

Questions regarding the role of protein phosphorylation in HIV replication and anti-acquired immune deficiency syndrome therapy

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Replication of the human immunodeficiency virus (HIV) post entry to T-lymphocytes (Figure 1) is dependent on the function of many viral proteins and to a great extent on the activity of the enzyme HIV reverse transcriptase (HIV-RT), a major target for anti-acquired immune deficiency syndrome (AIDS) therapy.¹ The enzyme has a triple action, acting as a reverse transcriptase, ribonuclease and DNA polymerase.² Three important questions arise in the HIV infection paradigm: 1) Is the activity of viral proteins, particularly that of HIV-1 RT, regulated by protein phosphorylation? 2) Why does the cell allow the virus to replicate once inside and not undergo apoptosis immediately once it detects abnormality, such as virus entry? 3) Why does resistance to anti-AIDS therapy develop and is this related to the activity of protein kinases?

The answer to the first question is yes. Many HIV viral proteins are subject to phosphorylation *in-vitro* and *in-vivo*. For instance, early studies showed that the HIV-1 matrix protein Gag is phosphorylated with protein kinase C (PKC) and other kinases, but no definite function has so far been ascribed to this modification.^{3,4} Phosphorylation of the HIV-1 capsid protein CAp24, by one or both kinases within the virion, on serines 109, 149 or 178, or both, seems essential for the viral uncoating process, indirectly affecting the process of reverse transcription.⁵ Phosphorylation of Vpr at serine 79 regulates its activity in the nuclear import of the HIV-1 preintegration complex.⁶ Casein kinase II (CKII) is known to phosphorylate HIV-1 Rev on

serines 5 and 8 and this seems necessary for transactivation,⁷ whilst phosphorylation of HIV vif seems important in viral activation and replication.⁸ Human immunodeficiency virus-1 protease is also phosphorylated by CKII.⁹ Phosphorylation of HIV-1 Vpu, which is involved in the binding and degradation of the viral receptor CD4, on serines 52 and 56, is required for the interaction of Vpu with the ubiquitin ligase, which triggers proteasome degradation of CD4, facilitating viral release from the infected cells.¹⁰⁻¹² Phosphorylation of viral proteins therefore, seems an important step for the infectivity and replication of the virus. Phosphorylation is known to regulate the activity of many cellular DNA/RNA polymerases.^{13,14} Recently, we showed that HIV-1 RT can be phosphorylated by a number of purified kinases *in-vitro*, most likely at a serine residue.¹⁵ We also showed that the enzyme can be utilized as a phospho-acceptor in metabolically labeled Sf9 eukaryotic cells.¹⁵ Others showed that *in-vitro* phosphorylation of HIV-1 RT with CKII augments its catalytic activity (we obtained similar results with PKC and AK, Idriss and Damuni (1992), unpublished observations), suggesting that phosphorylation may be a required step to accelerate the replication of the HIV-1.¹⁶ Whether the phosphorylation of HIV-1 RT, is in part responsible for switching the virus from latent to replicative mode, is still unclear. To date we have been unable to show that the enzyme is phosphorylated in T-lymphocytes (Jurkat cells). What kinases may be

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involved in the regulation of HIV-1 RT and therefore, viral replication? Not taking into account the 2 kinases that are present within the HIV virion,⁵ which have not been tested for RT phosphorylation, PKC, CKII and an auto-activated kinase (AK, catalytic domain of PAK2) are likely candidates. The latter 3 have been shown to phosphorylate the enzyme *in vitro*¹⁵ and at least CKII phosphorylation enhances RT activity.¹⁶

The second question is related to apoptosis, which is thought to be a major mechanism through which HIV-1 causes CD4+ T cell death post replication. Yet one wonders why does an infected cell not undergo apoptosis during the very early stages of infection (such as once viruses gain entry into the cell and during latency), so that it limits the spread of the virus? Cells normally undergo apoptosis through activation of biochemical pathways involving caspases,¹⁷ but other non-caspase dependent pathways may be at work.¹⁸ One consequence of viral infection during the early stages could be an elevation in the levels of inhibitory apoptotic proteins (IAPs) that inhibit apoptosis, allowing ample time for the virus to replicate, before it causes (apoptotic) cell death to it and other non-infected cells. Members of the IAP protein family are said to inhibit apoptosis through inhibition of protein caspases or caspase complexes.¹⁹ Enhanced expression/activity of certain IAPs apparently increases as a consequence of viral infection. Zhu et al²⁰ reported upregulation of Survivin, a member of the IAP family by HIV-1 Vpr. This potentially suggests involvement of IAPs in facilitating viral replication by prolonging cell viability. Human immunodeficiency virus-1 Nef stimulates anti-apoptotic signals in infected cells through phosphorylation of Bad, a pro-apoptotic member of the Bcl-2 family.²¹ It is interesting that perturbation of the mitochondrial membranes results in secretion of omi/Htr2A, an IAP binding protein, which promotes apoptosis.²² Perturbation of the mitochondrial membranes of infected lymphocytes is known to occur subsequent to highly active antiretroviral therapy (HAART) treatment in AIDS patients.^{23,24}

Another consequence of viral infection in the elevation of the levels of reactive oxygen species (ROS) such as nitric oxide (NO) within the cell and if HIV-1 infection suppresses apoptosis through IAPs, why does a cell escape NO-mediated damage, for example? One consequence of elevation in the levels of NO is the incorporation of the byproduct nitrotyrosine (NOY), a product of the biochemical reaction between NO with cellular tyrosine²⁵ onto -tubulin ultimately leading to cell death²⁶ and inhibition of myogenic differentiation.²⁷ This may define a potentially novel mechanism for elimination of abnormal cells (such as infected or cancerous cells) through the disruption of the

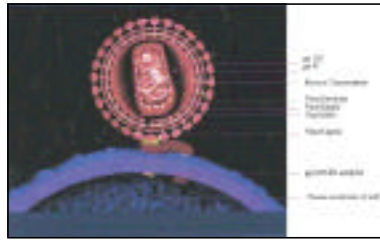


Figure 1 - Structure of the human immunodeficiency virus (HIV). The outer shell of the virus is known as the viral envelope. Embedded in the viral envelope is a protein known as env, which consists of an outer protruding cap glycoprotein (gp) 120, and a stem gp41. Glycoprotein 120 complexes with a cell receptor protein termed CD4 during viral entry into the cell. The viral envelope encloses matrix protein (p17), and within this is the viral core or capsid, which is made of another viral protein p24. The major macromolecules within the viral core are 2 single strands of HIV RNA, a nucleocapsid protein, and 3 enzymes reverse transcriptase, protease and integrase. Original image generated by Boehringer Ingelheim International GmbH, with labels added by author.

microtubule network.²⁸ The modifying enzyme tubulin tyrosine ligase (TTL) is capable of irreversibly incorporating NOY. I recently proposed this may act as a final apoptotic step, which springs into action when all other apoptotic steps fail. Cells may escape this 'last check point' (LCP) apoptotic step through phosphorylation-induced inactivation of TTL, which was proposed to be phosphorylated at a serine residue close to its ATP binding site, preventing ATP binding and inactivating the enzyme.^{29,30} Cells that escape nitrotyrosinated -tubulin-mediated LCP apoptosis are postulated to survive with a population of microtubules consisting of detyrosinated tubulin and facilitate viral replication. The tubulin tyrosination cycle therefore may be under the hierarchical control of reversible phosphorylation. Kinases activated by viral infection may perturb 'LCP' apoptosis³⁰ facilitating viral replication. Therefore, as far as the microtubule network is concerned, phosphorylation may prevent damage by ROS such as NO. In summary, a combination of synthesis of IAPs and inactivation of pro-apoptotic proteins through phosphorylation within cells seems to prolong cell viability post-infection with the HIV virus until such time the cell gets saturated with newly synthesized virions, after which an infected cell undergoes apoptosis.

Finally, why does anti-AIDS therapy fail to eradicate the disease and is phosphorylation involved in this phenomenon? Multidrug resistance

(MDR) to chemotherapeutic drugs is a major obstacle for the treatment of AIDS patients. By enlarge; P-glycoprotein (P-gp) has been responsible for this phenomenon in cancer, but apparently to a lesser extent in AIDS patients. The drug azidothymidine (AZT) is known to be a substrate for P-glycoprotein,³¹ so are many of the protease inhibitors, although they seem to confer a down-regulatory role on the efflux function of the protein. For instance, studies in primate model of AIDS showed a significant decrease in the expression of P-gp subsequent to HAART.³² Other drug resistance mechanisms are therefore involved. One major mechanism is mutation of HIV-RT, which prevents drug binding and this has been extensively reviewed elsewhere.³³⁻³⁵ However, post-translational modifications of viral proteins may also be important in regulating MDR in HIV infected cells. Phosphorylation of HIV-1 RT may play a direct role in preventing drug binding. Recently, phosphorylation of AZT-resistant HIV RT with CKII has been shown to diminish binding of AZTTP to RT.³⁶ potentially defining a novel mechanism for drug resistance. I propose phosphorylation of HIV-1 RT may be important not only as regulator of the enzyme's catalytic functions, but also in defining a novel post-mutation resistance mechanism, which is activated when mutation of residues within the enzyme is no longer practical, as it may lead to complete inhibition of the catalytic functions of HIV-1 RT due to gross structural alterations. It would be interesting to identify the maximum number of viable mutations that the enzyme can tolerate to confer resistance to drugs such as AZT without compromising its catalytic activities. It will equally be important to know whether this maximum number of viable mutations leads to exposure of phosphorylation consensus sequences allowing protein kinases, such as CKII, to add phosphates at phosphate-acceptor sites, leading to a second tier of MDR.

In conclusion, the idea that posttranslational modifications of HIV-1 RT confer resistance to chemotherapeutic drugs (such as AZT) is a plausible one and evidence in the literature exists to support this hypothesis.^{16,36} Understanding the full causes of MDR will lead to more effective anti-AIDS therapy in the future. Therefore, phosphorylation may play 3 crucial roles in AIDS (herein called Haifa hypothesis on AIDS) through activation of key viral proteins during infection and replication, through inhibition of TTL activity leading to inhibition of nitrotyrosinated tubulin-induced cell death and by preventing drug binding to HIV-1 RT through the addition of a phosphate at a site, which prevent drug binding to the enzyme (such as at the AZT binding site). Protein kinase C may be an important isotype in regulating RT activity since its activity is important during T-cell activation.

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