Overproduction of Polypeptides Corresponding to the Amino Terminus of the F-Box Proteins Cdc4p and Met30p Inhibits Ubiquitin Ligase Activities of Their SCF Complexes

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Ubiquitin ligases direct the transfer of ubiquitin onto substrate proteins and thus target the substrate for proteasome-dependent degradation. SCF complexes are a family of ubiquitin ligases composed of a common core of components and a variable component called an F-box protein that defines substrate specificity. Distinct SCF complexes, defined by a particular F-box protein, target different substrate proteins for degradation. Although a few have been identified to be involved in important biological pathways, such as the cell division cycle and coordinating cellular responses to changes in environmental conditions, the role of the overwhelming majority of F-box proteins is not clear. Creating inhibitors that will block the in vivo activities of specific SCF ubiquitin ligases may provide identification of substrates of these uncharacterized F-box proteins. Using Saccharomyces cerevisiae as a model system, we demonstrate that overproduction of polypeptides corresponding to the amino terminus of the F-box proteins Cdc4p and Met30p results in specific inhibition of their SCF complexes. Analyses of mutant amino-terminal alleles demonstrate that the interaction of these polypeptides with their full-length counterparts is an important step in the inhibitory process. These results suggest a common means to inhibit specific SCF complexes in vivo.

The ubiquitin (Ub)-proteasome proteolytic pathway regulates the abundance of polypeptides involved in a variety of processes such as the cell division cycle, immune response, and developmental pathways (for reviews see references 5, 12, and 30). With a wide range of proteins being targeted for degradation by the proteasome, the cell overcomes the problem of specificity through a hierarchical order of Ub-conjugating machinery. Free Ub is activated at the expense of ATP by a single Ub-activating enzyme, or E1. Activated Ub is then transferred to a member of a family of Ub-conjugating enzymes, or E2s. Eleven E2s are present in the budding yeast Saccharomyces cerevisiae, and likely more are present in multicellular eukaryotes. The association of E2 with a Ub ligase, or E3, provides a docking site for an E2 enzyme, and in yeast, the latter components, namely, Cdc53p (or cullin in higher eukaryotes), Skp1p, and Hrt1p (alternatively named Rbx1p or Roc1p). The latter provides a docking site for an E2 enzyme, and in yeast, the major E2 associated with SCF complexes is Cdc34p. These proteins are linked to a fourth variable protein component, called the F-box protein, which is responsible for substrate recognition. Recent crystallographic analyses have confirmed a wealth of genetic and biochemical evidence by demonstrating that the ~40-amino-acid F-box motif links the F-box protein with the common SCF components by binding Skp1p (35, 49). Components from yeast and human SCF complexes produced in insect cells have been demonstrated previously to possess Ub ligase activities (10, 16, 37, 43). Thus, SCF complexes define a family of E3 ligases that may be distinguished by a particular F-box protein, which in turn determines substrate specificity for that complex.

Recently, database analyses and two-hybrid screens have demonstrated the extensive nature of the F-box family of proteins in different eukaryotes: for example, 17 F-box proteins are present in budding yeast, 36 are present in humans, and 326 are present in the nematode Caenorhabditis elegans (4, 17, 31, 42, 44). Each F-box protein has the potential to form an SCF ligase and target specific proteins for degradation. Although the functions of a few F-box proteins have been determined, understanding the role of the remainder represents a significant challenge, especially in non-genetically tractable systems.

Some of the most extensive analysis on SCF complexes has been performed on those present in the yeast S. cerevisiae. Yeast contains multiple F-box proteins including Cdc4p, Met30p, and Grr1p, which have been demonstrated elsewhere to form SCF complexes and are referred to as SCF\textsuperscript{Cdc4p}, SCF\textsuperscript{Met30p}, and SCF\textsuperscript{Grr1p}, respectively (1, 21, 23, 28). These complexes may recognize multiple substrates and play key roles in controlling the abundance of cell division cycle regulators and transcription factors that coordinate cellular responses to environmental changes. Among the SCF\textsuperscript{Cdc4p} substrates is the cyclin-dependent kinase inhibitory protein Sic1p, and cdc4 mutants, which fail to degrade Sic1p, arrest at the...
G1/S boundary (1, 36). A single SCF<sub>Met30</sub> substrate has been defined, Met4p, which regulates methionine biosynthetic gene (<i>MET</i>) expression (33). However, at present, it is not clear whether polyubiquitin chain formation on Met4p is a signal for that protein’s degradation or regulates its activity by modulating its ability to bind other cofactors involved in <i>MET</i> gene transcription (15).

Here we report our analysis to identify inhibitors of specific SCF complexes. Using the SCF complexes present in yeast as a model, we sought to identify F-box fragments that act in a dominant-negative fashion when overproduced. We identified polypeptides corresponding to the amino-terminal domains of Cdc4p and Met30p that specifically inhibit their SCF complexes when overproduced. This dominant-negative activity is due, in part, to the physical association of the amino-terminal fragment with its full-length counterpart. Because the dominant-negative alleles correspond to the same region of an F-box protein and because SCF complex architecture is conserved from yeast to humans, we propose that overproducing the amino terminus of an F-box protein is a universal means to inhibit SCF complexes in other organisms.

**MATERIALS AND METHODS**

**Yeast strains and growth conditions.** The yeast strains used in this study are Y382 (MATa ade2 ade3 ura3 leu2-2 trp1-112 his3-119) (25), PY72 [MATa met4-1 (18myc::TRPI-L:LEU2 bar1Δ pep5::URA3)] (15), and NNMYCMET4 [MET4-(18myc)::LEU2 pep5::URA3], a modificative product of Y382 and PY72. Standard rich (yeast-peptone-dextrose) and defined minimal (Saccharomyces cerevisiae) media were prepared as described previously (32). Transformations were carried out as described previously (9). For plasmid selection, yeast cells were grown on defined minimal medium supplemented with the appropriate amino acids. For galactose induction, cells were first grown to early logarithmic phase in minimal medium containing sucrose instead of dextrose, then galactose-containing medium (2%) was added, and the culture was incubated for a further 3 h. Dominant-negative activity of glutathione S-transferase (GST) fusions was assayed when cells containing the pEG(KG) plasmid, which bears the <i>URA3</i> and <i>Leu2</i>-<i>d</i> markers, were plated onto medium lacking leucine. Strains containing a temperature-sensitive mutation were grown at the permissive temperature (23°C) and were incubated further under nonpermissive conditions (37°C). For two-hybrid interactions in P360-4A cells, patches derived from single colonies were replica plated onto medium lacking adenine.

**Plasmid constructions.** <i>Escherichia coli</i> cod DH5α was used to propagate plasmids. Plasmid manipulations used standard protocols (34). The vector pEG(KG) was used for expression of GST fusion proteins (26) from the <i>GAL1</i> promoter. All <i>GST-CDC4</i> and <i>MET30</i> fusion constructs were created by cloning PCR-generated DNA fragments with plasmid-borne <i>CDC4</i> or <i>MET30</i> DNA as template (MT1567 <i>CDC4</i> TRPI CEN, kindly provided by Mike Tyers, and pP58 <i>MET30</i> TRPI CEN, kindly provided by Steve Reed) (28) as previously described (22). GST-CDC4 and GST-MET30 fusions were created as follows. Primers annealing at the 5’ end of <i>CDC4</i> and <i>MET30</i> were designed to incorporate an XhoI restriction site, and primers annealing toward the 3’ end of these genes were designed to incorporate a SalI restriction site. The PCR products were cleaved with the appropriate restriction enzymes and ligated into pEG(KG), which had been restricted previously with the appropriate enzymes. Table 1 lists the primers used in this study. Table 2 lists the GST fusion constructs derived from using different sets of primers that are numbered to indicate the amino acids from the complete F-box protein that are encoded. Full-length <i>CDC4</i> was subcloned as follows. A ClaI-XhoI fragment containing the majority of <i>CDC4</i> was excised from MT1567 and ligated into pRS306 cleaved with the same restriction enzymes. The resulting plasmid was called pRS306-CDC4(178-1987). The 5’ portion of <i>CDC4</i> was generated as a PCR fragment with primers 5-01 and 5-02. Primer 5-01 incorporated an XhoI restriction site. The PCR product was restricted with XhoI and Clal and ligated into pRS306-CDC4(178-1987) cleaved with the same enzymes to create pRS306-CDC4(1-878). The 3’ portion of <i>CDC4</i> was

**TABLE 1. Oligonucleotide primers used in this study**

| Primer     | Sequence (5’ to 3’) |
|------------|---------------------|
| 12-001     | AAATCTAGACATGGGCTGTCATC |
| 12-002     | AAGTTCGACCATGCAACAA |
| 12-004     | AAATCTAGACATGGGCTGTCATC |
| 12-005     | AAATCTAGACATGGGCTGTCATC |
| 12-007     | AAATCTAGACATGGGCTGTCATC |
| 12-008     | AAATCTAGACATGGGCTGTCATC |
| 12-009     | AAATCTAGACATGGGCTGTCATC |
| 5-01       | AAAGGATCCTTTGATATGTTG |
| 5-02       | AAAGGATCCTTTGATATGTTG |
| 5-03       | AAAGGATCCTTTGATATGTTG |
| 5-04       | AAAGGATCCTTTGATATGTTG |
| 10-001     | AAAGGATCCTTTGATATGTTG |
| 10-002     | AAAGGATCCTTTGATATGTTG |
| pHA-SIC1-1a| AAAGGATCCTTTGATATGTTG |
| pHA-SIC1-2B| AAAGGATCCTTTGATATGTTG |
| PHA-CLN2-1A| AAAGGATCCTTTGATATGTTG |
| PHA-CLN2-2B| AAAGGATCCTTTGATATGTTG |
| NM11-001   | AAAGGATCCTTTGATATGTTG |
| NM11-002   | AAAGGATCCTTTGATATGTTG |
| NM11-003   | AAAGGATCCTTTGATATGTTG |
| NM11-004   | AAAGGATCCTTTGATATGTTG |
| NM10-004   | AAAGGATCCTTTGATATGTTG |
| MET30-4B   | AAAGGATCCTTTGATATGTTG |
| MET30-6B   | AAAGGATCCTTTGATATGTTG |
| NM25-004   | AAAGGATCCTTTGATATGTTG |
| NM25-005   | AAAGGATCCTTTGATATGTTG |
| NM25-006   | AAAGGATCCTTTGATATGTTG |
| NM25-007   | AAAGGATCCTTTGATATGTTG |
| NM25-010   | AAAGGATCCTTTGATATGTTG |
generated as a PCR fragment with primers 5-03 and 5-04. Primer 5-03 incorporated an XbaI restriction enzyme site, and primer 5-04 incorporated a SalI restriction enzyme site. The PCR product was restricted with these enzymes and ligated into prS306-CD4 (1-1987) treated with the same enzymes to create prS306-CD4C. prS306-CD4C was restricted with BamHI and SalI and ligated into pEG(KG) restricted with the same enzymes to generate pGST-CD4. MET30 was subcloned into the two-hybrid vector pACTII, kindly provided by Steve Elledge. Primer 10-001 annealing at the 5’ end of MET30 was designed to incorporate an NcoI restriction site, and the 10-002 primer annealing toward the 3’ end of MET30 was designed to incorporate an XhoI restriction site. The PCR product was cleaved with the appropriate restriction enzymes and ligated into pACTII, which had been restricted previously with the appropriate enzymes. Other two-hybrid constructs were made in either pGAD or pGBD, kindly provided by Elizabeth Craig. SKPI and CDC4 DNA fragments were generated by resticking the appropriate pEG(KG) construct with BamHI and SalI and ligated into the two-hybrid vector restricted with the same enzymes. pGAD-CD4C (50-779) and pGAD-CD4C (75-779) were derived by ligating a fragment generated by KpnI and SalI from pGAD-CD4C into either pGAD (50-779) or pGAD (75-779) restricted with the same enzymes.

SIC1 and CLN2 were subcloned into pH314HA3, kindly provided by Steve Elledge. Primers pHA-SIC1-1a, which annealed to the 5’ end of SIC1, and pHA-SIC1-2b, which annealed to the 3’ end of SIC1, were designed to incorporate a BamHI and a SacI restriction site, respectively. The PCR product, with MDM20 as a template (kindly provided by Mike Mendenhall), was cleaved with the appropriate enzymes and ligated into pH314HA3, which had been treated with the same enzymes. Primers pHA-CLN2-1a, which annealed to the 5’ end of CLN2, and pHACLN2-2b, which annealed to the 3’ end of CLN2, were designed to incorporate a BamHI and a SalI restriction site, respectively. The PCR product, with MT291 as a template (kindly provided by Mike Tyers), was cleaved with the appropriate enzymes and ligated into pH314HA3, which had been treated with the same enzymes.

pRD54, generously provided by Ray Deshaies, bears HA-HRT1 under the control of the GAL1 promoter. A fragment containing GAL1 HA-HRT1 was generated from pRD54 with restriction enzymes KpnI-Sacl and ligated into pRS314 to create prS314HAHRT1. For the green fluorescent protein (GFP) fusions, a GFP vector was initially constructed. With plasmid-borne GFP DNA (p1265GFP generously provided by Lucy Robinson), a PCR fragment was generated with primers NM25-004 and NM25-005, which contained BanHI and EcoRI restriction sites. The PCR fragment was treated with appropriate enzymes and ligated into pH314HA3, that was similarly restricted. PCR-generated DNA encoding Met30 (1-640p) and Met30 (1-225p) was generated with primers NM25-006 and NM25-007 and primers NM25-006 and NM25-010, respectively. These DNA fragments were restricted with SpeI and SacI and ligated into pGFP treated with the same enzymes. pGFP/Cdc4 (1-278p) was generated by restricting pGST/Cdc4 (1-278p) with BamHI and SacI and ligating the appropriate fragment into pGFP. CDC53-containing plasmids have been previously described (20).

**FACS analysis and microscopy.** Cells were prepared for fluorescence-activated cell sorting (FACS) analysis as described previously (38). For differential interference contrast and fluorescence microscopy, cells were stained with propidium iodide as described for FACS analysis and visualized with an Olympus UPlanApo objective on a DAPI (4’,6-diamidino-2-phenylindole) filter cube. Images were recorded with a Photometrix CH250/A cooled charge-coupled device camera (KAF1400 Kodak chip) with Scannersite IPLab Spectrum (version 3.2) software (Fairfax, Va.). For GFP, DAPI, and tetramethyl rhodamine isocyanate analysis, cells were grown to mid-logarithmic phase, induced by the addition of galactose, and grown for a further 5 to 6 h. Cells from 500 μl of this culture were resuspended in 50 μl of phosphate-buffered saline containing 0.5 mg of DAPI (Sigma) per ml. After 1 min of exposure to DAPI, the cells were viewed with a DAPI filter. The GFP signal was visualized with an S65T filter. To verify background fluorescence, the cells were visualized with a tetramethyl rhodamine isocyanate filter.

**Screen for mutant CDC4 (1-278p) and MET30 (1-225p) alleles.** A library of mutant pEG-CDC4 (1-278p) and pEG-MET30 (1-225p) plasmids was generated by passing the respective plasmids through the XLI-RED bacterial strain, per the manufacturer’s instructions (Stratagene). The library was transformed into Y382, and colonies that grew in the presence of galactose were isolated. Western immunoblot analysis was performed to identify plasmids that encoded products that migrated to the same extent as did wild-type GST-Cdc4 (1-278p) and GST-Met30 (1-225p). Mutant alleles were finally isolated by subcloning either the CDC4 (1-278p) or MET30 (1-225p)-encoding DNA fragments into wild-type pEG(KG) vector and retesting dominant-negative activity by transforming fragments into Y382 and assaying them for growth in the presence of galactose.

**DNA sequencing.** DNA was prepared according to QiaGen-Epipendorf kit specifications, and sequencing was carried out by the Arizona State University sequencing facility.

**Protein preparation and Western immunoblot techniques.** Protein preparations were performed as described before (24). Anti-FLAG, anti-MYC, and anti-GST antibodies were purchased from Sigma. Immunoprecipitations were performed with anti-FLAG resin, also purchased from Sigma.

**Gel filtration chromatography.** An 0.5-mg quantity of protein lysate was applied to a column (column volume: 12 ml) packed with a Superose 12 column (Amersham Biosciences) that had been previously equilibrated in 150 mM NaCl–50 mM Tris-HCl (pH 7.5)–0.5 mM EDTA. Protein from 1-ml fractions was precipitated with 10% trichloroacetic acid. The precipitate was washed with acetone, resolubilized in 20 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer, and subjected to Western immunoblot analysis. The proteins used to calibrate the column were thyroglobulin (669 kDa), apoferritin (440 kDa), aldolase (158 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa).

**RESULTS**

**Overproduction of Cdc4 (1-278p) inhibits SCF-Cdc4p complex activity and acts in a dominant-negative fashion.** We wished to identify a common means to inhibit specific SCF complexes. We believed that these proteins could serve as a potential source of polypeptides that act to inhibit their specific SCF complexes. Previously, we have reported our analysis to determine functional domains in Cdc4p in which we created a series of GST fusions corresponding to different portions of that F-box protein (25). During this work we demonstrated that overproduction of GST-Cdc4 (1-278p) is toxic to cells and induces a terminal morphology similar to loss of CDC4 activity. To further map a potential dominant-negative region in Cdc4p, we created an additional series of GST fusions covering the Cdc4p amino terminus (Fig. 1A). Although overproduction of residues 1 to 200 and 25 to 278 generated a slow-growth phenotype (data not shown), none of the other fusions had an effect on cell viability. Therefore, we focused our analysis on the GST-Cdc4 (1-278p) fusion.

Because overproduction of the GST fusion may impact on multiple pathways, further studies were performed to test if GST-Cdc4 (1-278p) specifically inhibited the SCF-Cdc4p complex. Overproduction of GST-Cdc4 (1-278p) resulted in cells that contained a single nucleus and had arrested in the G1 phase of the cell cycle, similar to cells containing the cdc4-3 temperature-sensitive mutation incubated at the nonpermissive temperature (Fig. 1B and C). If the arrest induced by GST-Cdc4 (1-278p) is due to the inhibition of the SCF-Cdc4p complex.
FIG. 1. Overproduction of GST-Cdc4(1-278)p inhibits SCF[Cdc4p] complex activity. (A) Schematic diagram of Cdc4p, illustrating the positions of the F box and WD-40 repeats, the GST-Cdc4p fusions made in this study, and a summary of their ability to permit growth when overproduced in Y382 cells. (B and C) Comparison of the terminal phenotypes of cells containing the cdc4-3 temperature-sensitive mutation and cells overproducing GST-Cdc4(1-278)p. Panel B is an overlay of differential interference contrast and fluorescent images of the indicated cells stained with propidium iodide, and panel C shows results of flow cytometry analysis. In panels B and C, the left panels show cdc4-3-containing cells and the right panels show Y382 cells overproducing GST-Cdc4(1-278)p. For both panel B and panel C, cells were grown to mid-logarithmic phase. cdc4-3-containing cells (strain A.2.7.A3p) were then shifted from 23 to 37°C for either 3 h (B) or the indicated length of time (C). Y382 cells containing pGST-Cdc4(1-278) were grown to mid-logarithmic phase and shifted to medium containing galactose for 8 h (B) or for the indicated time (C). (D) The terminal morphology of GST-Cdc4(1-278)p overproduction is dependent on SIC1. Shown are an overlay of differential interference contrast and fluorescent images of sic1Δ cells overproducing GST-Cdc4(1-278)p. PMY1 cells (sic1Δ) transformed with pGST-
complex, it should be dependent upon the presence of SIC1. Strains containing double mutations in SIC1 and genes encoding SCF\textsuperscript{Cdc4p} complex components are competent to pass through S phase but fail to undergo cytokinesis and arrest as large budded cells (11, 36). Indeed, this phenotype was copied when GST-Cdc4(1-278)p was overproduced in a sic1Δ cell (Fig. 1D).

We wished to assess the in vivo activity of the SCF\textsuperscript{Cdc4p} complex and that of a heterologous SCF complex when GST-Cdc4(1-278)p was overproduced. By Western immunoblot analysis, hemagglutinin (HA)-Sic1p was readily detectable when co-overproduced with GST-Cdc4(1-278)p but was not detectable when co-overproduced with GST (Fig. 1E). To determine the activity of a heterologous SCF complex, we measured the abundance of Cln2p, which is a substrate for the SCF\textsuperscript{Cln2p} complex (2, 37). As a control to detect a defect in Cln2p degradation, we used cells containing a mutation in CDC34, which encodes the E2 required for its proteolysis (8). HA-Cln2p could be clearly detected in cells lacking CDC34 activity, as determined by the appearance of multiple bands immunoreacting with anti-HA antibodies that likely correspond to stabilized, hyperphosphorylated Cln2p. However, we could not detect HA-Cln2p in cells producing GST-Cdc4(1-278)p (Fig. 1F). The abundance of Cln2p is cell cycle dependent (45). However, because cells containing the cdc34-1 mutation and cells overproducing GST-Cdc4(1-278)p have a common cell cycle arrest, the differences observed for Cln2p abundance are more likely due to differences in SCF\textsuperscript{Cln2p} activity. Thus, overproduction of GST-Cdc4(1-278)p does not affect the activity of the SCF\textsuperscript{Cln2p} complex whereas the cdc34-1 mutation does. Finally, we assessed whether elevated levels of Cdc4p would suppress the toxicity induced by GST-Cdc4(1-278)p overproduction. If GST-Cdc4(1-278)p affected only a specific pathway, its activity could be suppressed by co-overproduction of its F-box protein. Indeed, as determined by growth assay, co-overproduction of GST-Cdc4p but not of GST suppressed GST-Cdc4(1-278)p-mediated toxicity (Fig. 1E). Thus, together our data indicate that GST-Cdc4(1-278)p overproduction results in the stabilization of the SCF\textsuperscript{Cln2p} substrate Sic1p but not the substrate of a heterologous SCF complex. The stabilization of Sic1p is likely due to the inhibition of the SCF\textsuperscript{Cdc4p} Ub ligase.

**Overproduction of Met30(1-225)p acts to specifically inhibit the SCF\textsuperscript{Met30p} complex.** Because SCF complex architecture is highly conserved within and between species, we reasoned that polypeptides from the amino terminus of a different F-box protein would specifically inhibit its respective SCF complex. To test this notion, we constructed and analyzed a series of GST-Met30p fusions (Fig. 2A). **MET30** is essential, being required to regulate the activity of the transcription factor Met4p (15, 33, 40), and cells that have unregulated **MET4** activity arrest in G1 (29). We identified four fragments that were toxic when overproduced (Fig. 2A), but in our subsequent analysis GST-Met30(1-225)p and GST-Met30(150-225)p behaved identically to GST-Met30(1-277)p and GST-Met30(150-277)p. Because each of these fragments contains the Met30p F box, encoded by residues 180 to 225, the toxicity induced could be due to Skp1p titration rather than specific inhibition of the SCF\textsuperscript{Met30p} complex. To distinguish these possibilities, we tested which SCF component could suppress the toxicity induced by these amino-terminal fragments. As determined by growth assay, elevated levels of Met30p, but of no other SCF component or heterologous F-box protein, could suppress the toxicity induced by GST-Met30(1-225)p (Fig. 2B). However, elevated levels of Skp1p (Fig. 2C), but of no other SCF component (data not shown), could suppress the toxicity induced by GST-Met30(150-225)p. These data suggest that GST-Met30(1-225)p and GST-Met30(150-225)p affect cell viability by their action in different pathways. To test this hypothesis further, the different GST-Met30p fusions were expressed in a strain lacking the gene encoding the SCF\textsuperscript{Met30p} substrate Met4p. It has been previously shown that loss of **MET30** activity is suppressed by mutations in **MET4** (15, 33). Therefore, only GST-Met30p fusions that specifically inhibited **MET30** activity would be suppressed in a met4Δ strain. Indeed, as judged by growth assay, the met4Δ mutation suppressed the lethality associated with GST-Met30(1-225)p overproduction but not that of GST-Met30(150-225)p (Fig. 2D). Together, these genetic data indicate that GST-Met30(1-225)p specifically inhibits the **MET30** activity, whereas GST-Met30(150-225)p is not as specific. In order to confirm this notion, we assayed SCF\textsuperscript{Met30p} activity by monitoring Met4p abundance and modification. Previously, by Western immunoblot analysis, it has been demonstrated that Met4p may be detected as an unmodified species and as a species modified by Ub (15, 33). The abundance of both species is dependent on the activity of the SCF\textsuperscript{Met30p} Ub ligase complex. In the presence of GST-Met30(1-225)p, the abundance of unmodified Met4p was increased and that of the modified species was decreased (Fig. 2E), indicating that the GST fusion protein compromises SCF\textsuperscript{Met30p} complex activity. Finally, GST-Met30(1-225)p overproduction resulted in a G1 arrest, similar to that seen for unregulated **MET4** activity (Fig. 2F) (40). Thus, together our data indicate that GST-Met30(1-225)p overproduction inhibits SCF\textsuperscript{Met30p} complex activity, resulting in the accumulation of unmodified Met4p. In two cases, therefore, we have demonstrated that overproduction of polypeptides corresponding to CDC4(1-278)p were plated onto solid medium containing galactose and incubated overnight. (E) GST-Cdc4(1-278)p overproduction results in the accumulation of Sic1p. Lysates prepared from cells producing either HA-Sic1p or untagged Sic1p and the indicated GST fusion were subjected to Western immunoblot analysis with antibodies raised against GST (upper panel) or HA (middle panel). The lower panel is the signal obtained from a band cross-reacting to anti-HA antibodies and is used as a loading control. (F) GST-Cdc4(1-278)p overproduction does not result in Cln2p accumulation. Shown are results of Western immunoblot analysis of lysate prepared from cdc34-1Δ-containing cells (strain G101) and Y382 cells, producing the indicated GST fusion, to detect HA-Cln2p expressed from the GAL1 promoter. G101 cells were grown to early logarithmic phase at 23°C, shifted to 37°C for 3 h, and incubated in the absence of galactose for a further hour. Y382 cells were grown to mid-logarithmic phase and incubated in the presence of galactose for 8 h. The position of HA-Cln2p is as indicated by a bracket, and the asterisk indicates a cross-reacting band. (G) The toxicity of GST-Cdc4(1-278)p overproduction is suppressed by elevated levels of Cdc4p. Tenfold serial dilutions of saturated Y382 cultures overproducing the indicated proteins were plated onto medium containing galactose and incubated at 30°C for 4 days.
FIG. 2. Overproduction of GST-Met30(1-225)p inhibits SCF\textsuperscript{Met30p} complex activity. (A) Schematic diagram of Met30p, illustrating the positions of the F box and WD-40 repeats, the GST-Met30p fusions made in this study, and a summary of their ability to permit growth when overproduced in Y382 cells. (B to D) Suppression of GST-Met30(1-225)p- and GST-Met30(150-225)p-mediated toxicity. Tenfold serial dilutions of saturated cultures overproducing the indicated proteins were plated onto medium containing galactose and incubated at 30°C for 4 days. Strain Y382 is used in panels B and C, and strains KS410 (\textit{MET4}) and KS255 (\textit{met4}/H9004) are used in panel D. (E) GST-Met30(1-225)p overproduction results in decreased abundance of Met4p modification. Shown are results of Western immunoblot analysis with anti-MYC antibodies of lysate prepared from cells containing \textit{MET4}-(18myc) that were grown to mid-logarithmic phase and incubated in the presence of galactose for 5 h to induce the production of the indicated GST fusion. Relative exposure times are indicated. The bottom panel, used as a loading control, is a cross-reaction band. The arrow indicates unmodified Met4p; slower-migrating species are modified. (F) GST-Met30(1-225)p overproduction causes a G\textsubscript{1} arrest. Shown are results of flow cytometry analysis of Met30(1-225)p-overproducing cells incubated in galactose-containing medium for the indicated times.
the amino terminus of an F-box protein specifically inhibits the activity of their SCF complexes in vivo.

The dominant-negative F-box proteins are present as high-molecular-weight nuclear complexes. To understand how the amino-terminal F-box protein fragments confer inhibition, we determined their subcellular localization. Both full-length Cdc4p and Met30p are exclusively localized to the cell nucleus (3, 33). A single monopartite nuclear localization sequence, occurring at residues 82 to 85, has been reported elsewhere to be necessary for targeting Cdc4p to the nucleus (3). Consistent with this earlier report, GFP-Cdc4(1-278)p was detected exclusively in the nucleus (Fig. 3). No identifiable nuclear localization sequence occurs within yeast Met30p, unlike Met30p homologues in other organisms (27). Monitoring the subcellular localization of GFP fused to Met30p and Met30(1-225)p demonstrated that these proteins are nuclear, at least when overproduced with the GAL1 promoter (Fig. 3). We also saw no exclusion of GFP-Met30(1-640)p from the nucleus when GST-Met30(1-225)p was co-overproduced (data not shown). Thus, the dominant-negative F-box protein alleles localize exclusively to the nucleus and likely do not perturb the targeting of their full-length counterparts to the same location.

To assess if Cdc4(1-278)p associated with additional proteins present in the cell, we performed gel-filtration column chromatography of lysate prepared from cells expressing either GST or GST-Cdc4(1-278)p. A small amount of GST-Cdc4(1-278)p was detectable in the void volume, which may represent nonspecific aggregation or the interaction with proteins that are part of a high-molecular-weight complex. Indeed, SCF components have been demonstrated previously to interact with the proteasome (13, 41), and perhaps Cdc4p interacts with the proteasome through its amino terminus. The majority of GST-Cdc4(1-278)p was demonstrated to be present in fractions corresponding to approximately 350 kDa. In contrast, GST was present in fractions corresponding to approximately 44 kDa (Fig. 4). This indicates that GST-Cdc4(1-278)p is part of a multiprotein complex. To probe what proteins GST-Cdc4(1-278)p may associate with, we repeated the experiment with lysate from cells that coexpressed GST-Cdc4(1-278)p and GST-Cdc4p. These proteins cofractionated (Fig. 4), suggesting that the dominant-negative allele may associate with complexes containing its full-length counterpart.

The amino terminus of Cdc4p mediates multimerization. By virtue of their localization and the presence of GST-Cdc4(1-278)p in a high-molecular-weight complex of similar size as the SCFCdc4p complex (24), our physical characterization of the dominant-negative alleles indicated that they may associate with their SCF ligases. Indeed, we have observed that Met30p multimerizes through its amino terminus (C. Dixon and N. Mathias, unpublished observations), indicating that GST-Met30(1-225)p could associate with the SCFMet30p complex via its interaction with Met30p. By immunoprecipitation analysis, we explored whether Cdc4(1-278)p could associate with Cdc4p. To test if Cdc4p forms homomultimers in vivo, GST-Cdc4p was coproduced in yeast cells with either FLAG epitope-tagged Cdc4p or untagged Cdc4p. With the use of anti-FLAG antibody resin, GST-Cdc4p copurified with FLAG-Cdc4p but not with untagged Cdc4p (Fig. 5A). In the same experiment, GST-Cdc4(1-278)p specifically coprecipitated with FLAG-Cdc4p. To confirm these observations and to map the region of interaction, two-hybrid analysis was employed. Full-length Cdc4p, but not full-length Met30p, was capable of interacting with Cdc4(1-278)p but was incapable of interacting with fragments of Cdc4p lacking the amino terminus (Fig. 5B).
Therefore, Cdc4p forms homomultimers through the same region that possesses dominant-negative activity.

The interaction of Cdc4(1-278)p and Met30(1-225)p with their full-length counterparts is important for their dominant-negative activity. To assess if the interaction of Cdc4(1-278)p with Cdc4p was important for its dominant-negative activity, we generated alleles of Cdc4(1-278)p that were not toxic when overproduced. Three clones were isolated, and sequencing revealed that each contained single-amino-acid changes. When overproduced, two mutants, GST-Cdc4(1-278)(E229G)p and GST-Cdc4(1-278)(R256G)p, had little effect on the rate of cell growth, whereas one mutant, GST-Cdc4(1-278)(R261G)p, generated a slow-growth phenotype with cells displaying morphological changes similar to, yet milder than, that seen when wild-type Cdc4(1-278)p is overproduced (Fig. 6A and B). By Western immunoblot analysis, the steady-state level of each mutant protein was similar to that of wild-type Cdc4(1-278)p, indicating that the difference in their dominant-negative activity was not due to differences in their production (Fig. 6C). Using two-hybrid analysis, we observed that each mutant either failed to associate or had decreased association with full-length Cdc4p compared to wild-type Cdc4(1-278)p (Fig. 6D). The correlation of association with full-length counterpart and dominant-negative activity indicates that interaction with Cdc4p is important for the dominant-negative activity of Cdc4(1-278)p.

To test if a similar correlation could be established for the Met30p dominant-negative allele, the same approach was used and yielded six clones that failed to act in a dominant-negative fashion (Fig. 7A). Three clones contained a mutation within the F box (P188L, P188S, and C211Y), with the remainder containing mutations outside the F box (Y144C, Q167R, and S169P). Similar to that for the GST-Cdc4(1-278)p mutants, the steady-state abundance of GST-Met30(1-225)p was not greatly different from that of the wild type (Fig. 7B). By coimmunoprecipitation analysis, the Met30(1-225)p point mutants either failed to bind or had decreased ability to bind to full-length Met30p (Fig. 7C). In both cases, therefore, the mechanism of inhibition appears to rely on the ability of the dominant-negative allele to associate with its full-length counterpart.

**DISCUSSION**

We set out to identify portions of an F-box protein that act to specifically inhibit their SCF Ub ligase complexes. Because SCF complexes display remarkable conservation in terms of component composition and architecture, we believed that if a dominant-negative polypeptide could be identified, a similar region on a different F-box protein would have the same properties. Thus, a simple and common means of generating specific SCF inhibitors would be described that would aid the characterization of the numerous novel F-box proteins present in different eukaryotic species. We determined that a polypeptide corresponding to the amino terminus of two different F-box proteins inhibited their SCF complexes. Cdc4(1-278)p acts in a dominant-negative manner because its overproduction induces (i) a terminal phenotype identical to that of a cdc4 temperature-sensitive mutant, (ii) a terminal morphology that is dependent upon the presence of the SCF<sub>Cdc4p</sub> substrate Sic1p, (iii) Sic1p accumulation, and (iv) a defect that is specifically suppressed by elevated levels of Cdc4p. We determined that Met30(1-225)p acts in a dominant-negative manner be-
cause its overproduction induces (i) a lethality that is suppressed by mutations in \( MET4 \), (ii) a lethality that is suppressed by elevated levels of Met30p, (iii) a decrease in the SCFMet30p-dependent modification of Met4p, and (iv) a \( G_1 \) arrest that is shared with cells that have unregulated Met4p activity. Thus, our initial efforts to identify a common means to inhibit specific SCF complexes in vivo appear encouraging.

To further characterize the dominant-negative polypeptides, we began to determine the mechanism by which they caused their inhibitory effects. Both dominant-negative polypeptides occupied the same subcellular location as their full-length counterparts. Additionally, upon size fractionation, we demonstrated that GST-Cdc4(1-278)p is present as part of a high-molecular-weight complex that is of a similar size as that determined for the SCFCdc4p complex (24). We interpreted these data to indicate that the dominant-negative alleles could physically associate with their respective F-box protein. Indeed, as shown here and to be shown elsewhere (Dixon and Mathias, unpublished), both Cdc4p and Met30p homomultimerize and do so through their amino terminus. By generating mutant alleles of Cdc4(1-278)p and Met30(1-225)p, we observed a failure of these polypeptides to interact or a decrease in interaction with full-length F-box protein, which correlated with loss of their dominant-negative activity. Thus, we propose that this interaction is important for their inhibitory activity.

The above observations help explain two points. First, the requirement of the F box of Met30p for its multimerization suggests why the two dominant-negative polypeptides possess a significant structural difference: that for Met30p contains the F box whereas that for Cdc4p does not. Second, exclusive binding to their full-length counterpart explains how specificity is conferred by the dominant-negative polypeptides. It has been previously reported that removal of the F box has also resulted in the creation of a dominant-negative allele for the Slimb/SEL-10/\( \beta \)-TrCP/Fbw1 family of F-box proteins (18, 47, 48). However, this approach to generating dominant-negative F-box proteins is not universal, since overproduction of similar Met30p constructs results in dominant-active alleles (Dixon and Mathias, unpublished). Although in both cases substrate is bound to the F-box-less mutant and is resistant to degradation, the discrepancy in activities between the Slimb/SEL-10/\( \beta \)-TrCP/Fbw1 mutants and the Met30p mutants likely reflects how well these proteins shield their bound substrate, preventing its cellular activity. Thus, the use of F-box-less alleles may not always generate a dominant-negative effect. The approach described in this report relies on exploiting F-box fragments that mediate F-box protein multimerization. Since F-box proteins in other species also appear to multimerize through their amino terminus (39, 46), the approach described in this report may be more universally applicable than those approaches previously described.

What caveats need to be considered when using the ap-
Tenfold serial dilutions of saturated cultures overproducing the indicated proteins were plated onto medium containing galactose and incubated at 30°C for 4 days. (B) Western immunoblot analysis of lysate prepared from cells producing the indicated GST-Met30(1-225)p allele. (C) Co-immunoprecipitation analysis of GST-Met30(1-225)p alleles with HA-Met30p. Lysates prepared from Y382 cells producing the indicated GST-Met30(1-225)p allele with or without HA-Met30p, as indicated, were subjected to Western immunoblot analysis with anti-GST antibodies (upper panel) or incubated in the presence of anti-HA resin. Proteins immobilized on the resin were subjected to Western immunoblot analysis with antibodies raised against HA (middle panel) and GST (lower panel).
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REFERENCES

1. Bai, C., F. Sen, K. Hofmann, L. Ma, M. Goebel, J. W. Harper, and S. J. Elledge. 1996. SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. EMBO J. 15:629–638.

2. Barral, Y., S. Jentsch, and C. Mann. 1995. G1 cyclin turnover and nutrient uptake are controlled by a common pathway in yeast. Genes Dev. 9:399–409.

3. Blondel, M., J. M. Galan, Y. Chi, C. Lafourcade, C. Longaretti, R. J. De-la-Roche, and M. Peter. 2000. Nuclear-specific degradation of Far1 is controlled by the localization of the F-box protein Cdc4. EMBO J. 19:6085–6095.

4. Cenciarelli, C., D. S. Chiaur, D. Guardavaccaro, W. Parks, M. Vidal, and M. Pagano. 1999. Identification of a family of human F-box proteins. Curr. Biol. 9:1177–1179.

5. Ciechanover, A. 1998. The ubiquitin-proteasome pathway: on protein death and cell life. EMBO J. 17:1511–7160.

6. Craig, K. L., and M. Tyers. 1999. The F-box: a new motif for ubiquitin conjugating catalytic domains. Mol. Cell. Biol. 20:299–328.

7. Deshaies, R. J. 1999. SCF and Cullin/Ring H2-based ubiquitin ligases. Annu. Rev. Cell Dev. Biol. 15:435–467.

8. Deshaies, R. J., V. Chau, and M. Kirschner. 1995. Ubiquitination of the G1 cyclin Cln2p by a Cdc34p-dependent pathway. EMBO J. 14:303–312.

9. Elbe, R. 1992. A simple and efficient procedure for transformation of yeasts. BioTechniques 13:18–20.

10. Feldman, R. M., C. C. Correll, K. B. Kaplan, and R. J. Deshaies. 1997. A complex of Cdc4p, Skp1p, and Cdc34p/Cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sdp1. Cell 91:221–230.

11. Goh, P.-Y., and U. Surana. 1999. Cdc4, a protein required for the onset of S phase, serves an essential function during G2/M transition in Saccharomyces cerevisiae. Mol. Cell. Biol. 19:5512–5522.

12. Hersko, A., and A. Ciechanover. 1998. The ubiquitin system. Annu. Rev. Biochem. 67:425–479.

13. Jager, S., J. Strayle, W. Heinemeyer, and D. H. Wolf. 2000. Cdc4p, a cyclin: regulated proteolysis in the cell cycle. Cell 102:303–314.

14. Jansen, J., H. Pringle, B. Byers, and M. Goebl. 1993. Vectors for the selective expression of green fluorescent protein and Mike Tyers, Steve Reed, Steve Elledge, Elizabeth Craig, and Mark Goebel for plasmids and yeast strains.

15. Jansen, J., H. Pringle, B. Byers, and M. Goebl. 1993. Vectors for the selective expression of green fluorescent protein and the receptor component of the I kappaB tumor suppressor complex. Nature 362:525–526.

16. Jansen, J., H. Pringle, B. Byers, and M. Goebl. 1993. Vectors for the selective expression of green fluorescent protein and the receptor component of the I kappaB tumor suppressor complex. Nature 362:525–526.

17. Jansen, J., H. Pringle, B. Byers, and M. Goebl. 1993. Vectors for the selective expression of green fluorescent protein and the receptor component of the I kappaB tumor suppressor complex. Nature 362:525–526.

18. Jansen, J., H. Pringle, B. Byers, and M. Goebl. 1993. Vectors for the selective expression of green fluorescent protein and the receptor component of the I kappaB tumor suppressor complex. Nature 362:525–526.

19. Jansen, J., H. Pringle, B. Byers, and M. Goebl. 1993. Vectors for the selective expression of green fluorescent protein and the receptor component of the I kappaB tumor suppressor complex. Nature 362:525–526.

20. Jansen, J., H. Pringle, B. Byers, and M. Goebl. 1993. Vectors for the selective expression of green fluorescent protein and the receptor component of the I kappaB tumor suppressor complex. Nature 362:525–526.

21. Jansen, J., H. Pringle, B. Byers, and M. Goebl. 1993. Vectors for the selective expression of green fluorescent protein and the receptor component of the I kappaB tumor suppressor complex. Nature 362:525–526.

22. Jansen, J., H. Pringle, B. Byers, and M. Goebl. 1993. Vectors for the selective expression of green fluorescent protein and the receptor component of the I kappaB tumor suppressor complex. Nature 362:525–526.