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Participation of Tyrosine Phosphorylation in the Cytopathic Effect of Human Immunodeficiency Virus–1

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Protein tyrosine phosphorylation is a common mechanism of signaling in pathways that regulate T cell receptor–mediated cell activation, cell proliferation, and the cell cycle. Because human immunodeficiency virus (HIV) is thought to affect normal cell signaling, tyrosine phosphorylation may be associated with HIV cytopathicity. In both HIV-infected cells and transfected cells that stably express HIV envelope glycoproteins undergoing HIVgp41-induced cell fusion, a 30-kilodalton protein was phosphorylated on tyrosine with kinetics similar to those of syncytium formation and cell death. When tyrosine phosphorylation was inhibited by the protein tyrosine kinase inhibitor herbimycin A, envelope-mediated syncytium formation was coordinately reduced. These studies show that specific intracellular signals, which apparently participate in cytopathicity, are generated by HIV and suggest strategies by which the fusion process might be interrupted.

Despite intensive scrutiny, the mechanisms by which HIV infection depletes antigen-specific $CD4^+$ helper T cells and leads to the development of acquired immunodeficiency syndrome (AIDS) are incompletely understood (*1*). Immunodeficiency caused by HIV infection is likely to result from direct T cell killing (*2*) and the disruption of T cell function by the virus (*3*). Interference by HIV with antigen-specific helper cell triggering has been extensively documented in in vitro studies (*4*).

Tyrosine phosphorylation participates directly in the regulation of cell growth, in T cell receptor (TCR)–and CD4 receptor–mediated signaling, and in antigen-specific T cell proliferation. Normal antigen-specific triggering of helper T cells leads to the rapid tyrosine phosphorylation of the TCR ζ chain and other cellular substrates (*5*), and inhibition of tyrosine phosphorylation suppresses TCR-mediated cell proliferation (*6, 7*). Several enzymes controlling the cell cycle are regulated by tyrosine phosphorylation, and aberrant expression of certain kinases associated with cell cycle control, such as $p107^{wee1}$, also may lead to abnormal cell morphology (*8*). The kinase $p107^{wee1}$ contributes to the inhibitory tyrosine phosphorylation of the $p34^{cdc2}$ cell cycle kinase (*9–12*). Tyrosine dephosphorylation of $p34^{cdc2}$ is required for the G_2 to M phase transition in normal cell division.

We investigated the effects of HIV on T cell signal transduction pathways by examining the phosphotyrosine content of intracellular proteins. In response to infection of T cells with HIV-1 LAV, the amount of tyrosine phosphorylation of 30-, 40-, and 95-kD substrates increased in both Jurkat cells or in peripheral blood lymphocytes (PBLs) as compared to uninfected Jurkat cells or uninfected PBLs (Fig. 1) (*13*). Proteins of 40- and 95-kD become tyrosine-phosphorylated after cross-linking of CD3-TCR, but not CD4 or CD45 receptors in Jurkat cells or PBLs (*6, 7*). There have been no reports of a 30-kD substrate undergoing tyrosine phosphorylation in response to T cell activation (*6, 7*).

All of the substrates displayed their highest levels of tyrosine phosphorylation during the fourth day of infection (*14*), ranging from 5.0 to 8.0 times the initial levels for the 135-, 95-, and 40-kD proteins (Fig. 2A). No phosphorylation of the TCR ζ chain was observed. The increase in tyrosine phosphorylation of the 30-kD substrate (pp30) was the largest (23-fold) and slightly more persistent. Tyrosine phosphorylation of pp30 occurred early in the course of HIV infection compared to the production of viral proteins or the activity of reverse transcriptase (RT) in the medium, both of which were maximal after 5 days of culture (Fig. 2B).

To confirm that pp30 from HIV-infected cultures contained phosphotyrosine, we subjected pp30 to phosphoamino acid analysis. Jurkat cells were labeled with ^{32}P-orthophosphate 5 days after infection with HIV-1. The pp30 protein contained phosphotyrosine, phosphothreonine, and phosphoserine (Fig. 3) (*15*). This pattern of phosphorylation on tyrosine and threonine residues has been reported for at least one cell cycle kinase, $p34^{cdc2}$ (*9, 11, 16*).

The interaction of the HIV-envelope glycoproteins, which mediate CD4 binding (*17*), with the T cell signal transduction apparatus could potentially generate signaling abnormalities or lethal events. To study this possibility, we selected transfected Jurkat T cells that stably expressed HIV-envelope glycoproteins (*18*). We used an envelope-transfected cell line, HIV-env (2-8),

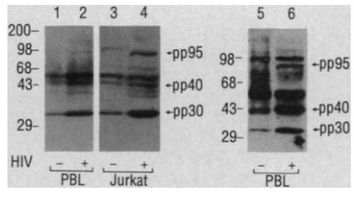

Fig. 1. Induction of tyrosine phosphorylation after infection of cells with HIV-1 LAV. Protein lysates from 5×10^6 peripheral blood lymphocytes (PBLs) (lanes 1, 2, 5, and 6) or Jurkat cells (lanes 3 and 4) either incubated with medium alone (−) or infected with medium containing HIV-1 LAV (+) were resolved on SDS-polyacrylamide gradient gels (10 to 20%), transferred for protein immunoblot analysis, probed with rabbit polyclonal antibody to phosphotyrosine, and developed with ^{125}I-labeled protein A (*13*). Size markers are indicated to the left of the gels (in kilodaltons); proteins with prominently augmented tyrosine phosphorylation (pp) are indicated by their molecular size (in kilodaltons) to the right.

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which produced all envelope glycoproteins including gp41. This cell line has coordinately down-regulated surface, but not intracellular, expression of CD4 and forms syncytia when cocultured with CD4-bearing Jurkat cells. A second envelope transfectant, HIV-env (2-2), which synthesized the same amount of gp160 as HIV-env (2-8) but did not process it to gp41 and gp120, was used to control for the requirement of gp41 in fusion or signal transduction.

To compare the effects on tyrosine phosphorylation, cell morphology, and cell death (*19*) resulting from the interaction of $CD4^+$ and envelope $gp41^+$ T cells with those observed after infection with HIV, we cocultured HIV-env (2-8) and $CD4^+$ Jurkat cells (*20*). Jurkat and HIV-env (2-8) cells had very similar basal patterns of tyrosine-phosphorylated substrates except for a small relative increase of tyrosine phosphate associated with pp30 in the envelope-transfected cell line (Fig. 4A). After 30 min of coculture, the phosphotyrosine content of 95-kD (pp95) and 55- to 60-kD (pp55) substrates increased. During this time period, cell viability remained high (109% of control), there was no increase in the tyrosine phosphorylation of pp30, and there was no evidence of syncytium formation (Fig. 4B). After 4 hours of coculture, tyrosine phosphorylation of pp95 and pp55 began to decline, enlarged cells appeared in the cultures, and some cell death was detected (93% viability). After 18 hours of coculture, the tyrosine phosphorylation of pp95 and pp55 further decreased, and tyrosine phosphorylation of the 30-kD substrate increased eightfold. Cell viability was 66%, and syncytia had formed. Longer culture led to a further increase in pp30 tyrosine phosphate and the death of over half the cells (47% viability).

Cell fusion, duplicating HIV-1 infection, did not lead to phosphorylation of the TCR ζ chain (*21*). Cells expressing only gp160 [HIV-env (2-2)] did not fuse and, when cultured alone or with Jurkat cells, displayed no increased phosphorylation of pp30 (*20*). Although these studies did not distinguish the cell population in which tyrosine phosphorylation occurred, the entire process, including pp30 phosphorylation, was completely inhibited by soluble CD4 or by antibodies to Leu-3a (*22*), which suggests a requirement for interaction between HIV-env gp120-gp41 on one cell with CD4 on a second cell (*22*).

To determine whether inhibition of cellular tyrosine phosphorylation could alter the consequences of HIV-env–CD4 interaction, we used the benzenoid ansamycin antibiotic, herbimycin A, an inhibitor of protein tyrosine kinases (PTKs) (*23*). Herbimycin appears to directly inhibit kinase activity and to target kinases for degradation. In T cells, herbimycin inhibits the function of at least two Src-related PTKs, *lck* and *fyn* (*7*). When HIV-env gp120-gp41–expressing T cells were cocultured with $CD4^+$ T cells in the absence of herbimycin A, tyrosine phosphorylation of the 30-kD substrate was induced (Fig. 5A); in contrast, cells incubated with herbimycin A for 18 hours before coculture no longer showed augmented tyrosine phosphorylation of the 30-kD substrate (*24*, *25*). The morphological changes associated with coculture and cell fusion were also attenuated in the herbimycin A–treated cocultures (Fig. 5B). At least some of the residual morphologic abnormality observed in these cocultures may have resulted from the effects of herbimycin alone (Fig. 5B). Herbimycin A similarly reduced the cytopathic effect in HIV-infected Jurkat cells, although the amounts of viral proteins in herbimycin-treated infections were also lower (*26*). The effects of herbimycin may result from its inhibition of syncytia formation as

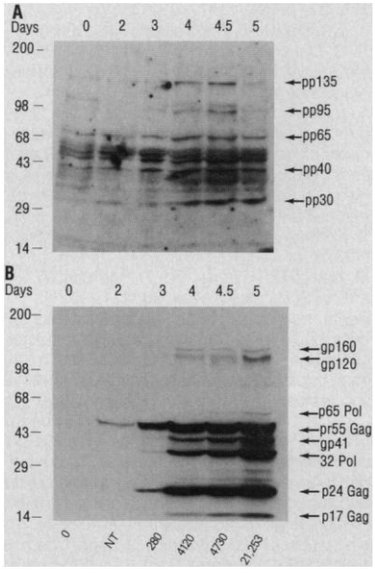

Fig. 2. Time course of tyrosine phosphorylation of proteins and accumulation of viral proteins. Jurkat cells were exposed to HIV-1 LAV (multiplicity of infection 1:500) for 2 hours, and equal portions were placed into separate cultures and harvested at sequential time points after the indicated number of days. Two samples were harvested on the day of peak syncytia formation (day 4). Protein immunoblots were analyzed with (**A**) polyclonal rabbit antibodies to phosphotyrosine or (**B**) a polyclonal pooled serum from humans infected with HIV-1; migration of the major tyrosine-phosphorylated substrates (A) or major viral proteins (B) is as indicated. Numbers below (B) indicate RT activity in counts per minute at the time of harvest. NT, not tested. Molecular size markers in kilodaltons are at the left.

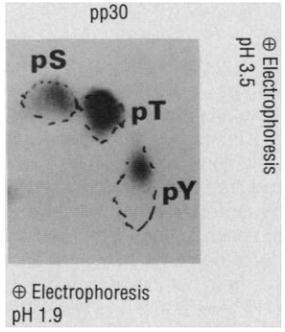

Fig. 3. Phosphoamino acid analysis of pp30. HIV-LAV–infected Jurkat cells (10^8) were labeled for 5 hours with 10 mCi of ^{32}P-orthophosphate 5 days after infection. The 30-kD substrate was isolated by gel electrophoresis, transferred to Immobilon (Millipore), and hydrolyzed in 5.7 N HCl. The hydrolysates were resolved by two-dimensional thin-layer electrophoresis (*11*). pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine.

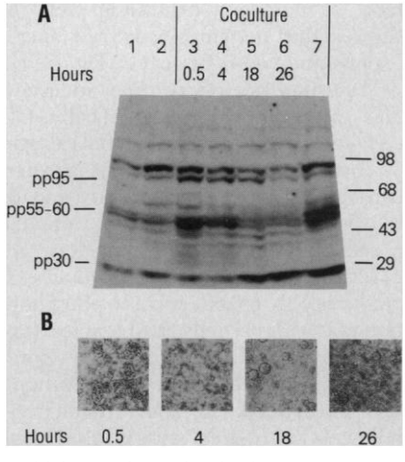

Fig. 4. Changes in tyrosine phosphorylation of pp30 during HIV-envelope–induced syncytium formation and cell death. Jurkat cell line HIV-env (2-8) was transfected to stably express the envelope glycoproteins gp41, gp120, and gp160 of HIV-1 Hxb2 (*29*). HIV-env (2-8) cells were grown either alone or with an equal number of Jurkat cells for the indicated length of time after coculture. (**A**) Equal numbers of viable cells (2×10^6) were lysed and subjected to protein immunoblot analysis to detect tyrosine-phosphorylated substrates. Lanes 1 and 2, Jurkat and HIV-env (2-8) cells, respectively, immediately before coculture; lane 3, cells 30 min after coculture; lane 4, after 4 hours; lane 5, after 18 hours; lane 6, after 26 hours; lane 7, an independent coculture of HIV-env and Jurkat cells serving as a reference for substrate tyrosine phosphorylation. The autoradiogram was scanned by laser densitometry to quantitate the relative amount of tyrosine-phosphorylated pp30 in the cocultured lanes, which was determined to be 100%, 110%, 835%, and 1111% at 30 min, 4 hours, 18 hours, and 26 hours after coculture. Molecular size markers in kilodaltons on the right. (**B**) Photographs (×10 magnification) and culture viability were taken immediately before harvest. Time is shown below in hours.

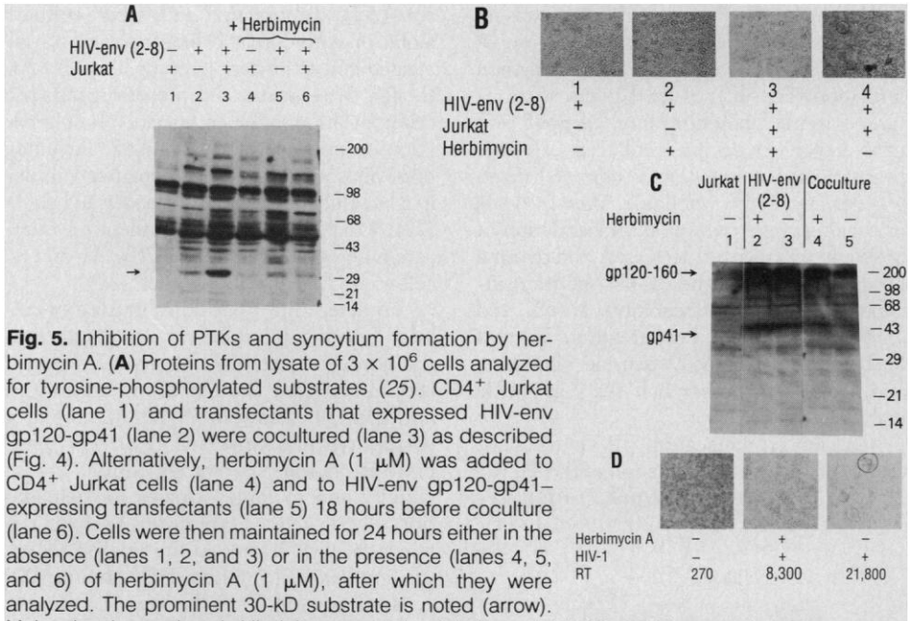

Fig. 5. Inhibition of PTKs and syncytium formation by herbimycin A. (**A**) Proteins from lysate of 3×10^6 cells analyzed for tyrosine-phosphorylated substrates (*25*). $CD4^+$ Jurkat cells (lane 1) and transfectants that expressed HIV-env gp120-gp41 (lane 2) were cocultured (lane 3) as described (Fig. 4). Alternatively, herbimycin A (1 μM) was added to $CD4^+$ Jurkat cells (lane 4) and to HIV-env gp120-gp41–expressing transfectants (lane 5) 18 hours before coculture (lane 6). Cells were then maintained for 24 hours either in the absence (lanes 1, 2, and 3) or in the presence (lanes 4, 5, and 6) of herbimycin A (1 μM), after which they were analyzed. The prominent 30-kD substrate is noted (arrow). Molecular size markers in kilodaltons are at the right here and in (C). (**B**) Photographs of the cultures (×10 magnification) showing typical syncytia in the cocultures (photo 4) and the effects of herbimycin A in diminishing the abnormal cell morphology (photo 3). Although HIV-env gp120-gp41 transfectants have a relatively normal cell morphology (photo 1), incubation with herbimycin A alone induced some cellular enlargement (photo 2). No effect on cell morphology or fusion was caused by the solvent dimethyl sulfoxide added in the same amount required to deliver the drug. (**C**) Protein immunoblot analysis for HIV-associated proteins. Lysates from 3×10^6 $CD4^+$ Jurkat cells (lane 1), HIV-env gp120-gp41 transfectants in the presence (lane 2) or absence (lane 3) of herbimycin A, or their cocultures in the presence (lane 4) or absence (lane 5) of herbimycin A were analyzed for gp41, gp120, and gp160 protein expression with a serum taken from humans infected with HIV. (**D**) Effects of herbimycin on HIV-infected cells. Jurkat cells (5×10^6) were infected with HIV-1 LAV at a multiplicity of infection of 1:500 or were infected with medium without virus. Three days later, one of a parallel set of infections was treated with herbimycin A (1 μM) and maintained for 48 hours; control cultures were maintained in medium alone (*26*). On day 5, RT activity in 50 μl of culture supernatant was determined (in counts per minute) (*9*), and the cells were photographed (×10 magnification).

observed in lymphocyte function–associated antigen–1 negative cultures (*27*), or, less likely, may be a result of a small antiproliferative effect of the drug (Fig. 5D). These considerations were not relevant to the transfected cells, since a protein immunoblot of HIV-env–expressing cell lines showed that herbimycin did not alter the expression of gp41 or gp160 (Fig. 5C).

Tyrosine phosphorylation induced by HIV infection or, more specifically, CD4–envelope gp41 interaction, indicates specific intracellular signaling that apparently participates in HIV-1–mediated cytopathicity. Because phosphorylation is initiated by a cell line that produces envelope gp41-gp120-gp160 and not by a cell line that produces only gp160, tyrosine phosphorylation is at least partially attributable to gp41. Some, but not all, of the substrates normally phosphorylated after TCR activation are also phosphorylated after interaction of HIV-env gp120-gp41 with CD4. The ability of herbimycin A to reduce HIV-associated morphological abnormalities while blocking pp30 tyrosine phosphorylation could be explained if tyrosine phosphorylation is required for intracellular transmission of a lethal signal, which may involve pp30. The evidence that inhibition of PTK attenuates the cytopathic effect of envelope-CD4 interaction potentially suggests a new direction for efforts to spare $CD4^+$ T cells in HIV-infected individuals. The tyrosine phosphorylations described here may be representative of the cellular activation process (*28*) that has been consistently associated with HIV cytopathicity.

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25. The relative tyrosine phosphate content of pp30 for each of the lysates in Fig. 5A was determined by laser densitometry to be 31% (lane 1); 100% (lane 2); 326% (lane 3); 77% (lane 4); 165% (lane 5); and 133% (lane 6).
26. In Fig. 5D, total cell number and viability (%), determined at harvest for each of the culture conditions, were 10^7 (97%) in media alone; 5.5×10^6 (81%) with HIV; and 7.5×10^6 (91%) with HIV + herbimycin A.
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Observational Learning in *Octopus vulgaris*

Graziano Fiorito and Pietro Scotto

Untrained *Octopus vulgaris* (observers) were allowed to watch conditioned *Octopus* (demonstrators) perform the task of selecting one of two objects that were presented simultaneously and differed only in color. After being placed in isolation, the observers, in a similar test, consistently selected the same object as did the demonstrators. This learning by observation occurred irrespective of the object chosen by the demonstrators as the positive choice and was more rapid than the learning that occurred during the conditioning of animals. The task was performed correctly without significant errors and further conditioning for 5 days. These results show that observational learning can occur in invertebrates.

Octopuses, like many other invertebrate species, are capable of a learned change in behavior as a result of experience (*1–4*), demonstrating that *Octopus* can integrate information to produce adaptive behavioral patterns (*5*). Among vertebrates living in social groups, learning can be facilitated by observation of another member of the same species (conspecific) performing a behavioral act (*6, 7*).

Octopus vulgaris does not have social habits, which implies that it has little experience observing the behavior of conspecifics. However, some cephalopods are social, and specialized behavioral patterns are exhibited when octopuses come in contact with each other (*8*). Therefore, we tested whether octopuses can learn to perform a task by observation of other trained octopuses.

Individuals of *Octopus vulgaris* were conditioned to discriminate between two stimuli that were identical in shape and size but differed in color (*9*). Training was followed by a session of trials while an untrained *Octopus* visually observed the choices made by the conditioned demonstrator. Experiments to investigate the ability of *Octopus vulgaris* (*10*) to learn by observation were conducted in three phases: (i) training of demonstrators, (ii) observation of the task by untrained octopuses, and (iii) testing of observers.

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Training of demonstrators (*11*) was realized by a series of trials where two balls were presented to the animal. We selected one ball to be the correct choice. When the animal attacked the correct ball it was rewarded, and each attack of the incorrect object was punished. The training of a demonstrator was complete when the animal made no mistakes in five trials.

Two groups (red balls and white balls) of demonstrators were trained. For the red group, the red ball was considered to be the correct choice. Its selection was rewarded with a small piece of fish attached to the side of the ball that was not visible to the animal. Selection of the white ball was punished by an electric shock (*9*). For the second group of octopuses the conditions were reversed. For the octopuses ($n = 30$) conditioned to choose the red ball, full training was reached at 16.83 ± 1.35 trials (mean ± standard error of the mean). The white group ($n = 14$) was trained after 21.50 ± 1.46 trials. Full training was reached at a significantly different number of trials for the two groups (Student t test = 2.11, df = 42, $P < 0.05$) (*12*).

During the observational phase, an untrained *Octopus vulgaris* housed in the tank adjacent to its demonstrator observed four trials during which the demonstrator attacked the ball it had been taught to attack (Fig. 1). During this phase no errors were detected in the demonstrator groups (Fig. 2, A and C), even though they were no longer rewarded for making the correct response.

From the analysis of videorecordings we noted that observer octopuses increased their attention (*13*) during each of the four trials. In particular, we noted that the observers followed the action patterns of their demonstrators with movements of the head and eyes. When these movements during observational trials were compared with those seen during intertrial times, we detected a significative increase in their number [Wilcoxon matched-pairs test: $z = 5.23$, $P < 0.01$, n = (44 observers minus 2 null differences) = 42]. The observers also spent more time outside of their homes and displayed other behavioral patterns that have been recognized for *Octopus vulgaris* when in the presence of a conspecific (*8, 13*).

For the testing phase, observer octopuses were tested (*14*) with a session of five trials of simultaneous presentation of both white and red balls randomly positioned. No reward or punishment was given at this time for any choice made. The *Octopus vulgaris* ($n = 30$) that observed demonstrators attacking the red ball chose the red ball significantly more often than they did the white ball [129 red, 13 white in 150 trials; $\chi^2 = 90$, $P < 0.01$ (Fig. 2B)]. Animals ($n = 14$) that observed demonstrators of the white group chose the white ball [7 red, 49 white in 70 trials; $\chi^2 \approx 28$, $P < 0.01$ (Fig. 2D)].

During the testing phase, the red and white observers made 14 and 30% of cumulative errors, respectively. However, for both groups of observers if one considers only the number of attacks to the wrong ball, errors were ~9%. Failure to attack

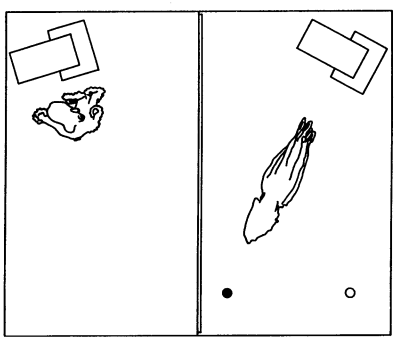

Fig. 1. Schematic of the experimental apparatus and protocol. An *Octopus vulgaris* is shown (right side of the figure) attacking a ball (the red one) and acting as a demonstrator for the other animal (observer, left side) that is standing outside of its home and watching its conspecific during the whole session through a transparent wall. Each tank had an independent supply of running water. Octopuses were allowed to visually interact for 2 hours before the start of the observational phase. Mean duration of the trials, which depended on the demonstrator's performances, was 40 s, and intertrial intervals were fixed at 5 min.