Retinoblastoma protein phosphorylation does not require activation of p34CDC2 protein kinase

Gerald A. EVANS*† and William L. FARRAR‡

*Biological Carcinogenesis and Development Program, Program Resources Inc./DynCorp

and ‡Laboratory of Molecular Immunoregulation, Cytokine Mechanisms Section, Biological Response Modifiers Program, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD 21702-1201, U.S.A.

Of the many intracellular events that occur after mitogenic stimulation of cells, the phosphorylation of the retinoblastoma protein (RB) in early G₁-phase appears to play a pivotal role in controlling cell-cycle progression. RB phosphorylation results in release from a proliferative block imposed by hypophosphorylated RB. Several investigators have presented evidence, using models produced *in vitro*, that the serine kinase p34CDC2 phosphorylates RB and is responsible for regulating RB phosphorylation. Using human T-cells as a model, we show that lectin treatment of resting T-cells results in detectable RB phosphorylation by 24 h after treatment. Further, using immunoprecipitation and immunoblotting, no detectable p34CDC2 could be seen until 48 h after lectin stimulation. Analysis of the relative histone H1 activity of p34CDC2, purified by immunoprecipitation, revealed that RB phosphorylation does not parallel increases in p34CDC2 activity as T-cells progress into S-phase, supporting the contention that p34CDC2 activation as a histone H1 kinase is not a critical regulator of RB phosphorylation. Further treatment of activated T-cells, arrested in G₁-phase, with interleukin 2 results in a 95 % increase in RB phosphorylation within 4 h with no detectable increase in the histone H1 kinase activity of p34CDC2. Together, these data suggest that p34CDC2 activation is not required for early cell-cycle phosphorylation of RB.

INTRODUCTION

As normal cells progress through the cell cycle in response to mitogenic stimulation, a series of events occur which control the ability of the cell to move from a state of growth arrest in G₀/G₁-phase to a state of active DNA synthesis in S-phase and subsequent nuclear and cytoplasmic division. A key event which appears to have a fundamental role in controlling this process is the phosphorylation on serine and threonine residues of the protein product of the retinoblastoma susceptibility gene (RB). Several investigators have shown that elimination of functional RB by deletion, mutation [1] or binding to the transforming proteins of adenovirus [2], polyomavirus [3] or papillomavirus [4] can lead to cellular transformation and carcinogenesis. These findings suggest that RB acts on a suppressor of cell-cycle progression. Analysis of RB during the cell cycle has revealed that it undergoes heavy phosphorylation as the cell progresses through G₁- and into S-phase [5–8]. It has been postulated that under normal circumstances, the hypophosphorylated form of RB, which exists only during early G₁-phase, functions as a suppressor of cell growth, and phosphorylation decreases the suppressive function of RB and allows cell-cycle progression after mitogenic stimulation [7]. Therefore understanding the events that control RB phosphorylation is a key to deducing the mechanisms that control normal cell growth.

A protein serine/threonine kinase that is a candidate RB kinase is p34CDC2 [9,10]. CDC2 is a homologue of the cell-division-cycle-control genes first studied in fission yeast [11,12]. In frog and starfish oocytes, CDC2 is the catalytic component of M-phase-promoting factor which governs mitotic progression [13–17]. In these organisms as well as in human cells, CDC2 or a related kinase has an important role in controlling mitosis. In human HeLa cells, the CDC2 protein kinase exists as a 34 kDa

catalytic protein which has virtually no detectable kinase activity toward histone H1 in early G₁-phase of the cell cycle [18]. On cell-cycle progression, the kinase activity of CDC2 toward histone H1 increases rapidly with maximal activity occurring at mitotic metaphase [18,19]. Several laboratories have presented evidence that p34CDC2 phosphorylates RB at physiologically relevant sites on the protein. Maturation-promoting factor (MPF), which has p34CDC2 as its catalytic component, was shown to phosphorylate RB *in vitro* on the same sites that are phosphorylated *in vivo* with synthetic peptides to mimic CDC2 phosphorylation sites on RB [9]. Further, using recombinant RB and immunopurified CDC2, Lin *et al.* [10] have shown that CDC2 phosphorylates RB on the same sites that are phosphorylated *in vivo* using proteolytic digestion and two-dimensional peptide mapping. Together, these results implicate p34CDC2, or a related kinase with similar substrate specificity, as the kinase that phosphorylates RB *in vivo*. A major indicator of the mitotic activity of CDC2 is its relative ability to phosphorylate histone H1. In this paper, using human T-cells as a model, we have analysed RB phosphorylation and compared the state of phosphorylation with p34CDC2 activation as a histone H1 kinase. We present evidence that suggests that RB phosphorylation does not require p34CDC2 activation.

MATERIALS AND METHODS

Tissue culture media and reagents

Normal RPMI 1640 medium, 100 × penicillin/streptomycin and 100 × glutamine were purchased from Mediatech, Washington DC, U.S.A. Phosphate-free RPMI 1640 medium was purchased from Advanced Biotechnologies Inc., Columbia, MD, U.S.A. Fetal calf serum was purchased from Gibco Laboratories, Life Technologies Inc., Grand Island, NY, U.S.A. Phyto-

Abbreviations used: RB, retinoblastoma protein; MPF, maturation-promoting factor; PHA, phytohaemagglutinin; IL-2, interleukin 2; TBS/Az, 10 mM-Tris/HCl (pH 7.5)/150 mM-NaCl/0.1 % Na₂S₂O₈.

† To whom correspondence should be addressed.

haemagglutinin (PHA) was purchased from Wellcome Diagnostics, Temple Hill, Dartford, Kent, U.K. Recombinant human interleukin 2 (IL-2) was purchased from Hoffmann-LaRoche, Nutley, NJ, U.S.A. All other chemicals and reagents were from standard vendors.

Lymphocyte preparation and cell culture

Human peripheral blood mononuclear cells were obtained by leucopheresis of normal volunteers at the blood bank of the National Institutes of Health. After density sedimentation of the mononuclear cells with lymphocyte separation medium (Organon Teknika, Durham, NC, U.S.A.), the lymphocytes were purified by counterflow centrifugal elutriation as previously described [20], except that pyrogen-free phosphate-buffered saline [20] was used in the elutriation procedure. The purified lymphocytes were 75–85% positive toward an antibody specific for the CD3 component of the T-cell receptor (CD3⁺) with the remaining cells consisting of 5–15% B-cells and 5–7% null cells [20]. These lymphocyte preparations contained <1% monocytes, as assessed by non-specific esterase staining [20].

T-lymphocytes were cultured in the presence of PHA or activated with PHA and cultured in the presence of IL-2. Lymphocytes were cultured up to 4 days in the presence of PHA (1 µg/ml) at a density of 1×10^6 cells/ml in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, glutamine and antibiotics and analysed as described below. To prepare activated lymphocytes for stimulation with IL-2, newly isolated T-cells were cultured with PHA for 48 h. Cells were then recovered by centrifugation and washed twice with low-pH RPMI 1640 medium by bubbling CO₂ through the cell suspension. Cells were resuspended in RPMI 1640 medium supplemented with 1% fetal calf serum, glutamine and antibiotics and cultured for 24 h to establish a G₁-phase-enriched population (quiescent). Activated T-cells were recovered by centrifugation, washed twice with RPMI 1640 medium plus CO₂ and once with RPMI 1640 medium alone. T-cells were resuspended at 1×10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum, glutamine, antibiotics and cultured in the presence of recombinant purified IL-2 (1 µg/ml) for up to 4 h and analysed as described below.

For all experiments described above, cell proliferation was monitored by measuring [³H]thymidine incorporation [21].

Cell labelling, lysis and immunoprecipitation

For phosphate labelling *in vivo*, cultured T-cells were washed twice with phosphate-free RPMI 1640 medium and resuspended at 35×10^6 cells/ml in phosphate-free RPMI 1640 medium supplemented with 5% dialysed fetal calf serum, glutamine and antibiotics and containing 0.5 mCi of [³²P]orthophosphate/ml (Dupont/New England Nuclear, Boston, MA, U.S.A.). Labelling was carried out for 90 min at 37 °C. Labelled cells (35×10^6) were recovered by centrifugation at 12000 g for 1 min and lysed with 1 ml of extraction buffer (10 mM-Tris, pH 7.6, 1% Triton X-100, 100 mM-NaCl, 5 mM-EDTA, 30 mM-sodium pyrophosphate, 50 mM-NaF, 50 µM-sodium vanadate, 1 mM-phenylmethanesulphonyl fluoride and 0.1% BSA) at 4 °C for 1 h. Lysates were clarified by centrifugation at 12000 g for 20 min at 4 °C. Clarified lysate (250 µg), as determined by the method of Bradford [22], or, if labelled, a volume of lysate corresponding to equal counts, was incubated with 2 µg of an RB-specific monoclonal antibody, mAb245 (Bethesda Research Laboratories, Life Technologies Inc., Gaithersburg, MD, U.S.A.) or with 2 µl of p34CDC2-specific antiserum G6 [18] for 1 h at 4 °C. For mAb245 immunoprecipitation, 100 µl of 20% protein A-conjugated Sepharose (Pharmacia LKB Biotechnologies, Uppsala, Sweden) in extraction buffer was mixed with 5 µg of anti-mouse IgG polyclonal rabbit serum (Kirkegaard and Perry,

Gaithersburg, MD, U.S.A.) and incubated for 1 h at 4 °C. Anti-mouse IgG-protein A-Sepharose was mixed with antibody containing lysate and incubated for 1 h at 4 °C. For the G6 antibody preparations, 100 µl of 20% (w/v) protein A-Sepharose was added directly to the antibody-containing lysates and incubated as described. Immunoprecipitates were washed three times with extraction buffer and three additional times with extraction buffer without BSA. Immunoprecipitated proteins were either eluted from the Sepharose pellet with 50 µl of 2 × SDS loading buffer and subjected to SDS/PAGE [23] and autoradiography or immunoblot, or used for histone H1 kinase assays described below. Autoradiography was performed using preflashed film, and exposures were verified to be within the film's linear exposure range by comparing the relative change in protein phosphorylation seen between treatments with that seen from several different exposure times. Quantification of autoradiographs was performed using an LKB Ultrascan laser densitometer. Autoradiographs presented as well as those analysed by laser densitometry were exposures that represented the phosphorylation trend seen in multiple experiments.

Immunoblotting

Electrophoresed immunoprecipitates were transferred to Immobilon poly(vinylidene difluoride) paper (Millipore Corp., Bedford, MA, U.S.A.) in buffer containing 25 mM-Tris/HCl (pH 8.3), 192 mM-glycine, 20% (v/v) methanol and 0.01% SDS [24] at 150 mA for 1 h using an LKB semi-dry blotter. Blots were blocked overnight at 4 °C in 10 mM-Tris/HCl (pH 7.5)/150 mM-NaCl/0.1% NaN₃ (TBS/Az) containing 1% (w/v) BSA. For anti-CDC2 immunoblotting, a 1:1000 dilution of G6 serum in TBS/Az/1% BSA was added and incubated for 1–2 h at room temperature. Blots were washed six times for 10 min each wash with TBS/Az/0.1% BSA. TBS/Az containing 0.1% BSA and 0.5 µg of biotinylated protein A/ml (Vector Laboratories Inc., Burlingame, CA, U.S.A.) was added and incubated for 1 h at room temperature. Blots were washed six times as described and incubated with a 1:10000 dilution of streptavidin-conjugated alkaline phosphatase (Bethesda Research Laboratories) in TBS/Az/0.1% BSA for 1 h at room temperature. Blots were washed three times as described and once with TBS/Az containing 0.5% Tween-20 (Bio-Rad Laboratories, Richmond, CA, U.S.A.) and developed according to manufacturer's directions.

Histone H1 kinase assay

Washed G6 immunoprecipitations of CDC2 were collected from PHA- and IL-2-stimulated T-cells at different time-points after mitogen stimulation. Immunoprecipitates were mixed with 50 µl of a solution containing 25 mM-Hepes (pH 7.5), 10 mM-MgCl₂, 10 µM-ATP with 10 µCi of [³²P]ATP/ml (Dupont/New England Nuclear) and 100 µg of histone H1/ml (type IHS; Sigma Chemical Co., St. Louis, MO, U.S.A.). Reactions were carried out for 10 min at 23 °C and stopped by adding 50 µl of 2 × SDS loading buffer and boiling for 2 min. Samples were electrophoresed and autoradiographed, and histone phosphorylation was quantified using an LKB Ultrascan laser densitometer.

RESULTS

Changes in RB phosphorylation do not correlate with changes in CDC2 activity in PHA-stimulated T-cells

RB, a suppressor of cell-cycle progression, is only active as a hypophosphorylated protein. After mitogen stimulation, RB is phosphorylated in G₁-phase resulting in a putative release from the proliferative block imposed by the hypophosphorylated

Fig. 1. RB phosphorylation in PHA-treated T-cells occurs in the absence of detectable p34CDC2

Human T-cells were treated with PHA for the indicated times and labelled with [³²P]orthophosphate. Radiolabelled cell lysates were immunoprecipitated with an anti-RB monoclonal antibody or an anti-p34CDC2 serum (G6) and subjected to SDS/PAGE and autoradiography or immunoblot. (a) Autoradiograph of phosphorylated RB. (b) Autoradiograph of phosphorylated p34CDC2. (c) G6 immunoblot of p34CDC2 purified by immunoprecipitation using G6 serum as described in the Materials and methods section. RB and p34CDC2 are indicated on the left. U, M and L refer to the upper, middle or lower bands respectively of p34CDC2 shown in (b) and (c). The experiment was repeated three times with three different T-cell batches with equivalent results.

species [5–8]. In order to understand fully the mechanisms by which mitogen stimulates cell-cycle progression, it is important to understand the manner in which the state of RB phosphorylation is controlled. The most well-characterized mitogenic activity of p34CDC2 is that activity which, on mitogen stimulation, increases to a maximum during G₂M-phase of the cell cycle. This activity is represented by an increased ability to utilize histone H1 as a substrate *in vitro*. On comparing data presented in the literature, it appears that the initial increase in histone H1 kinase activity occurs as RB phosphorylation approaches a maximum, suggesting that the activated form of CDC2 may not be the principal kinase that phosphorylates RB. Certain data, however, have implicated the mitogenically active form of CDC2 (i.e. MPF) as the RB kinase [9]. Because no report in the literature has addressed this issue with evidence obtained *in vitro*, we have attempted to correlate CDC2 histone H1 kinase activity (i.e. the mitogenically active form of CDC2) with the relative state of RB phosphorylation in a human T-cell model.

Treatment of quiescent T-cells with lectin (PHA) triggers a series of events which leads to movement of the T-cell through G₁- into S-phase by 48 h after treatment [25], with maximal DNA synthesis, and S-phase progression, occurring by 72 h [25]. Analysis of the level of RB phosphorylation in PHA-treated T-cells shows that the first observable phosphorylation can be seen

Fig. 2. Changes in the activity of p34CDC2 do not correlate with changes in RB phosphorylation

RB phosphorylation (■) was determined by densitometric analysis of autoradiographs of phosphorylated RB and this was compared with the activity of p34CDC2 toward histone H1 (□) as described in the Materials and methods section. The data represent the fold increase in RB phosphorylation and p34CDC2 activity above that seen in untreated cells. The experiment was repeated three times with three different T-cell batches with equivalent results.

by 24 h after PHA treatment (Fig. 1a) and peaks at 48 h after PHA treatment (Figs. 1a and 2). Interestingly, on analysing the relative amount of p34CDC2 protein by immunoprecipitation and immunoblot, there is no detectable protein by 24 h after PHA treatment (Fig. 1c). This is also supported by the work of Furukawa *et al.* [26] which shows that immunodetectable p34CDC2 is first detected at approx. 36 h after PHA stimulation. These data suggest that significant RB phosphorylation occurs during extremely low-level production of p34CDC2 protein.

On comparing the relative histone H1 kinase activity of p34CDC2 in T-cells as they progress through the cell cycle in response to PHA treatment, it becomes clear that changes in the activity of p34CDC2 do not parallel changes in the level of RB phosphorylation. A distinctive feature of p34CDC2 activation as a histone H1 kinase is dephosphorylation [18,19,27,28]. The state of phosphorylation of p34CDC2 is shown in Fig. 1(b). Maximal phosphorylation is seen at 48 h after PHA treatment with dephosphorylation and subsequent activation occurring by 72 h after PHA treatment (Figs. 1b and 2). In contrast, the maximal RB phosphorylation can be seen by 48 h and is decreasing by 72 h after PHA treatment (Figs. 1a and 2). Further evidence for the state of phosphorylation of these proteins is represented by shifts in electrophoretic mobility. On average, increased phosphorylation of RB results in slower migration and this can be seen in Fig. 1(a). CDC2 exists in three forms, designated upper, middle and lower. The upper and middle band re-

Fig. 3. Early G_1 -phase phosphorylation of RB does not require p34CDC2 activation

T-cells treated with PHA for 72 h were washed free of endogenous IL-2 and growth was arrested in G_1 -phase by incubation in 1% fetal calf serum for 24 h. Growth-arrested cells were then treated with or without IL-2 for the indicated times and RB phosphorylation (■) and p34CDC2 (□) activity were determined as described in the Materials and methods section. The data represent the fold increase in RB phosphorylation and p34CDC2 activity above that seen in untreated cells. The experiment was repeated three times with three different T-cell batches with equivalent results.

present phosphorylated CDC2 and the lower band is the unphosphorylated species. Immunoblot analysis of CDC2 in Fig. 1(c) reveals a distinctive increase in the amount of the lower unphosphorylated band by 72 h treatment, which correlates with the dephosphorylation results obtained with ^{32}P labelling. The relationship between RB phosphorylation and p34CDC2 activity can best be seen by a direct comparison of the histone H1 kinase activity of the p34CDC2 enzyme complex and RB phosphorylation. Fig. 2 compares the densitometric determination of RB phosphorylation with the activity of p34CDC2 determined by analysing the level of histone H1 phosphorylation in an immune complex kinase assay as described in the Materials and methods section. From this analysis, it can be clearly seen that RB is maximally phosphorylated well before maximum activation of p34CDC2 (Fig. 2). Further, as p34CDC2 approaches maximum activity, RB phosphorylation is approx. 50% of maximum (Fig. 2). This comparison supports the contention that the activation of p34CDC2 as a histone H1 kinase is not principally involved in the regulation of RB phosphorylation.

Early cell-cycle phosphorylation of RB does not require CDC2 activation in IL-2-stimulated T-cells

Because the phosphorylation of RB during early G_1 -phase

appears to be most critical in controlling the cell-cycle-suppressive function of RB, we analysed the relative levels of RB phosphorylation and p34CDC2 activity in IL-2 stimulated pre-activated T-cells. IL-2 is the principal mitogen that directly stimulates T-cells to move from G_1 - to S-phase [29]. Movement into S-phase begins within 10 h of treatment with IL-2, as judged by thymidine-uptake data and maximum RB phosphorylation [25]. Analysis of the relative phosphorylation of RB shows that initial increases of 20% and 95% can be seen by 2 and 4 h after IL-2 treatment respectively (Fig. 3). The histone H1 kinase activity of p34CDC2, however, remains unchanged through 4 h of IL-2 stimulation (Fig. 3). This again supports the contention that p34CDC2 activation is not required for early G_1 -phase phosphorylation of RB.

DISCUSSION

The data presented here suggest that p34CDC2 activation is not required for early cell-cycle phosphorylation of RB. Using a model *in vitro*, it has been shown that RB phosphorylation by MPF [9] and immunopurified CDC2 [10] occurs on the same sites *in vitro* that are phosphorylated *in vivo*. Our data, however, using a model *in vivo* show distinct discrepancies between RB phosphorylation and CDC2 activity. Most notably, RB phosphorylation occurs without detectable p34CDC2 protein (Fig. 1) and in the absence of p34CDC2 activation in response to IL-2 (Fig. 3). Further, the major regulator of RB phosphorylation as T-cells progress through the cell cycle is not simply p34CDC2 activity. This is indicated by a lack of correlation between changes in RB phosphorylation and changes in p34CDC2 activity as measured by its ability to phosphorylate histone H1 (Fig. 2).

Several scenarios exist that may explain the results presented here. It has been reported that several forms of CDC2 exist during the cell cycle [30] and that complex-formation with secondary proteins alters substrate specificity [18,19]. This leaves open the possibility that a CDC2-containing protein complex lacking histone H1 kinase activity may phosphorylate RB. The identification of several CDC28 homologues in the fission yeast system indicates the existence of a family of cell-cycle-controlling genes. Because CDC2 is the mammalian homologue of fission yeast CDC28, it seems likely that a family of CDC2-related proteins function within higher eukaryotic systems as well. Recent isolation of several CDC2-like protein kinases in human and rodent cells supports this assumption. Bunnell *et al.* [31] have recently identified a 58 kDa protein kinase which is homologous to p34CDC2 which functions during the late stages of mitosis. Most intriguing is the identification of CDK2, which some believe may function as a G_1 -specific CDC2 [32]. One or another of these CDC2-like protein kinases may be responsible for regulating RB phosphorylation in early G_1 -phase.

In attempting to link early cell-cycle events that follow mitogenic stimulation to those events that directly control cell growth, many investigators have tried to package RB phosphorylation neatly with p34CDC2 activation. On the basis of our results, this cannot be done. However, with the recent identification of additional CDC2-like protein kinases, it appears likely that a member of this family controls the state of RB phosphorylation and may provide that critical link between growth-factor-receptor-mediated events and cell-cycle progression.

We thank Dr. John O'Shea, Dr. Dan Longo and Dr. J. J. Oppenheim for their critical review of this manuscript before its submission. This project was funded at least in part with Federal funds from the Department of Health and Human Services under contract number NO1-CO-74102 with Program Resources, Inc./DynCorp. The content of this publication does not necessarily reflect the views or policies of the

Department of Health and Human Services, nor does mention of trade names, commercial products or organizations imply endorsement by the U.S. Government.

REFERENCES

1. Lee, W.-H., Bookstein, R. & Lee, E. Y.-H. P. (1988) *J. Cell. Biochem.* **38**, 213–227
2. Whyte, P., Williamson, N. C. & Harlow, E. (1989) *Cell* **56**, 67–75
3. Ludlow, J. W., DeCaprio, J. A., Huang, C. M., Lee, W. H., Paucha, E. & Livingston, D. M. (1989) *Cell* **56**, 57–65
4. Dyson, N., Howley, P. M., Munger, K. & Harlow, E. (1989) *Science* **243**, 934–936
5. DeCaprio, J. A., Ludlow, J. W., Lynch, D., Furukawa, Y., Griffin, J., Piwnica-Worms, H., Huang, C. M. & Livingston, D. M. (1989) *Cell* **58**, 1085–1095
6. Buchkovich, K., Duffy, L. A. & Harlow, E. (1989) *Cell* **58**, 1097–1105
7. Chen, P.-L., Scully, P., Shew, J.-Y., Wang, J. Y. J. & Lee, W.-H. (1989) *Cell* **58**, 1193–1198
8. Mihara, K., Cao, X. R., Yen, A., Chandler, S., Driscoli, B., Murphree, A. L., T'Ang, A. & Fung, Y. K. T. (1989) *Science* **246**, 1300–1303
9. Taya, Y., Yasuda, H., Kamijo, M., Nakaya, K., Nakamura, Y., Ohba, Y. & Nishimura, S. (1989) *Biochem. Biophys. Res. Commun.* **164**, 580–586
10. Lin, B. T. Y., Gruenwald, S., Morla, A. O., Lee, W. H. & Wang, J. Y. J. (1991) *EMBO J.* **10**, 857–864
11. Nurse, P., Thuriaux, P. & Nasmyth, K. (1976) *Mol. Gen. Genet.* **146**, 167–178
12. Simanis, V. & Nurse, P. (1986) *Cell* **45**, 261–268
13. Draetta, G., Brizuela, L., Potashkin, J. & Beach, D. (1987) *Cell* **50**, 319–325
14. Dunphy, W., Brizuela, L., Beach, D. & Newport, J. (1988) *Cell* **54**, 423–431
15. Gautier, J., Norbury, C., Lohka, M., Nurse, P. & Maller, J. (1988) *Cell* **54**, 433–439
16. Labbe, J., Lee, M., Nurse, P., Picard, A. & Doree, M. (1988) *Nature (London)* **335**, 251–254
17. Arion, D., Meijer, L., Brizuela, L. & Beach, D. (1988) *Cell* **55**, 371–378
18. Draetta, G. & Beach, D. (1988) *Cell* **54**, 17–26
19. Brizuela, L., Draetta, G. & Beach, D. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4362–4366
20. Wahl, L. M., Katona, I. M., Wilder, R. L., Winter, C. C., Haraoui, B., Sher, I. & Wahl, S. M. (1984) *Cell. Immunol.* **85**, 373–383
21. Mills, G. B., Cragoe, E. J., Gelfand, E. W. & Grinstein, S. (1985) *J. Biol. Chem.* **260**, 12500–12507
22. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
23. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
24. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
25. Evans, G. A., Wahl, L. M. & Farrar, W. L. (1992) *Biochem. J.* **282**, 759–764
26. Furukawa, Y., Piwnica-Worms, H., Ernst, T. J., Kanakura, Y. & Griffin, J. (1990) *Science* **250**, 805–808
27. Morla, A., Draetta, G., Beach, D. & Wang, J. Y. J. (1989) *Cell* **58**, 193–203
28. Draetta, G., Piwnica-Worms, H., Morrison, D., Druker, B., Roberts, T. & Beach, D. (1988) *Nature (London)* **336**, 738–744
29. Cantrell, D. A. & Smith, K. A. (1984) *Science* **224**, 1311–1316
30. Giordana, A., Whyte, P., Harlow, E., Franza, B. R., Beach, D. & Draetta, G. (1989) *Cell* **58**, 981–990
31. Bunnell, B. A., Heath, L. S., Adams, D. E., Lahti, J. M. & Kidd, V. J. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7467–7471
32. Elledge, S. J. & Spottswood, M. R. (1991) *EMBO J.* **10**, 2653–2659

Received 13 February 1992/7 May 1992; accepted 12 May 1992