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Inhibition of Human T-Cell Leukemia Virus Type 2 Rex Function by Truncated Forms of Rex Encoded in Alternatively Spliced mRNAs

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Three mRNA species encoding the x-III open reading frame are expressed in human T-cell leukemia virus type 2 (HTLV-2)-infected cells. An mRNA composed of exons 1, 2, and 3 produces the essential posttranscriptional regulator Rex; shorter 1-3 and 1-B mRNAs encode a family of x-III proteins of unknown function that represent truncated forms of Rex. This report presents an analysis of the functional interactions between Rex and the x-III proteins, results of which suggest a role for the x-III proteins as negative regulators of Rex function. Cotransfection assays demonstrated that the x-III proteins were able to inhibit the ability of Rex to activate the expression of a Rex-dependent mRNA. Analysis of intracellular compartmentalization in actinomycin D-treated cells showed that coexpression of the x-III proteins resulted in the sequestration of Rex into the nuclear compartment. Subcellular fractionation studies showed that Rex was preferentially localized in the cytoplasmic or nuclear fraction depending on its phosphorylation status and that coexpression of Rex with the x-III proteins changed the phosphorylation pattern of Rex and the intracellular distribution of the x-III proteins. In vitro protein binding assays demonstrated the formation of Rex-Rex homomultimeric complexes; however, mixed Rex/x-III multimers were not detected. These findings indicated a correlation between phosphorylation and intracellular trafficking of Rex and suggested that the mechanism underlying the inhibitory effects of the x-III proteins might result from an interference with these processes.

Although human T-cell leukemia/lymphotropic virus types 1 (HTLV-1) and 2 (HTLV-2) are closely related and show similar transforming potential in vitro, their in vivo cellular tropism and pathogenicity appear to be distinct (reviewed in references 13 and 20). Despite indications that HTLV-2 is associated with hematological malignancies and neurological syndromes, the etiologic role of the virus in human disease has not been clearly established; in contrast, HTLV-1 has been conclusively linked to adult T-cell leukemia/lymphoma and a degenerative spastic myelopathy (reviewed in reference 13). The genetic basis for the different in vivo tropism and pathogenicity of the two viruses remains to be understood.

HTLV-1 and HTLV-2 have been taxonomically grouped with the oncogenic retroviruses simian T-cell leukemia/lymphotropic viruses and bovine leukemia virus on the basis of their relatively complex genome and expression (reviewed in reference 18). The most distinctive genetic feature of retroviruses of the HTLV/bovine leukemia virus group is the presence of the X region located in the 3′ portion of the genome. Studies of HTLV-2 expression demonstrated that a combination of alternative splicing and multicistronic translation greatly expands the coding potential of the X region (14–16). The best-characterized products of the X region of HTLV-2 are the essential viral regulatory proteins Tax (Tax_2) and Rex (Rex_2), which are expressed from a doubly spliced dicistronic mRNA containing exons 1, 2, and 3. Tax_2 and Rex_2 are translated from AUG codons located in exon 2, and their major portions are encoded in the x-IV and x-III open reading frames (ORFs), respectively, located in exon 3 (45, 49, 50) (Fig. 1). Tax_2 is a transcriptional activator of the viral promoter (12, 52) and is essential for the transformation of human T lymphocytes by HTLV-2 (44). Rex_2 regulates the expression of the viral mRNAs at the posttranscriptional level by interacting directly with an RNA element termed the Rex-responsive element (RXRE-II) (5, 25, 31, 35, 43). The Rex_2–RXRE-II interaction relieves the repressive effect of sequences present in the 5′ long terminal repeat (LTR) that inhibit the export of incompletely spliced viral mRNAs from the nucleus to the cytoplasm (6, 7). In addition, Rex_2 is able to inhibit RNA splicing in vitro at an early step of spliceosome formation (1). The Rex products of HTLV-1 and HTLV-2 and their *cis*-acting elements RXRE-I and RXRE-II are structurally similar and functionally interchangeable (31). Mutational analyses have defined regions that are essential for the biological properties of HTLV-1 Rex (Rex_1), including an amino-terminal nuclear localization signal (NLS)/RXRE-binding domain (8, 51), a multimerization domain (9), and an activation domain (27). These regions contain core motifs that are highly conserved in the amino acid sequence of Rex_2; the positions of these domains in Rex_2 are indicated in Fig. 1.

Both HTLV-1 and HTLV-2 produce alternatively spliced mRNAs encoding x-III ORF-derived proteins that correspond to carboxy-terminal portions of Rex. In HTLV-2, these truncated x-III ORF products are expressed from two singly spliced mRNAs containing exon 1 directly linked to either exon 3 or to exon B (Fig. 1). Initiation of translation from the first AUG codon located within the x-III ORF gives rise to two major protein isoforms of 22 and 20 kDa and a minor 18-kDa protein differing by posttranslational modification; translation from the second AUG of the x-III ORF produces a minor 17-kDa product (Fig. 1) (15, 16). HTLV-1 produces a single truncated x-III ORF product termed $p21^{Rex}$ (3, 4, 36). While Rex plays an essential role in the posttranscriptional control of viral expression, the function of $p21^{Rex}$ and the x-III proteins remains obscure. As shown in the right-hand portion of Fig. 1, the putative multimerization and activation domains of Rex_2 are also present in the 22-, 20-, and 18-kDa x-III proteins. The

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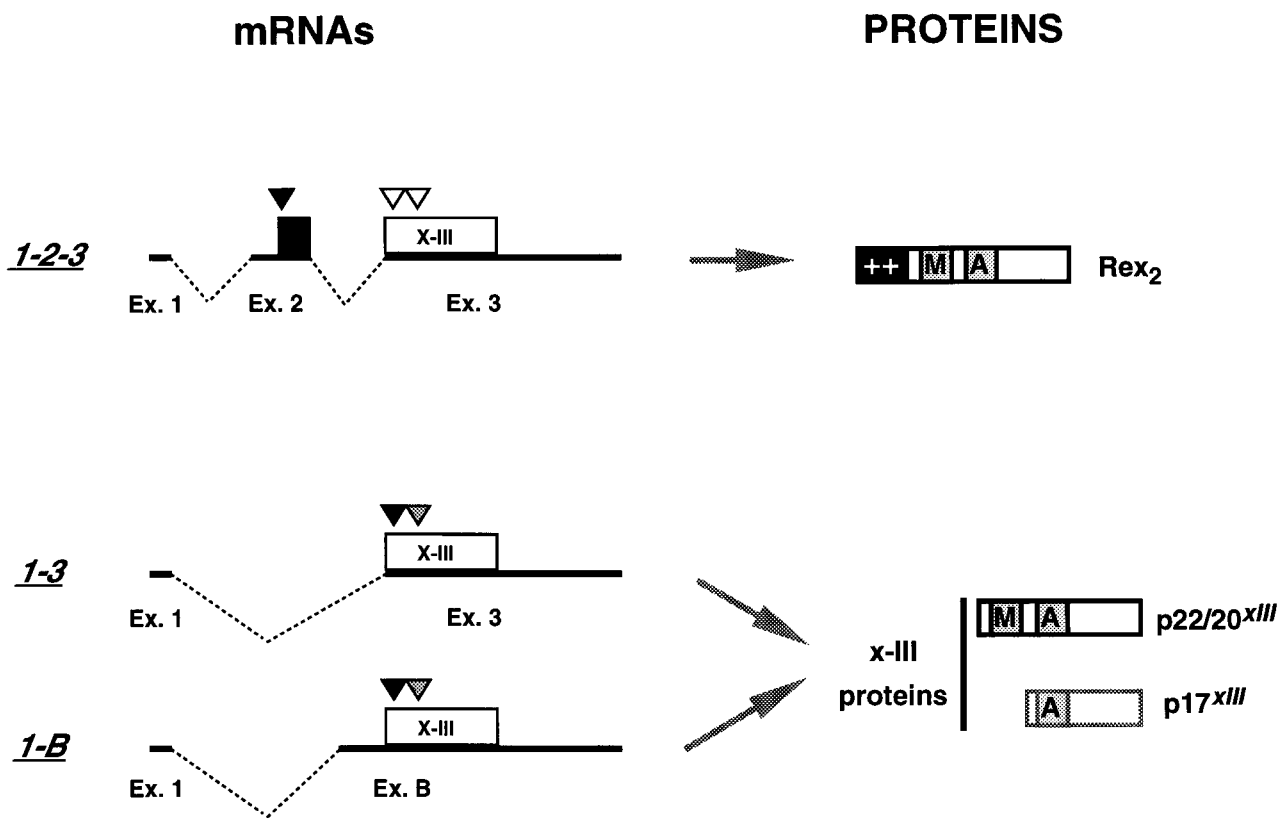

FIG. 1. Translation of the x-III ORF from alternatively spliced HTLV-2 mRNAs. The left-hand panel shows the schematic structure of alternatively spliced HTLV-2 mRNAs expressing x-III-derived proteins. Solid lines represent exons, and boxes represent ORFs. Positions of AUG initiation codons within the x-III ORF are indicated by solid triangles (major initiation site), shaded triangles (minor initiation site), and open triangles (site not detectably utilized). Rex_2 is translated from the doubly spliced 1-2-3 mRNA by initiation at an AUG (solid triangle in exon 2) that is linked in frame with the x-III ORF in exon 3. The dicistronic 1-2-3 mRNA also encodes Tax, which is translated from a different AUG that lies in frame with the x-IV ORF in exon 3 (not shown). AUG codons located within the x-III ORF in exon 3 (open triangles) are not utilized as initiation sites in the context of the 1-2-3 mRNA. mRNAs 1-3 and 1-B have identical coding potential, producing a family of x-III-derived protein isoforms (termed x-III proteins for simplicity) and the $p28^{xII}$ protein encoded in the partially overlapping x-II ORF (15). The x-III plasmids used in the present studies were truncated at the 3′ terminus of the x-III ORF to avoid expression of $p28^{xII}$. The major x-III proteins, termed $p22/20^{xIII}$, and a minor 18-kDa product are translated from the first AUG codon of the ORF (nt 7247 [solid triangle]) and differ by phosphorylation. A minor 17-kDa band is translated from the second AUG codon of the x-III ORF (nt 7337 [shaded triangle]). It is noteworthy that Rex_2 and its truncated forms, represented by the x-III proteins, are produced from distinct mRNAs. The right-hand portion of the figure shows the location of the positively charged NLS/RNA binding domain (solid box, ++), multimerization domain (shaded box, M), and activation domain (shaded box, A) within Rex_2. The x-III proteins contain the activation and multimerization domains but lack the amino-terminal domain of Rex_2.

presence of functional domains within the x-III proteins led us to ask whether they may play a role in controlling Rex_2 function. In this study, we present evidence for the functional interaction between Rex_2 and the x-III proteins at several levels, resulting in an interference with Rex_2 function, subcellular compartmentalization, and phosphorylation.

MATERIALS AND METHODS

Recombinant plasmids. Eukaryotic expression plasmids coding for Rex_2 and the x-III proteins were derived from the vector pLdK3pA, which contains the human immunodeficiency virus (HIV) promoter, a polylinker, and the simian virus 40 pA signal/site (33) and are designated by the pLs prefix. Plasmid $pLsRex_2$-AU1, which produced Rex_2 tagged at the carboxy terminus with the AU-1 epitope, contained the Rex_2-coding portion of cDNA 1-2-3 (nucleotides [nt] 5102 to 5183 of exon 2 and nt 7214 to 7660 of exon 3) followed by a sequence encoding the AU-1 epitope (amino acids DTYRYI). Plasmid pLsX-III-AU1, which produced the x-III proteins tagged at the carboxy terminus with the AU-1 epitope, contained a portion of cDNA 1-3 corresponding to exon 1 (nt 1 to 449) and part of exon 3 (nt 7214 to 7660) followed by a sequence encoding the AU-1 epitope. pLsX-III-HA1 was identical to pLsX-III-AU1, except that the x-III ORF was tagged at the carboxy terminus with the HA1 epitope (amino acids YDVPDYASL). pCgagRXRE-II contained the HIV-1 LTR promoter and gag gene linked to a 445-bp fragment of HTLV-2 spanning the RXRE (nt 316 to 760 of the R-U5 region). $pGST-Rex_2$-AU1 was generated by cloning a PCR-amplified fragment coding for AU-1-tagged Rex_2 into the *Bam*HI site of pGex2T (Pharmacia) in the same reading frame as the glutathione *S*-transferase (GST) gene.

Cells and transfections. HeLa cells constitutively producing the HIV-1 Tat protein (HLtat cells [47]) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum and transfected by the calcium phosphate coprecipitation technique (22). The HLtat cell line was chosen for its high-level expression of genes cloned into pLdK3pA vector. Plasmid DNAs used in transfections were purified by anion-exchange chromatography (Qiagen).

Bioassays for Rex function. HLtat cells were cotransfected with pCgagRXRE-II (1 μg), pL3CAT (1 μg, included as a standard for transfection efficiency [57]), and $pLsRex_2$-AU1 in the absence or presence of different amounts of pLsX-III-AU1. After 22 h, the cells were lysed in a buffer containing 100 mM Tris (pH 7.5) and 0.5% Triton X-100. Lysates were subjected to one freeze-thaw cycle and cleared by centrifugation at 12,000 × *g* for 5 min. Portions of the lysates were combined with sample buffer, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis in 16% T–2.6% C gels, and transferred to nitrocellulose membranes. The blots were probed with a monoclonal antibody specific for the AU-1 epitope (Babco) or HIV-1 Gag (Cellular Products), developed by using a chemiluminescence system (ECL; Amersham), and exposed to autoradiography film (Hyperfilm; Amersham). Portions of the cell lysates were also analyzed by p24 Gag antigen capture assay (Cellular Products) to quantitate the amount of Gag protein. Cotransfection of 0.2 μg of $pLsRex_2$-AU1 resulted in an approximately 500-fold induction of Gag production from 1 μg of pCgagRXRE-II. This 500-fold stimulation of Gag expression was considered to reflect 100% Rex_2 activity. Gag production in the presence of Rex_2 and different amounts of x-III proteins were likewise calculated and expressed as percentages of full Rex_2 activity. As reported previously (54), we observed a reduction of chloramphenicol acetyltransferase (CAT) production in the presence of Rex and Rex-related plasmids that did not reflect differences in transfection efficiencies.

Therefore, the activation values derived from Gag quantitation were not normalized for CAT.

Immunofluorescence and actinomycin D treatment. At 20 h after transfection, HLtat cells were treated for 4 h with 10 μg of actinomycin D per ml. The cells were then fixed with 3.7% formaldehyde for 20 min, permeabilized with 0.1% Nonidet P-40 for 10 min, and analyzed by indirect immunofluorescence with the anti-AU-1 or anti-HA1 monoclonal antibody and fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin antibody (Sigma).

Metabolic labeling and immunoprecipitation. At 22 h after transfection, HLtat cells were incubated for 1 h in either phosphate-free, methionine/cysteine-free, or leucine-free Dulbecco's modified Eagle's medium containing 1% fetal calf serum and then metabolically labeled with either 200 μCi of [^{32}P]orthophosphate (Amersham) per ml, 300 μCi of [^{35}S]methionine-[^{35}S]cysteine mixture (Promix; Amersham) per ml, or 300 μCi of [^{3}H]leucine (Amersham) per ml. After 3 h, the cells were lysed in 1× RIPA buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 μg of leupeptin per ml) and sheared five times through a 26-gauge needle; alternatively, nuclear and cytoplasmic fractions were prepared and then diluted in 1× RIPA buffer (see below). Following preclearing with normal mouse serum and protein A-Sepharose, the metabolically labeled proteins were immunoprecipitated with anti-AU-1 mouse monoclonal antibody. Immune complexes were sedimented with protein A-Sepharose, solubilized, and analyzed by SDS-PAGE in 16% T–2.6% C gels. The gels were enhanced with 1 M sodium salicylate, dried, and exposed to X-ray film.

Subcellular fractionation. Cells were metabolically labeled with [^{35}S]methionine-[^{35}S]cysteine, lysed under nondenaturing conditions in the presence of 0.5% Nonidet P-40, and separated into nuclear and cytoplasmic fractions by sedimentation on a 24% sucrose cushion (28). The resulting cytoplasmic and nuclear fractions were combined with 9 volumes of 1× RIPA, immunoprecipitated, and analyzed by SDS-PAGE as described above. The unphosphorylated form of Rex_2 and the components of mitochondrial complex III were used as nuclear and cytoplasmic markers, respectively, to control for the quality of the fractionation technique. Anti-mitochondrial complex III serum was a gift of Roberto Bisson.

Protein binding assays. Recombinant GST-Rex_2-AU1 fusion protein was produced in *Escherichia coli* BL21 transformed with pGST-Rex_2-AU1. Bacteria were lysed by sonication, and recombinant GST-Rex_2 was purified by binding to glutathione-Sepharose (53) and incubated with extracts of transfected, metabolically labeled HLtat cells prepared in a nondenaturing buffer as described previously (19). Assays carried out with unfused GST protein were included as negative controls. The complexes attached to the beads were washed, solubilized, and analyzed by SDS-PAGE in 16% T–2.6% C gels.

RESULTS

Suppression of Rex_2 function by the x-III proteins. To analyze the possible interactions of the x-III proteins with Rex_2 at the functional level, we developed a quantitative bioassay to measure Rex_2 activity by using the reporter plasmid pCgagRXRE-II. This plasmid contains the HIV-1 LTR and *gag* gene, linked to the RXRE of HTLV-2 and the simian virus 40 polyadenylation signal/site. Upon transfection of the HLtat cell line, *gag*–RXRE-II mRNA is transcribed, but its basal level expression is greatly impaired by the presence of inhibitory sequences within the *gag* gene (48). Production of Gag protein from this plasmid is strictly dependent on rescue of the *gag*–RXRE-II mRNA through binding of Rex_2 to the RXRE-II element. Cotransfection of pCgagRXRE-II with increasing amounts of the Rex_2 expression plasmid pLsRex_2-AU1 resulted in a dose-dependent increase of HIV-1 Gag expression (data not shown), which could easily be monitored by immunoblot analysis with an anti-Gag antibody and quantitated by a Gag antigen capture assay.

To evaluate the effects of the x-III proteins on Rex_2 activity, HLtat cells were transfected with constant amounts of pCgag RXRE-II reporter plasmid and pLsRex_2-AU1 in the absence or presence of serial dilutions of a plasmid coding for the x-III proteins (pLsX-III-AU1). Cells were harvested 22 h after transfection and assayed for Gag production by a Gag-specific antigen capture assay (Fig. 2A) or immunoblotting (Fig. 2B) and assayed for production of AU1-tagged Rex_2 and x-III proteins by immunoblotting with an AU1-specific monoclonal antibody (Fig. 2C).

Figure 2B shows an anti-Gag immunoblot demonstrating the production of Gag protein (detected in its 55-kDa precursor form) from pCgagRXRE-II in the presence of the Rex_2 plasmid and in the absence of the x-III proteins (lane 4). Coexpression of increasing amounts of the x-III proteins along with Rex_2 resulted in a dramatic dose-dependent inhibition of Gag production (lanes 1 to 3). To obtain a quantitative evaluation of the amount of Gag protein produced in these transfections, portions of the lysates were analyzed by a Gag antigen capture assay. Activation of Gag expression was calculated from the concentrations of Gag protein measured in the cell lysates (see Materials and Methods); the fold induction of Gag expression by Rex_2 in the absence of the x-III proteins was considered to be 100% activation and was used as a reference to express other values as percentages of activation, as shown in the bar graph in Fig. 2A. Cotransfection of 0.2 μg of pLsRex_2-AU1 with 2.5 μg (lane 1), 1.25 μg (lane 2), or 0.625 μg (lane 3) of pLsX-III-AU1 resulted in a reduction of Rex_2 activity corresponding to 3% (lane 1), 13% (lane 2), and 45% (lane 3), respectively, of the full Rex_2 response (lane 4).

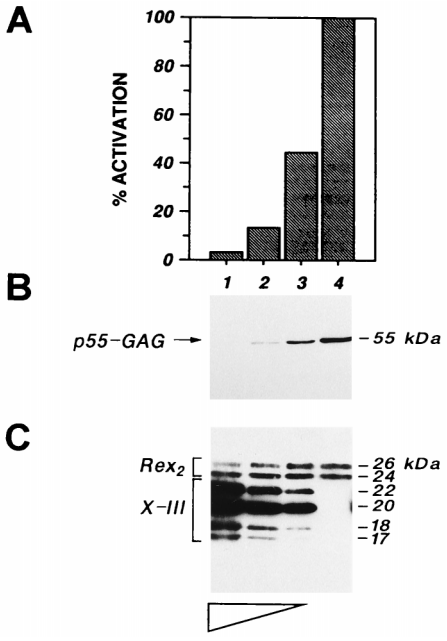

FIG. 2. Suppression of Rex_2 function by the x-III proteins. HLtat cells were seeded in 35-mm petri dishes and cotransfected with pCgagRXRE-II (1 μg), pL3CAT (1 μg), and pLsRex_2-AU1 (0.2 μg) in the absence (lane 4) or presence of 2.5 μg (lane 1), 1.25 μg (lane 2), or 0.625 μg (lane 3) of pLsX-III-AU1. The total amount of DNA in all transfections was brought to 7.5 μg with pBluescript (Stratagene) as a carrier. (A) Results of Gag antigen capture assay of lysates of cells prepared as described in Materials and Methods. Activation values are expressed as a percentage of the Gag production stimulated by Rex_2 in the absence of x-III proteins (100% activity [lane 4]). (B and C) Immunoblots of the same lysates, carried out with an anti-Gag monoclonal antibody (B) or anti-AU-1 monoclonal antibody (C) to detect Rex_2 and the x-III proteins. Indicated are the 55-kDa HIV Gag precursor (B) and the different isoforms of Rex_2 (26 and 24 kDa) and the x-III proteins (22, 20, 18, 17 kDa) (C).

Figure 2C shows the amounts of Rex_2 and x-III proteins produced in each transfection, detected by immunoblot analysis with a monoclonal antibody specific for the AU-1 tag epitope, which was expressed at the carboxy terminus of both Rex_2 and the x-III proteins. In agreement with previous reports (24, 25, 49), Rex_2 was detected as two major bands of 26 and 24 kDa (Fig. 2C) which are known to differ by their extent of phosphorylation. As we described previously (15), four x-III protein isoforms of 22, 20, 18, and 17 kDa were detected in cells cotransfected with pLsX-III-AU1 (Fig. 2C, lanes 1 to 3). It is noteworthy that although the Rex_2 plasmid contained the internal x-III AUGs, it did not express detectable levels of the

x-III proteins (lane 4). Since all of the transfections were performed with the same amount of pLsRex$_2$-AU1, production of Rex$_2$ protein also served as an indicator of transfection efficiency. Comparison of the levels of Rex$_2$ in lanes 1 to 4 showed that similar amounts of the protein were produced in all of the transfections, indicating that the inhibition of Gag protein production in the presence of the x-III proteins was not due to differences in transfection efficiency and that the x-III proteins did not affect Rex$_2$ synthesis. However, interestingly, in the presence of the highest excess of x-III proteins, a selective reduction of the hyperphosphorylated 26-kDa form of Rex$_2$ was observed while the amount of the hypophosphorylated 24-kDa Rex$_2$ remained unchanged (Fig. 2C, lane 1); this observation prompted us to further investigate the possible effects of the x-III proteins on Rex$_2$ phosphorylation (see below).

To determine which of the x-III proteins was responsible for inhibition of Rex$_2$ function, similar cotransfection assays were performed with expression plasmids carrying a point mutation in the first (pLsX-III-Δ3-AU1) or the second (pLsX-III-Δ4-AU1) internal AUG codon of the x-III ORF (nt 7247 and 7337, respectively). Results of these studies showed that pLsX-III-Δ4-AU1, which produced the major p22/20$^{x\text{III}}$ proteins, retained the inhibitory effects of pLsX-III-AU1 while pLsX-III-Δ3-AU1, which produced only p17$^{x\text{III}}$, did not influence Rex$_2$ activity (data not shown). These observations indicated that the inhibitory effect exerted by the x-III proteins was expressed by its major 22- and 20-kDa isoforms.

Given that Rex$_1$ and Rex$_2$ are functionally interchangeable (31), we were interested in testing the ability of the x-III proteins to inhibit Rex$_1$-mediated activation of RXRE-I. HLtat cells were transfected with the Rex$_1$ expression plasmid pL3Rex (54) and the RXRE-I reporter plasmid pCgagRXRE (2) in the absence or presence of increasing amounts of pLsX-III-AU1. Immunoblots and Gag antigen capture assays of lysates prepared from the transfected cells demonstrated that the x-III proteins were able to inhibit Rex$_1$ activity with an efficiency similar to that observed for the Rex$_2$/RXRE-II system (data not shown).

By analogy to the Rex/RXRE regulatory pathway of HTLV, the HIV-1 Rev protein activates the expression of unspliced and singly spliced HIV-1 mRNAs through interaction with an RNA target termed the Rev-responsive element (RRE) (reviewed in ref. 38). Cross-activation experiments have shown that Rex can activate RRE-containing RNAs (41) and that some synthetic *trans*-dominant repressors of Rex function are also able to inhibit the Rev/RRE response (11, 42). This prompted us to test the possible influence of the x-III proteins on the functional activity of Rev. HLtat cells were transfected with a plasmid coding for the HIV-1 LTR, *gag* gene, and RRE (plasmid pCgagA2pA [2]) in the absence or presence of the Rev expression plasmid pBsRev (19) and either pLsX-III-AU1 or a plasmid coding for a *trans*-dominant mutant Rev protein (plasmid pLBL [33]). Lysates of the cells were analyzed by SDS-PAGE and immunoblotted to detect Gag (Fig. 3A), the x-III proteins (Fig. 3B), and Rev (Fig. 3C). The results of the anti-Gag immunoblot showed that coexpression of the x-III proteins did not interfere with the ability of Rev to activate the *gag*-RRE mRNA (Fig. 3A, compare lane 2 with lanes 3 and 4); in contrast, coexpression of the *trans*-dominant mutant Rev protein resulted in a clear inhibition of Gag protein production (Fig. 3A, lanes 5 and 6). Anti-AU-1 and anti-Rev immunoblots confirmed the expression of the x-III proteins (Fig. 3B) and *trans*-dominant Rev (Fig. 3C); the level of wild-type Rev protein produced in these assays was too low to be detected by immunoblotting.

Taken together, the results of these functional assays demonstrated that the x-III proteins acted as specific potent inhibitors of Rex function. To find clues to the mechanism underlying this inhibition, we investigated the effect of the x-III proteins on the following properties of Rex$_2$ which are considered to be essential for its activity: intracellular trafficking, phosphorylation, and multimerization.

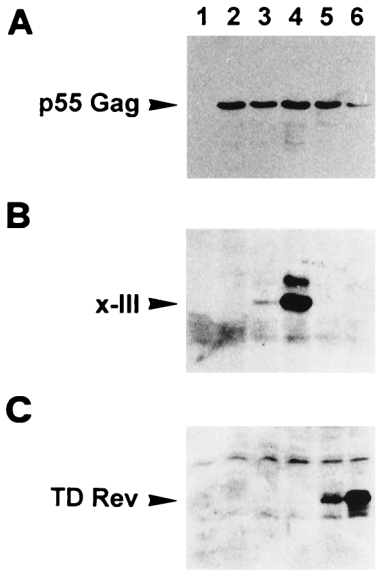

FIG. 3. The x-III proteins do not inhibit HIV-1 Rev function. HLtat cells were seeded in 35-mm petri dishes and transfected with 3 μg of pCgagA2pA in the absence or presence of 0.07 μg of pBsRev (lanes 1 and 2, respectively) and 0.15 or 1.2 μg of either pLsX-III-AU1 (lanes 3 and 4, respectively) or pLBL (lanes 5 and 6, respectively). The total amount of DNA in all transfections was brought to 7.5 μg with pBluescript as a carrier. Shown are immunoblots of the cell lysates carried out with anti-Gag monoclonal antibody to detect the 55-kDa Gag protein (A), anti-AU-1 monoclonal antibody to detect the x-III proteins (B), and rabbit anti-Rev antibody (19) to detect *trans*-dominant mutant (TD) Rev (C).

Intracellular trafficking properties of Rex$_2$ and the x-III proteins. Recent studies demonstrated that Rex$_1$ shuttles between the nucleus and the cytoplasm, a property thought to be tightly linked to its role in nucleocytoplasmic mRNA transport (32, 37, 55). Further investigations provided evidence that a nuclear export factor interacting with the activation domain of Rex controls the export leg of this shuttling process (10, 37). Given that the x-III proteins contain the activation domain of Rex$_2$ (Fig. 1), we hypothesized that the functional inactivation of Rex$_2$ by the x-III proteins might reflect an interference with the intracellular trafficking of Rex$_2$. To test this, we transfected HLtat cells with either pLsRex$_2$-AU1 alone, pLsX-III-HA1 alone, or both pLsRex$_2$-AU1 and pLsX-III-HA1. The cells were then fixed, permeabilized, and probed with anti-AU-1 antibody or anti-HA1 antibody to selectively visualize either Rex$_2$ or the x-III proteins, respectively. Preliminary experiments carried out by conventional fluorescence microscopy showed that when expressed alone, the x-III proteins showed a diffuse distribution throughout the cell (15). When coexpressed with Rex$_2$, the x-III proteins exhibited an increased accumulation in the nuclear compartment and in areas corresponding to nucleoli; in contrast, the nucleolar/nuclear distribution of Rex$_2$ did not appear to be affected by coexpression of the x-III proteins (data not shown). Additional experiments carried out by confocal microscopy confirmed that Rex$_2$ accumulated primarily in nucleoli/nuclei when expressed alone (Fig. 4, Rex$_2$ panel) or in combination with the x-III proteins (data not shown). When expressed alone, the x-III proteins

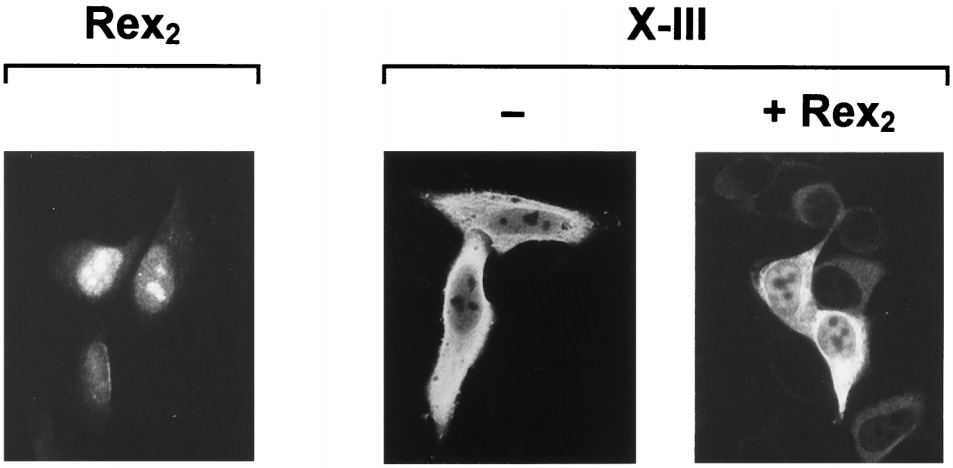

FIG. 4. Redistribution of the intracellular compartmentalization of the x-III proteins in the presence of Rex_2. HLtat cells were seeded in chamber slides (Nunc Inc.) and cotransfected with 1 μg of pLsRex_2-AU1 and either 3 μg of pBluescript or 3 μg of pLsX-III-HA1. After 22 h, the cells were harvested for indirect immunofluorescence by using anti-AU-1 monoclonal antibody (Rex_2 panel) or anti-HA1 monoclonal antibody (x-III panels). The cells were then analyzed by confocal microscopy with a Nikon RCM 8000 confocal microscope.

were detected primarily in the cytoplasm, showed a very weak signal in the nucleus, and appeared to be excluded from nucleoli (Fig. 4, x-III − panel). When coexpressed with Rex_2, the x-III proteins exhibited a slightly increased accumulation in the nucleus, particularly in areas immediately surrounding the nucleoli (Fig. 4, x-III + Rex_2 panel). A similar redistribution of x-III localization was also observed when the proteins were coexpressed with Rex_1 (data not shown). Given that the x-III proteins lack an NLS, the ability of Rex_2 to change the level of nuclear accumulation of the x-III proteins suggested a possible direct Rex_2–x-III interaction.

We next investigated the effect of the x-III proteins on the ability of Rex_2 to shuttle between the nucleus and cytoplasm. This shuttling process can be studied by treating Rex_2-expressing cells with actinomycin D, a treatment that results in disruption of the nucleolar structure and redistribution of the Rex signal into the cytoplasmic compartment. Cells transfected with plasmids expressing Rex_2-AU1 and/or the x-III-HA1 proteins were incubated for 4 h in the absence or presence of 10 μg of actinomycin D per ml and examined by indirect immunofluorescence with anti-AU-1 and anti-HA1 antibodies. Results of anti-AU-1 immunofluorescence assays showed that when expressed alone, Rex_2 showed a strong cytoplasmic signal upon treatment with actinomycin D [Fig. 5, compare (−) and +ActD panels]. Interestingly, the simultaneous expression of Rex_2 and the x-III proteins resulted in a marked decrease in the amount of Rex_2 detected in the cytoplasmic compartment in actinomycin D-treated cells (Fig. 5, +ActD +x-III panel). Incubation of replica plates with anti-HA1 antibody verified the production of the HA1-tagged x-III proteins (data not shown). The observation that the x-III proteins blocked the redistribution of Rex_2 into the cytoplasm of actinomycin D-treated cells suggested that they might interfere with the export leg of the Rex_2 shuttling pathway.

Alteration of the phosphorylation pattern of Rex_2 in the presence of the x-III proteins. Studies of the functional significance of Rex_2 phosphorylation indicate that the hyperphosphorylated 26-kDa band is the form that is competent for binding to RXRE in vitro (25) and for inhibiting splicing in vitro (1). These observations prompted us to determine whether the x-III proteins also bear this modification and whether their expression might influence the phosphorylation of Rex_2. HLtat cells were transfected with pLsRex_2-AU1 or pLsX-III-AU1, metabolically labeled with [^{3}H]leucine or [^{32}P]orthophosphate, and subjected to immunoprecipitation with the anti-AU-1 antibody followed by SDS-PAGE analysis. Figure 6 shows that the relative intensities of the bands corresponding to Rex_2 (compare lanes 1) as well as the x-III proteins (compare lanes 2) differed depending on whether [^{3}H]leucine or [^{32}P]orthophosphate was used for the metabolic labeling. Both major 22- and 20-kDa isoforms of the x-III proteins were phosphorylated, although the 22-kDa product appeared to incorporate more ^{32}P label than the 20-kDa band. Pulse-chase labeling experiments carried out with [^{3}H]leucine showed that the 22-kDa hyperphosphorylated band became more abundant during the chase while the 20-kDa band decreased in intensity (data not shown). This observation supported the results of a mutational analysis which suggested that

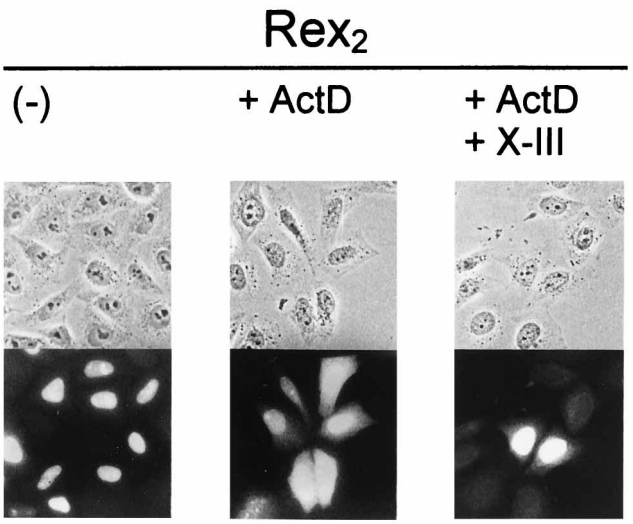

FIG. 5. Sequestration of Rex into the nuclear compartment of actinomycin D-treated cells in the presence of the x-III proteins. HLtat cells were seeded in 35-mm petri dishes and cotransfected with 1 μg of pLsRex_2-AU1 and either 6.5 μg of pBluescript or 6.5 μg of pLsX-III-HA1. At 20 h after transfection, the cells were incubated for an additional 4 h in the absence or presence of 10 μg of actinomycin D (ActD) (Sigma) per ml and then harvested for indirect immunofluorescence by using anti-AU-1 monoclonal antibody (shown here) or anti-HA1 monoclonal antibody (not shown). The cells were examined with a Zeiss Axioplan fluorescence microscope.

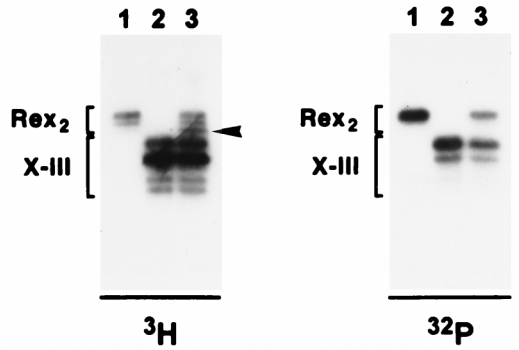

FIG. 6. Alteration of the Rex_2 phosphorylation pattern in the presence of the x-III proteins. HLtat cells were seeded in 35-mm petri dishes and transfected with pLsRex$_2$-AU1 (1.5 μg [lanes 1]) or pLsX-III-AU1 (6 μg [lanes 2]) or cotransfected with both plasmids (1.5 μg and 6 μg, respectively [lanes 3]); the total amount of DNA in each transfection was brought to 7.5 μg with pBluescript as a carrier. The cells were then metabolically labeled with [^{3}H]leucine (^{3}H panel) or [^{32}P]orthophosphate (^{32}P panel), lysed, and immunoprecipitated with anti-AU-1 monoclonal antibody. Shown is an SDS-PAGE analysis of the immunoprecipitated proteins. The arrowhead indicates the position of unphosphorylated Rex.

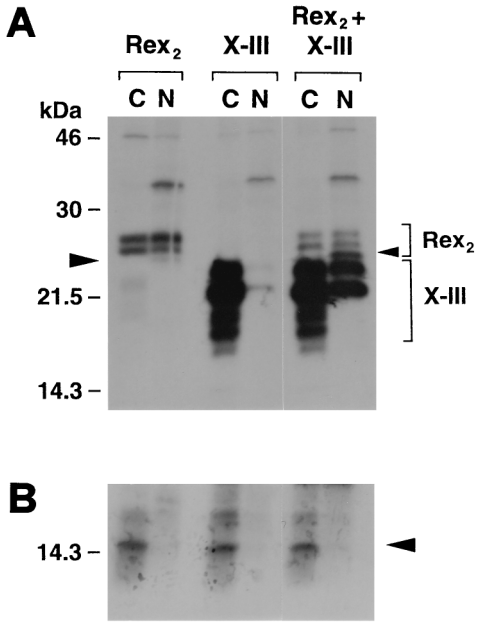

FIG. 7. Intracellular compartmentalization of the x-III proteins and the different phosphorylated forms of Rex_2. HLtat cells were seeded in 60-mm petri dishes and transfected with either 4 μg of pLsRex$_2$-AU1, 4 μg of pLsRex$_2$-AU1 and 12 μg of pLsX-III-AU1, or 12 μg of pLsX-III-AU1; the total amount of DNA was brought to 16 μg with pBluescript as a carrier. The cells were metabolically labeled with [^{35}S]methionine-[^{35}S]cysteine and then separated into cytoplasmic (C) and nuclear (N) fractions as described previously (28); each fraction was subjected to immunoprecipitation under denaturing conditions with anti-AU-1 monoclonal antibody (A) or an antiserum raised against the multienzymatic complex III purified from bovine mitochondria (B). Shown is an SDS-PAGE analysis of the immunoprecipitated proteins. Arrowheads indicate the positions of unphosphorylated Rex as a nuclear marker band (A) and a 15-kDa component of mitochondrial complex III as a cytoplasmic marker band (B).

the 22-kDa band is derived from the 20-kDa product by post-translational modification (15). Taken together, these findings showed that by analogy to Rex_2, the major x-III proteins exhibited different migration properties that correlated with their extent of phosphorylation. Lanes 1 of Fig. 6 show the two major 26- and 24-kDa forms of Rex_2. As described previously (24, 25), the 26-kDa band represented a highly phosphorylated form of Rex_2; longer exposures of the gels confirmed that the 24-kDa band was also weakly phosphorylated (data not shown). In agreement with earlier studies (25), a faster-migrating minor band representing a completely unphosphorylated form of Rex_2 could be observed in some metabolic labeling experiments. This unphosphorylated Rex_2 band was not detected by immunoblotting, possibly because of its poor recovery in the cell lysis procedure used for the immunoblots and/or its rapid turnover into the phosphorylated forms.

Figure 6 also shows a comparison of the expression pattern of Rex_2 and the x-III protein isoforms when coexpressed (lanes 3). Interestingly, the concomitant expression of Rex_2 and x-III proteins resulted in a shift of the phosphorylation pattern of Rex_2, with a marked relative increase of the faster-migrating, unphosphorylated form (indicated by the arrow in panel ^{3}H, lane 3), which was barely detectable in the absence of the x-III proteins (panel ^{3}H, lane 1). As noted in the immunoblot analysis carried out for the functional assays, a high excess of x-III proteins that virtually abrogated Rex-dependent Gag production also resulted in a relative decrease in the intensity of the hyperphosphorylated 26 kDa form of Rex_2 (Fig. 2C, lane 1). These data indicated that the x-III proteins changed the phosphorylation pattern of Rex_2 toward a relative overrepresentation of the hypophosphorylated and unphosphorylated forms.

Intracellular compartmentalization of the x-III proteins and the different phosphorylated forms of Rex_2. The observations that the x-III proteins were able to sequester Rex_2 into the nuclear compartment in actinomycin D-treated cells and to change its phosphorylation state led us to analyze the intracellular compartmentalization of the different phosphorylated forms of Rex_2 in the absence and presence of the x-III proteins. Cells were transfected with a Rex_2–AU1-expressing plasmid and/or a plasmid expressing x-III–AU1 proteins, metabolically labeled with [^{35}S]methionine-[^{35}S]cysteine, lysed under nondenaturing conditions, and separated into nuclear and cytoplasmic fractions as described previously (28). The fractions were then immunoprecipitated under denaturing conditions with anti-AU-1 antibody (Fig. 7A) or an antiserum raised against mitochondrial proteins, which served as a control for the absence of cytoplasmic contaminants in the nuclear fraction (Fig. 7B). SDS-PAGE analysis of the immunoprecipitated proteins revealed that when Rex_2 was expressed alone, the faster-migrating (unphosphorylated) form (see arrowhead) was found exclusively in the nuclear fraction and thus served as an internal standard indicating the absence of artifactual nuclear leakage; the hyperphosphorylated 26-kDa and hypophosphorylated 24-kDa forms appeared to be more evenly distributed in the two compartments (Fig. 7A, Rex_2 lanes). Coexpression of Rex_2 and the x-III proteins resulted in a decrease in the relative amount of hyperphosphorylated 26-kDa Rex_2, especially in the nuclear fraction, while the hypophosphorylated form was less affected; in contrast, the relative amount of the unphosphorylated form of Rex_2 showed a clear increase (Fig. 7A, Rex_2 + x-III lanes). This overall rearrangement of the ratio of phosphorylated to unphosphorylated Rex_2 in the presence of the x-III proteins confirms and extends the data obtained with unfractionated cell lysates detected by metabolic labeling (Fig. 6) and by immunoblotting (Fig. 2). Interestingly, in the presence of Rex_2, a partial redistribution of the x-III proteins into the nuclear fraction was observed (Fig. 7A, Rex_2 + x-III lanes) compared with their almost exclusive cytoplasmic localization in the absence of Rex_2 (Fig. 7A, x-III lanes). This observation is in agreement with the partial redistribution of the x-III proteins into the nuclear compartment that was observed in immunofluorescence analyses of cells transfected with both Rex_2 and the x-III proteins (Fig. 4).

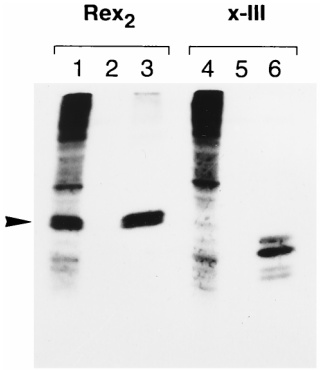

FIG. 8. Binding assays of Rex_2 and the x-III proteins. Lysates of metabolically labeled HLtat cells expressing Rex_2-AU1 or x-III-AU1 proteins were incubated with GST-Rex_2 in binding assays as described in Materials and Methods and analyzed by SDS-PAGE. Lanes: 1, binding of Rex_2 (indicated by the arrowhead) to GST-Rex_2; 4, lack of binding of GST-Rex_2 to the x-III proteins; 2 and 5, control assays with unfused GST; 3 and 6, immunoprecipitations of a portion of each radiolabeled HLtat cell lysate with anti-AU-1 monoclonal antibody.

Similar fractionation studies carried out with Rex_1 and the x-III proteins indicated that coexpression of Rex_1 resulted in a partial accumulation of the x-III proteins in the nucleus; however, this redistribution effect was not as striking as that seen in cotransfections of Rex_2 and the x-III proteins (data not shown). The effect of the x-III proteins on the level of Rex_1 phosphorylation could not be assessed by this approach, since, in contrast to Rex_2, Rex_1 is detected as a single band by SDS-PAGE.

Taken together, these data linked the phosphorylation of Rex_2 to its intracellular compartmentalization, suggesting a possible role of phosphorylation in the control of Rex's nucleocytoplasmic shuttling and functional properties.

Multimerization properties of Rex_2 and the x-III proteins. As shown in Fig. 1, the x-III proteins contain a domain that, by analogy to HTLV-1, is predicted to be important for the ability of Rex_2 to form multimeric complexes. In addition, the data obtained by immunofluorescence and fractionation studies indicated a change in the subcellular distribution of the x-III proteins upon coexpression with Rex_2, suggesting their direct interaction. It appeared possible, therefore, that the inhibition of Rex_2 function by the x-III proteins was partly due to their ability to form inactive Rex_2–x-III complexes. This hypothesis was tested by analyzing the direct in vitro association of bacterially produced GST-Rex_2 with radiolabeled Rex_2 and x-III proteins produced in transfected HLtat cells. Recombinant GST-Rex_2 fusion protein was expressed in bacteria and purified by affinity chromatography with Sepharose-immobilized glutathione. Purified GST-Rex_2 attached to the resin was incubated under nondenaturing conditions with extracts of metabolically labeled HLtat cells that had been transfected with pLsRex_2-AU1 or pLsX-III-AU1. Multimeric complexes between in vivo-labeled proteins and bacterially produced GST-Rex_2 were washed, solubilized from the resin, and analyzed by SDS-PAGE and fluorography. The results of this assay showed that specific binding of Rex_2 to GST-Rex_2 was readily detected (Fig. 8, lane 1), providing direct evidence for the ability of Rex_2 to form homomultimeric complexes even under in vitro conditions. In contrast, binding of the x-III proteins to GST-Rex_2 was not observed (lane 4), nor did cotransfection of Rex_2 and x-III proteins result in a decreased GST-Rex_2/Rex_2 binding (data not shown), suggesting that the x-III proteins did not efficiently multimerize with Rex_2 or affect its ability to form homomultimers. Lanes 2 and 5 show that neither Rex_2 nor the x-III proteins recognized unfused GST protein attached to the resin, indicating the specificity of the binding between Rex_2 and GST-Rex_2. Lanes 3 and 6 show immunoprecipitations of portions of the HLtat lysates with AU1-specific monoclonal antibody, carried out to confirm the presence of Rex_2 and the x-III proteins in the HLtat cell extracts. These results provide direct evidence of the capability of Rex_2 to form homomultimeric complexes, a process that is presumed to be necessary for the function of the protein, based on extensive studies of HTLV-I Rex and the homologous protein of HIV, Rev (38). The finding that the x-III proteins failed to bind GST-Rex_2 under the same experimental conditions suggested that the x-III proteins did not interfere with the normal homomultimerization process of Rex_2.

DISCUSSION

In previous studies, we showed that alternatively spliced HTLV-2 mRNAs produce x-III proteins that can be regarded as aminoterminally-deleted forms of Rex_2 (15). The present study demonstrates that these products act as *trans*-dominant inhibitors of Rex_2 activity in a functional bioassay that tests the ability of Rex_2 to rescue the expression of a *gag*–RXRE-II reporter mRNA (Fig. 2). Additional assays showed that the x-III proteins were also able to work as *trans*-dominant inhibitors of Rex_1 activity but were inactive against the Rev-RRE system of HIV-1 (Fig. 3).

The present knowledge of Rex structure and function suggests three possible mechanisms whereby a mutant Rex protein could inhibit wild-type Rex: (i) competition for required cellular factors by a mutant containing an intact activation domain but lacking a functional RNA binding domain or multimerization domain; (ii) formation of inactive mixed multimers between wild-type Rex and a mutant lacking a functional RNA binding domain or activation domain; and (iii) competition for binding to RXRE by a mutant with a defective multimerization or activation domain. The fact that the x-III proteins lack an RNA binding domain but possess potentially functional multimerization and activation domains allowed us to exclude the third mechanism and focus on other biological properties of Rex_2 that are thought to be tightly linked to its function, i.e., intracellular trafficking, phosphorylation, and multimerization.

Studies of Rex_2 subcellular compartmentalization in cells treated with actinomycin D, an inhibitor that is used to study nuclear retention versus import-export competence, demonstrated that the x-III proteins were able to induce a sequestration of Rex_2 into the nuclear compartment of actinomycin D-treated cells (Fig. 5). Studies of *trans*-dominant Rev mutants that contain intact NLS/RNA binding and multimerization domains but a defective activation domain demonstrated that these proteins are retained in the nucleus of actinomycin D-treated cells (19, 34) whereas wild-type Rev (19, 28, 34, 39) and Rex_1 (32, 55) are redistributed to the cytoplasm. These results were extended by the observation that coexpression of *trans*-dominant mutant Rev and wild-type Rev in actinomycin D-treated cells increases the nuclear retention of Rev (55, 56), leading to the proposal that such mutants form heterodimers with Rev that are unable to interact with a nuclear export factor that binds to the activation domain (10). Similar recent studies on the effect of $p21^{Rex}$ on Rex_1 trafficking demonstrated that this protein is also able to increase the nuclear retention of Rex_1 in actinomycin D-treated cells (32). Unlike *trans*-dominant mutant Rev proteins, $p21^{Rex}$ lacks the NLS/RNA binding and multimerization domains but possesses an intact activation domain, leading to the hypothesis that it disrupts Rex_1 trafficking by competing for the nuclear export factor (32). This latter mechanism could also explain the ob-

served nuclear sequestration of Rex_2 in the presence of the x-III proteins. Unfortunately, no data have been reported regarding the possible effects of $p21^{Rex}$ on Rex_1 function, phosphorylation, or multimerization; therefore, it remains unclear whether $p21^{Rex}$ and the x-III proteins can be regarded as functional homologs.

Coexpression of Rex_2 with the x-III proteins resulted in a clear reduction in the ratio between hyperphosphorylated and hypo- or unphosphorylated Rex_2 (Fig. 6 and 7); the decrease in the expression of the 26-kDa hyperphosphorylated form of Rex_2 correlated well with the decrease in Rex_2 function (Fig. 2). This disturbance of the Rex_2 phosphorylation pattern is likely to have important consequences for its activity, since recent studies have shown that both the binding of Rex_2 to RXRE-II (25) and its ability to inhibit splicing in vitro (1) correlate with its phosphorylation. The finding that the nucleocytoplasmic compartmentalization of Rex_2 was connected to its phosphorylation status (Fig. 7) suggested a possible control of Rex_2 intracellular trafficking and function by phosphorylation. Phosphorylation has already been shown to play a key role in directing the nuclear import of the HIV matrix protein (21), the T antigen of SV40 (40), and the cellular transcriptional activator alpha interferon-stimulated gene factor 3 (46). The finding that the x-III proteins are efficiently phosphorylated (Fig. 6) raises the question whether their effect on Rex_2 phosphorylation is due to competition for protein kinases or whether it results from their disruption of the intracellular compartmentalization of Rex_2.

On the basis of the results of our in vitro protein binding assays (Fig. 8), it appears unlikely that the x-III proteins form inactive heteromultimeric complexes with Rex_2. However, these negative results do not rule out the possibility that Rex_2 and the x-III proteins do interact but with a lower affinity than the Rex_2-Rex_2 interaction or that their interaction might be revealed only by using in vivo assays such as the two-hybrid system. Nevertheless, taken together, our data argue against the formation of inactive x-III–Rex_2 multimers and suggest that the inhibitory effect of the x-III proteins is likely to result from an interference with the intracellular trafficking and phosphorylation of Rex, presumably by sequestering cellular factors involved in nuclear export and/or protein kinases. This proposal is consistent with data obtained by Katahira et al., who studied a chimeric Rex_1 mutant in which the amino-terminal NLS/RXRE binding domain was replaced by the SV40 NLS (29). Although this chimera did not interact with RXRE RNA, it was able to inhibit Rex function in a *trans*-dominant manner. The inhibitory activity of the mutant required an intact activation domain but was not affected by mutation of the multimerization sequences, indicating that its action results from competition for cellular cofactors. Extensive mutational analysis of different functional domains in Rex_2 and the x-III proteins will be necessary to establish precise cause-effect correlations among Rex_2 function, phosphorylation, and trafficking and to directly link the inhibitory effect of the x-III proteins to a specific cellular process.

The inhibitory properties of the x-III proteins and the SV40-Rex_1 chimera are in apparent contrast to the phenotype of Rex_1 mutants described by Rimsky et al. (42), carrying amino acid substitutions in the NLS/RXRE binding domain. These mutants, which contained missense mutations in amino acids 5 to 7 (RRR→DL; mutant M1) or 14 to 15 (KR→DL; mutant M2), were found to be impaired in their biological activity and nuclear targeting, probably due to disruption of the NLS/RNA binding properties of the protein. However, unlike the SV40-Rex_1 chimera and the x-III proteins of HTLV-2, mutants M1 and M2 did not exhibit a *trans*-dominant phenotype, indicating that they were also impaired in their ability to interact with wild-type Rex_1 or with cellular proteins necessary for the Rex response, e.g., export factors or kinases. One possible explanation for these apparently contradictory results is that the introduction of acidic residues in the NLS/RXRE binding domain might disrupt the overall conformation of Rex_1 and impair the function of domains located further downstream. To address this point, it will be necessary to analyze additional mutations in this domain.

Previous studies showed that the AUG codons from which the x-III proteins are translated are not required for virus replication and transformation in vitro (23). However, recent in vivo analyses carried out on rabbits infected with HTLV-2 pointed out that the proximal part of the X region, which was previously shown to be dispensable for viral replication and transformation in vitro (26), is indeed important for maintaining high viral replication in vivo (17). It is noteworthy that similar discrepancies between in vivo and in vitro data have been described for the *nef* gene of simian immunodeficiency virus, whose function is essential only during natural infection (30). Therefore, it is possible that the x-III proteins of HTLV-2 play an important role in infection in vivo but that their functions may not be easily recognized in experimental systems based on in vitro propagation assays. The fact that Rex_2 and the x-III proteins must be expressed from distinct mRNAs suggests that production of Rex_2 and its inhibitors might be regulated differently in the viral life cycle by control of the alternative splicing pattern of viral mRNAs, thus contributing a genetic switch to control the expression of Rex-dependent viral mRNAs. Further studies are required to investigate the production of the x-III proteins in the context of natural infection and their possible effect on the life cycle/transforming potential of HTLV-2.

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