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Identification and Properties of an Atypical Catalytic Subunit ($p34^{PSK\text{-}J3}$/cdk4) for Mammalian D Type G1 Cyclins

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Summary

Murine D type cyclins associate with a catalytic subunit ($p34^{PSK\text{-}J3}$) with properties distinct from known cyclin-dependent kinases (cdks). Mouse $p34^{PSK\text{-}J3}$ shows less than 50% amino acid identity to $p34^{cdc2}$, $p33^{cdk2}$, and $p36^{cdk3}$, lacks a PSTAIRE motif, and does not bind to $p13^{suc1}$. Cyclin D1-$p34^{PSK\text{-}J3}$ complexes accumulate in macrophages during G1 and decline in S phase, whereas complexes involving cyclins D2 and D3 form in proliferating T cells. Although histone H1 kinase activity is not detected in cyclin D or PSK-J3 immunoprecipitates, cyclin D-$p34^{PSK\text{-}J3}$ complexes assembled in vitro stably bind and phosphorylate the retinoblastoma gene product (pRb) and an Rb-like protein (p107) but do not interact with pRb mutants that are functionally inactive. Thus, $p34^{PSK\text{-}J3}$ is a cyclin D-regulated catalytic subunit that acts as an Rb (but not H1) kinase.

Introduction

Growth factors act during the first gap phase (G1) of the cell division cycle to regulate the accumulation of critical gene products that are required for entry into S phase (Pardee, 1989). It is during this interval that environmental cues are most effective in influencing cells either to proliferate, to differentiate, or to exit the cell cycle. However, once chromosomal DNA replication ensues, cells become relatively refractory to growth factor–induced signals and execute an autonomous program that terminates with mitosis. Check point controls ensure that S phase ends before cell division begins (Hartwell and Weinert, 1989), thereby guaranteeing the obligatory temporal relationship between DNA replication and mitosis.

The regulation of a subset of mammalian G1 cyclins by growth factors provides an avenue for linking extrinsic signals with the cell cycle clock (Sherr, 1991). Cyclins are regulatory subunits of kinases that govern key cell cycle transitions, the best-characterized being cyclin B, which forms complexes with $p34^{cdc2}$ to regulate both mitotic entry and exit (Murray and Kirschner, 1989; Hunt, 1989; Nurse, 1990; Maller, 1991). Cyclin A can associate independently with $p34^{cdc2}$ and with a related cyclin-dependent kinase (cdk), $p33^{cdk2}$, during the S and G2 phases of the cell cycle (Giordano et al., 1989; Pines and Hunter, 1990, 1991; Tsai et al., 1991; Pagano et al., 1992b; Rosenblatt et al., 1992; Elledge et al., 1992). Although the biologic role of cyclin A is not well understood, disruption of cyclin A function by microinjection of antibodies or antisense oligonucleotides into cells during G1 can prevent chromosomal DNA replication (Girard et al., 1991; Pagano et al., 1992b; Zindy et al., 1992), and other evidence suggests that it may act to ensure the temporal relationship between S phase and mitosis (Walker and Maller, 1991). Together, cyclin A and cdk2 form higher-order quaternary complexes with a protein (p107) related to the retinoblastoma gene product (pRb) and with the transcription factor E2F, suggesting that the cyclin may indirectly govern gene expression during S phase (Cao et al., 1992; Devoto et al., 1992; Ewen et al., 1992; Faha et al., 1992; Pagano et al., 1992a; Shirodkar et al., 1992). Yet other G1 cyclins (*CLN1*, *CLN2*, and *CLN3*) in the budding yeast Saccharomyces cerevisiae interact with $p34^{CDC28/cdc2}$ to ensure passage through START and commitment to S phase (Nash et al., 1988; Cross, 1988; Hadwiger et al., 1989; Richardson et al., 1989). Although these genes appear to be functionally redundant, they are subject to different regulatory controls (Wittenberg et al., 1990; Chang and Herskowitz, 1990; Cross and Tinkelenberg, 1991; Nasmyth and Dirick, 1991) and act differentially in governing the timing of cell cycle initiation in mother and daughter cells (Lew et al., 1992). The yeast *CLN* genes have no exact homologs in mammalian cells, but cyclins C, D1, D2, D3, and E may represent analogous regulators (Motokura et al., 1991; Xiong et al., 1991; Matsushime et al., 1991b; Lew et al., 1991; Koff et al., 1991; Léopold and O'Farrell, 1991). Cyclin E is first expressed just prior to the G1/S transition (Lew et al., 1991), and, like cyclin A, it can associate with $p33^{cdk2}$ (Koff et al., 1991). By contrast, the timing of expression of the three D type cyclins is highly variable in different cell types and can be influenced by stimulation with growth factors (Matsushime et al., 1991a, 1991b; Inaba et al., 1992). Catalytic partners of the C and D type cyclins have not been identified, but several other $p34^{cdc2}$-related kinases have been characterized (Hanks, 1987; Bunnell et al., 1990; Meyerson et al., 1992; Okuda et al., 1992). We now show that one such enzyme, distinct from known cdks, can associate with and be regulated by cyclins of the D type.

Results

Molecular Cloning of *cdk2*-Related cDNAs from Mouse Macrophages

When deprived of colony-stimulating factor 1 (CSF-1) for 18 hr, bone marrow–derived mouse macrophages arrest

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ATG GCT GCC ACT CGA TAT GAA CCC GTG GCT GAA ATT GGT GTC GGT GCC TAT GGG   54
Met Ala Ala Thr Arg Tyr Glu Pro Val Ala Glu Ile Gly Val Gly Ala Tyr Gly   18
        Thr Ser

ACG GTG TAC AAA GCC CGA GAT CCC CAC AGT GGC CAC TTT GTG GCC CTC AAG AGT  108
Thr Val Tyr Lys Ala Arg Asp Pro His Ser Gly His Phe Val Ala Leu Lys Ser   36

GTG AGA GTT CCT AAT GGA GGA GCA GCT GGA GGG GGC CTT CCC GTC AGC ACA GTT  162
Val Arg Val Pro Asn Gly Gly Ala Ala Gly Gly Gly Leu Pro Val Ser Thr Val   54
                            Gly Gly                     Ile

CGT GAG GTG GCC TTG TTA AGG AGG CTG GAG GCC TTT GAA CAT CCC AAT GTT GTA  216
Arg Glu Val Ala Leu Leu Arg Arg Leu Glu Ala Phe Glu His Pro Asn Val Val   72

CGG CTG ATG GAT GTC TGT GCT ACT TCC CGA ACT GAT CGG GAC ATC AAG GTC ACC  270
Arg Leu Met Asp Val Cys Ala Thr Ser Arg Thr Asp Arg Asp Ile Lys Val Thr   90
                                                    Glu

CTA GTG TTT GAG CAT ATA GAC CAG GAC CTG AGG ACA TAC CTG GAC AAA GCA CCT  324
Leu Val Phe Glu His Ile Asp Gln Asp Leu Arg Thr Tyr Leu Asp Lys Ala Pro  108
                    Val

CCA CCG GGC CTG CCG GTT GAG ACC ATT AAG GAT CTA ATG CGT CAG TTT CTA AGC  378
Pro Pro Gly Leu Pro Val Glu Thr Ile Lys Asp Leu Met Arg Gln Phe Leu Ser  126
                    Ala                                                 Arg

GGC CTG GAT TTT CTT CAT GCA AAC TGC ATT GTT CAC CGG GAC CTG AAG CCA GAG  432
Gly Leu Asp Phe Leu His Ala Asn Cys Ile Val His Arg Asp Leu Lys Pro Glu  144

AAC ATT CTA GTG ACA AGT AAT GGG ACC GTC AAG CTG GCT GAC TTT GGC CTA GCT  486
Asn Ile Leu Val Thr Ser Asn Gly Thr Val Lys Leu Ala Asp Phe Gly Leu Ala  162
                        Gly

AGA ATC TAC AGC TAC CAG ATG GCC CTC ACG CCT GTG GTG GTT ACG CTC TGG TAC  540
Arg Ile Tyr Ser Tyr Gln Met Ala Leu Thr Pro Val Val Val Thr Leu Trp Tyr  180

CGA GCT CCT GAA GTT CTT CTG CAG TCT ACA TAC GCA ACA CCC GTG GAC ATG TGG  594
Arg Ala Pro Glu Val Leu Leu Gln Ser Thr Tyr Ala Thr Pro Val Asp Met Trp  198

AGC GTT GGC TGT ATC TTT GCA GAG ATG TTC CGT CGG AAG CCT CTC TTC TGT GGA  648
Ser Val Gly Cys Ile Phe Ala Glu Met Phe Arg Arg Lys Pro Leu Phe Cys Gly  216

AAC TCT GAA GCC GAC CAG TTG GGG AAA ATC TTT GAT CTC ATT GGA TTG CCT CCA  702
Asn Ser Glu Ala Asp Gln Leu Gly Lys Ile Phe Asp Leu Ile Gly Leu Pro Pro  234

GAA GAC GAC TGG CCT CGA GAG GTA TCT CTA CCT CGA GGA GCC TTT GCC CCC AGA  756
Glu Asp Asp Trp Pro Arg Glu Val Ser Leu Pro Arg Gly Ala Phe Ala Pro Arg  252
                        Asp                                     Pro

GGG CCT CGG CCA GTG CAG TCA GTG GTG CCA GAG ATG GAG GAG TCT GGA GCG CAG  810
Gly Pro Arg Pro Val Gln Ser Val Val Pro Glu Met Glu Glu Ser Gly Ala Gln  270

CTG CTA CTG GAA ATG CTG ACC TTT AAC CCA CAT AAG CGA ATC TCT GCC TTC CGA  864
Leu Leu Leu Glu Met Leu Thr Phe Asn Pro His Lys Arg Ile Ser Ala Phe Arg  288

GCC CTG CAG CAC TCC TAC CTG CAC AAG GAG GAA AGC GAC GCA GAG TGA 912
Ala Leu Gln His Ser Tyr Leu His Lys Glu Glu Ser Asp Ala Glu  .  303
                                    Asp     Gly Asn Pro      .
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Figure 1. Nucleotide and Predicted Protein Sequence of Mouse PSK-J3

Amino acid differences between the mouse and human proteins are indicated below the numbered lines. The Gly-X-Gly-X-X-Gly motif (residues 13–18), the PV/ISTVRE region (residues 50–56), and Thr-172 are underlined.

their growth in early G1 so that cells restimulated with the growth factor reenter the cell cycle synchronously and begin to replicate their cellular DNA 8–10 hr later (Tushinski and Stanley, 1985; Matsushime et al., 1991b). To isolate cDNAs that might encode catalytic partners of mammalian D type cyclins, a cDNA library prepared from the mRNA of BAC1.2F5A mouse macrophages synchronized in mid-G1 phase was screened with a human *cdk2* cDNA probe, and the nucleotide sequences of candidate clones were determined. We obtained cDNAs corresponding to murine *cdc2* and *cdk2*, as well as two clones predicted to encode more distantly related kinases, PCTAIRE-1 and PSK-J3. Full-length cDNAs of cdk3 and PCTAIRE-1 and -3 (which take their names from the amino acid sequences in the conserved PSTAIRE motif of cdc2) were isolated and supplied by colleagues (Meyerson et al., 1992; Okuda et al., 1992). Neither cdc2, cdk2, cdk3, nor PCTAIRE-1 and -3, when transcribed and translated in vitro, were found to form complexes with D type cyclins.

Human PSK-J3 was previously cloned from HeLa cells, based on its homology to mixed oligonucleotide probes representing highly conserved regions of other mammalian kinases (Hanks, 1987). The amino acid sequences of murine and human PSK-J3 (Figure 1) share features typical of other serine/threonine kinases, but the putative enzyme has not been found to exhibit histone H1 or casein kinase activity, either in specific immunoprecipitates from mammalian cell lysates or when expressed and recovered from bacteria (S. K. H., unpublished data). The predicted molecular mass of the PSK-J3 gene product is 33.7 kd, and its amino acid sequence is 95% identical to its human cognate (Figure 1) but shows only 44%, 48%, and 48% identity to human cdc2 (cdk1), cdk2, and cdk3, respectively (Meyerson et al., 1992). A Gly-X-Gly-X-X-Gly se-

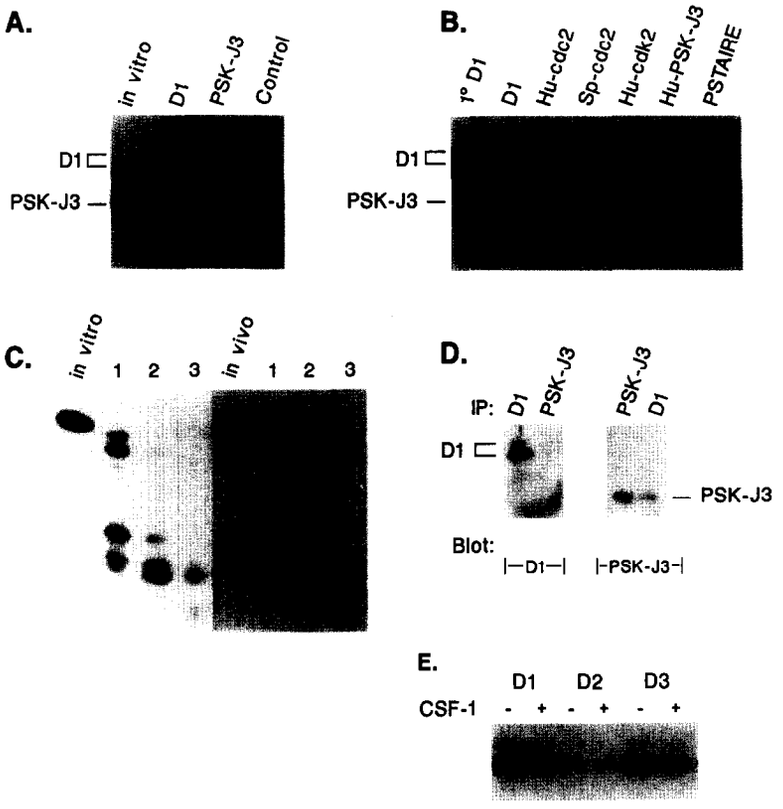

Figure 2. Immunologic and Biochemical Properties of PSK-J3

(A) The electrophoretic mobility of $p34^{PSK-J3}$ translated in vitro (lane 1) corresponds to that of the polypeptide immunoprecipitated from BAC1.2F5A macrophages (lane 3). Lane 2 defines the mobility of the cyclin D1 gene products ($p36^{D1}$ and a posttranslationally modified form of slower mobility collectively labeled D1) as well as the position of a coprecipitating p34 protein. Immunoprecipitation in lane 4 (Control) was performed with nonimmune serum. The autoradiographic exposure time was 2 days.

(B) Metabolically labeled primary cyclin D1 immunoprecipitates from BAC1.2F5 cells were dissociated in SDS, and equal aliquots were either left untreated (1° D1) or were reprecipitated with antisera to cyclin D1 (lane 2), human (Hu), S. pombe (Sp) $p34^{cdc2}$ (lanes 3 and 4), human $p33^{cdk2}$ (lane 5), human $p34^{PSK-J3}$ (lane 6), or anti-PSTAIRE (lane 7). The autoradiographic exposure time was 8 days.

(C) V8 protease digests of $p34^{PSK-J3}$ translated in vitro (left four lanes) versus those of the metabolically labeled p34 band coprecipitating with antiserum to cyclin D1 from BAC1.2F5A macrophages (in vivo, right four lanes) are shown. Lanes 1, 2, and 3 contained samples digested with 1/1000, 1/100, and 1/10 dilutions of protease stock solution; digestions were performed in situ in the gels. Exposure times were 2 hr (left lanes) versus 3 days (right lanes). In longer exposures, additional lower molecular weight bands of corresponding mobilities were detected in lanes 3.

(D) Immunoprecipitates (IP, noted at top) prepared with antisera to cyclin D1 (D1) or PSK-J3 were separated on gels and immunoblotted with the antisera indicated below the panels (Blot). Exposure time was 3 days.

(E) GST–cyclin D fusion proteins (D1, D2, or D3) adsorbed to glutathione–Sepharose beads were incubated for 30 min at 25°C with radiolabeled PSK-J3 translated in vitro in the presence of BAC1.2F5 cell lysates derived from CSF-1 stimulated (+) or 18 hr starved (−) cells. Radiolabeled proteins released from the washed beads were resolved on denaturing gels. Exposure time was 18 hr.

quence at residues 13–18 is followed by a Lys at codon 35, which is predicted to represent the site of ATP binding (Hanks et al., 1988). In cdc2, Thr-14 and Tyr-15 are known sites of inhibitory phosphorylation within the glycine-rich motif, and although these residues are each conserved in cdk2 and cdk3 (Meyerson et al., 1992), PSK-J3 contains Ala in place of Thr at residue 16 (Figure 1). PSK-J3 also lacks the PSTAIRE motif (single-letter amino acid code) characteristic of known cdks, but instead contains PV/ISTVRE at the corresponding position (residues 50–56). A threonine residue at codon 172 is analogous in position and context to Thr-161 of cdc2, whose phosphorylation is required for activation of the enzyme (Gould et al., 1991; Ducommun et al., 1991).

PSK-J3 Forms Complexes with Cyclin D1

Antisera to the cyclin D1 protein (designated $p36^{D1}$) precipitate two polypeptides of about 36 kd from metabolically labeled BAC1.2F5A macrophages (Figure 2A, lane 2), which are also detected after immunoblotting the denatured proteins (Matsushime et al., 1991b). Both forms of cyclin D1 are phosphoproteins, and the biochemical differences between them have not as yet been defined. The same antisera also coprecipitated an ~34 kd polypeptide from asynchronously proliferating cells (Figure 2A, lane 2) that is identical in electrophoretic mobility on SDS-containing gels to the major form of the PSK-J3 gene product transcribed and translated in vitro (lane 1) or precipitated from metabolically labeled BAC1.2F5A cells with antiserum to PSK-J3 (lane 3). The anti-PSK-J3 serum does not react with that fraction of $p34^{PSK-J3}$ that is bound to cyclin D1 (see below) and did not reciprocally precipitate 36 kd polypeptides that comigrated with cyclin D1 (lane 3).

When complexes precipitated with antiserum to cyclin D1 (Figure 2B, lane 1) were denatured in SDS, diluted in buffer, and reprecipitated with polyvalent antisera to cyclin D1 (lane 2), bacterially produced human cdc2 (lane 3), Schizosaccharomyces pombe cdc2 (lane 4), human cdk2 (lane 5), human PSK-J3 (lane 6), or anti-PSTAIRE (lane 7), the 34 kd polypeptide was quantitatively reprecipitated only by antiserum to PSK-J3. To establish that p34 was, in fact, the product of the *PSK-J3* gene and not an antigenically related but nonidentical protein, lysates of synchronized BAC1.2F5A macrophages labeled with [^{35}S]methionine during the late G1 interval were immunoprecipitated with antiserum to cyclin D1, and coprecipitating p34 was isolated on gels and subjected to digestion with V8 protease. Comparison of V8 digests of the p34 species recov-

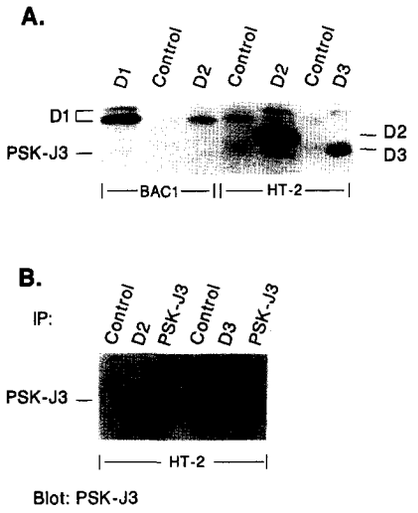

Figure 3. PSK-J3 Binds to Cyclin D1 in Macrophages and to Cyclins D2 and D3 in T Cells

(A) BAC1.2F5 cells or HT-2 T cells (as indicated below the plate) were metabolically labeled and immunoprecipitated with antisera to cyclins D1, D2, and D3, as indicated above the panel. Results with preimmune sera (Control) from rabbits immunized with either cyclin D2 or D3 are shown to the left of each lane containing D2 or D3 immunoprecipitates. Exposure time was 3 days.

(B) Immunoprecipitates prepared from HT-2 T cell lysates with the antisera designated above the panel (IP, top) were separated on gels and blotted with antiserum to PSK-J3. Exposure time was 3 days.

ered from in vivo complexes with cyclin D1 with those of authentic $p34^{PSK-J3}$ transcribed and translated in vitro showed that the two were identical (Figure 2C). Side-by-side comparison of V8 digests of $p34^{PSK-J3}$ (both the in vitro and in vivo products) and $p33^{cdk2}$ translated in vitro revealed that the two proteins yielded readily distinguishable peptide fragments (data not shown).

Figure 2D shows that when matched lysates of unlabeled, asynchronously proliferating BAC1.2F5A macrophages were precipitated with antiserum to either cyclin D1 (lanes 1 and 4) or PSK-J3 (lanes 2 and 3) and then immunoblotted with the same antiserum (lanes 1 and 3) or with the heterologous serum (lanes 2 and 4), $p34^{PSK-J3}$ was detected in anti-cyclin immunoprecipitates (lane 4). Comparison of lanes 3 and 4 suggested that a significant fraction of the immunoprecipitable PSK-J3 protein coprecipitated with antiserum to cyclin D1, initially suggesting that much of the PSK-J3 protein in cells might be bound to this cyclin. However, when lysates of BAC1.2F5A cells were either directly immunoprecipitated with antiserum to cyclin D1 or were first precleared with an excess of antiserum to PSK-J3 and then reprecipitated with anti-cyclin D1 serum, immunoblotting of the precipitated proteins with antiserum to human PSK-J3 revealed indistinguishable quantities of $p34^{PSK-J3}$ in the two cyclin D1 immunoprecipitates. Therefore, those PSK-J3 molecules that had bound to cyclin D1 were not removed by antiserum to $p34^{PSK-J3}$. Similar results were obtained with a second antiserum raised against the full-length mouse $p34^{PSK-J3}$ protein expressed in bacteria. In reciprocal experiments, no cyclin D1 coprecipitated with the antiserum to PSK-J3 (Figure 2D, lane 2; also see Figure 2A, lane 3), consistent with the interpretation that the antiserum reacted only with the unbound form of the enzyme.

Radiolabeled $p34^{PSK-J3}$, transcribed and translated in vitro in a reticulocyte lysate system, was able to bind to glutathione S-transferase (GST) fusion proteins prepared with D type cyclins (Figure 2E). Because the binding of cyclin A to $p34^{cdc2}$ requires posttranslational modifications that can occur in cell extracts, the in vitro binding reactions were carried out in the presence of lysates from macrophages that were either proliferating in the presence of CSF-1 (+ lanes) or growth arrested by CSF-1 starvation for 18 hr (− lanes). Under either condition, radiolabeled $p34^{cdc2}$ and $p33^{cdk2}$ did not bind to the D type cyclin fusion proteins but were able to form readily detectable complexes with GST–cyclin A (data not shown). Addition of cell lysates was required for the latter binding reactions, but they were not necessary to drive the in vitro associations of D type cyclins with PSK-J3. Therefore, phosphorylation at Thr-172 or other posttranslational modifications are either not prerequisites for binding or must occur in reticulocyte lysates. Both $p34^{cdc2}$ and $p33^{cdk2}$ also bound to S. pombe $p13^{suc1}$–Sepharose beads, whereas PSK-J3 did not (negative data not shown, but see Figure 6B below for confirmatory results), underscoring another difference in the properties of these proteins (also see Meyerson et al., 1992).

Radiolabeled $p34^{PSK-J3}$ bound more efficiently in vitro to cyclins D1 and D3 than to cyclin D2 (Figure 2E), suggesting that it might not be able to form complexes with cyclin D2 in vivo. Whereas BAC1.2F5A cells predominantly express cyclin D1, which can be immunoprecipitated either with antiserum to cyclin D1 or D2 (Figure 3A, lanes 1–3), cyclin D2 mRNA and protein are expressed at much lower levels (Matsushime et al., 1991a), and the protein cannot be detected using our standard metabolic labeling procedures (Figure 3A, lane 3). The fact that the antiserum to cyclin D2 cross-reacted with cyclin D1 in the same extract (lane 3) validated the negative result. Cyclin D3 mRNA is not expressed in macrophages but can be detected in many other cell types (Matsushime et al., 1991b; Inaba et al., 1992). Interleukin 2–dependent T lymphocytes express both cyclins D2 (lane 5) and D3 (lane 7); the bands in the 36 kd range in these lanes do not correspond to cyclin D1, whose mRNA is not expressed in HT-2 cells (Matsushime et al., 1991b). A 34 kd protein with the mobility of PSK-J3 was coprecipitated with antiserum to cyclin D2 (lane 5), but could not be observed in cyclin D3 immunoprecipitates, given the similar mobility of $p34^{D3}$ itself (lane 7). However, when cyclin D2 or D3 immunoprecipitates were separated on gels and blotted with antiserum to PSK-J3, we detected immunoreactive proteins of the appropriate mass (Figure 3B). Therefore, it seems likely that each of the D type cyclins has the potential to associate with $p34^{PSK-J3}$ in different cell types. The relatively weak PSK-J3 signals observed in Figure 3B, lanes 2 and 5, may reflect the fact that the formation of complexes between $p34^{PSK-J3}$ and cyclin Ds are periodic so that cyclin D2 and D3 precipitates might well contain more $p34^{PSK-J3}$ during certain phases of the cell cycle (see below). We do not exclude

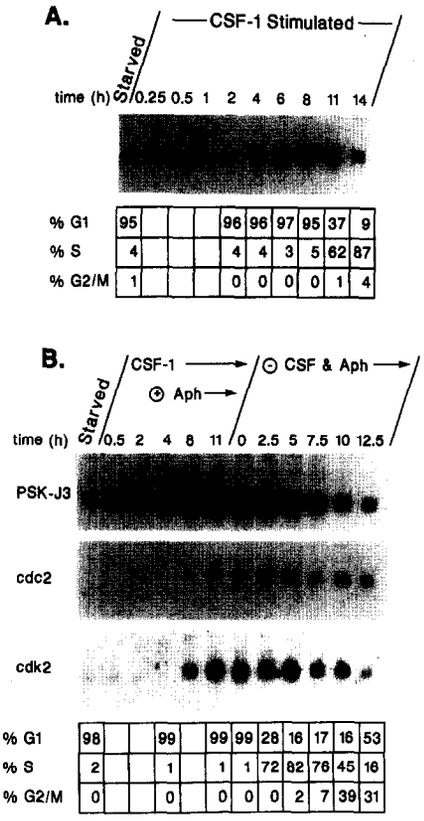

Figure 4. Cell Cycle Analysis of PSK-J3, cdc2, and cdk2 mRNAS in BAC1.2F5 Macrophages

(A) Cells were arrested by growth factor starvation for 18 hr (Starved) and then stimulated with CSF-1 for the indicated periods of time. Equal amounts of RNA extracted from the cells were separated on gels and analyzed by Northern blotting with a radiolabeled PSK-J3 cDNA probe. Exposure time was 3 days.

(B) Cells starved as above were stimulated with CSF-1 for 11 hr. Aphidicolin was added for the last 5 hr to rearrest the cells at the G1/S boundary. Both CSF-1 and aphidicolin were then removed, and the cells were allowed to complete the S, G2, and M phases in the complete absence of the growth factor. RNAs were harvested from parallel cultures at the indicated times and analyzed by sequential Northern blotting using full-length mouse PSK-J3, cdc2, and cdk2 cDNAs. Exposure times were 3 days (PSK-J3), 5 days (cdc2), and 7 days (cdk2), respectively. Hybridization was performed first with cdc2, next with cdk2, and last with the PSK-J3 probe, all of which were of similar complexity and specific radioactivity.

Cells from indicated samples from experiments shown in (A) and (B) were analyzed for DNA content by flow cytometry, and the percentage of cells in each phase of the cell cycle was calculated using a computer program.

the possibility that D type cyclins might interact with other kinase partners as well.

Formation of Complexes between Cyclin D1 and PSK-J3 Is Maximal Near the G1/S Boundary

Northern blotting analysis revealed that CSF-1-starved, G1-arrested BAC1.2F5 macrophages expressed a major 1.7 kb *PSK-J3* mRNA. When CSF-1-deprived cells were restimulated with the growth factor, the level of *PSK-J3* mRNA significantly increased as cells approached the G1/S boundary 8–10 hr later and then decreased as cells progressed into S phase (Figure 4A). However, metabolic labeling of the synchronized macrophages with [^{35}S]methionine during sequential 3 hr cell cycle intervals and immunoprecipitation of radiolabeled p34$^{PSK\text{-}J3}$ revealed that its apparent rate of synthesis did not increase as cells passed through G1 (negative data not shown). The discrepancy between this result and that predicted from Northern blot analysis might reflect cell cycle–dependent regulation of PSK-J3 degradation or, more simply, the failure of this antiserum to precipitate p34$^{PSK\text{-}J3}$ molecules that formed complexes with cyclin D1 (see above).

If CSF-1-stimulated cells were rearrested before S phase by addition of aphidicolin, an inhibitor of DNA polymerase, the levels of *PSK-J3* mRNA were maximal at the G1/S boundary but subsequently declined after aphidicolin and CSF-1 were removed (Figure 4B). After release of an aphidicolin blockade, the cells can complete division at the same rate either in the presence or absence of CSF-1, underscoring the requirement for the growth factor only during G1 (Matsushime et al., 1991a, 1991b). Under these conditions, however, *PSK-J3* mRNA levels during S phase decreased at a slower rate than that observed in non–drug-treated cells. Sequential hybridization of the same blot showed that *cdc2* and *cdk2* mRNAs were not highly expressed in CSF-1-starved cells but were induced just prior to S phase (Figure 4B). Note from the different autoradiographic exposure times in Figure 4B that the relative levels of *cdc2* and *cdk2* mRNAs were also lower than that of *PSK-J3*.

CSF-1-deprived, early G1-arrested BAC1.2F5A macrophages do not express D type cyclin mRNAs or proteins, but when restimulated to enter the cell cycle with CSF-1, cyclin D1 is detected within 2 hr of growth factor treatment, reaches a maximum after 4–6 hr, and remains elevated throughout the cell cycle as long as CSF-1 is present. Removal of CSF-1 is followed by the rapid degradation of cyclin D1 mRNA and protein, and cells that are not continuously stimulated for the entire G1 interval are unable to enter S phase (Tushinski and Stanley, 1985; Matsushime et al., 1991b). When CSF-1-deprived macrophages were restimulated with the growth factor and metabolically labeled with [^{35}S]methionine during subsequent 3 hr intervals, increasing amounts of radiolabeled p34$^{PSK\text{-}J3}$ were seen to coprecipitate with cyclin D1 as the cells progressed through G1, and maximal complex formation was detected at the G1/S transition and in early S phase (Figure 5A). Similar results were observed if unlabeled cyclin D1 immunoprecipitates were immunoblotted with antiserum to PSK-J3 (data not shown). Therefore, in spite of our failure to observe cell cycle–dependent changes in the rate of synthesis of p34$^{PSK\text{-}J3}$ in metabolic labeling experiments performed with an antiserum that detects only the unbound form of the protein, the accumulation of p34$^{PSK\text{-}J3}$ in complexes with cyclin D1 and its decline during S phase (Figure 5A) more closely correlated with its periodic mRNA expression (see Figure 4).

To study the stability of these complexes, growth-arrested cells were brought synchronously into cycle and metabolically labeled for 30 min with [^{35}S]methionine during the late G1 interval. The cells were then transferred to medium lacking the radiolabeled precursor, and parallel

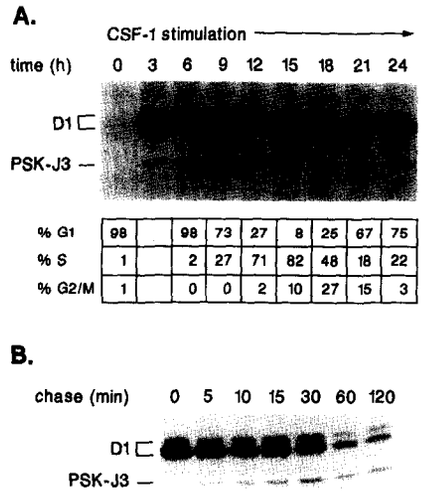

Figure 5. Cell Cycle Expression and Turnover of $p34^{PSK-J3}$ in Macrophages

(A) BAC1.2F5A cells starved for 18 hr were stimulated with CSF-1 for the indicated times and the percentage of cells in each phase of the cell cycle was determined by flow cytometric analysis of their DNA content. Cells labeled for sequential 3 hr intervals were harvested at the indicated times (noted above lanes), and complexes immunoprecipitated with antiserum to cyclin D1 were separated on a gel. Exposure time was 3 days.

(B) Cells starved for 18 hr were stimulated with CSF-1 for 6 hr until they were in mid-G1. Cultures were incubated for 30 min in methionine-free medium and then metabolically labeled for 30 min with [^{35}S]methionine. The medium containing the radiolabeled precursor was removed and replaced by fresh medium containing a 100-fold excess of unlabeled methionine for the indicated times. Cell lysates were immunoprecipitated with antiserum to cyclin D1, and the denatured complexes were separated on gels. Exposure time was 3 days.

cultures were lysed at various times during the "chase." Figure 5B shows that the initial half-life of bulk cyclin D1 was about 20 min, corresponding to similar turnover values obtained with unsynchronized cells or with those in mid-S phase (data not shown). However, radiolabeled PSK-J3 in these complexes appeared to be more stable. PSK-J3 molecules could have reassociated with unlabeled cyclin D1 synthesized during the chase period. Alternatively, if only a minority of cyclin molecules form complexes with $p34^{PSK-J3}$, this bound subpopulation might be protected from proteolytic degradation. Consistent with this interpretation, both forms of $p36^{D1}$ failed to turn over at the same rapid rate during the second hour of chase (Figure 5B).

Cyclin D Can Activate PSK-J3 Kinase Activity

Immune complexes prepared with antiserum to PSK-J3 or to cyclin D1 paradoxically lack detectable histone H1 or casein kinase activity (Matsushime et al., 1991b; S. K. H., unpublished data). As an alternative strategy for unmasking PSK-J3 kinase activity, we attempted to determine whether cyclin D–$p34^{PSK-J3}$ complexes formed in vitro might phosphorylate pRb or the pRb-related polypeptide, p107 (Ewen et al., 1991). The rationale for this assay stems from recent observations that cyclins D2 and D3, but not cyclin D1, can bind directly to GST–pRb and GST–p107 fusion proteins in vitro (M. E. E., C. J. S., H. M., H. K. Sluss, and D. M. Livingston, submitted). The interactions between cyclins D2 and D3 with pRb and p107 depend both upon the integrity of pRb and p107 sequences required for binding SV40 T antigen as well as upon the C-terminal domains of both proteins distal to the T antigen–binding "pocket." For example, a pRb pocket mutant (Cys-706 to Phe) that cannot bind T antigen and lacks growth-suppressive activity in vivo does not bind cyclins D2 or D3 with high affinity in vitro. Similarly, pRb (or p107) C-terminal deletion mutants that bind T antigen in vitro but are inactive as tumor suppressors in vivo (Qin et al., 1992) also fail to bind cyclins D2 and D3. Because only cyclins D1 and D3 bound $p34^{PSK-J3}$ with high affinity in vitro (see Figure 2E), and only cyclins D2 and D3 interact strongly with pRb under similar binding conditions (see above), stable ternary complexes should only be formed in vitro between cyclin D3, GST–pRb (or GST–p107), and $p34^{PSK-J3}$. Other combinations, including those with the GST–pRb and GST–p107 mutants and/or cyclins D1 and D2, should not efficiently yield such complexes.

Cyclins D1, D2, and D3, produced in insect cells using baculovirus vectors, were mixed with radiolabeled $p34^{PSK-J3}$ synthesized by translation in vitro. As controls, we used insect lysates infected with a wild-type baculovirus as well as reticulocyte lysates programmed with a brome mosaic virus mRNA. Various combinations of insect and reticulocyte extracts were then incubated with GST–pRb or GST–p107 fusion proteins adsorbed to glutathione–Sepharose beads, and proteins eluted from washed beads were electrophoretically separated on denaturing polyacrylamide gels. Staining of the gels with Coomassie brilliant blue confirmed that cyclins D2 and D3, but not cyclin D1, bound efficiently to GST–pRb or GST–p107 beads and that their binding was greatly diminished when the pRb Phe-706 or the p107 C-terminal truncation mutants were substituted for their wild-type counterparts (M. E. E., C. J. S., H. M., and D. M. Livingston, submitted). As predicted, [^{35}S]methionine-labeled $p34^{PSK-J3}$ bound efficiently to wild-type GST–pRb and GST–p107 fusion proteins in the presence of cyclin D3, but not cyclins D1 or D2, and its association was markedly decreased when pRb or p107 mutants that are unable to bind cyclin D3 were substituted (Figure 6A).

When complexes formed in vitro were incubated with [γ-^{32}P]ATP, pRb or p107 in ternary complexes with cyclin D3 and $p34^{PSK-J3}$ was phosphorylated at significantly higher levels relative to controls (Figure 6A). No such stimulation was observed when a reticulocyte lysate containing the brome mosaic virus protein was substituted for that containing $p34^{PSK-J3}$ (see Figure 6B below). Addition of histone H1 to complexes containing cyclin D3, PSK-J3, and GST–pRb yielded barely detectable levels of H1 kinase activity, about 10-fold less than that detected with the pRb and p107 mutant proteins; indistinguishably low levels of H1 kinase activity were detected when reticulocyte extracts lacking $p34^{PSK-J3}$ were substituted for those containing the transcribed enzyme (negative kinase data not shown). Moreover, a kinase-defective PSK-J3 mutant containing a methionine for lysine substitution at the predicted ATP binding site (codon 35) formed complexes with cyclin D3

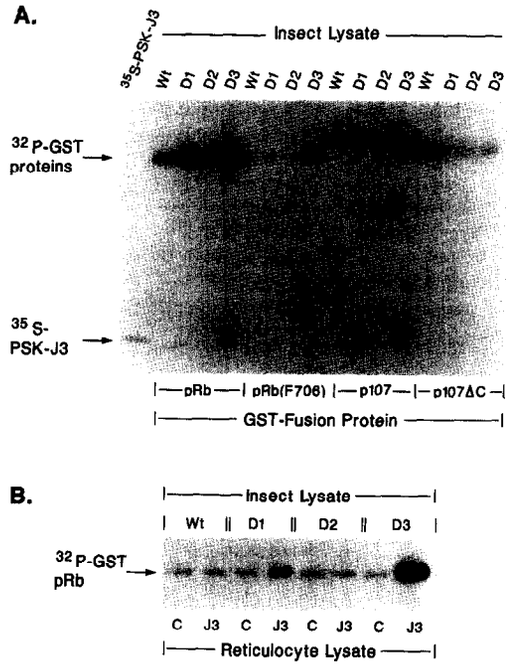

Figure 6. A Complex of PSK-J3 and Cyclin D3 Binds and Phosphorylates pRb and p107

(A) Lysates from insect cells infected either with a wild-type (Wt) baculovirus or with recombinants encoding the indicated D type cyclins (as labeled at the top of the panel) were mixed with [^{35}S]methionine-labeled p34^{PSK-J3} translated in vitro and with 0.5 μg of the indicated GST–pRb or GST–p107 proteins adsorbed to glutathione–Sepharose (labeled at bottom). After binding for 30 min at 25°C, the beads were washed and incubated at 30°C for 20 min with divalent cation and [γ-^{32}P]ATP. The products of the sequential binding and kinase reactions were then separated on SDS gels. The positions of the radiolabeled proteins are noted at the left of the panel. Exposure time was 20 min.

(B) Binding and kinase reactions were performed as in (A) with the indicated insect lysates (top, each containing ~2 μg of cyclin D per 0.25 ml) and with reticulocyte lysates (10 μl) programmed to translate either p34^{PSK-J3} or a control (C) brome mosaic virus protein (bottom). After mixing, the combined insect and reticulocyte extracts were precleared at 4°C using both p13^{suc1} beads and a C-terminally truncated GST–p107 fusion protein (0.5 μg) adsorbed to glutathione–Sepharose to remove adventitious kinase activity. The truncated GST–p107 protein does not specifically bind the PSK-J3–cyclin D3 complex (see [A]). GST–pRb-Sepharose (0.5 μg) was then added to the cleared lysates, and kinase reactions were performed as above. The radiolabeled pRb proteins were resolved on an SDS gel. Exposure time was 1 hr.

and pRb but was devoid of kinase activity in this assay (data not shown). Under these conditions, then, pRb and p107 appeared to be preferred substrates for the cyclin D–PSK-J3 kinase.

We reasoned that the relatively high background levels of kinase detected in these assays might be due to other cdks present in the reticulocyte or insect cell lysates, and so p13^{suc1} beads and the p107 C-terminal deletion mutant linked to glutathione–Sepharose were used to preclear the combined lysates prior to their incubation with GST–pRb. This significantly decreased the overall levels of background kinase activity but did not affect the intensity of the phosphorylation signal obtained with ternary complexes formed with cyclin D3, p34^{PSK-J3}, and pRb (Figure 6B; compare exposure times for Figures 6A and 6B). Under these conditions, uniformly and markedly lower levels of nonspecific kinase activity were obtained using insect lysates from cells infected with wild-type baculovirus or with recombinant viruses expressing cyclin D1 or D2. Similarly, reticulocyte lysates containing transcribed and translated brome mosaic virus protein were poorly active, even when cyclin D3 was present (Figure 6B).

To improve the specificity of the enzyme assay further, we produced murine p34^{PSK-J3} in insect cells and used it in lieu of the in vitro translated protein as a source of the enzyme. After mixing insect lysates producing the various D type cyclins with those containing equal quantities of PSK-J3, we incubated the different combinations either with GST–pRb beads or with a mutant GST–pRb protein (Cys-706 to Phe) that does not efficiently bind any of the D type cyclins. Following binding, the washed beads were assayed for kinase activity. Again, under these conditions, only complexes containing pRb, cyclin D3, and p34^{PSK-J3} showed high levels of in vitro kinase activity, whereas other combinations of reactants, including all those containing pRb (Phe-706), lacked significant kinase activity (Figure 7A). When the phosphorylated pRb protein was eluted from the gel and subjected to two-dimensional phosphoamino acid analysis, 84% of radiolabeled phosphate was incorporated into phosphoserine and the remainder into phosphothreonine. Thrombin cleavage of the radiolabeled fusion protein released an unlabeled, intact GST fragment and generated a series of pRb-derived phosphopeptides, thereby demonstrating that the sites of in vitro phosphorylation were confined to the pRb domain (data not shown). In agreement with these findings, when intact p105^{Rb} was immunoprecipitated from BAC1.2F5A lysates and the immune complexes were washed and incubated with insect lysates containing both cyclin D3 and p34^{PSK-J3}, but not either subunit alone, mouse pRb in the immune complexes was specifically phosphorylated (Figure 7B). Neither immunoglobulin heavy nor light chains were phosphorylated in the same reaction mixture. The ability of the cyclin D3–p34^{PSK-J3} complex to bind and phosphorylate pRb specifically in these assays therefore indicates that p34^{PSK-J3} functions as a bona fide cyclin D–dependent kinase. We suggest that cdk4 might be a more appropriate name for this enzyme.

Discussion

The existence of many cyclins and cdc2-related kinases and their ability to interact combinatorially with one another point to an unexpected degree of complexity in their potential to control cell cycle transitions. Although the complex of p34^{cdc2} and cyclin B appears to act as a regulator of mitotic entry and exit in all eukaryotic cells (Nurse, 1990), mammalian G1 cyclins have no exact cognates in yeast, and their appearance in higher eukaryotes may reflect levels of specialization inherent in more complex multicellular organisms. A property of the D type cyclins is that they are ubiquitously but differentially expressed in various cell types. For example, cyclins D1 and D2, but not D3, are synthesized in proliferating, CSF-1-dependent macrophages, whereas mature, interleukin 2–dependent

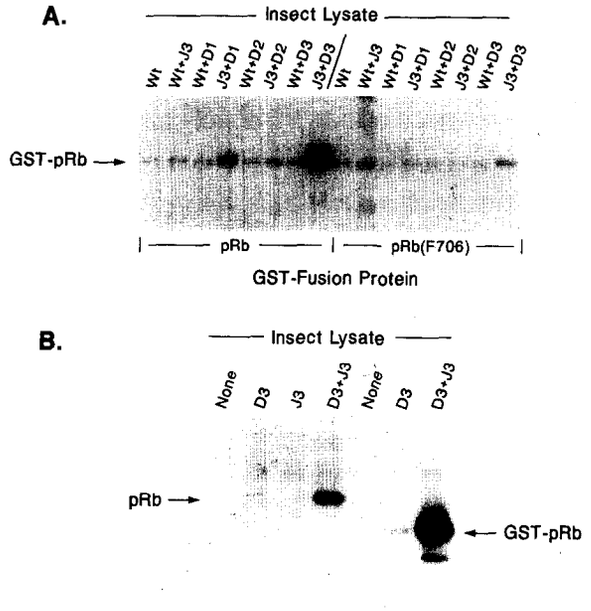

Figure 7. Recovery of Cyclin D3–p34^{PSK-J3} pRb Kinase Activity from Insect Cells

(A) Lysates of insect cells infected either with a wild-type (Wt) baculovirus or with recombinant vectors encoding D type cyclins or p34^{PSK-J3} were produced, and mixtures containing the indicated proteins (top; 0.25 μg of each in a total volume of 0.25 ml) were added to 0.5 μg of GST–pRb or GST–pRb (Phe-706) Sepharose beads. After incubation at 25°C for 30 min, the beads were washed and incubated at 30°C for 20 min in buffer containing divalent cation and [γ-^{32}P]ATP. Labeled products were electrophoretically separated on denaturing gels and detected by autoradiography (exposure time 40 min).

(B) Asynchronized BAC1.2F5A cells were immunoprecipitated with a mixture of two monoclonal antibodies (XZ133 and XZ104) directed to pRb (Hu et al., 1991), and the washed precipitates (four lanes at left) were incubated with 0.25 ml insect lysates (indicated at the top) containing cyclin D3 and/or p34^{PSK-J3} as in (A). The immunoprecipitates were washed, and after incubation with buffer containing divalent cation and [γ-^{32}P]ATP, pRb was resolved on a denaturing gel. As a control, 0.5 μg of GST–pRb was assayed in parallel (right two lanes). The exposure time was 90 min.

T cells express cyclins D2 and D3 but not D1. The regulation of cyclin D genes as part of the delayed early response to growth factor stimulation (Matsushime et al., 1991b) suggests that they may function to integrate extrinsic signals for growth and differentiation with the cell cycle regulatory machinery during the G1 interval.

The ability of the PSK-J3 kinase to interact with D type cyclins underscores further differences between these regulators and cyclins A and B. The known human cyclin-dependent kinases, p34^{cdc2} (cdk1), p33^{cdk2}, and p36^{cdk3}, can complement mutant *CDC28/cdc2* genes in yeast, but a series of less conserved *cdc2*-related genes, including PSSALRE, three PCTAIRES, and PLSTIRE, cannot (Meyerson et al., 1992). PSK-J3 (a "PV/ISTVRE" kinase) is more distantly related to p34^{cdc2} (44% overall amino acid identity) than are the latter enzymes and, as expected, does not complement mutant *cdc28* genes in S. cerevisiae (S. K. H., unpublished data). Moreover, whereas mammalian C, D1, and E type cyclins can rescue *CLN*-deficient yeast strains, cyclin D1 was the least active and, paradoxically, functioned less efficiently than cyclin B (Xiong et al., 1991; Lew et al., 1991; Koff et al., 1991). S. pombe p34^{cdc2} can also bind directly to another regulatory protein, p13^{suc1}, but PSK-J3 does not. However, critical sites of regulatory phosphorylation in p34^{cdc2}, including the counterparts of Tyr-15 and Thr-161, are conserved in position and context in PSK-J3, raising the possibility that its kinase activity might be subject to similar controls by other phosphatases and kinases. Although our results indicate that p34^{PSK-J3} can associate with the three different D type cyclins in vivo, we cannot exclude that complexes might also form with other protein kinase partners.

In contrast with mRNAs encoding D type cyclins, PSK-J3 mRNA is expressed in CSF-1-deprived, growth-arrested macrophages, and its level increases as cells progress through G1 and then declines during S phase. Under the same inductive conditions, cyclin D1 mRNA and protein levels are maximally expressed by mid-G1 and remain elevated as long as CSF-1 is present (Matsushime et al., 1991b). The induction of cyclin D1 precedes the markedly lesser increase in cyclin D2, which peaks at the G1/S boundary and then ebbs. The cell cycle–dependent association of cyclin D1–p34^{PSK-J3} complexes seen using antiserum to the cyclin mirrored *PSK-J3* mRNA expression. Although no increase in the rate of synthesis of p34^{PSK-J3} was observed when the enzyme was directly precipitated with an homologous antiserum, these antibodies did not recognize PSK-J3 in its bound form. Indeed, this probably accounts for our failure to detect either cyclin D1 or specific kinase activity in such precipitates. Like p34^{cdc2}, the steady-state abundance of p34^{PSK-J3} in macrophages might remain invariant, but its rates of synthesis and turnover could be cell cycle regulated (McGowan et al., 1990; Welch and Wang, 1992). In contrast, expression of the bulk regulatory cyclin D1 subunit pool is CSF-1 regulated and nonperiodic.

Because antiserum to PSK-J3 precipitated only the uncomplexed form of the protein, whereas antiserum to cyclin D1 precipitated only the bound species, immunoblotting of the p34^{PSK-J3} molecules recovered from both classes of precipitates provides a means of estimating the ratio of the bound and free catalytic subunits. A substantial fraction of p34^{PSK-J3} (estimated to involve more than 50% of the molecules detected during mid- to late G1) appeared to be complexed to cyclin D1. Kinetic labeling experiments performed during the late G1 interval when complex formation was maximal revealed that the majority of newly synthesized cyclin D1 molecules are rapidly degraded with a half-life of 20 min. However, the coprecipitating PSK-J3 molecules were more stable, suggesting either that labeled PSK-J3 subunits reassociated with unlabeled cyclins synthesized during the chase period or that the subpopulation of cyclin D1 that was bound to p34^{PSK-J3} was protected from proteolytic attack. Indeed, the turnover of labeled cyclin during the second hour of chase was significantly slower than that in the first, resulting in the progressive equalization of the prelabeled cyclin D1 and PSK-J3 molecules that were coprecipitated with anti-cyclin serum. If the pool of cyclin D1 that is larger and has more rapid

turnover represents a fraction that is unbound to catalytic subunits, the concentration of cyclin D1 may not be rate limiting for complex formation. In macrophages, both the rapidly and more slowly migrating forms of cyclin D1 undergo phosphorylation during G1, and the amount of the slower migrating species increases during this interval and decreases during S phase (Matsushime et al., 1991b). These posttranslational modifications might potentially contribute to complex formation in vivo.

Although cyclin D or PSK-J3 immunoprecipitates lack detectable histone H1 kinase activity, complexes of cyclin D3 and PSK-J3 can bind and phosphorylate pRb and p107 in vitro. The specificity of these reactions depended upon the preferential ability of GST–pRb and p107 fusion proteins to form complexes with cyclins D2 and D3 (M. E. E., C. J. S., H. M., and D. M. Livingston, submitted) as well as upon the relative propensity of cyclins D1 and D3 to bind PSK-J3 efficiently in vitro. Although immobilized pRb and p107 were far better substrates for the cyclin D3–$p34^{PSK\text{-}J3}$ complex than soluble histone H1, they need not be physiologic targets of the enzyme in vivo. Moreover, the fact that a biologically inactive pRb mutant was poorly phosphorylated may reflect its inability to bind to cyclin D3–$p34^{PSK\text{-}J3}$ rather than its true potential as an enzymatic substrate. Even so, the stability of ternary complexes helped physically to direct the kinase toward pRb. Recent experiments indicate that cotransfection of expression vectors encoding pRb and cyclin D2 into human Rb-negative Saos-2 osteosarcoma cells leads to pRb hyperphosphorylation, whereas transiently expressed pRb alone remains unphosphorylated (M. E. E., C. J. S., H. M., and D. M. Livingston, submitted). The biologically inactive pRb Phe-706 mutant, which was poorly phosphorylated by the cyclin D3–$p34^{PSK\text{-}J3}$ complex in vitro, is also a poor substrate for cyclin D2–induced phosphorylation in the Saos-2 system. Together, these data suggest that there may indeed be a physiological interplay between various cyclin D–$p34^{PSK\text{-}J3}$ complexes and pRb, a tumor suppressor protein whose phosphorylation appears necessary for the G1/S transition (Buchkovich et al., 1989; DeCaprio et al., 1989; Ludlow et al., 1990).

Experimental Procedures

cDNA Cloning of Murine PSK-J3

A cDNA library from synchronized mouse BAC1.2F5 macrophages in mid-G1 (Matsushime et al., 1991b) was screened under relaxed hybridization conditions with a full-length human *cdk2* cDNA probe isolated from a lymphoma cell cDNA library. Hybridization was performed for 40 hr at 37°C in buffers containing 35% formamide and 0.5 M Na^+, and filters were washed in 0.02 M Na^+ and 0.1% SDS at the same temperature. Two clones corresponding in nucleotide sequence to portions of human PSK-J3 (Hanks, 1987) were obtained, the longest of which was missing the N-terminal 70 amino acids of the predicted kinase. This *PSK-J3* cDNA was used as a probe to screen cDNA libraries from mouse NIH 3T3 fibroblasts, T cells, and Wehi-3B myeloid cells (Stratagene, La Jolla, California) for additional clones, and authentic *PSK-J3* cDNAs were isolated from each. A 1.4 kb clone containing the complete PSK-J3 coding sequence was isolated from Wehi-3B cells. DNA sequences were determined by dideoxynucleotide chain termination (Sanger et al., 1977) using double-stranded plasmid DNA templates.

Cell Culture and Cell Cycle Analysis

Murine BAC1.2F5A macrophages were grown in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum, glutamine, antibiotics, and 25% L cell conditioned medium as a source of CSF-1. Mouse HT-2 T cells were propagated in complete medium containing interleukin 2. Spodoptera frugiperda (Sf9) cells were cultured at 27°C in Grace's medium supplemented with 10% fetal calf serum, yeastolate, lactalbumin hydrolysate, and gentamicin. BAC1.2F5A cells were arrested in early G1 by CSF-1 starvation for 18 hr. Where indicated, cells stimulated to advance synchronously through the cell cycle were rearrested at the G1/S boundary by addition of 25 μg/ml aphidicolin. Cell cycle analysis was performed by flow cytometric measurement of DNA content, and the percentages of cells within the G1, S, and G2/M phases were determined with the computer program PEAK as described previously (Matsushime et al., 1991b).

Antisera to $p34^{PSK\text{-}J3}$, $p35^{D2}$, and $p34^{D3}$

Antisera to the complete coding sequences of cyclins D2 and D3 were prepared using GST fusion proteins as previously described (Matsushime et al., 1991b). Bacterially produced recombinant proteins were purified by elution from SDS–polyacrylamide gels after cleavage of the GST moiety by factor Xa and were used for immunization of rabbits. cDNAs encoding the proteins were inserted into pBluescript and transcribed and capped in vitro (Stratagene Transcription System). Following treatment of the products with DNAase I, extraction with phenol–chloroform, ethanol precipitation, and resuspension, 0.5 μg of each mRNA was used as a template for in vitro translation in a rabbit reticulocyte system (Promega). Unique [^{35}S]methionine-labeled D type cyclins synthesized in each reaction were immunoprecipitated with different antisera. The antisera to cyclins D1 and D2 cross-reacted with the two proteins, but neither reacted with cyclin D3; antiserum to cyclin D3 did not precipitate cyclins D1 or D2. Antiserum to PSK-J3 was prepared by immunizing rabbits with a bacterially produced TrpE fusion protein containing the C-terminal half of the human protein (amino acid residues 140–303). Radiolabeled mouse $p34^{PSK\text{-}J3}$ was precipitated by antiserum to its human cognate, but not by antisera raised to S. pombe or human cdc2 or by an antiserum to the human cdk2 C-terminus (the latter generously supplied by Dr. David Morgan, University of California, San Francisco). Reciprocally, the antiserum to PSK-J3 did not react with radiolabeled $p34^{cdc2}$ or $p33^{cdk2}$. Monoclonal antibodies XZ133 and XZ104 to pRb (Hu et al., 1991) were generously provided by Dr. Edward Harlow. Rabbit immune immunoglobulin G to a PSTAIRE peptide was purchased from Upstate Biotechnology Incorporated (Lake Placid, New York).

Metabolic Labeling, Immunoprecipitation, and Peptide Mapping

Subconfluent adherent BAC1.2F5 cells in 100 mm diameter dishes were starved for 30 min in 3 ml of methionine-free medium and metabolically labeled for the indicated times with 200 μCi/ml [^{35}S]methionine (1000 Ci/mmol; Amersham, Arlington Heights, Illinois). Cleared cell lysates were immunoprecipitated with rabbit antisera, and radiolabeled proteins separated on denaturing polyacrylamide gels containing SDS were detected by autofluorography of the dried gel slabs (Anderson et al., 1984). In some experiments, immunoprecipitates prepared with antisera to PSK-J3 or to D type cyclins were immunoblotted with either the same or another antiserum at 1:150 to 1:300 dilution, and sites of antibody binding were detected with ^{125}I-labeled Staphylococcus aureus protein A (Amersham) (Downing et al., 1988).

For peptide mapping of $p34^{PSK\text{-}J3}$, CSF-1-starved BAC1.2F5A cells were stimulated with CSF-1 for 7 hr to advance them synchronously into G1 phase, and the cultures were starved in methionine-free medium for 30 min and labeled for 2 hr with 500 μCi/ml [^{35}S]methionine (2 ml per 100 mm diameter dish). Cell lysates were immunoprecipitated with antiserum to cyclin D1, and radiolabeled p34 was separated on a 10% polyacrylamide gel containing SDS and visualized by autoradiography of the unfixed gel. Authentic $p34^{PSK\text{-}J3}$ prepared by translation in vitro was purified in parallel. Gel slices containing individual p34 bands from each lane were excised and reinserted into individual wells of a stacking gel overlaying an SDS-containing 15% polyacrylamide slab. The dried gel slices were allowed to swell for 10 min in situ and overlaid with 20 μl of 0.125 M Tris–HCl (pH 6.8), 0.1% SDS, 1 mM EDTA, 20% glycerol, and 2.5 mM dithiothreitol. This solution was in turn overlaid with 10 μl of the same buffer containing 10% glycerol and a 1/10, 1/100, or 1/1000 dilution of a 10 mg/ml aqueous stock solution

of endoproteinase Glu-C (Boehringer Mannheim). Electrophoresis was performed at 20 mA, and fixed dried gels were processed for autofluorography as above.

Production of D Type Cyclins in Insect Cells

BamHI fragments containing complete mouse cyclin D or PSK-J3 coding sequences were inserted into the BamHI cloning site of the baculovirus transfer vector, pAcYM1 (Matsuura et al., 1987). Vector plasmids were cotransfected into Sf9 cells together with Autographa californica nuclear polyhedrosis virus DNA (Summers and Smith, 1987). Four days later, virus-containing supernatants were harvested, and recombinant viruses were isolated after three successive rounds of plaque purification. Sf9 cells infected with viruses were assayed for expression of recombinant protein by labeling with [^{35}S]methionine or by immunoblotting. Recombinant viruses producing D type cyclins were expanded by reinfection. For routine production of lysates containing the recombinant proteins, 8×10^6 Sf9 cells were infected at a multiplicity of 30, lysed 48 hr postinfection with 1 ml of EBC buffer (50 mM Tris–HCl [pH 8.0], 120 mM NaCl, 0.5% Nonidet P-40) containing 5 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 0.1 mM sodium fluoride, 10 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM EDTA, and 1 mM dithiothreitol, and taken for binding and kinase assays. The quantities of D type cyclins and p34$^{PSK\text{-}J3}$ were estimated by Coomassie brilliant blue staining of the electrophoretically separated proteins by comparison with protein standards of known concentration. The concentrations of proteins used in the various kinase assays are indicated in the figure legends.

In Vitro Binding and Kinase Assays

Bacterial GST expression vectors encoding pRb, p107, or mutant derivatives (pRb Phe-706 and a C-terminally truncated p107 protein missing residues 817–936 [Ewen et al., 1991]) were introduced into Escherichia coli strain BL21 (pLysS), and bacterial stock cultures grown overnight in LB broth containing ampicillin were diluted 1:10 in 100 ml of fresh broth and grown for 90 min at 37°C. Exponentially growing cells were induced for 2.5 hr with isopropyl-β-D-thiogalactopyranoside, pelleted at 4°C, suspended in 5 ml of TNEN buffer (20 mM Tris–HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40), and disrupted by sonication. After centrifugation to remove debris, supernatant fluids were diluted with 1 vol of TNEN buffer and divided into 1 ml aliquots. Glutathione–Sepharose beads (1.0 ml; Pharmacia) were washed three times in 5 ml of TNEN containing 40 mg/ml bovine serum albumin and suspended in an equal volume of the same buffer. Washed beads (30 μl) were added to each 1 ml bacterial supernatant and incubated for 30 min at 4°C on a rotating stirrer. The beads were then washed three times with low salt TNEN (TNEN containing 100 mM NaCl) and suspended in an equal buffer volume. [^{35}S]methionine-labeled PSK-J3 transcribed in vitro (see above) (10 μl) was combined with 0.25 ml of insect cell lysate containing D type cyclin (~1–2 μg) and 30 μl of GST–pRb or GST–p107 Sepharose (~0.5 μg of protein) and incubated at 25°C for 30 min with occasional shaking. The beads were washed four times with low salt TNEN, denatured in gel sample buffer, and separated on 10% polyacrylamide gels containing SDS.

For determination of kinase activity, complexes bound to Sepharose beads and washed as described above were washed once more with 50 mM HEPES (pH 7.5) and suspended in 20 μl of the same buffer containing 10 mM $MgCl_2$, 1 mM dithiothreitol, 25 μM unlabeled ATP, and 10 μCi of [γ-^{32}P]ATP (6000 Ci/mmol, New England Nuclear). In some experiments, 0.5 mg/ml histone H1 was added as substrate. The samples were incubated for 20 min at 30°C (with linear kinetics of incorporation during the interval), denatured in SDS sample buffer, and applied to polyacrylamide gels. To eliminate background kinase activity contaminating both the unprogrammed insect and reticulocyte lysates, [^{35}S]methionine-labeled translated proteins (10 μl) were mixed as above with 0.25 ml of insect lysate, and samples were first precleared by addition of 30 μl of p13^{suc1} beads (Oncogene Sciences) and 30 μl of glutathione–Sepharose beads containing the p107 C-terminally truncated protein, both of which fail specifically to bind either D type cyclins or p34$^{PSK\text{-}J3}$. Lysates were incubated at 4°C for 90 min, and the beads were removed by centrifugation. The supernatants were then incubated with GST–pRb–Sepharose and assayed for kinase activity as described above.

Acknowledgments

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GenBank Accession Numbers

The accession numbers for mouse cyclins D2 and D3 are M86182 and M86183, respectively. The accession number for mouse cdk4/PSK-J3 is L01640.

Note Added in Proof

Repeated immunization of rabbits with full-length mouse p34$^{PSK\text{-}J3/cdk4}$ produced in bacteria has now yielded antisera that coprecipitate cyclin D1 from BAC1.2F5A macrophage cell lysates. The molar ratio of p34$^{PSK\text{-}J3/cdk4}$ to p36^{D1} in these complexes is approximately 1:1.

When insect lysates containing p34$^{PSK\text{-}J3/cdk4}$ and either cyclins D1, D2, or D3 were incubated directly with GST–pRb and [γ-^{32}P]ATP in a kinase reaction, GST–pRb was phosphorylated by the catalytic subunit in association with any of the three D type cyclins. Therefore, the specific requirement for cyclin D3 observed in Figures 6 and 7 reflects its preferential capacity to form stable complexes in vitro with both pRb and p34$^{PSK\text{-}J3/cdk4}$ and not the inability of cyclins D1 or D2 to activate this catalytic subunit.