

The Amino Terminus of the Retinoblastoma (Rb) Protein Associates with a Cyclin-dependent Kinase-like Kinase via Rb Amino Acids Required for Growth Suppression¹

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Abstract

We have shown previously that a novel cell cycle-regulated histone H1 kinase activity, retinoblastoma kinase (RbK), associates with and phosphorylates the amino terminus of the Rb protein in G₂-M. We have shown also that the amino terminus of p107, a Rb-related protein, does not associate with a similar kinase *in vitro* or *in vivo*. Here, we report that a RbK-like kinase associates with the amino terminus of p130, another Rb-related protein, only marginally. Moreover, the association of RbK with Rb *in vitro* is shown to require a discrete portion of the Rb amino terminus, amino acids 89–202. This region has been shown previously to be subject to inactivating mutations in retinoblastoma and to be required for Rb-mediated growth suppression *in vitro*. Taken together, these data indicate that the formation of Rb-RbK complexes may play an important role in Rb-mediated growth suppression. We have mapped two *in vitro* sites of Rb phosphorylation by RbK to sites that are phosphorylated *in vivo* and are targets of cyclin-dependent kinase phosphorylation *in vitro*. As such, at least some sites of RbK phosphorylation overlap with those of other proline-directed serine and threonine kinases. Consistent with this latter observation, we report that the *trans*-activation domain of *c-myc* is phosphorylated specifically by RbK *in vitro* at a site (serine 62) that is phosphorylated *in vivo* during G₂-M, cell-cycle phases in which RbK activity is maximal.

Introduction

Functional inactivation of the retinoblastoma gene *Rb-1* is associated with the genesis of a variety of human neoplasias, including retinoblastoma, osteosarcoma, and small cell lung, bladder, and breast carcinomas (1–5). The *Rb* gene spans approximately 200 kilobase pairs of DNA within the q14 band of human chromosome 13 (6–8). *Rb-1* encodes a family of ubiquitously expressed nuclear phosphoproteins (termed p105-Rb) that differ from one another by their extent of posttranslational modification (4, 9). The amino acid sequence of p105-Rb has been well conserved throughout vertebrate evolution, and *Rb-1* has been shown to be a member of a family of related genes that includes at least two additional members, *p107* and *p130* (10–14). Although mutational inactivation of *p107* and *p130* has not been associated with the etiology of human cancer as yet, *in vitro* analyses indicate that these Rb-related genes can function as negative regulators of tumor cell growth (15–18).

The Rb protein is phosphorylated in a cell cycle-regulated manner (19–24). In quiescent and senescent cells or cells in early to mid-G₁, the vast majority of p105-Rb is unphosphorylated. In synchronously growing cells, Rb is phosphorylated prior to the onset of DNA synthesis and is subject to additional phosphorylations as cells progress through S-phase and G₂. Rb is dephosphorylated abruptly at the end of mitosis, probably by a type I protein phosphatase activated in anaphase. p105-Rb has been shown to be associated with and phosphorylated by the cyclin and cdk⁴ families of cell cycle-regulated kinases (for review, see Ref. 25). These include the G1 cyclins, cyclins D and E, and the mitotic cyclin, cyclin A, in conjunction with cdk1 (*cdc2*), cdk2, and cdk4. Recent evidence suggests that Rb participates with the D-type cyclins in an important checkpoint in mammalian G₁ that may be functionally akin to “start” in yeast cells (25–27). Disruption of this checkpoint via Rb inactivation or deregulation of G1 cyclin and cdk activity is associated with precocious S-phase entry and tumorigenesis. However, Rb may regulate the progression of cells through additional cell-cycle checkpoints negatively as overexpression of Rb in synchronous populations of S-phase cells leads to growth arrest in G₂ (28).

Molecular analyses of Rb alleles carried by human tumor cells indicate that the carboxyl-terminal two-thirds of the Rb protein is a frequent target of mutational inactivation (4, 5, 29–36). Rb exons 13–22 have been shown to be subject to

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⁴ The abbreviations used are: cdk, cyclin-dependent kinase; RbK, retinoblastoma kinase; GST, glutathione S-transferase; MAP, mitogen-activated protein; cdc, cell division cycle, erk, extracellular signal-regulated kinase.

point mutations that result in missense substitutions or splicing aberrations that delete entire exons. These data imply that this carboxyl-terminal portion of p105-Rb contains at least one significant domain of Rb function. Indeed, subsequent analyses have determined that this portion of the Rb protein, referred to widely as the Rb "pocket," is a site of interaction of Rb with a variety of cellular proteins that are either regulators (e.g., cyclins and cdk) or targets (e.g., transcription factors) of Rb function (37–42). In addition to mutational inactivation, this portion of Rb is also a target of inactivation via complex formation with viral oncoproteins, such as adenovirus E1A, SV40 large-T antigen, and human Papillomavirus E7 (43–46). The binding of Rb by these viral proteins blocks its capacity to interact with its natural effectors, leading to deregulated cell growth and virus-induced transformation.

Rb is believed to regulate cell cycle progression at least in part through its physical or functional interaction with a variety of transcription factors, including E2F1–3, myoD, Elf-1, Sp1/Sp3, c-myc, and ATF-2 (47–55). The functional interaction of Rb with transcription factors can lead to transcriptional repression (e.g., E2F1–3 and Elf-1) or the stimulation of transcription (e.g., Sp1/Sp3, and ATF-2). As might be predicted given the role of *Rb* as a tumor-suppressor gene, the transcriptional activity of genes associated with cell proliferation has been shown to be inhibited by Rb whereas genes associated with growth inhibition and/or cell differentiation are often stimulated by Rb. Whether Rb limits cell proliferation via additional mechanisms remains to be determined.

Consistent with the proposition that the Rb pocket is required for Rb-mediated growth suppression, microinjection of recombinant partial Rb proteins or exogenous expression of the Rb pocket has been demonstrated to be sufficient for the inhibition of cell cycle progression of some Rb-negative tumor cells (56–58). Yet, portions of Rb outside the Rb pocket have been shown recently to play a significant role in tumor suppression, *in vitro* Rb-mediated growth control, and the regulation of the transcriptional activity of one target of Rb function, Sp1 (52, 59–61). For example, Dryja *et al.* (59) and Hogg *et al.* (60) have identified retinoblastoma patients that carry mutated *Rb-1* alleles that have suffered discrete inactivating mutations within the Rb amino terminus. Inheritance of such alleles has been shown to lead to both bilateral and unilateral tumors, albeit with low penetrance. Qian *et al.* (61) created and analyzed a panel of internally deleted Rb cDNAs carrying nonoverlapping deletions throughout the Rb coding sequence. Transfection of these constructions into SAOS-2 cells, a human osteosarcoma cell line that expresses a defective Rb protein, enabled these investigators to determine that deletions within the Rb pocket as well as deletions within the Rb amino terminus abrogate Rb-mediated growth suppression. Importantly, these functionally defective, amino-terminal mutants were shown to have lost the ability to suppress SAOS-2 growth *in vitro*, despite the continued formation of physical complexes of Rb with important targets of Rb function, such as E2F. Finally, Udvadia *et al.* (52) reported, that in addition to mutations within the Rb pocket, a discrete portion of the Rb amino terminus is also required for functional interactions of Rb with Sp1, a tran-

scription factor for which *trans*-activation is stimulated ("superactivated") by Rb coexpression. Taken together, these data argue strongly that the Rb amino terminus plays an important role in mediating and/or regulating Rb function.

Given that the Rb amino terminus contributes to Rb-mediated growth suppression, Sterner *et al.* (62) sought to identify and characterize cellular proteins that interact with this portion of human and mouse Rb specifically. In so doing, they showed that a set of cellular proteins, including a novel cell cycle-regulated serine/threonine kinase termed RbK, associated specifically with this portion of Rb. RbK was shown: (a) to associate with Rb *in vitro* and *in vivo*; (b) to be biochemically and immunochemically distinct from known Rb-associated kinases; and (c) to be a potent Rb and histone H1 kinase *in vitro*. Interestingly, the formation of Rb-RbK complexes and/or RbK activity was shown to be restricted to post-S-phase cells, with maximal levels of RbK activity detected in early G₂ through metaphase. In contrast, a kinase with similar properties was not detected in association with the amino terminus of p107. In this report, we extend our analyses of Rb-RbK complexes and RbK activity. We show that the amino terminus of p130 associates with histone H1 kinase activity marginally *in vitro*, and we have defined a portion of the Rb amino terminus, amino acids 89–149, which is necessary and sufficient for association with RbK. Moreover, we have mapped two sites of Rb phosphorylation by RbK and show that these sites are coincident with sites of phosphorylation *in vivo* and sites that are phosphorylated by cdk *in vitro*. Finally, we report that the *trans*-activation domain of c-myc, a Rb-binding protein, is a target of RbK phosphorylation *in vitro* at a site that is phosphorylated *in vivo* in a cell cycle-dependent manner.

Results

The Amino Termini of Rb, p107, and p130 Are Distinguished by Their Capacity to Associate with and Be Phosphorylated by at Least One Mitotic Histone H1 Kinase (RbK). We have characterized previously a cell cycle-regulated Rb and histone H1 kinase activity (RbK) that associates specifically with the amino termini of human and mouse Rb proteins in G₂-M (62). In doing so, we have demonstrated that: (a) RbK is a Rb-associated kinase that is distinct from previously described Rb kinases, such as G1 and mitotic cdk kinases; and (b) the amino terminus of p107, a Rb-related protein, does not associate with a similar kinase *in vitro* or *in vivo*. To determine whether the amino terminus of p130, another Rb-related protein, associates with a RbK-like activity *in vitro*, we prepared a GST-p130 fusion protein and assessed its capacity to associate with protein kinases in an *in vitro* protein-binding and kinase assay in parallel with similar fusion proteins carrying the amino termini of Rb and p107. Whole-cell extracts prepared from metaphase-arrested ML-1 human myeloid leukemia cells were first pre-cleared of GST-binding proteins by incubation with GST-bound glutathione-Sepharose beads and then depleted of mitotic cyclins and cdk by incubation with an excess of p13^{SUC}-agarose beads. Depleted extracts were then incubated with glutathione-Sepharose beads bound with equal amounts (5 μg; Fig. 1) of GST-Rb, GST-p107, or GST-p130

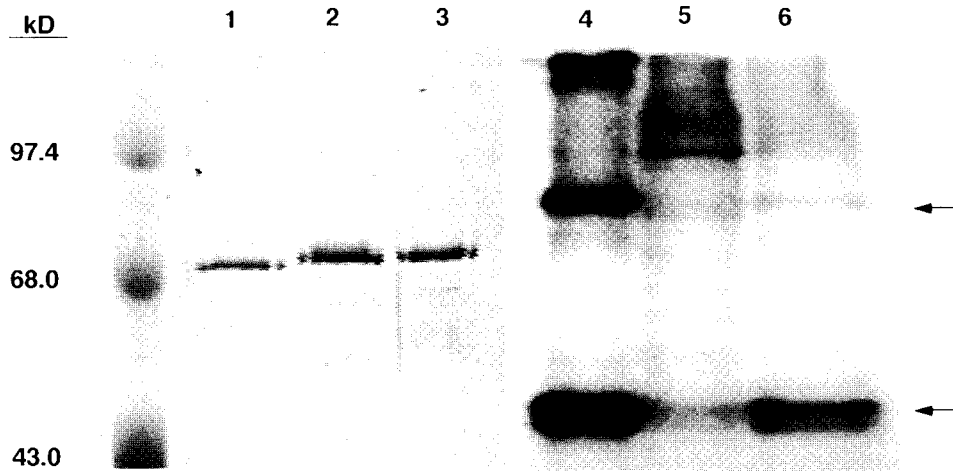


Fig. 1. Differential association and phosphorylation of Rb-related proteins and histone H1 by RbK activity. *Left*, Coomassie blue-stained polyacrylamide gel of purified amino-terminal GST fusion proteins used for the *in vitro* protein binding and kinase assay shown in the *right panel*. Five micrograms GST-Rb (*Lane 1*), GST-p107 (*Lane 2*), or GST-p130 (*Lane 3*) fusion proteins were applied to each lane of a 4/8% acrylamide gel. Molecular weight markers are indicated on the *left*. *Right*, *in vitro* protein binding and kinase assay using the amino termini of Rb, p107, and p130. Mitotic ML-1 extracts were precleared of GST- and p13^{SUC}-binding proteins and then incubated with equivalent amounts (5 μ g) of GST-Rb (*Lane 4*), GST-p107 (*Lane 5*), or GST-p130 (*Lane 6*) fusion proteins. Following extensive washing and the addition of histone H1, kinase assays were performed as described previously (62). Resulting radiolabeled proteins were resolved on a 4/8% acrylamide gel that was dried subsequently and exposed to film for 1.5 h at -80°C . Arrows indicate the positions of radiolabeled GST-fusion proteins (*upper*) and histone H1 (*lower*).

amino-terminal fusion proteins. Following extensive washes to remove loosely associated proteins, bead-bound kinase activity was detected in an *in vitro* kinase assay using histone H1 as substrate. As we have demonstrated previously (62), at least one potent histone H1 and Rb kinase, RbK, associates with GST-Rb beads (Fig. 1). As shown in Fig. 1, consistent with our previous results, an analogous portion of the p107 amino terminus did not associate with detectable amounts of p13^{SUC}-insensitive histone H1 kinase activity and was not itself phosphorylated *in vitro*. In parallel assays, minimal amounts of histone H1 kinase activity were detected in association with GST-p130 beads, and p130 itself was phosphorylated negligibly *in vitro*. In contrast to similar assays using GST-Rb and GST-p107, we have noted that the abundance of GST-p130-associated histone H1 kinase activity is quite variable in metaphase ML-1 extracts, often being completely undetectable. We have not determined the basis for this variation in p130-associated histone H1 kinase activity as yet. Nonetheless, it is readily apparent in Fig. 1 that RbK-like kinases associate with and phosphorylate the amino termini of Rb, p107, and p130 with distinctly different efficiencies. We presume that the differential association of RbK with Rb, p107, and p130 reflects biochemical and perhaps functional differences between their respective amino termini.

A Discrete Region of the Rb Amino Terminus That Is the Target of Loss-of-Function Mutations in Retinoblastoma Is Required for the Recovery of Wild-Type Levels of RbK Activity. Given that RbK associates with the amino terminus of Rb, we wished to delimit Rb amino acids that are necessary for their association *in vitro*. In doing so, we hoped to determine whether the association of Rb with RbK requires Rb amino acids that are deleted or are targets of mutation in retinoblastoma and/or that are necessary for Rb-mediated growth suppression *in vitro*. Additionally, our *in*

Table 1 Relative RbK histone H1 kinase activity recovered using wild-type and mutated amino-terminal human GST-Rb fusion proteins in *in vitro* protein binding and kinase assays

GST-Rb fusion protein ^a	Relative recovered RbK histone H1 kinase activity (%) ^b
Wild-type Rb	
1-485	100
Mutated Rb	
$\Delta 89-140$	0
$\Delta 128-167$ (exon 4)	37
$\Delta 140-202$	30
$\Delta 202-249$	100
$\Delta 309-343$	72
$\Delta 343-389$	100

^a A wild-type GST-Rb fusion protein prepared from the first 485 amino acids of a human Rb cDNA has been described previously (62). Similar fusion proteins carrying deletions of the indicated amino acids were prepared as described in "Materials and Methods."

^b *In vitro* protein-binding and kinase assays were performed as described previously (62) using identical amounts (5 μ g) of wild-type and mutated GST-Rb fusion proteins, metaphase ML-1 extracts, and histone H1 as kinase substrate. Following resolution of kinase assays on SDS-PAGE gels, radiolabeled histone H1 was excised and quantified by scintillation counting. Relative recovered RbK activity of mutated GST-Rb fusion proteins was calculated in comparison with that recovered using wild-type GST-Rb (set to 100%). Results shown are mean values obtained from three to five independent *in vitro* protein-binding and kinase assays.

in vitro protein-binding and kinase assays suggested that the preferential association of RbK with Rb should be a consequence of one or more primary amino acid sequences that are divergent within the amino termini of p107 and p130.

In rare instances, mutations within the Rb amino terminus predispose individuals to tumorigenesis. Dryja *et al.* (59) and Hogg *et al.* (60) have identified retinoblastoma patients within two distinct families that are predisposed to tumor development due to the inheritance of defective *Rb-1* alleles carrying

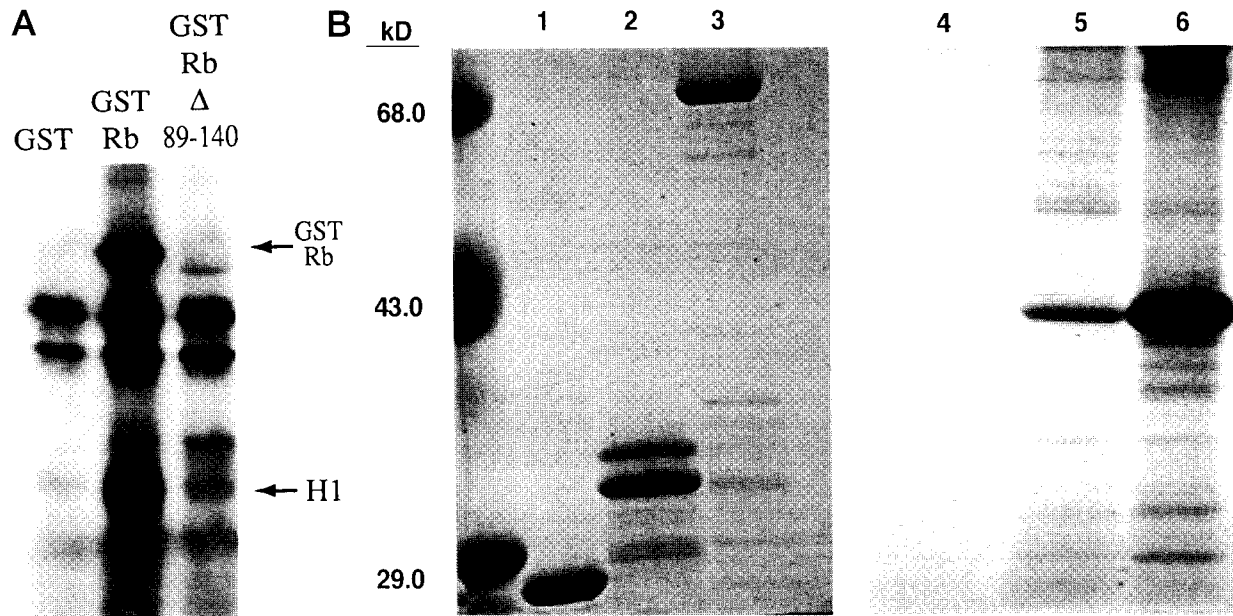


Fig. 2. Identification of Rb amino acids that are necessary and sufficient for association with RbK *in vitro*. A, absence of RbK activity in protein-binding and *in vitro* kinase assays using a mutated GST-Rb protein lacking Rb amino acids 89–140. Mitotic A549 extracts were precleared successively with GST-bound glutathione-agarose and p13^{SUC}-Sepharose and equilibrated subsequently with GST, wild-type human GST-Rb, or an amino-terminal human GST-Rb protein lacking amino acids 89–140. Radiolabeled proteins were visualized as in Fig. 1. The relative abundance of phosphorylated histone H1 (Table 1) was quantified as described in Table 1. GST-Rb and histone H1 are indicated by arrows on the right. B, Rb amino acids 89–149 are sufficient to associate with RbK *in vitro*. Left, Coomassie blue-stained polyacrylamide gel of GST (Lane 1), GST-Rb₆₆ (Lane 2), and full-length amino-terminal GST-Rb (Lane 3) fusion proteins (5 μ g each) used in an *in vitro* protein binding and kinase assay shown in the right panel. GST-Rb₆₆ is a truncated Rb fusion protein that contains Rb amino acids 89–149. Molecular weight markers are indicated on the left. Right, *in vitro* protein binding and kinase assay. Mitotic ML-1 extracts were prepared and examined in an *in vitro* protein binding and kinase assay as in Fig. 1. Extracts were applied to equal quantities (5 μ g) of GST (Lane 4), GST-Rb₆₆ (Lane 5), or full-length, amino-terminal GST-Rb (Lane 6) fusion proteins. Following extensive washes, full-length, amino-terminal GST-Rb was added to GST and GST-Rb₆₆ binding and kinase reactions as a substrate for RbK phosphorylation. Resulting radiolabeled proteins were resolved on a 4/8% acrylamide gel that was dried subsequently and exposed to film for 4.5 h at -80°C . The arrow at the right indicates the position of radiolabeled GST-Rb.

amino-terminal deletions of exon 4. In contrast to the vast majority of familial retinoblastoma patients, for whom predisposition to tumor formation is inherited with high penetrance (>90%), individuals inheriting defective *Rb* alleles with such amino-terminal deletions exhibit incomplete (20–30%) penetrance. Such incompletely penetrant alleles are presumed to reflect the synthesis of Rb proteins with diminished, but not absent, tumor suppressor activity. To determine whether the loss of exon 4 amino acids alters the efficiency with which Rb associates with RbK *in vitro*, we prepared a GST-Rb fusion protein lacking exon 4 sequences (amino acids 128–167) from a Rb cDNA isolated from the immortalized lymphocytes of a child heterozygous for exon 4. This internally deleted, amino-terminal GST-Rb protein was shown to be expressed as efficiently in bacteria as was wild-type Rb (data not shown) and was then examined for *in vitro* RbK-binding activity. As shown in Table 1, deletion of exon 4-encoded amino acids results in a GST-Rb fusion protein that exhibits significantly diminished (37%) RbK-binding activity relative to wild-type GST-Rb.

To confirm this observation and expand our examination of the Rb amino terminus, we constructed a series of GST-Rb fusion proteins from a battery of internally deleted Rb cDNAs that have been examined previously for their capacity to inhibit the proliferation of Rb-negative tumor cells *in vitro* (61). Each of these mutated amino-terminal GST-RB fusion

proteins was shown to express stable, truncated fusion proteins of the predicted sizes in bacteria (data not shown), and each was examined subsequently for RbK-binding activity. Consistent with results reported for the deletion of exon 4 amino acids, deletion of Rb amino acids between positions 89 and 202 led to the abrogation (amino acids 89–140; Fig. 2A and Table 1) or diminution (amino acids 140–202; Table 1) of RbK-binding activity. Deletions within the Rb amino terminus downstream of amino acid 202 did not alter the abundance of Rb-associated RbK activity significantly *in vitro*. Thus, we conclude that the recovery of wild-type levels of RbK activity requires sequences between Rb amino acids 89–202, a region of the Rb protein previously shown to be a target of mutation in retinoblastoma and to be required for Rb-mediated growth suppression *in vitro*.

To delimit Rb amino acids that are sufficient for association with RbK *in vitro* further, we used Rb oligonucleotides and the PCR to prepare a GST-Rb fusion protein (GST-Rb₆₆) carrying a portion (amino acids 89–149) of the Rb amino terminus that is necessary for RbK association. This truncated GST-Rb fusion protein was incubated with precleared extracts prepared from metaphase-arrested ML-1 cells in parallel with a full-length, amino-terminal GST-Rb fusion protein and GST alone. Bead-bound proteins were then examined for kinase activity in an *in vitro* kinase assay using full-length, amino terminal Rb as substrate. As shown in

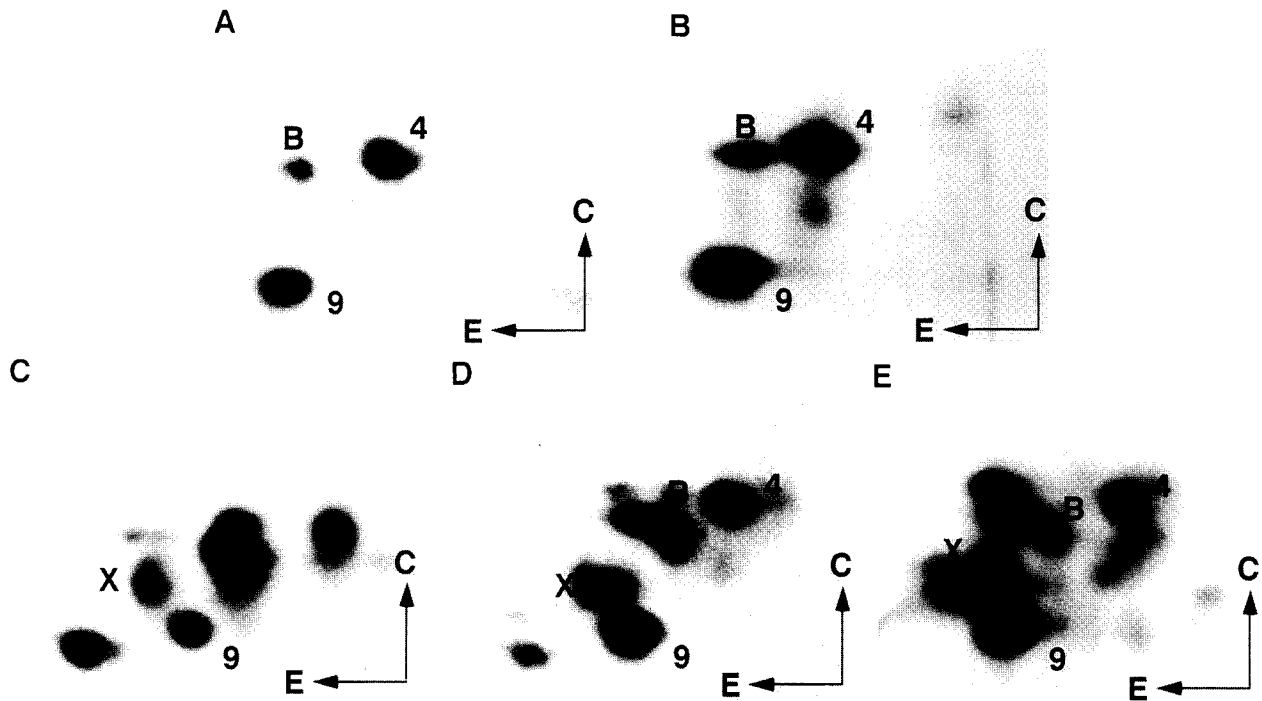


Fig. 5. Comparison of two-dimensional tryptic phosphopeptide maps of Rb synthetic peptides. Phosphorylated Rb synthetic peptides were eluted from polyacrylamide gels, digested exhaustively with trypsin, and resolved by electrophoresis (E) and ascending chromatography (C) in the directions indicated. A, phosphopeptides resulting from RbK phosphorylation of Rb 238–259. Two thousand cpm trypsin-digested Rb 238–259 were applied to a thin-layer plate prior to electrophoresis. B, phosphopeptides resulting from phosphorylation of Rb 238–259 by $p13^{suc}$ -bound mitotic kinases harvested from ML-1 cells. Five thousand cpm trypsin-digested Rb 238–259 were applied to a thin-layer plate prior to electrophoresis. C, phosphopeptides resulting from the phosphorylation of Rb 353–381 by $p13^{suc}$ -associated kinases harvested from CV-1 cells overexpressing cyclin A and cdc2. Five thousand cpm trypsin-digested Rb 353–381 were applied to a thin-layer plate prior to electrophoresis. D, mixture of trypsin-digested Rb 238–259 (1000 cpm) and Rb 353–381 (1000 cpm) phosphopeptides. E, phosphopeptides resulting from RbK phosphorylation of GST-Rb. Five thousand cpm trypsin-digested GST-Rb were applied to a thin-layer plate prior to electrophoresis. Common phosphopeptides are designated as described by Lees *et al.* (63), except for the phosphopeptide labeled X.

detection of eight major and six minor phosphopeptides. On careful scrutiny, several phosphopeptides seemed to be similar to those mapped previously by Lees *et al.* (63) to Rb amino acids 238–259 and 353–381. These same phosphopeptides have been shown previously to result following the phosphorylation of Rb by a preparation of mitotic cdk (63). To substantiate the supposition that RbK phosphorylates Rb at previously mapped sites of *in vivo* phosphorylation, two experiments were performed: (a) we compared two-dimensional phosphopeptide maps of wild-type GST-Rb and a point-mutated fusion protein that carries alanine substitutions at three sites of *in vivo* phosphorylation (amino acids 249, 356, and 373); and (b) synthetic peptides corresponding to amino acids 238–259 and 353–381 were synthesized and used as substrates in *in vitro* kinase assays, and resulting phosphopeptides were analyzed following exhaustive digestion with trypsin. As shown in Fig. 4B, *in vitro* phosphorylation of a GST-Rb fusion protein carrying alanine substitutions at amino acids 249, 356, and 373 results in the recovery of two major tryptic phosphopeptides, only one of which is coincident with phosphopeptides derived from wild-type GST-Rb. This result suggests that one or more sites of *in vivo* Rb phosphorylation are also sites of RbK phosphorylation *in vitro*. Alternatively, the introduced amino acid substitutions might perturb the phosphorylation of Rb by RbK at

distal sites. As shown in Fig. 5, the former possibility is substantiated by results from *in vitro* kinase assays using a synthetic Rb peptide corresponding to amino acids 238–259. Phosphorylation of this synthetic peptide by RbK (Fig. 5A) or $p13^{suc}$ -bound mitotic kinases (Fig. 5B) results in a common set of three phosphopeptides that are identical to that published previously for the phosphorylation of this peptide by purified cdk (63). As such, we have labeled each phosphopeptide as designated previously by Lees *et al.* (63). Comparison of these phosphopeptides with those resulting from *in vitro* phosphorylation of GST-Rb (Fig. 5E) by RbK shows that each phosphopeptide resulting from phosphorylation of peptides 238–259 is observed in *in vitro* RbK kinase assays using amino-terminal GST-Rb. Phosphoamino acid analyses of RbK-phosphorylated peptide 238–259 resulted in the recovery of the predicted radiolabeled amino acids (serine and threonine; Table 2; Ref. 63). In contrast, a Rb synthetic peptide, corresponding to amino acids 225–237, which is not phosphorylated *in vivo* and has been shown to not be a target of mitotic cdk *in vitro*, was not phosphorylated by RbK in parallel *in vitro* kinase assays (Table 2; Ref. 63).

Surprisingly, parallel *in vitro* kinase assays using another synthetic Rb peptide previously shown to be a target of cdk (Rb 353–381) did not result in appreciable phosphorylation

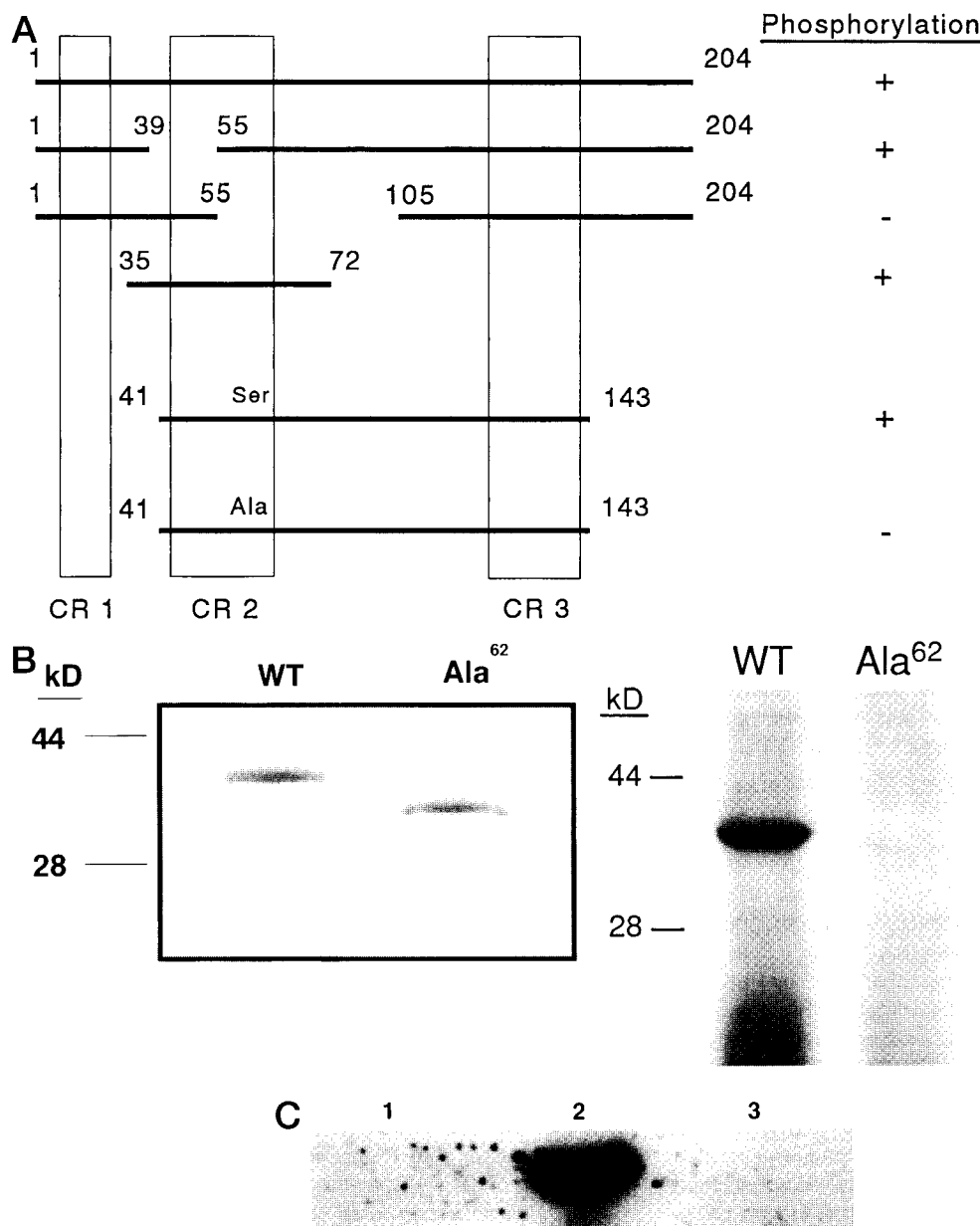


Fig. 6. Phosphorylation of the c-myc trans-activation domain by RbK. **A**, schematic diagram of GST-myc fusion proteins used in RbK *in vitro* kinase assays. c-myc amino acids included in each GST fusion protein are indicated by horizontal lines, with their respective amino acid end points. Conserved regions (CR1, CR2, and CR3) within the trans-activation domains of all *myc* family members are indicated by vertical boxes. A GST-myc fusion carrying an alanine substitution for serine 62 is indicated at the bottom. Phosphorylation results for each GST fusion protein are indicated at the right. **B**, phosphorylation of wild-type and point-mutated GST-myc fusion proteins. *Left*, Coomassie blue-stained gel of wild-type and point-mutated GST-myc fusion proteins used in an *in vitro* RbK kinase assay. The point-mutated GST-myc fusion carries an alanine substitution for serine 62 that results in an increase in its mobility in acrylamide gels. *Right*, *in vitro* RbK kinase assay using wild-type and point-mutated GST-fusion proteins. The GST fusion proteins depicted in the left panel were used as substrates in an *in vitro* RbK kinase assay. **C**, phosphorylation of c-myc 51–66 synthetic peptide by RbK *in vitro*. A synthetic peptide corresponding to c-myc amino acids 51–66 was used as a substrate in an *in vitro* RbK kinase assay following the incubation of GST or GST-Rb beads with mitotic ML-1 extracts. Kinase reactions were resolved by thin-layer electrophoresis. *Lane 1*, GST plus peptide; *Lane 2*, GST-Rb plus peptide; *Lane 3*, GST-Rb without peptide.

substrates, only wild-type c-myc protein was phosphorylated by RbK. To ensure that serine 62 is not only required for RbK phosphorylation but is also a site of phosphorylation, we performed an *in vitro* kinase assay using a synthetic peptide corresponding to c-myc amino acids 51–66 (Table 2). Incubation of this c-myc-derived peptide with RbK activity bound to GST-Rb beads resulted in its phosphorylation (Fig. 6C). Consistent with the supposition that serine 62 is the only *in vitro* target of RbK phosphorylation within c-myc, amino acid analysis of this *in vitro*-phosphorylated, c-myc-derived synthetic peptide resulted in the detection of only radiolabeled phosphoserine (Table 2). We conclude from these results that the c-myc trans-activation domain is phosphorylated specifically *in vitro* by RbK, a cell cycle-regulated kinase, at

a site that is phosphorylated *in vivo* in a cell cycle-dependent manner.

Discussion

This report extends the biochemical and functional characterization of RbK, a novel amino-terminal, Rb-associated histone H1 kinase, and RbK activity. We have shown previously that RbK activity is conferred by at least one serine and threonine kinase that has maximal Rb association and/or kinase activity in mammalian G₂-M (62). We have shown also that: (a) RbK is not a cyclin A-, B-, or E-associated cdk; (b) RbK is immunochemically distinct from the MAP kinases erk1 and erk2; and (c) RbK does not associate with p13^{suc} in

in vitro (62). Here, we show that RbK activity seems to associate preferentially with the Rb amino terminus as analogous portions of two Rb-related proteins, p107 and p130, associate with little or no histone H1 kinase activity. The specificity of Rb-RbK complex formation seems to be conferred by sequences within a 113-amino acid segment of the Rb amino terminus (amino acids 89–202) that is conserved poorly in p107 and p130. RbK phosphorylates the amino terminus of Rb *in vitro* on at least some sites that are phosphorylated *in vivo* and that are also targets of phosphorylation by cdk. In addition to histone H1 and Rb, we have shown that RbK phosphorylates the c-myc *trans*-activation domain specifically *in vitro* at a site that is phosphorylated *in vivo* in a cell cycle-dependent manner.

Although microinjection and transfection experiments of a single cell line, SAOS-2, have suggested that the Rb pocket is sufficient for the negative regulation of cell cycle progression (56–58), it is increasingly apparent that the Rb amino

terminus plays a significant role in mediating or regulating cell cycle events subsequent to the G₁-S-phase transition. This proposition is supported by evidence from several quarters: (a) Karantza *et al.* (28) reported that the conditional expression of wild-type Rb in synchronous populations of S-phase cells leads to the arrest of cell cycle progression in G₂; the importance of the Rb amino terminus in mediating this block to cell cycle progression was underscored by the observation that growth arrest in G₂ was also induced by the conditional expression of a Rb pocket mutant (28); (b) some viral oncoproteins, such as adenovirus E1A, complex with both unphosphorylated and phosphorylated Rb proteins (70, 71); this result suggests that isoforms of p105-Rb that are present throughout the cell cycle are functionally active; (c) mutants of E1A that are disabled in their capacity to physically interact with Rb are capable of inducing DNA synthesis in quiescent cells but are not able to stimulate mitosis (72); and (d) protein complexes between Rb and E2F have been noted throughout S-phase in synchronized populations of growing cells (73, 74). Finally, we note that a role for tumor suppressor genes in regulating cell cycle progression subsequent to S-phase is not without precedent, because p53 participates in the management of a cell cycle checkpoint in G₂-M (75–77). Whether Rb regulates transcription in G₂-M and/or additional functions remains to be determined.

Because the formation of active Rb-RbK kinase complexes seems to be restricted to late cell cycle stages, we speculate that, in conjunction with RbK, Rb participates in

terminus plays a significant role in mediating or regulating cell cycle events subsequent to the G₁-S-phase transition. This proposition is supported by evidence from several quarters: (a) Karantza *et al.* (28) reported that the conditional expression of wild-type Rb in synchronous populations of S-phase cells leads to the arrest of cell cycle progression in G₂; the importance of the Rb amino terminus in mediating this block to cell cycle progression was underscored by the observation that growth arrest in G₂ was also induced by the conditional expression of a Rb pocket mutant (28); (b) some viral oncoproteins, such as adenovirus E1A, complex with both unphosphorylated and phosphorylated Rb proteins (70, 71); this result suggests that isoforms of p105-Rb that are present throughout the cell cycle are functionally active; (c) mutants of E1A that are disabled in their capacity to physically interact with Rb are capable of inducing DNA synthesis in quiescent cells but are not able to stimulate mitosis (72); and (d) protein complexes between Rb and E2F have been noted throughout S-phase in synchronized populations of growing cells (73, 74). Finally, we note that a role for tumor suppressor genes in regulating cell cycle progression subsequent to S-phase is not without precedent, because p53 participates in the management of a cell cycle checkpoint in G₂-M (75–77). Whether Rb regulates transcription in G₂-M and/or additional functions remains to be determined.

In this and a previous report, we have characterized the biochemical and functional attributes of RbK kinase activity. Interestingly, the capacity of Rb to associate with RbK is not shared equally among Rb-related proteins. Consistent with the notion that Rb may functionally cooperate with RbK to regulate transcription in G₂-M, we have demonstrated that Rb and RbK complexes phosphorylate the c-myc *trans*-activation domain at a site, serine 62, that is phosphorylated *in vivo* (68, 69). Interestingly, similar observations have been reported recently for at least one cdk complex-related protein (78). In these experiments, cyclin A-associated kinase activity tethered to p107 has been shown to phosphorylate the c-myc *trans*-activation domain at serine 62 *in vitro* (78). Whether the phosphorylation of serine 62 *in vivo* is a physiologically relevant event remains to be established. Nonetheless, physical complexes and interactions between Rb and c-myc and p107 and c-myc have been detected both *in vitro* and *in vivo* (47, 68). The phosphorylation of serine-62 in some, but not all, cell

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Whether Rb regulates transcription in G₂-M and/or additional functions remains to be determined. Consistent with the observation that the amino termini of p107 and p130 seem not to associate with RbK-like kinases, this 113-amino acid portion of the Rb amino terminus is conserved poorly among Rb family members. In contrast, this lack of conservation does not extend as readily to flanking regions of the Rb amino terminus, where significant segments of amino acid identity between Rb, p107, and p130 are localized (11, 12). More importantly, the portion of the Rb amino terminus that is required for association with RbK has

tween p107 and RbK have not been detected *in vitro* or *in vivo*, and only marginal histone H1 kinase activity has been noted in association with the p130 amino terminus *in vitro*. These observations provide the first biochemical evidence that the amino termini of Rb family members are functionally distinct. The specificity of Rb and RbK interactions seems to be dependent on sequences within a 113-amino acid portion of the Rb amino terminus that is encoded by exons 3–6. Consistent with the observation that the amino termini of p107 and p130 seem not to associate with RbK-like kinases, this 113-amino acid portion of the Rb amino terminus is conserved poorly among Rb family members. In contrast, this lack of conservation does not extend as readily to flanking regions of the Rb amino terminus, where significant segments of amino acid identity between Rb, p107, and p130 are localized (11, 12). More importantly, the portion of the Rb amino terminus that is required for association with RbK has

been resolved. Consistent with the notion that Rb may functionally cooperate with RbK to regulate transcription in G₂-M, we have demonstrated that Rb and RbK complexes phosphorylate the c-myc *trans*-activation domain at a site, serine 62, that is phosphorylated *in vivo* (68, 69). Interestingly, similar observations have been reported recently for at least one cdk complex-related protein (78). In these experiments, cyclin A-associated kinase activity tethered to p107 has been shown to phosphorylate the c-myc *trans*-activation domain at serine 62 *in vitro* (78). Whether the phosphorylation of serine 62 *in vivo* is a physiologically relevant event remains to be established. Nonetheless, physical complexes and interactions between Rb and c-myc and p107 and c-myc have been detected both *in vitro* and *in vivo* (47, 68). The phosphorylation of serine-62 in some, but not all, cell

types has been correlated with an increased abundance of c-myc-mediated transcription (67, 68). In accord with this correlation, the functional consequence of Rb and c-myc interactions *in vivo* has been reported to be a stimulation of c-myc-mediated transcription (65). It is not as yet clear what physiological role this burst of c-myc transcriptional activity might play in G₂ (68). Serine 62 has been shown previously to be an *in vitro* target of other proline-directed serine and threonine kinases, such as MAP and cdc2 kinases (66, 67, 69). That the site of RbK phosphorylation of c-myc overlaps with a site of phosphorylation by proline-directed kinases is consistent with results of phosphopeptide maps of GST-Rb following phosphorylation by RbK; RbK phosphorylates the Rb amino terminus on at least two sites of *in vivo* phosphorylation that are also targets of cdk's *in vitro*. It is not as yet established whether phosphorylation of these Rb sites regulates Rb function or whether they are phosphorylated *in vivo* as a consequence of the association of RbK with the Rb amino terminus. The answers to these and other questions will be facilitated undoubtedly by the cloning of RbK.

Materials and Methods

Cells. ML-1 human myeloid leukemia cells were obtained from Dr. Stephen H. Friend (Massachusetts General Hospital Cancer Center, Charlestown, MA), and A549 human lung adenocarcinoma cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco's MEM (GIBCO-BRL, Gaithersburg, MD.) supplemented with 10% heat-inactivated FCS (Hyclone Laboratories, Logan, UT) and 50 ng/ml mezlocillin (Mezlin; Miles, Inc., West Haven, CT) at 37°C in a humidified incubator under 5% CO₂.

Construction of GST Fusion Proteins. The construction of wild-type human amino-terminal Rb (pGEX2TK-HuRb) and p107 (pGEX2TK-107) GST fusion proteins has been described previously (62). A mutated human Rb cDNA carrying alanine substitutions for amino acids 249, 356, and 373 (pRbMM1/SVE; a generous gift of Dr. Dennis J. Templeton, Case Western Reserve University, Cleveland, OH) was used as substrate for the PCR to create a GST-fusion protein analogous to the wild-type GST-Rb fusion mentioned above. Briefly, a Perkin-Elmer thermal cycler, the above cDNA, Vent polymerase (New England Biolabs, Inc., Beverly, MA), and the following Rb oligonucleotides were used in the PCR: exon 1, 5'-GTCATGCCGCCAAAACCCCGAAAA-3'; and exon 11, 5'-TAACTGGAGTGTGTGGAGGAATTATAT-3'. Following amplification, restriction enzymes that cleave at unique sites within the Rb amino terminus were used to exchange this mutated partial Rb cDNA for the corresponding wild-type sequences in pGEX2TK-HuRb. Similarly, mutated Rb cDNAs carrying internal deletions throughout the Rb amino terminus (also gifts of Dr. Dennis J. Templeton; Ref. 61) were used as substrates for the PCR to

create a GST fusion protein. Rb cDNAs of internal deletions were

was also facilitated by the exchange of restriction enzyme fragments with unique ends. A GST-Rb fusion carrying a deletion of exon 4-encoded amino acids (128-167) was prepared following the cloning of a mutated Rb cDNA from EBV-immortalized lymphocytes (RDS 327-07; obtained from Drs. Philip J. Rosenfeld and Thaddeus P. Dryja, Massachusetts Eye and Ear Infirmary, Boston, MA) of a child with familial retinoblastoma. Lymphocyte polyadenylated RNA, oligodeoxythymidylic acid, reverse transcriptase, and RNase H were used to prepare first-strand cDNA, and the Rb exon 1 and 11 primers indicated above were then used to amplify the mutated Rb cDNA from these cells by the PCR. This exon 4-deleted Rb cDNA was cloned subsequently into pGEX2TK-HuRb as described above using appropriate restriction enzymes. A GST fusion protein that carries Rb amino acids 89-149 was prepared using the PCR, pGEX-2T (Pharmacia/Biotech, Inc., Uppsala, Sweden), and the following Rb oligonucleotides: 5'-TCTGTGGATGGAGTATTG-3' and 5'-CTTGACATAGCATATCAACTTTGG-3'. A GST fusion protein carrying the amino-terminal 418 amino acids of p130 was prepared using the PCR, a full-length cDNA (a generous gift of Dr. Peter Whyte, McMaster University, Hamilton, Ontario, Canada), pGEX-3X (Pharmacia/Biotech), and the following oligonu-

cleotides: 5'-ATGCGGTCGGGAGGTGACCAGT-3' and 5'-CTGGAGTCA-CACAAGGGCTATTCTCC-3'. The nucleotide sequences at the junctions of all GST fusion proteins were confirmed by double-stranded DNA sequencing using Sequenase (United States Biochemical Corp., Cleveland, OH), [³⁵S]dATP, and appropriate oligonucleotide primers. GST fusion proteins prepared from c-myc, GST-myc 1-204 and deletions thereof, and GST-myc 41-143 and alanine 62 substitution were obtained from Drs. Rene Bernards (Netherlands Cancer Institute, Amsterdam, the Netherlands) and Michael C. Ostrowski (Ohio State University, Columbus, OH). The expression of GST fusion proteins was induced in BL21 bacteria by the addition of isopropyl-1-thio-β-D-galactopyranoside, and fusion proteins were harvested and quantified as described previously (62, 81).

Mitotic Arrest, Extract Preparation, and *in Vitro* Protein-binding and Kinase Assays. ML-1 and A549 cells were arrested in metaphase by incubation of cells in 0.4 μg/ml nocodazole (Sigma Chemical Co., St. Louis, MO) for 16 h, and extracts were prepared as described previously (62). Depletions of mitotic extracts with GST and p13^{SUC} beads and *in vitro* protein-binding and kinase assays were also performed as described previously (62). *In vitro* kinase assays that used an epitope-tagged cyclin A/cdc2 kinase used recombinant vaccinia virus-infected cell extracts (a gift of Dr. Dennis J. Templeton) that were prepared as described previously (82). Ascites fluid prepared from a monoclonal antibody that recognizes the epitope tag (anti-EE; Ref. 82) was used to immunoprecipitate kinase activity prior to the addition of radiolabeled αATP and substrate.

For *in vitro* kinase assays using Rb- and c-myc-derived synthetic peptides, 30 μg peptides were added to kinase reactions. c-myc peptide 51-66 was a generous gift of Dr. Roger J. Davis, (University of Massachusetts, Worcester, MA). Rb synthetic peptides were prepared on automated peptide synthesizers and purified by high-performance liquid chromatography on reverse-phase columns in a 10-40% gradient of acetonitrile. Eluted synthetic peptides (80-98% pure) were analyzed by mass spectrophotometry to confirm the accuracy of synthesis. Where indicated, synthetic peptides were subjected to peptide sequencing to ensure sequence fidelity.

Two-dimensional Phosphopeptide Mapping and Phosphoamino Acid Analysis. Phosphoamino acid and phosphopeptide analyses were performed essentially as described previously (63, 83) with the following minor modification. Radiolabeled synthetic peptides were precipitated with trichloroacetic acid and resuspended in 6 N HCl prior to incubation in a boiling water bath for 2 h. Following lyophilization, unlabeled phosphoamino acid standards were added, and phosphorylated amino acids were resolved by two-dimensional TLC.

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