HIV RNA copies per milliliter (*10*).

It has been generally believed that the immune response to HIV is responsible for the decline of virus concentration from the high levels seen early in infection (*1*, *11*). In particular, appearance of a cytotoxic T lymphocyte (CTL) response specific to certain HIV epitopes close to the time of the peak of HIV virus level has been observed (*3*, *12*, *13*). The results from this model raise the possibility that the appearance of the HIV-specific immune response is a consequence of the high level of virus but is not a major cause of the decline from this high level. If this hypothesis is true, and the prevailing view that the HIV-specific immune response "controls" HIV replication is not, one might expect to find some patients in whom virus levels decline from a high peak even in the absence of specific immune responses. Suggestive evidence can be found in the literature. One patient (of five studied, patient AD11) experienced a 100-fold drop in virus concentration during primary HIV infection even though there was no detectable HIV-specific antibody or CTL response (*3*). Another patient (again of a total of five studied, HOBR) had no CTL response while viremia was being "controlled," albeit "less efficiently" than in patients with a response (*13*). Lastly, data from three children infected during childbirth showed no evidence of CTL responses or neutralizing antibodies, even though the increase in virus concentration consistent with primary viremia fell (*14*).

Estimates have suggested that infected cells and free virions have a very short half-life in HIV-infected persons (*15*, *16*), a mean of about 2 days for the sum of the lifetimes of virus-producing cells and free virions. We have chosen slightly slower rates of removal of free virions (average life span, 1/2 day) and virus-producing cells (average life span, 3 days) because for much of the period of infection being modeled (up to the peak in free-virus concentration), no HIV-specific immune response is thought to occur. No introduction of an immune response at the time of the peak (by increasing the values of σ and δ) has been modeled because it was felt to be important to study whether the virus could decline in the absence of any change in the rate of removal of free virions and virus-infected cells.

In conclusion, simple modeling of the population dynamics of acute HIV infection in the absence of any assumption of a growing immune response suggests the presence of a transient peak of virus concentration. These results, and the lack of a detectable HIV-specific immune response at the time of the decline from the initial virus peak in some patients, raise the possibility that the decline in virus level seen in acute HIV infection is not a reflection of the capacity of the HIV-specific immune response to control virus replication.

REFERENCES AND NOTES

1. S. J. Clark *et al.*, *N. Engl. J. Med.* **324**, 954 (1991).
2. E. S. Daar, T. Mougdil, R. D. Meyer, D. D. Ho, *ibid.*, p. 961.
3. R. A. Koup *et al.*, *J. Virol.* **68**, 4650 (1994).
4. A. R. McLean, V. C. Emery, A. Webster, P. D. Griffiths, *AIDS* **5**, 485 (1991).
5. D. Shenzle, *Stat. Med.* **13**, 2067 (1994); A. S. Perelson, in *Mathematical and Statistical Approaches to AIDS Epidemiology: Lecture Notes in Biomathematics*, C. Castillo-Chavez, Ed. (Springer-Verlag, New York, 1989), vol. 82, pp. 350–370; A. S. Perelson, D. E. Kirschner, R. J. De Boer, *Math. Biosci.* **114**, 81 (1993).
6. M. Piatak *et al.*, *Science* **259**, 1749 (1993).
7. S. D. Gowda *et al.*, *J. Immunol.* **142**, 773 (1989).
8. M. Peakman *et al.*, in *Cell Activation and Apoptosis in HIV Infection*, J. M. Andrieu and W. Lu, Eds. (Plenum, New York, 1995).
9. T. L. Katzenstein *et al.*, in preparation.
10. S. Kinloch-de Loes *et al.*, *N. Engl. J. Med.* **333**, 408 (1995).
11. R. A. Koup, D. D. Ho, *Nature* **370**, 416 (1994).
12. J. T. Safrit, C. A. Andrews, T. Zhu, D. D. Ho, R. A. Koup, *J. Exp. Med.* **179**, 463 (1994); J. T. Safrit, A. Y. Lee, C. A. Andrews, R. A. Koup, *J. Immunol.* **153**, 3822 (1994).
13. P. Borrow, H. Lewicki, B. H. Hahn, G. M. Shaw, M. B. A. Oldstone, *J. Virol.* **68**, 6103 (1994).
14. K. Luzuriaga *et al.*, *J. Infect. Dis.* **167**, 1008 (1993).
15. X. Wei *et al.*, *Nature* **373**, 117 (1995).
16. D. D. Ho *et al.*, *ibid.*, p. 123.
17. W. E. Paul, *Fundamental Immunology* (Raven, New York, 1985).
18. C. A. Michie, A. McLean, C. Alcock, P. C. L. Beverly, *Nature* **360**, 264 (1992).
19. D. S. Dimitrov *et al.*, *J. Virol.* **67**, 2182 (1993).

20. This report arises in part from useful discussions with J. Downing, A. Mocroft, C. Sabin, V. Emery, S. Jolles, A. Cozzi Lepri, H. Meadows, and G. Janossy.

14 August 1995; accepted 21 November 1995

Dependence of Cyclin E–CDK2 Kinase Activity on Cell Anchorage

Fang Fang,*† Gertraud Orend,* Nobumoto Watanabe, Tony Hunter, Erkki Ruoslahti‡

Most nonmalignant cells are anchorage-dependent; they require substrate attachment for growth and, in some instances, survival. This requirement is lost on oncogenic transformation. The cyclin E–CDK2 complex, which is required for the G_1-S transition of the cell cycle, was activated in late G_1 phase in attached human fibroblasts, but not in fibroblasts maintained in suspension. In transformed fibroblasts the complex was active regardless of attachment. The lack of cyclin E–CDK2 activity in suspended cells appeared to result from increased expression of CDK2 inhibitors and a concomitant decrease in phosphorylation of CDK2 on threonine-160. Suppression of cyclin E–CDK2 activity may thus underlie the anchorage dependence of cell growth.

Most untransformed mammalian cells fail to proliferate when they are prevented from attaching to a solid substrate, a phenomenon known as anchorage dependence of growth (*1*). Loss of anchorage dependence is the property of transformed cells that correlates best with tumorigenicity in vivo (*2*). Anchorage dependence has been attributed to the arrest of cell cycle progression in late G_1 phase resulting from a lack of substrate adhesion (*3*). Because the G_1 cyclins—cyclin D and cyclin E—together with their associated cyclin-dependent kinases (CDKs) are key regulators in the G_1 phase (*4*), we investigated whether the activation of CDK complexes with cyclin D or cyclin E is controlled by cell anchorage.

F. Fang, G. Orend, E. Ruoslahti, La Jolla Cancer Research Foundation, Cancer Center, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA.
N. Watanabe and T. Hunter, Molecular Biology and Virology Laboratory, Salk Institute for Biological Studies, La Jolla, CA 92037, USA.

*These authors contributed equally to this work.
†Present address: Department of Pathology, Massachusetts General Hospital, Boston, MA 02114, USA.
‡To whom correspondence should be addressed.

Both KD and IMR90 untransformed human diploid fibroblasts were synchronized in G_0 by serum deprivation, split into attached and suspended cultures (*5*), and exposed to fetal bovine serum. Both cell lines failed to initiate DNA synthesis when cultured in suspension (*6*), confirming that nonadherent cells are arrested before entering S phase. Although attached and suspended cells differ in size and shape, the protein contents per cell were similar (*6*). Immunoblot analysis of KD cell extracts showed that the expression of cyclin D1 and cyclin D3 (cyclin D2 is absent from KD cells) did not differ between attached and suspended cells during the G_1 phase (Fig. 1A). The amounts of CDK4, CDK5, and CDK6, all of which can associate with cyclin D, were also similar under both culture conditions. Moreover, in vitro retinoblastoma protein (Rb) kinase assays (*7*) showed that cyclin D1 and CDK6 immunoprecipitates from G_1 phase cells were equally active under the two culture conditions (Fig. 1B). These observations confirm previous data (*3*) showing that cell cycle progression through mid G_1 phase,

which requires cyclin D–CDK activity, is independent of anchorage.

The amounts of CDK2 and cyclin E protein were similar in suspended and attached KD cells (Fig. 2A) and IMR90 cells (6). However, whereas a high level of cyclin E–CDK2 activity was present in attached KD and IMR90 cells around the G_1-S transition, little or no such activity was apparent when these cells were maintained in suspension (Fig. 2B). Another anchorage-dependent human fibroblast line, FS, gave similar results (6). The amount of CDK2 protein in CDK2 immunocomplexes was similar in attached and suspended cells (Fig. 2C). In contrast, the HUT12 cell line, an anchorage-independent, chemically transformed variant of KD cells (8), showed the same level of kinase activity in cyclin E immunoprecipitates regardless of adhesion conditions (Fig. 2B). Cyclin A activity was undetectable in the suspended cells, as well as in the attached cells arrested by hydroxyurea; however, it was readily measurable 3 hours after reattachment of suspended cells or removal of hydroxyurea from attached cells (6).

The lack of cyclin E–CDK2 activity in suspended cells was not attributable to a defect in complex formation, because cyclin E immunoprecipitates obtained from attached and suspended cells contained similar amounts of CDK2 protein, as determined by immunoblot analysis (Fig. 3A). CDK2 migrated as a doublet on a SDS-polyacrylamide gel; more of the faster migrating form was present in attached KD and FS cells, whereas the slower migrating form predominated in suspended cells. Only the faster migrating CDK2 was associated with cyclin E in HUT12 cells, whether attached or suspended. Because the faster migrating form of CDK2 corresponds to molecules that are phosphorylated on Thr^{160} (or both Tyr^{15} and Thr^{160}) (9), our observations indicated that phosphorylation of CDK2 on Thr^{160} is reduced in suspended cells. This conclusion was confirmed by isolating CDK2 from suspended and hydroxyurea-arrested attached cells after labeling with [^{32}P]orthophosphate (10). The extent of CDK2 phosphorylation in the attached cells was five times that in the suspended cells (6). In addition, phosphopeptide mapping (11) demonstrated that CDK2 in the attached and suspended cells shared the same phosphorylation sites, but the extent of phosphorylation at these sites in the attached cells was five times that in the suspended cells (Fig. 3B). These results rule out the possibility that phosphorylation on Thr^{14} and Tyr^{15}, which are the inhibitory phosphorylation sites in CDK2, is the mechanism of cyclin E–CDK2 inhibition in suspended cells. Instead, decreased phosphorylation on Thr^{160}, a stimulatory phosphorylation site essential for CDK2 activity (12), may be one cause of the low cyclin E–CDK2 activity in suspended cells.

Phosphorylation of CDK2 on Thr^{160} is catalyzed by the CDK-activating kinase (CAK), which is a heterotrimer of CDK7, cyclin H, and MAT (13). A high level of CAK activity was detected in CDK7 and cyclin H immunoprecipitates from the nuclear fraction of both attached and suspended KD and FS cells (6), indicating that CAK activity is not reduced in the suspended cells. Given that CAK is localized within the nucleus (14), nuclear localization of cyclin E–CDK2 is obviously a key requirement for activation of the complex. Indeed, subcellular fractionation revealed that CDK2 and cyclin E, as well as CDK7 and cyclin H, were almost exclusively present in the nucleus in late G_1 in both attached and suspended cells (6). This result excludes abnormal intracellular localization as the mechanism of cyclin E–CDK2 inhibition in suspended cells. In addition, the total amount of the Cdi (KAP) phosphatase, which catalyzes the dephosphorylation of CDK2 phosphorylated on Thr^{160} (15), as well as the amount of this protein bound to cyclin E–CDK2, was not affected by cell attachment (6).

The decreased cyclin E–CDK2 activity in suspended cells was possibly attributable to an increase in the amount of a CDK2 inhibitor. Three such inhibitors—$p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$—have been identified (16); they inhibit CDK2 kinase activity directly as well as prevent the phosphorylation of Thr^{160} (17). We investigated whether the concentrations of any of these inhibitors differed between attached and suspended cells. The amounts of $p21^{CIP1}$ and $p27^{KIP1}$ in suspended KD and FS cells were four times

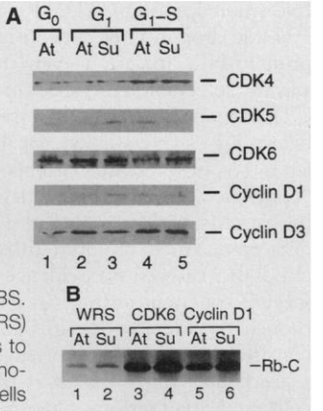

Fig. 1. Progression of G_1 phase in adherent and nonadherent KD cells. Cells were synchronized in G_0 and then split into attached (At) or suspended (Su) cultures and exposed to serum as described (5). (**A**) Expression of CDKs and D-type cyclins in G_1 phase. Lane 1, serum-deprived cells; lanes 2 and 3, synchronized cells cultured in DMEM supplemented with 20% FBS for 7 hours; lanes 4 and 5, synchronized cells cultured in DMEM supplemented with 20% FBS and 2 mM hydroxyurea for 24 hours. Immunoblots were probed with polyclonal antibodies to CDK4, CDK5, CDK6, cyclin D1, and cyclin D3 (7). (**B**) Rb kinase activities of cyclin D1 and CDK6 complexes. Immunoprecipitates were isolated from lysates of synchronized KD cells that had been cultured for 10 hours in DMEM supplemented with 20% FBS. Immunoprecipitation was performed with whole rabbit serum (WRS) (lanes 1 and 2); antibodies to CDK6 (lanes 3 and 4); or antibodies to cyclin D1 (lanes 5 and 6). Kinase activity associated with the immunoprecipitates was measured as described (7). Equal numbers of cells were used for each immunoprecipitation. Rb-C, fusion protein of glutathione-S-transferase and the COOH-terminal fragment of human pRb. More than 95% of the cells cultured in suspension were viable in each experiment as judged by trypan blue exclusion. CDK4 immunoprecipitates showed equal but low activity for attached and suspended KD cells that had been exposed to 20% FBS for 10 hours (6).

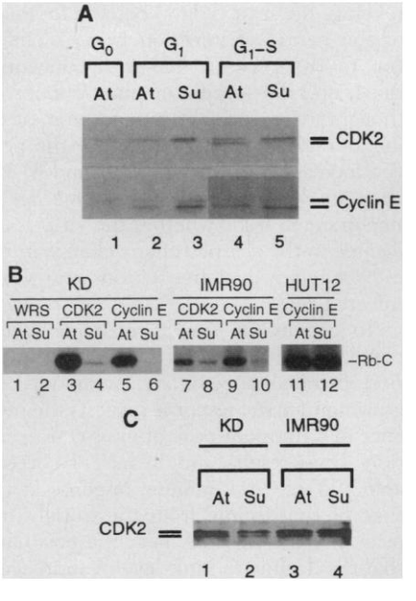

Fig. 2. Effect of cell anchorage on the concentration and kinase activity of cyclin E and CDK2. (**A**) Expression of CDK2 and cyclin E in G_1 phase. Lane 1, KD cells deprived of serum for 60 hours (5); lanes 2 and 3, synchronized cells cultured in DMEM supplemented with 20% FBS for 7 hours; lanes 4 and 5, cells cultured in DMEM supplemented with 20% FBS and 2 mM hydroxyurea for 17 hours. Immunoblots were probed with polyclonal antibodies to CDK2 and to cyclin E (7). (**B**) Kinase activity of cyclin E–CDK2. Attached (At) or suspended (Su) KD (lanes 1 to 6), IMR90 (lanes 7 to 10), or HUT12 (lanes 11 and 12) cells were cultured for 24 hours in DMEM containing 20% FBS and 2 mM hydroxyurea (5). CDK2 (lanes 3, 4, 7, and 8) and cyclin E (lanes 5, 6, 9, 10, 11, and 12) were immunoprecipitated and assayed for Rb kinase activity (7). Immunoprecipitates prepared with whole rabbit serum (WRS) (lanes 1 and 2) were used as controls. (**C**) Detection of CDK2 protein. The CDK2 immunoprecipitates isolated from KD or IMR90 cells as described in (B) were subjected to immunoblot analysis with antibodies to CDK2. The control immunoblot with WRS was negative (6).

those in the corresponding attached cells; the amount of $p57^{KIP2}$ was similar under the two conditions (Fig. 4A). The increased inhibitor concentrations were primarily attributable to increased expression rather than decreased degradation in the G_1 phase (*18*), because the attached cells contained only slightly more $p27^{KIP1}$ in G_0 than in G_1 under our experimental conditions (6). The amount of $p27^{KIP1}$ in cyclin E immunoprecipitates from suspended cells was two- to fourfold that found in attached cells (Fig. 4B). Consistent with the increase in $p21^{CIP1}$, the amount of CDK2 co-immunoprecipitated with $p21^{CIP1}$ from suspended FS cells was three times that observed with attached cells. However, despite the fact that more CDK2 was associated with $p21^{CIP1}$ in suspended FS cells, the in vitro histone H1 kinase activity of $p21^{CIP1}$ immunoprecipitates prepared from lysates of suspended cells was almost undetectable, whereas a substantial amount of kinase activity was present in $p21^{CIP1}$ immunoprecipitates isolated from attached cells (6), probably because cyclin E–CDK2–inhibitor complexes with a low inhibitor to cyclin E–CDK2 ratio retain activity (*19*). The increased amounts of $p27^{KIP1}$ and $p21^{CIP1}$ associated with cyclin E–CDK2 could account for the decreased phosphorylation of CDK2 on Thr^{160}, as well as on Thr^{14} and Tyr^{15}, that we observed in suspended cells; both inhibitors block CDK2 phosphorylation at Thr^{160} (*17*) and $p21^{CIP1}$ blocks phosphorylation at Tyr^{15} (*20*).

The three CDK2 inhibitors were present in HUT12 cells at concentrations comparable to those in KD and FS cells. Furthermore, the amounts of $p21^{CIP1}$ and $p27^{KIP1}$ were greater in suspended than in attached HUT12 cells (Fig. 4A). This result was unexpected because cyclin E–CDK2 activity was high in both attached and suspended HUT12 cells. However, CDK2 and cyclin E concentrations in HUT12 cells were four to five times those in KD cells (Fig. 4C) and IMR90 cells (6). The same was also true of the amount of cyclin E–CDK2 complex (6). Thus, the cyclin E–CDK2 complex would appear to be present in excess relative to the inhibitors in suspended HUT12 cells and therefore remains uninhibited; increased expression of cyclin E and CDK2 may be important in the oncogenic transformation of HUT12 cells.

Together, our results indicate that the cell cycle is blocked in suspended cells at the level of cyclin E–CDK2 activity and that this block is attributable to increased expression of CDK2 inhibitors. This mechanism resembles the cell cycle arrest induced by transforming growth factor–β, for which a similar fourfold increase in $p27^{KIP1}$ is sufficient to arrest mink Mv1Lu cells in G_1 with inactive cyclin E–CDK2 complexes (*21*). Anchorage dependence has previously been linked to inhibition of cyclin A expression; rodent NIH 3T3 and NRK fibroblasts were shown to lack cyclin A when grown in suspension, and forced expression of exogenous cyclin A rendered NRK cells partially independent of anchorage (*22*). Our data suggest that the absence of cyclin A in unattached cells is a consequence of cell cycle arrest in late G_1 phase, rather than the cause of the arrest, because cyclin A is induced and functions in early S phase, after the activation of cyclin E–CDK2 (*4*, *23*). The anchorage independence of NRK cells induced by overexpression of cyclin A may result from an ability of cyclin A to replace cyclin E functionally, or it may reflect the fact that a major function of activated cyclin E–CDK2 is to induce cyclin A expression.

Cell anchorage is mediated by the binding of cell surface integrins to extracellular matrix proteins (*24*). The integrins are connected to focal adhesion kinase and various cytoskeletal components inside the cell (*25*), but it is unclear how the engagement of integrins eventually regulates progression of the cell cycle. Our results suggest that cyclin E–CDK2 kinase is the ultimate target of this complex regulatory pathway. Given that epithelial and endothelial cells undergo apoptosis when denied anchorage (*24*), this pathway also may control cell survival.

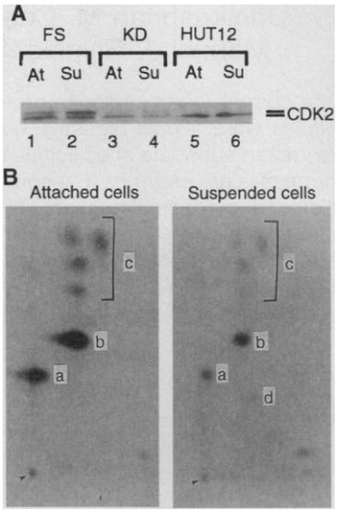

Fig. 3. Cyclin E–CDK2 complex formation (**A**) and phosphorylation of CDK2 (**B**) in attached and suspended cells. In (A) attached (At) and suspended (Su) FS (lanes 1 and 2), KD (lanes 3 and 4), and HUT12 (lanes 5 and 6) cells were cultured for 17 hours in DMEM supplemented with 20% FBS and 1 mM hydroxyurea. Immunoprecipitation of cell lysates prepared with equal numbers of cells was performed with antibodies to cyclin E. Proteins were separated by electrophoresis on precast 4 to 20% SDS-polyacrylamide gels (Novex) and subjected to immunoblot analysis with antibodies to CDK2. In (B) attached (left panel) and suspended (right panel) KD cells were labeled with [^{32}P]orthophosphate (*10*). CDK2 was immunoprecipitated with antibodies to cyclin E, and visualized by electrophoresis and autoradiography. Phosphopeptide mapping of CDK2 was performed as described (*11*). Spots a to d indicate phosphopeptides containing Thr^{14} and Tyr^{15} (a), Tyr^{15} (b), and Thr^{160} (c), as well as a minor unidentified peptide (d). Electrophoresis at pH 1.9 was performed in the horizontal dimension with the anode on the left (origin shown by arrowhead in the left corner), followed by chromatography in the vertical dimension.

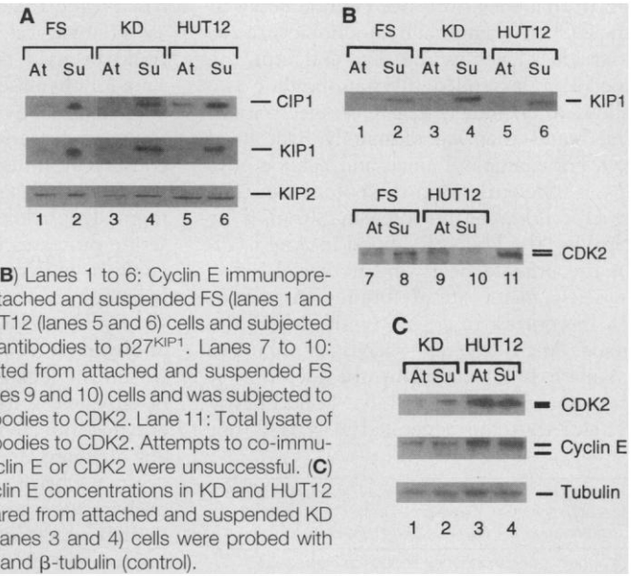

Fig. 4. CDK2 inhibitors in attached (At) and suspended (Su) FS, KD, and HUT12 cells. Cells were cultured for 17 hours in DMEM supplemented with 20% FBS and 1 mM hydroxyurea. (**A**) Immunoblot analysis of $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$ in whole cell lysates of FS (lanes 1 and 2), KD (lanes 3 and 4), and HUT12 (lanes 5 and 6) cells. (**B**) Lanes 1 to 6: Cyclin E immunoprecipitates were isolated from attached and suspended FS (lanes 1 and 2), KD (lanes 3 and 4), and HUT12 (lanes 5 and 6) cells and subjected to immunoblot analysis with antibodies to $p27^{KIP1}$. Lanes 7 to 10: $p21^{CIP1}$ was immunoprecipitated from attached and suspended FS (lanes 7 and 8) and HUT12 (lanes 9 and 10) cells and was subjected to immunoblot analysis with antibodies to CDK2. Lane 11: Total lysate of HUT12 cells probed with antibodies to CDK2. Attempts to co-immunoprecipitate $p57^{KIP2}$ with cyclin E or CDK2 were unsuccessful. (**C**) Comparison of CDK2 and cyclin E concentrations in KD and HUT12 cells. Whole cell lysates prepared from attached and suspended KD (lanes 1 and 2) and HUT12 (lanes 3 and 4) cells were probed with antibodies to CDK2, cyclin E, and β-tubulin (control).

REFERENCES AND NOTES

1. I. MacPherson and L. Montagnier, *Virology* **23**, 291 (1964); M. Stoker, C. O'Neill, S. Berryman, V. Waxman, *Int. J. Cancer* **3**, 683 (1968); J. Folkman and A. Moscona, *Nature* **273**, 345 (1978).
2. S.-I. Shin, V. H. Freedman, R. Risser, R. Pollack, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 4435 (1975).
3. H. Otsuka and M. Moskowitz, *J. Cell Physiol.* **87**, 213 (1975); J. Campisi and E. E. Medrano, *ibid.* **114**, 53 (1983); T. M. Guadagno and R. K. Assoian, *J. Cell Biol.* **115**, 1419 (1991); E. K.-H. Han, T. M. Guad-

agno, S. L. Dalton, R. K. Assoian, *ibid.* **122**, 461 (1993).
4. M. Philippe and J. M. Roberts, *Cell* **66**, 1217 (1991); F. Fang and J. W. Newport, *ibid.*, p. 731; C. J. Sherr, *ibid.* **73**, 1059 (1993); E. A. Nigg, *BioEssays* **17**, 471 (1995); J. Pines, *Biochem. J.* **308**, 697 (1995); A. Koff *et al.*, *J. Cell Biol.* **130**, 755 (1995); M. Ohtsubo, A. M. Theodoras, J. Schumacher, J. M. Roberts, M. Pagano, *Mol. Cell. Biol.* **15**, 2612 (1995).
5. Cells were cultured in 10-cm dishes until they were 70% confluent, synchronized by incubating in serum-free medium [Dulbecco's minimum essential medium (DMEM) supplemented with bovine serum albumin (1 mg/ml), bovine insulin (5 μg/ml), transferrin (5 μg/ml), and sodium selenite (5 ng/ml)] for 48 to 72 hours, and then stimulated by transfer into DMEM containing 20% fetal bovine serum (FBS). For the attached cultures, cells were allowed to reattach to tissue culture dishes. For the suspended cultures, cells were gently scraped from tissue culture dishes, separated by centrifugation, and resuspended in DMEM supplemented with 20% FBS and 1% Methocel [J. Rheinwald and H. Green, *Cell* **2**, 287 (1974)]. Hydroxyurea, an inhibitor of DNA synthesis, was added at 1 or 2 mM to the cultures to prevent any of the cells from progressing into the S phase of the cell cycle. The cell suspension was sealed in a 50-ml Corning tube, gassed with sterile 5% CO_2, and incubated in a rolling incubator at 37°C. Methocel (1%) had no effect on the protein kinase activity of CDK2 in attached cells.
6. F. Fang, G. Orend, N. Watanabe, T. Hunter, E. Ruoslahti, unpublished data.
7. Immunoprecipitations and in vitro kinase assays were performed as described [H. Matsushime *et al.*, *Mol. Cell. Biol.* **14**, 2066 (1994)], with the exception that 1 μg of glutathione-S-transferase–pRb(768–928) fusion protein was used in each kinase reaction. Antibodies were from the following sources: Antisera to CDK2 (SC-163), CDK4 (SC-260), CDK5 (SC-173), CDK6, and CDK7 (SC-529) were provided by M. Pagano or were from Santa Cruz Biotechnology; antiserum to cyclin D1 was from S. Reed, Santa Cruz Biotechnology (SC-450), or Upstate Biotechnology (06-137); antibodies to cyclin E (SC-248, SC-198), cyclin H (SC-855), cyclin D2 (SC-181), and cyclin D3 (SC-182) were from Santa Cruz Biotechnology; antiserum to $p21^{Cip1}$ was provided by S. Elledge or obtained from Oncogene Sciences (OP64, OP68) or Pharmingen (15091A); antiserum to $p27^{Kip1}$ was from Transduction Laboratories (K25020) or Santa Cruz Biotechnology (SC-776, SC-528); and antiserum to $p57^{Kip2}$ was provided by W. Harper.
8. T. Kakunaga, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1334 (1978).
9. Y. Gu, J. Rosenblatt, D. O. Morgan, *EMBO J.* **11**, 3995 (1992).
10. KD cells were synchronized in serum-free medium for 2 days, and then cultured attached or suspended for 20 hours in DMEM supplemented with 10% FBS and 1 mM hydroxyurea as described (*5*). The cells were transferred to phosphate-free DMEM supplemented with 20% dialyzed FBS and 1 mM hydroxyurea, and incubated for 1 hour. The cells in each 10-cm dish were then switched to 5 ml of fresh phosphate-free DMEM containing 20% dialyzed FBS, 1 mM hydroxyurea, and 5 mCi of [^{32}P]orthophosphate, and incubated for 6 hours. Suspended cells were incubated similarly except that the phosphate-free medium contained 1% Methocel. Cells were later collected and lysed with radioimmunoprecipitation assay buffer. Immunoprecipitation was performed as described (*7*).
11. W. J. Boyle, P. van der Geer, T. Hunter, *Methods Enzymol.* **201**, 110 (1991).
12. G. Draetta, *Trends Cell Biol.* **3**, 287 (1993).
13. J. Shuttleworth, R. Godfrey, A. Colman, *EMBO J.* **9**, 3233 (1990); R. Y. C. Poon, K. Yamashita, J. P. Adamczewski, T. Hunt, J. Shuttleworth, *ibid.* **12**, 3123 (1993); M. J. Solomon, J. W. Harper, J. Shuttleworth, *ibid.*, p. 3133; R. P. Fisher and D. O. Morgan, *Cell* **78**, 713 (1994); T. P. Mäkelä *et al.*, *Nature* **371**, 254 (1994); R. Y. C. Poon and T. Hunter, *Curr. Biol.* **11**, 1243 (1995).
14. J.-P. Tassan, S. J. Schultz, J. Bartek, E. A. Nigg, *J. Cell Biol.* **127**, 467 (1994).
15. R. Y. C. Poon and T. Hunter, *Science* **270**, 90 (1995).
16. T. Hunter and J. Pines, *Cell* **79**, 573 (1994); S. J. Elledge and J. W. Harper, *Curr. Opin. Cell Biol.* **6**, 847 (1994); C. J. Sherr and J. M. Roberts, *Genes Dev.* **9**, 1149 (1995).
17. K. Polyak *et al.*, *Cell* **78**, 59 (1994); O. Aprelikova, Y. Xiong, E. T. Liu, *J. Biol. Chem.* **270**, 18195 (1995).
18. M. Pagano *et al.*, *Science* **269**, 682 (1995).
19. H. Z. Zhang, G. J. Hannon, D. Beach, *Genes Dev.* **8**, 1750 (1994).
20. $p21^{CIP1}$ inhibited Tyr^{15} phosphorylation in cyclin A–CDK2 complexes by Wee1 in vitro (R. Y. C. Poon and T. Hunter, unpublished data).
21. I. Reynisdóttir, K. Polyak, A. Iavarone, J. Massagué, *Genes Dev.* **9**, 1831 (1995).
22. T. M. Guadagno, M. Ohtsubo, J. M. Roberts, R. K. Assoian, *Science* **262**, 1572 (1993).
23. J. Pines and T. Hunter, *Nature* **346**, 760 (1991); F. Girard, U. Strausfeld, A. Fernandez, N. J. C. Lamb, *Cell* **67**, 1169 (1991); M. Pagano, R. Pepperkok, F. Verde, W. Ansorge, G. Draetta, *EMBO J.* **11**, 961 (1992).
24. J. E. Meredith, B. Fazeli, M. A. Schwartz, *Mol. Biol. Cell* **4**, 953 (1993); S. M. Frisch and H. Francis, *J. Cell Biol.* **124**, 619 (1994); E. Ruoslahti and J. C. Reed, *Cell* **77**, 477 (1994).
25. M. D. Schaller and J. T. Parsons, *Trends Cell Biol.* **3**, 258 (1993); E. A. Clark and J. S. Brugge, *Science* **268**, 233 (1995).
26. We thank J. Wang for the glutatathione-S-transferase–pRb (768–928) fusion protein, and M. Pagano, S. Reed, S. Elledge, W. Harper, R. Brent, and R. Poon for antibodies and other help with this work. Supported by grants CA 60725, CA 67224, and CA 28896 and Cancer Center Support Grant CA 30199 (E.R.), as well as grant CA 37980 (T.H.), from the National Cancer Institute. T.H. is an American Cancer Society research professor. N.W. is a staff scientist of the Tsukuba Life Science Center at the Institute of Physical and Chemical Research (RIKEN) in Tsukuba, Japan, and is a recipient of a long-term fellowship from the Human Frontier Science Program. G.O. was supported by a fellowship from the Deutsche Forschungsgemeinschaft, Germany.

5 October 1995; accepted 15 November 1995

Angiotensin II–Forming Activity in a Reconstructed Ancestral Chymase

Unnikrishnan M. Chandrasekharan, Subramaniam Sanker, Manuel J. Glynias, Sadashiva S. Karnik, Ahsan Husain*

The current model of serine protease diversity theorizes that the earliest protease molecules were simple digestive enzymes that gained complex regulatory functions and restricted substrate specificities through evolution. Among the chymase group of serine proteases are enzymes that convert angiotensin I to angiotensin II, as well as others that simply degrade angiotensins. An ancestral chymase reconstructed with the use of phylogenetic inference, total gene synthesis, and protein expression had efficient and specific angiotensin II–forming activity (turnover number, about 700 per second). Thus, angiotensin II–forming activity is the more primitive state for chymases, and the loss of such activity occurred later in the evolution of some of these serine proteases.

Chymases are a family of closely related mast cell serine proteases that are involved in diverse functions such as peptide hormone processing (*1–4*), the inflammatory response (*5*), and parasite expulsion (*6*). Although all chymases resemble chymotrypsin in that they hydrolyze peptide bonds at the COOH-termini of hydrophobic aromatic residues (such as Phe, Tyr, and Trp), the use of an extended substrate-binding site allows a remarkable degree of selectivity in the bonds that are ultimately hydrolyzed (*7*). For example, human and baboon chymases efficiently form the potent vasoconstrictor hormone angiotensin (Ang) II by cleaving the Phe^8–His^9 bond in Ang I (*2*). In the primate heart and its vessels, chymase is a major Ang II–forming enzyme (*8*, *9*). In contrast to chymotrypsin, which degrades Ang II, the high specificity of human chymase is illustrated by the fact that it does not cleave the Tyr^4–Ile^5 bond in Ang II, and thus the generated Ang II is not degraded (*2*). Rat chymase-1 readily degrades Ang I and Ang II (*3*).

Is the specificity observed in some chymases (that is, the formation of Ang II without its subsequent degradation) an early or late feature of evolution? Parsimony-based reconstruction of ancestral ribonuclease (*10*) suggested that a similar approach could be used to reconstruct the original state for chymase evolution. We created an alignment of chymase and related leukocyte serine protease sequences by using several conserved primary and secondary structural motifs as anchors (Fig. 1). The most parsimonious phylogenetic tree of leukocyte serine proteases was inferred by parsimony analysis, which has been shown to accurately predict known phylogeny (*11*). The bootstrap value (95%) supports the segregation of chymases as a distinct group of enzymes within the leukocyte serine protease family of enzymes. Within the chymase group of enzymes, human chymase, baboon chymase, dog chymase, and mouse chymase-5 form a distinct subgroup that we have classified as α-chymases (bootstrap value, 98%). Although we classified the remaining known mammalian chymases as β-chymases, the

Department of Molecular Cardiology, Mail Code FF3-30, Research Institute, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195, USA.

*To whom correspondence should be addressed.