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19. The αNTD (residues 1 to 235) with a point mutation Arg^{45}→Ala (αNTD_{R45A}) was subcloned by polymerase chain reaction into the T7 RNAP-based PET-15b expression vector (Novagen) for overexpression of NH_2 terminally expressed hexahistidine-tagged (His_6) protein. Using *E.coli* BL-21(DE3) host cells, we achieved excellent expression (>20 mg of protein per liter of cell culture after purification to homogeneity from the soluble fraction). After cell lysis through a continuous-flow French press and a low-speed spin, the soluble fraction was loaded onto a Ni^{2+}-chelating affinity column, and His_6-αNTD was eluted with 100 mM imidazole. The protein was then treated overnight at 4°C with thrombin to quantitatively remove the His_6-tag, then was loaded onto a MonoQ (Pharmacia) ion-exchange column. After elution with a NaCl gradient, the protein was homogeneous as judged by Coomassie-stained SDS-polyacrylamide gels.
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Cell Cycle Arrest by Vpr in HIV-1 Virions and Insensitivity to Antiretroviral Agents

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Expression of human immunodeficiency virus–type 1 (HIV-1) Vpr after productive infection of T cells induces cell cycle arrest in the G_2 phase of the cell cycle. In the absence of de novo expression, HIV-1 Vpr packaged into virions still induced cell cycle arrest. Naturally noninfectious virus or virus rendered defective for infection by reverse transcriptase or protease inhibitors were capable of inducing Vpr-mediated cell cycle arrest. These results suggest a model whereby both infectious and noninfectious virions in vivo, such as those surrounding follicular dendritic cells, participate in immune suppression.

The HIV-1 *vpr* gene encodes a 14-kD nuclear protein (Vpr) that is expressed within infected cells and is packaged into virions (*1*). Vpr is nonessential for viral replication in T cell lines and activated peripheral blood lymphocytes (PBLs) in vitro but it is necessary for efficient infection of nondividing cells such as macrophages (*2, 3*). Simian immunodeficiency virus–induced disease progression in macaques was attenuated when either *vpr* alone or both *vpr* and the related gene *vpx* were mutated, indicating that Vpr plays an important role in viral pathogenesis (*4*). Functions ascribed to Vpr include transport of the viral core into the nucleus of nondividing cells (*3*) and up-regulation of viral gene expression (*5*). One particularly intriguing function of Vpr is the ability to induce cell cycle arrest at the G_2 checkpoint in a variety of mammalian cells, including human PBLs (*6–8*). This cell cycle arrest is characterized by alterations in the activation and phosphorylation state of Cdc2 kinase (*7, 8*) and resembles the G_2 checkpoint induced by genotoxic agents (*9*). Over time, virally infected cells arrested in the G_2 phase by Vpr die by apoptosis (*10*).

In previous studies of Vpr-mediated cell cycle arrest, we used HIV-1 bearing the murine Thy 1.2 reporter gene to allow concurrent visualization of infected cells by cell surface staining for Thy 1.2 and cell cycle status by staining with propidium iodide (*9, 10*). In some experiments, there was significant cell cycle arrest even in cells that were not productively infected (Thy 1.2^-), particularly at early time points before detectable Thy 1.2 expression (*11*). We tested the possibility that virion-associated Vpr can mediate cell cycle arrest by generating a vesicular stomatitis virus G protein (VSV-G) pseudo-typed HIV-1 that carried Vpr but was incapable of synthesizing it because of a frameshift mutation within *vpr* [$HIV\text{-}1_{NL4\text{-}3\text{-}thy}$ env(-)-vprX]. Epitope-tagged Vpr was supplied to the virion by cotransfection, resulting in trans-complementation [$HIV\text{-}1_{NL4\text{-}3\text{-}thy}$ env(-)vprX + BSVpr] (Fig. 1A) (*12*). Virion particles formed in this fashion contained Vpr in amounts comparable to wild-type virus [$HIV\text{-}1_{NL4\text{-}3\text{-}thy}$ env(-)]. Infection with virions complemented in trans with Vpr (VprX mutant + Vpr in trans) resulted in cell cycle arrest in the Thy 1.2^+ population (G_1/G_2 = 0.26) compared with the Thy 1.2^- population (G_1/G_2 = 1.8) and with infection by the *vprX* mutant (G_1/G_2 = 1.9) (Fig. 1B). The level of arrest induced by virion-associated Vpr alone was typically less than that observed with wild-type virus capable of de novo Vpr production (Fig. 1B), probably because of higher levels of Vpr expressed in cells de novo. Similar results were observed after infection of primary human PBLs and SupT1 T cells with an HIV-1 vector that packages Vpr but whose genome is defective for de novo expression of all HIV-1 genes (*13*). Virions pseudotyped with a CXCR-4 tropic HIV-1 envelope also induced cell cycle arrest (*14*) (Fig. 2B). The extent of G_2 arrest induced by virion-associated Vpr varied depending on the virion preparation and cell type. However, HIV-1 particles prepared without envelope, but still containing Vpr within the viral core, did not induce G_2 arrest (*15*). Thus, cell cycle arrest required both Vpr and viral entry into cells and was not the result of contaminating soluble Vpr in our virion preparations. Significantly, the cell cycle arrest observed with virion-associated Vpr was observed at low multiplicities of infection (MOI = 0.15; Fig. 1B) (*16*).

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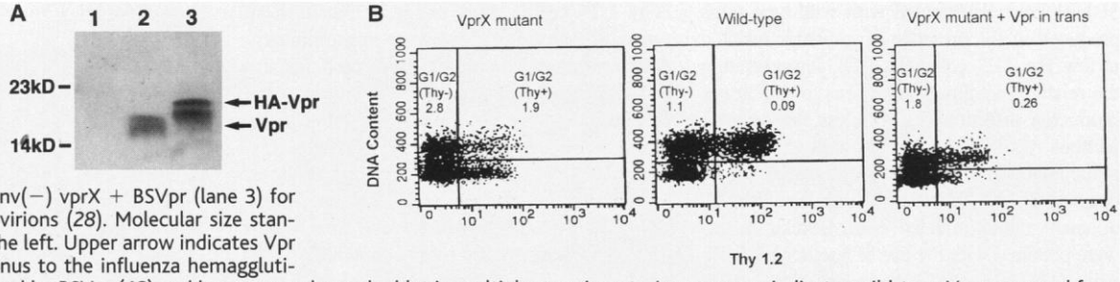

Fig. 1. Virus complemented with Vpr in trans can induce cell cycle arrest. (**A**) Western blot analysis of HIV-$1_{NL4-3-thy}$ env(−) vprX (lane 1), HIV-$1_{NL4-3-thy}$ env(−) (lane 2), and HIV-$1_{NL4-3-thy}$ env(−) vprX + BSVpr (lane 3) for the presence of Vpr in virions (*28*). Molecular size standards are indicated on the left. Upper arrow indicates Vpr fused at the NH_2-terminus to the influenza hemagglutinin nonapeptide expressed by BSVpr (*12*) and has appeared as a doublet in multiple experiments. Lower arrow indicates wild-type Vpr expressed from HIV-$1_{NL4-3-thy}$ env(−). (**B**) Cell cycle profile, 24 hours after infection, of HeLa cells infected with HIV-$1_{NL4-3-thy}$ env(−) vprX (*vprX* mutant), HIV-$1_{NL4-3-thy}$ env(−) (wild type), or HIV-$1_{NL4-3-thy}$ env(−) vprX + BSVpr (*vprX* mutant + Vpr in trans) (*29*).

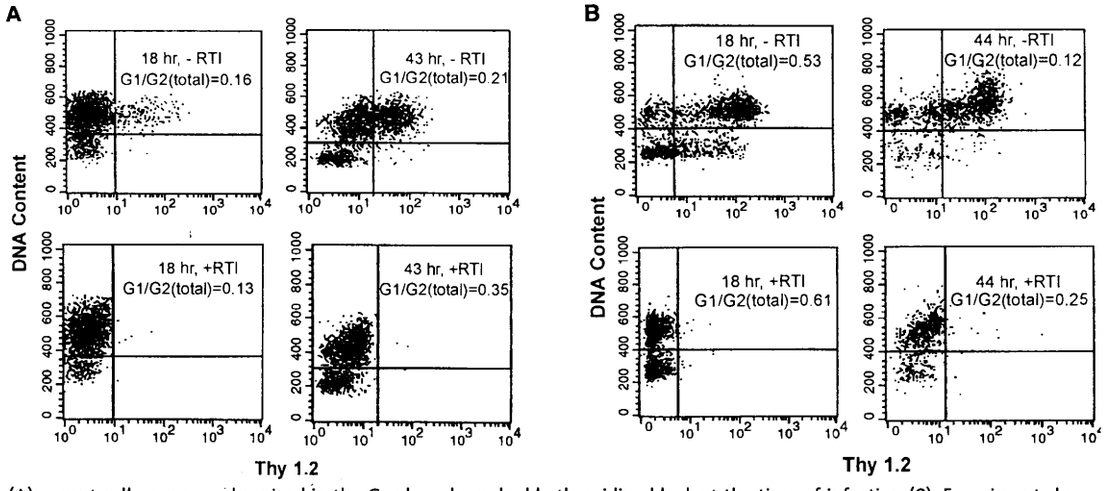

Fig. 2. Vpr can induce cell cycle arrest in the presence of RTI. (**A**) HeLa cells were infected with wild-type virus pseudotyped with VSV-G. One set of samples was treated with AZT and nevirapine (+RTI) (*18*). At the indicated times after infection, cells were analyzed for Thy 1.2 expression and DNA content (*29*). Data shown are representative of three separate experiments. (**B**) HeLa CD4 cells were infected with wild-type virus pseudotyped with HIV-1_{LAI} envelope (*18*) and treated as in (A) except cells were synchronized in the G_1 phase by a double thymidine block at the time of infection (*9*). Experiment shown is representative of three separate experiments.

We tested whether infection with viruses blocked at reverse transcription by zidovudine (AZT) and nevirapine, which are nucleoside and nonnucleoside reverse transcriptase inhibitors (RTIs) (*17*), respectively, would still result in cell cycle arrest. Infection with wild-type virus in the absence of RTI produced Thy 1.2^+ cells that were arrested in the G_2 phase (Fig. 2A). Most of the Thy 1.2^- cells were also arrested at the 18-hour time point, suggesting early arrest before expression of viral genes. Infection with wild-type virus in the presence of RTI resulted in few Thy 1.2^+ cells, which indicates that these drugs were effective in preventing productive infection (*18*) (Fig. 2A). Nevertheless, there was a significant increase in the G_2 population evident 18 hours after infection and continuing to 43 hours after infection. At the later time point, the level of arrest was lower than that observed in non-RTI–treated infected cells: G_1/G_2 ratios of 0.35 and. 0.21, respectively. This difference was consistent in three separate experiments and is likely due to higher levels of Vpr synthesized in the non-RTI–treated infected cells. Similar results were observed when HeLa CD4 cells were infected with wild-type virus pseudotyped with the CXCR-4 tropic HIV-1_{LAI} envelope (Fig. 2B).

Protease inhibitors (PIs) are anti-HIV-1 drugs that prevent viral replication by blocking cleavage of the Gag precursor, resulting in production of immature noninfectious particles (*19*). Compared with untreated virus preparations, wild-type virus or the *vprX* mutant produced in the presence of the PI indinavir contained reduced amounts of processed Gag proteins (*20*) (Fig. 3A). Wild-type virus, but not the *vprX* mutant, packaged Vpr within the virions in the presence or absence of indinavir (Fig.

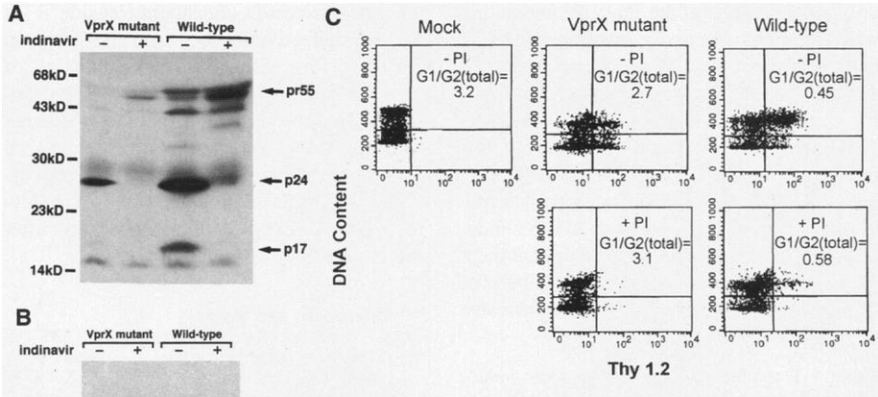

Fig. 3. Ability of virions containing Vpr to cause cell cycle arrest is not affected by treatment with PI. VprX mutant or wild-type virus was produced in the absence (−) or presence (+) of indinavir (*20*). (**A**) The presence of Gag in virions was analyzed by Western blot analysis (*28*). (**B**) The presence of Vpr in virions was analyzed by Western blot analysis (*28*). (**C**) Cell cycle profile, 48 hours after infection, of HeLa cells that were mock infected or infected with *vprX* mutant or wild-type virus produced in the absence (−PI) or presence (+PI) of indinavir (*29*).

3B). HeLa cells infected with wild-type virus produced in the presence of indinavir resulted in few Thy 1.2^+ cells (Fig. 3C), demonstrating the relative inability of this virus to establish a productive infection. Nevertheless, these noninfectious viral particles were still capable of inducing cell cycle arrest, as indicated by the decreased G_1/G_2 ratio relative to mock-infected or *vprX* mutant-infected cells. Similar results were obtained with the use of another PI, nelfinavir (*11*). In several experiments, the level of cell cycle arrest induced by wild-type virus treated with indinavir was generally comparable to the G_2 arrest mediated by wild-type virus treated with RTI [G_1/G_2 ratios of 0.58 (Fig. 3C) and. 0.35 (Fig. 2A), respectively].

Most viral particles contained within HIV-1 preparations are noninfectious (*21*). In addition, a high proportion of noninfectious HIV-1 particles are found in infected patients in vivo (*22*), which raises the possibility that naturally occurring noninfectious particles could contribute to cell cycle arrest. We examined the potential of these particles to induce G_2 arrest by examining the degree of cell cycle arrest in the Thy 1.2^- population in an HIV-1–infected culture over time. Mock-infected HeLa cell cultures contained 35 to 37% of cells in the G_2 phase at any one time (Table 1). At 18 hours after infection, 98% of the Thy 1.2^+ population from HIV-1–infected cultures were in the G_2 phase. Similarly, 85% of the Thy 1.2^- cells were in the G_2 phase. The increase over mock-infected cells in the proportion of Thy 1.2^- cells in G_2 was maintained at 42 hours after infection. It is unlikely that arrest of the Thy 1.2^- population was due to productively infected cells that had yet to express Thy 1.2 on their cell surface because the percentage of Thy 1.2^+ cells remained constant in this experiment at 49% at the two time points. Therefore, our interpretation of these experiments is that naturally existing noninfectious virus is capable of causing cell cycle arrest. In three independent experiments, we calculated that there were approximately equivalent numbers of G_2-arrested Thy 1.2^- cells and G_2-arrested Thy 1.2^+ cells, which suggests that in these virus preparations there were approximately equal numbers of noninfectious particles and infectious particles capable of causing cell cycle arrest. We note that our estimate of noninfectious particles that can induce arrest is less than the generally accepted estimate of noninfectious particles present in virus preparations (*21*). This is likely due to multiple factors responsible for loss of infectivity (*21*), some of which might preclude entry of Vpr into cells.

Table 1. Noninfectious HIV-1 virions can induce cell cycle arrest. HeLa cells were infected with wild-type virus sufficient to productively infect 49% of the cells. At the indicated times after infection, cells were analyzed for Thy 1.2 expression and DNA content (*29*). Results are expressed as percent of cells in G_1 and G_2. Similar results were obtained in three independent experiments.

Cell cycle stage	18 hours after infection			42 hours after infection		
	Mock	Thy 1.2^-	Thy 1.2^+	Mock	Thy 1.2^-	Thy 1.2^+
G_1	64	14	1.5	63	27	1.2
G_2	36	85	98	37	73	98

One consequence of Vpr activity may be the suppression of effective immune responses through, as we proposed (*8, 10*), inhibition of clonal expansion of T cells in response to antigen, or transcriptional dysregulation (*23*). By blocking key early steps in the immune response, Vpr would contribute to immune dysfunction and serve an important function for the virus by facilitating the persistence of virus-producing cells. Our results raise the possibility that high proportions of both infectious and noninfectious HIV-1 particles found in the blood and lymph nodes of infected individuals (*22*)—for example, those surrounding antigen-presenting follicular dendritic cells (*24*)—could arrest $CD4^+$ T cells and contribute to the $CD4^+$ immune dysfunction observed throughout the course of HIV-1 disease (*25*). Importantly, the cell cycle arrest observed with virion-associated Vpr is achievable at low MOIs, which indicates that the arrest of $CD4^+$ T cells in vivo could be highly efficient. In addition, our results indicate that the current anti–HIV-1 drugs, including nucleoside and nonnucleoside RTIs and PIs, effectively block infectivity but do not affect this virion-associated function of Vpr. Thus, in patients undergoing HIV-1 antiviral therapy, any HIV-1 particles produced from residual infected cells at reservoir sites (*26*) would likely still have a T cell suppressive effect. This could contribute to the slow recovery of T cell function observed after effective antiviral suppression (*27*).

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11. B. Poon and I. S. Y. Chen, unpublished observations.
12. All virus stocks were generated by calcium phosphate-mediated transfection [J. Sambrook, E. F. Fritsch, T. Maniatis, in *Molecular Cloning*, N. Ford, C. Nolan, M. Ferguson, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, ed. 2, 1989), pp.16.32–16.34] of 293T cells. VSV-G pseudotyped HIV-$1_{NL4\text{-}3\text{-}thy}$ env(−)vprX and HIV-$1_{NL4\text{-}3\text{-}thy}$ env(−) were generated after transfection with pCMV-VSV-G and either NLthyΔBglVprX or NLthyΔBgl and then concentrated (*9*). Virus stocks of HIV-$1_{NL4\text{-}3\text{-}thy}$ env(−)vprX + BSVpr were generated after transfection with BSVpr [V. Planelles *et al.*, *J. Virol.* **70**, 2516 (1996)], pCMV-VSV-G, and NLthyΔBglVprX.
13. Virus defective for de novo expression of all HIV-1 genes but that packages Vpr was obtained with pHR'CMV-thy, in which the *lacZ* gene from pHR'-CMV-lacZ [L. Naldini *et al.*, *Science* **272**, 263 (1996)] was replaced by the Thy 1.2 gene, pCMVΔR8.2 [L. Naldini, U. Blomer, F. H. Gage, D. Trono, I. M. Verma, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 11383 (1996)], and pCMV-VSV-G, and concentrated (*9*). PBLs isolated from normal uninfected donors were stimulated for 72 hours in RPMI 1640 with 20% fetal calf serum (FCS) and phytohemagglutinin (5 mg/ml) and were maintained after infection in RPMI 1640 with 10% FCS and recombinant interleukin-2 (20 units/ml). At 72 hours after infection, infection of PBLs with virus containing Vpr resulted in a decrease in the G_1/G_2 ratio of Thy 1.2^+ cells compared with mock-infected cells (1.70 and 6.58, respectively). At 72 hours after infection, infection of SupT1 T cells resulted in a decrease in the G_1/G_2 ratio of Thy 1.2^+ cells compared with mock-infected cells (0.43 and 1.7, respectively).
14. Virions pseudotyped with HIV-1_{LAI} envelope were obtained after transfection with pHR'CMV-thy, pCMVΔR8.2, and pLET-LAI [D. Camerini, V. Planelles, I. S. Y. Chen, *Science* **264**, 1160 (1994)] and concentrated by ultracentrifugation at 40,000*g* for 1 hour. At 72 hours after infection of HeLa CD4 cells, the levels of infection were low (3.3% Thy 1.2^+ cells) but, in Thy 1.2^+ cells, infection with virus containing Vpr resulted in a decrease in the G_1/G_2 ratio compared with mock-infected cells (1.4 and 2.4, respectively).
15. Nonenveloped viral particles were obtained after transfection with NLthyΔBgl and concentrated (*9*). Vpr was detected at comparable levels in the enveloped and nonenveloped viral preparations by Western blot analysis (*28*). At 48 hours after infection, HeLa cells infected with nonenveloped HIV-1 had a cell cycle profile comparable to that of mock-infected cells (G_1/G_2 ratios of 2.1 and 2.25, respectively). Infection with VSV-G–enveloped HIV-1 in parallel resulted in a G_1/G_2 ratio of 0.15.
16. Infection of HeLa cells with HIV-1 that packages Vpr (*13*) sufficient to productively infect 12% of the cells (12% Thy 1.2^+) resulted in a decrease in the G_1/G_2 ratio of Thy 1.2^+ cells compared with mock-infected cells (0.61 and 2.1, respectively). Similarly, infection of SupT1 T cells to obtain 6% Thy 1.2^+ cells resulted in a decrease in the G_1/G_2 ratio of Thy 1.2^+ and Thy 1.2^- cells compared with mock-infected cells (0.66 and 0.76 and 1.4, respectively).
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18. For experiments involving RTI, cells were treated with 5 μM 3′-azido-3′-deoxythymidine (Sigma) and 5 μM nevirapine for 2 hours before infection, and RTIs were maintained in the medium during and after infection. RTI treatment of mock-infected or VSV-G or HIV-1_{LAI} pseudotyped *vprX* mutant-infected cells resulted in G_1/G_2 ratios of 1.64, 1.27, and 1.35, respectively, compared with 1.75 for non-RTI–treated cells. Virions pseudotyped with HIV-1 envelope were obtained

after transfection with NLthyΔBgl or NLthyΔBglVprX and pLET-LAI. Supernatant was collected 48 hours after transfection and concentrated by ultracentrifugation at 40,000*g* for 1 hour.
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28. Concentrated virus was lysed in 2× loading buffer and subjected to electrophoresis on SDS–15% polyacrylamide gels (*9*). Western blotting was performed with a rabbit polyclonal antibody for Vpr (provided by N. Landau, Aaron Diamond AIDS Research Center, New York) or human anti-HIV hyperimmune plasma (provided by P. Krogstad and Y. Bryson, UCLA) and developed with the enhanced chemiluminescence assay (Amersham, Arlington Heights, IL).
29. The method of double staining for surface marker Thy 1.2 and DNA content was performed as described in (*9*, *10*). All stained cells were acquired on a FACScan II apparatus (Becton-Dickinson) and analyzed with Cell Quest software.
30. We thank D. S. An, J. Zack, and P. Krogstad for valuable reagents and advice and Vaheideh Gudeman for technical support. Supported by NIH grant CA70018 and Center for AIDS Research grant AI28697. B.P. and K.G.-F. were supported by NIH postdoctoral training grant T32/A107388.

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Specific Covalent Labeling of Recombinant Protein Molecules Inside Live Cells

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Recombinant proteins containing four cysteines at the *i*, *i* + 1, *i* + 4, and *i* + 5 positions of an α helix were fluorescently labeled in living cells by extracellular administration of 4′,5′-bis(1,3,2-dithioarsolan-2-yl)fluorescein. This designed small ligand is membrane-permeant and nonfluorescent until it binds with high affinity and specificity to the tetracysteine domain. Such in situ labeling adds much less mass than does green fluorescent protein and offers greater versatility in attachment sites as well as potential spectroscopic and chemical properties. This system provides a recipe for slightly modifying a target protein so that it can be singled out from the many other proteins inside live cells and fluorescently stained by small nonfluorescent dye molecules added from outside the cells.

Attachment of fluorescent or other useful labels onto proteins has traditionally been accomplished by in vitro chemical modification after purification (*1*). Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* can be genetically fused with many host proteins to produce fluorescent chimeras in situ (*2*, *3*). However, GFP is potentially perturbative because of its size (238 amino acids), can usually only be fused at the NH_2- or COOH-terminus of the host protein, offers a limited variety of colors, and is of no assistance for spectroscopic readouts other than fluorescence. We therefore designed and synthesized a tight-binding pair of molecular components: a small receptor domain composed of as few as six natural amino acids that could be genetically incorporated into proteins of interest, and a small (<700-dalton), synthetic, membrane-permeant ligand that could be linked to various spectroscopic probes or crosslinks. The ligand has relatively few binding sites in nontransfected mammalian cells but binds to the designed peptide domain with a nanomolar or lower dissociation constant. An unexpected bonus is that the ligand is nonfluorescent until it binds its target, whereupon it becomes strongly fluorescent.

Our approach exploits the facile and reversible covalent bond formation between organoarsenicals and pairs of thiols. Trivalent arsenic compounds bind to the paired thiol groups of proteins containing closely spaced pairs of cysteines or the cofactor lipoic acid (*4*, *5*). Such binding, which is responsible for much of the toxicity of arsenic compounds, is completely reversed by small vicinal dithiols such as 2,3-dimercaptopropanol [British anti-Lewisite (BAL)] or 1,2-ethanedithiol (EDT), which form tighter complexes with the organoarsenical than do cellular dithiols (*6*, *7*). If a peptide domain could be designed with even higher affinity than that of the antidotes for an organoarsenical ligand, the ligand could be administered in the presence of excess antidote and specifically bind the desired peptide domain without poisoning other proteins. To achieve this unusual affinity, we designed a peptide domain with four cysteines already organized to bind an organic molecule containing two appropriately spaced trivalent arsenics (Fig. 1). If the distance between the two pairs of cysteines matched the spacing between the arsenics, the two dithiol-arsenic interactions should be highly cooperative and entropically favorable. The four cysteines were placed at the *i*, *i* + 1, *i* + 4, and *i* + 5 positions of an α helix, so that the four thiol groups would form a parallelogram on one side of the helix. We chose acetyl-WEAAAREACCRECCARA-amide (*8*) as a model peptide for in vitro tests, on the basis of the known propensity of peptides of the form acetyl-W(EAAAR)$_n$A-amide (*9*) to form α helices.

Fourteen biarsenical ligands were synthesized and tested for their ability to bind the

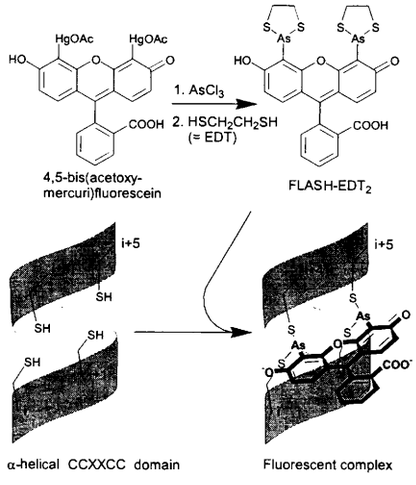

Fig. 1. Synthesis of FLASH (*20*) and proposed structure of its complex with an α-helical tetracysteine-containing peptide or protein domain. Although the structure is drawn with the *i* and *i* + 4 thiols bridged by one arsenic and the *i* + 1 and *i* + 5 thiols bridged by the other, we cannot rule out the isomeric complex in which one arsenic links the *i* and *i* + 1 thiols while the other links the *i* + 4 and *i* + 5 thiols.

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