

3. Abstract

Effective replication of HIV-1, the virus responsible for AIDS, depends on the activation of T-cells, its main target. This relies mostly on the changes occurring in the proteins involved in the biological processes or signaling cascades occurring in the cell. Phosphorylation (the addition of phosphates) is one of the most important changes. We will express each of the nine viral proteins in the cell. The phosphorylation status of many protein targets involved in activation pathways will be then analyzed by specific antibodies that recognize the phosphorylated proteins. Students involved in the proposed research will learn the techniques that allow for the analysis of phosphorylation in individual cells. Changes in the phosphorylated state of any of the targets will help in understanding the processes exploited by the virus for its own benefit. This will also provide insights on the development of possible new drugs for antiviral therapy.

4. Project Description

Project Objective: The proposed research is aimed at characterizing the effect of the proteins of HIV-1 on a set of proteins in the infected cell that are involved in important biological pathways. The characterization of this effect will be based on phosphorylation, a hallmark of cell-process activation that consists of the addition of phosphates on a protein. The proposed study has three-fold biological objectives: to characterize the effect that the individual HIV-1 proteins have on cellular proteins involved in biological processes leading to efficient viral infection. This will help in understanding how the virus exploits the target cell for its own benefit. Secondly, it will provide insights on novel pathways to act upon for the design of drugs for gene therapy. Thirdly, it will assist in the educational process of students and their development as young scientists by becoming acquainted with cutting-edge technology in the field of Biotechnology and Molecular Cell and Viral Biology.

Background: The fight against HIV-1 is difficult mainly due to the virus's capacity to evolve. Classical therapies are aimed at disrupting the functions of the viral enzymes. The host proteins that the virus exploits to its own benefit remain attractive targets for therapy, since they should not be under selective pressure to avoid the therapy and thus may have a more deleterious effect on the virus. Successful viral infection (from the virus point of view) is based on a complex crosstalk between the viral machinery and the machinery of the target infected cell. This crosstalk comprises a set of signaling cascades or chains of changes involving genes, their products (the proteins) and the phosphorylation state of the proteins in the infected cell. Several factors have been described that demonstrate the intricate relationship between virus and host cell. While the host tries to block infection, the virus develops ways to evade the attack. The APOBEC3G host enzyme, for example, introduces too many mutations in the viral genome (that comprises all the genes of the virus) during viral replication for the virus to survive. To counteract this effect, the virus wisely uses one of its own proteins, Vif, that binds to APOBEC3G and inactivates it (1). Another example is NFATc, a cellular protein that the virus exploits to activate its main target cell, the T-cells, allowing for efficient infection and replication (2,3).

Significance: The analysis of activation pathways is critical for understanding the intricate relation between virus and host. This activation is mainly due to phosphorylation. Studying the effect of each of the individual proteins of HIV-1 on the cell in general and on the phosphorylation status of cell proteins involved in important biological pathways in particular, will further our understanding of the viral life cycle on one hand and may identify targets for gene therapy (4) and drug discovery on the other.

Preliminary studies: The crosstalk between viral and cellular factors involves interactions between proteins, and most certainly activation of cellular proteins by the proteins of the virus. This activation is mainly based on the addition of a phosphate on the protein, a process referred to as phosphorylation. Jean-François Fortin from Stanford University and I have recently shown that MAPKp38 is over-activated (phosphorylated) in HIV-1 infected cells (5). MAPKp38 is an important protein involved in a wide variety of cellular processes such as growth, differentiation, activation of genes and development. Furthermore, preliminary results in collaboration with Dr Cynthia Bristow from Rockefeller University show an increased basal level of AKT phosphorylation in cells that permit HIV-1 infection compared to a very similar cell that resists infection. AKT is another important protein involved in the pathways that regulate biological processes such as survival, proliferation, cell growth, cell motility and metabolism.

FACS (fluorescence activated cell sorter) is a powerful machine that enables to analyze individual cells within large population of cells based on their fluorescence characteristics. In classical FACS, the analysis of cells is based on fluorescent antibodies that recognize proteins on the surface of the cell. The preliminary studies described above were made possible thanks to a development in FACS that is

based on the analysis of intracellular markers rather than the classical surface markers. This technique, referred to as Phospho-flow, was developed in the laboratory of Garry P Nolan at Stanford University (6, 7), and consists of the treatment of cells in such a way that will be able to internalize antibodies without disrupting the cell shape. As a result, the technique allows for the analysis of the phosphorylation status of proteins inside the cell. Importantly, because of the use of the FACS machine, the analysis can be performed at the single cell level.

Plan: The proposed research involves four main steps:

#1. Introduce the HIV-1 genes into a vector (transfer device) that carries a fluorescent marker.

The vector, called PBMN-i-eGFP, was kindly provided by Garry P. Nolan from Stanford University. This vector carries the green fluorescent protein GFP and allows for the introduction of an additional gene., in this case of HIV-1. At the end of step #1 we will obtain nine vectors, each containing one of the nine genes of the virus.

#2. Produce particles carrying the individual genes of HIV-1, and transfer of the genes into T-cells..

The vector carrying the viral gene and GFP (step #1) is necessary but not sufficient to produce a particle that can transfer the gene into a target cell. In order to do so, we will use a 'packaging cell line', a cell that packages all the genetic information into a whole particle. This, in turn, will be used to transfer each of the genes of HIV-1 into target cells. The target cell used in this proposal is a SupT1 cell, a T-cell, part of the immune system and a natural target of HIV-1.

#3. Select for cells that express each of the viral proteins (based on fluorescence intensity) and demonstrate that fluorescence intensity correlates viral protein expression.

As mentioned in step#1, the particle produced carries both the GFP and one of the genes of HIV-1. Consequently, after transfer, the cells will become fluorescent. With the help of the FACS machine, we will collect the population of fluorescent cells. Those will be analyzed by Western Blot, a biochemical technique that enables detection of individual proteins based on antibody recognition. We will do so with the nine populations selected, each expressing one of the genes of HIV-1. A particle that does not include any of the viral genes will be used as a negative control.

#4. Analyze the phosphorylation status of cellular proteins involved in pathways leading to cell activation.

As mentioned, HIV-1 exploits the activation of many biological pathways in the cell to its own benefit. Here, we will analyze a set of cellular proteins involved in these pathways. We will treat the cells that express the proteins of HIV-1 (step#3) in such a way that antibodies can enter them. Then we will use fluorescent antibodies that recognize the phosphorylated proteins. As a result, the fluorescence intensity of the cells carrying phosphorylated proteins will increase. In order to discern between the GFP (green fluorescence) and the antibodies, the latter will be coupled to a red fluorescent dye. Both green and red fluorescence can be captured and analyzed by FACS. At the end of the study we will compile the results on the activation of targets, creating a 'biosignature' panel or picture of the phosphorylation status of the cell in the presence of the viral proteins.

Cited Literature:

1. Sheehy, A.M., Gaddis, N.C., Choi, J.D., and Malim, M.H. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature*, 2002.418(6898): p. 646-50.
2. Kinoshita, S., Su, L., Amano, M., Timmerman, L.A., Kaneshima, H., Nolan, G.P. The T cell activation factor NF-ATc positively regulates HIV-1 replication and gene expression in T cells. *Immunity*, 1997. 6(3): p. 235-44.
3. Kinoshita, S., Chen, B.K., Kaneshima, H., and Nolan, G.P. Host control of HIV-1 parasitism in T cells by the nuclear factor of activated T cells. *Cell*, 1998.95(5): p. 595-604.

4. Wolkowicz, R. and Nolan, G.P. Gene therapy progress and prospects: novel gene therapy approaches for AIDS. *Gene Ther*, 2005. 12(6): p. 467-76.
5. Fortin, J-F., Wolkowicz, R. and Nolan, G.P. HIV-1 infection sensitizes p38 signaling at the single cell level. (submitted to *J Virol*).
6. Krutzik, P.O., Irish, J.M., Nolan, G.P. and Perez, O.D. Analysis of protein phosphorylation and cellular signaling events by flow cytometry: techniques and clinical applications. *Clin Immunol*, 2004. 110(3): p. 206-21
7. Perez, O.D. and Nolan, G.P. Phospho-proteomic immune analysis by flow cytometry: from mechanism to translational medicine at the single-cell level. *Immunol Rev*, 2006.210: p. 208-28

Timeline:

1. (January 1, 2007 - April 30, 2007). The nine genes of HIV-1 will be introduced one by one into vectors (transfer device) that will enable their transfer into the mammalian T-cell target, a cell part of the immune system.
2. (May 1, 2007 - June 30, 2007). The vectors carrying the viral genes will be used to produce viral particles which enable the transfer of genes into target cells and allow for their expression in a stable manner.
3. (July 1, 2007 - September 30, 2007). Cells that express the viral proteins will be selected based on fluorescence. The amount of protein expressed in these cells will be analyzed in order to corroborate that: the fluorescence intensity correlates with the viral protein level expressed.
4. (October 1, 2007 - November 30, 2007). Cells expressing the individual viral proteins will be treated with specific antibodies that recognize the phosphorylated (activated) proteins inside the cell. This will allow for a detailed analysis on the effect of the viral proteins on the phosphorylation status of important cellular proteins involved in biological pathways, at the single cell level.
5. (December 1, 2007 - December 31, 2007). Preparation of a manuscript describing the data obtained during the proposed research. This manuscript will be probably sent for publication in a peer review journal such as the 'Journal of Virology' or the 'Journal of Biological Chemistry'.

Mission of UGP:

The proposed project is based on the involvement of the students in the laboratory in every aspect and steps of the research. The project will certainly enhance the education and training of the students and help them in developing as young scientists. They will gain important skills in DNA recombinant technology, mammalian cell culture, production of viral particles for gene transfer, and FRCS analysis for the study of cells at the single cell level. This experience will not only strongly enhance their capabilities as scientists but also encourage them to think and plan experiments. Moreover, the proposed research will allow me to keep in line with the newest technologies and discoveries in the field, increasing my teaching capabilities with up-to date information.

Results obtained during the proposed research will be of high-quality scientific standards and fulfill the requirement for peer review journal publication. Naturally, the students involved in the research will be included as authors. When the opportunity arises the study will be presented in conferences.

The results from this study will form the foundation for a more extended and complex research. Cellular proteins shown to be affected by any of the viral proteins will become the subject of a future proposal aimed at blocking this effect. I will apply for an NIH R21 grant intended to encourage exploratory/developmental research, which provides \$275,000 over a two-year period, and it is due on May 1st 2007. Acceptance of UGP application will certainly help in producing the results needed for forming the framework for extended studies, increasing, in turn, the chances of acceptance of NIH grants.

5. Experience and Qualifications

Position title: Assistant Professor in Biology

Experience: During my Ph.D. I became acquainted with all the basic techniques used in Molecular Cell Biology including extensive tissue culture (mammalian cells) and DNA recombinant technology. As a Molecular Virologist at Stanford University I extended my experience into cell analysis at the single cell level, through the use of FACS (Fluorescence Activating Cell Sorter) fundamental for the research proposed here. I also gained experience in the production of virus and infection of mammalian cells, again, critical for the success of the proposed research. Techniques of peptide library expression and biological screens gained during my eight years of experience at Stanford will be of extreme help in the future projects that will derive from the results obtained during the proposed research.

Education:

Institution	Degree	Year	Field	Project
Tel Aviv University, Tel Aviv, Israel.	B.Sc.	1981-1984	Biology	Basic Life Sciences
Tel Aviv University, Tel Aviv, Israel.	M.Sc.	1984-1987 *	Microbiology	Overproduction of the Biotin synthetase gene in E.coli.
The Weizmann Institute of Science, Rehovot, Israel.	Ph.D.	1993-1998	Molecular Cell Biology	DNA-binding activity of the tumor suppressor p53
Stanford University Medical Center, Palo Alto, CA.	Post-Doc	1998-2002	Molecular Pharmacology	Use of peptide libraries and production of replication deficient HIV-based virions
Stanford University Medical Center, Palo Alto, CA.	Research Associate	2002-2006	Microbiology & Immunology	Blockage of HIV-1 infection by novel gene therapeutic approaches

* (1988-1992 compulsory military service)

Publications:

1. Fortin, J-F., Wolkowicz, R. and Nolan, G.P. HIV-1 infection sensitizes p38 signaling at the single cell level. (submitted to *J Virol*).
2. Rabmowitz, M., Myers, L., Banjevic, M., Char, A., Sweetkind-Singer, J., Haberer, J., McCarn, K., Wolkowicz, R. Accurate prediction of HIV-1 drug response from the reverse transcriptase and protease amino acid sequences using sparse models created by convex optimization. *Bioinformatics*, 2006. 1;22(5): p. 541-9.
3. Wolkowicz, R. and Nolan, G.P. Gene therapy progress and prospects: novel gene therapy approaches for AIDS. *Gene Ther*, 2005. 12(6): p.467-76.
4. Wolkowicz, R., Jager, G.C. and Nolan, G.P. A random peptide library fused to CCR5 for selection of mimetopes expressed on the mammalian cell surface via retroviral vectors. *J Biol Chem*, 2005. 280(15): p.15195-201.
5. Wolkowicz, R., Nolan, G.P., Curran, M.A. Lentiviral vectors for the delivery of DNA into mammalian cells. *Methods Mol Biol*, 2004. 246: p. 391-411.
6. Hale MB, Nolan GP, Wolkowicz R. Oligonucleotide-directed site-specific integration of high complexity libraries into ssDNA templates. *Nucleic Acids Res*, 2004.29;32(2):e22.
7. Wolkowicz R, Nolan GP. Retroviral technology--applications for expressed peptide libraries. *Front Biosci*, 2003. 1;8: p. d603-19

Awards:

1998-1999 Stanford Dean's fellowship
2000 NIH personal grant (Garry Nabel)

6. Budget Request Form

Item Requested	Amount Requested
1. Payment of Student Assistants	
a. # of hrs. x \$ /hr.	\$
b. # of hrs. x \$ /hr.	\$
2. Equipment or Supplies (attach vendor quote)	
a. VWR incubator CSA model 1545	\$ 1,677.00
b. Two Mini-PROTEAN 3 Electrophoresis Cells	\$ 904.00
c. Beckman X-15R centrifuge package	\$ 7,255.00
3. Professional Services	
a.	\$
b.	\$
4. Travel (attach hotel, airfare, travel quotes)	
a.	\$ -
b.	\$ -
5. Course Release (one or two) Spring 2007 semester only o One course = \$4,517 a Two course = \$9,034	\$
Chair/Director approval signature	
6. Summer Stipend (for June 2007) one month solo	\$
7. Other (please describe)	
a.	\$
b.	\$
TOTAL BUDGET REQUESTED	\$ 9,836.00

TOTAL BUDGET MAY NOT EXCEED \$10,000