R5 and X4 HIV envelopes induce distinct gene expression profiles in primary peripheral blood mononuclear cells

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HIV envelope binds to and signals through its primary cellular receptor, CD4, and through a coreceptor, either CC chemokine receptor 5 (CCR5) or CXC chemokine receptor 4 (CXCR4). Here, we evaluate the response of peripheral blood mononuclear cells to a panel of genetically diverse R5 and X4 envelope proteins. Modulation of gene expression was evaluated by using oligonucleotide microarrays. Activation of transcription factors was evaluated by using an array of oligonucleotides encoding transcription factor binding sites. Responses were strongly influenced by coreceptor specificity. Treatment of cells from CCR5Δ32 homozygous donors with glycoprotein (gp)120 derived from an R5 virus demonstrated that the majority of responses elicited by R5 envelopes required engagement of CCR5. R5 envelopes, to a greater extent than X4 envelopes, induced the expression of genes belonging to mitogen-activated protein kinase signal transduction pathways and genes regulating the cell cycle. A number of genes induced by R5, but not X4, envelopes were also up-regulated in the resting CD4+ T cell population of HIV-infected individuals. These results suggest that R5 envelope facilitates replication of HIV in the pool of resting CD4+ T cells. Additionally, signaling by R5 gp120 may facilitate the transmission of R5 viruses by inducing a permissive environment for HIV replication.

glycoprotein 120 | microarray | tropism | viral replication | signal transduction

HIV-1 infection of CD4+ T cells begins with the fusion of the viral envelope with the outer membrane of a target cell. Fusion is initiated when the viral envelope glycoprotein (gp)120 binds first to CD4 and then to a coreceptor, primarily CC chemokine receptor 5 (CCR5) or CXC chemokine receptor 4 (CXCR4) (1–4). These interactions between gp120 and cell-surface receptors transduce signals in the target cell. Ligation of CD4 by gp120 increases the tyrosine kinase activity of p56lck, a src-related protein kinase associated with the cytoplasmic domain of CD4 on CD4+ T cell membranes (5). Engagement of CCR5 and CXCR4 by gp120 also mediates signaling events (6–9), as evidenced by the rapid mobilization of Ca^{++}, and the phosphorylation of intracellular substrates including PYK2 and FAK (10, 11). In this respect, the HIV envelope protein delivers a unique near simultaneous dual signal to CD4+ T cells. No protein in the human proteome has been shown to deliver an analogous signal. The response to this stimulus depends on the state of differentiation and activation of the targeted cell. A number of reports demonstrate that envelope induces activated CD4+ T cells to apoptose *in vitro* (12–15). Envelope proteins have also been shown to disrupt antigen-specific CD4+ T cell responses (16). We have focused on the impact of envelope-mediated signaling on resting CD4+ T cells. These cells constitute one of the latent reservoirs of HIV that prevents eradication of virus from infected individuals, even after prolonged treatment with potent antiretroviral drugs (17, 18). *In vitro* exposure of resting CD4+ T cells derived from HIV-infected individuals to envelope results in a burst of viral replication (19). Insight into the mechanisms that produce this burst was obtained from a study of the gene expression profiles in peripheral blood mononuclear cells (PBMCs) treated with gp120 derived from an R5 strain of HIV (14). Envelope treatment induced the expression of multiple factors that promote viral replication. Of note, nuclear factor of activated T cells (NFAT) was among the transcription factors that gp120 induced. NFAT binds to the HIV LTR (20) and induces viral replication in resting CD4+ T cells (21, 22). Treatment of resting CD4+ T cells with gp120 induces the dephosphorylation and nuclear translocation of NFAT (23). These observations are consistent with gene expression profiles obtained from cell lines, which suggest HIV infection influences cellular pathways that facilitate viral replication (24, 25).

Here, we further investigated envelope-mediated signaling by comparing the response of PBMCs to gp120s derived from both R5 and X4 strains of HIV. R5 and X4 viruses exhibit markedly different properties *in vivo*. Regardless of the route of transmission, the viruses that first appear in the acute phase of infection are R5 (26–28). Throughout the early stages of disease, R5 viruses predominate. In 40%–50% of infected individuals, the dominant quasispecies switches to a predominantly X4 phenotype. This switch, which often occurs only after 8–10 years of infection, is a harbinger of accelerated progression of clinical disease. Explanations for these distinct properties of R5 vs. X4 viruses will likely enhance our understanding of HIV pathogenesis.

With these issues in mind, we undertook a study of the commonalities and differences between the transcriptional programs induced by envelope proteins derived from R5 vs. X4 viruses. We treated resting PBMCs with five different envelope proteins, including gp120s derived from both R5 and X4 viruses, representing three of the major HIV subgroups (A, B, and C). Freshly isolated PBMCs from seven healthy donors were treated with these envelopes, and the induction of transcription-related genes was evaluated by using high-density oligonucleotide microarrays. To better understand the role of CCR5-signaling vs. CD4-signaling, we also treated PBMCs derived from individuals homozygous for the CCR5Δ32 deletion with R5 and X4 gp120s.

Results

Coreceptor Specificity Plays a Dominant Role in the Response of PBMCs Exposed to Envelope. PBMCs from seven healthy donors were exposed to five recombinant gp120s and a mock protein

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Abbreviations: CCR5, CC chemokine receptor 5; CXCR4, CXC chemokine receptor 4; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PBMC, peripheral blood mononuclear cell.

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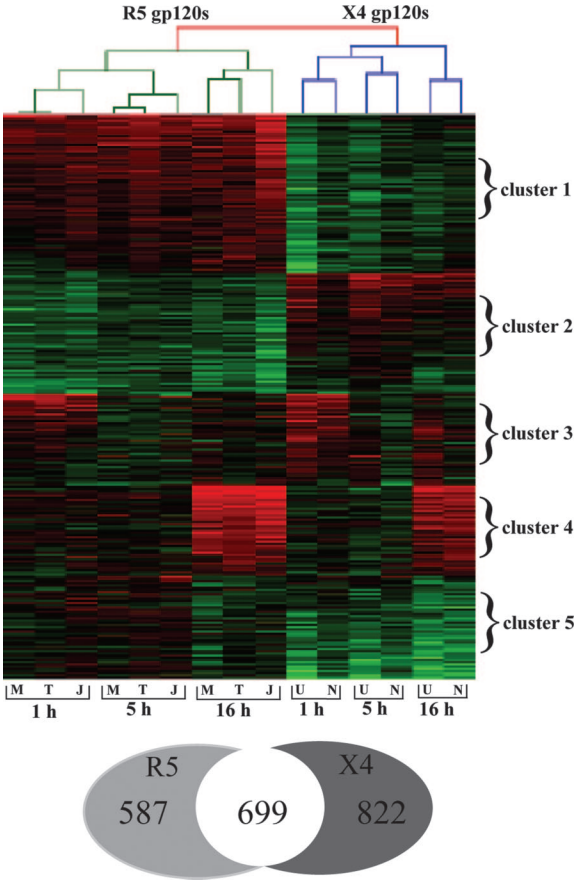

Fig. 1. Modulation of gene expression in response to treatment with genetically diverse R5 and X4 envelope proteins. Freshly isolated PBMCs from seven donors were treated with three R5 envelopes [92MW959 (M) (subgroup C), 92TH14–12 (T) (subgroup B), and JR-FL (J) (subgroup B)] and two X4 gp120s [92UG021–9 (U) (subgroup A) and NL4–3 (N) (subgroup B)] at three time points (1, 5, and 16 h). Statistical significance (*t* score) is reported relative to mock-treated PBMCs (red, *t* score >2; black, *t* score = 0; green, *t* score <−2). Hierarchical clustering analysis was used to cluster samples, and *K*-means clustering was used to group genes. A Venn diagram comparing overlap of the gene induced by R5 gp120s and X4 gp120s is included.

Table 1. Ten most significant enriched biological categories associated with genes induced by R5 and X4 gp120s

Category	*P* value	No. of genes
R5-induced genes (cluster 1) (1–16 h)		
Nucleic acid metabolism	7.79 e^{-8}	146
Protein serine/threonine kinase	1.45 e^{-7}	35
Response DNA damage	3.89 e^{-7}	23
Transcription	8.43 e^{-7}	65
IgG binding	1.57 e^{-5}	5
cAMP-dependent kinase	4.64 e^{-5}	18
Cell proliferation	9.44 e^{-5}	58
Caspase activity	6.75 e^{-4}	5
Protein tyrosine kinase	1.31 e^{-3}	20
GTPase regulator	2.01 e^{-3}	17
X4-induced genes (cluster 2) (1–16 h)		
Protein kinase	2.54 e^{-7}	168
Cell proliferation	3.16 e^{-6}	52
Heavy metal bind	1.02 e^{-5}	5
Cytoskeleton organization	4.42 e^{-5}	39
Intracellular signal	2.48 e^{-4}	42
GTPase regulator	9.71 e^{-4}	15
Hydrolase activity	2.77 e^{-3}	65
Actin filament processing	5.54 e^{-3}	9
Protein tyrosine kinase	6.77 e^{-3}	15
Pyrophosphatase activity	7.2 e^{-3}	22
Genes induced by R5 and X4 gp120s (clusters 4 and 5) (early stage, 1–5 h)		
Cell cycle	1.10 e^{-7}	49
Peptide binding	5.5 e^{-6}	14
Intracellular signaling	5.9 e^{-4}	34
Transcription activity	9.72 e^{-5}	40
Receptor binding	6.21 e^{-4}	23
Apoptosis	1.11 e^{-3}	20
Cell–cell signaling	1.4 e^{-3}	22
Cytokine	1.98 e^{-3}	7
Chemokine	2.01 e^{-3}	5
Response chemical stimuli	1.3 e^{-2}	11
Genes induced by R5 and X4 gp120s (clusters 4 and 5) (late stage, 16 h)		
Chemokine ligand	6.19 e^{-9}	11
Cell–cell signaling	3.91 e^{-8}	31
Cytokine activity	6.11 e^{-6}	16
Organogenesis (hemopoietic or lymphoid)	1.31 e^{-5}	58
Response to external stimuli	1.33 e^{-5}	47
Response to chemical stimuli	9.97 e^{-5}	16
Cell cycle	3.14 e^{-4}	38
Intracellular signaling cascade	5.86 e^{-3}	30
Wound healing	8.2 e^{-3}	7
Transcription	1.4 e^{-2}	31

Category enrichment was determined using the DAVID bioinformatics tool.

preparation. We used three R5 gp120s, JR-FL (subgroup B), 92MW959 (subgroup C), and 92TH14–12 (subgroup B), and two X4 envelopes, 92UG21–9 (subgroup A) and NL4–3 (subgroup B). Cells were harvested at three time points (1, 5, and 16 h), and transcription profiles were generated with Affymetrix U95A chips. A mixed model ANOVA was used to identify ≈ 2,000 genes that were significantly modulated in response to envelope treatment ($P < 0.01$; absolute fold change > 1.5). To represent significant differences between envelope treatments at each time point relative to mock-treated cells, *t* scores, which depict the direction and significance of modulation of each gene, were generated and used for clustering genes and sample groups (Fig. 1). A data set, which is published as supporting information on the PNAS web site, lists the Affymetrix U95A probe sets identifier along with the *t* scores for the sample groups and cluster membership. We report these responses for each envelope at each time point (1, 5, and 16 h). The hierarchical clustering of sample groups shows that the tropism of the envelope proteins is the primary factor driving gene expression (Fig. 1). Gene profiles generated by exposure to R5 envelopes clustered together, and gene profiles generated by exposure to X4 envelopes clustered together. The sample groups were further subclustered based on time, as indicated by the dendrogram. This clustering occurred despite the genetic diversity that distinguished the envelopes used, such that receptor tropism, not subgroup, explained most of the differences in gene expression after envelope treatment. The majority of genes modulated were specific for either R5 or X4 envelopes, with 587 genes modulated exclusively by R5 gp120s and 822 modulated exclusively by X4 envelopes (Fig. 1).

We identified five major clusters. Cluster 1 includes genes that were up-regulated only by R5 envelopes. Genes in cluster 1 were slightly down-modulated or not altered by X4 envelopes. Cluster 2 includes genes up-regulated only by X4 envelopes and either down-modulated or unchanged in response to R5 envelope. Cluster 3 includes genes up-regulated at early time points (1 and 5 h) by both R5 and X4 envelopes. Cluster 4 included genes up-regulated late (16 h) in response to both R5 and X4 envelopes. Cluster 5 includes genes down-modulated by X4 envelopes and either unmodified or slightly up-regulated by R5 envelopes.

Genes Induced by R5 Envelopes Are Not Up-Regulated in CCR5Δ32 PBMCs. The results described above indicate that coreceptor signaling plays a dominant role in envelope-mediated induction of gene expression in PBMCs. We hypothesized that the absence of CCR5 on the cell surface should obviate the modulation of the

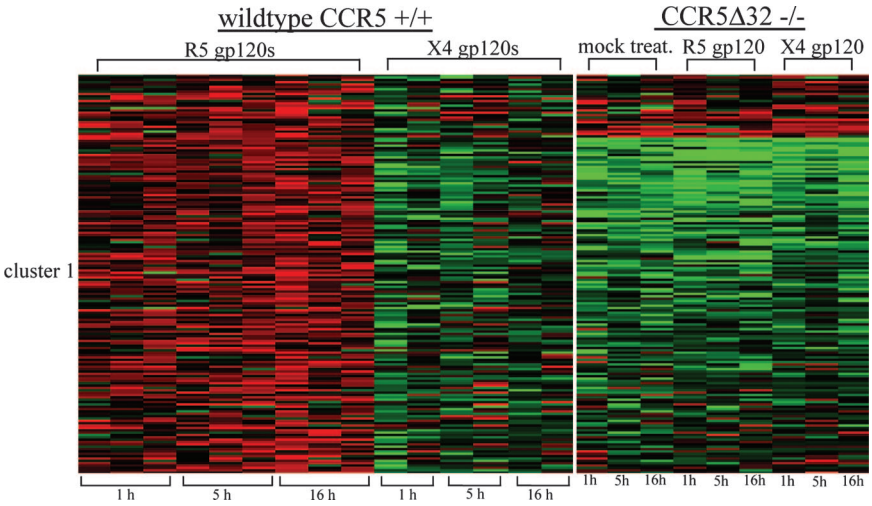

Fig. 2. Genes induced by R5 gp120s in wild-type PBMCs are not induced in CCR5Δ32 PBMCs. The effect of R5 gp120 on those genes up-regulated by R5 gp120s in wild-type PBMCs (from Fig. 1, cluster 1) in PBMCs from CCR5Δ32 homozygous donors. R5 and X4 gp120 proteins were used to stimulate cells, and gene expression was determined at three time points (1, 5, and 16 h). Statistical significance (*t* score) is reported relative to mock-treated wild-type PBMCs (red, *t* score >2; black, *t* score $= 0$; green, *t* score <-2).

majority of genes in response to R5 envelope. To test this hypothesis, we treated PBMCs derived from individuals homozygous for the CCR5Δ32 allele (2, 3) with both R5 and X4 gp120s. PBMCs derived from three individuals homozygous for the CCR5Δ32 allele were treated with an R5 gp120, an X4 gp120, or a mock protein preparation for 1, 5, and 16 h. Expression analysis was carried out as described above with differences (*t* scores) reported relative to the mock treatment of wild-type CCR5 PBMCs. The vast majority of genes specifically induced by R5 envelopes in wild-type PBMCs (cluster 1) were not induced by an R5 gp120 in CCR5Δ32 PBMCs (Fig. 2), demonstrating that those genes are up-regulated in response to gp120 engagement of CCR5. Engagement of CD4 alone was insufficient to induce the up-regulation of genes specifically modulated by R5 gp120s in wild-type PBMCs.

R5 and X4 Envelopes Modulate Genes Associated with Cell Proliferation, Cell Cycle, and Transcription Factors. To better understand the differential impact of R5 vs. X4 gp120-mediated signaling, we searched for enriched biological categories of genes in cluster 1 (R5-induced genes), cluster 2 (X4-induced genes), and cluster 3 (genes induced by both R5 and X4 gp120s) by using the functional annotation tool DAVID (29, 30). Table 1 lists the ten categories that achieved the highest degree of significance for each cluster. For cluster 3, enriched biological categories were identified for both early and late time points. Of note, the term "transcription" and derivatives thereof achieved a high degree of significance and was among the terms most frequently associated with gp120-modulated genes. A number of the terms listed in Table 1 are of particular interest, insofar as genes belonging to these categories can have a profound impact on viral replication.

R5 and X4 gp120s Activate Transcription Factors Differentially. HIV replication is strongly influenced by the activation state of host cell transcription factors. We next asked whether R5 and X4 envelopes differentially altered the DNA-binding activity of transcription factors. We used a blot array that tests the DNA-binding activity of >90 transcription factors (Fig. 3). We treated freshly isolated PBMCs for 4 h with either an R5 or an X4 envelope and compared the DNA-binding activity of the represented transcription factors relative to mock-treated PBMCs. Several transcription factors were differentially activated by R5 vs. X4 gp120. In particular, GATA-3 was activated by X4 gp120 but not by R5 gp120. GATA-3, which is abundantly expressed in T lymphocytes, binds directly to the HIV LTR and induces LTR-mediated transcription (31). In contrast, STAT-4 was exclusively activated by R5 gp120. STAT-4 is a member of the STAT family of transcription factors and plays a central role in the polarization of T lymphocytes toward a Th1 phenotype (32).

MAP Kinase Signaling Pathways Are Strongly Impacted by Envelope-Mediated Signal Transduction. R5 envelopes induce mitogen-activated protein kinase (MAPK)-related genes (13, 14). MAPKs comprise a family of serine/threonine kinases involved in lymphocyte survival and differentiation and thus may be particularly relevant to HIV replication and HIV-mediated cytopathicity (7, 33,

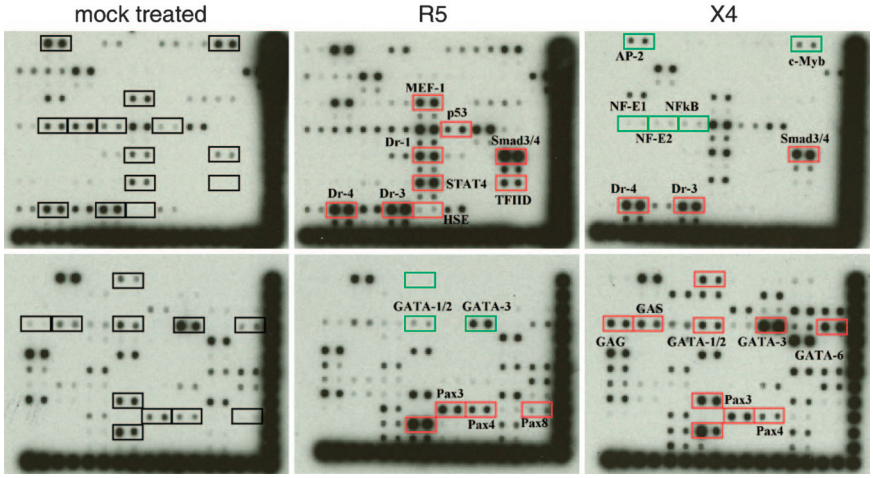

Fig. 3. Transcription factor/DNA probe-binding array. Immobilized DNA probes encoding ≈90 transcription factor binding sites (represented in duplicate) were probed with nuclear extracts from freshly isolated PBMCs treated with R5 and X4 gp120s. Mock-treated nuclear lysates were included for reference. Transcription factors whose binding activity was modulated in envelope-treated cells are highlighted in boxes. Red boxes, increased DNA binding activity; green boxes, decreased DNA binding activity; black boxes, corresponding transcription factors in the mock-treated control sample are included for reference. Transcription factors discussed in the text are labeled. Results shown are representative of two separate experiments.

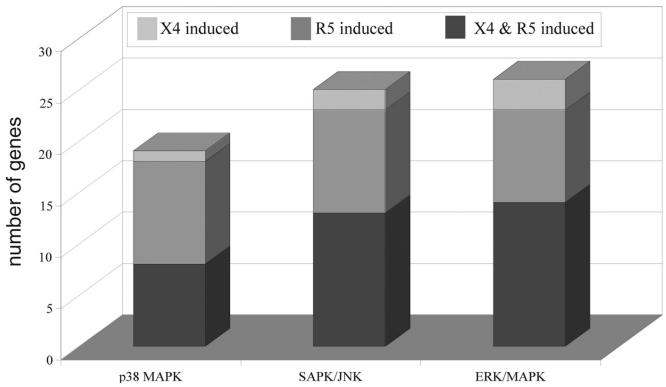

Fig. 4. MAPK signaling pathway genes are induced to a greater extent by R5 envelopes than by X4 envelopes. The aggregate number of genes in each of the three main MAPK signaling pathways [p38, stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK), and ERK/MAPK] that were modulated by R5 and X4 envelopes are shown. See Table 2 for additional information.

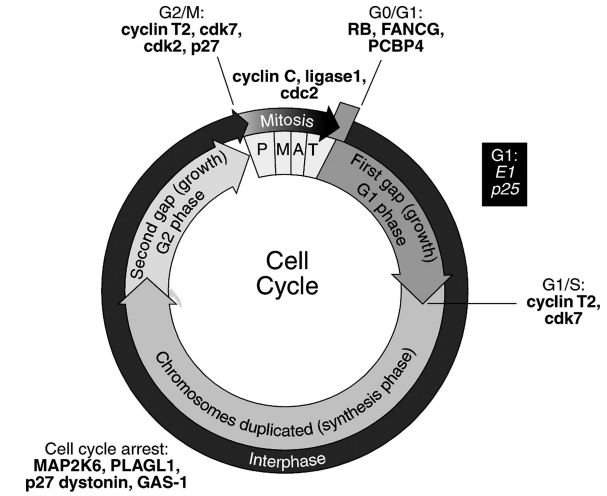

Fig. 5. Cell cycle-related genes are modulated to a greater extent by R5 gp120s than by X4 gp120s. The stages of the cell cycle where these genes are involved are indicated. Genes induced by R5 envelopes are listed in bold, and genes induced by X4 envelopes are boxed and listed in italics.

34). Both R5 and X4 envelopes exerted a strong impact on the MAPK pathway by modulating a total of 67 genes, as determined by the DAVID functional annotation knowledge base (data not shown). Fifty-two MAPK genes were modulated by R5 gp120s, and 41 MAPK genes were modulated by X4 gp120s. Approximately 40% of the MAPK pathways were induced exclusively by R5 gp120s. The MAPK family can be subdivided into three kinase cascades: extracellular signal-regulated kinase 1 (ERK1)/ERK2, c-Jun N-terminal kinase/stress-activated protein kinase, and p38 (35). We observed a striking dichotomy in the induction of these kinase cascades by R5 vs. X4 envelopes (Fig. 4 and Table 2). Many of the MAPK genes induced by R5 gp120s belonged to the p38 subfamily. Only one p38 kinase gene was modulated by X4 gp120s. In addition, the MAPK genes induced by R5 envelopes were enriched in genes associated with regulation of transcription, cell proliferation, and apoptosis.

Cell Cycle Genes Are Differentially Modulated by R5 Vs. X4 Envelopes. Among the gene categories strongly modulated by gp120 were "cell cycle" and "cell proliferation" (Table 1). A limited number of cell cycle genes were modulated by X4 gp120s (Fig. 5). In contrast, R5 gp120s impacted a larger number of genes that were associated with all stages of the cell cycle, including genes involved in regulating G_1/S and G_2/M checkpoints in the cell cycle (Fig. 5). Cell cycle-associated genes involved in the G_0 to G_1 checkpoint that were modulated by R5 envelopes included RB, FANCG, and PCBP4. R5 envelopes also modulated the G_1/S restriction-point genes cyclin T2, cdk7, and G_2/M restriction point genes, including cdk7, cdk2, p27, and cyclin T2. Additionally, R5 envelopes modulated genes associated with cell cycle arrest (including MAP2K6, PLAGL1, p27 dystonin, and growth arrest-specific-1) and mitosis (including cyclin C, ligase1, and cdc2).

Genes Induced by R5 Envelopes Are Also Induced in the Resting CD4$^+$ T Cell Pool. Latently infected resting CD4$^+$ T cells comprise one of the primary HIV reservoirs that are an obstacle to the eradication of HIV from infected individuals (17). We previously reported that, in viremic patients, this reservoir exhibits a distinct expression profile favorable to HIV-1 gene expression (29). In a separate study, we postulated that R5 gp120 induces a cascade of signals in nonproliferating cells that favors virus replication (14). We considered the possibility that the expression profile of resting CD4$^+$ T cells derived from viremic individuals might share features in common with PBMCs treated with gp120. We compared the group of genes specifically up-regulated in the CD4$^+$ T cell reservoir of viremic individuals with all of genes included in Fig. 1 that were modulated in response to either R5 and/or X4 envelopes. Of the 326 genes up-regulated in the CD4$^+$ T cell reservoir of viremic patients, 47 genes were also up-regulated exclusively by treatment of PBMCs of uninfected individuals with R5 gp120s (Fig. 6 and Table 3). Remarkably, no other cluster was enriched in those 326 genes. A Fisher exact test was applied to calculate the probability of sharing this many genes between the two gene lists by chance alone. The 326 annotated genes specifically up-regulated in the CD4$^+$ T cell reservoir of viremic individuals represent 3.8% of the genes on the microarray and thus, by chance alone, 3.8% of the genes in cluster 1 would be expected to overlap. However, we find that 9.0% of the genes in cluster 1 overlap with the genes specifically up-regulated in the CD4$^+$ T cell reservoir of viremic individuals, yielding a Fisher's exact P value of 4.3×10^{-8}. Consistent with our hypothesis that envelope signaling facilitates the replication of virus from the resting CD4$^+$ T cell reservoir (13, 14, 19), cell cycle and transcription are among the descriptive terms significantly associated with those 47 genes (Fig. 6).

Discussion

It is well established that upon exposure to HIV, most individuals are infected by R5 viruses (28, 36). X4 viruses appear transiently

Table 2. List of genes modulated by R5 and X4 gp120s as determined by INGENUITY PATHWAY ANALYSIS software

MAPK cascade	R5-induced genes	X4-induced genes
p38 MAPK	**ATF-2, DUSP10, HSB2, MAP2K6, MAP3K7 MEF2C, TGFBR1, TGFB3, CREB1, STAT1,** ELK1, FASLG, MAPK11, MAPK13, MAX	**MAPKAPK3**, ELK1, FASLG, MAPK11, MAPK13, MAX
SAPK/JNK	**ATF-2, DUSP10, MAP3K1, MAP3K7, MAP3K12, GNA13, MAPK8, MAPK5, PIK3R2, TRA,** CDC42, CRKL, DUSP4, ELK1, GADD45A, MAPK2K7, MAPK10, PIK3CD, PIK3R1, PIK3R3	**DUSP8, HRAS,** CDC42, CRKL, DUSP4, GADD45A, MAPK2K7, MAPK10, PIK3CD, PIK3R1, PIK3R3
ERK/MAPK	**DUSP6, FOS, HSB2, PRKCB1, CREB1, PIK3R2, PPP1CB, STAT1, YMHAZ,** BAD, DUSP4, DUSP9, ELK1, PIK3CD, PIK3R1, PIK3R3, PPP2R5B, PPP2R5D, RAPGEF1, SRC, TLN2	**HRAS, PRKCG, MYCN,** BAD, DUSP4, DUSP6, ELK1, PIK3CD, PIK3R1, PPP2R5B, PPP2R5D, RAPGEF1, SRC, TLK2

Genes uniquely modulated by either R5 or X4 gp120s are listed in bold. JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase.

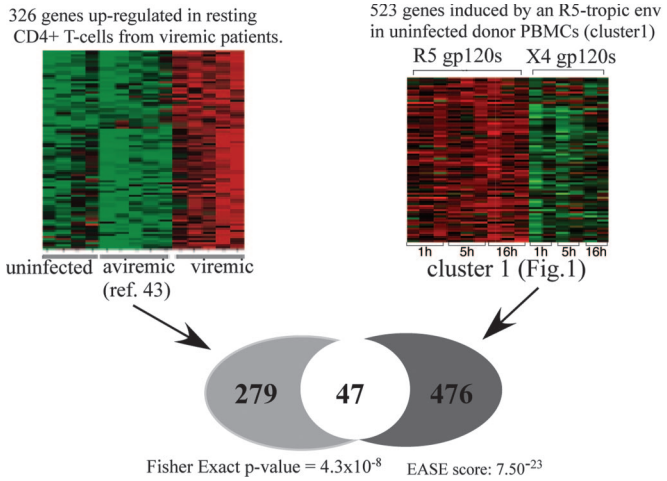

Fig. 6. Genes up-regulated *in vivo* in resting CD4$^+$ T cells of viremic individuals have significant overlap with the genes induced by R5 gp120s *in vitro* (cluster 1). A heat map of genes up-regulated in resting CD4$^+$ T cells from HIV-infected viremic individuals (from ref. 29) are presented along with the heat map of genes induced *in vitro* by R5 gp120s (from Fig. 1, cluster 1). A Venn diagram indicating the extent of gene overlap between the two data sets is included. A Fisher exact test and an EASE score representing the probability that this overlap occurred by chance are provided. See Table 3 for additional information.

throughout the course of disease, but, in general, these viruses only predominate late in disease and then only in a minority of infected individuals (28). The basis for this pattern is unclear. Considering that viral tropism is a property intrinsic to gp120, we carried out a comparative analysis of the impact of R5 and X4 envelope-mediated signal transduction on the transcriptional program of PBMCs. Although the signaling properties of R5 and X4 envelopes have been studied and compared (6–8), the impact of R5 vs. X4 envelopes on the metabolic state of PBMCs is poorly understood. We evaluated changes in the transcriptional program of PBMCs in response to treatment with recombinant envelope proteins derived from three major subgroups (A, B, and C), including both R5 and X4 envelopes. We found that the response of PBMCs was markedly influenced by the coreceptor tropism of the envelope with which they were treated.

We extended this analysis by treating PBMCs derived from individuals homozygous for the CCR5Δ32. Few of the genes uniquely induced by R5 gp120s in wild-type PBMCs were up-regulated in CCR5Δ32 PBMCs, suggesting that envelope signaling through CD4 alone induces few transcriptional changes in common with those elicited by the dual engagement of CD4 and CCR5. Comparing all of the genes modulated by R5 gp120 in CCR5Δ32 PBMCs with all of the genes modulated by R5 gp120 in wild-type PBMCs, we did not observe any group of commonly modulated genes, underscoring the unique response of cells to the near simultaneous stimulus delivered by R5 envelopes through CD4 and CCR5.

Table 3. The four most significant biological categories enriched in the group of overlapping genes and their EASE scores

Descriptive term	Gene symbol	EASE score
Ser/Thr kinase	Weel, LIMK1, SMG1, SRP72, RIOK3, PRPF4B, CAMK2G	4.2 e^{-4}
DNA repair	ERCC5, RAD51C, PMAS2L3, MSH6	2.9 e^{-3}
Transcription	CCNC, SP100, SIM2, MDS1, TAF11, YY1, PMS2L3, TIAL1, ATF2, JMJD2C, NCOA3, ZNF339, RUNX1	6.7 e^{-3}
Cell cycle	CCNC, PMS2L3, RIOK3, MSH6, MPHOSPH9, Weel, CAMK2G	1.1 e^{-2}

We identified functional categories associated with the genes uniquely induced by either R5 or X4 envelopes. Both types of envelopes significantly impacted genes associated with cell proliferation and protein tyrosine kinases, consistent with our previous demonstration that envelopes can induce viral replication in resting cells (19). However, R5 envelopes were more pronounced in their capacity to modulate the p38 MAPK cascade. The expression of p38 MAPK has been shown to positively correlate with HIV replication (37) and inhibitors of p38 kinase inhibit viral replication (37, 38). Thus, the more pronounced activation of genes included in the p38 signaling cascade identifies one potential mechanism that favors R5 viral replication.

Resting CD4$^+$ T cells play a critical role in two key phases of HIV infection. They are one of the earliest targets of productive infection during the initial phases of transmission (39). Additionally, they serve as one of the long lived reservoirs that harbors latent virus. We have carried out an extensive characterization of the CD4$^+$ T cell reservoir (40), including a gene-profiling analysis (29), in which we reported that the transcription program of resting CD4$^+$ T cells of individuals with measurable viremia is markedly different from that of either aviremic or uninfected individuals. Considering that the frequency of infected cells within the population of resting CD4$^+$ T cells is $<0.1\%$, the differences in gene expression between CD4$^+$ T cells of viremic individuals vs. aviremic or uninfected individuals were likely the result of indirect effects associated with ongoing immune responses in the viremic patients. Additionally, viral gene products, including envelope protein, may have contributed to the alterations in the transcriptional program that we observed. We asked whether the envelope-modulated transcription program in PBMCs from uninfected individuals shared common genes with the altered transcription program identified in the resting CD4$^+$ T cell reservoir of HIV-infected viremic individuals. Of the 326 genes specifically up-regulated in the resting CD4$^+$ T cell population derived from viremic individuals, 47 were shared with the set of genes modulated exclusively by R5 envelopes ($P < 0.001$). No significant overlap was observed with any of the four other gene clusters we identified. Considering that the persistent viremia in these patients drives chronic activation of the immune system, it is possible that the effect of that activation on resting CD4$^+$ T cells is similar, to a degree, to envelope-mediated stimulation of these cells. Alternatively, we are observing a direct response to envelope in viremic individuals. If this latter scenario is correct, it raises the possibility that envelope engagement of resting CD4$^+$ T cells promotes low level viral replication from this reservoir. As previously noted, we have demonstrated that envelope possesses this capacity to induce virus replication *in vitro* in PBMCs from HIV-infected individuals (19).

Finally, evidence derived from simian immunodeficiency virus (SIV)-infected macaques suggests that the very first CD4$^+$ T cells productively infected after transmission across mucosal surfaces are resting cells, as defined by the absence of cell-surface activation markers (39). Haase refers to these cells, however, as "ostensibly resting," because true resting cells lack the requisite nucleotide pools and activated transcription factors necessary to produce virus. The data presented herein, along with our previous studies, raises the possibility that by creating a metabolic state that is more conducive to viral replication in resting cells, envelope signaling may facilitate viral transmission. In other words, the ability of R5 envelope to effectively trigger the activation of genes involved in promoting viral replication may be critically important to the strong bias toward R5 viruses in the initial transmission of HIV.

In summary, we have demonstrated that HIV envelopes induce transcription programs in PBMCs that are strongly influenced by the coreceptor tropism of the envelope used. Both R5 and X4 envelopes induce factors relevant to viral replication, including the

expression of genes involved in cell proliferation, the cell cycle, transcription factors, and MAPKs. However, within each of these categories, R5 and X4 envelopes induced different subsets of genes. These differences may influence the susceptibility of $CD4^+$ T cells to productive infection by R5 vs. X4 virus. Genes induced by R5 envelopes, in particular, may facilitate replication of virus in resting $CD4^+$ T cells, and as such, R5 envelope-mediated signaling may contribute to the establishment and/or maintenance of viral reservoirs, and the productive infection of resting cells at mucosal surfaces.

Methods

Cells and Reagents. PBMCs were obtained by apheresis from normal volunteers. Cells were resuspended in RPMI medium 1640/10% FBS. PBMCs expressing CCR5Δ32 alleles were not used unless specified. Expression and purification of gp120 was performed as described in ref. 41. Cell lines were cultured in hollow fiber cartridges (FiberCell Systems, Frederick, MD). Proteins were determined to be endotoxin-free by using a Limulus amoebocyte assay (BioWhittaker). A mock protein prepared in an identical manner was derived from untransfected CHO cells.

Exposure of PBMCs to gp120. Freshly isolated PBMCs (5×10^7) were incubated in 10% FBS/RPMI medium 1640 at 37°C with gp120 or mock protein (50 ng of gp120 per 10^6 cells) for times specified. Donor treatments are listed in Table 4, which is published as supporting information on the PNAS web site.

Preparation of cRNA for Oligonucleotide Arrays. cRNA was prepared as described in refs. 13 and 14. Total RNA from 5×10^7 cells per time point was extracted by using TRIzol (Life Technologies, Carlsbad, CA). cRNA was generated from 15 μg of RNA by using the Superscript Choice (Life Technologies) followed by *in vitro* transcription (Enzo Diagnostics) with biotin-labeled dNTPs. cRNAs were then prepared for hybridization to Human Genome U95A chips according to manufacturer's instructions (Affymetrix, Santa Clara, CA).

Transcription Factor Blot Array. To obtain nuclear lysates, PBMCs were washed with cold PBS and lysed by using NE-PER Nuclear Extraction Reagents (Pierce). Protein concentrations were determined by the bicinchoninic assay (Pierce). Blot arrays were hybridized according to the manufacturer's instructions (Panomics, Redwood City, CA), followed by chemiluminescent visualization.

Microarray Statistical Analysis and Functional Annotation of Genes. Image processing was performed on a total of 101 Affymetrix U95A arrays from a total of 10 donors by using MICROARRAY SUITE 5.0 software. Sample patient information can be found in Table 4. To determine significant differences in gene expression levels between the three time points, the two envelope tropisms, the five envelope types, and the CCR5 mutation status, log base 2 gene expression measurements for each gene on each array were modeled by using a multifactor mixed-model nested ANOVA by using the SAS statistical package. Expression values <20 were truncated to 20. Significantly modulated genes were defined as those with absolute fold change >1.5 and an ANOVA P value <0.01. Approximately 2,000 genes were selected. t scores, relative to mock treatment, were used for K-means clustering to group genes and hierarchical clustering to group envelope responses at each time point by using PARTEK DISCOVERY SUITE software. Functional annotation and biological term enrichment analysis was performed by using the DAVID knowledge base (31) and INGENUITY PATHWAYS ANALYSIS 3.0 software.

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1. Alkhatib, G., Broder, C. C. & Berger, E. A. (1996) *J. Virol.* **70,** 5487–5494.
2. Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R. E., Hill, C. M., *et al.* (1996) *Nature* **381,** 661–666.
3. Dragic, T., Litwin, V., Allaway, G. P., Martin, S. R., Huang, Y., Nagashima, K. A., Cayanan, C., Maddon, P. J., Koup, R. A., Moore, J. P. & Paxton, W. A. (1996) *Nature* **381,** 667–673.
4. Feng, Y., Broder, C. C., Kennedy, P. E. & Berger, E. A. (1996) *Science* **272,** 872–877.
5. Juszczak, R. J., Turchin, H., Truneh, A., Culp, J. & Kassis, S. (1991) *J. Biol. Chem.* **266,** 11176–11183.
6. Freedman, B. D., Liu, Q. H., Del Corno, M. & Collman, R. G. (2003) *Immunol. Res.* **27,** 261–276.
7. Lee, C., Liu, Q. H., Tomkowicz, B., Yi, Y., Freedman, B. D. & Collman, R. G. (2003) *J. Leukocyte Biol.* **74,** 676–682.
8. Stantchev, T. S. & Broder, C. C. (2001) *Cytokine Growth Factor Rev.* **12,** 219–243.
9. Weissman, D., Rabin, R. L., Arthos, J., Rubbert, A., Dybul, M., Swofford, R., Venkatesan, S., Farber, J. M. & Fauci, A. S. (1997) *Nature* **389,** 981–985.
10. Cicala, C., Arthos, J., Ruiz, M., Vaccarezza, M., Rubbert, A., Riva, A., Wildt, K., Cohen, O. & Fauci, A. S. (1999) *J. Immunol.* **163,** 420–426.
11. Davis, C. B., Dikic, I., Unutmaz, D., Hill, C. M., Arthos, J., Siani, M. A., Thompson, D. A., Schlessinger, J. & Littman, D. R. (1997) *J. Exp. Med.* **186,** 1793–1798.
12. Alimonti, J. B., Ball, T. B. & Fowke, K. R. (2003) *J. Gen. Virol.* **84,** 1649–1661.
13. Arthos, J., Cicala, C., Selig, S. M., White, A. A., Ravindranath, H. M., Van Ryk, D., Steenbeke, T. D., Machado, E., Khazanie, P., Hanback, M. S., *et al.* (2002) *Virology* **292,** 98–106.
14. Cicala, C., Arthos, J., Selig, S. M., Dennis, G., Jr., Hosack, D. A., Van Ryk, D., Spangler, M. L., Steenbeke, T. D., Khazanie, P., Gupta, N., *et al.* (2002) *Proc. Natl. Acad. Sci. USA* **99,** 9380–9385.
15. Banda, N. K., Bernier, J., Kurahara, D. K., Kurrle, R., Haigwood, N., Sekaly, R. P. & Finkel, T. H. (1992) *J. Exp. Med.* **176,** 1099–1106.
16. Douek, D. C., Picker, L. J. & Koup, R. A. (2003) *Annu. Rev. Immunol.* **21,** 265–304.
17. Finzi, D., Blankson, J., Siliciano, J. D., Margolick, J. B., Chadwick, K., Pierson, T., Smith, K., Lisziewicz, J., Lori, F., Flexner, C., *et al.* (1999) *Nat. Med.* **5,** 512–517.
18. Siliciano, R. F. (1999) *Curr. Infect. Dis. Rep.* **1,** 298–304.
19. Kinter, A. L., Umscheid, C. A., Arthos, J., Cicala, C., Lin, Y., Jackson, R., Donoghue, E., Ehler, L., Adelsberger, J., Rabin, R. L. & Fauci, A. S. (2003) *J. Immunol.* **170,** 2449–2455.
20. Pessler, F. & Cron, R. Q. (2004) *Genes Immun.* **5,** 158–167.
21. Cron, R. Q., Bartz, S. R., Clausell, A., Bort, S. J., Klebanoff, S. J. & Lewis, D. B. (2000) *Clin. Immunol.* **94,** 179–191.
22. Kinoshita, S., Chen, B. K., Kaneshima, H. & Nolan, G. P. (1998) *Cell* **95,** 595–604.
23. Cicala, C., Arthos, J., Censoplano, N., Cruz, C., Chung, E., Martinelli, E., Lempicki, R. A., Natarajan, V., Vanryk, D., Daucher, M. & Fauci, A. S. (2006) *Virology* **345,** 105–114.
24. Jensen, M. A., Li, F. S., van 't Wout, A. B., Nickle, D. C., Shriner, D., He, H. X., McLaughlin, S., Shankarappa, R., Margolick, J. B. & Mullins, J. I. (2003) *J. Virol.* **77,** 13376–13388.
25. van 't Wout, A. B., Lehrman, G. K., Mikheeva, S. A., O'Keeffe, G. C., Katze, M. G., Bumgarner, R. E., Geiss, G. K. & Mullins, J. I. (2003) *J. Virol.* **77,** 1392–1402.
26. Hartley, O., Klasse, P. J., Sattentau, Q. J. & Moore, J. P. (2005) *AIDS Res. Hum. Retroviruses* **21,** 171–189.
27. Moore, J. P., Kitchen, S. G., Pugach, P. & Zack, J. A. (2004) *AIDS Res. Hum. Retroviruses* **20,** 111–126.
28. Philpott, S. M. (2003) *Curr. HIV Res.* **1,** 217–227.
29. Chun, T. W., Justement, J. S., Lempicki, R. A., Yang, J., Dennis, G., Jr., Hallahan, C. W., Sanford, C., Pandya, P., Liu, S., McLaughlin, M., *et al.* (2003) *Proc. Natl. Acad. Sci. USA* **100,** 1908–1913.
30. Dennis, G., Jr., Sherman, B. T., Hosack, D. A., Yang, J., Gao, W., Lane, H. C. & Lempicki, R. A. (2003) *Genome Biol.* **4,** R60.
31. Yang, Z. & Engel, J. D. (1993) *Nucleic Acids Res.* **21,** 2831–2836.
32. Berenson, L. S., Ota, N. & Murphy, K. M. (2004) *Immunol. Rev.* **202,** 157–174.
33. Popik, W., Hesselgesser, J. E. & Pitha, P. M. (1998) *J. Virol.* **72,** 6406–6413.
34. Popik, W. & Pitha, P. M. (2000) *J. Virol.* **74,** 2558–2566.
35. Dong, C., Davis, R. J. & Flavell, R. A. (2002) *Annu. Rev. Immunol.* **20,** 55–72.
36. Zhu, T., Mo, H., Wang, N., Nam, D. S., Cao, Y., Koup, R. A. & Ho, D. D. (1993) *Science* **261,** 1179–1181.
37. Shapiro, L., Heidenreich, K. A., Meintzer, M. K. & Dinarello, C. A. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 7422–7426.
38. Muthumani, K., Wadsworth, S. A., Dayes, N. S., Hwang, D. S., Choo, A. Y., Abeysinghe, H. R., Siekierka, J. J. & Weiner, D. B. (2004) *AIDS* **18,** 739–748.
39. Haase, A. T. (2005) *Nat. Rev. Immunol.* **5,** 783–792.
40. Chun, T. W. & Fauci, A. S. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 10958–10961.
41. Mossman, S. P., Bex, F., Berglund, P., Arthos, J., O'Neil, S. P., Riley, D., Maul, D. H., Bruck, C., Momin, P., Burny, A., *et al.* (1996) *J. Virol.* **70,** 1953–1960.