Previous studies have indicated that a ~1,500-kDa complex, designated the cyclosome or anaphase-promoting complex, has a regulated cyclin-ubiquitin ligase activity that targets cyclin B for degradation at the end of mitosis. The cyclosome is inactive in the interphase of the embryonic cell cycle and is converted to the active form in late mitosis in a phosphorylation-dependent process initiated by protein kinase Cdc2-cyclin B. We show here that the active, phosphorylated form of the cyclosome from clam oocytes binds to p13\textsuperscript{suc1}, a protein known to associate with Cdc2. The following evidence indicates that the binding of the cyclosome to p13\textsuperscript{suc1} is not mediated via the Cdc2-cyclin B complex: (a) activated cyclosome binds to p13\textsuperscript{suc1}- Sepharose following its separation from Cdc2-cyclin B by gel filtration chromatography; (b) cyclosome from interphase extracts, activated by a kinase in which cyclin B has been replaced by an N-terminally truncated derivative fused to glutathione-S-transferase, binds well to p13\textsuperscript{suc1}-Sepharose but not to glutathione-agarose. An alternative possibility, that the phosphorylated cyclosome binds directly to a phosphate-binding site of p13\textsuperscript{suc1}, is supported by the observation that the cyclosome is efficiently eluted from p13\textsuperscript{suc1}-Sepharose by phosphate-containing compounds. This information was utilized to develop a procedure for the affinity purification of the cyclosome. A factor abundant in the fraction not adsorbed to p13\textsuperscript{suc1}-Sepharose stimulates the activity of purified cyclosome. It is suggested that binding of Suc1 may have a role in the regulation of cyclosome activity.

Recent studies have indicated that a large ~1,500-kDa complex, referred to as the “cyclosome” (1) or anaphase-promoting complex (APC)\textsuperscript{1} (2), plays an important role in the degradation of cyclin B at the end of mitosis. Cyclin B is the activating subunit of protein kinase Cdc2. The activation of Cdc2 is required for cells to undergo mitosis, while the inactivation of Cdc2, caused by the specific and regulated proteolysis of its cyclin B subunit, is essential for exit from mitosis (reviewed in Ref. 3). Studies in cell-free extracts that reproduce embryonic cell cycles showed that cyclin B is degraded by the ubiquitin pathway (4, 5), a system in which proteins are targeted for degradation by ligation to ubiquitin (reviewed in Ref. 6). Cyclin ubiquitylation and degradation depends on a partially conserved 9-amino acid motif, the “destruction box,” which is usually located ~40–50 amino acid residues from the N terminus of mitotic cyclins (4).

We have been studying the mechanisms of cyclin degradation by fractionation of a clam oocyte cell-free system (7). Three components were identified to be involved in the ligation of ubiquitin to cyclin B: the ubiquitin-activating enzyme E1, a specific ubiquitin-carrier protein E2-C (7, 8), and a cyclin ubiquitin ligase activity associated with particulate material. E1 and E2-C are constitutively active, but the particle-associated ligase activity is cell cycle-regulated; it is inactive in the interphase, but becomes activated at the end of mitosis (7). The particle-associated component was extracted with salt, partially purified, and found to be a ~1,500-kDa complex, the cyclosome (1). The cyclosome acts on both cyclin A and cyclin B and requires intact destruction box sequences of both cyclins.

The activity of the cyclosome is regulated by reversible phosphorylation, as indicated by the findings that the active, mitotic form of the cyclosome can be converted to the inactive form by treatment with an okadaic acid-sensitive phosphatase (9) and that the inactive, interphase form of the cyclosome can be converted to the active form by incubation with protein kinase Cdc2-cyclin B (1, 9). Activation of the cyclosome by protein kinase Cdc2-cyclin B includes a time lag (1, 9), which may serve to prevent the premature inactivation of the kinase in the cell cycle. Based on these findings, we suggested the cyclosome has a regulated cyclin-ubiquitin ligase activity, which targets cyclin B for degradation at the end of mitosis (1).

A similar particle, termed the APC, was identified in Xenopus egg extracts by King et al. (2). By the use of antibodies that cross-react with Xenopus proteins, these investigators further identified two subunits of the complex as homologues of the products of budding yeast CDC16 and CDC27 genes, which are required for exit from mitosis and the degradation of mitotic cyclins (10). Other subunits of the cyclosome/APC particle have been identified in a variety of organisms (11–16) and appear to be strongly conserved in evolution. The cyclosome is also involved in the degradation of anaphase inhibitor proteins, which contain destruction box sequences (17–19). The subunits of the cyclosome include several components involved in ubiquitin conjugation, ubiquitin ligase activity, and the destruction box sequences that target the destruction of cyclins.

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\textsuperscript{1} The abbreviations used are: APC, anaphase-promoting complex; Suc\textsubscript{1}, suppressor of Cdc2; Cks, cyclin kinase subunit; Cdk, cyclin-dependent kinase; BSA, bovine serum albumin; rm-BSA, reduced carboxymethylated BSA; STI, soybean trypsin inhibitor; DTT, dithiothreitol; GST, glutathione S-transferase; NPP, p-nitrophenyl phosphate; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; AMP-PNP, 5′-adenyl ϕ,γ-imidodiphosphate.
of the complex responsible for its specific actions, as well as those responsible for the regulation of its activity, have not yet been identified.

To define the mode of action and mechanisms of regulation of the cyclosome, its extensive purification is essential. The Xenopus cyclosome/APC particle was purified by immunoprecipitation (2, 15), but such preparations are not suitable for biochemical studies. In the present report we describe the binding of the cyclosome to $p_{13}^{ suc1}$ and its use for affinity purification. $p_{13}^{ suc1}$ was originally identified in the fission yeast by its ability to suppress certain temperature-sensitive mutations of Cdc2 (20). A homologous protein in budding yeast, designated Cks, was found to bind strongly to the cyclin-dependent kinase (21). The Suc1/Cks family of proteins is essential for viability and is highly conserved in evolution (reviewed in Refs. 22 and 23), but its exact functions remained unknown. Genetic evidence in yeasts indicates multiple roles in the cell cycle, including entry into mitosis, exit from mitosis, and transition between G1 and S phases of the cell cycle (24, 25).

Recent biochemical studies with immunodepleted extracts of *Xenopus* eggs further indicated that Suc1/Cks is required in at least two stages of the embryonic cell cycle: in the activation of the Cdc2-cyclin B complex by tyrosine dephosphorylation of Cdc2, and in exit from mitosis due to cyclin B degradation (26). The structural changes of Suc1/Cks proteins (27, 28) and of their complex with Cdc2 (29) have been solved. In addition to the Cdk binding site they contain a highly conserved phosphate-binding site. It has been suggested (but not yet demonstrated) that the latter site may bind to some phosphorylated proteins, and thus Suc1/Cks may have a role in targeting Cdk-cyclin kinases to certain phosphorylated proteins (26, 29).

We show here that $p_{13}^{ suc1}$ selectively binds the active, phosphorylated form of the cyclosome. Our evidence suggests that this binding is due to interaction of the cyclosome with the phosphate-binding site of $p_{13}^{ suc1}$. This information was utilized to develop an affinity procedure for the purification of the cyclosome.

**EXPERIMENTAL PROCEDURES**

Ubiquitin from bovine erythrocytes, recombinant STI, and *p* -nitrophenylphosphate (pNPP) were obtained from Sigma, and okadaic acid was obtained from Boehringer Mannheim. Ubiquilin aldehyde was prepared as described (30). E1 was purified from human erythrocytes (31). Recombinant clam E2-C was expressed in bacteria as described (8) and purified by gel filtration as described (32). $p_{13}^{ suc1}$ was coupled to cyanogen bromide-activated Sepharose-4B at a concentration of 0.025 M, which was 5–40%125I-cyclin ligated to ubiquitin. One unit of ligase activity was defined as that converting 1% 125I-cyclin to ubiquitin conjugates under the conditions described above.

Assay of Cyclin-Ubiquitin Ligase Activity—Reactions were conducted in a volume of 10 μl: 40 mM Tris-HCl (pH 7.6), 1 mg/ml recombinant STI, 20 μM MgCl₂, 0.5 mM dithiothreitol, 50 μg/ml creatine phosphokinase, 50 μM ubiquitin, 1 μM ubiquitin aldehyde, 0.025 M, 1 μM okadaic acid where specified, and 1–2 μM (10–18 pmoles) of 125I-labeled cyclin B-13–91/1 protein A. A N-terminal fragment of cyclin B has been shown to be a suitable substrate for destruction box-specific, cell cycle-regulated ubiquitinylation and degradation of mitotic cyclins (1, 4, 7). When enzyme activity associated with Sepharose beads was determined, samples were agitated during incubation. Following incubation at 18 °C for 1 h, the samples were subjected to electrophoresis on a 12.5% polyacrylamide-SDS gel. Results were quantified with a PhosphorImager (Molecular Dynamics). The amount of radioactivity in all cyclin-ubiquitin conjugates was expressed as the percentage of total radioactivity in each lane (1). Reactions were conducted in the range linear with enzyme concentration, which was 5–40% 125I-cyclin ligated to ubiquitin. One unit of ligase activity was defined as that converting 1% 125I-cyclin to ubiquitin conjugates under the conditions described above.

Miscellaneous Assays—Activity of protein kinase cyclin B-Cdc2 was measured by the phosphorylation of histone H1 following adsortion to $p_{13}^{ suc1}$-Sepharose. Virtually all such activity in extracts of meiotic M phase clam oocytes is due to cyclin B-Cdc2 complexes (data not shown). Histone H1 kinase assays were conducted as described (35), except that the concentration of ATP was 300 μM, and reactions were conducted at 18 °C. One unit of enzyme activity is defined as that causing the incorporation of 1 pmol of phosphate into histone H1 under these conditions. Protein concentration was determined with the Bio-Rad protein assay, using bovine serum albumin as standard. To estimate the amount of protein bound to beads, proteins were first eluted from beads by mixing with 4 M guanidine hydrochloride for 1 h at room temperature.

**RESULTS**

**Binding of Active Cyclosome to $p_{13}^{ suc1}$—Sepharose—$p_{13}^{ suc1}$ from fission yeast and homologous Cks proteins from other sources for affinity purification.
organisms bind strongly to Cdc2 and to some other members of the Cdk family of proteins (22, 23). p13<sup>suc1</sup>-Sepharose beads are therefore commonly used to isolate Cdk-cyclin complexes (36).

In preliminary experiments, we found that the active form of the cyclosome<sup>2</sup> bound tightly to p13<sup>suc1</sup>-Sepharose. We then tried to examine the nature of the interaction between p13<sup>suc1</sup> and the cyclosome and to exploit this binding for the affinity purification of the cyclosome.

In the experiment shown in Fig. 1, a crude fraction from M phase clams oocytes was preincubated (or not) with ATP, as indicated in the figure, and then was applied to p13<sup>suc1</sup>-Sepharose or BSA-Sepharose beads as described under “Experimental Procedures.” Except that samples of 200 μg of protein were added to 20 μl of beads. Following adsorption at 0 °C for 1 h, the supernatants were collected, and the beads were washed four times with 1-ml portions of buffer B, followed by two washes with buffer C (see “Experimental Procedures”). Cyclin-ubiquitin ligation was determined in samples of 5% of the washed beads (lanes 1–3) and 2.5% of the supernatants (lanes 4–6), as described under “Experimental Procedures.” Cyc, position of free 125I-cyclin. Numbers on the right indicate the position of molecular mass markers (kDa).

![Fig. 1. Binding of activated cyclosome from M phase extracts to p13<sup>suc1</sup>-Sepharose.](image)

Salt extract of fraction 1 from M phase clam oocytes was preincubated (or not) with ATP, as indicated in the figure, and then was applied to p13<sup>suc1</sup>-Sepharose or BSA-Sepharose beads as described under “Experimental Procedures,” except that samples of 200 μg of protein were added to 20 μl of beads. Following adsorption at 0 °C for 1 h, the supernatants were collected, and the beads were washed four times with 1-ml portions of buffer B, followed by two washes with buffer C (see “Experimental Procedures”). Cyclin-ubiquitin ligation was determined in samples of 5% of the washed beads (lanes 1–3) and 2.5% of the supernatants (lanes 4–6), as described under “Experimental Procedures.” Cyc, position of free 125I-cyclin. Numbers on the right indicate the position of molecular mass markers (kDa).

Although we followed cyclin-ubiquitin ligase activity in most experiments, the term “cyclosome” is used in this paper for brevity. As shown below, a high molecular mass complex is indeed bound to p13<sup>suc1</sup>.

The activity associated with p13<sup>suc1</sup>-Sepharose beads. Binding of cyclosome was apparent specific for p13<sup>suc1</sup>-Sepharose, because under similar conditions, there was no significant binding to control (BSA-Sepharose) beads (Fig. 1, lane 3), and all activity remained in the supernatant (lane 6). Mode of Binding of Cyclosome to p13<sup>suc1</sup>—We have considered several alternative possibilities to account for the binding of the cyclosome to p13<sup>suc1</sup>. It is possible that binding is not direct but that the cyclosome binds Cdc2-cyclin B, which in turn is bound to p13<sup>suc1</sup>. The cyclin B subunit of the Cdc2-cyclin B protein kinase complex is a substrate of the cyclosome for ubiquitin ligation, and thus cyclin B-Cdc2 may be tightly bound to the active site of the ligase. The Cdc2-cyclin B protein kinase is also an activator (although not necessarily a direct one) of the cyclosome, and it is possible that the kinase is tightly bound to site(s) of the cyclosome that it may phosphorylate. In both of these cases, p13<sup>suc1</sup> would bind to the Cdc2 subunit of the Cdc2-cyclin B complex, which is tightly associated with the cyclosome. A third possibility is that p13<sup>suc1</sup> may bind directly to the active, phosphorylated form of the cyclosome, possibly by its phosphate-binding site (27–29).

We have first examined the possibility that the cyclosome is bound to p13<sup>suc1</sup> via Cdc2-cyclin B by asking the question whether, following activation of the cyclosome, the presence of Cdc2-cyclin B is still required for binding to p13<sup>suc1</sup>-Sepharose. In the experiment shown in Fig. 2, the cyclosome was first converted to the active form by preincubation of M phase extract with ATP and then was separated from Cdc2-cyclin B by gel filtration on Superox-6 in the presence of salt. As shown previously (9), this procedure separates the active cyclosome (~1,500 kDa) from most of Cdc2-cyclin B (~100 kDa). Following gel filtration, the binding of each fraction to p13<sup>suc1</sup>-Sepharose beads was examined. It may be seen in Fig. 2 that the profile of cyclin-ubiquitin ligation activity adsorbed to p13<sup>suc1</sup>-Sepharose closely followed that before adsorption. The recovery of activity was about 20% in each fraction, regardless of the amount of residual Cdc2-cyclin B. Thus, for example, cyclin-ubiquitin ligase activity bound to p13<sup>suc1</sup>-Sepharose beads in fractions 20–22, which had no detectable Cdc2-cyclin B kinase activity, to an extent similar to that in fractions 24–26, which contained a low amount of residual kinase activity. In all fractions, cyclin-ubiquitin ligase activity was completely removed from the supernatants following adsorption to p13<sup>suc1</sup>-Sepharose beads (data not shown). These findings suggest that following the activation of the cyclosome, the continued presence of Cdc2-cyclin B is not required for the binding of the cyclosome to p13<sup>suc1</sup>.

It is possible that the active form of the cyclosome is associated with a tightly bound molecule of Cdc2-cyclin B, which is below the detection limit of our kinase assay and is not dissociated during gel filtration. We therefore continued to examine this problem by another approach, using interphase extracts. In interphase extracts (prepared from emetine-arrested two-cell clam embryos; Ref. 7) cyclin B is mostly degraded, levels of protein kinase Cdc2-cyclin B are about 100-fold lower than in M phase extracts, and the cyclosome is inactive. The cyclosome can be converted to the active form by incubation of interphase extracts with protein kinase Cdc2-cyclin B and ATP (1, 7). In the experiment shown in Table 1, interphase extract was incubated with a derivative of Cdc2 kinase containing recombinant cyclin B that lacked the N-terminal 88 amino acid residues and was fused to glutathione S-transferase (GST). This truncated derivative of cyclin B can form active protein kinase with Cdc2, which can activate the cyclosome, but it cannot be ubiquitinyl-
Experimental Procedures.

Cyclin-ubiquitin ligase activity associated with beads was assayed as described under “Experimental Procedures,” and then was applied to a Superose-6 HR 10/30 column (Pharmacia Biotech Inc.) equilibrated with 50 mM Tris-HCl (pH 7.2), 250 mM KCl, 0.2 mg/ml rcm-BSA, and 1 mM DTT. Fractions of 0.5 ml were collected at a flow rate of 0.4 ml/min. Column fractions were concentrated by ultrafiltration with Centricon-30 concentrators (Amicon), diluted 20-fold with buffer C (see “Experimental Procedures”), and absorbed onto 40 p13 suc1-Sepharose beads, which was presumably due to the action of the low amounts of Cdc2-cyclin B (assayed by the phosphorylation of histone H1, as described under “Experimental Procedures.”

Salt extract of fraction 1 from M phase clam oocytes (6 mg of protein) was preincubated with ATP, as described under “Experimental Procedures,” except that the time of preincubation was 90 min. Where indicated, 4,000 units of GST-cdc2/cyclin B-Cdc2 were added in preincubation. Subsequently, samples were brought to 250 mM KCl, as described under “Experimental Procedures,” and absorbed onto 40 μl of beads indicated in the table. Cyclin-ubiquitin ligase and histone H1 kinase activities associated with beads were assayed as described under “Experimental Procedures.”

| Addition of GST-Δ88 cyclin B-Cdc2 | Beads | Activity associated with beads | units | units |
|-----------------------------------|-------|-------------------------------|-------|-------|
|                                   | p13   | Cyclin-ubiquitin ligase        | 40    | 180   |
|                                   | p13   | H1 kinase                      | 205   | 1,600 |
|                                   | GSH   |                               | 0     | 1,660 |
|                                   | BSA   |                               | 0     | 19    |

Activated cyclosome binds to p13 suc1-Sepharose following separation from Cdc2-cyclin B by gel filtration chromatography. Salt extract of fraction 1 from M phase clam oocytes (6 mg of protein) was preincubated with ATP, as described under “Experimental Procedures,” and then was applied to a Superose-6 HR 10/30 column (Pharmacia Biotech Inc.) equilibrated with 50 mM Tris-HCl (pH 7.2), 250 mM KCl, 0.2 mg/ml rcm-BSA, and 1 mM DTT. Fractions of 0.5 ml were collected at a flow rate of 0.4 ml/min. Column fractions were concentrated by ultrafiltration with Centricon-30 concentrators (Amicon), diluted 20-fold with buffer C (see “Experimental Procedures”), and concentrated again to final volume of 45–60 μl. 70% of every second fraction was adsorbed onto 30 μl of p13 suc1-Sepharose beads, and then the beads were washed as described in the legend to Fig. 1. Cyclin-ubiquitin ligase activity was assayed in samples of column fractions before adsorption (○) and adsorbed to beads (●) and was expressed as the total activity in each fraction. Cdc2-cyclin B kinase activity associated with p13 suc1-Sepharose beads (Table I). This was accompanied by a complete disappearance of cyclin-ubiquitin ligase activity from the supernatant (data not shown). This finding confirms the conclusion that the active form of the cyclosome binds preferentially to p13 suc1. This observation also appears to be incompatible with the notion that the cyclosome is bound to p13 suc1 via cyclin B to the active ubiquitination site, since Δ88-cyclin B is neither a substrate nor a competitive inhibitor, and thus it cannot be bound to the ubiquitination active site of the cyclosome.

In this experiment, we have also taken advantage of the fact that GST-Δ88-cyclin B-Cdc2 can be bound to glutathione-agarose beads via its GST moiety. If binding of the cyclosome to p13 suc1 is mediated by Cdc2-cyclin B bound to any site or subunit of the cyclosome, it would be expected that, following incubation of interphase extracts with GST-Δ88-cyclin B-Cdc2, the cyclosome would be bound to GSH-agarose beads along with the kinase. However, no significant binding of cyclin-ubiquitin ligase activity to GSH beads could be detected under conditions identical to those promoting cyclosome binding to p13 suc1-Sepharose (Table I). By contrast, the binding of GST-Δ88-cyclin B-Cdc2 to GSH beads (assayed by H1 kinase activity) was as efficient as its binding to p13 suc1 beads. The cumulative evidence from the above experiments does not support the notion that Cdc2-cyclin B mediates an indirect binding of the cyclosome to p13 suc1. A direct binding of active, phosphorylated cyclosome to the phosphate-binding site of p13 suc1 is suggested by the nature of compounds that promote the elution of the cyclosome from p13 suc1 beads, as described below.

Elution of Cyclosome from p13 suc1-Sepharose Beads—We have next examined different experimental conditions for the elution of the cyclosome from p13 suc1 beads. The aim of these
Affinity Purification of Cyclosome on p13\textsuperscript{Suc1}

Elution of cyclosome from p13\textsuperscript{Suc1}-Sepharose by phosphate compounds or high pH

Salt extract of fraction 1 from M phase oocytes was preincubated with ATP as described under “Experimental Procedures.” Samples of 500 

eluted 300 μl of p13\textsuperscript{Suc1}-Sepharose beads, for 60 min at 0 °C. Subsequently, beads were washed twice with 1-ml portions of buffer A, followed by two washes with buffer C (see “Experimental Procedures”). The beads were then suspended in 0.5-ml portions of the solutions indicated in the table (first column), which also contained 1 mg/ml rat BSA and 1 mM DTT. In experiment 1, all solutions contained 50 mM Tris-Cl, pH 7.2. In addition, solutions of potassium phosphate and p-nitrophosphoryl bovine were also adjusted to pH 7.2. Elution was carried out at 0 °C for 30 min, with stirring every 5 min. Subsequently, beads were separated from eluates by centrifugation (700 rpm, 3 min).

The beads were further washed three times with 1-ml portions of buffer C and were suspended in 50 μl of buffer C containing 2 mg/ml rat BSA. Eluates were concentrated with Centricon-30 concentrators, diluted 20-fold with buffer C, and concentrated again to 50 μl. Cyclin-ubiquitin ligase activity associated with beads or eluates was expressed as the percentage of the initial activity of salt extract.

| Elution with | Cyclin-ubiquitin ligase recovered |
|--------------|----------------------------------|
|              | Beads (%) | Eluate (%) |
| **Experiment 1** |           |            |
| 50 mM Tris-Cl, pH 7.2 | 16.0 | 0 |
| +300 mM KCl | 12.6 | 0.2 |
| +600 mM KCl | 3.8 | 1.2 |
| +150 mM potassium phosphate | 5.0 | 6.7 |
| +50 mM p-nitrophosphoryl bovine | 2.3 | 16.5 |
| **Experiment 2** |           |            |
| 50 mM Tris-Cl, pH 7.2 | 16.9 | 0.4 |
| 50 mM Tris-Cl, pH 9.0 | 2.6 | 10.3 |

The Fraction Not Adsorbed to p13\textsuperscript{Suc1}-Sepharose Contains Factor(s) That Stimulate the Activity of Affinity-purified Cyclosome—As noted above, although cyclin-ubiquitin ligase activity disappeared from the supernatants following adsorption of the cyclosome to p13\textsuperscript{Suc1}-Sepharose beads, it was only partially eluted when the higher molecular mass factor, since when the higher molecular mass peak was subjected to gel filtration chromatography on Superose-6, stimulatory activity eluted in two peaks: a sharp peak at about 100 kDa and a higher molecular mass broad peak at about 400–800 kDa (Fig. 3B). At least part of the higher molecular mass peak was subjected to repeated separation on Superose-6 in the presence of 0.5 M KCl, part of activity was converted to the lower molecular mass form (data not shown).

The mode of action of the stimulatory factor from the flow-through fraction is not known. It does not seem to be involved in the phosphorylation process responsible in the conversion of the interphase form of the cyclosome to the mitotic form (see “Discussion”). The purification and characterization of this factor is the subject of continued work. In the present study, the stimulatory activity of this factor was used for the detection of purified cyclosome at high sensitivity.
Affinity Purification of Cyclosome on p13

with pH 9 buffer released most of the cyclin-ubiquitin ligase activity from p13-Sepharose. However, only about one-third of the total protein that was adsorbed to the beads and a small fraction of the H1 kinase were eluted at pH 9, and the rest remained adsorbed to p13-Sepharose beads. This relative selectivity in the elution of the cyclosome at pH 9 caused its enrichment in the eluate and its separation from most of protein kinase Cdc2-cyclin B. The recovery of cyclin-ubiquitin ligation activity in the pH 9 eluate was about 13% when assayed without the flow-through and 66% when assayed in the presence of the flow-through. Thus, the overall purification achieved by this procedure was around 30-fold when assayed in the presence of the flow-through stimulatory factor (Table III).

It is notable that while the flow-through stimulated the activity of the cyclosome in the pH 9 eluate about 5-fold, it stimulated only slightly the activity of the enzyme bound to p13 beads (Table III and see “Discussion”). It is also noteworthy that when assayed in the presence of the flow-through, the amount of cyclin-ubiquitin ligation activity in the pH 9 eluate was 2-fold higher than that associated with the p13-Sepharose beads prior to elution (Table III). It might be that the catalytic efficiency of immobilized enzyme associated with beads is lower than that of the free cyclosome in solution or that immobilized enzyme is not accessible to added flow-through factor.

To further purify the cyclosome, the affinity-purified preparation was subjected to gel filtration chromatography on Superose-6 (Fig. 4). The activity of cyclin-ubiquitin ligation was assayed in the presence or absence of the flow-through stimulatory factor. Following gel filtration, activity without the flow-through was very low and was stimulated more than 10-fold by the flow-through (Fig. 4A). The elution position of affinity-purified cyclosome was similar to that observed previously with crude preparations (1, 9), with an apparent molecular size of about 1,500 kDa. This similarity in size indicates that most of the cyclosome is bound to p13 and is eluted as an entire complex, although the possible loss of some loosely bound subunits cannot be ruled out (see “Discussion”). The purity of the preparation was examined by silver staining. As expected, the pH 9 eluate (Fig. 4B, OR lane) contained numerous protein bands. However, many of these were removed from the region at the cyclosome by the gel filtration procedure. While the preparation following gel filtration is not homogenous, at least nine protein bands in the subunit region of 60–200 kDa cannot be ruled out (see “Discussion”). The purity of the preparation was examined by silver staining. As expected, the pH 9 eluate (Fig. 4B, OR lane) contained numerous protein bands. However, many of these were removed from the region at the cyclosome by the gel filtration procedure. While the preparation following gel filtration is not homogenous, at least nine protein bands in the subunit region of 60–200 kDa cannot be ruled out (see “Discussion”).

**Fig. 3.** The activity of affinity-purified cyclosome is stimulated by factor(s) present in the fraction not adsorbed to p13-Sepharose. Affinity purification was carried out as described under “Experimental Procedures,” using pH 9 buffer for elution. A, influence of increasing concentrations of flow-through fraction. Cyclin-ubiquitin ligation was assayed with 0.5% of the column fraction in the presence of the indicated concentrations of the flow-through fraction. The flow-through fraction (7.3 mg of protein) was separated on a Superose-6 column under conditions identical to those described for Fig. 2. Cyclin-ubiquitin ligation was determined with 0.5% of the column fractions in the presence of a constant amount affinity-purified cyclosome preparation. The amount of cyclin-ubiquitin ligation obtained by cyclosome alone (19.2 units) was subtracted from all results. The elution positions of marker proteins (kDa) are indicated by arrows.
and 6). In the latter case, only a small amount of the monoubiquitylated derivative was found, indicating a low affinity of the destruction box mutant for the enzyme, resulting in poor processivity in the addition of multiple ubiquitin molecules.

The selectivity of affinity-purified cyclosome for destruction box-containing cyclins was further examined by the competition of different unlabeled cyclin derivatives on the ligation to ubiquitin of $^{125}$I-labeled cyclin B-(13–66) fragment (Fig. 5, lanes 9–13). The actual amount of protein recovery is probably lower, and thus the estimate of purification is a minimal value.

### Table III

| Fraction                  | Protein (μg) | H1 kinase (units $\times 10^{-3}$) | Cyclin-ubiquitin ligase |
|---------------------------|-------------|-----------------------------------|------------------------|
|                           | Amount      | Yield %                           | Specific activity (units/μg) | Purification (fold) |
|                           | No addition | With flow-through                  |
| Fraction 1 (salt extract) | 12,000      | 1,165                             | 86,400                  | 100              | 86,400                  | 100          | 7.2 | 1    |
| Flow-through              | 6,480       | 15                                | 429                     | 0.5              |
| p13$^{3997}$-Sepharose beads$^a$ | 670         | 1,165                             | 22,500                  | 26.1             |
| pH 9 eluate               | 250$^b$     | 104                               | 11,030                  | 12.8             |

$^a$ A sample of washed beads was withdrawn before the elution step.

$^b$ Protein in the pH 9 eluate could not be determined directly because of the presence of carrier STI (see “Experimental Procedures”). It was estimated by the decrease in protein associated with p13$^{3997}$ beads following elution. The actual amount of protein recovery is probably lower, and thus the estimate of purification is a minimal value.

### Fig. 4

**A**. Gel filtration chromatography of affinity-purified cyclosome. A sample of affinity-purified cyclosome, prepared from 12 mg of protein of salt extract, was separated on Superose-6 under conditions similar to those described for Fig. 2, except that rem-BSA was replaced by STI (0.2 mg/ml) as the carrier protein. A, cyclin-ubiquitin ligation activity was estimated in samples of column fractions in the presence (●) or absence (○) of 100 μg/ml of flow-through fraction. The elution position of a 670-kDa marker protein is indicated by the arrow. B, samples of 10% of the column fractions were subjected to electrophoresis on an 8% polyacrylamide-SDS gel and stained with silver. OR, a sample of 0.5% of the original affinity-purified preparation before gel filtration. Numbers at the top indicate fraction numbers, and numbers on the right indicate the positions of molecular mass marker proteins (kDa). Protein bands that co-migrate with cyclosome activity (see Fig. 4A) are indicated by dots between fractions 20 and 21.

### Fig. 5

**Selectivity of affinity-purified cyclin-ubiquitin ligase for destruction box-containing mitotic cyclins.** Cyclin-ubiquitin ligation was carried out as described under “Experimental Procedures,” except that $^{125}$I-labeled substrate was cyclin B-(13–66)/protein A in lanes 1–3 and its corresponding AARL destruction box mutant in lanes 4–6. Where indicated, 2.5 μg of protein of crude mitotic salt extract of fraction 1 (Crude) or 5 μl of affinity-purified cyclosome preparation (Pure) were added as the source of enzyme. In lanes 9–13, the indicated unlabeled cyclin derivatives were added at the following concentrations: 200 μg/ml (lanes 9 and 10), 50 μg/ml (lanes 11–13). Cyc, position of the free labeled cyclin fragment; Contam., contamination in the preparations of labeled cyclin fragments.
finity-purified cyclosome retains selectivity for destruction box-containing cyclins, as observed previously with a partially purified preparation (1).

**DISCUSSION**

This study was initiated by the observation that the active form of the cyclosome binds strongly to p13^{suc1}-Sepharose beads (Fig. 1). Since the best known property of p13^{suc1} and of homologous Cks proteins is their ability to bind Cdk (22, 23, 29), we have first examined the possibility that cyclosome binding is mediated via Cdc2-cyclin B, which is both a substrate of the enzyme and its activator. We did not find evidence for such an indirect binding, since activated cyclosome bound to p13^{suc1}-Sepharose following its separation from Cdc2-cyclin B (Fig. 2), and cyclosome from interphase extracts activated by GST-Δ88-cyclin B bound well to p13^{suc1}-Sepharose, but not to GSH-Sepharose (Table I). An alternative possibility, that the cyclosome is bound directly to the phosphate-binding site of p13^{suc1} (27–29) is suggested by its elution by anions such as phosphate or sulfate and more effectively by the phosphate ester p-nitrophenyl phosphate (Table II). Since activation of the cyclosome is due to its phosphorylation (9, 15), the latter suggestion provides a straightforward explanation for the observation that only the active form of the cyclosome binds to p13^{suc1}-Sepharose. A possible role of the anionic-binding site of Cks proteins in their binding to phosphoproteins has been suggested (26, 29), but we are not aware of any previous instance in which this has been shown to take place. It appears that many other phosphorylated proteins may also bind to p13^{suc1}-Sepharose, since incubation of M phase extracts with ATP and okadaic acid, which promotes the accumulation of phosphorylated proteins, caused a more than 2-fold increase in the binding of total proteins to p13^{suc1}-Sepharose beads (data not shown).

Although many different (presumably phosphorylated) proteins bind to p13^{suc1}-Sepharose, considerable purification of the cyclosome is obtained by the present affinity procedure. This is aided by the fact that only a part of the proteins bound to p13^{suc1} are eluted at pH 9, along with the cyclosome (Table III). Furthermore, the large size of the cyclosome allows its efficient separation from many other proteins in a subsequent step of gel filtration chromatography (Fig. 4). The final preparation, although not homogenous, appears to be highly purified (Fig. 4B). It does not contain significant amounts of Cdc2-cyclin B protein kinase activity, and no Cdk was detectable by immunoblotting with anti-FSTAIRE antibody (data not shown). It thus appears that this purification procedure may be valuable for future studies on the mode of action of the cyclosome and its regulation by protein kinase Cdc2-cyclin B.

In the course of this study, we have observed that the activity of affinity-purified preparations of the cyclosome is greatly stimulated by a factor abundant in the fraction not adsorbed to p13^{suc1}-Sepharose. In gel filtration chromatography of this flow-through fraction, stimulatory activity eluted in two peaks, the higher molecular size of which may be an aggregate of the smaller factor (Fig. 3). At present, we do not know the mode of action of this stimulatory factor; its purification and characterization are being pursued in our laboratory. The stimulatory factor is apparently not a protein kinase based on the following observations: (a) the activity of the factor is not inhibited by staurosporin, an agent that inhibits completely the activation of the interphase form of the cyclosome by protein kinase Cdc2-cyclin B; (b) the factor stimulates cyclosome activity also when ATP is replaced by the nonhydrolyzable βγ analog AMPNNP (data not shown). This ATP analog is not a substrate for protein kinases, but it can be used in the E1 reaction (which involves the scission of the α–β bond of ATP) that is needed for the cyclin-ubiquitin ligation assay. We have furthermore found that the activity of the stimulatory factor was not affected by phosphatase treatment, while affinity-purified cyclosome was inactivated by phosphatase treatment (data not shown), as observed previously with partially purified cyclosome (9). It thus seems that the stimulatory factor is not involved in the protein kinase pathway that converts the interphase form of the cyclosome to the mitotic phosphorylated form; rather, it stimulates the activity of phosphorylated cyclosome. The factor is also not involved in the selective recognition of destruction box-containing cyclins, since similar selectivity was observed in the absence of the factor (Fig. 5) as in its presence (data not shown). The stimulatory factor may be an easily dissociable subunit of the cyclosome, which is dissociated or inactivated during enzyme purification. Such a subunit may be present in both cyclosome-associated and free forms, as reported previously for some subunits of the cyclosome from fission yeast (16) and budding yeast (14). If the factor is a dissociable subunit, it may be still present in cyclosome associated with p13^{suc1}-Sepharose, the activity of which is stimulated only slightly by the flow-through fraction (Table III). Alternatively, it is possible that cyclosome immobilized on beads is not accessible to added factor. Further work is required to characterize this stimulatory factor. In the present study, the use of the flow-through factor was essential to detect highly purified cyclosome following gel filtration, and the activity was almost completely dependent on the addition of the stimulatory factor (Fig. 4A).

Although the present data bear directly only on the characterization of the binding of the cyclosome to p13^{suc1} and its use for affinity purification, they may also give a clue as to the possible role of p13^{suc1} in cyclin degradation. As noted above, genetic evidence in yeasts indicated that p13^{suc1} and other homologous Cks proteins are required at multiple stages of the cell cycle, including exit from mitosis and the degradation of cyclin B (24, 25). Biochemical experiments in immunodepleted extracts of *Xenopus* eggs also indicated that Cks is required for cyclin degradation (26), but these experiments could not distinguish whether Cks is required for the activity of this system or for the activation of the cyclin degradation machinery. We found no evidence to indicate that p13^{suc1} is required for the activity of the mitotic, phosphorylated form of the cyclosome (data not shown), but it may be involved in the activation process. Since p13^{suc1} can bind both protein kinase Cdc2 and the phosphorylated cyclosome, it is possible that it may direct the kinase to the phosphorylated cyclosome. Assuming that multiple phosphorylations are required for full activation of the cyclosome, initial phosphorylations may cause tighter binding of protein kinase Cdc2 to the cyclosome via p13^{suc1} and thus may accelerate the rate of further phosphorylations. Such a model may also account, at least in part, for the lag kinetics of cyclosome activation by protein kinase Cdc2-cyclin B. Work in our laboratory is now aimed at examining the possible role of Suc1 protein in the regulation of cyclosome activity.

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