p45^{SKP2} antibodies (lanes 3 and 5) or the corresponding control pre-immune serum (lanes 4 and 6). Immunoprecipitates were Western blotted with anti-HA mAb 12CA5 (lanes 3–6). (D) U2-OS cells were transfected with 10 µg of each of the indicated mammalian expression plasmids. Cell extracts were prepared and aliquots were subjected to immunoprecipitation with anti-NT antibodies, followed by Western blotting with anti-HA mAb 12CA5 (upper panel, lanes 1–4). Aliquots of whole cell lysates were Western blotted with anti-HA mAb 12CA5 (middle panel, lanes 1–4) or anti-NT antibodies (lower panel, lanes 1–4). The arrow in the upper panel indicates co-immunoprecipitated CUL-1-HA. The arrowhead indicates the IgG band. Dashes on the left indicate molecular weight markers (from top to bottom in kDa: 109, 78, 46.7). (E) U2-OS cells were transfected with the indicated mammalian expression plasmids and processed as described in (D), except that immunoprecipitations were performed with anti-HA mAb 12CA5, followed by Western blotting with anti-NT antibodies (upper panel, lanes 1–4). Aliquots of whole cell lysates were Western blotted with anti-HA mAb 12CA5 (middle panel, lanes 1–4) or anti-NT antibodies (lower panel, lanes 1–4). The arrow indicates co-immunoprecipitated MT-p45^{SKP2} (lane 4). The bracket indicates the IgG band. (F) U2-OS cells were transfected with mammalian expression plasmids encoding GFP–CUL-1 (upper panels) or GFP–CUL-1 and MT-p45^{SKP2} (lower panels), triple stained with anti-GFP antibodies (left), anti-myc mAb 9E10 (middle) and DAPI (right) and examined by indirect immunofluorescence microscopy. Note the relocalization of GFP–CUL-1 to the nucleus in the presence of MT-p45^{SKP2}.

Fig. 3. Human CUL-1 associates with cyclin A, p45^{SKP2} and p19^{SKP1} *in vivo* and forms a multiprotein complex that includes p45^{SKP2} and p19^{SKP1}. (A) Untransfected U2-OS cells were immunoprecipitated with anti-cyclin A (lane 1), anti-(fl)p45^{SKP2} (lane 3) or anti-(C)p19^{SKP1} antibodies (lane 5). Controls included the corresponding pre-immune serum (lanes 2, 4 and 6) or anti-cyclin B1 antibodies (lane 7). Immunoprecipitates were processed for Western blotting with affinity-purified anti-(C)CUL-1 antibodies. The arrow indicates co-immunoprecipitated endogenous CUL-1 and the bracket identifies the IgG band. Dashes indicate molecular weight markers (from top to bottom in kDa: 109, 78, 46.7). (B) U2-OS cells were transfected with 10 µg each of mammalian expression plasmids encoding FT-p19^{SKP1}, MT-p45^{SKP2} and CUL-1-HA (lanes 2 and 5), or a combination lacking FT-p19^{SKP1} (lanes 3 and 6). Aliquots of cell extracts were subjected to immunoprecipitation with anti-flag mAb M5, and immune complexes were released with flag peptide, subjected to re-immunoprecipitation with anti-NT antibodies and processed for Western blotting with anti-HA mAb 12CA5 (lanes 1–3). Aliquots of whole cell lysates were processed directly for Western blotting with a mixture of anti-HA mAb 12CA5, anti-NT antibodies and anti-flag mAb M5. The brackets in the left panel identify the IgG signal and the arrow indicates co-immunoprecipitated CUL-1-HA. Dashes indicate molecular weight markers [from top to bottom in kDa: 109, 78, 46.7 (lanes 1–3); 78, 46.7, 34.5 and 28.8 (lanes 4–6)].

is localized predominantly in the nucleus (M.Gstaiger and W.Krek, unpublished).

Expression of p45^{SKP2}, p19^{SKP1} and CUL-1 during the somatic cell cycle

Since complexes between p45^{SKP2} and vertebrate homologs of budding yeast CDC34 ubiquitin conjugation pathway components can be detected *in vivo*, we next wished to determine whether complex formation is regulated in a cell cycle-dependent manner.

To this end, human T98G cells (Stein, 1979) were synchronized in G₀/G₁ by serum deprivation and released from arrest by the addition of serum. Extracts were prepared at selected time points, equalized for total protein amounts and then analyzed by immunoblotting using antibodies specific for p45^{SKP2}, p19^{SKP1} and CUL-1 (Figure 5A, lower panels). As a control, the same samples were also probed with anti-cyclin A and anti-cyclin E antibodies. Cell synchrony was monitored, in parallel, by flow cytometry (Figure 5A, upper panel).

As shown in the fluorescence-activated cell sorting (FACS) analysis figures, T98G cells respond to serum withdrawal by arresting efficiently in G₀/G₁. Following addition of 20% serum, these cells progress synchronously into the first S phase (20–24 h later), through mitosis (32–36 h) and further into the next S phase (40–44 h).

The total abundance of p19^{SKP1} and CUL-1 varied little during the cell cycle. Both p19^{SKP1} and CUL-1 protein were detected in extracts prepared from serum-deprived

cells and their abundance increased only modestly following serum addition. In contrast, p45^{SKP2} protein levels varied dramatically as cells advanced through the cell cycle. In fact, the appearance of p45^{SKP2} coincided well with progression of cells through the S/G₂ phases, i.e. 24–28 h after serum addition. Around 36 h following serum addition, cells had moved through M phase and p45^{SKP2} levels had decreased greatly, then increased again as cells started a new round of S phase, thereby mirroring the expression profile of cyclin A (Pines and Hunter, 1990). In contrast, cyclin E protein appeared, as expected, earlier than cyclin A (~12–16 h after serum addition) and declined as cells advanced through S/G₂ (Dulic *et al.*, 1992; Koff *et al.*, 1992) (data not shown). Thus, one level of regulation for p45^{SKP2} complex formation with CUL-1, p19^{SKP1} or CDC34 is provided by the S phase-specific expression of a p45^{SKP2} subunit.

To determine whether p45^{SKP2} protein expression is increased in an S phase-enriched cell population of normal, untransformed cells, human diploid fibroblasts were deprived of serum for 3 days (G₀) and then released from the arrest by the addition of serum. Extracts were prepared from serum-deprived cells and from cells released for 28 h. This time coincides with progression of cells through S phase as determined by FACS analysis (data not shown). Cell extracts, equalized for total protein amounts, were then analyzed by immunoblotting using anti-(fl)p45^{SKP2} antibodies (Figure 5B, upper panel, lanes 1–3). As in synchronized T98G cells, p45^{SKP2} protein abundance is

Fig. 4. p45^{SKP2} and human CDC34 localize to the nucleus and form specific complexes *in vivo*. (A) U2-OS cells were transfected with 10 µg of MT-p45^{SKP2}(wt) and 10 µg of HA-CDC34 or HA-UBC9 mammalian expression plasmids. Aliquots of cell extracts were processed as described in Figure 2D. The arrow indicates the position of co-immunoprecipitated HA-CDC34 (lane 5). The arrowhead identifies the IgG band. Note that HA-UBC9 did not co-immunoprecipitate with MT-p45^{SKP2} (see lane 4). Aliquots of whole cell lysates were immunoblotted with anti-myc mAb 9E10 (middle panel) or anti-HA mAb 12CA5 (lower panels). (B) U2-OS cells were transfected with mammalian expression plasmids encoding MT-p45^{SKP2} (upper panel) or HA-CDC34 (lower panel), stained with anti-NT antibodies (upper panel, left) or anti-HA mAb 12CA5 (lower panel, left), and examined by indirect immunofluorescence microscopy. The right panels show the corresponding DAPI staining.

increased dramatically in an S phase-enriched cell population of human diploid fibroblasts compared with serum-starved cells (Figure 5B, compare lanes 2 and 3). Immunoblotting of these lysates with an anti-tubulin antibody showed that equivalent amounts of proteins were present in each lane (Figure 5B, lower panel, lanes 1–3). Thus, p45^{SKP2} abundance is subject to cell cycle regulation in both normal and transformed cells.

To compare the abundance of p45^{SKP2} in normal versus transformed cells, cell extracts were prepared from exponentially growing T98G, U2-OS and WI-38 cells, equalized for protein content and analyzed by immunoblotting using anti-(fl)p45^{SKP2} antibodies. As shown in Figure 5C, normal human diploid lung fibroblasts contain significantly less p45^{SKP2} protein than transformed cells from different origins (Figure 5C, compare lane 2 with lanes 1 and 3).

The leucine-rich repeat domain of p45^{SKP2} contains sequences required for stable p45^{SKP2}–cyclin A–CDK2 interaction

To examine whether the p45^{SKP2}-bound cyclin A–CDK2 provides another level of cell cycle control contributing potentially to the timely formation of a p45^{SKP2}–p19^{SKP1}–CUL-1 complex, we needed first to define sequences on p45^{SKP2} which contribute to stable cyclin A–CDK2 complex formation. To this end, a series of p45^{SKP2} deletion mutants were constructed (Figure 6A). The

mutants sustained deletions of either the most C-terminal 40 (Kpn) or 200 amino acid residues (Xba), or the entire LRR domain. Another mutant, ΔF6, sustained a deletion of six residues within the F-box, residues which are evolutionarily conserved among F-box proteins (Bai *et al.*, 1996). Prior experiments revealed that mutations of some of these residues in CDC4 abolished CDC4–SKP1 interactions (Bai *et al.*, 1996).

Mammalian expression plasmids encoding wild-type (wt) MT-p45^{SKP2} and the aforementioned mutant derivatives were then transfected into U2-OS cells. All p45^{SKP2} species under investigation were synthesized to equivalent levels, as shown by Western blotting of equalized amounts of the corresponding whole cell lysates (Figure 6B, upper panel). When anti-NT immunoprecipitates of cell lysates were tested for associated kinase activity, only MT-p45^{SKP2}(wt) displayed histone H1 kinase activity (Figure 6B, lower panel, lane 2). Thus, stable association of p45^{SKP2} with a cellular kinase, most likely cyclin A–CDK2 (see below Figure 6E), requires certain carboxy-terminal sequences of p45^{SKP2}.

Inspection of the extreme C-terminus of p45^{SKP2} revealed the presence of a ‘KxL’ motif (Figure 6C), which has been implicated as a cyclin–CDK recognition determinant in other known cyclin–CDK-binding proteins (e.g. see Vlach *et al.*, 1997, and references therein). For example, it was shown that conversion of the ‘RxL’ motif

Fig. 5. S phase-specific expression of human p45^{SKP2}. (A) T98G cells were arrested in G₀/G₁ by serum deprivation and stimulated to re-enter the cell cycle by readdition of serum. At the indicated times thereafter, whole cell extracts were prepared, equalized for protein content and processed for Western blotting with the indicated antibodies. In parallel, one aliquot of cells was processed for FACS analysis to determine cell cycle distribution (uppermost panel). Western blots from two independent experiments are shown. Experiment 1 (panels 1–3, from top to bottom): anti-cyclin A, anti-(fl)p45^{SKP2} and anti-(C)p19^{SKP1} antibodies. Experiment 2 (panels 4 and 5, from top to bottom): anti-(C)CUL-1 and anti-(fl)p45^{SKP2} antibodies. FACS analysis data for experiment 2 are not shown. (B) Normal human diploid fibroblasts, either exponentially growing (upper panel, lane 1), arrested in G₀/G₁ by serum deprivation (upper panel, lane 2) or enriched in S phase (upper panel, lane 3), were lysed, and aliquots of cell extracts, equalized for protein content, were processed for Western blotting using either anti-(fl)p45^{SKP2} (upper panel, lanes 1–3) or anti-tubulin mAb Tu27b (lower panel, lanes 1–3). Note the absence of p45^{SKP2} in G₀/G₁ synchronized cells. (C) Exponentially growing T98G cells (lane 1), WI-38 cells (lane 2) and U2-OS cells (lane 3) were lysed, and equivalent amounts of protein extracts were processed for Western blotting using anti-(fl)p45^{SKP2} antibodies.

of p27 to 'AxA' abolishes p27–cyclin interaction (Vlach *et al.*, 1997). In addition, the p45^{SKP2} mutant MT-p45^{SKP2}(Kpn), which failed to bind cyclin A–CDK2 (Figure 6B), harbors a deletion extending to, but not including, the indicated 'KxL' motif, which resides within the last leucine-rich repeat of p45^{SKP2} (Zhang *et al.*, 1995). Based, therefore, on sequence similarity and previous results, two additional p45^{SKP2} mutants were constructed, MT-p45^{SKP2}(AxA) and MT-p45^{SKP2}(ΔA3). The former mutant sustains a conversion of Lys362 and Leu364 to alanine and the latter a deletion of amino acid residues 362–364 of p45^{SKP2}. These MT-p45^{SKP2} mutant species were then tested, in parallel with MT-p45^{SKP2}(wt) and (ΔF6), for their ability to bind an endogenous histone H1 kinase. All p45^{SKP2} species under investigation were synthesized to equivalent levels following transfection into U2-OS cells (Figure 6D, upper panel). In the relevant anti-NT immunoprecipitates, MT-p45^{SKP2}(wt) and (ΔF6) were associated with a kinase capable of phosphorylating histone H1, while AxA was not (Figure 6D, lower panel, lanes 2–4). Identical results were obtained with MT-p45^{SKP2}(ΔA3), as well as with other MT-p45^{SKP2} mutants that sustained either eight or 35 residue deletions in the F-box domain (data not shown). Thus, the existence of stable p45^{SKP2}–kinase complexes depends on specific

C-terminal residues of p45^{SKP2} and is independent of the F-box domain. In fact, complex formation appears to depend upon the integrity of the intact 'KxL' motif residing within the LLR domain of p45^{SKP2}. The observation that MT-p45^{SKP2}(Kpn), which sustains a deletion right up to but not including the 'KxL' motif of p45^{SKP2}, was also defective in cyclin A–CDK2 binding (see Figure 6B, lane 3) suggests that certain neighboring amino acid residues are, in addition, required for stable p45^{SKP2}–cyclin A–CDK2 binding. Similar findings were reported for E2F-1–cyclin A–CDK2 interactions (Krek *et al.*, 1994).

To establish whether the failure of MT-p45^{SKP2}(AxA) to bind a cellular kinase correlates with the absence of cyclin A, expression plasmids encoding MT-p45^{SKP2}(wt), (ΔF6) and (AxA) were transfected into U2-OS cells alone or in combination with an HA-tagged cyclin A. Anti-HA immunoprecipitates were then analyzed by immunoblotting for the presence of co-immunoprecipitated MT-p45^{SKP2} species (Figure 6E). In agreement with the kinase assays shown above, HA-cyclin A immunoprecipitated MT-p45^{SKP2}(wt) and (ΔF6) (Figure 6E, lanes 2 and 3), but not MT-p45^{SKP2}(AxA) (Figure 6E, lane 4), suggesting that the failure of MT-p45^{SKP2}(AxA) to display associated kinase activity is due to the absence of a bound cyclin A. Identical results were obtained when the MT-p45^{SKP2}

Fig. 6. Identification of sequences required for stable p45^{SKP2}-cyclin A-CDK2 interaction. (A) Schematic representation of wild-type (wt) p45^{SKP2} and various mutant derivatives. (B) U2-OS cells, transfected with 20 μg of each indicated expression plasmid, were lysed and aliquots processed for Western blotting with anti-NT antibodies (upper panel, lanes 1–5) or for immunoprecipitation with anti-NT antibodies. Immunoprecipitates were processed further for *in vitro* kinase reactions in the presence of histone H1 substrate (lower panel, lanes 1–5). The bracket designates the position of phosphorylated histone H1. (C) Alignment of the putative cyclin A-CDK2-binding domain in p45^{SKP2} with other known cyclin-CDK complex-binding proteins. Highly conserved residues are in bold. (D) U2-OS cells transfected with 20 μg of each indicated plasmid were processed as described in (B). The bracket designates the position of phosphorylated histone H1. (E) pcDNA3 vector plasmid (lane 1) or expression plasmids carrying MT-p45^{SKP2}(wt) (lane 2), MT-p45^{SKP2}(ΔF6) (lane 3) or MT-p45^{SKP2}(AxA) (lane 4) were transfected into U2-OS cells together with an expression plasmid encoding HA-cyclin A. Whole cell lysates were processed for immunoprecipitation with cross-linked anti-HA mAb 12CA5 (upper panel), followed by Western blotting with anti-NT antibody. Aliquots of lysates were processed for Western blotting with anti-HA mAb 12CA5 (middle panel) or anti-NT antibodies (lower panel). Note that HA-cyclin A failed to co-immunoprecipitate with MT-p45^{SKP2}(AxA).

species under investigation were tested for their ability to bind endogenous cyclin A and CDK2 (data not shown).

p19^{SKP1} binding to p45^{SKP2} is dependent on p45^{SKP2}-cyclin A-CDK2 complex formation

Now equipped with mutants defective in cyclin A-CDK2 association, we tested whether binding of p45^{SKP2} to p19^{SKP1} or CUL-1 is linked to the physical presence of cyclin A-CDK2. To this end, mammalian expression plasmids encoding MT-p45^{SKP2}(wt) and the mutant derivatives, (AxA) and (ΔF6), were transfected either alone or in combination with FT-p19^{SKP1} into U2-OS cells, followed by [³⁵S]methionine labeling. As shown in Figure 7A, FT-p19^{SKP1} was detected readily in anti-NT immunoprecipitates when co-synthesized with MT-p45^{SKP2}(wt) (lane 3), but not with the MT-p45^{SKP2} mutant derivatives (AxA) or (ΔF6) (lanes 5 and 7, respectively). In the reciprocal experiment, anti-flag antibody immunoprecipitated FT-p19^{SKP1} and MT-p45^{SKP2}(wt) efficiently (lane 9), but not

the p45^{SKP2} mutant derivatives under investigation (lanes 11 and 13). Identical results were obtained with other p45^{SKP2} mutants defective in cyclin A-CDK2 binding, i.e. (ΔLRR), (Kpn) and (Xba) (data not shown). Therefore, p19^{SKP1} binding to p45^{SKP2}, much like the CDC4-SKP1 interaction, appears to require an intact F-box motif since F-box deletion mutants of p45^{SKP2} failed to bind p19^{SKP1}. However, p19^{SKP1}-p45^{SKP2} complex formation is not only dependent on the presence of certain sequences within the F-box. Additionally, it requires specific p45^{SKP2} C-terminal sequences. Interestingly, the same sequences required for p19^{SKP1}-p45^{SKP2} interaction are also required for stable p45^{SKP2}-cyclin A-CDK2 complex formation, suggesting that the former interaction depends on the latter.

To test whether CUL-1 binding to p45^{SKP2} is dependent on the p45^{SKP2}-bound cyclin A-CDK2 or on an intact F-box motif, mammalian expression plasmids encoding MT-p45^{SKP2}(wt), (AxA) and (ΔF6) were transfected either alone or in combination with CUL-1-HA into U2-OS

Fig. 7. Recruitment of p19^{SKP1} to p45^{SKP2} requires the p45^{SKP2}-bound cyclin A-CDK2. (A) [³⁵S]Methionine-labeled U2-OS cells, previously transfected with 10 μg of expression plasmids encoding MT-p45^{SKP2}(wt), (AXA) and (ΔF6) or 15 μg of FT-p19^{SKP1} expression plasmid (as indicated by the plus and minus signs at the top of each lane) were lysed and subjected to immunoprecipitation with either anti-NT antibodies (lanes 1–7) or anti-flag mAb M5 (lanes 8–13). Arrows indicate the respective positions of MT-p45^{SKP2} and FT-p19^{SKP1}. Dashes indicate molecular weight markers (from top to bottom in kDa: 109, 78, 46.7, 34.5, 28.8). (B) U2-OS cells were transfected with 10 μg of expression plasmids encoding MT-p45^{SKP2}(wt), (AXA) and (ΔF6) or 15 μg of CUL-1-HA expression plasmid, as indicated by the plus and minus signs at the top of each lane. Aliquots of lysates were either subjected to immunoprecipitation with anti-NT antibodies, followed by Western blotting with a mixture of anti-HA mAb 12CA5 and anti-NT antibodies (upper panel), or directly processed for Western blotting with anti-HA mAb 12CA5 (middle panel) and anti-NT antibodies (lower panel). Note that lanes 10 and 11 are loaded in the reverse order with respect to the plus and minus signs at the top of the figure. The arrow indicates co-immunoprecipitated CUL-1-HA, brackets designate the position of immunoprecipitated MT-p45^{SKP2} and the arrowhead identifies the IgG band. Dashes indicate molecular weight markers (from top to bottom in kDa: 109, 78, 46.7).

cells, and the anti-NT immunoprecipitates were analyzed by immunoblotting for the presence of co-immunoprecipitated CUL-1-HA (Figure 7B). Two conclusions can be drawn from the results shown. First, p45^{SKP2}-CUL-1 complex formation does not require p45^{SKP2}-bound cyclin A-CDK2 since CUL-1-HA was immunoprecipitated effi-

ciently along with MT-p45^{SKP2}(wt) or the cyclin-CDK2 binding-defective mutant MT-p45^{SKP2}(AxA) (Figure 7B, lanes 4 and 6). Second, MT-p45^{SKP2}(ΔF6), which is defective in FT-p19^{SKP1} binding, is also impaired in CUL-1-HA binding (Figure 7B, lane 8). A longer exposure of the Western blot shown in Figure 7B (upper panel) revealed the presence of a residual CUL-1-HA signal, suggesting that MT-p45^{SKP2}(ΔF6) is largely (albeit not wholly) defective in CUL-1 binding (data not shown). Equivalent levels of CUL-1-HA and the various MT-p45^{SKP2} species were expressed as shown by Western blotting of equal amounts of whole cell lysates (Figure 7B, lanes 9–16). Identical results were also obtained when MT-p45^{SKP2}(ΔA3) and the F-box mutants MT-p45^{SKP2}(ΔF8) and (ΔF35) were tested for FT-p19^{SKP1} and CUL-1-HA binding (data not shown). Thus, p45^{SKP2}-CUL-1 interaction, although independent of the presence of cyclin A-CDK2 and p19^{SKP1}, is affected by mutations within the F-box.

Discussion

The data presented here show that human CUL-1 associates with the F-box protein p45^{SKP2}, p19^{SKP1} and cyclin A. These components assemble into a large multiprotein complex, the formation of which requires, at a minimum, an intact F-box motif and the p45^{SKP2}-bound cyclin A-CDK2. The molecular composition of this multiprotein complex, i.e. the presence of an F-box protein, a cullin and a human homolog of yeast SKP1, p19^{SKP1}, closely resembles a recently described budding yeast E3 ubiquitin protein ligase complex required, in combination with ubiquitin-conjugating enzyme CDC34, for the ubiquitination of the CDK inhibitor SIC1 (see Figure 8). Due to the specificity mediated by the F-box protein, this new type of E3 complex was designated SCF^{CDC4}, for SKP1/CDC53(cullin)/F-box protein complex (Feldman *et al.*, 1997; Skowyra *et al.*, 1997). We propose that the requirement for p45^{SKP2} function for the onset of S phase in mammalian cells reflects, in analogy to budding yeast, a requirement for ubiquitination and degradation of selected S phase regulatory proteins, facilitated by this potential p45^{SKP2}-directed E3 ubiquitin protein ligase complex. In support of this view, we found that human ubiquitin-conjugating enzyme CDC34, a functional homolog of budding yeast CDC34, co-immunoprecipitated with p45^{SKP2}. We hypothesize that all eukaryotic cells engage a common set of components, key elements of which are SCF-type E3s and CDC34, to trigger selective ubiquitination of G₁ and S phase regulatory proteins, thereby promoting entry into and progression through S phase.

Several experimental points support the proposal that p45^{SKP2} is a component of a potential human SCF-type E3 ligase. First, two yeast F-box proteins, CDC4 and GRR1, were shown to complex independently with CDC53 and SKP1 and function as specific SIC1 and CLN receptors, respectively (Skowyra *et al.*, 1997). In fact, purified CDC4-CDC53-SKP1 complexes mixed together with CDC34 and an E1 were found to be necessary and sufficient for the multi-ubiquitination of SIC1 *in vitro*. We found that p45^{SKP2} also interacts with a member of the cullin family, CUL-1, *in vivo*. Association was established by direct co-immunoprecipitation *in vitro* and *in vivo*

Fig. 8. A schematic drawing comparing the constituents of the CDC34–SCF^{CDC4} complex from *S.cerevisiae* with the p45^{SKP2}–p19^{SKP1}–CUL-1 complex in human cells (this report). Each complex is found in association with the ubiquitin-conjugating enzyme CDC34. In analogy to budding yeast, the p19^{SKP1}–CUL-1–p45^{SKP2} complex may function as an SCF to recruit specific substrates for ubiquitination by CDC34. The potential SCF^{p45SKP2} also contains an associated cyclin A–CDK2. The architecture of these complexes is hypothetical since the physical interactions among the components have yet to be defined in detail (for further details, see Discussion).

(Figures 2 and 3). In addition, p45^{SKP2} was capable of relocating CUL-1 to the nucleus when both proteins were co-synthesized (Figure 2F). Although the exact function(s) of cullins remain to be determined, the yeast homolog of CUL-1, CDC53, has been clearly implicated in the degradation of critical cell cycle regulatory proteins. Genetic experiments suggest that CDC53 is essential for the degradation of SIC1 and CLN2 and functions in concert with CDC34 (Schwob *et al.*, 1994; Mathias *et al.*, 1996; Willems *et al.*, 1996). It forms specific complexes with CDC4, SKP1 and CDC34 and is a required component for multi-ubiquitination of SIC1 in an *in vitro* system (Feldman *et al.*, 1997; Skowyra *et al.*, 1997).

Second, p19^{SKP1}, another constituent of the cyclin A–CDK2–p45^{SKP2} complex (Zhang *et al.*, 1995), is a human homolog of SKP1, which is required for ubiquitination and degradation of multiple cell cycle regulatory proteins in budding yeast (Bai *et al.*, 1996). We present evidence that p19^{SKP1} also binds CUL-1 *in vivo*, and together they can exist with p45^{SKP2} in a single complex (Figure 3). These protein–protein interactions are, therefore, not mutually exclusive and are consistent with the notion that p45^{SKP2}, CUL-1 and p19^{SKP1} form a functional entity. The simplest interpretation of this evidence is that the p45^{SKP2}–CUL-1–p19^{SKP1} complex may be a vertebrate representative of an SCF-type E3 ubiquitin ligase complex.

Third, in *Schizosaccharomyces pombe*, the F-box protein Pop1, which is structurally related to CDC4, has been shown to be required for ubiquitin-mediated proteolysis of Rum1 and Cdc18, based on the lack of polyubiquitinated forms of these proteins in *pop1* mutants. Moreover, Pop1 formed complexes with Cdc18 (Kominami and Toda, 1997).

Finally, the human homolog of ubiquitin-conjugating enzyme CDC34 co-immunoprecipitated with p45^{SKP2} when co-expressed in human U2-OS cells. No such co-immunoprecipitation was seen when UBC9 substituted for CDC34 in this assay, arguing that the observed interaction is specific (Figure 4A). This finding parallels known functional interactions between CDC34 and SCF E3 ubiquitin protein ligases in budding yeast (Mathias *et al.*, 1996; Willems *et al.*, 1996; Feldman *et al.*, 1997; Skowyra *et al.*, 1997).

This combined evidence places p45^{SKP2} (in complex with CUL-1 and p19^{SKP1}) on a putative CDC34-dependent ubiquitin conjugation pathway in humans. Given that p45^{SKP2} function is required for the onset of S phase, one wonders whether the targets of this pathway include proteins whose degradation is required for execution of S phase.

In mammalian cells, several proteins involved in cell cycle regulation have been reported to be actively degraded at defined points during the cell cycle. Cyclin D1 (Diehl *et al.*, 1997), cyclin E (Clurman *et al.*, 1996; Won and Reed, 1996), members of the Kip/Cip family of CDK inhibitors [i.e. p27^{KIP1} (Pagano *et al.*, 1995; Sheaff *et al.*, 1997; Vlach *et al.*, 1997) and p21^{WAF1} (Maki and Howley, 1997)], E2F transcription factors (Hateboer *et al.*, 1996; Hofmann *et al.*, 1996; Campanero and Flemington, 1997) and p53 (Scheffner *et al.*, 1993) have been shown to be degraded actively by the ubiquitin–proteasome pathway. In addition, p27 ubiquitination was found to be stimulated by human CDC34 *in vitro* (Pagano *et al.*, 1995) and was degraded specifically in whole cell extracts derived from S phase cells (Brandeis and Hunt, 1996). It is unknown at present what the relevant E3 ligases are for each of these established ubiquitin–proteasome targets. Whether ubiquitination of any of the aforementioned proteins involves p45^{SKP2} now becomes relevant.

Using highly synchronized cell populations, we were able to demonstrate that p45^{SKP2} protein expression is cell cycle regulated, peaking during S phase and decaying as cells progress through M phase. The observed expression profile mirrors that of cyclin A (Pines and Hunter, 1990) and is entirely consistent with previous data suggesting a requirement for p45^{SKP2} function in S phase. The increase in p45^{SKP2} protein abundance at G₁/S may be related directly to the observed increase in p45^{SKP2} mRNA around that time (Zhang *et al.*, 1995). p45^{SKP2} protein abundance drops off during M phase progression, around the same time as does cyclin A protein, but whether p45^{SKP2} is, like cyclin A, actively ubiquitinated during M phase is unclear at present (Glotzer *et al.*, 1991). It is certainly a possibility, particularly because the proteasome inhibitor LLnL is able to rescue the drop off of p45^{SKP2} protein levels in M phase (A.Marti and W.Krek, unpublished).

Notably, p45^{SKP2} does not contain any recognizable destruction box motif, as do cyclin A and B (Glutzer *et al.*, 1991).

Neither p19^{SKP1} nor CUL-1 protein expression is periodic. In fact, both species were detected readily even in serum-deprived cells. Thus, the timely formation of a p45^{SKP2}-p19^{SKP1}-CUL-1 complex is determined, at least in part, by p45^{SKP2}'s own cell cycle-dependent expression. If indeed certain F-box proteins serve a substrate recognition/ubiquitination function as proposed here for p45^{SKP2}, it is tempting to speculate that cell cycle-dependent synthesis of selected F-box proteins may contribute to control the timing of their action as components of distinct SCF-type E3s. If this were the case, it would be reminiscent of the sequential activation of distinct cyclin-dependent protein kinases by the time-dependent synthesis of their cognate cyclin subunits.

Another level of cell cycle regulation of multiprotein complex formation may be provided by the p45^{SKP2}-bound cyclin A-CDK2. Several mutants of p45^{SKP2}, including deletion and point mutants defective in cyclin A-CDK2 binding, also failed to bind p19^{SKP1}, suggesting a model in which one function of the p45^{SKP2}-bound cyclin A-CDK2 is to recruit p19^{SKP1} to the F-box protein p45^{SKP2}. Such a model would be consistent with the observed concerted appearance of p45^{SKP2} and p19^{SKP1} in cyclin A-CDK2 complexes (Zhang *et al.*, 1995). A potential requirement for a cyclin-CDK function in SKP1-CDC4 complex formation has not been observed. This interaction appears to be only dependent on an intact F-box (Bai *et al.*, 1996). Interestingly however, recent evidence suggests that GRR1-SKP1 interaction also requires, in addition to the F-box, certain C-terminal sequences of GRR1 (Li and Johnston, 1997). Whether this requirement is linked to a stably bound kinase, as is the case for p45^{SKP2}-cyclin A-CDK2 interaction, is not known at present. Consistent with the notion that the F-box of p45^{SKP2} is important for p19^{SKP1} binding is the fact that p45^{SKP2} mutants lacking highly conserved residues within the F-box failed to bind p19^{SKP1}. Whether interdependency between F-box protein-p19^{SKP1} complex formation and cyclin-CDK function also applies to other F-box proteins remains to be determined. If p45^{SKP2} indeed functioned to drive S phase-specific protein destruction, then cyclin A-CDK2-triggered multiprotein complex formation would ensure interdependency and tight coordination of the activities of the cell cycle clock and those of a potential ubiquitin conjugation pathway.

The significance of a cyclin A-CDK2-triggered recruitment of p19^{SKP1} into a p45^{SKP2}-CUL-1 complex can only be speculated upon at present. One outcome could be stimulation of F-box-substrate interaction. However, other possibilities exist. For example, p19^{SKP1} could also function to facilitate ubiquitin transfer from the E2 to the substrate. The identification of physiological substrates of this potential E3 ligase complex will eventually allow one to test such possibilities.

Interestingly, the same F-box mutants of p45^{SKP2} that were defective in p19^{SKP1} binding were also defective in CUL-1 interaction. Binding of the latter did not require a p45^{SKP2}-bound cyclin A-CDK2, suggesting that the cyclin A-CDK2 binding-defective mutants studied here are not impaired in all aspects of protein-protein interaction.

Whether the F-box domain of p45^{SKP2} is necessary and sufficient for CUL-1 binding is not known. Notably, however, p19^{SKP1} is also not required for CUL-1 recruitment (Figure 7B). As a result, one could speculate on the possibility that at least one function of the F-box may be to provide a structural foundation for multiprotein complex assembly, a possible prerequisite for proper functioning of this class of E3 ligases.

In many transformed cells, a cyclin A-CDK2-p45^{SKP2}-p19^{SKP1} complex replaces the quaternary cyclin A-CDK2-p21^{WAF1}-PCNA complex found in normal cells (Zhang *et al.*, 1995). The reported subunit rearrangement affecting cyclin A-CDK2 complexes may be related to the fact that p45^{SKP2} abundance is greatly increased in these transformed cells (Zhang *et al.*, 1995). One possible consequence would be direct competition between p45^{SKP2} and p21^{WAF1} for cyclin A-CDK2 binding. Taken at face value, one could argue that the interaction of p45^{SKP2} and p21^{WAF1} with cyclin A-CDK2 is mutually exclusive. In this regard, p21^{WAF1} contains a copy of the 'RXL' motif, implicated as a cyclin-CDK recognition determinant (Adams *et al.*, 1996). The set of residues in p45^{SKP2} which we found necessary for stable cyclin A-CDK2 binding also contain such a motif. In fact, 24mer peptides corresponding to the 'RXL' motif of p27, p21 or E2F-1 compete efficiently with p45^{SKP2} for cyclin A-CDK2 binding *in vitro* (W.Krek, unpublished). This raises the possibility that p45^{SKP2} may occupy the same surface on cyclin A as does p21^{WAF1}. Mutual exclusiveness for cyclin-CDK binding has already been observed in the case of the 'RXL' motif-containing proteins p21^{WAF1} and p107. In fact, it was observed that the ratios of p21^{WAF1}-cyclin A-CDK2 complexes to p107-cyclin A-CDK2 complexes can change in response to DNA-damaging agents (Zhu *et al.*, 1995). Given this, it is tempting to speculate that increased abundance of p45^{SKP2} in certain transformed cells may interfere with efficient inhibition of cyclin A-CDK2 complexes by CKIs in response to S phase inhibitory signals, leading to execution of DNA replication under inappropriate conditions.

Previous reports linked cyclin A-CDK2 function to the regulation of specific transcriptional events (Dynlacht *et al.*, 1994; Krek *et al.*, 1994, 1995; Xu *et al.*, 1994; Zhu *et al.*, 1995). The data shown here point to a possible role for this enzyme in promoting S phase entry via a potential p45^{SKP2}-directed E3 ligase. The identification of physiological targets for this cyclin A-CDK2-linked effector pathway will provide a better understanding of the role of cyclin A-CDK2 in coordinating S phase.

Materials and methods

Plasmid constructions

To generate pcDNA3-MT-p45^{SKP2}(wt), pGST-p45^{SKP2} (Zhang *et al.*, 1995) was digested with *NcoI*-*ScaI* and the resulting 1.4 kb fragment was treated with Klenow polymerase to generate blunt ends. pcDNA3-MT-E2F-1(wt) was digested with *Bam*HI-*EcoRV* and the vector fragment harboring the myc tag was isolated, treated with Klenow polymerase and calf intestinal phosphatase (CIP), and combined with the full-length 1.4 kb p45^{SKP2} cDNA fragment. The myc epitope, 5'AGCTTACCATGGAGCAGAAGCTGATCTCCGAGGAGGACCTGAACATGG3', was cloned into the *Hind*III-*Bam*HI site of pcDNA3 (Invitrogen). To generate pcDNA3-NT-p45^{SKP2}(wt), full-length p45^{SKP2} cDNA was excised from pcDNA3-MT-p45^{SKP2}(wt) by partial *Bam*HI-*XhoI* digestion and cloned into pcDNA3 previously digested with *Bam*HI-*XhoI*. To generate

pcDNA3-p45^{SKP2}(wt) (untagged version), pcDNA3-MT-p45^{SKP2}(wt) was digested with *SmaI*-*XhoI* and the resulting fragment was subcloned into the *EcoRV*-*XhoI* sites of pcDNA3. The same fragment was also subcloned into the *EcoRV*-*XhoI* sites of pSP72 (Promega) to generate pSP72-p45^{SKP2}(wt).

pcDNA3-MT-p45^{SKP2}(Kpn) was generated by replacing a *KpnI*-*XhoI* fragment from pcDNA3-MT-p45^{SKP2}(wt) with a double-stranded (ds) oligonucleotide with the sequence 5'CTGAAGTATTTATC3' encoding a stop codon flanked by a *KpnI* and an *XhoI* site. pcDNA3-MT-p45^{SKP2}(Xba) was generated by replacing an *XbaI*-*XhoI* fragment from pcDNA3-MT-p45^{SKP2}(wt) with a ds oligonucleotide (5'CTAGACTGAA-GTATTC3') encoding a stop codon flanked by an *XbaI* and an *XhoI* site. pcDNA3-MT-p45^{SKP2}(Δ LRR), an in-frame deletion of the LRR, was generated by first replacing a *BamHI*-*KpnI* fragment from pSP72-p45^{SKP2}(wt) with a ds oligonucleotide (5'GATCCAGATCTCTCTGTG-TAC3') containing a *BglII* site flanked by *BamHI* and *KpnI* sites, generating pSP72-p45^{SKP2}(Δ LRR). From this plasmid, a *BspEI*-*XhoI* fragment was isolated and subcloned into the *BspEI*-*XhoI* sites of pcDNA3-MT-p45^{SKP2}(wt) to generate pcDNA3-MT-p45^{SKP2}(Δ LRR).

A two-step PCR method was used to generate F-box and cyclin A-CDK2 binding-defective mutants of p45^{SKP2}. The template used was pcDNA3-MT-p45^{SKP2}(wt). PCR was first performed separately with either an upstream primer and an antisense oligonucleotide containing the appropriate mutation or with a downstream primer and the sense oligonucleotide containing the appropriate mutation. The two PCR products purified from an agarose gel were then combined with the upstream and downstream primers and further amplified. The final PCR products were digested with the relevant restriction endonucleases and the resulting fragments used to replace the corresponding wild-type fragment in pcDNA3-MT-p45^{SKP2}(wt). The primers used to generate specific p45^{SKP2} mutants are the following: pcDNA3-MT-p45^{SKP2}(Δ F6), 5'TCTGAAGTCTGCTCAGGC3'/5'ACAGGAAAAATCCGGAAGA-GAGTCCCA3'/5'CTTCCGGATTTTCTGTCTGTGCTC3'/5'ATTGACAATGGGATCCGA3'; pcDNA3-MT-p45^{SKP2}(Δ F8), 5'TCTGAAGTCTGTGCTCAGGC3'/5'CAGGAGGCACAGCTCATCCGGAAG-3'/5'GGATGAGCTGTGCTCCCTGAGCTG3'/5'ATTGACAATGGGATCCGA3'; pcDNA3-MT-p45^{SKP2}(Δ F35), 5'TCTGAAGTCTGTGCTCAGGC3'/5'CCGGATCAGACCTTAGACC3'/5'TCTAAGGTCTGAT-3'/5'ATTGACAATGGGATCCGA3'; pcDNA3-MT-p45^{SKP2}(Δ xA), 5'G-AACCTCTCCTGGTGTGTT3'/5'CAAAAGTGTGCTGTGCTGTAG-TGTGGGAATTTCT3'/5'TCCACACTAGCAACAGCACAAAGTTT-TTGAATC3'/5'CTTTATCCGTCCTTCGG3'; pcDNA3-MT-p45^{SKP2}(Δ A3), 5'GAACCTCTCCTGGTGTGTT3'/5'TCCACACTACAAGTT-TTGAATC3'/5'CAAAAGTGTGCTGTGTTGGAATTTCT3'/5'CTT-TTATCCGTCCTTCGG3'. All PCR-derived regions in the newly generated mutants were confirmed by direct sequencing.

To generate a mammalian expression vector encoding full-length p19^{SKP1}, namely pcDNA3-p19^{SKP1}, a partial *EcoRI*-*XhoI* fragment was isolated from pGST-p19^{SKP1} (Zhang *et al.*, 1995) and subcloned into the *EcoRI*-*XhoI* sites of pcDNA3. An amino-terminal flag epitope-tagged (FT) p19^{SKP1} expression vector was generated by isolating a *HindIII*-*XhoI* fragment of pcDNA3-p19^{SKP1} and subcloning it into the *HindIII*-*XhoI* sites of pFLAG-MAC (Invitrogen). To generate pcDNA3-FT-p19^{SKP1}, a partial *EcoRV*-*XhoI* fragment was isolated from pFLAG-MAC-p19^{SKP1} and subcloned into the *EcoRV*-*XhoI* sites of pcDNA3.

Amino-terminal HA-tagged mammalian expression constructs of human UBC9 and CDC34 were generated by one-step PCR using *Pfu* polymerase. The UBC9 and CDC34 amplification products were subcloned into pcDNA3 containing a previously subcloned HA tag-encoding sequence located between the *HindIII*-*BamHI* sites (Krek *et al.*, 1993).

To generate pcDNA3-CUL-1, the human CUL-1 clone (Kipreos *et al.*, 1996) was digested with *EspI*-*DraI*, treated with Klenow polymerase to generate blunt ends, and subcloned into the pBlueBacHis (Invitrogen) vector that had been digested with *XhoI* and treated with Klenow polymerase and CIP. From the resulting plasmid, an *XhoI*-*SalI* fragment was isolated and used to replace the carboxy-terminal *XhoI* fragment of pcDNA3-CUL-1-HA (Pause *et al.*, 1997), thereby replacing the HA tag with the original C-terminus of CUL-1. pGFP-CUL-1-HA was constructed by subcloning a *BamHI*-*NoI* fragment into the same sites of pcDNA3 containing a previously subcloned GFP-coding sequence located between the *HindIII*-*BamHI* sites.

To generate pcDNA3-HA-cyclin A, a *BamHI*-*EcoRI* fragment isolated from a pGST-cyclin A plasmid was subcloned into the same sites of pcDNA3 harboring an HA-tag between the *HindIII*-*BamHI* sites.

To generate baculovirus expression vectors for p45^{SKP2}, CUL1 and CUL2, the corresponding *BamHI*-*XhoI* fragments of pcDNA3-NT-

p45^{SKP2}, pcDNA3-CUL-1-HA and pcDNA3-CUL-2-HA were isolated and subcloned into the same site of pBlueBacHis2A (Invitrogen).

Preparation and affinity purification of rabbit polyclonal antibodies against p45^{SKP2}, p19^{SKP1}, CUL-1 and cyclin A

Polyclonal rabbit serum to full-length human p45^{SKP2}, anti-(fl)p45^{SKP2}, was raised against a GST-p45^{SKP2} fusion protein purified from bacteria (Kaelin *et al.*, 1991). To raise anti-cyclin A antibody, full-length human cyclin A was subcloned into the T7 phage expression vector pET28a and introduced into *E. coli* strain DH5 α . Overexpression of cyclin A in *E. coli* and subsequent purification from bacterial lysates was done as previously described (Krek *et al.*, 1992). Purified fusion proteins were used to immunize rabbits.

For the production of peptide antibodies recognizing cloned human p45^{SKP2} (anti-NT), the peptide MHVFKTPGPADAMHRKHLQE, corresponding to the first 18 amino acids of the predicted N-terminus of human p45^{SKP2}, was synthesized. For anti-(C)p19^{SKP1} antibody production, the peptide DFTEEEEAQVRKENQWCEEK, corresponding to the carboxy-terminal region of human p19^{SKP1}, was synthesized and injected into rabbits. Likewise, anti-(C)CUL-1 peptide antibodies were raised against the synthetic peptide EKEYLERVDGEKDTYSYLA, corresponding to the carboxy-terminal region of human CUL-1. Each peptide was coupled to keyhole limpet hemocyanin (Pierce) by glutaraldehyde coupling (Harlow and Lane, 1988) and injected into rabbits.

Polyclonal rabbit serum to p45^{SKP2} was affinity purified by incubation with first a GST affinity column, followed by a GST-p45^{SKP2} fusion protein affinity column, previously prepared by covalently cross-linking the respective proteins to glutathione-Sepharose with dimethylpimelidate (Harlow and Lane, 1988). Anti-peptide antibodies were affinity purified by coupling 10 mg of synthetic peptide to 1 g of CH-Sepharose 4B (Pharmacia) according to the manufacturer's protocol. Incubation and elution of antibodies were carried out as described (Krek *et al.*, 1992).

Cell culture, transfection and cell cycle analysis

Human U2-OS, T98G, WI-38, MRC-5 cells and human diploid fibroblasts (HDF) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Gibco). NIH-3T3 cells were grown in DMEM supplemented with 5% bovine calf serum (Hyclone). Insect Sf9 cells were maintained in Grace's medium containing 10% heat-inactivated FCS (Summers and Smith, 1987) and infected with the indicated recombinant baculoviruses as previously described (Desai *et al.*, 1992).

Human T98G glioblastoma cells (Stein, 1979) or HDFs were growth arrested by incubation of cells for ~72 h in DMEM without serum. Cells were serum stimulated by addition of FCS to a final concentration of 20%.

Human U2-OS cells were transiently transfected at 50% confluency using the CaCl₂ method as previously described (Krek *et al.*, 1993) with the indicated amounts of plasmid DNA and harvested ~24 h after removal of the precipitate.

For cell cycle analysis, T98G cells or HDFs were harvested by trypsinization and processed for flow cytometric analysis as described previously (Krek and Nigg, 1991a).

Metabolic labeling and immunochemical techniques

In vivo labeling of proteins with [³⁵S]methionine (Amersham) was performed as described (Krek and Nigg, 1991b).

For immunoprecipitation, U2-OS cells from a 10 cm dish were washed twice in ice-cold phosphate-buffered saline (PBS) and immediately lysed for 30 min on ice in 800 μ l of TNN buffer [50 mM Tris (pH 7.5), 250 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF, 0.2 mM Na₃VO₄, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μ g/ml aprotinin]. Extracts were centrifuged at 4°C for 15 min at top speed in an Eppendorf microfuge and supernatants pre-incubated for 30 min at 4°C with 25 μ l of a 50% (w/w) slurry of protein A-Sepharose (Pharmacia), followed by centrifugation. Where appropriate, aliquots of the supernatants were removed for protein quantification before primary antibodies were added to the lysates. Affinity-purified primary antibodies were used at the following concentrations: 3 μ g/ml of anti-NT, 2 μ g/ml of anti-(fl)p45^{SKP2}, 7 μ g/ml of anti-(C)p19^{SKP1}, 1.25 μ g/ml of anti-cyclin B1 (Santa Cruz) antibodies, 2 μ l of anti-cyclin A antiserum, 50 μ l of tissue culture supernatant of 12CA5 (anti-HA mAb), 1.25 μ g/ml of anti-mouse IgG and 3.5 μ g/ml of M5 (anti-Flag mAb; IBI). After incubation on ice for 60 min with the appropriate antibodies, 50 μ l of protein A-Sepharose was added and incubation was continued for another 60–180 min. Alternatively, immunoprecipitation was performed with anti-HA mAb 12CA5, cross-linked to protein A-Sepharose in the presence of 20 mM dimethylpimeli-

midate (Pierce). Immune complexes were collected by centrifugation and washed four times in TNN buffer. For secondary immunoprecipitations, washed immunobeads were treated for 15 min on ice with 15 µg of Flag-peptide (IBI), followed by addition of 500 µl of TNN buffer. Samples were then incubated further with affinity-purified antibodies, and immune complexes were isolated as described above. Pellets were resuspended in 50 µl of SDS sample buffer and boiled for 5 min. Solubilized proteins were analyzed on SDS-polyacrylamide gels and detected either by autoradiography or fluorography.

Electrophoretic transfer of proteins to nitrocellulose membranes (Optitrans; Schleicher and Schuell) was carried out for 6–8 h at 450 mA, 4°C, using 1× blotting buffer [50 mM Tris (pH 7.5), 380 mM glycine, 0.1% SDS, 20% methanol]. Nitrocellulose membranes were blocked with 5% non-fat dry milk in TBST [10 mM Tris (pH 8.0), 33 mM NaCl, 0.1% Tween-20] for 3 h at room temperature. Membranes were then incubated overnight at 4°C or for 3–4 h at room temperature with either anti-HA mAb HA 11 (Babco) (1:3000 dilution of 1.0 µg/µl), anti-myc mAb 9E10 (1:50 dilution of tissue culture supernatant), or affinity-purified anti-NT (1:500 dilution of 0.37 µg/µl), anti-(fl)p45^{SKP2} (1:500 dilution of 0.23 µg/µl), anti-(C)p19^{SKP1} (1:500 dilution of 0.8 µg/µl), anti-(C)CUL-1 (1:500 dilution of 0.42 µg/µl), anti-tubulin mAb Tu27b (1:10 dilution of tissue culture supernatant) antibodies, or anti-cyclin A antiserum (1:4000 dilution). Membranes were washed three times for 20 min with TBST and incubated for 1 h in 5% non-fat dry milk in TBST containing peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG (Amersham) at a 1:3000 dilution. Signals were detected by ECL (Amersham).

Immunofluorescence analysis

Cells were washed with PBS, fixed for 5 min in 3% paraformaldehyde/2% sucrose in PBS, rinsed quickly twice with PBS, incubated for another 5 min with 0.5% Triton X-100 in PBS and washed again quickly twice with PBS. Affinity-purified anti-NT antibodies were used at 2 µg/ml. Tissue culture supernatants of mouse mAb 9E10 (anti-myc) or mouse mAb 12CA5 (anti-HA) were used undiluted. Anti-GFP antibodies (Clontech) were used at 1:500 dilution. Incubations of primary antibodies were carried out for 60–90 min at room temperature in a humidified atmosphere. After two quick and three 5 min washes in PBS, secondary antibodies (diluted 1:100) were applied together with 4',6'-diamidino-2-phenylindole (DAPI) stain diluted 1:1000 from a 10 mg/ml stock solution. All dilutions were done in 5% FCS in PBS. Secondary reagents used were affinity-purified Texas red-conjugated goat anti-rabbit IgG (Amersham) and fluorescein-conjugated goat anti-mouse IgG (Amersham). After 20 min incubation with the secondary reagents, cells were washed as described above, mounted in Mowiol (Gibco) and viewed with a Zeiss fluorescence microscope using a 40× oil immersion objective.

Miscellaneous techniques

Protein concentrations were determined using the BioRad protein determination assay. *In vitro* kinase reactions were carried out as described by Krek and Nigg (1991a). The procedure for preparing whole cell extracts has been described (Krek *et al.*, 1993). Recombinant baculoviruses were generated using the Bac-N-Blue Transfection Kit (Invitrogen), following the instructions of the manufacturer. *In vitro* mixing reactions using insect Sf9 cell extracts expressing His₆-NT-p45^{SKP2}, His₆-CUL-1-HA or His₆-CUL-2-HA were performed at 4°C for 60 min in 500 µl mixtures of TNN buffer. Reaction mixtures subsequently were processed for immunoprecipitation with the indicated antibody.

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