Regulation of CD4 Expression via Recycling by HRES-1/RAB4 Controls Susceptibility to HIV Infection*

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A novel 2986-base transcript encoded by the antisense strand of the HRES-1 human endogenous retrovirus was isolated from peripheral blood lymphocytes. This transcript codes for a 218-amino acid protein, termed HRES-1/Rab4, based on homology to the Rab4 family of small GTPases. Antibody 13407 raised against recombinant HRES-1/Rab4 detected a native protein of identical molecular weight in human T cells. HRES-1 nucleotides 2151–1606, located upstream of HRES-1/Rab4 exon 1, have promoter activity when oriented in the direction of HRES-1/Rab4 transcription. The human immunodeficiency virus, type 1 (HIV-1) tat gene stimulates transcriptional activity of the HRES-1/Rab4 promoter via trans-activation of the HRES-1 long terminal repeat. Transfection of HIV-1 tat into HeLa cells or infection of H9 and Jurkat cells by HIV-1 increased HRES-1/Rab4 protein levels. Overexpression of HIV-1/Rab4 in Jurkat cells abrogated HIV infection, gag p24 production, and apoptosis, whereas dominant-negative HRES-1/Rab4S27N had the opposite effects. HRES-1/Rab4 inhibited surface expression of CD4 and targeted it for lysosomal degradation. HRES-1/Rab4S27N enhanced surface expression, recycling, and total cellular CD4 content. Infection by HIV elicited a coordinate down-regulation of CD4 and up-regulation of HRES-1/Rab4 in PBL. Moreover, overexpression of HRES-1/Rab4 reduced CD4 expression on peripheral blood CD4+ T cells. Stimulation by HIV-1 of HRES-1/Rab4 expression and its regulation of CD4 recycling reveal novel coordinate interactions between an infectious retrovirus and the human genome.

Endogenous retroviruses (ERV)³ belong to the larger family of retrotransposable elements that make up as much as 40% of the human genome (1). Human ERV (HERV) have the basic structures of the integrated proviral form of infectious retroviruses with long terminal repeats (LTRs) flanking sequences homologous to gag, pol, and env genes (2). Human ERV have generally been found to be defective proviruses having accumulated deletions or stop codons in gag, pol, and/or env open reading frames (3). Human ERV are commonly designated as HERV followed by a single letter amino acid code corresponding to a tRNA. The 3’ terminus of tRNA is predicted to initiate reverse transcription by annealing to an 18-nucleotide-long primer-binding site (PBS) at the 5’-LTR. ERV copy numbers vary from one to several hundred per haploid genome (4).

ERV may represent a key molecular link between the host genome and infectious viral particles. They constitute a large reservoir of viral genes that may be activated by mutations caused by radiation or chemicals or recombination with exogenous retroviruses. Although exogenous retroviruses are infectious, with a replication cycle that requires integration of proviral DNA into host cell DNA, ERV are transmitted genetically in a classical Mendelian fashion through the germ line as proviral DNA. Expression of ERV can influence the outcome of infections in different ways that are both beneficial and detrimental to the host (2). These include provision of genes for recombination with exogenous viruses, interference with virion assembly, modulation of immune responses to exogenous viruses, and blocking cellular receptors for viral entry (5).

Human immunodeficiency virus, type 1 (HIV-1), uses two receptors for cellular attachment and viral entry. Initial viral attachment occurs through the binding of the envelope protein gp120 to the CD4 molecule expressed on the surface of T lymphocytes and macrophages. Viral binding to CD4 is necessary but insufficient to mediate viral entry. Interaction between CD4 and gp120 increases the affinity of virions for coreceptor molecules CXCR4 and CCR5. Genetic polymorphisms or deletions within CCR5 diminish or abrogate viral binding to the receptor, which leads to a lower susceptibility to infection and slower disease progression in persons carrying these mutations (6). CXCR4-using viruses are generally more pathogenic, via depletion of CD4+T cells, than are CCR5-using viruses. CD4 appears repeat; GST, glutathione S-transferase; GFP, green fluorescent protein; PDBu, phorbol 12,13-dibutyrate; HBSS, Hanks’ balanced salt solution; IRES, internal ribosome entry site; PBS, primer-binding site; TFR, transferrin receptor; PE, phycoerythrin; CAT, chloramphenicol acetyltransferase; PBMC, peripheral blood mononuclear cells; Ab, antibody.

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§ The abbreviations used are: ERV, endogenous retroviruses; HERV, human ERV; HIV, human immunodeficiency virus; HIV-1, human immunodeficiency virus, type 1; PBL, peripheral blood lymphocytes; LTR, long terminal repeat; GST, glutathione S-transferase; GFP, green fluorescent protein; PDBu, phorbol 12, 13-dibutyrate; HBSS, Hanks’ balanced salt solution; IRES, internal ribosome entry site; PBS, primer-binding site; TFR, transferrin receptor; PE, phycoerythrin; CAT, chloramphenicol acetyltransferase; PBMC, peripheral blood mononuclear cells; Ab, antibody.
to play a role in HIV entry distinct from merely serving as the attachment protein for the virus. CD4 undergoes endocytosis following T cell activation via the activation of protein kinase C and subsequent phosphorylation of CD4 (7, 8). HIV binding was also reported to induce phosphorylation of CD4 via a protein kinase C-dependent pathway; however, internalization of HIV does not require endocytosis of CD4 (9, 10).

In this study, we investigated potential interactions between a newly identified gene product of the human T-cell leukemia virus type I-related endogenous retroviral sequence, HRES-1, and HIV-1. HRES-1 was previously isolated based on homology to the long terminal repeat and \textit{gag} regions of human T cell leukemia virus type I (11). Hybridization analysis with genomic DNA samples of selected phylogenetic stages revealed that HRES-1 was confined to the primate lineage (11). HRES-1 is a single copy sequence in the haploid genome that has been mapped to 1q42 at the long arm of chromosome 1 (12). A 684-bp flanking region 5' to p28 contains a TATA box, a polyadenylation signal, a potential histidyl tRNA primer-binding site (PBS), and characteristic inverted repeat sequences at locations that are typical of a retroviral LTR (2, 11). HRES-1 is one of the few human ERV that remain transcriptionally active (3, 11). By utilizing HRES-1-derived probes, a novel 2986-base-long transcript encoded by the antisense strand of the HRES-1 locus was isolated from human peripheral blood lymphocytes (PBL). The sequence of this cDNA showed considerable homology to the 735-base-long \textit{Rab4a} gene and is thus termed HRES-1/\textit{Rab4}. Antibody raised against recombinant HRES-1/\textit{Rab4} detected a native protein of identical molecular weight in PBL and Jurkat and H9 T cell lines. The first coding exon of HRES-1/\textit{Rab4} is embedded in the HRES-1 endogenous retroviral sequence. HRES-1 nucleotides 2151–1606, located upstream from HRES-1/\textit{Rab4} exon 1, showed strong promoter activity when oriented in the direction of HRES-1/\textit{Rab4} transcription. The \textit{tat} gene of HIV-1 stimulates transcriptional activity of the HRES-1/\textit{Rab4} promoter via trans-activation of the HRES-1 LTR and increases HRES-1/\textit{Rab4} protein levels. In turn, HRES-1/\textit{Rab4} regulates recycling and surface expression of CD4 and thus controls susceptibility to infection by HIV-1.

**MATERIALS AND METHODS**

**Northern Blot Analysis**—Total RNA was extracted from Jurkat cells, fractionated in 1% glyoxal gels, transferred to nylon mem-
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A

HRES-1/Rab4 cDNA

Exon1
1  CGGACGGAGG GTGAGGAGCC CTGGGCTGGC GCGGGCTTG GAGTCGGCTG
51  GCCGCCGAGC GCCGCTTGGC GCCGTGGGACC GGTCGGGCC
101  CTCCTCCTCG CGCGCGGCGG CGCCAGGCTGTT CCGCGCTGCT
151  GCCGCCGAGC GCCGCTTGGC GCCGTGGGACC GGTCGGGCC
Exon2
201  TCCCAAGAGCT TCCCAAGAGCT TCCCAAGAGCT TCCCAAGAGCT TCCCAAGAGCT
251  AGTTCTCGTG TATTTGAAAAC GCTTACAGCTG GCTTACAGCTG GCTTACAGCTG
301  CAGTTTTGTTG AAAAAAAGTT CAACAGATGAC TCAAATCATG CAAATAGAT
351  GGAATTGTTCT GAACAGATGAC TCAAATCATG CAAATAGAT
Exon3
401  AAATATGGGA TACACAGAGA GAAAGAAGAT CTTAATGGGT
451  TATTTTGGAG GCGCGCGGGCT GCGCGCGGGCT GCGCGCGGGCT
501  AGAAAGCTCTA AAACCTGATCA CTCATATAGC GAAAGAAGAT
551  CGAGCTCGAGA TATACCTGAGA TATACCTGAGA TATACCTGAGA
601  GCAGATCGTG GAGTTATAGT CTTAAGGAA AAAAAAAAAT
651  TGACCGCTGAG TTTTTGGAAC CACGCGCTGAC CACGCGCTGAC
Exon4
701  AGGCTTTTGTG ACAGCTCGAGA GAAAGAAGAT CTTAATGGGT
751  GACGCTCGAGA GAAAGAAGAT CTTAATGGGT
801  AGGCTTTTGTG ACAGCTCGAGA GAAAGAAGAT CTTAATGGGT
851  AGGCTTTTGTG ACAGCTCGAGA GAAAGAAGAT CTTAATGGGT
901  CACATTCGTT GAACAGATGAC TCAAATCATG CAAATAGAT
951  TTCTACTCGTG TATTTTGGAAC CACGCGCTGAC CACGCGCTGAC
Exon5
1001  GTGCGCGCGA CCAGCTCGAGA GGCGCGCGA CCAGCTCGAGA
1051  ATGGCAACACA CGGCTTGAGT TGGATGTAAC AAAAAAAAAT
1101  TCCTTCTCGTG TGGATGTAAC AAAAAAAAAT
1151  CCGACAGCTG CGGCTTGAGT TGGATGTAAC AAAAAAAAAT
1201  GGGACTACTAGT CGGCTTGAGT TTCTGAACTAGTTTGAT
1251  ATTTAAAAAAC AGTATTTTTT TTCAAGTTTT
1301  AATTAAAAAAC AGTATTTTTT TTCAAGTTTT
1351  ATGTTGTTTTT TTCAAGTTTT
1401  GTGATCCACA CTTGAAATAC TAGTACGTCG GACATTCGTC GATTTTTT
1451  AATTAAAAAAC AGTATTTTTT TTCAAGTTTT
1501  GTTGGTTTTT TTCAAGTTTT
1551  TACACTCGTG CTTGAAATAC TAGTACGTCG GACATTCGTC GATTTTTT
1601  TTCTCCTCCTT TGGGACTACTAGT CGGCTTGAGT TTCTGAACTAGTTTGAT
1651  TAGTTGTTTTT TTCAAGTTTT
1701  TCTTACTCGTG CTTGAAATAC TAGTACGTCG GACATTCGTC GATTTTTT
1751  AATTAAAAAAC AGTATTTTTT TTCAAGTTTT
1801  GGGAACTCGTG CTTGAAATAC TAGTACGTCG GACATTCGTC GATTTTTT

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branes, and hybridized to $^{32}$P-labeled human β-actin (13) or HRES-1-derived fragments, as described earlier (11). Hybridization and washing were done under high stringency conditions.

**Screening of Expression Library and Cloning and Sequencing of cDNA**—A human lymphocyte cDNA library prepared in Agt11 phage (Stratagene, La Jolla, CA) was screened with HRES-1 probes under high stringency conditions, as described earlier (14). Positive clones were transferred into the EcoRI site of Bluescript KS+ plasmid (Stratagene) and sequenced in both strands. Nucleotide sequence of the 2986-base-long HRES-1/Rab4 cDNA was submitted to GenBank™ (accession number AY585832).

**Prokaryotic Expression of Recombinant Protein**—Full-length HRES-1/Rab4 protein was expressed as a fusion protein with glutathione S-transferase (GST), as described earlier (15). BamHI and XhoI sites were generated by PCR-mediated mutagenesis immediately 5' of the first methionine codon and 3' of the stop codon of HRES1/Rab4 and cloned into BamHI- and XhoI-cleaved pGEX-6P1 plasmid vector (Amersham Biosciences). Optimum stimulation of expression of the recombinant fusion protein was obtained with 1 mM isopropyl thio-$\beta$-galactoside after 2 h. HRES1/Rab4-GST fusion protein was affinity-purified through binding of GST to glutathione-coated agarose beads (Sigma). HRES-1/Rab4 protein was cleaved from GST by precision protease (Amersham Biosciences) and was separated from the agarose bead-bound GST by centrifugation.

**Construction and Transfection of Eukaryotic Expression Vectors**—HRES-1/Rab4 was overexpressed in Jurkat cells using a doxycycline-inducible GFP-encoding bi-cistronic expression vector system (16). Jurkat cells were maintained in RPMI 1640 medium containing 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 μM l-glutamine, transfected by electroporation with pUHD172–1neo plasmid (kindly provided by Dr. Hermann Bujard, University of Heidelberg) (17), and selected for neomycin resistance in the presence of 1 mg/ml G418.
HRES-1/Rab4 Regulates CD4 Expression

A

HRES-1/Rab4
151  CCGAACCAGGCGGCGCAATCACGGGGGAGGCGAAGGCGGGGCC 200

Rab4a
1  .CGAACCAGGCGGCGCAATCACGGGGGAGGCGAAGGCGGGGCC 47

B

HRES-1/Rab4
1  MSQTAMESETDFPLFKLVLVGNAGTGKSCLLLHQPFEKKFKDSNHITGVEP 50

Rab4a
1  .MSQTAMESETDFPLFKLVLVGNAGTGKSCLLLHQPFEKKFKDSNHITGVEP 45
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Jur-TA cells are neomycin (G418)-resistant Jurkat cells transfected with rtTA-containing pUHD172–1neo and thus produce the reverse trans-acting factor (rtTA) capable of interacting with promoters harboring tetracycline operator sequences only in the presence of tetracycline analogs. Jur-TA cells were electroporated with GFP-producing pTR5-DC/GFP*TK/hygcontrol (construct 4480), wild-type (construct 6678), and dominant-negative mutant HRES-1/Rab4S27N-containing vector (construct 9035); and doubly transfected cells were selected in 400 µg/ml G418 and 200 µg/ml hygromycin. Percentage of GFP-positive cells in an aliquot of the transfected cultures was periodically checked by flow cytometry. After 24 h of incubation with 1 µg/ml doxycycline, which did not affect cell viability, the most brightly fluorescent 1% of cells was sorted using a flow cytometer and subsequently maintained in the presence of 200 µg/ml G418 and 100 µg/ml hygromycin. Bulk-sorted aliquots of 4480, 6678, and 9035 construct-transfected cells were utilized in all experiments. Transfected cells were not cloned to avoid artifacts related to variations in sites of vector integration. Control and HIV-1 tat gene-transfected HeLa and Jurkat cells (18) were obtained from the NIH AIDS Research and Reference Program.

Transduction of HRES-1/Rab4 by Adeno-associated Virus Vector (pAAV-IREs-hrGFP)—The HRES-1/Rab4 cDNA was inserted upstream of the internal ribosomal entry site of the pAAV-IREs-hrGFP vector (Stratagene, La Jolla, CA). HRES-1/Rab4-expressing adeno-associated virus was produced by transfection of HEK293 cells with HRES-1/Rab4-containing pAAV-IREs-hrGFP, pAAV-RC, and pHelper plasmids, which supply the necessary exogenous gene products for virus production (Stratagene). At 1:1 multiplicity of infection, >99% of cells infected with pAAV-HRES-1/Rab4-IREs-hrGFP, pAAV-HRES-1/Rab4S27N-IREs-hrGFP, or pAAV-IREs-hrGFP control virus were GFP-positive. Maximal GFP expression was observed 24 h after virus infection. GFP and HRES-1/Rab4 expressions were diminished by 50% after 48 h and became undetectable 5 days post-infection.

Site-directed Mutagenesis—Dominant-negative HRES-1/Rab4 was created by replacing serine 27 with asparagine (S27N) using the Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, California). Briefly, 25 ng of the wild-type HRES-1/Rab4 cDNA plasmid (template) was incubated with 125 ng of sense (5′-GCAGGAACCTGGCAAAAT-TGCCCTACTTCTCATCAG-3′) and antisense primers (5′-CTGATGAGCATAATTGCGAGTTCTTCTGC-3′) with mutagenic residues underlined) and dNTPs and subjected to 18 PCR cycles with Pfu Turbo DNA polymerase with denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 68 °C for 12 min. DpnI was used to digest the parental supercoiled double-stranded methylated DNA for 1 h at 37 °C. Transformations were performed in Escherichia coli XL-1 Blue cells using DpnI-treated DNA. Mutagenesis was confirmed by sequencing of the resultant plasmids.

Transient Transfections and Reporter Gene Assays—HeLa cells were transfected with 1.6 µg of pBLCAT3–HRES-1 promoter chloramphenicol acetyltransferase (CAT) reporter gene constructs (19) at 80% confluency in 9.5-cm² wells using 20 µl of PLUS reagent and 4.8 µl of Lipofectamine reagent (Invitrogen). To normalize transfection efficiency, each plasmid was cotransfected with 1.6 µg of the pRSVβ-gal plasmid (β-galactosidase reporter gene driven by the Rous sarcoma virus promoter) (20). The HRES-1/Rab4-containing plasmids did not affect promoter activity of pRSVβ-gal, as determined by the amount of transfected cell lysate used for CAT assays. For each experiment, the promoterless pBLCAT3 vector was used as a negative control, and pRSV-CAT was used as positive control. After 4 h of exposure to the DNA/Lipofectamine complex in serum- and antibiotic-free media, the DNA complex was removed, and cells were further cultured for an additional 36 h in complete growth media. Cells were harvested in 150 µl of 250 mM Tris, pH 7.8, and solubilized by three rounds of freezing and thawing. For the β-galactosidase assay, 30 µl of lysate was incubated with 270 µl of reaction mixture (1 mM MgCl₂, 50 mM β-mercaptoethanol, 3 mM o-nitrophenyl β-d-galactopyranosidase, and 0.1 mM NaN₃, pH 7.5, at 37 °C) and terminated with addition of 500 µl of 1 M Na₂CO₃ once the reaction turned yellow. Absorbance values were measured at 420 nm and used...
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FIGURE 4. The HRES-1/Rab4 promoter lies within nucleotides 1601–2151. A, detection of HRES-1/Rab4 promoter activity using CAT reporter gene assays. Left panel, physical maps of pBLCAT3-based constructs containing upstream (nucleotides 1606–2151) and HRES-1 LTR internal promoter DNA segments (nucleotides 1–1200). Nucleotide positions of promoter DNA sequences correspond to the HRES-1 sequence (GenBank™ accession number X16660). EagI (E), HindIII (H), and SstII (S) restriction sites within the HRES-1/Rab4 promoter sequence are shown as landmarks. Shaded boxes indicate promoter sequence in each construct. HRES-1/Rab4 promoter-CAT reporter constructs were transiently transfected into HeLa (middle panel) and HeLa tat cells (right panel), using pBLCAT3 and pRSVCAT as negative and positive controls, respectively, in all experiments. Each plasmid was cotransfected with the pRSVβ-gal to standardize transfection efficiency. Column diagrams represent the mean ± S.E. of at least six experiments. p values indicate significant differences in CAT activity relative to construct 6225. A representative transfection is shown for each cell type. B, Western blot analysis of HRES-1/Rab4 protein levels in HeLa, HeLa tat, H9, and HIV-infected H9 cells. Protein lysates from 2 × 106 cells per lane were separated in SDS-PAGE, blotted to nitrocellulose, and developed with antibodies to directed against HRES-1/Rab4 (SC 312), HIV-tat, actin, and transaldolase (TAL).

to calculate the quantity of each transfected cell lysate to be used in the CAT assay. Cell lysates were heated at 65 °C for 10 min to inactivate acetylases, and CAT assays were performed at 37 °C in 50 μl of reaction mixture of 250 mM Tris, pH 7.8, normalized cell lysate, 0.4 mM acetyl-coenzyme A, 0.025 μCi of [14C]chloramphenicol. Acetylated chloramphenicol was extracted with ethyl acetate, dried in a vacuum centrifuge, resuspended in ethyl acetate, spotted on a silica gel (Analtech, Newark, DE), and resolved in an equilibrated chromatography tank containing 19:1 chloroform/methanol. A 445 SI Phosphoimager with ImageQuant software (Amersham Biosciences) was used to determine the ratio of acetylated [14C]chloramphenicol. All assays were conducted within the range of linearity of CAT activities with respect to incubation time, based on the β-galactosidase assay (21).

Retroviral Proteins and Antibodies—Retroviral reagents were obtained from the AIDS Research and Reference Program, National Institutes of Health. Infectious stock of the strain HIV-1IIIb was harvested from 24-h supernatants of freshly infected H9 cells (ATCC CRL-8543), and infectious titer was determined by an in situ infectivity (MAGI) assay (22). Supernatants with titers of 2.1 × 105 infectious units (IU)/ml were filtered through a 0.45-μm filter, concentrated by ultrafiltration, and aliquots were stored at −70 °C. Infections were performed with cell-free virus supernatants containing 100 ng of p24 core protein measured by an enzyme-linked immunosorbent assay following the manufacturer’s recommendations (NEK-060, PerkinElmer Life Sciences), corresponding to 2.1 × 105 IU per 5 × 106 cells or a multiplicity of infection of 0.04. Thus, 1 ng of p24 core protein corresponded to 2000 infectious virions, in accordance with earlier findings (23). Virus infection was carried out in 1 ml of serum-free RPMI 1640 medium for 2 h in the presence of 10 μg/ml Polybrene (Sigma). Subsequently, cells were washed in serum-free RPMI and resuspended in 10 ml of fresh RPMI 1640 medium containing 20% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml amphotericin B, and 2 mM L-glutamine. HIV-1 SF2 p25/245 gag contained the gag 24 protein (24). HIV-1/IIIB Gag4 contained the p17 C terminus, beginning at amino acid position 146, all of p24, and the p15 N terminus (Repligen, Cambridge, MA). To monitor production of viral proteins, HIV-1 gag p24-specific polyclonal sheep antibody (25) and tat-specific rabbit antibody 705 (AIDS Research and Reference Program, National Institutes of Health) were utilized.

Separation of CD4+ T Cells from Human Peripheral Blood—PBMC were separated from peripheral blood (26), and 107 cells were incubated with 17.5 μl of anti-CD4 beads to isolate CD4+ T cells (catalog number 111.45, Dynal, Lake Success, NY). After stimulation with 5 μg/ml concanavalin A and 200 units/ml interleukin-2 (Sigma) for 3 days, beads were removed by washing in PBS with 2% fetal calf serum over a Dynal magnet, and 107 CD4+ T cells (≥98% pure) were infected with HIV-1, as described above.

Monitoring of Cell Survival by Flow Cytometry—HIV-induced apoptosis was assessed by observing cell shrinkage and quantified by flow cytometry after staining with Cy-5-conjugated annexin V (Ann V-Cy5; Bio Vision, Mountain View, CA) in 10 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl2, as described earlier (26, 27). Staining with Ann V-Cy5 (FL-5) was used to monitor cell viability in parallel with assessing expression of GFP (FL-1) and surface antigens with PE-conjugated CD4 (FL-2), PE-conjugated fusin/CXCR4 (FL-4), and PE- or PE-Cy5-conjugated transferrin receptor (TFR/CD71) antibodies (FL-3).

Receptor Recycling—Internalization of surface receptors was induced by treatment with 100 nM phorbol 12,13-dibutyrate (PDBu) for 1 h at 37 °C (7, 8). Subsequently, cells were washed twice and kept at 4 °C to assess internalization or returned to 37 °C to allow receptor recycling in the absence or presence of
doxycycline. After termination of recycling, cells were kept on ice until analysis of surface expression by flow cytometry.

**Permeabilization of Cells for Detection of Intracellular Antigen by Flow Cytometry**—10^6 Jurkat cells were centrifuged, resuspended in Hank’s balanced salt solution (HBSS, Cellgro) containing 4% paraformaldehyde, and incubated for 10 min at room temperature. After centrifugation, cells were resuspended in 500 μl of HBSS with 0.1% saponin, 10 mM HEPES, pH 7.4, and 1% fetal calf serum and incubated for 10 min at room temperature. After centrifugation, cells were resuspended in 100 μl of HBSS with 0.1% saponin, 10 mM HEPES, pH 7.4, 1% bovine serum albumin, and PE-conjugated IgG1 monoclonal antibody KC57 RD1 for detection of HIV-1 gag p24 (Beckman Instruments, Fullerton, CA) or isotype control antibody and incubated for 30 min at room temperature. Cells were washed in 900 μl of HBSS with 0.1% saponin and 10 mM HEPES, pH 7.4, and resuspended in 500 μl of HBSS with 1% paraformaldehyde and kept on ice up to 24 h prior to analysis. For detection of intracellular HRES-1/Rab4, cells were stained with primary rabbit antibodies SC312 (Santa Cruz Biotechnology, Santa Cruz, CA) or 13407, followed by washing and incubation of secondary PE-conjugated donkey anti-rabbit IgG.

**Western Blot Analysis**—Whole cell protein lysates were resuspended in SDS-PAGE sample buffer (2 x 10^5 cells per 10 μl), separated on a 12% SDS-polyacrylamide gel, blotted to nitrocellulose, and probed with primary rabbit antibodies directed to HRES-1/Rab4 (SC312 or Ab 13407), sheep antibody to HIV-1 gag p24, rabbit anti-HIV-1 tat antibody 705, rabbit Ab 170 specific for transaldolase, or β-actin-specific mouse Ab 1501R (Chemicon, Temecula, CA), as described previously (14, 29). CD4 was detected with rabbit antibody SC-7219 (Santa Cruz Biotechnology) and monoclonal antibody 4B12 (NovoCastra NCL-1-CD4-368, Newcastle-upon-Tyne, UK). Lck was detected with mouse monoclonal antibody sc-433 (Santa Cruz Biotechnology). For detection of rabbit antibodies, horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) was utilized. Sheep and mouse primary antibodies were detected with biotin-conjugated goat anti-mouse or anti-sheep IgG, respectively, followed by incubation with horseradish peroxidase-conjugated streptavidin (Jackson ImmunoResearch). Expression of HRES-1/Rab4, HIV-1 tat, HIV-1 gag p24 was visualized by enhanced chemiluminescence (Western Lightning Chemiluminescence Reagent Plus, PerkinElmer Life Sciences), followed by detection of transaldolase and β-actin using 4-chloronapthol. Automated densitometry was used to quantify the relative levels of protein expression using a Kodak Image Station 440CF with Kodak 1D image analysis software (Eastman Kodak Co.).

**Confocal Microscopy**—For TFR staining, 10^6 cells were incubated for 30 min in serum-free RPMI medium at 37 °C, washed once, and incubated in uptake medium (RPMI containing 20 mM HEPES, 0.5% bovine serum albumin, pH 7.4) with 50 μg/ml Alexa 647-conjugated transferrin (Molecular Probes, Eugene, OR) for 30 min at 37 °C, and then pipetted onto poly-l-lysine-coated (0.1 mg/ml poly-l-lysine; Sigma) coverslips for permeabilization and staining with Rab4 antibody. For CD4, CD5, fusin, and CD3ε staining, 10^6 cells were stained with Alexa Fluor 647- or PE/Cy5-conjugated antibodies on ice for 20 min in complete medium, and internalization was induced with 100 nM PDBu for 1 h at 37 °C. Cells were then washed and kept on ice or allowed to recycle at 37 °C. After internalization and recycling, cells were adhered to coverslips precoated with 0.1 mg/ml poly-l-lysine for 10 min at room temperature and fixed in 4% paraformaldehyde in Hank’s balanced salt solution (HBSS). Cells were permeabilized with 0.1% saponin, 0.01 μg/ml HEPES buffer, and 1% fetal bovine serum in HBSS and stained with 4 μg/ml rabbit anti-Rab4a antibody in 0.1% saponin, 0.01 μg/ml HEPES buffer, and 1% bovine serum albumin in HBSS for 30 min. After three washes the cells were incubated in 12.5 μg/ml Texas Red-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch) for 30 min, washed four times, and the coverslips mounted onto glass slides in ProLong Gold (Molecular Probes). Imaging was performed on a Bio-Rad MRC-1024 ES confocal microscope equipped with a krypton/argon laser capable of delivering excitation at 488, 568, and 647 nm using the Bio-Rad Lasersharp 2000 software. All images were recorded with a 60 x 1.4 numerical aperture oil immersion objective with appropriate discriminatory filter sets. To minimize the noise and to keep a low photobleaching rate, we selected an acquisition time of 1 s per scan and averaged 10 scans to produce each 512 x 512-pixel image.

The images registered from the same confocal plane for the green, red, and blue signals were saved and superimposed. For every pixel in the original image, the system plots a corresponding point related to the intensities of the green versus red, green versus blue, and red versus blue channels. Colocalization was quantified by correlation calculation in each pixel of paired images of ≥15 cells exhibiting green, red, and blue color fluorescence using the Simple PCI software version 5.3.0.1102 (Compix, Sewickly, PA).

**Proteasome, Cysteine Protease, and Lysosomal Inhibitors**—Proteasome inhibitors, obtained from Biomol (Plymouth Meeting, PA), were dissolved in Me₂SO, as described earlier (30). 10^6 cells/ml were incubated with proteasome inhibitors gliotoxin (1 and 10 μM), MG-132 (2.5 and 25 μM), clastolactacystin β-lactone (1 and 15 μM), epoxomycin (1 and 5 μM), and MG-262 (500 nm and 1 μM), or 0.1% Me₂SO for 4, 8, 12, and 24 h. Cysteine protease inhibitors E64 and loxistatin (EST), clalpin inhibitor, and N-acetyl-leucine-leucine-methionine-CHO (ALLM) were obtained from Calbiochem. These protease inhibitors were also resuspended in Me₂SO and used to treat cells for 12 and 24 h at the following maximal concentrations: 1 μM E64, 10 μM EST, and 10 μM ALLM. Lysosomal acidification was inhibited with NH₄Cl (1, 10, and 40 mM) or chloroquine (2.5, 25, and 100 μM). Following incubation, cells were harvested, and CD4 and β-actin protein expression was analyzed from 12.5 μg of cytosolic lysate by Western blot analysis.

**Statistical Analysis**—Statistical analyses were performed using Prism version 3.0 for Windows (GraphPad Software, San Diego). Data were expressed as the means ± S.E. of individual experiments. Changes were considered significant at p value <0.05.

**RESULTS**

Characterization of Novel HRES-1-encoded Antisense Transcript and Protein, Term HRES-1/Rab4—Northern blot analysis with HRES-1 probes of total RNA from Jurkat cells detected...
HRES-1/Rab4 Regulates CD4 Expression

A

B

C

D

actin

HRES-1/Rab

HIV gag p24

4480
6678
9035

Doxy
- + - + - + - + - + -

HIV
- - + - + - + - + - +

HRES-1/Rab4
1.0 10 5.6 54 63 0 5 53 0

HIV gag p24
1.0 1.0 0.14 0.03 2.11 1.96

4480
6678
9035

Uninfected
19.5
116 (63.3%)

HIV-infected
22.2
109 (57.7%)

Doxycycline
41.3
484 (84.5%)

4480
6678
9035

Control
4480 C
4480 D
6678 C
6678 D
9035 C
9035 D

% Apoptosis

HIV-infected

P = 0.0005

P = 0.0006
0.8-, 1.2-, 2.5-, 3.0-, and 6-kb transcripts (Fig. 1). The 3.0-kb transcript was isolated from a normal human peripheral blood cDNA library based on hybridization to a 6-kb HRES-1 probe (11). This novel 2986-base-long cDNA (GenBank™ accession number AY585832; Fig. 2) corresponding to the 3.0-kb transcript was found to be encoded by the antisense strand of the HRES-1 locus (Fig. 2). The sequence of this 2986-base-long cDNA showed considerable homology to the 735-base-long \textit{Rab4a} gene (GenBank™ accession number M28211.gb.pr1, Fig. 3) and was termed HRES-1/Rab4. HRES-1/Rab4 codes for five additional amino acids and two discordant residues, 163 (Asp \rightarrow Asn) and 209 (Thr \rightarrow Ala) (Fig. 3). The predicted molecular weight of HRES-1/Rab4 is 24,389, whereas that of \textit{Rab4a} is 23,901. The 5' and 3'-untranslated regions in the HRES-1/Rab4 cDNA were markedly different from those of \textit{Rab4a} (Fig. 3).

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure6}
\caption{Effect of HRES-1/Rab4 on surface expression of TFR/CD71, CXCR4/fusin, and CD4. Jurkat cells carrying doxycycline-inducible GFP-producing control (4480 C and 4480 D), HRES-1/Rab4 and GFP-producing expression vector (6678 C and 6678 D), or dominant-negative HRES-1/Rab4S27N and GFP-producing expression vector (9035 C and 9035 D) were preincubated with or without doxycycline for 24 h and stained with PE-Cy5-conjugated monoclonal antibodies specific for TFR, fusin, or CD4. Values in dot plots indicate mean channel FL-1 (GFP) and FL-2 fluorescence (TFR, fusin, or CD4), respectively. Bar charts represent mean ± S.E. of four independent experiments. \textit{p} values reflect comparison to untreated control and doxycycline-treated 4480 cells, respectively. \textit{B}, effect of HRES-1/Rab4 on recycling of TFR/CD71 and CD4. Jurkat cells carrying doxycycline-inducible GFP-producing control, HRES-1/Rab4 and GFP-producing expression vector, or dominant-negative HRES-1/Rab4S27N and GFP-producing expression vector were preincubated with or without doxycycline for 24 h (9035 C and 9035 D), and receptor internalization was induced by treatment with 100 nM PDBu for 1 h at 37 °C. Cells were washed twice at 4 °C to remove PDBu. Subsequently, cells were plated on ice (0 min) or returned to 37 °C to allow receptor recycling. Aliquots were removed at the time points indicated (10, 30, 60, or 120 min), stained with anti-CD4 or anti-TFR antibodies at 4 °C, and kept on ice until analyzed by flow cytometry. MFI, mean fluorescence intensity. Control, base-line surface expression in the absence of PDBu. Results are representative of three independent experiments.}
\end{figure}

\begin{figure}[h]
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\caption{Effect of HRES-1/Rab4 on infection of Jurkat T cells by HIV-1. \textit{A}, Western blot analysis of HRES-1/Rab4 protein levels in Jurkat cells carrying doxycycline-inducible expression vectors. GFP-producing control (4480), HRES-1/Rab4 and GFP-producing expression vector (6678), or dominant-negative HRES-1/Rab4S27N and GFP-producing cells (9035) were incubated for 24 h in the absence or presence of doxycycline. As a loading control human β-actin was monitored. \textit{B}, Western blot analysis of gag p24 protein levels in HIV-infected Jurkat cell lines with altered HRES-1/Rab4 expression. Protein lysates were prepared 4 days post-infection, separated by SDS-PAGE, blotted to nitrocellulose, and developed with antibodies directed to HIV gag p24 and actin. Based on automated densitometry, relative expression of HIV gag p24 with respect to HIV-infected 4480 cells (100%) is shown below each lane. As positive controls, H9 cells were also infected with HIV-1. C, detection of intracellular HIV-1 gag p24 by flow cytometry. Day 4 post-infection, cells were permeabilized and stained with PE-conjugated K57-RD1 monoclonal antibody. H9 cells permanently infected with HIV-1 were used as a positive control. Histograms of HIV-1-infected cells (open curves) are overlaid on control cells (shaded curves). Values over histograms indicate mean channel fluorescence; percentage of positive cells is shown in parentheses. \textit{p} values reflect comparison to untreated control and doxycycline-treated 4480 cells (4480 D), respectively.}
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\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure6}
\caption{Effect of HRES-1/Rab4 on recycling of TFR/CD71 and CD4. Jurkat cells carrying doxycycline-inducible GFP-producing control, HRES-1/Rab4 and GFP-producing expression vector, or dominant-negative HRES-1/Rab4S27N and GFP-producing expression vector were preincubated with or without doxycycline for 24 h (9035 C and 9035 D), and receptor internalization was induced by treatment with 100 nM PDBu for 1 h at 37 °C. Cells were washed twice at 4 °C to remove PDBu. Subsequently, cells were plated on ice (0 min) or returned to 37 °C to allow receptor recycling. Aliquots were removed at the time points indicated (10, 30, 60, or 120 min), stained with anti-CD4 or anti-TFR antibodies at 4 °C, and kept on ice until analyzed by flow cytometry. MFI, mean fluorescence intensity. Control, base-line surface expression in the absence of PDBu. Results are representative of three independent experiments.}
\end{figure}

0.8-, 1.2-, 2.5-, 3.0-, and 6-kb transcripts (Fig. 1). The 3.0-kb transcript was isolated from a normal human peripheral blood cDNA library based on hybridization to a 6-kb HRES-1 probe (11). This novel 2986-base-long cDNA (GenBank™ accession number AY585832; Fig. 2A) corresponding to the 3.0-kb transcript was found to be encoded by the antisense strand of the HRES-1 locus (Fig. 2B). The sequence of this 2986-base-long cDNA showed considerable homology to the 735-base-long \textit{Rab4a} gene (GenBank™ accession number M28211.gb.pr1, Fig. 3A) and was termed HRES-1/Rab4. HRES-1/Rab4 codes for five additional amino acids and two discordant residues, 163 (Asp \rightarrow Asn) and 209 (Thr \rightarrow Ala) (Fig. 3B). The predicted molecular weight of HRES-1/Rab4 is 24,389, whereas that of \textit{Rab4a} is 23,901. The 5' and 3'-untranslated regions in the HRES-1/Rab4 cDNA were markedly different from those of \textit{Rab4a} (Fig. 3A). HRES-1 was previously mapped to human...
HRES-1/Rab4 Regulates CD4 Expression

A

4480 D

6678 D

9035 D

B

4480 D

6678 D

9035 D
chromosome 1 region q42 (12). All eight coding exons of the HRES-1/Rab4 cDNA were localized within contig NT031728.1 mapped to the 1q41–1q42 genomic locus (Fig. 1A). The HRES-1 fragment restricted with SmaI-EagI (SEa), corresponding to HRES-1/Rab4 exon 1, detected four transcripts (0.8, 1.2, 2.5, and 3.0 kb), suggesting that these transcripts were encoded by the antisense strand. The EcoRI-Smal HRES-1 probe (EcS) hybridized to a 6-kb transcript previously detected by an HRES-1/p28 strand-specific RNA probe (11). Thus, transcription of HRES-1/p28 is directed toward the centromere, whereas the HRES-1/rab4 gene is transcribed in the direction of the telomere at human chromosome 1q41-1q43 (www.ncbi.nlm.nih.gov).

To test for expression of native HRES-1/Rab4, an antibody was raised in rabbit against full-length recombinant protein (rHRES-1/Rab4) expressed as a fusion protein with GST in E. coli. The affinity-purified and cleaved rHRES-1/Rab4 was used to raise rabbit antibody Ab 13407 (Fig. 3C). This antibody recognized the rHRES-1/Rab4 protein produced in E. coli or Jurkat cells and detected the native protein of identical molecular weight in both PBL and Jurkat cells. Antibody SC312, raised against a 20-amino acid C-terminal segment of Rab4a, recognized HRES-1/Rab4 and Rab4a of slightly lower molecular mass. The predicted molecular weight of HRES-1/Rab4 is 24,389, whereas that of Rab4a is 23,901. Because the HRES-1 is a single copy sequence in the haploid genome (12), the previously identified Rab4 may originate from another chromosomal locus or correspond to an alternative translation product of the polymorphic HRES-1/Rab4 genomic locus.

The tat Gene of HIV-1 Stimulates the Promoter Activity and Expression of HRES-1/Rab4—The first coding exon of HRES-1/Rab4 is embedded in the HRES-1 endogenous retroviral sequence (Fig. 1A). Based on alignment with full-length cDNA and primer extension analysis (data not shown), transcription start site of HRES-1/Rab4 was mapped to HRES-1 position 1611. In CAT reporter gene assays, the HindIII-EagI fragment of HRES-1 (nucleotides 2151–1606) exhibited strong promoter activity when oriented in the direction of HRES-1/Rab4 tran-
HRES-1/Rab4 Regulates CD4 Expression

Described (Fig. 4A). Insertion of the HRES-1 LTR (residues 1200 to 1) downstream of the CAT gene augmented promoter activity in HeLa cells transfected with HIV-1 tat (HeLa-tat), whereas it diminished promoter activity in control HeLa cells (Fig. 4A). CAT activity of the HRES-1/Rab4 promoter-CAT-LTR construct (plasmid 6413) was enhanced in HeLa-tat cells by 2.9 ± 0.2-fold (p < 0.0001). Moreover, elevated HRES-1/Rab4 protein levels were noted in HeLa-tat, Jurkat-tat (not shown), and HIV-infected H9 human T cells (Fig. 4B). HRES-1/Rab4 protein levels were increased in HeLa-tat, as compared with control HeLa cells, by 2.77 ± 0.2-fold (p < 0.0001). The results clearly indicate that HIV-tat can increase expression of HRES-1/Rab4 via trans-activation of HRES-1 LTR internal promoter. Although the HRES-1 LTR was originally considered to harbor an HIV-1 TAR (trans-activation target) sequence (11, 31), subsequent analysis by Berkhout and co-workers (32, 33) identified TAR as a larger domain of the HIV-1 LTR, which is not fully contained in HRES-1. Thus, up-regulation of HRES-1 expression by HIV-1 may not occur through the typical tat-TAR interaction (32, 33).

HRES-1/Rab4 Inhibits HIV-induced Gag p24 Production and Apoptosis—Because HRES-1/Rab4 expression is up-regulated by HIV-1 tat, its potential role in viral pathogenesis was investigated. Jurkat cells overexpressing HRES-1/Rab4 were generated using a doxycycline-inducible GFP-encoding bi-cistronic expression vector system (34). Jurkat cells containing pUHD172–1neo (Jur-TA) stably transfected with the pTR5-DC/GFP*TK/hygro vector alone (4480) produced GFP in the presence of 1 μg/ml doxycycline and were utilized as negative control. Jur-TA cells stably transfected with the pTR5-DC/HRES-1Rab4*GFP*TK/hygro vector (6678) produced more expression of CD4 than control cells. Base-line expression of CD4 in Jur-TA cells was monitored in Jurkat cells overexpressing HRES-1/Rab4 (Fig. 5A). HRES-1/Rab4 profoundly reduced HIV gag p24 production detected by Western blot (Fig. 5B) and the percentage of HIV-1 infected cells determined by flow cytometry of intracellular gag p24 staining (Fig. 5C) and diminished HIV-induced apoptosis by 51% (p < 0.0005) and 57% (p < 0.0057) in the absence or presence of doxycycline, respectively (Fig. 5D). Low levels of HIV gag p24 were detected by Western blot (Fig. 5B), but none by flow cytometry of 6678-transfected cells (Fig. 5C). To further substantiate specificity of HRES-1/Rab4-induced changes on infection by HIV-1, Jurkat cells overexpressing a dominant-negative form of HRES-1/Rab4 were generated. Substitutions in the GTP-binding domains of all rab proteins and other members of the ras superfamily, such as S22N mutant Rab4 (35), were potent trans-dominant inhibitors of vesicular transport (36). Therefore, S27N substitution was created in the HRES-1/Rab4-producing pTR5-DC/GFP*TK/hygro vector by site-directed mutagenesis. The mutated plasmid was transfected into Jur-TA cells. Following doxycycline induction, GFP-positive cells were sorted by flow cytometry. Expression of HRES-1/Rab4S27N was monitored by Western blot analysis and automated densitometry with respect to β-actin internal control (Fig. 5A). In comparison to native HRES-1/Rab4 protein levels of 4480 control cells, HRES-1/Rab4S27N was expressed in 9035 cells at 5.05 ± 0.6-fold elevated levels in the absence of doxycycline (p < 0.0001) and 49.96 ± 2.2-fold elevated levels in the presence of doxycycline (p < 0.0001; based on four independent experiments). To avoid artifacts related to variations in sites of vector integration, transfected cells were not cloned, but bulk-sorted aliquots of cells overexpressing HRES-1/Rab4 or HRES-1/Rab4S27N or GFP alone were utilized in all experiments. Following pretreatment with doxycycline for 24 h, each cell type was infected with HIV-1. HRES-1/Rab4 abrogated production of HIV-1 gag p24 as determined by Western blot analysis (Fig. 5B). Along the same line, HRES-1/Rab4 reduced the percentage of HIV-1-infected cells determined by flow cytometry of intracellular gag p24 staining (Fig. 5C) and diminished HIV-induced apoptosis (Fig. 5D). By contrast, HRES-1/Rab4S27N enhanced gag p24 production and HIV-induced apoptosis, and the latter was markedly enhanced in 9035 cells treated with doxycycline (p = 0.0003; Fig. 5D).

HRES-1/Rab4 Regulates Surface Expression of CD4 via Recycling—Rab proteins belong to the family of small GTPases that regulate endosome recycling. Rab4a has been shown to regulate recycling of early endosomes carrying the TFR in epithelial cells (37) or GLUT4 in adipocytes (38). Therefore, we examined whether the impact of HRES-1/Rab4 on HIV infection was mediated via recycling and expression of surface receptors. TFR expression was not affected in the absence of doxycycline; however, it was reduced by HRES-1/Rab4 in the presence of doxycycline (Fig. 6A; p < 0.0034). Similar results were obtained by using Alexa 647-conjugated transferrin and PE-Cy5-conjugated CD71 monoclonal antibody for detection of TFR (data not shown). Strikingly, expression of CD4 was reduced on the surface of 6678 cells as compared with 4480 cells, even in the absence of doxycycline (−62 ± 2.9%, p < 0.0001). Addition of doxycycline further reduced expression of CD4 on 6678 cells (−72 ± 0.9%, p = 0.022). By contrast, surface expression of CD4 was enhanced on HRES-1/Rab4S27N-producing 9035 cells (Fig. 6A). CD4 expression was not affected by increased production of GFP in control 4480 cells (Fig. 6A). Expression of HIV coreceptor fusin/CXCR4 (Fig. 6A) and CD45RO (data not shown) was not influenced by HRES-1/Rab4. Thus, coordinate suppression by HRES-1/Rab4 and up-regulation by HRES-1/Rab4S27N of CD4 expression indicate a specific role for HRES-1/Rab4 in regulation of CD4 expression.

CD4 undergoes protein kinase C-mediated endocytosis following T cell activation (7). Thus, CD4 internalization was induced by treatment with 100 nM PDBu for 1 h at 37 °C. Subsequently, PDBu was removed by washing twice, and cells were kept at 4 °C to assess internalization or returned to 37 °C to allow recycling. Recycling of TFR/CD71, CD4, fusin/CXCR4, CD45RO, and CD3e to the cell surface was monitored in Jurkat cells overproducing wild-type HRES-1/Rab4 or HRES-1/Rab4S27N with respect to control cells. Base-line expression of TFR was reduced after induction of HRES-1/Rab4 by doxycycline for 24 h (Fig. 6A), whereas the rate of TFR recycling after serum deprivation was not affected by HRES-1/Rab4 or HRES-1/Rab4S27N (Fig. 6B). Surface expression of CD4 was profoundly reduced in cells overexpressing HRES-1/Rab4, thus preventing further reduction by PDBu and evaluation of recy-
clinging (Fig. 6B). Although base-line expression of CD4 was markedly enhanced by HRES-1/Rab4S27N, after internalization and recycling for 120 min, surface expression of CD4 exceeded base-line levels by 36% (p < 0.0117) in the presence of doxycycline (Fig. 6B, 9035D cells). After internalization and recycling, TFR levels remained below base-line levels by 25% on cells overproducing HRES-1/Rab4S27N in the presence of doxycycline (Fig. 6B, 9035D cells). Thus, recycling of CD4, but not TFR, was selectively enhanced in cells overproducing HRES-1/Rab4S27N. 

Colocalization of HRES-1/Rab4 with TFR and CD4—Following serum starvation for 1 h, internalization and recycling of TFR were monitored by staining with Alexa 647-conjugated transferrin. Alternatively, CD4 was internalized by treatment with 100 nM PDBu for 1 h at 37 °C, washed, and allowed to recycle at 37 °C for 30 min. Cells were adhered to poly-L-lysine-coated coverslips and permeabilized, and Rab4 was visualized by subsequent staining with Ab SC312 and Texas Red-conjugated goat anti-rabbit IgG. TFR colocalized with HRES-1/Rab4 to overlapping intracellular blebs irrespective of HRES-1/Rab4 or HRES-1/Rab4S27N expression (Pearson's correlation coefficient, r = 0.96; Fig. 7A). Following PDBu treatment, CD4 also colocalized with HRES-1/Rab4 to intracellular blebs (r = 0.99; Fig. 7B). The patterns of colocalization were markedly different after recycling. CD4 recycled to the membrane of control and HRES-1/Rab4S27N-expressing cells and displayed a uniform ring pattern. CD4 failed to recycle to the cell membrane and remained confined to discrete foci in cells overproducing HRES-1/Rab4 (Fig. 7C). Overall intensity of CD4 fluorescence was also diminished in cells overproducing HRES-1/Rab4 (Fig. 7C).

Overexpression of HRES-1/Rab4 Causes Lysosomal Degradation of CD4—Flow cytometry and confocal microscopy suggested that HRES-1/Rab4 may affect total CD4 protein levels. Western blot analyses revealed that HRES-1/Rab4 expression reduced CD4 protein content to 29.6% (p = 0.0004) and 25.9% (p = 0.002) of control cells in the absence and presence of doxycycline, respectively (Fig. 8A). By contrast, HRES-1/Rab4S27N increased CD4 protein content 1.9-fold (p = 0.016) and 3.1-fold (p = 0.012) in the absence and presence of doxycycline, respectively (Fig. 8A). Levels of Lck, noncovalently associated with CD4 in the cell membrane (39), was unchanged. Treatment with the cell-permeable proteasome inhibitors clasto-lactacystin β-lactone, epoxomycin, gliotoxin, MG-132, and MG-262, cysteine protease inhibitors E64 and EST, or calpain inhibitor ALLM did not influence CD4 protein levels (data not shown). However, the lysosomal inhibitor chloroquine normalized CD4 levels diminished by HRES-1/Rab4 in a dose- and time-dependent manner (Fig. 8B). Another lysosomal inhibitor, NH4Cl, augmented CD4 content to a lesser extent. CD4 levels of control cells and of cells overexpressing HRES-1/Rab4S27N were not significantly influenced by chloroquine or NH4Cl (not shown). Thus, the abrogation of CD4 expression by HRES-1/Rab4 may have resulted from inhibition of endocytic recycling and targeting of CD4 for lysosomal degradation.
HRES-1/Rab4 Regulates CD4 Expression

A

|        | CD4 T cells |            | PBMC |
|--------|-------------|------------|------|
| HIV-1  |             |            |      |
| Day 2  | - + + +     | - + + +    |      |
| Day 4  | - + + +     | - + + +    |      |
| Day 6  | - + + +     | - + + +    |      |
| Day 8  | - + + +     | - + + +    |      |

HIV gag p24

CD4

HRES-1/Rab4

Actin

B

|        | CD4 T cells |            | PBMC |
|--------|-------------|------------|------|
| HIV-1  |             |            |      |
| Day 2  | - + + +     | - + + +    |      |
| Day 4  | - + + +     | - + + +    |      |
| Day 6  | - + + +     | - + + +    |      |
| Day 8  | - + + +     | - + + +    |      |

CD4

HRES-1/Rab4

Actin

CD4/Actin level 1 1.06 0.57 1.21 1 1.51 0.52 1.28

C

Graphs showing CD4 and HRES-1/Rab4 expression levels under different conditions.
HRES-1/Rab4 Regulates CD4 Expression

Coordinate Regulation of CD4 and HRES-1/Rab4 Expression in Peripheral Blood Lymphocytes—To investigate the effect of HIV-1 on expression of HRES-1/Rab4 in peripheral blood lymphocytes, PBMC and affinity-purified CD4 T cells were infected by HIV-1. Western blot analysis showed a coordinate down-regulation of CD4 and up-regulation of HRES-1/Rab4 in HIV-infected cells (Fig. 9A). Infection of CD4 T cells and PBMC with HRES-1/Rab4-producing AAV resulted in down-regulation of CD4 expression with respect to uninfected cells or cells infected with control AAV or HRES-1/Rab4S27N-producing AAV (Fig. 9, B and C).

DISCUSSION

ERV constitute a large reservoir of viral genes that can influence the outcome of infections by exogenous retroviruses via provision of genes for recombination with proviral DNA, interference with virion assembly, blocking cellular receptors for viral entry, and modulation of immune responses to exogenous viruses (2, 5). Although most human ERV have truncated and mutated proviral DNA, few of them are transcriptionally active (3). The present data provide evidence that both the sense and antisense strands of the human ERV HRES-1 can be transcribed in human cells. Bidirectional transcription has been documented at several genomic loci (40, 41), including another ERV, HERV-H (6), and the 1q42 locus harboring HRES-1 (42). Although the sense strand of HRES-1 encodes a 28-kDa gag-like protein, HRES-1/p28, expressed in T lymphocytes and salivary gland epithelial cells (5, 11, 29), the antisense strand provides exon 1 and transcriptional regulatory elements of the HRES-1/Rab4 protein. The tat gene of HIV-1 stimulates expression of HRES-1/Rab4 protein via trans-activation of the HRES-1 LTR. In turn, HRES-1/Rab4 modulates surface expression of CD4 and, thus, infection of T cells by HIV-1.

The present data identify HRES-1/Rab4 as a regulator of CD4 recycling. Rab proteins belong to the family of small GTPases that regulate receptor endosome recycling (43). In particular, Rab4a has been shown to influence recycling of the TFR in epithelial cells (37) or GLUT4 in adipocytes (38). Unlike fusin, CD5, and CD45RO, surface expression of CD4 and TFR was influenced by HRES-1/Rab4. Although HRES-1/Rab4 colocalized with both TFR and CD4, surface expression of TFR was only reduced in doxycycline-stimulated cells overproducing HRES-1/Rab4 up to 100-fold. By contrast, CD4 levels were coordinately down-regulated by HRES-1/Rab4 and up-regulated by HRES-1/Rab4S27N. CD4 levels were markedly diminished by 3-fold overexpression of HRES-1/Rab4. Similar increase of HRES-1/Rab4 expression was elicited by HIV infection or transfection of HIV-1-tat, suggesting that HIV-1 may utilize HRES-1/Rab4 to regulate CD4 expression. Along this line, production of HIV-1 gag p24 and apoptosis of HIV-infected cells were reduced by HRES-1/Rab4 and enhanced by HRES-1/Rab4S27N. Surface expression or recycling of CXCR4/fusin was not influenced by HRES-1/Rab4, suggesting that infection by HIV-1 was selectively modulated via CD4 expression. CD4 and TFR mRNA were not affected by HRES-1/Rab4 or HRES-1/Rab4S27N (data not shown), suggesting that changes in surface expression were solely mediated on the protein level.

CD4 appears to play a role in HIV entry beyond merely serving as the attachment protein for the virus. CD4 undergoes endocytosis following T cell activation (7), and HIV entry may depend on the internalization of CD4. In turn, CD4 internalization is dependent on the activation of protein kinase C and subsequent phosphorylation of CD4 (8). Accordingly, treatment with PDBu, an activator of protein kinase C, markedly reduced surface expression of CD4. Protein kinase C-mediated down-regulation of CD4 involves altered endosomal sorting (44). CD4 is constitutively internalized into early endosomes and recycled to the cell surface. In the presence of phorbol ester, CD4 is diverted from the constitutive recycling early endosome pathway to the late endosome/lysosome pathway, and as a consequence, there is a reduction in the recycling of internalized CD4. Indeed, after removal of PDBu, CD4 expression increased. Re-appearance of CD4 on the cell surface was not influenced by inhibition of protein synthesis with cycloheximide (data not shown), suggesting that internalized CD4 was not synthesized de novo but recycled back to the cell surface. Overexpression of HRES-1/Rab4 markedly reduced surface expression of CD4 and targeted it for lysosomal degradation. CD4 levels suppressed by overexpression of HRES-1/Rab4 were normalized by lysosomal but not proteasomal inhibitors. In contrast, surface expression, recycling, and total cellular CD4 content was enhanced by HRES-1/Rab4S27N. Thus, HRES-1/Rab4 plays a dominant role in CD4 expression in T cells by regulating its traffic between the recycling and late endosome/lysosome pathway.

The present data show that HIV stimulates expression of HRES-1/Rab4 which, in turn, abrogates recycling of CD4 to the cell surface. Indeed, profound down-regulation of CD4 expression on the surface of HIV-infected cells may be mediated, at least in part, via inhibition of CD4 recycling by HRES-1/Rab4. Previously, HIV-induced CD4 down-regulation has been attributed to nef-initiated internalization and retention (45), lysosomal degradation (46, 47), and vpu-initiated degradation by the proteasome (48–50). HIV nef also influences apoptosis signal processing, T-cell receptor expression (51), and formation of the immunological synapse (52). This study reveals a role for HRES-1/Rab4 in lysosomal degradation of CD4. HRES-

FIGURE 9. Western blot analysis of HRES-1/Rab4 and CD4 levels in human CD4 T cells and PBMC infected by HIV-1. Protein lysates of HIV-infected (+) and uninfected control cells (−) were analyzed for expression of CD4, HRES-1/Rab4, HIV gag p24, and actin on days 2, 4, 6, and 8 post-infection. Based on automated densitometry, using actin as base line, relative expression of HRES-1/Rab4 and CD4 in HIV-infected cells with respect to uninfected cells (normalized at 1.0) is shown below each lane. Data represent three independent experiments. A, expression of HRES-1/Rab4 overexpression on CD4 levels in CD4 T cells and PBMC. Cells were infected with pAAV-ERV-HRES-4GFP control virus (AAV) and HRES-1/Rab4-p producing (AAV/HRES-1/Rab4) or HRES-1/Rab4S27N-producing AAV (AAV/HRES-1/Rab4S27N) and analyzed by Western blot 24h later. Data represent three independent experiments. B, flow cytometry of CD4 expression in PBMC infected with control AAV (open histogram) and HRES-1/Rab4-producing AAV or HRES-1/Rab4S27N-producing AAV (shaded histogram overlays). The percentage of CD4+ cells and relative surface expression of CD4 (mean channel fluorescence, in parentheses) are indicated. Data represent three independent experiments.
HRES-1/Rab4 Regulates CD4 Expression

1/Rab4 belongs to the family of small GTPases that regulate receptor endosome recycling (43) and may be involved in Nef-induced lysosomal degradation of CD4 (46, 47). Wild-type HRES-1/Rab4 inhibited whereas HRES-1/Rab4527TN enhanced HIV infection and virion production, suggesting that regulation of HRES-1/Rab4 expression may play an active role in the life cycle of HIV-1. This notion is supported by coordinate up-regulation of HRES-1/Rab4 and down-regulation of CD4 expression in HIV-infected CD4 T cells and PBMC. HRES-1/Rab4 promoter activities and protein levels are increased in cells infected by HIV-1 or transfected by HIV-tat. In turn, enhanced expression of HRES-1/Rab4 may contribute to down-regulation of CD4 recycling to the cell surface, thus preventing re-infection by HIV-1, allowing for increased virion production, and protecting virus-infected cells against killing by cytotoxic T cells (53, 54). Thus, stimulation of HRES-1/Rab4 expression by HIV-1 and regulation of HIV coreceptor CD4 recycling by HRES-1/Rab4 represent novel mechanisms of coordinate interaction between infectious viral particles and ERV of the human genome.

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