Monoubiquitinated Histone H1B Is Required for Antiviral Protection in CD4$^+$T Cells Resistant to HIV-1[†]

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ABSTRACT: Linker histone H1B (H1B) coeluted with an antiviral activity during the purification of HIV-1 resistance factor (HRF) from supernatants of HRF(+) cells. Western blot analysis of the supernatant using α-H1 and α-ubiquitin antibodies detected the same band of roughly 46 kDa; this band was absent from the control supernatant. Depletion of histone from biologically active material did not affect its potential, suggesting that ubiquitinated H1B is not required for the HRF-mediated antiviral protection in HIV-1 susceptible target cells; however, specific silencing of histone H1B via RNAi in HRF(+) cells reduced the biological activity of cell culture supernatants by 96% and reversed the HIV-1 resistance phenotype of HRF(+) cells. Exposure to HRF induced ubiquitination and secretion of H1B from target HIV-1 susceptible cells, suggesting that ubiquitinated H1B is a cofactor of HRF, possibly regulating its expression and secretion from CD4$^+$T cells induced to resist HIV-1 infection.

It is now clear that human T lymphocytes and macrophages not only are susceptible to HIV-1 infection but also express multiple factors mediating viral resistance (*1*−*10*). We devised a system for induction of resistance to HIV-1 by both primary and transformed CD4-positive T cells, enabling investigation of the acquired resistance phenotype (*4*, *11*). We have isolated cell lines that both are resistant to HIV-1 and secrete a soluble factor capable of inhibiting HIV-1 replication in susceptible cells. Investigation of the virological aspects of resistant cells and the HIV-1 resistance factor (HRF)[1] indicated that virus binds, enters, and reverse transcribes efficiently but is blocked in transcription. Parallel investigation of the cellular aspects of the response indicated that like susceptible cells, resistant cells carry both CD4 and CXCR4 HIV-1 entry receptors. In recent studies, we have extended our comparison of sister cells lines distinguished by HIV-1 susceptibility or HIV-1 resistance and secretion of HRF to more fully define the overall resistance phenotype (*12*). Our goal was to identify cellular genes that are necessary for cellular resistance and HRF function as well as that encoding HRF itself. We found that HRF(+) cells exhibited a selective down- or upmodulation of genes involved in transcription, several of which are implicated in either the susceptibility of cells to HIV-1 or the promotion of HIV-1 transcription itself, which indicated global changes in the cellular mechanisms that might be involved in the maintenance of the acquired resistance phenotype.

This study was conducted to investigate the proteins secreted by HRF(+) cells as part of our long-term goal of isolating and purifying the protein(s) conferring the antiviral state upon susceptible cells. Performing ion exchange chromatography upon supernatants of HRF(+) cells and testing fractions for biological activity in HIV-1 inhibition assays, we found that the linker histone H1B was present in the active fraction. A review of the biological properties of histones as a group revealed that extracellular histones have been shown to participate in or mediate antimicrobial functions in several systems (*13*−*24*). The extracellular form of the core histone, H2A, had powerful activity against Gram-positive bacteria (*15*). Also, histone H2A-derived peptides, hipposin and bufforin, inhibited a wide spectrum of Gram-positive and Gram-negative bacteria (*14*, *18*). Antibacterial peptides derived from histone H1A were also isolated from stimulated human granulocytes (*21*). Most interestingly, extracellular histone H2B was copurified with α-defensins and other antibacterial peptides during chromatographic separation of human T cell culture supernatants (*13*). Taken together, these studies make clear that histones assume extracellular antimicrobial functions or accessory functions in several systems and recommended further investigation of H1B secreted by HRF(+) T lymphocytes.

With these precedents in mind, we investigated the structure of H1B and its role in the HIV-1 resistance phenotype of HRF(+) cells. Extracellular H1B secreted by HRF(+) but not HRF(−) cells was monoubiquitinated, a modification shown to alter histone regulatory functions in cellular gene expression (*25*, *26*). We therefore investigated four possible, nonexclusive roles for H1B in the expression

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[1] Abbreviations: HRF, HIV-1 resistance factor; H1B, histone H1B; RSA, rapid suppression assay; PMA, phorbol 12-myristate 13-acetate; LTR, long terminal repeat; MOI, multiplicity of infection; siRNA, short interfering RNA.

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of HRF. Exposing HIV-1 susceptible cells to the HRF(+) cell culture supernatant, we tested whether induction of HIV-1 resistance is associated with ubiquitination and secretion of H1B. Using immunodepletion of H1B from HRF(+) supernatants, we tested whether H1B is HRF or is required for its biological activity. Using siRNA, we tested whether transcription of H1B is required for HRF expression or secretion and last whether H1B is required for the resistance phenotype of HRF(+) cells. Our studies demonstrate that H1B is not HRF itself but is essential for HIV-1 resistance in HRF(+) cells and for expression or secretion of HRF itself. These studies elucidate some of the complex events required for the establishment and maintenance of HIV-1 resistance in human $CD4^+$T lymphocytes.

EXPERIMENTAL PROCEDURES

Cell Cultures and Antisera. HIV-1 resistant cell line HRF-(+) was described previously under the name R1c2-12 (*4*, *11*, *27*). HIV-1 susceptible cell line HRF(−) is a clone of the SupT1 cell line and was described previously under the name S-3 (*4*). The 1G5 cell line was obtained through NIH AIDS Research and Reference Reagent Program. Continuous cultures of HRF(+), HRF(−), and 1G5 cell lines were maintained in RPMI culture medium supplemented with 5% fetal bovine serum (FBS), antibiotics, and glutamate at 37 °C in a 5% CO_2/95% air-humidified incubator. Supernatants used for isolation of HRF or for Western blot analysis were obtained from cells cultured at a concentration of 2×10^6 cells/mL for 24 h in protein-free hybridoma medium (Sigma).

Goat polyclonal antibodies specific to an epitope at the amino terminus of histone H1, to B23 (nucleophosmin), and to caspase-3, mouse monoclonal antibodies to α-tubulin, and rabbit polyclonal antibodies to ubiquitin and relevant secondary antibodies were purchased from Santa Cruz Biotechnology, Inc.; the pooled serum of AIDS patients was a kind gift from D. Volsky, and SIM.2 mouse monoclonal antibody to CD4 was obtained through NIH AIDS Research and Reference Reagent Program.

Rapid Suppression Assay (RSA). RSA was conducted by inhibition of phorbol 12-myristate 13-acetate (PMA) induction of 1G5 cells that are stably transfected with an inducible luciferase gene driven by an HIV-1 long terminal repeat (LTR) (*28*). The biological activity of HRF was calculated as a percent inhibition of expression of the luciferase gene. 1G5 cells were washed in PBS and resuspended in hybridoma medium to a concentration of 5×10^6 cells/mL. Aliquots (100 μL) of 1G5 cells were supplemented with a 0.01−1% volume of tested supernatants or protein fractions brought to a final volume of 200 μL and incubated for 3 h at 37 °C. Subsequently, all cells were induced with PMA at a concentration of 5 ng/mL. Two control tubes that contained 1G5 cells were resuspended in hybridoma medium with or without PMA. Three hours later, all cells were collected by centrifugation and lysed in the same tubes using reporter lysis buffer (Promega). Twenty microliters of lysate was mixed with the beetle luciferin substrate (Promega) and tested in a luminometer (Turner Designs Instruments).

Ion Exchange Chromatography. HRF-containing supernatants were collected, centrifuged at 2000 rpm to remove particulate materials, filtered through a 0.45 μm Millipore filter, and concentrated by lyophilization from 300 mL aliquots using a Labconco lyophilizer. Protein powder was resuspended in 10 mL of distilled water and subjected to dialysis against 10 mM Tris-HCl (pH 8.0) using benzoylated cellulose tubing with a molecular mass cutoff of 1.2 kDa (Sigma). Dialyzed material was concentrated again by lyophilization and stored at 4 °C. Subsequently, the desalted lyophilisate of HRF(+) or HRF(−) cell supernatants was dissolved in 10 mM Tris-HCl (pH 8.0) and separated over the course of 25 min. A 0 to 100% linear gradient of buffer B [Tris-HCl (pH 8.1) and 1 M NaCl] was administered at a flow rate of 3.0 mL/min through a 5 mL ion exchange High Trap Q XL column by an Acta Prime chromatography system (all from Pharmacia). Eluted protein peaks were dialyzed as described above and tested by RSA. Subsequently, active protein fractions identified by RSA were pooled and analyzed by tandem mass spectrometry analysis at the Protein Core Facility at Columbia University.

Mass Spectrometry Analysis. We applied nanoelectrospray ionization coupled with a single desalting and concentration step followed by direct analysis of the unseparated peptide mixture by mass spectrometry (*29*). Briefly, the protein sample was digested overnight with 100 ng of modified endoproteinase Lys-C (sequencing-grade, Roche Molecular Biochemicals, Indianapolis, IN) in 0.025 M Tris (pH 8.5) at 32 °C. Peptides were extracted from the gel with a 50% acetronitrile/2% TFA mixture and subjected to reversed phase HPLC using a C-18 ZipTip column (Millipore) followed by elution from the tip with 10 μL of 50% acetronitrile. Immediately prior to injection into the mass spectrometer, the acetronitrile was removed on a Speed-Vac concentrator and the volume was reduced to 2 μL. One microliter of peptide extract was analyzed by nano-LC−MS/MS using a Micromass Q-Tof hybrid quadrupole time-of-flight mass spectrometer with a nanoelectrospray source. The MS/MS spectra were searched using the MASCOT algorithm with two criteria: (1) two or more MS/MS spectra match the same protein entry in the database that was searched and (2) matched peptides will be derived from the same type of enzymatic digestion performed on the identified protein.

Depletion of Histone from the HRF(+) Supernatant. M-280 tossyl-activated Dynabeads (1×10^8, Dynal Biotechnology) were coated with 50 μg of a goat polyclonal α-histone antibody (Santa Cruz Biotechnology, Inc.) according to the manufacturer's protocol. Ten milliliter of the HRF(+) serum protein-free supernatant was concentrated and desalted as described above, resuspended as a 20× concentrate, and incubated with 4×10^7 α-histone-coated magnetic beads in the presence of 0.01% hexadecyltrimethylammonium bromide (Sigma). Following the overnight incubation at 4 °C, beads were collected on a magnetic particle separator, and the histone protein-free HRF(+) supernatant was subjected to RSA and Western blot analysis. Histone-coated magnetic beads were resuspended in 1× SDS loading buffer, and histone was released from immunocomplexes by heating at 65 °C for 1 h.

siRNA Silencing of Histone mRNA in HRF(+) Cells. The *Homo sapiens* histone H1B mRNA sequence (GenBank entry NM005322) was subjected to BLAST analysis to exclude common coding sequences. On the basis of this analysis, we selected a 115 bp mRNA fragment unique to histone H1B as a template for dsRNA synthesis. Two primers [sense (5′-ATGTCGGAAACCGCTCCTGCC-3′) and antisense

(5′-TCGCTTTGCGCTTAGCAGCGC-3′), with appended T7 promoter sequences (5′-TAATACGACTCACTATAGG-3′)] were used to amplify a histone cDNA fragment from mRNA of HRF(+) cells. Subsequently, the cDNA fragment was cloned into the pCR^{TA} vector (Invitrogen) and amplified in *Escherichia coli* DH5α cells. The histone cDNA fragment enlarged by the T7 polymerase promoter was excised from the plasmid by EcoRI digestion, separated through agarose gel electrophoresis, and purified through Ultrafree-DA columns (Millipore). Three micrograms of this material served as a template for dsRNA synthesis using the ShortCut RNAi kit based on the manufacturer's protocol (New England Biolabs Inc.). Ten micrograms of dsRNA was subjected to processing by shortcut RNase III for 20 min at 37 °C and purified by ethanol precipitation. The concentration of siRNA fragments was calculated on the basis of UV absorbance and by band intensity comparison of siRNA fragments run next to serial dilutions of the 21 bp RNA standard (New England Biolabs Inc.) on a 20% TBE−polyacrylamide gel (Invitrogen).

For the initial histone knockout experiments, 3×10^6 HRF-(+) cells were transfected with 4 and 8 nM histone siRNA fragments by lypofectamine 2000 (Invitrogen) in hybridoma medium. Complete growth medium was added after 4 h, and cells were cultured at a concentration of 1×10^6 cells/mL for an additional 48 h. After this time, cells were collected to confirm histone H1B mRNA degradation by RT-PCR (Invitrogen) based on the manufacturer's protocol, and supernatants were submitted to RSA. The 681 bp histone cDNA was amplified with the pair of primers: sense (HIS-F), 5′-ATGTCGGAAACCGCTCCTGCC-3′; and antisense (HIS-R), 5′-CTACTTCTTTTTGGCAGCCGCCTTC-3′. Amplification of the β-actin transcript was performed with the pair of primers described previously (*30*).

Assay of the Effect of H1B siRNA Silencing on the Susceptibility of HRF(+) Cells to HIV-1 Replication. To test the correlation of histone H1B to HRF activity, 21×10^6 HRF(+) and 12×10^6 HRF(−) cells were infected with HIV-1 NL4-3 (*31*) at an MOI of 1. Three days later, the HRF(+) culture was divided into two parts: one served as a control for restrictive infection and another was subjected to siRNA silencing of histone H1B at 8 nM as described above. Degradation of histone mRNA was confirmed by RT-PCR with the pair of primers (HIS-F and HIS-R) as described above. HIV-1 infection was monitored by the levels of extracellular antigen p24 by ELISA (Coulter), by RT-PCR of the single spliced *vif* transcript using a pair of primers [sense (NL616), 5′-GTGTGGAAAATCTCTAGCAGTG-GCGC-3′; and antisense (VFSP), 5′-AACCAGTCCT-TAGCTTTCCTTGAAATATAC-3′ (*32*)], and by indirect immunofluorescence using pooled AIDS sera.

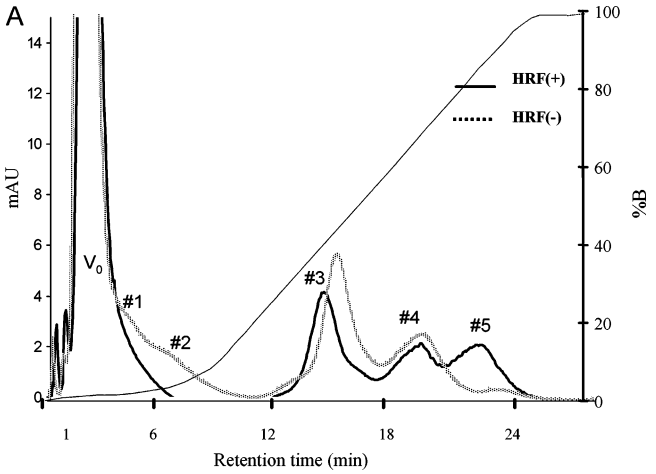
Analysis of the Ubiquitinated H1B Subcellular Compartment Distribution in HRF(+) Cells. To trace the distribution of ubiquitinated H1B in HIV-1 resistant cells, we performed subcellular fractionation essentially as described previously (*33*). Briefly, 2×10^7 HRF(+) cells were subjected to swelling in a hypotonic buffer [10 mM Tris-HCl (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM dithiothreitol] supplemented with protease inhibitors (Sigma) and lysed in a glass Dounce homogenizer (type A). The efficiency of cell breakage was monitored by dye exclusion using a phase-contrast microscope. Lysed cells were centrifuged at 500*g* for 5 min to remove nuclei (pellet P_1). The supernatant was fractionated further by centrifugation at 50000*g* for 20 min, yielding a pellet (P_{50}) containing cell membranes and a supernatant (S_{50}) containing cytosol. The S_{50} supernatant was subjected to the next round of centrifugation at 200000*g* for 1 h to pellet a heterogeneous population of microsomal vesicles and polysomes (P_{200}). Nuclear membranes we isolated as described previously (*34*) with minor modifications. Briefly, crude nuclei were resuspended in extraction buffer [20 mM HEPES (pH 7.9), 25% glycerol, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT] and lysed by 20 strokes in a glass Dounce homogenizer (type B). Nuclear membranes were harvested by centrifugation at 13000*g* for 5 min. All fractions were concentrated by lyophilization, and concentrates were resuspended in 1× SDS loading buffer, heated at 56 °C for 20 min, resolved through 8 to 16% gradient SDS−PAGE, and subjected to a Western blot procedure using α-histone and α-ubiquitin antibodies as described above. Separation of cellular compartments was confirmed by antibodies detecting α-tubulin, CD4, and nucleophosmin.

Induction of Histone H1B Ubiquitination and Secretion from Cells Exposed to HRF Treatment. HRF(−) and HRF-(+) cells were cultured adjacent to each other in a double-chamber apparatus (Corning). After 24, 48, and 72 h, HRF(+) cells were removed and HRF(−) cells were washed three times in PBS and cultured for an additional 24 h in the absence of HRF. Subsequently, HRF(−) cells and cell culture supernatants were collected for further analysis. Lyophilized culture supernatants were dissolved in 100 μL of H_2O and denatured in 6× SDS loading buffer, and cells were lysed in RIPA buffer and subjected to Western blot analysis using α-histone and α-ubiquitin antibodies as described above.

RESULTS

Histone H1B Is Copurified with Antiviral Activity during Ion Exchange Chromatography. We performed chromatography protocols coupled with mass spectrometry analysis in efforts to isolate an antiviral factor from cell culture supernatants of HRF(+) cells. Supernatants were harvested from overnight cultures grown in protein-free hybridoma medium. Desalted lyophilisates of HRF(+) and control HRF-(−) supernatants were separated via an ion exchange High Trap Q XL column. This procedure resulted in the separation of the concentrated supernatants into several protein peaks, one of which was present in HRF(+) but not in the HRF-(−) supernatant (Figure 1A). Eluted protein fractions were tested by our rapid suppression assay (RSA) for inhibition of HIV-1 infection. The biological activity was detected in a single protein peak that was absent from HRF(−) material (Figure 1B). The active fraction was subjected to proteolytic digestion by LysC endopeptidase, and peptides derived from this digestion were analyzed by nanoelectrospray tandem mass spectrometry followed by database searching with multiple peptide sequence tags (*29*). This procedure identified three peptides derived from linker histone H1B and one derived from ubiquitin (Table 1). Although histones are usually considered to be nuclear proteins, several studies have detected extracellular histones (*16*, *17*, *23*, *24*). We next

A



B

Tested material	% inhibition
HRF(+) supernatant	85
HRF(-) supernatant	0.1
Pooled HRF(+) V_0	0.7
Pooled HRF(+) #1	0.7
Pooled HRF(+) #2	0.1
Pooled HRF(+) #3	0.5
Pooled HRF(+) #4	0.5
Pooled HRF(+) #5	95

FIGURE 1: Separation of HRF(+) and HRF(−) cell culture supernatants by anion exchange chromatography. (A) Concentrated 300 mL aliquots of HRF(+) and HRF(−) supernatants were applied to a strong (5 mL) anion exchange High Trap QXL column (Pharmacia) and separated over the course of 25 min with a 0 to 100% linear gradient of buffers: buffer A being Tris-HCl (pH 8.1) and buffer B being 1 M NaCl at a flow rate of of 3.0 mL/min, followed by a 10 min wash in 100% B with the Acta-Prime chromatography system (Pharmacia); collected fractions are indicated by numbers. (B) Summary of testing of the biological activity in the HRF(+) and HRF(−) supernatants and collected protein fractions from anion exchange chromatography. All values are calculated as the percent inhibition of luciferase expression by a 0.1% volume of the tested sample.

investigated the role of histone H1B in the anti-HIV-1 activity of HRF.

Ubiquitinated Histone H1B Is Secreted by HIV-1 Resistant Cells but Not by HIV-1 Susceptible Cells. To investigate the correlation between expression of extracellular H1B and HIV-1 resistance, we subjected supernatants of HRF(+) and HRF(−) cells to electrophoresis and Western blot staining with the anti-H1B antibody (Figure 2A). Immunoblot analysis revealed that HRF(+) and HRF(−) cells secrete H1B with different mobilities; H1B secreted by HRF(−) cells migrated at its native mobility of approximately 30 kDa (*16*, *35*), but HRF(+) cells produced histone migrating with the mobility of a 46 kDa protein. Since one of the peptides identified by mass spectrometry analysis was derived from ubiquitin (Table 1), we reasoned that the observed increase in the molecular mass of histone secreted by HRF(+) cells might be explained by postsynthetic ubiquitination. To explore this possibility, the membrane blot was stripped and reprobed with the anti-ubiquitin antibody (Figure 2A). Overlaying the autoradiograms showed that H1B and ubiquitin migrated with the same mobility in samples from HRF(+) but not HRF(−) cells, consistent with mass spectrometry analysis and our proposal that histone secreted by HIV-1 resistant cells is ubiquitinated. To exclude the possibility that the presence of nuclear proteins in cell culture supernatants might result from apoptosis, we employed indirect immunofluorescence to test both HRF(+) and HRF(−) cells for the presence of a mediator of apoptosis, caspase 3 (*36*). We found that neither cell line expressed an elevated level of caspase 3, suggesting that the secretion of ubiquitinated histone H1B by HRF(+) cells was independent of apoptosis (data not shown), and pointing to an active mechanism regulating export of this protein. Such a mechanism, including direct disintegration or micellization of the cell membrane leading to the formation of transient holes consistent with ion channel activities, was proposed for the extracellular secretion of the core histone H2A (*15*, *37*, *38*).

On the basis of this information, we studied the distribution of ubiquitinated H1B in different cellular compartments representing nucleus (P_1) cell membranes (P_{50}), cytosol (S_{50}), nuclear membranes (NM), and polysomes (P_{200}). All subcellular fractions from HRF(+) cells were subjected to electrophoresis and Western blot staining with anti-H1 and anti-ubiquitin antibodies, and the accuracy of compartment separation was tested by antibodies raised to CD4, α-tubulin, and nucleophosmin (Figure 2B). Consistent with our hypothesis of active extracellular transport of ubiquitinated H1B, we found the highest concentration of this protein in the cell membrane fraction (P_{50}, Figure 2B). A small portion of ubiquitinated H1B was also associated with nucleus, cytosol, and nuclear membrane but not with fraction P_{200} representing the site of protein synthesis, which suggested that H1B is ubiquitinated in the nucleus followed by rapid translocation to the cell membrane and subsequent export to the extracellular compartment.

As we reported before, the exposure to HRF(+) cells or their culture supernatant conferred the resistance to virus upon HIV-1 susceptible cells (*4*, *11*), and experiments described above showed that ubiquitinated H1B is present in HRF(+) cells and their biologically active cell culture supernatant which allowed us to speculate that the induction of antiviral resistance in HIV-1 susceptible cells coincides with ubiquitination and export of H1B. To prove this hypothesis, we used the same approach as described previously (*4*, *11*) and cultured over time both HRF(+) and HRF(−) cells in double-chamber apparatus. Following this exposure to HRF, cells were washed and cultured alone for an additional 24 h and tested for the presence of ubiquitinated H1B in both intra- and extracellular compartments (Figure 2C,D). Consistent with our previous observations, we detected ubiquitinated H1B in total lysates of HRF(+) but not HRF(−) control cells. However, 3 days of exposure to HRF induced ubiquitination and secretion of H1B, thus linking the induction of resistance to HIV-1 with ubiquitination and secretion of H1B and suggesting that ubiquitinated H1B might be HRF itself.

Ubiquitinated Histone H1B Is Dispensable for HRF Function in the Extracellular Compartment of HIV-1 Resistant Cells. Since ubiquitinated H1B is present in the active fraction of the HRF(+) supernatant, it may constitute HRF, associate with HRF, or copurify with HRF. To test these possibilities, we removed histone from the HRF(+) cell supernatant by immunodepletion using magnetic beads coated with the anti-histone H1B antibody. Complete deple-

Table 1: Amino Acid Sequences and Molecular Weights of Peptides Obtained from LC−MS/MS Analysis of Bioactive Fraction after IXC Separation

no.	peptide	derivation	*m/z*	obsd MW	calcd MW
1	SETAPAETATPAPVEK	histone H1B	799.93 (2+)	1597.85	1597.78
2	ATGPPVSELITK	histone H1B	606.84 (2+)	1211.67	1211.68
3	ALAAGGYDVEK	histone H1B	547.31 (2+)	1092.61	1092.55
4	TITLEVEPSDTIENVK	ubiquitin	894.36 (2+)	1788.72	1786.92

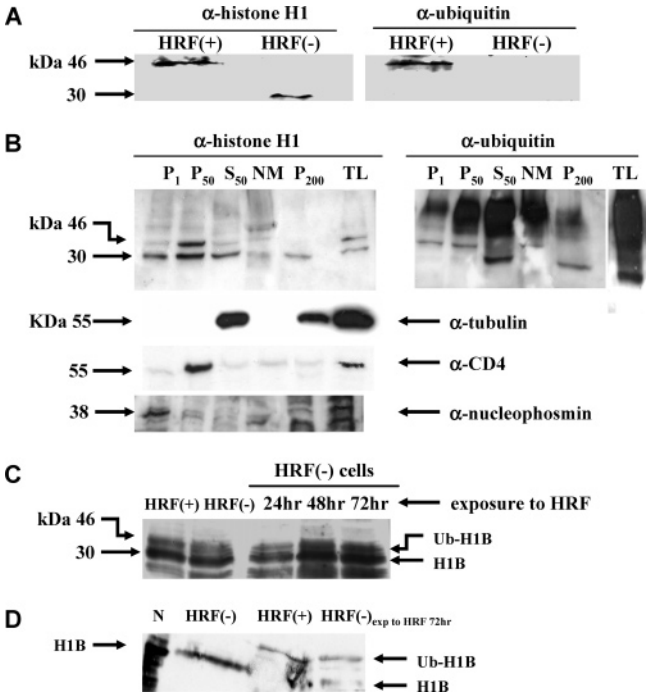
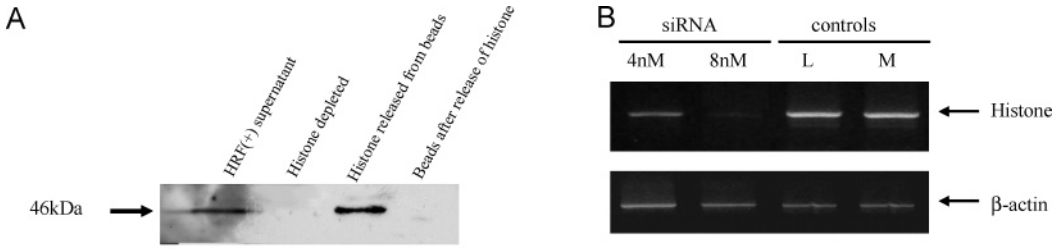


FIGURE 2: Ubiquitinated histone H1 associates with the membrane fraction and is secreted into extracellular space by HRF(+) cells. Western blot analysis of HRF(+) and HRF(−) cell culture supernatants (A and D), subcelluar fractions from HRF(+) cells (B), and total cell lysates of HRF(−) cells cultured in the presence of HRF (C). (A and D) Supernatants from HRF(+) and HRF(−) or HRF(−) cells cultured in the presence of HRF for 72 h were resolved through 10% SDS−PAGE and probed with anti-H1B and anti-ubiquitin antibodies. (B and C) Subcellular protein fractions or total cell lysates were resolved through 8−16% SDS−PAGE and probed with antibodies to H1B, ubiquitin, α-tubulin, CD4, and nucleophosmin: P_1, nuclei; P_{50}, membranes; S_{50}, cytosol; NM, nuclear membranes; P_{200}, polysomes and microsomal vesicles; TL, total lysate.

tion of histone was confirmed by Western blot analysis using the same anti-histone antibody (Figure 3A). The histone-free fraction was compared to the starting material for HRF activity by RSA, and they exhibited equivalent biological activity (Figure 3C). These results indicate that H1B is not HRF and that H1B is not required for HRF-mediated antiviral protection in HIV-1 susceptible target cells.

HRF and H1B May Be Copurified in Ion Exchange Chromatography. Given its secretion by HRF(+) but not HRF(−) cells, the possibility remains, however, that ubiquitinated H1B plays a role in the expression of HRF. We tested this possibility by silencing H1B transcription in HRF(+) cells using siRNA gene targeting (*39*) and an assay of HRF expression. Two doses of siRNA histone fragments were delivered to HRF(+) cells via liposomes, and 2 days later, both cells and culture supernatants were collected for analysis. First, we confirmed the absence of histone mRNA by RT-PCR (Figure 3B). The level of expression of histone mRNA was reduced by 61 and 94% based on band densitometry analysis by 4 and 8 nM siRNA fragments, respectively, compared to controls. The specificity of histone mRNA degradation was confirmed by the analysis of expression of the β-actin gene which was unaffected in all the systems that were tested (Figure 3B). Supernatants of each cell group were then tested for the biological activity by RSA (Figure 3C). There was a significant reduction in HRF activity in supernatants derived from cells subjected to histone silencing; 4 nM histone siRNA inhibited HRF activity by 85%, and an 8 nM solution reduced HRF activity by 96%. These findings indicate that H1B expression is required for HRF activity in cell supernatants. However, the site of H1B interactions is unclear. H1B may be required for HRF expression, HRF function, or HRF secretion. As the first step in resolving these possibilities, we tested the maintenance of HIV-1 resistance in HRF(+) cells themselves, reasoning that the resistance phenotype depends on HRF function or expression but not its secretion.

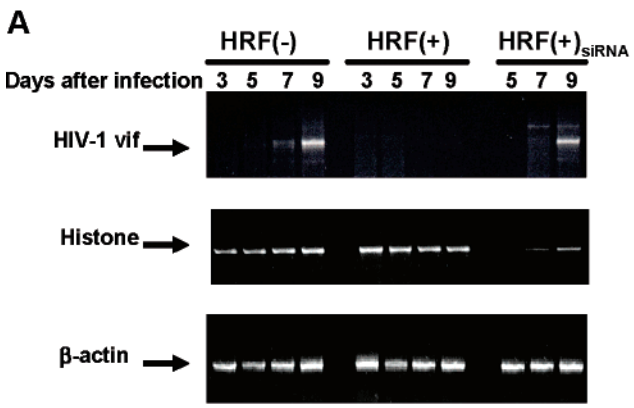
Expression of H1B Is Required for the Preservation of the HIV-1 Resistance Phenotype in HRF(+) Cells. HRF(+) and control HRF(−) cells were infected with HIV-1/NL4-3 at a multiplicity of infection of 1. Three days later, the HRF(+) culture was divided into two parts; one was subjected to histone H1B-specific silencing by 8 nM siRNA, and the other served as a control for the maintenance of HIV resistance. Cells were then tested over time for H1B expression and HIV-1 production. Degradation of histone mRNA was confirmed by RT-PCR which exhibited 100% inhibition of histone expression 2 days after siRNA transfection (Figure 4A, day 5 after infection). The level of expression of this gene was still reduced by 82% (day 7 after infection) and 52% (day 9 after infection). Replication of HIV-1 was tested by RT-PCR amplification of a single spliced *vif* transcript, detection of the p24 core antigen in cell culture supernatants, and the indirect immunofluorescence of infected cells. Consistent with our previous findings (*4*, *11*), the transcription of HIV-1 *vif* was inhibited in HRF(+) but not HRF(−) cells (Figure 4A). However, histone silencing in HIV-1-infected HRF(+) cells resulted in a reversal of resistance. Six days after introduction of histone siRNA, the level of expression of the *vif* transcript was comparable to that in HIV-1 susceptible HRF(−) cells (Figure 4A). Results of RNA analysis were confirmed by analysis of the viral protein by ELISA and by immunofluorescent staining of cells for HIV-1 antigens (Figure 4B,C). Five days after virus challenge and 2 days after histone silencing, the level of the p24 protein in supernatants from HRF(+) cells was comparable to that in unsilenced HRF(+) cells, indicating that complete knockout of histone expression did not result in the immediate reversal of virus inhibition. However, 9 days after infection, histone silencing



C

Tested material	% inhibition
HRF(+) supernatant	85
HRF(-) supernatant	0.1
Histone depleted HRF(+) supernatant	90
HRF(+) supernatant after treatment of cells with 4nM of histone siRNA	15
HRF(+) supernatant after treatment of cells with 8nM of histone siRNA	3.7

FIGURE 3: Ubiquitinated histone H1B is indispensable for HRF activity in the producing cells. (A) Western blot analysis of HRF(+) supernatants subjected to histone depletion. (B) RT-PCR analysis of histone transcripts after gene target specific RNA interference; mock controls include lypofectamine alone (L) or medium alone (M). (C) Summary of testing of the biological activity in the analyzed samples. All values are calculated as the percent inhibition of luciferase expression by a 0.1% volume of the tested sample.



B

Days after infection	HIV-1 p24 core antigen ng/ml HRF(-)	HRF(+)	HRF(+)$_{siRNA}$
3	1.48	0.15	NA
5	3.23	0.25	0.22
7	3.40	0.76	4.98
9	6.96	0.80	8.06

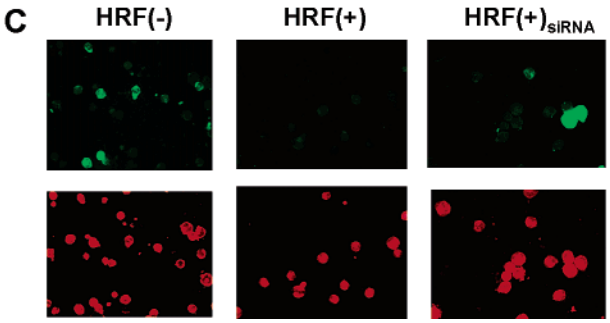


FIGURE 4: Interference with histone H1B expression in HRF(+) cells overturns the resistance to HIV-1. (A) RT-PCR analysis of single spliced vif, histone, and β-actin mRNA. (B) Levels of secreted HIV-1 p24 core antigen measured by ELISA; NA means not applicable for this time point. (C) Immunofluorescence staining of HIV-1-infected HRF(+) and HRF(−) cells collected 9 days after the virus challenge; upper level cells were stained with pooled serum from AIDS patients followed by goat anti-human FITC antibody; the lower level is Evans blue.

permitted HIV-1 expression at the level of protein expression of 8.06 ng/mL, exceeding that in HIV-1 susceptible HRF-(−) cells. These results gave us confidence that H1B and its posttranslational modification in HIV-1 resistant cells are a part of an active mechanism awaiting its definition.

Overall, our research indicates that although histone H1B itself does not hold the biological activity of HRF, its expression is essential for the HIV-1 resistance phenotype in HRF(+) cells. Furthermore, we have a strong basis for believing that H1B might be involved in the control of the expression and secretion of HRF itself.

DISCUSSION

The findings presented here indicate that monoubiquitinated histone H1B is required for the maintenance of antiviral resistance and secretion of antiviral factors in a T lymphocyte cell line. Secretion of monoubiquitinated H1B, possibly in association with HRF, is another element distinguishing cells

induced to HIV-1 resistance from their sister susceptible cells such as those we have recently reported (*12*).

The presence of H1B eluting at a high salt concentration from a positively charged matrix during anion exchange chromatography was somewhat surprising. Our calculations indicate that H1B accounts for ~40% of peak 5 (data not shown). All histones are basic proteins (*40*), and under chromatography conditions, they should not bind to the column. Since H1B peptides were detected reproducibly, we considered that its unusual fractionation could be explained by the bound DNA in the sample derived from HRF(+) cells (*24*), a possibility we excluded because of the absence of methylene blue staining (*41*) of nylon membranes tested after the Western blot procedure (not shown). However, the observed aberrant fractionation of H1B may be due to ubiquitination or interaction with a strongly negatively charged protein.

The presence of this nuclear protein in the extracellular compartment could indicate poor cell culture conditions which promoted leakage of the nuclear proteins or apoptosis leading to cell death. The high viability of both cell cultures and the absence of detection of the mediator of apoptosis caspase 3 excluded these possibilities, suggesting that the presence of H1B outside the cells was not related to apoptosis. Instead, we believe that expression of monoubiquitinated H1B is part of the overall difference between the phenotype of HIV-1 resistant HRF(+) cells and the susceptible parental or sister cell lines.

Our recent evaluation of differential expression of genes in HRF(+) versus HRF(−) cells (*12*) showed selective down- or upmodulation of genes involved in transcription regulation. Several products of differentially expressed genes can regulate directly or indirectly the LTR-mediated transcription or nuclear export of viral proteins and participate in the initial virus−cell interactions, subsequent replication, and efficient transmission. However, H1B mRNA expression as determined by RT-PCR is not different in HRF(+) and HRF(−) cells (not shown). The observed global changes in the cellular mechanisms preventing virus infection could be induced originally by HRF, and the postsynthetic ubiquitination of H1B rather than H1B expression may be the key difference in the maintenance of the acquired resistance phenotype.

The depletion of H1B from cell culture supernatants failed to alter HRF activity, indicating that H1B itself is not HRF and that its presence is not required for the acquisition of resistance by HIV-1 susceptible target cells by their exposure to the HRF(+) supernatant. We then investigated whether ubiquitination and release of H1B in HRF(+) cells are a consequence of the global changes in the cellular metabolism induced by the acquisition of resistance (*12*) or whether they play a role in HRF expression. The exclusive detection of a monoubiquitinated form of H1B in HRF(+) and HRF(−) cells exposed to HRF cell culture supernatants presents strong support for this hypothesis. Although the functional significance of this modification is yet to be determined, it has been suggested that ubiquitinated histones may participate in gene activation (reviewed in ref *42*). The *in vitro* studies showed that histone H1 is ubiquitinated by $TAF_{II}250$, a component of general transcription factor TFIID (*25*), which suggested that ubiquitination of linker histones might sequentially regulate transcription of genes transactivated by selected transcription factors. We further investigated the associations of H1B in HRF(+) cells through targeted siRNA silencing of H1B expression. This method was demonstrated to be very effective in an extensive decrease in the level of gene expression in a variety of cellular systems (*39*, *43*−*47*). The silencing of H1B expression in HRF(+) cells did not result in apoptosis or nuclear degeneration over the time period that was assayed, consistent with slow turnover of H1B in the nucleus, but it resulted in the sequential reduction of HRF activity first in the extracellular compartment followed by the reversal of the resistance phenotype in cells themselves. This genetic approach to assessing the linkage between monoubiquitinated H1B expression and HRF expression is complementary to the structural approach by chromatography. However, gene silencing is free from some difficulties in the interpretation of the cofractionation of H1B and HRF. The reversal of HIV-1 resistance by H1B silencing indicates a strong association in the expression of the histone and the expression of the antiviral factor.

Functions of histones have been enlarged from structural scaffolding for nucleosomes to include innate antimicrobial responses (*14*, *15*, *18*, *19*, *21*, *48*), stimulation of myoblasts during skeletal muscle regeneration (*16*), or growth factor-like activation of androgen-dependent cells (*22*). Histone H1 has been shown to function in adenovirus infection to mediate nuclear import of viral DNA, including disassembly of viral capsids and recruitment of H1 nuclear import factors (*49*, *50*). Multiple functions of histones raise the possibility that H1B serves as a chaperone for secretion of HRF. The association of ubiquitinated H1B with the membranes supports this hypothesis. However, our data indicate that this may not be an exclusive role of H1B in the HRF activity, or it would require HRF(+) cells to take up extracellular HRF to mediate its antiviral function. The induction of ubiquitination and secretion of histone H1B from HIV-1 susceptible cells that were cultured in the presence of HRF provides a molecular link between these diverse functions.

Combining these scientific precedents with our results allowed us to conclude that the induction or maintenance of the antiviral phenotype in $CD4^+$T cells involves histone H1B. We showed that H1B itself is not HRF, that H1B is not required for HRF-mediated antiviral protection in HIV-1 susceptible target cells, but that the expression of H1B is indispensable for HRF activity in both intra- and extracellular compartments. We hypothesize that monoubiquitination of H1B participates directly or through other cellular cofactors in the expression of HRF; the subsequent secretion of monoubiquitinated H1B into the extracellular compartment might also facilitate the transport of HRF. Identification of this elusive antiviral factor will improve the understanding of the nature of H1B−HRF interactions.

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