T Cell Activation Antigen, CD26, as a Cofactor for Entry of HIV in CD4$^+$ Cells

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The CD4 molecule is essential for binding HIV particles, but is not sufficient for efficient viral entry and infection. The cofactor was shown to be dipeptidyl peptidase IV (DPP IV), also known as CD26. This serine protease cleaves its substrates at specific motifs; such motifs are also highly conserved in the V3 loops of HIV-1, HIV-2, and related simian isolates. Entry of HIV-1 or HIV-2 into T lymphoblastoid and monocytoid cell lines was inhibited by a specific monoclonal antibody against DPP IV or specific peptide inhibitors of this protease. Co-expression of human CD4 and CD26 in murine NIH 3T3 cells rendered them permissive to infection by HIV-1 and HIV-2. These observations could provide the basis for developing simple and specific inhibitors of HIV and open a possibility for vaccine development.

The human immunodeficiency virus (HIV) infects lymphocytes, monocytes, and macrophages by binding to its principal receptor, the CD4 molecule, through the viral envelope glycoprotein gp120 (*1*). The V3 loop of gp120 is critical for HIV infection, since mutations in it modify cell tropism, generate virions with reduced infectivity, and affect syncytium formation (*2*).

Although there is no direct evidence for cleavage of the V3 loop during HIV entry or syncytium formation, several observations have suggested that the V3 loop may interact with an accessory cell surface component that is probably a protease (*3*). TL2, a cell surface protease similar to trypsin, had been suggested as a potential candidate for the cleavage of the V3 loop. The activity of this tryptase is inhibited by gp120, by V3 loop peptides containing the GPGR motif, by the Kunitz-type inhibitor trypstatin, and by the protease inhibitors leupeptin and antipain. Furthermore, V3 loop peptides, trypstatin, and polyclonal antibody against tryptase TL2 were reported to inhibit syncytium formation. In all of these studies, however, syncytium formation rather than HIV entry or infection were investigated. Cell surface proteins that bind HIV-1 gp41 have been reported but their role in HIV infection has not been demonstrated clearly (*4*).

In spite of their variability, the V3 loops of HIV and simian immunodeficiency virus (SIV) isolates contain highly conserved RP, KP, and GP dipeptide motifs (*5*) (Fig. 1). These motifs also fulfill the substrate specificity of the dipeptidyl peptidase IV (DPP IV) enzymatic activity of the T cell activation antigen, CD26 (*6*). The conservation of these motifs suggests that there is constant selective pressure to preserve them because of their role in virus infection. In the case of HIV-1 isolates from Europe and North America, the dipeptide motif GP found in the crown of the V3 loop is conserved in more than 90% of the samples tested (Fig. 1) and remains unchanged in vivo over several years in a given individual in spite of other substitutions occurring in the crown of the V3 loop. In vitro substitutions of the glycine or the proline residue in the GP motif generate virions with suppressed infectivity, whereas infection in the presence of a neutralizing monoclonal antibody to V3 (anti-V3) results in the production of escape mutant virions, in which the proline is substituted by glutamine (*1*). The V3 loop sequence of the SIV CPZ (chimpanzee) isolate is related to that of HIV-1, and accordingly it contains both the NH_2-terminal RP motif and the GP motif at the crown of the loop. On the other hand, the SIV MND V3 loop is quite divergent but it contains an EP motif that can also serve as a substrate for DPP IV.

(1) CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHC

(2) CTRPYNNTRQRTRIGPGQAFYTTGKIIGDIRQAHC

(3) CKRPGNKTVVPITLMSGLVFHSQPINKRPRQAWC
K

(4) CRRPGNKTVLPVTIMSGLVFHSQPINDRPKQAWC

(5) CHRPGNNTRGEVQIGPGMTFYNIENVVGDTRSAYC

(6) CHRKGNRSVVSTPSATGLLFYHGLEPGKNLKKGMC

Fig. 1. Conserved motifs (boxed letters) in the consensus V3 loops of HIV-1, HIV-2, and SIV isolates. (**1**) 527 European and North American isolates, (**2**) 108 African isolates, (**3**) 25 HIV-2 isolates, (**4**) 8 SIV isolates, (**5**) SIV-CP2, and (**6**) SIV-MND. The study on HIV-1 and 22 of the HIV-2 isolates was reported by Myers *et al.* (*5*). The three other new isolates of HIV-2 were UCI, ALT, and EHO. The sequence of the group of eight SIV isolates (MM 251, MM 142, MM 239, MNE, SMMH4, SMMPBJ, MAC 32, and AGM-TYO), SIV MND, and SIV CPZ were from the Los Alamos HIV sequence Database (*5*). Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

These observations suggested to us that DPP IV may serve as a cofactor of CD4 by interacting with the V3 loop. DPP IV activity has been detected in various tissues, especially in T lymphocytes, monocytes, and platelets. The expression of CD26 is enhanced considerably upon T cell activation (*8*). Recently, CD26 was reported to be identical to the adenosine deaminase binding protein (*9*).

HIV entry into a permissive T cell line (CEM) was monitored by measuring the intracellular concentration of the HIV major core protein p25 after digestion of extracellular virus by trypsin treatment; this procedure eliminates more than 99% of the input virus (*10*). The HIV-1 preparation used in this entry assay was characterized as a highly virulent HIV stock that could synchronously infect at least 90% of cells (*10*). Although the literature contains reports of different effects of V3 peptides (*3*, *11*) on infection and syncytium formation, in our hands V3 loop peptides inhibited the penetration of HIV particles by 88 to 96%. This effect was specific as other gp120 peptides did not have a significant effect (Table 1). Furthermore, peptides corresponding to the crown of the V3 loop also inhibited the entry of HIV particles. These results, and the fact that the V3 loop is not directly involved in the binding of gp120 to the CD4 molecule (*1*), were consistent with the hypothesis that the V3 loop interacts with a cell surface protein other than

Table 1. Inhibition of HIV-1 LAI entry by V3 loop peptides. CEM cells (5×10^5) were first incubated (37°C, 30 min) with peptides [sequence details are as described (*30*)] before the addition of HIV-1 LAI and further incubation at 37°C for 1 hour. HIV entry was then estimated as described (*10*). The mean ± SD of three independent experiments is shown. Dose-dependent inhibitory effects were also observed at 100 and 50 μM. All peptides were purified by high performance liquid chromatography and were at least 90% homogeneous. The three peptides of IIIB type were obtained through the AIDS Research and Reference Reagent Program, NIAID, NIH, from S. Pincus. The other peptides were obtained from l'Association Nationale de la Recherche sur le SIDA, France.

Peptide (200 μM)	Amino acid residues	Internalized p25 (pg/10^5 cells)
None	–	168 ± 12
gp120-IIIB (LAI)	350–378	156 ± 9
gp120-IIIB (LAI)	418–441	138 ± 9
V3-IIIB (LAI)	295–321	20 ± 8
V3-1286	301–333	7 ± 10
V3-crown (LAI)	307–327	62 ± 14
V3-crown HXB2 (LAI)	307–327	7 ± 11

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the CD4 molecule. We therefore investigated HIV entry in the presence of monoclonal antibodies (mAb) specific for T cell surface antigens including CD26 (Table 2). As expected, mAb 110/4 (which binds the V3 loop) and mAb OKT4A (which reacts with the gp120 binding site in the CD4 molecule), inhibited HIV entry by 85 and 93%, respectively. Comparable inhibition (89%) was observed with mAb 1F7, which binds CD26. A somewhat lower level of inhibition (64%) was observed with another antibody to CD26 (BA5), OKT4, directed against an epitope in the CD4 molecule outside the gp120 binding site, resulted in partial inhibition of HIV entry, probably due to steric hindrance. Other mAbs (*12*) specific for neutral endopeptidase CD10, tyrosine phosphatase CD45, and α- or β-lymphocyte functional antigen 1 did not affect HIV entry.

The tripeptide IPI (isoleucyl-prolyl-isoleucine), an inhibitor of DPP IV–like enzymatic activity (*13*), inhibited HIV entry in a dose-dependent manner in contrast to peptides GGG, APL, FPA, RRR, and RSR, which had no effect. The peptides GPGG, KPR, and RPGFSPFPR (which contain the conserved GP, KP, and RP motifs) resulted in marked inhibition of HIV entry (Fig. 2). Peptides that inhibited HIV entry also inhibited the cleavage of a substrate of DPP IV–like enzymatic activity, GP-pNA. The endopeptidase and exopeptidase inhibitors aprotinin, leupeptin, antipain, pepstatin, and bestatin had no apparent effect on HIV entry or DPP IV–like enzymatic activity (*14*). The observation that HIV entry was insensitive to leupeptin and antipain indicates that TL2 is not a cofactor in our system. Consistent with these observations, the tripeptide IPI inhibited HIV entry in lymphoblastoid (MOLT4 and Jurkat) and monocytoid (U937) cells and freshly isolated $CD4^+$ T lymphocytes activated by phytohemagglutinin at concentrations comparable to those observed in CEM cells. It should be emphasized that CEM, MOLT4, Jurkat, and U937 cells expressed CD26 on their surfaces (Fig. 3).

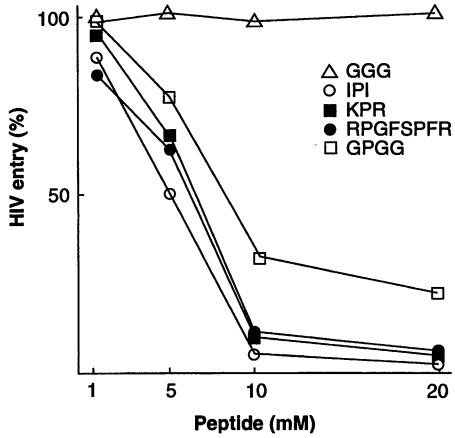

Fig. 2. Inhibition of HIV entry by IPI and peptides containing GP-, RP-, and KP-dipeptide motifs. HIV-1 entry in the presence of different peptides was carried out as described (*10*). The mean value of three independent experiments is shown (with standard deviations in the range of 8 to 15%). The peptides were purchased from Sigma and were purified to 99% homogeneity by high-performance liquid chromatography. All peptides that inhibited HIV entry also inhibited DPP IV activity assayed either on the surface of intact cells or in cell extracts.

Table 2. Inhibition of HIV-1 entry by mAbs specific for DPP IV–CD26. HIV-1 LAI entry was measured as described (*10*). The mAbs OKT4A and OKT4 were purchased from Ortho Diagnostics Systems; mAb 110/4 was generously provided by Genetic Systems, Seattle; mAbs BA5, ALB1, UCHL1, 25.3, and BL5 were obtained from Immunotech S.A., Marseille; mAb 1F7 was a gift provided by C. Morimoto, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts. CEM cells were incubated for 30 min with mAbs before assay of HIV entry. All the mAbs, with the exception of mAb 110/4, were found to bind CEM cells by fluorescence-activated cell sorting (FACS) analysis. The results represent the mean ± SD of three independent experiments. The percent inhibition of HIV entry by mAb 1F7 at 0.5 and 0.1 μg/ml was 64 and 30%, respectively.

mAb (1 μg/ml)	Specificity	Internalized p25 (pg/10^5 cells)
None	–	170 ± 5
OKT4A	CD4	12 ± 2
OKT4	CD4	90 ± 10
110/4	V3 loop	25 ± 8
1F7	CD26	19 ± 4
BA5	CD26	62 ± 11
ALB1	CD10	175 ± 7
UCHL1	CD45RO	172 ± 9
25.3	α-LFA-1	180 ± 11
BL5	β-LFA-1	168 ± 9

To determine whether CD26 is implicated in HIV-2 entry, we used HIV-2 EHO (*15*), a highly cytopathic, HIV-2–related virus (*16*). After 1 hour of infection to allow HIV-2 entry in the presence of different inhibitors, cells were trypsinized to eliminate input virus and were cultured to assay for the production of virus. Peptides IPI and KPR (at 10 mM) and mAb 1F7 resulted in 81, 96, and 85% inhibition of HIV-2 EHO production, respectively, whereas the corresponding control agents (peptide GGG and anti-CD10) had no apparent effect. In a similar experiment, IPI, KPR, and mAb 1F7 treatment during HIV-1 LAI entry resulted in 96, 94, and 89% inhibition of virus production, respectively (*15*).

Previous studies have indicated that the additional cell surface component required for HIV entry, like the CD4 molecule, is species specific (*17*). We found DPP IV–like peptidase activity on the surface of $CD4^-$ human and murine cell lines (*18*). However, peptidase activity on murine cells (such as NIH 3T3 and L929 cells) was resistant to inhibition by IPI. In further studies with extracts from human (HeLa) and murine (NIH 3T3) cells, the murine enzyme was less sensitive to inhibition by IPI than the human one. The concentration of IPI that inhibited activity by 50% (IC_{50}) was at least 100-fold higher for murine compared to human DPP IV–like activity assayed on GP- or RP-pNA. The

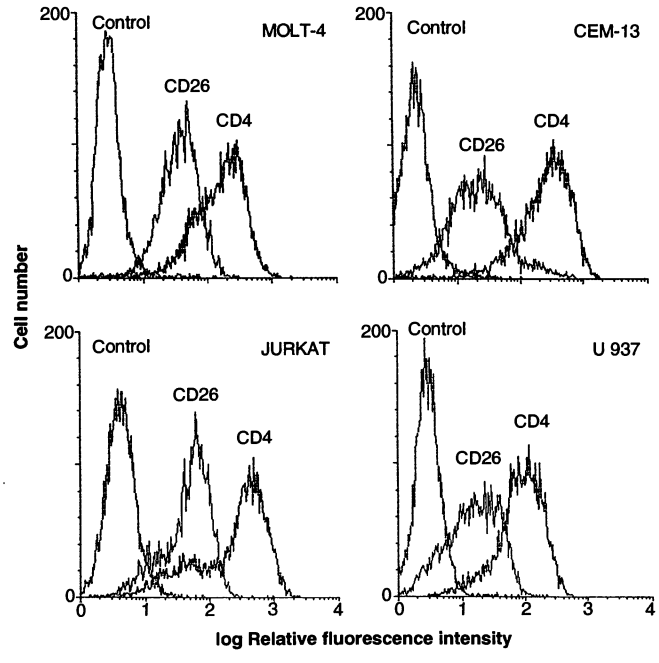

Fig. 3. Presence of CD26 on Molt-4, CEM-131, Jurkat, and U937 cells. Fluorescence-activated cell sorting analysis of cell lines was carried out with mAb 71/11 against human ribosomal associated protein kinase (Hovanessian, Institut Pasteur) as a control, and mAbs BA5 (Immunotech, SA, Marseille) and OKT4A (Ortho Diagnostics Systems) against CD26 and CD4, respectively.

activity of arginine-peptidase was similar between human and murine sources and was relatively insensitive to IPI (*18*). Our results suggest that CD26 sequence differences between these species are responsible for the inability of HIV to infect murine cells expressing the human CD4 molecule.

The inability of murine CD26 to serve as a cofactor with human CD4 was further demonstrated by the experiment described in Fig. 4A. NIH 3T3 cells were transfected with human CD4- and CD26-expressing plasmid vectors, either individually or together. Transfected cells were then challenged with HIV-1 LAI and the production of virus was measured by using the supernatants to infect CEM cells. At 7 days after infection, no significant virus production was detectable in supernatants of NIH 3T3 cells expressing either CD4 or CD26. On the other hand, culture supernatant from transfected cells expressing both CD4 and CD26 produced at least 30-fold higher levels of infectious virus in CEM cells. Addition of IPI (Fig. 2) and heparin (*10*) to cells expressing both CD4 and CD26 resulted in inhibition of HIV infection. As the murine CD26 peptidase activity is resistant to IPI, the blockage of HIV infection during viral challenge of NIH 3T3 cells should be due to the inhibitory effect of IPI on the transfected human CD26.

NIH 3T3 cells expressing either CD4 or CD26 were found to be slightly infectable, as they produced infectious virus that, when amplified on CEM cells, resulted in the production of detectable amounts of virus at day 11 after infection. However, the amount of virus produced from such transfected cells was found to be at least 70- and 650-fold less, respectively, compared to that from transfected cells expressing both CD4 and CD26 together (Fig. 4A). The fact that no infectious virus was found in the culture supernatant of control NIH 3T3 cells that were infected in parallel with HIV-1 illustrates that the presence of infectious virus in the culture supernatant from NIH 3T3 cells expressing both CD4 and CD26 was due to the replication of virus in these cells.

In a similar type of experiment but using the HIV-2 EHO isolate, infection occurred in cells expressing both human CD4 and CD26 molecules. As expected, such infection was blocked by tripeptide inhibitors (IPI and KPR) and heparin (Fig. 4B). Several reports have suggested that certain HIV-2 preparations can infect $CD4^-$ human cells at very low efficiencies (*19*). Accordingly, expression of CD26 alone in NIH 3T3 cells resulted in a slight production by HIV-2 EHO, which was at least fivefold lower than that produced by cells expressing both CD4 and CD26 (Fig. 4B). Overall, these results confirm that CD26 is essential for HIV entry into CD4-expressing cells.

In order to confirm that production of virus in NIH 3T3 cells expressing human CD4 and CD26 was due to HIV replication, transfected cells were challenged with HIV-1 LAI in the absence or the presence of 5 μM of the reverse transcriptase inhibitor, azidothymidine (AZT). Cells were then washed extensively before trypsinization and passaging in fresh culture medium. After 24 hours, virus in the supernatants of these cultures was assayed by infection of CEM cells. At 7 days after infection, syncytia were observed only in CEM cells infected with the supernatant of $CD4^+$, $CD26^+$ transfected cells that had been challenged with the HIV virus in the absence of AZT. The concentration of p25 in the CEM supernatant was 450 and 8 pg/ml in cultures infected by virus in the absence or in the presence of AZT, respectively. These results indicate that HIV replication was indeed required in NIH 3T3 cells expressing both CD4 and CD26 in order to produce infectious virus. Such observations also illustrate that HIV production by transfected cells expressing human CD4 and CD26 was not due to any artifactual consequences of the experimental approach used in these experiments.

To investigate the mechanism by which peptide inhibitors inhibit viral entry, we studied the binding of ^{125}I-labeled gp120 to CEM cells in the presence of mAbs and peptide inhibitors (Fig. 5). As expected, mAbs OKT4A and 110/4 (*2*) completely abolished the binding of ^{125}I-labeled gp120 to CEM cells. In contrast OKT4 only partially inhibited the binding, consistent with the slight inhibition of HIV entry by this antibody. A partial inhibition of gp120 binding was observed with mAb 1F7 (most probably a result of steric hindrance), suggesting that CD26 is located in the vicinity of the CD4 molecule. The peptide inhibitors

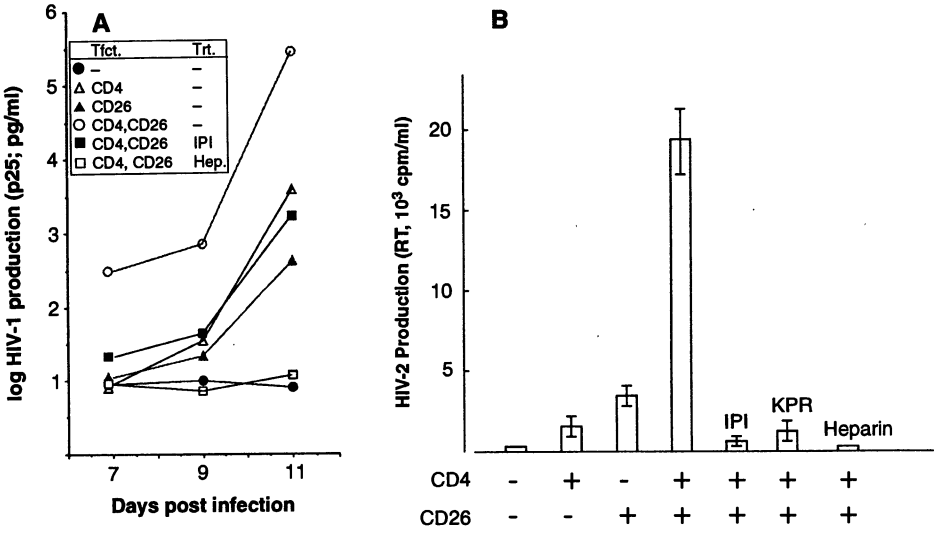

Fig. 4. Coexpression of human CD4 and CD26 in murine NIH 3T3 cells renders them permissive to infection by (**A**) HIV-1 and (**B**) HIV-2. NIH 3T3 cells were transfected with human CD4- and CD26-expressing plasmid vectors, either independently or together, by calcium phosphate coprecipitation (*32*). At 48 hours, transfected cells were challenged with HIV-1 LAI or HIV-2 EHO in the absence or presence of IPI (20 mM) and heparin (100 μg/ml). Six hours later, cells were trypsinized to eliminate extracellular virus and then replated in fresh culture medium. Twenty-four hours after viral challenge, CEM cells (5×10^6) were exposed to 1 ml of each culture supernatant for 90 min before dilution in 5 ml of culture medium (RPMI-1640 containing 10% fetal bovine serum). (**A**) HIV-1. The production of virus in CEM cell cultures was assayed by measuring the concentration of p25 (the mean of two independent experiments) in the culture medium on days 7, 9, and 11. Tfct., transfection; Trt, treatment; Hep, heparin (**B**) HIV-2. The production of virus in CEM cell cultures was assayed by measuring the reverse transcriptase activity in the culture medium on day 7. The mean of two independent experiments is shown. HIV-2 production in $CD4^+$ $CD26^+$ NIH 3T3 cells was confirmed by the presence of viral proteins as shown by immunoblot analysis of crude CEM cell extracts with $HIV\text{-}2^+$ serum. NIH 3T3 cells transfected with the CD4-expressing plasmid resulted in the expression of CD4 on 6 to 8% of cells 48 hours after trypsin treatment, as monitored by FACS analysis of trypsinized cells with mAb OKT4 (*32*). Since trypsin treatment of cells eliminates cell surface CD26 antigen, the expression of CD26 in transfected cells was monitored by assaying DPP IV activity in immune complex preparations with mAb 1F7 (*31*). DPP IV enzymatic activity was detectable only in extracts of NIH 3T3 cells transfected with the CD26-expressing plasmid. By this procedure, the DPP IV activity values of the human CD26 for the cleavage of GP-pNA in control, $CD4^+$, $CD26^+$, and $CD4^+$ $CD26^+$ cells were 0, 0, 416, and 385 pmol/hour per 10^6 cells, respectively (*31*). For comparison, the DPP IV activity in similarly prepared immune complex preparations derived from extracts of CEM cells was 2960 pmol/hour per 10^6 cells.

IPI and KPR had no apparent effect on the binding, thus indicating that their inhibition of viral entry is a post-binding event.

The observations that peptides containing motifs conserved in the V3 loops of HIV-1 or HIV-2 isolates can block CD26 peptidase activity and HIV entry are strongly in favor of an interaction between the V3 loop and CD26. The V3 loop peptides (24 to 33 amino acids in length) did not affect the DPP IV activity of CD26 in the assay in which GP- or RP-pNA was used as an exopeptidase substrate. This can be explained by the higher affinity of GP-pNA for the enzyme as compared with V3 loop peptides (*20, 21*). However, for HIV entry, the V3 loop can compete with the gp120 of the HIV particles since it is competing with a similar structure.

The observation that peptide inhibitors blocked entry of HIV without affecting the binding of gp120 to the CD4 receptor suggests that the CD4 molecule simply serves as an efficient virus attachment site on the cell surface that then allows the interaction between the V3 loop and CD26. Binding of gp120 to CD4 may induce conformational changes, making the V3 loop more accessible. This is the observation that the binding of V3 loop–specific mAb to gp120 is enhanced by soluble CD4 (sCD4) (*22*). Although HIV entry was significantly blocked by both mAb IF7 as well as tripeptides IPI and KPR, DPP IV activity was inhibited only by the tripeptides. Monoclonal antibody IF7 had no apparent effect on DPP IV activity (*23*). On the other hand, these tripeptides did not affect binding, whereas mAb 1F7 exerted a partial inhibitory effect that was probably due to steric hindrance. These two types of inhibitors therefore might interfere in the process of HIV entry by two independent mechanisms: IPI and KPR could inhibit the active site involved in DPP IV, whereas the gp120 recognition site in CD26.

We have been unable to detect the cleavage of gp120 during HIV-1 entry in CEM cells. Similarly, gp120 is not cleaved when incubated with a purified preparation of DPP IV or when incubated with cell extracts manifesting high levels of DPP IV activity (*21*). Therefore, it remains possible that CD26 is acting as a coreceptor without actually cleaving its target.

Under our conditions, peptides that inhibited HIV entry also inhibited cell surface DPP IV activity at similar concentrations. However, further studies based on point mutations in the catalytic domain of CD26 are necessary to determine a requirement for the catalytic domain.

Previously, several authors (*24*) have reported cleavage occurring in the V3 loop of pure preparations of gp120 into 50- and 70-kD fragments by thrombin, tryptase, and cathepsin E. Cleavage was enhanced in the presence of sCD4, suggesting that the binding of gp120 to sCD4 may expose the V3 loop and make it a potential target for the action of different proteases. A phenylmethyl-sulfonyl-fluoride (PMSF)–resistant cleavage of gp120 in purified HIV-1 preparations that were mixed with sCD4 has also been reported; however, it is most unlikely that this cleavage is a consequence of CD26 functioning, as DDP IV activity is inhibited by low concentrations of PMSF (*25*). Under our experimental conditions, the IC_{50} value of PMSF on the DDP IV activity was 0.2 mM.

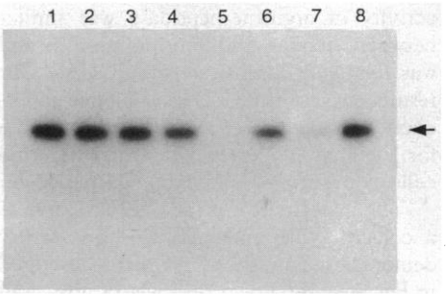

Fig. 5. The binding of gp120 to CEM cells in the presence of tripeptide inhibitors and anti-CD26. CEM cells (5×10^6) were incubated (37°C, 1 hour) with ^{125}I-labeled gp^{120} (50 ng; 10 Ci/mg) in the presence or absence of 20 mM KPR or IPI, or mAbs at 1 μg/ml. Cells were then washed with PBS containing 2 mM EDTA, and cytoplasmic extracts were analyzed by polyacrylamide gel electrophoresis in the presence of SDS. Recombinant gp120 was radioiodinated with the Bolton-Hunter reagent as described (*11*). No apparent internalization of gp120 occurred under these experimental conditions. Trypsin treatment resulted in complete loss of ^{125}I-labeled gp120 bound to cells. Lane 1, no added inhibitors or antibodies; lane 2, KPR; lane 3, IPI; lane 4, OKT4; lane 5, OKT4A; lane 6, 1F7; lane 7, 110/4; lane 8, ALB1. The arrowhead indicates the position of gp120.

The involvement of CD26 in HIV entry and infection would be consistent with other findings. In freshly isolated CD4$^+$ T lymphocytes, CD26 is expressed on the surface of quiescent cells but its level is enhanced considerably upon T cell activation (*26*), a process routinely associated with enhanced expression of cell surface CD26 (*27*).

Recently, Valle-Blazquez *et al.* (*28*) have reported that HIV preferentially infects freshly isolated CD26$^-$/CD4$^+$ T lymphocytes in vitro. However, in this work, the CD26$^+$ cells were selected by anti-CD26–coated beads, which may result in down modulation of CD26 and lead to artifacts. Furthermore, only one sample was used for this in vitro infection experiment; viral infection was detectable after amplification by the polymerase chain reaction (PCR) technique, probably due to low infection of such cells, which as the authors state might have been contaminated by as much as 5% with CD26$^+$ cells. In our experiments, all cell lines studied for HIV entry were shown to be CD26$^+$ (Fig. 3).

Several reports have shown a selective decrease in the proportion of CD26-expressing CD4$^+$ T lymphocytes in HIV-1–infected individuals (*28*). Such observations are consistent with the requirement of CD26 for HIV entry and the fact that HIV-producing cells in vitro die by apoptosis. The transmembrane glycoprotein gp41/gp120 complex is essential for entry of HIV particles, and when expressed on the membrane of infected cells, it is responsible for initiation of apoptosis by interaction with the CD4 molecule (*29*). By analogy with the mechanism of HIV entry described here and previous observations indicating that modulation of CD26 results in signal transduction (*27*), it remains plausible to suggest that CD26 is also implicated in the mechanism of triggering apoptosis through the gp41/gp120 complex.

The inhibition of HIV-1 and HIV-2 entry by IPI and KPR indicates the possibility of developing simple but specific inhibitors that could block the function of CD26 and thus be used as effective therapeutic agents in AIDS patients. Finally, the domains in the V3 loops of HIV-1 and HIV-2 containing the strongly conserved dipeptide motifs could be considered as potential targets for the development of polyvalent vaccines.

REFERENCES AND NOTES

1. D. Klatzman *et al.*, *Nature* **312**, 767 (1984); A. G. Dalgleish *et al.*, *ibid.*, p. 763 (1984); L. E. Eiden and J. D. Lifson, *Immunol. Today* **13**, 201 (1992).
2. J. Goudsmit *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4478 (1988); E. Kinney-Thomas *et al.*, *AIDS* **2**, 25 (1988); J. J. De Jong *et al.*, *J. Virol.* **66**, 6777 (1992); B. Chesebro *et al.*, *ibid.*, p. 6547; P. S. Linsley *et al.*, *ibid.* **62**, 3695 (1988); E. O. Freed *et al.*, *ibid.* **65**, 190 (1991); K. A. Page, S. M. Stearns, D. R. Littman, *ibid.* **66**, 524 (1992); E. Helseth *et al.*, *ibid.* **64**, 2416 (1990). In the literature, mAb 110/4 specific for the V3 loop of HIV-1 LAI is considered not to affect the binding of gp120 to CD4. However, this is a misinterpretation of the data reported by Linsley *et al.*, since they demonstrate that with mAb 110/4 there may be as much as 65% inhibition of binding of crude preparation of gp120 to CD4$^+$ cells. In our binding experiments (Fig. 5), the sensitivity of the assay (10^{-9} M) is probably much higher due to the use of pure ^{125}I-labeled gp120.
3. P. E. Stephens *et al.*, *Nature* **343**, 219 (1990); H. Kido, Y. Yokogoshi, N. Katunuma, *J. Biol. Chem.* **263**, 18104 (1988); A. Koito *et al.*, *Intern. Immunol.* **1**, 613 (1989); T. Hattori *et al.*, *FEBS Lett.* **248**, 48 (1989); H. Kido, A. Fukutomi, N. Katunuma, *ibid.* **286**, 233 (1991).
4. N. M. Qureshi, D. H. Coy, R. F. Garry, L. A. Henderson, *AIDS* **4**, 553 (1990); C. F. Ebenbichler *et al.*, *ibid.* **7**, 489 (1993); L. A. Henderson and N. M. Qureshi, *J. Biol. Chem.* **268**, 15291 (1993).
5. G. Myers *et al.*, *Human Retroviruses and AIDS* (Los Alamos National Laboratory, Los Alamos, NM, 1992); *ibid.* (1991); S. W. Barnett, M.

Quiroga, A. Werner, D. Dina, J. A. Levy, *J. Virol.* **67**, 1006 (1993); R. Kreutz *et al.*, *AIDS Res. Hum. Retroviruses* **8**, 1619 (1992); L. Milich, B. Margolin, R. Swanstrom, *J. Virol.* **67**, 5623 (1993). The deduced amino acid sequence of the V3 loop of HIV-2 EHO is the following: CKRPGNKMVVPIRTVSGILFHSQPINKRPKQAWC (M. A. Rey-Cuillé *et al.*, unpublished data).

6. A. N. Barclay *et al.*, *The Leucocyte Antigen. Facts Book* (Harcourt Brace Jovanovich, London, 1993); C. Morimoto *et al.*, *J. Immunol.* **143**, 3430 (1989); M. Hegen, G. Niedobitek, C. E. Klein, H. Stein, B. Fleischer, *ibid.* **144**, 2908 (1990); T. Tanaka *et al.*, *ibid.* **149**, 481 (1992).
7. G. J. LaRosa *et al.*, *Science* **249**, 932 (1990); A. J. Leigh Brown, *AIDS* **5**, S35 (1991); K. Holmbäck *et al.*, *J. Virol.* **67**, 1612 (1993); R. J. Grimalia *et al.*, *ibid.* **66**, 1875 (1992); T. Masuda *et al.*, *J. Immunol.* **145**, 3240 (1990); S. H. Chiou, E. O. Freed, A. T. Panganiban, W. R. Kenealy, *AIDS Res. Hum. Retroviruses* **8**, 1611 (1992).
8. E. Schön *et al.*, *Scand. J. Immunol.* **29**, 127 (1989); T. Mattern, W. Scholz, A. C. Feller, H. D. Flad, A. J. Ulmer, *ibid.* **33**, 737 (1991).
9. J. Kameola, T. Tanaka, Y. Nojima, S. F. Schlossman, C. Morimoto, *Science* **261**, 466 (1993); M. E. Morrison *et al.*, *J. Exp. Med.* **177**, 1135 (1993).
10. The procedure used was essentially as described [J. M. Harouse *et al.*, *Science* **253**, 320 (1991)]. HIV entry was monitored by the intracellular concentration of p25 after elimination of more than 99% of extracellular input virus by trypsin treatment. Briefly, CEM cells (5×10^6) were suspended in 1 ml of HIV-1 preparation corresponding to 0.1 μg of p25. After 1 hour of incubation at 37°C, the remaining virus adsorbed on cells was removed by washing cells in phosphate-buffered saline (PBS) containing 2 mM EDTA and then treating with trypsin (2.5 mg/ml for 10 min at room temperature). Digestion was stopped by the addition of 10× RPMI-1640 containing 10% (v/v) fetal bovine serum. Cytoplasmic extracts were prepared by disruption of cells in extraction buffer containing 20 mM tris-HCl, pH 7.6, 0.15M NaCl, 5 mM $MgCl_2$, 0.2 mM PMSF, aprotinin (100 units/ml), and 0.5% Triton X-100. After centrifugation at 1000*g*, the concentration of p25 in the supernatant was measured by an enzyme-linked immunosorbent assay (ELISA). For the assay of HIV production, trypsinized cells were suspended in RPMI-1640 containing 10% fetal bovine serum and cultured for 3 days. The supernatants of such cultures were tested for the production of virus by measuring the concentration of p25. In all the experiments, mAbs and peptides were added 30 min before the addition of virus.

HIV entry, which occurs at 37°C but not at 4°C, is blocked by polyanions such as heparin, dextran-sulfate, and poly(A) poly(U) without affecting the binding of gp120 to $CD4^+$ CEM cells. [B. Krust, C. Callebaut, A. G. Hovanessian, *AIDS Res. Hum. Retroviruses* **9**, 1081 (1993)]. For this reason, in order to verify that trypsin treatment eliminates extracellular virus, we used poly(A) poly(U) and/or heparin (at 100 μg/ml each) in every assay of HIV entry to monitor the background levels of p25. Inhibition of HIV entry in the presence of polyanions was also monitored by immunoblot analysis (with anti-p25) of crude extracts of cells before and after trypsin treatment. Before trypsin treatment of cells, no apparent difference was detectable in the level of p25 between untreated and polyanion-treated cells, whereas after trypsin treatment p25 was detectable only in untreated cells. Under our experimental conditions, therefore, trypsin treatment efficiently eliminated extracellularly associated virus.

For the assay of HIV entry, the HIV-1 LAI stock propagated on CEM cells was used. This virus stock results in a synchronous infection of more than 90% of cells by the input virus [A. G. Laurent-Crawford and A. G. Hovanessian *J. Gen. Virol.* **74**, 2619 (1993)]. The virus preparation was in RPMI-1640 medium supplemented with 10% fetal bovine serum and Polybrene (2 μg/ml).

This technique, once optimized for the trypsin treatment, could be used reproducibly to assay the effect of HIV entry inhibitors. Under our experimental conditions, there was routinely a very strong correlation between the amount of virus entered into the cells after 1 hour of infection and the amount of virus produced at 3 days after culture of similarly trypsinized cells. If in spite of trypsin treatment there was some residual virus still remaining bound to cells, then it would have been impossible to see a systematic inhibition of HIV entry and infection in the presence of tripeptide inhibitors that did not affect the binding of gp120 as we describe in this paper.

11. A. De Rossi *et al.*, *Virology* **184**, 187 (1991); Q. J. Sattentau and J. P. Moore, *J. Exp. Med.* **174**, 407 (1991); P. N. Nehete, R. B. Arlinghaus, K. J. Sastry, *J. Virol.* **67**, 6841 (1993).
12. N. H. Dang *et al.*, *J. Immunol.* **145**, 3963 (1990); Y. Torimoto *et al.*, *ibid.* **147**, 2514 (1991); R. Pardi *et al.*, *Immunol. Today* **13**, 224 (1992).
13. H. Umezawa *et al.*, *J. Antibiot.* **37**, 422 (1984).
14. The effect of exopeptidase and endopeptidase inhibitors (at 100 μg/ml) was tested on the entry of HIV-1 LAI in CEM cells. One hour after virus infection, extracellular HIV particles were eliminated by trypsin treatment (*10*) and cells were cultured in fresh medium for 3 days. The supernatant of each culture was tested for the production of HIV by measuring the concentration of p25. The effect of the different inhibitors was less than 20%.
15. M. A. Rey *et al.*, *Virology* **173**, 258 (1989). CEM cells were infected with HIV-2 EHO (*16*) or HIV-1 LAI in the presence of peptides (10 mM) or mAbs (1 μg/ml) for 1 hour at 37°C. Infected cells were then treated with trypsin to eliminate extracellular virus (*10*) and cells were cultured for 3 days in RPMI 1640 supplemented with 10% fetal bovine serum. The production of virus (the mean of three independent experiments) was measured by the assay of reverse transcriptase (RT) activity for HIV-2 EHO (182,640 RT cpm/ml for the control infection). For HIV-1 LAI, virus production was measured either by the assay of p25 concentration for HIV-1 LAI (146 ng/ml for the control infection) or by RT activity (855,760 cpm/ml for the control infection).
16. M. A. Rey-Cuillé, J. Galabru, A. G. Laurent-Crawford, B. Krust, A. G. Hovanessian, in preparation.
17. P. J. Maddon *et al.*, *Cell* **47**, 333 (1986); P. A. Ashorn, E. A. Berger, B. Moss, *J. Virol.* **64**, 2149 (1990); D. B. Weiner, *Pathobiology* **59**, 361 (1991); T. Dragic *et al.*, *J. Virol.* **66**, 4794 (1992); C. C. Broder, D. S. Dimitrov, R. Blumenthal, E. A. Berger, *Virology* **193**, 483 (1993).
18. B. Bauvois, J. Sancéau, J. Wietzerbin, *Eur. J. Immunol.* **22**, 923 (1992). Consequently, the DPP IV peptidase activity (*31*) from NIH 3T3 cells has a distinct response to IPI compared to that from human cell lines studied in this manuscript (HeLa, MOLT4, and CEM).
19. P. R. Clapham, D. Blanc, R. A. Weiss, *Virology* **181**, 703 (1991); R. A. Weiss and P. R. Claplam, in *Science Challenging AIDS*, G. B. Rossi, E. Beth-Giraldo, L. Chieco-Bianchi, F. Dianzani, G. Giraldo, P. Verani, Eds. (Karger, Farmington, CT, 1992), pp. 107–115.
20. A. J. Kenny *et al.*, *Biochem. J.* **155**, 169 (1976); B. Bauvois, J. Gavrilovic, J. Wietzerbin, in *Lymphatic Tissues and in Vivo Immune Responses*, B. A. Imhof, S. Berri-Aknin, S. Enzine, Eds. (Dekker, New York, 1991); T. Nagatsu *et al.*, *Anal. Biochem.* **74**, 466 (1976); M. Kudo, T. Nakamura, J. Koyama, *J. Biochem.* **97**, 1211 (1985); B. Bauvois, *Biochem. J.* **252**, 723 (1988).
21. The V3 peptides and gp120 did not affect DPP IV peptidase activity of CD26 in the assay in which GP- or RP-pNA was used as an exopeptidase substrate (*31*). Incubation of ^{125}I-labeled gp120 (see Fig. 5) with a purified preparation of human placental DPP IV (Calbiochem Biochemicals for Research) or with CEM cell extracts manifesting a high level of DPP IV peptidase activity (*31*) under peptidase assay conditions did not result in the cleavage of gp120. The ^{125}I-labeled gp120 was cleaved by thrombin as expected (*19*).
22. J. A. McKeating, J. Cordell, C. J. Dean, P. Baffe, *Virology* **191**, 732 (1992).
23. T. Tanaka, J. Kameoka, A. Yaron, S. F. Schlossman, C. Morimoto, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 4586 (1993). Addition of mAb 1F7 in the DPP IV assay in the presence of cell extracts as described (*31*) does not affect the cleavage of GP-pNA substrate. Furthermore, immune complex preparations of mAb 1F7 with CD26 manifest DPP IV-like enzymatic activity.
24. B. Ardman, M. Kowalski, J. Bristol, W. Haseltine, J. Sodroski, *J. Acq. Immun. Def. Synd.* **3**, 206 (1990); G. J. Clements *et al.*, *AIDS Res. Hum. Retroviruses* **7**, 3 (1991).
25. A. Werner and J. A. Levy, *J. Virol.* **67**, 2566 (1993); I. De Meester *et al.*, *Clinica Chimica Acta.* **210**, 23 (1992).
26. S. D. Gowda, B. S. Stein, N. Mohagheghpour, C. J. Benike, E. G. Engleman, *J. Immunol.* **142**, 773 (1989); J. A. Zack *et al.*, *Cell* **61**, 213 (1990).
27. D. A. Hafler, M. Chofflon, D. Benjamin, N. H. Dang, J. Breitmeyer, *J. Immunol.* **142**, 2590 (1989); E. Schön *et al.*, *Eur. J. Immunol.* **17**, 1821 (1987); N. H. Dang *et al.*, *ibid.* **145**, 3963 (1990); Y. Torimoto *et al.*, *ibid.* **147**, 2514 (1991); E. Munoz, M. V. Blasquez, J. A. Madueno, G. Rubio, J. Pena, *Immunology* **77**, 43 (1992).
28. A. De Pasquale *et al.*, *Acta Haemat.* **81**, 19 (1989); M. Valle-Blazquez *et al.*, *J. Immunol.* **149**, 3073 (1992); G. Vanham *et al.*, *J. Acq. Imm. Def. Synd.* **6**, 749 (1993).
29. C. Terai, R. S. Kornbluth, C. D. Pauza, D. D. Richman, D. A. Carson, *J. Clin. Invest.* **87**, 1710 (1991); A. G. Laurent-Crawford *et al.*, *Virology* **185**, 829 (1991); A. G. Laurent-Crawford *et al.*, *AIDS Res. Human Retroviruses* **9**, 761 (1993).
30. The amino acid sequences of the peptides used in Table 1 are the following: gp120-IIIB (350–378): REQFGNNKTIIFKQSSGGDPEIVTHSFNC; gp120-IIIB (418–441): CRIKQIINMWQKVGKAMYAPPISG; V3-IIIB (295–321): TRPNNNTRKSIRIQRGPGRAFVITGKIGNMRQAH; V3-1286 (301–333): CTRPNNNTRKRIYLGPGRAWYTTKAIIGDIRQA; V3-crown LAI (307–327): CNTRKSIRIQRGPGRAFVTIGK; V3-crown HXB2 (307–327): CNTRKRIRIQRGPGRAFVTIGK.
31. The DPP IV–like peptidase activity was measured either on the surface of intact cells (5×10^6) or in extracts from MOLT4 cells (25 μl) in a total reaction volume of 0.5 ml in peptidase buffer [100 mM Hepes pH 7.6, 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 8 mM glucose, 1% bovine serum albumin, and 0.5 mM of either GP-pNA or RP-pNA (*18*)]. Incubation was at 37°C for 2 to 4 hours and the reaction was stopped by the addition of 1 M sodium acetate pH 4.5 (1 ml). After centrifugation at 12,000*g* for 5 min, the production of pNA in the supernatant was measured by absorption at 405 nm. For the preparation of cell extracts, cells lysed in buffer E (75 μl per 10^7 cells) were kept at 4°C for 10 min before centrifugation at 12,000*g* for 10 min. The supernatant diluted with one volume of buffer BI was then centrifuged once again at 12,000*g* for 10 min and the supernatant stored at −80°C. Buffer E contains 20 mM tris HCl, pH 7.6, 150 mM NaCl, 5 mM $MgCl_2$, 0.2 mM PMSF, aprotinin (100 U/ml) and 0.5% Triton X-100. Buffer BI contains 20 mM tris-HCl, 400 mM NaCl, 50 mM KCl, 1 mM EDTA, 0.2 mM PMSF, aprotinin (100 U/ml), 5 mM β-mercaptoethanol, 1% Triton X-100, and 20% glycerol. Assay of DPP IV peptidase activity in transfected NIH 3T3 cells was carried out after immunoprecipitation of crude cell extracts with mAb 1F7 and protein A–Sepharose in buffer BI [A. G. Hovanessian *et al.*, *EMBO J.* **6**, 1273 (1987)]. After three washes with buffer BI, immune complex preparations were washed in peptidase buffer and assayed for DPP IV peptidase activity as above.
32. NIH 3T3 cells were transfected 3 days after seeding at 1.2×10^6 per 25-cm^2 flask. The culture medium (Dulbecco's) was replaced with fresh medium and, after 1 hour incubation, 10 μg of each plasmid (pLXSN expressing human CD4 under the control of Mo-MuLV LTR; pKG5 expressing human CD26 under the control of the SV40 early promoter) or a mixture containing 5 μg of each was precipitated on the cells by the

calcium phosphate coprecipitation technique. After 48 hours, transfected cells were washed with medium containing 2 mM EDTA and then with culture medium. Cells were then challenged with HIV-1 LAI (corresponding to 0.5 μg of p25, which is equivalent to about 40 particles per cell) for 6 hours in the absence or presence of inhibitors. Cells were first washed in medium containing 2 mM EDTA and then washed once with trypsin before incubation (5 min at 37°C) in 5 ml of trypsin. Cells were then replated in 75-cm^2 flasks containing fresh culture medium and incubated at 37°C for 24 hours. One-milliliter portions of each supernatant were then used to infect CEM cells (5 × 10^6). The production of HIV-1 (as determined by ELISA of p25) in CEM cultures was measured by assaying culture supernatants 7, 9, and 11 days later. The expression of CD4 antigen on the cell surface of transfected cells was carried out 48 hours after trypsin treatment by FACS analysis with mAb OKT4. [P. R. Rao, M. A. Talle, P. C. Kung, G. Goldstein, *Cell Immunol.* **80**, 310 (1983)]. The expression of human CD26 was determined after immunoprecipitation (see Fig. 4, legend).

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Distinct Roles for Cyclin-Dependent Kinases in Cell Cycle Control

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The key cell-cycle regulator Cdc2 belongs to a family of cyclin-dependent kinases in higher eukaryotes. Dominant-negative mutations were used to address the requirement for kinases of this family in progression through the human cell cycle. A dominant-negative Cdc2 mutant arrested cells at the G_2 to M phase transition, whereas mutants of the cyclin-dependent kinases Cdk2 and Cdk3 caused a G_1 block. The mutant phenotypes were specifically rescued by the corresponding wild-type kinases. These data reveal that Cdk3, in addition to Cdc2 and Cdk2, executes a distinct and essential function in the mammalian cell cycle.

Cell division is controlled by way of a complex network of biochemical signals that are similar in all eukaryotic cells. Together, these signals regulate specific transitions in the cell cycle. The best characterized transitions are those from G_1 to S phase and from G_2 to mitosis. In yeast, passage through both transition points is regulated by the same protein kinase, the product of the *CDC28* or *cdc2*$^+$ gene for *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, respectively (*1*). The Cdc2-CDC28 catalytic subunit requires association with a cyclin regulatory subunit for kinase activity (*2*), and different cyclins are involved in the G_1/S transition (G_1 cyclins) and the G_2/M transition (mitotic cyclins). Multicellular eukaryotes appear to have developed a higher degree of regulation. They express multiple cyclins, like yeast, but also contain multiple catalytic subunits that can interact with these cyclins. Whereas $p34^{cdc2}$ is active and essential at the G_2/M transition (*3*, *4*), a closely related kinase, $p33^{cdk2}$, has been implicated in the initiation of DNA replication (*3*, *5*, *6*).

Twelve human protein kinases have been described that share extensive amino acid sequence identity with $p34^{cdc2}$ (*7*–*10*). These kinases are named temporarily after their amino acid sequence in the PSTAIRE-region (*11*), a domain that is conserved between yeast and human Cdc2. Alternatively, they are designated as cyclin-dependent kinases either when a cyclin partner is identified or when they complement yeast *cdc2-cdc28* mutations. In mammalian cells, Cdc2 associates mainly with A- and B-type cyclins; Cdk2 associates with cyclins A, E, and D; and Cdk4 (formerly PSK-J3), Cdk5 (previously PSSALRE), and Cdk6 (previously PLSTIRE) associate with D-type cyclins (*5*, *8*, *10*, *12*, *13*). Although Cdk3 has never been found in association with cyclins, because of high sequence identity with both Cdc2 and Cdk2 and the ability to complement *cdc28* mutations in yeast, it is classified as a cyclin-dependent kinase (*7*).

The existence of a family of Cdc2-related genes suggests that other kinases, in addition to Cdc2 and Cdk2, may regulate distinct steps in the cell cycle. To investigate the requirement for the other kinases in cell cycle progression, we examined the phenotypic consequences of the inactivation of each kinase. We generated dominant-negative mutations for each Cdc2-related kinase and expressed these mutant forms in human cells. When expressed at high levels, dominant-negative mutations inactivate the function of the wild-type protein by competing for essential interacting molecules (*14*). Data from previous structure-function studies predicted that the mutation of Asp^{145} in Cdk2 (Asp^{146} in Cdc2) might generate dominant-negative mutants. This residue is conserved in all protein kinases and is part of an amino acid stretch, KLAD*FGLAR (*11*) (* marks point of mutation), that is identical in all Cdc2-related genes (*7*, *15*). The equivalent Asp residue in 3′,5′-adenosine monophosphate (cAMP)–dependent kinase is known to be essential in the phospho-transfer reaction (*16*). On the basis of the crystal structure data, this residue presumably chelates Mg^{2+} and orients the β- and γ-phosphates of magnesium adenosine triphosphate (Mg^{2+}ATP) in the catalytic cleft of the enzyme (*17*). Moreover, an Asp to Asn point mutation at this position has been identified in one of the two dominant-negative mutant alleles that have been found for CDC28 in yeast (*18*). Finally, this residue is located outside the regions of cdc2 that are implicated in binding cyclin and $p13^{suc1}$ subunits (*19*).

To determine whether dominant-negative inhibition could lead to specific loss of function, we tested the effects of the Asp to Asn mutation in Cdc2 and Cdk2. For each kinase, four versions were generated: wild type (wt) and mutant, each untagged or modified with an influenza hemagglutinin (HA) epitope tag at the COOH-terminus to allow discrimination between endogenous and exogenous kinases (*20*). When expressed from the inducible *GAL4* promoter in yeast, wild-type tagged and untagged forms of Cdc2 and Cdk2 were able to rescue the *cdc28-4* allele at the nonpermissive temperature (36°C), indicating that the tagged kinases were functional (*21*). The corresponding mutant forms could not rescue *cdc28* mutations at the nonpermissive temperature. Moreover, these mutants interfered with proliferation when induced at the permissive temperature (*21*).

The wild-type and mutant kinases were cloned under the control of the cytomegalovirus (CMV) promoter and were transiently transfected into human U2OS osteosarcoma cells (*20*, *22*). The expression levels of the wild-type and mutant proteins were similar (Fig. 1, B and C). However, in vitro histone H1 kinase activity was only associated with the wild-type kinases (Fig. 1D). The epitope-tagged forms of both wild-type and mutant Cdc2 appeared to associate with

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