Human Immunodeficiency Virus Type 1 Vpu Has a CD4- and an Envelope Glycoprotein-Independent Function

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Vpu is a 16-kDa membrane-associated phosphoprotein that is expressed from the same, singly spliced message as the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein precursor, gp160. Previous studies suggest that Vpu functions in the late stages of viral replication, possibly in virus egression from the cell. Recently, it has been demonstrated that Vpu functions to allow gp160 to be more efficiently processed by disrupting CD4-gp160 complexes generated by transfection of HeLa cells. We show here that the lack of expression of intact Vpu results in a 90% reduction in infectious virus produced over a single round of replication from HeLa cells in the absence of CD4 expression. This reduction persists when HIV-1 particles are pseudotyped with the HIV-2 or amphotropic murine leukemia virus envelope glycoprotein. Pulse-chase analysis of HIV-1 capsid protein (p24) in the absence of CD4 and envelope glycoprotein demonstrates that the rate of virus release is reduced when Vpu is not expressed. Our findings indicate that Vpu has a function involving particle release not dependent on CD4 or envelope glycoprotein expression.

Human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of AIDS (2, 9, 15) and is a member of the lentivirus subfamily of retroviruses. In addition to the *gag*, *pol*, and *env* genes, HIV-1 contains at least six other genes designated *tat*, *rev*, *vif*, *vpr*, *vpu*, and *nef*. The related lentivirus HIV-2 contains a similar array of accessory genes but lacks an obvious *vpu* open reading frame.

Vpu is an 81-amino-acid, membrane-associated, 16-kDa phosphoprotein (3, 17, 20). Although Vpu is not absolutely required for viral replication *in vitro*, the loss of a functional *vpu* gene reduces the amount of virus produced by a factor of 5 to 10 (14, 21, 22). This reduction does not reflect lower levels of viral protein synthesis, but rather the reduction reflects a decrease in the efficiency of virus released from the infected cell, which suggests that Vpu plays a role in virus exit from the cell (14, 22). However, the mechanistic role that Vpu plays in virus release is not understood. Moreover, because Vpu is expressed from the same, singly spliced mRNA as the envelope glycoprotein precursor gp160 (18), Vpu has been proposed to function late in the viral life cycle.

Proteolytic processing of gp160 is required to generate the surface glycoprotein gp120 and the transmembrane glycoprotein gp41, which remain noncovalently associated. This mature envelope glycoprotein (Env-gp) facilitates virus fusion and entry into cells that express the receptor molecule for HIV-1 (CD4) (4, 13).

Recently, Vpu was shown to enhance the intracellular processing of gp160 in HeLa cells expressing CD4 (23). This increase in processing apparently results from the disruption of gp160-CD4 complexes by Vpu. It was therefore suggested that the role of Vpu in viral replication is to disrupt gp160-CD4 complexes formed in the cell and to allow more efficient processing of gp160 to gp120 and gp41. It is not clear whether this activity of Vpu affects the overall production of virus particles or the infectivity of produced particles. However, if this is the only function of Vpu, then the phenotype of a *vpu* mutant should be Env-gp and CD4 dependent.

We used several complementary approaches to examine Vpu function. Surprisingly, the results indicate that Vpu functions in the absence of both CD4 and Env-gp. A mutation in Vpu resulted in an approximately 90% reduction in viral titer over one round of replication in cells that lack CD4. In addition, pseudotyped HIV-1 particles that contain heterologous Env-gp from viruses that lack Vpu still required Vpu for efficient replication. Finally, pulse-chase analysis demonstrated that capsid protein (p24) release from cells is reduced when functional Vpu is not expressed. This reduction in virus release is observed even in the absence of both CD4 and Env-gp in virus-producing cells. Together, these results suggest that in addition to the previously observed interaction of Vpu with gp160-CD4 complexes (23), Vpu is involved in virion assembly and particle release in a manner mechanistically independent of CD4 and Env-gp expression.

MATERIALS AND METHODS

Transfections and infections of cells. Cells were cultured in Dulbecco's modified Eagle's medium with 7% calf serum. CD4-LTR/ β -gal indicator cells (12) were cultured in the same medium, but with 100 μ g of hygromycin B per ml and 400 μ g of G418 per ml. A total of 15 μ g of DNA was used for transfection of 6×10^5 HeLa cells on 60-mm-diameter tissue culture dishes by using the calcium phosphate coprecipitation method (10). A 1:1 ratio of pGB108 or pBG135 to the Env expression construct was used for transfections. To prepare virus for infection, medium was collected from the transfected cells and was subjected to low-speed centrifugation to remove cell debris. One milliliter of the supernatant was recovered, and DEAE-dextran was added to a final concentration of 8 μ g/ml. Three hundred microliters of this supernatant (3- and 10-fold dilutions) was used to infect 4×10^4 CD4-LTR/ β -gal cells on 24-well tissue culture dishes. Three hours later, fresh medium was added, and successful infection was monitored by assaying Tat-inducible β -galactosidase expression 2 days later, as described previously (12).

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DNA constructions. pGB108 has been described previously (7). pBG135 was made by partial digestion of pGB108 with *SspI* and insertion of an 8-nucleotide *NotI* linker into the *SspI* site in the *vpu* open reading frame (nucleotide position 6153 in pNL4-3). pBG139 was derived from pIIIenv3-1 (19) (provided by W. Haseltine). The *XhoI-XhoI* fragment of pIIIenv3-1 (containing the partial long terminal repeat [LTR] and the *env* gene) was inserted into the *XhoI* site of pSP70 (Promega) to create pBG132. pBG124 was created by inserting an 8-bp *BssHII* linker into the *SspI* site in the *vpu* gene of pAL7 (5). The *SacI-KpnI* fragment of pBG124, containing the *vpu* open reading frame with the 8-bp *BssHII* linker inserted into the *SspI* site, was inserted into the corresponding region of pBG132 to create pBG134. The *EcoRV-NaeI* fragment of p763 (provided by B. Sugden), containing the simian virus 40 poly(A) site, was inserted into the *EcoRI* site of pBG134 (after the *EcoRI* site was filled in with Klenow and appropriate deoxynucleotide triphosphates) to create pBG130. The *SacI-KpnI* of pAL7 was inserted into the *SacI-KpnI* site of pBG130 to create pBG137. The *SacI-SacI* fragment of pIIIenv3-1 (containing part of the 5' LTR and regions 5' to *vpu*) was inserted into the *SacI* sites of pBG130 and pBG137 to create pBG138 and pBG139, respectively. pCMVHIV-2env has been previously described (7). pSV-A-MLV-*env* (provided by D. Littman) has been previously described (16). pSV₂tat72 has been previously described (6).

Syncytium assay. The syncytium assay was performed as previously described (8). Briefly, 15 μ g of DNA was used for transfection of 8×10^5 HeLa T4⁺ cells on gridded 60-mm-diameter tissue culture dishes as described above. A 1:1 ratio of pBG138 or pBG139 to pSV₂tat72 was used for transfections. Forty-eight hours later, the transfected cells were stained and syncytia with more than five nuclei were counted as described previously (8).

Pulse-chase analysis. Ten micrograms of each DNA was used to transfect 6×10^5 HeLa cells on 60-mm tissue culture dishes. Forty-eight hours after transfection, HeLa cells were incubated in methionine-free medium for one-half hour and then were pulsed with [³⁵S]methionine (ICN) at a final concentration of 125 μ Ci/ml for one-half hour. The labelling medium was removed, the cells were washed twice with $1 \times$ phosphate-buffered saline (PBS), and 2 ml of fresh cell culture media was added. After 0, 60, 120, and 210 min, 1.5 ml of medium was removed and subjected to low-speed centrifugation to remove cellular debris. One milliliter was recovered and immunoprecipitated with polyclonal rabbit anti-p24 antiserum (American Bio-Technologies, Inc.) at a 1:750 dilution as previously described (8). The cells, pulsed for 120 min, were lysed, after supernatant collection, in 1 ml of phospholysis buffer (8) and were immunoprecipitated in parallel with the supernatant samples to detect the levels of intracellular p24. The samples were subjected to electrophoresis on a 15% polyacrylamide gel and were analyzed by autoradiography.

RESULTS

Vpu has a CD4-independent function. We initially attempted to determine whether a mutation in *vpu* had an effect on virus production in the absence of CD4. To accomplish this, we used a transcomplementation strategy to compare virus replication over one round (5, 11, 16). GB108 is an *env* mutant derivative of the infectious molecular clone NL4-3 (1), and aside from the *env* gene, encodes all of the HIV-1 open reading frames, including *vpu*. BG135 is a *vpu* mutant derivative of GB108 attributable to an 8-bp insertion

FIG. 1. HIV-1 expression constructs. pBG139 expresses the HIV-1 Env-gp and Vpu from the HIV-1 LTR. pBG138 is identical to pBG139, except it contains an 8-bp insertion to create a frameshift mutation in the *vpu* gene. pGB108 is a derivative of pNL4-3 with a substitution mutation in the *env* gene (7). pBG135 is identical to pGB108, except it has an 8-bp insertion to create a frameshift mutation in the *vpu* gene. SV, simian virus 40.

in the *vpu* open reading frame (Fig. 1). pBG139 expresses the HIV-1 Env-gp and Vpu from the HIV-1 LTR. pBG138 is identical to pBG139, except that it contains a frameshift mutation in the *vpu* gene because of an 8-bp insertion (Materials and Methods; Fig. 1). The *vpu* open reading frame in BG135 and pBG138 is likely to produce a truncated 31-amino-acid protein. However, a similar mutation in the *vpu* gene has been analyzed previously and appears to be phenotypically Vpu⁻ (20, 21). Viral DNA was cotransfected along with an envelope expression construct into CD4⁻ HeLa cells. Three days later, virus was harvested and was used to infect CD4⁺-LTR/ β -gal indicator cells (provided by M. Emerman). CD4⁺-LTR/ β -gal indicator cells are HeLa cells that express CD4. This cell line also contains the *Escherichia coli lacZ* gene expressed from the HIV-1 LTR (12). Efficient β -galactosidase expression is, therefore, Tat dependent. Upon infection with the transcomplemented virus, Tat is expressed from the *env* mutant proviral DNA. Infected CD4⁺-LTR/ β -gal cells can be screened for expression of β -galactosidase by using 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside, and the resulting blue cells can be counted to determine the virus titer. On the basis of this assay, the infectious titer obtained in the absence of Vpu was approximately 90% lower than when GB108 was cotransfected with pBG138 (Table 1). It should be noted that this phenotype was observed with virus produced from HeLa cells that do not express CD4. This indicates that there is a CD4-independent function for Vpu. It is extremely unlikely that the HeLa cells used in this assay express CD4, because they are not susceptible to infection by HIV-1. In addition, direct examination of the virus-producing HeLa cells used in

TABLE 1. Effect of *vpu* mutation on titer of HIV-1 particles produced with HIV-1 Env-gp from CD4⁺ HeLa cells

Transfected DNA	Relative viral titer $\pm$ SD ^a
pBG138 +	
pGB108	70 $\pm$ 9
pBG135	5 $\pm$ 3
pBG139 +	
pGB108	100 ^b
pBG135	51 $\pm$ 9

^a All data represent experiments performed in parallel and are the average of three assays. Infectious units were measured by infecting CD4-LTR/ β -gal cells as described in the text.

^b Transfection of pGB108 and pBG139 resulted in a titer of approximately 3,300 blue cell transforming units/ml.

our experiments found no detectable CD4, as evidenced by Western blot (immunoblot) analysis (17a).

To ensure that the reduction in virus titer was due to the absence of Vpu and not to the perturbation of BG135 sequences other than *vpu*, pBG139 was also used to complement BG135. Cotransfection of BG135 and pBG139 into HeLa cells gave a titer similar to cotransfection with GB108 and pBG138 (Table 1). This indicates that the observed reduction in virus titer was due solely to the absence of Vpu.

The CD4-independent function of Vpu does not involve an interaction with the HIV-1 Env-gp. To determine whether Vpu interacts with the HIV-1 Env-gp, we used a heterologous envelope expression construct to complement either GB108 or BG135. HIV-2 does not contain a *vpu* gene, so Vpu is not likely to functionally interact with HIV-2 Env-gp. If Vpu functions solely by interaction with the HIV-1 Env-gp, then pseudotyping of HIV-1 particles with HIV-2 Env-gp might be expected to alleviate the requirement for Vpu. Thus, there should be no difference in titer when pCMVHIV-2env (Fig. 2) is cotransfected with either BG135 (*vpu* mutant) or GB108. However, the results presented in Table 2 indicate that the reduction in titer due to the absence of Vpu persists even when HIV-2 Env-gp pseudotypes HIV-1 particles. Therefore, Vpu is still required for efficient replication of this HIV-1 pseudotype.

We also performed the envelope complementation experiment by using an amphotropic murine leukemia virus (A-MLV) Env-gp expression vector. A-MLV is more distantly related to HIV-1 than to HIV-2, so the A-MLV Env-gp is very unlikely to interact with Vpu. In addition, A-MLV utilizes a receptor other than CD4 to infect cells. pSV-A-MLV-*env* (provided by D. Littman) expresses the A-MLV

TABLE 2. Effect of *vpu* mutation on titer of HIV-1 particles produced with HIV-2 and A-MLV Env-gps from CD4⁺ HeLa cells

Transfected DNA	Relative virus titer $\pm$ SD ^a
pCMVHIV-2env +	
pGB108	100 ^b
pBG135	9 $\pm$ 3
pSV-A-MLV- <i>env</i> +	
pGB108	100 ^c
pBG135	14 $\pm$ 7

^a All data represent experiments performed in parallel and are the average of three assays. Infectious units were measured by infecting CD4-LTR/ β -gal cells as described in the text.

^b Transfection of pGB108 and pCMVHIV-2env resulted in a titer of approximately 700 blue cell transforming units/ml.

^c Transfection of pGB108 and pSV-A-MLV-*env* resulted in a titer of approximately 71,000 blue cell transforming units/ml.

Env-gp under the control of the MLV LTR (Fig. 2) (16). The data presented in Table 2 indicate that Vpu is required for efficient replication of this HIV-1 pseudotype as well. This indicates that there is a requirement for Vpu that does not involve an interaction with HIV-1 Env-gp. It should also be noted that CD4⁺ cells were again used for the production of pseudotyped particles.

Vpu is required for Env-independent assembly or budding. Because Vpu function was apparently not dependent on HIV-1 Env-gp expression, we wanted to determine whether the reduction in infectious virus produced by the *vpu* mutant could be due to a defect in particle assembly or release, as could be expected on the basis of published results (14, 20, 22). Moreover, we attempted to determine the relative efficiency of virus release with and without Vpu expression in the absence of Env-gp. We performed a pulse-chase and immunoprecipitation analysis on viral protein produced from HeLa cells transfected with either BG135 (*vpu* mutant) or GB108 (Materials and Methods). Because GB108 and BG135 do not encode an intact *env* gene, it was possible to study particle release in the absence of Env-gp expression.

At 60, 120, and 210 min after initiation of the chase, viral protein was detected for GB108, but no released p24 was detected from cells transfected with BG135 (Fig. 3). After prolonged exposure of the gel, we were able to detect a small amount of p24 at the 210-min time point in media from cells transfected with BG135 (data not shown). We would expect a low level of p24 in the media of cells transfected with BG135 because a low level of infectious virus is detectable in the absence of Vpu. Because intracellular levels of p24 were equivalent, the vast reduction in the amount of p24 in the supernatant of cells transfected with BG135 indicates that particle formation or release is less efficient in the absence of Vpu. We hypothesize that Vpu may interact with the Gag and Gag-Pol polyproteins in some manner to enhance assembly or budding.

Vpu does not affect syncytium formation by HIV-1 Env-gp. The previous experiment demonstrates a vast reduction in the rate of budding particles in the absence of Vpu. This greatly reduced number of budding particles could account for the approximately 90% reduction in infectious virus detectable over one round of replication. However, it could also mask any effects the absence of Vpu expression may have on the Env-gp of HIV-1. We therefore wanted to assess whether the presence of Vpu might affect HIV-1 Env-gp function. Many of the properties of Env-gp can be examined

FIG. 2. HIV-2 and A-MLV expression constructs. pCMVHIV-2env expresses the HIV-2 Env-gp from the human cytomegalovirus (CMV) immediate-early enhancer and promoter (7). pSV-A-MLV-*env* expresses the A-MLV Env-gp from the MLV LTR and contains the simian virus 40 (SV) enhancer promoter and simian virus 40 poly(A) (16).

FIG. 3. Kinetics of p24 release from cells transfected with a *vpu* mutant. HeLa cells were mock (M) transfected or transfected with either pBG135 (*Vpu*⁻) or pGB108 (*Vpu*⁺), and 48 h later the cells were pulse labelled with [³⁵S]methionine for 30 min, followed by a chase with fresh cell culture medium for various times. After 0, 60, 120, and 210 min, medium was removed, immunoprecipitated with polyclonal rabbit anti-p24 antiserum (American Bio-Technologies, Inc.), and analyzed on a 15% polyacrylamide gel. The cells from the 120-min time point were lysed and immunoprecipitated to detect intracellular (IC) levels of p24.

by syncytium formation after transfection of CD4⁺ cells with an Env-gp expression construct (8). To determine whether the presence of Vpu might modulate Env-gp-induced syncytium induction, we transfected either pBG138 or pBG139 into HeLa T4⁺ cells and scored the ability of each construct to form syncytia in the presence of Tat expression. We found no difference in syncytium-forming ability of the HIV-1 Env-gp in the presence or absence of Vpu (Table 3).

DISCUSSION

The results presented here indicate that the disruption of gp160-CD4 complexes is not the only role of Vpu in HIV-1 replication. Analysis of a *vpu* mutant indicated that a *Vpu*⁻ phenotype is Env-gp and CD4 independent. Our experiments and those of others (14, 20, 22) suggest that Vpu may facilitate translocation of the Gag and Gag-Pol polyproteins to the proper intracellular membranes where assembly occurs, function directly in assembly of the Gag and Gag-Pol polyproteins, or facilitate egression of viral particles from the cell.

We have shown that production of virus was lower in the absence of *vpu* expression regardless of whether the Env-gp used was from HIV-1, HIV-2, or A-MLV. This result indicates that the function of Vpu is not solely manifested through interaction with the HIV-1 Env-gp. Because HIV-2 and HIV-1 are related viruses, it is formally possible that Vpu may be able to interact with both HIV-1 and HIV-2

envelope glycoproteins. However, we think this is unlikely, because HIV-2 does not contain a gene with obvious sequence similarity to *vpu*. Moreover, A-MLV almost certainly does not have a *vpu* gene. The observation that Vpu is still required to produce infectious virus for HIV-1 particles pseudotyped with A-MLV Env-gp provides unambiguous evidence that Vpu has a function that does not involve interaction with the HIV-1 Env-gp.

We have expressed the HIV-1 Env-gp in HeLa T4 cells with and without Vpu and screened these cells for syncytium formation and have found no decrease in the number of syncytia in the absence of Vpu. This experiment suggests that there is no effect on the expression or function of Env-gp at the surface of cells in the absence of Vpu. However, the syncytium assay is an indirect assay for Env-gp expression and may not be sensitive enough to detect subtle differences in the level of Env-gp expressed on the cell surface. The relative levels of expression of Env, Vpu, and CD4 in this syncytium assay may not be equivalent to those in experiments previously reported by Willey et al. (23) and could be responsible for our inability to detect a CD4- and Env-gp-dependent function for Vpu. Willey et al. have shown that overexpression of Env compared with CD4 can eliminate Env-gp dependence upon Vpu for proper expression (23).

Results recently published by Yao et al. demonstrated that Vpu expression decreases syncytium formation by an infectious molecular clone of HIV-1 in MT4 cells (24). They attributed this lower level of syncytium formation to the loss of Env-gp from the cell surface because of incorporation onto budding particles. If increased efficiency of particle release is responsible for decreased syncytium formation in the presence of Vpu, then it is unlikely that Vpu expression would affect syncytium formation in the absence of particle budding. The syncytium formation assays described here were performed in the absence of particle formation, and under those conditions, we did not detect an effect of Vpu expression on syncytium formation. Thus, our results are consistent with those of Yao et al. (24).

On the basis of previous work suggesting Vpu is involved in the late stages of HIV-1 replication (14, 20, 22), we conducted a pulse-chase experiment to examine the release of p24 from cells in the presence or absence of Vpu and in the absence of Env-gp and CD4. We were readily able to detect p24 in the culture media of cells transfected with *vpu*⁺ viral DNA as early as 60 min after the pulse. However, we only detected an extremely small amount of p24 in the media of cells transfected with *vpu* mutant viral DNA at 210 min after the pulse. This suggests that Vpu interacts with the Gag and Gag-Pol polyproteins, either directly or indirectly, to enhance assembly or budding of viral particles. Because Env-gp and CD4 were both absent in the p24-producing cells, this result provides strong direct evidence that there is an Env-gp- and CD4-independent function for Vpu.

The role for Vpu discussed here and the previously published role of Vpu in dissociation of gp160-CD4 complexes (23) may not be mutually exclusive. Vpu has been shown by indirect immunofluorescence to localize to a perinuclear region of the cell (14). Perinuclear localization could allow Vpu to interact with both gp160-CD4 complexes and Gag and Gag-Pol polyproteins during virus production. We are currently conducting experiments to reconcile these two models for Vpu function.

We would also like to note that shortly after the original submission of this manuscript, Cohen et al. (25), using approaches that overlap with those described in this paper,

TABLE 3. Effect of a *vpu* mutation on syncytium formation^a

Transfected DNA ^b	<i>Vpu</i> ^c	Syncytia/μg of DNA ± SD
pBG138	-	1,100 ± 200
pBG139	+	850 ± 200

^a All data represent experiments performed in parallel and are the average of five assays. Transfections containing pBG138 or pBG139 alone yielded approximately 40 syncytia per μg of DNA. Mock transfections as well as pSV2tat72 alone yielded approximately 10 syncytia per μg of DNA.

^b These transfections also contained pSV2tat72.

^c -, mutant; +, wild type.

also demonstrated that Vpu has a CD4- and Env-gp-independent function.

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REFERENCES

- Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin. 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and non-human cells transfected with an infectious molecular clone. *J. Virol.* **59**:284-291.
- Barre-Sinoussi, F., F. R. Chermann, M. T. Nugeyre, S. Chamaret, J. Gruest, C. Dautet, C. Axler-Blin, F. Vizin-Brun, C. Rouzioux, W. Rozenbaum, and C. Montagnier. 1983. Isolation of T-lymphotropic retrovirus from a patient at risk for acquired immunodeficiency syndrome (AIDS). *Science* **220**:868-871.
- Cohen, E. A., E. F. Terwilliger, J. G. Sodroski, and W. A. Haseltine. 1988. Identification of a protein encoded by the *vpu* gene of HIV-1. *Nature (London)* **334**:532-534.
- Dalglish, A. G., P. C. L. Beverly, P. R. Clapham, D. H. Crawford, M. F. Greaves, and R. A. Weiss. 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature (London)* **312**:763-767.
- Delwart, E. L., G. L. Buchsacher, Jr., E. O. Freed, and A. T. Panganiban. 1992. Analysis of HIV-1 envelope mutants and pseudotyping of replication-defective HIV-1 vectors by genetic complementation. *AIDS Res. Hum. Retroviruses* **8**:1669-1677.
- Frankel, A. D., and C. O. Pabo. Cellular uptake of the Tat protein from human immunodeficiency virus. *Cell* **55**:1189-1193.
- Freed, E. O., E. L. Delwart, G. L. Buchsacher, Jr., and A. T. Panganiban. 1992. A mutation in the human immunodeficiency virus type 1 transmembrane glycoprotein gp41 dominantly interferes with fusion and infectivity. *Proc. Natl. Acad. Sci. USA* **89**:70-74.
- Freed, E. O., D. J. Meyers, and R. Risser. 1989. Mutational analysis of the cleavage sequence of the human immunodeficiency virus type 1 envelope glycoprotein precursor gp160. *J. Virol.* **63**:4670-4675.
- Gallo, R. C., S. Z. Salahuddin, M. Popovic, G. M. Shearer, M. Kaplan, B. F. Haynes, T. J. Palker, R. Redfield, J. Blaske, B. Safai, G. White, P. Foster, and P. D. Markham. 1984. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science* **224**:500-503.
- Graham, F. L., and A. J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**:456-467.
- Helseth, E., M. Kowalski, D. Gabuzda, U. Olshevsky, W. Haseltine, and J. Sodroski. 1990. Rapid complementation assays measuring replicative potential of human immunodeficiency virus type 1 envelope glycoprotein mutants. *J. Virol.* **64**:2416-2420.
- Kimpton, J., and M. Emerman. 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated β -galactosidase gene. *J. Virol.* **66**:2232-2239.
- Klatzmman, D., E. Champagne, S. Chamaret, J. Gruest, D. Guetard, T. Hercend, J.-C. Gluckman, and L. Montagnier. 1984. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. *Nature (London)* **312**:767-768.
- Klimkait, T., K. Strebel, M. D. Hoggan, M. A. Martin, and J. M. Orenstein. 1990. The human immunodeficiency virus type 1-specific protein *vpu* is required for efficient virus maturation and release. *J. Virol.* **64**:621-629.
- Levy, J. A., A. D. Hoffman, S. M. Kramer, J. A. Landis, J. M. Shimabukuro, and L. S. Oshiro. 1984. Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. *Science* **225**:840-842.
- Page, K. A., N. R. Landau, and D. R. Littman. 1990. Construction and use of a human immunodeficiency virus vector for analysis of virus infectivity. *J. Virol.* **64**:5270-5276.
- Schubert, U., T. Schneider, P. Henklein, K. Hoffmann, E. Berthold, H. Hauser, G. Pauli, and T. Porstmann. 1992. Human-immunodeficiency-virus-type-1-encoded Vpu protein is phosphorylated by casein kinase II. *Eur. J. Biochem.* **204**:875-883.
- Schwartz, M. Personal communication.
- Schwartz, S., B. K. Felber, E.-M. Fenyo, and G. N. Pavlakis. 1990. Env and Vpu proteins of human immunodeficiency virus type 1 are produced from multiple bicistronic mRNAs. *J. Virol.* **64**:5448-5456.
- Sodroski, J., W. C. Goh, C. Rosen, K. Campbell, and W. A. Haseltine. 1987. Role of the HTLV-III/LAV envelope in syncytium formation and cytopathicity. *Nature (London)* **322**:470-474.
- Strebel, K., T. Klimkait, F. Maldarelli, and M. A. Martin. 1989. Molecular and biochemical analyses of human immunodeficiency virus type 1 *vpu* protein. *J. Virol.* **63**:3784-3791.
- Strebel, K., T. Klimkait, and M. A. Martin. 1988. A novel gene of HIV-1, *vpu*, and its 17-kilodalton product. *Science* **241**:1221-1223.
- Terwilliger, E. F., E. A. Cohen, Y. Lu, J. G. Sodroski, and W. A. Haseltine. 1989. Functional role of human immunodeficiency virus type 1 *vpu*. *Proc. Natl. Acad. Sci. USA* **86**:5163-5167.
- Willey, R. L., F. Maldarelli, M. A. Martin, and K. Strebel. 1992. Human immunodeficiency virus type 1 Vpu protein regulates the formation of intracellular gp160-CD4 complexes. *J. Virol.* **66**:226-234.
- Yao, X. J., S. Garzon, F. Boisvert, W. A. Haseltine, and E. A. Cohen. 1993. The effect of *vpu* on HIV-1-induced syncytia formation. *J. Acquired Immune Defic. Syndr.* **6**:135-141.
- Yao, X. J., H. Göttinger, W. A. Haseltine, and E. A. Cohen. 1992. Envelope glycoprotein and CD4 independence of *vpu*-facilitated human immunodeficiency virus type 1 capsid export. *J. Virol.* **66**:5119-5126.