Functional Conservation of HTLV-1 Rex Balances the Immune Pressure for Sequence Variation in the Rex Gene

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Naturally occurring mutations in Human T-cell Leukemia Virus Type 1 (HTLV-1) Tax protein lead to loss of recognition by cytotoxic T-lymphocytes. Most of these mutations also abolish or severely impair the transactivation function of Tax. Ninety percent of the rex gene, which encodes the viral regulator of mRNA splicing (Rex), overlaps with the tax gene. In this paper, we report that four previously described point mutations in tax that abolished CTL recognition and activity did not alter either the dimerisation function or the ability to export viral mRNA of the corresponding Rex proteins. Rex proteins containing two other amino acid changes were likewise functional. However, five Rex deletion mutants, predominantly but not exclusively found in HAM/TSP patients, had all lost these functions. We conclude that, although the Tax protein is subject to strong CTL-mediated selection, there are stronger functional constraints on amino acid variation in Rex. This may limit the variation in the tax/rex nucleotide sequence which results in immune evasion.

Key Words: HTLV-1; Rex; immune selection; naturally occurring mutants; impaired function; consensus sequence.

INTRODUCTION

Strong recognition of HTLV-1 Tax antigen by cytotoxic T-lymphocytes (CTL) in infected individuals is well established (Jacobson et al., 1990; Parker et al., 1994). Many of the CTL epitopes in Tax are restricted by HLA-A2 MHC class-I molecules and several naturally occurring mutations have been identified within the sequences encoding these epitopes (Niewiesk et al., 1995). Where these result in an amino acid change, the altered peptide is no longer recognized by CTL raised to the index protein sequence (Niewiesk et al., 1995), providing evidence for immune driven selection of viral sequence variants. In addition, the ability of Tax proteins bearing these mutations to transcriptionally activate three different promoter sequences was shown to be deficient compared with wild-type Tax (Niewiesk et al., 1994).

HTLV-1 Tax and Rex are encoded by overlapping reading frames (90% of the coding sequence for Rex overlaps that encoding Tax). Rex is an essential posttranscriptional regulatory protein that mediates the nuclear export and subsequent translation of incompletely spliced viral mRNAs that encode structural proteins (Inoue et al., 1987). However, Rex has not been shown to be a target for direct immune recognition by CTL (Daenke et al., 1996), although the mutations imposed by CTL recognition of Tax in some cases transpose to the Rex protein.

It is unclear, therefore, whether Rex function is compromised by the immune reactivity to Tax. A calculation of the rate and ratio of naturally occurring synonymous and nonsynonymous changes (Dn/Ds) in the overlapping tax/rex nucleotide sequence, showed no evidence of consistent positive or negative selection on Rex (Niewiesk et al., 1996).

In vitro mutational analysis of Rex has shown that small changes to the Rex protein sequence can lead to dramatic effects on its biological function. Small deletions within the amino terminus abrogate nuclear targeting and RxRE binding (Grassmann et al., 1991). Single and multiple lysine substitutions for arginines in the N-terminal domain do not affect nuclear targeting but reduce or abolish RxRE binding (Hammes et al., 1993). However, these mutations fall outside the tax/rex overlapping sequence which is subject to immune pressure. Mutagenesis of residues in the activation or dimerisation domains leads to the establishment of a dominant negative phenotype that lacks function and inhibits wtRex activity (Rimsky et al., 1989; Bohnlein et al., 1991; Weichselbraun et al., 1992). The presence and consequences of mutations such as these in vivo has not been addressed. We therefore sought to analyze the Rex function of ten naturally occurring sequence isolates of tax/rex, four of which contain point mutations that allow escape from immune recognition of Tax by CTL and, in two cases, abolish HTLV-1 transactivation function of Tax (Niewiesk et al., 1995). In addition, we investigated five deletion mutants of Rex which were commonly identified in provi-
ral DNA or cDNA from HTLV-1-infected patient samples. From a panel of eight patient samples, we have identified the grand consensus sequence for the complete rex gene from healthy and symptomatic HTLV-1-infected individuals.

MATERIALS AND METHODS

PCR amplification and cloning for sequencing

Subjects have been described in detail elsewhere (Niewiesk et al., 1994; Parker et al., 1994). The first letter of the subject code denotes either a patient with tropical spastic paraparesis (T) or a healthy HTLV-1 carrier (H). The consensus sequence for the tax/rex overlapping nucleotide region has been published (Niewiesk et al., 1994). Genomic DNA was isolated from frozen PBL using standard procedures. PCR amplification of the N-terminal sequence for rex (5146–5205) used primers p27up (5′-CGTCTAGACTCCTCAAGCGAG-3′; 5121 to 5141 containing a Xbal site) and E3A (5′-GGTCATATCCTGGAGCGTGC-3′; 5715 to 5696). One microgram of template genomic DNA was amplified using buffer containing 50 mM KCl, 10 mM Tris (pH 8.3), 1 mM MgCl2, 0.2 mM dNTPs, 2.5U Taq polymerase (Biotaq; BioLine), and 1 μM each primer. Thirty cycles of 94°C, 1 min; 55°C, 1 min; 72°C 30 sec were used. PCR products were gel purified and cloned into M13mp18 for sequencing. Full-length cDNA sequences were amplified using primers p27up and TSRII (5′-TACAGATGGAATACGAGC-RT (Pharmacia). Spliced and unspliced mRNA template sequencing to check for PCR incorporation errors. Forty-eight hours following transfection (Lipofectin reagent;Tax activity of the deletion mutants was assayed fol-

RESULTS

Rex consensus sequence and point mutants

The grand consensus sequence of the tax/rex overlapping region (7324–7833) has been published (Niewiesk et al., 1994). However, the N-terminal coding sequence of Rex 5146–5205 has not been investigated for sequence

mRNA splicing inhibition assay

Approximately 5 × 10^5 Cos7 cells were cotransfected with 2 μg pFE-Rex and 2 μg pgTAT-LTR/R’ (Malim et al., 1989; Hanly et al., 1989). After 72 hr, cells were harvested and cytoplasmic mRNA was extracted using the Quickprep mRNA kit (Pharmacia). cDNA was prepared from 2.5 μg mRNA by reverse transcription with MoMuLV RT (Pharmacia). Spliced and unspliced mRNA template in the cytoplasm was detected by RT-PCR using primers TAT1 5′-ATGGAGCCAGTAGATCCTA-3′; TAT2 5′-GGGCTGTCCGGTCCCTC-3′; TAT3 5′-CTGGACATTACCAGCTTGCG-3′; 5715 to 5696). One microgram of template genomic DNA was amplified using buffer containing 50 mM KCl, 10 mM Tris (pH 8.3), 1 mM MgCl2, 0.2 mM dNTPs, 2.5U Taq polymerase (Biotaq; BioLine), and 1 μM each primer. Thirty cycles of 94°C, 1 min; 55°C, 1 min; 72°C 30 sec were used. PCR products were gel purified and cloned into M13mp18 for sequencing. Full-length and deletion mutant rex cDNA sequences were amplified using primers p27up and TSRII (5′-TACAGATGGAATACGAGC-RT (Pharmacia). Spliced and unspliced mRNA template sequencing to check for PCR incorporation errors. Forty-eight hours following transfection (Lipofectin reagent;Tax activity of the deletion mutants was assayed fol-

Dimerization assay

Dimerization of Rex variants was assayed in a modification of the yeast GAL4 assay (Bogerd et al., 1993). Rex cDNA sequences were appended to sequences encod-
TABLE 1

Comparison of Isolate Sequences with the Grand Consensus of Rex

<table>
<thead>
<tr>
<th>Mutant name</th>
<th>PM1</th>
<th>PM2</th>
<th>PM3</th>
<th>PM4</th>
<th>PM5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuc. position</td>
<td>5174</td>
<td>5175</td>
<td>5193</td>
<td>7354</td>
<td>7359</td>
</tr>
<tr>
<td>Grand consensus</td>
<td>G</td>
<td>A</td>
<td>A</td>
<td>T</td>
<td>G</td>
</tr>
<tr>
<td>HD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HH</td>
<td>A</td>
<td>G</td>
<td>A</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td>HM</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>HN</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>TB</td>
<td>A</td>
<td>G</td>
<td>A</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td>TD</td>
<td>A</td>
<td>G</td>
<td>A</td>
<td>G</td>
<td>A</td>
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<tr>
<td>TG</td>
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<td></td>
</tr>
<tr>
<td>TE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rex aa change</td>
<td>R → Q&lt;sup&gt;b&lt;/sup&gt;</td>
<td>F → L</td>
<td>T → A</td>
<td>V → M</td>
<td>P → S</td>
</tr>
</tbody>
</table>

<sup>a</sup> Part of this data reproduced from Niewiesk et al. (1994).

<sup>b</sup> Sequence variants at 5174 and 5175 were found, always occurred together and resulted in the single aa change from R → Q.

Variation in patient samples. A comparison of isolate sequences from patients HD, HH, HM, HN, TB, TD, TG, Ti, and TE showed a consensus change from the published Seiki sequence (Seiki et al., 1983) at position 5193 (T → A) (Table 1). This synonymous substitution was found in every isolate sequenced from patient material and in 2/12 isolates from MT2 cells. In addition, a frequent substitution found independently in two patients (TB and TD) was the GA (5174, 5175) transposition to AG which changed the amino acid from Arg to Gln. The grand consensus sequence for full length rex is shown in Table 1, where nucleotide positions differing from the index sequence (Seiki et al., 1983) are identified.

Point mutations identified originally in CTL epitope coding regions of Tax (Niewiesk et al., 1995) and which alter the Rex coding sequence (PM1-5) are shown in Table 1. PM1, PM2, and PM3 are located in the region intervening the N-terminal RNA binding element of Rex and the first multimerization domain. PM4 is within the second multimerisation domain and immediately adjacent to TFH 119–121 which is important for multimerization (Bogerd et al., 1993). PM5 changes the native Rex translation termination codon to Trp and results in a 20 amino acid extension at the C-terminus (extended protein sequence = 190-WFFATPASGPSSPMFPTSE*-210). This sequence was the consensus sequence in four individuals (Niewiesk et al., 1994).

Identification of tax/rex deletion mutants

Five naturally occurring deletion mutants of rex were identified. The deletion span and resulting translation products are shown in Table 2. Deleted sequences were identified in five TSP patients, from proviral DNA and mRNA templates. No deleted tax/rex sequences were identified in healthy patient samples or from the MT-2 cell line.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Deletion span</th>
<th>Patient</th>
<th>Rex ATG</th>
<th>Tax ATG</th>
<th>Rex STOP</th>
<th>Tax STOP</th>
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<tbody>
<tr>
<td>DM1</td>
<td>7454–8107</td>
<td>TE</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>DM2</td>
<td>7409–8331</td>
<td>TG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM3</td>
<td>7438–7450</td>
<td>TD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM4</td>
<td>7624–8223</td>
<td>TE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM5</td>
<td>7635–8207</td>
<td>TE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>TS</sup> Stop codon.
All PM-Rex but Not DM-Rex Proteins Form Heterodimers

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Cat activity fold induction</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>wtRex:wtRex</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Cat only</td>
<td>0.02</td>
<td>0.25</td>
</tr>
<tr>
<td>p5SVB</td>
<td>2.06</td>
<td>0.11</td>
</tr>
<tr>
<td>PM1:wtRex</td>
<td>1.12</td>
<td>0.09</td>
</tr>
<tr>
<td>PM2:wtRex</td>
<td>1.12</td>
<td>0.09</td>
</tr>
<tr>
<td>PM3:wtRex</td>
<td>1.07</td>
<td>0.16</td>
</tr>
<tr>
<td>PM4:wtRex</td>
<td>0.76</td>
<td>0.18</td>
</tr>
<tr>
<td>PM5:PM5</td>
<td>1.43</td>
<td>0.2</td>
</tr>
<tr>
<td>DM1:wtRex</td>
<td>0.07</td>
<td>0.04</td>
</tr>
<tr>
<td>DM2:wtRex</td>
<td>0.07</td>
<td>0.04</td>
</tr>
<tr>
<td>DM3:wtRex</td>
<td>0.07</td>
<td>0.01</td>
</tr>
<tr>
<td>DM4:wtRex</td>
<td>0.06</td>
<td>0.04</td>
</tr>
<tr>
<td>DM5:wtRex</td>
<td>0.10</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Note. Cos7 cells were transfected with pG5BCAT plasmid, wtRex-VP16, and pGAL4-wtRex or pGAL4-PM1-5 or pGAL4-DM1-5 plasmids. Heterodimer formation was assayed by CAT activity in transfected lysates 48 hr after transfection. Figures represent the fold-induction of CAT activity relative to wtRex homodimer formation. SEM of three independent assays is given.

Dimerization of Rex mutant proteins

Each point mutant of Rex was tested for the ability to form heterodimers with wild-type (wt) Rex (Table 3). All the point mutants were able to dimerize with comparable stability to homodimeric wtRex. No difference was seen when mutants fused with GAL4 were tested for heterodimer formation with wtRex-VP16 fusions or vice versa (data not shown). PM5, which possesses the extended C-terminus, was also tested for homodimer formation. In this case, the CAT activity showed an increase over the wild type induction (t test P < 0.05), indicating a more stable interaction of the PM5 Rex subunits. Combinations of each PM1-4 mutant paired with PM5 were similar to wild type in activity (data not shown).

Deletion mutants DM1-5 were also tested for heterodimer formation with wild-type Rex (Table 3). None of the deletion mutants were able to form heterodimers with wtRex.

Export of RxRE mRNA

Rex protein was detected as a 30-kDa band in Cos7 cells transfected with PM1-5 by Western blot (Fig. 1a). PM 1, 2, 4, and 5 were expressed at comparable levels to wtRex. Loading of samples in each lane was shown to be equivalent by staining for total protein (data not shown). Protein expressed from PM3 was detected as two bands of 30 and 25 kDa. The mutation in PM3 causes an amino acid change from Val to Met which may be used as an alternative initiation codon to produce a protein of 25 kDa (Ciminale et al., 1995). Both protein forms were expressed in equal amounts by PM3. Mis-sense mutation of the N-terminal wtRex ATG resulted in a similar translation product to PM3 with the main translation product being 25 kDa in size (Fig. 1b). The minor 26-kDa band may represent a posttranslational modification of the 25-kDa band. A 21-kDa minor band of variable intensity was seen in the translation products of all the point mutants. The identity of this band is uncertain, but could represent a degradation product of Rex (Hammes et al., 1993) or a p21Rex translation product.

The expression vector pgTat-LTR/R (Hanly et al., 1989) possesses two tat exons separated by an intron in which the HTLV-1 RxRE has been cloned. The presence or absence of the RxRE containing intron is detected by RT-PCR. In the absence of Rex, the tat exons were spliced...
DISCUSSION

The role of cytotoxic T-cells in selecting viral isolates which escape immune detection is well documented in LCMV (Pircher et al., 1990), HTLV-1 Tax (Niewiesk et al., 1995), and HIV-1 Nef (Price et al., 1997). In HTLV-1 Tax, in addition to evading CTL recognition, escape mutants lose the ability to transactivate various promoters. Although there may be complementation of Tax function from intra- or intercellular sources in vitro (Marriott et al., 1991), the significance of this to the viral swarm in vivo is unclear. CTL specific for Rex are not detected in PBL from patients who mount a strong response to Tax (Daenke et al., 1996). Sequestration of Rex in the nuclear compartment is unlikely to prevent its entry into the class I presentation pathway as other viral nuclear proteins are efficiently presented to CTL (Bastin et al., 1987; Culmann-Penciolelli et al., 1995). Unlike Tax, there is no evidence that Rex is chronically expressed in cells carrying proviral sequences and it may be a rare target for immune recognition. It is not known whether Rex can evade detection by CTL by avoiding processing and/or presentation of peptide epitopes to T-cells, although there are no obvious indications in the protein sequence that this might be the case. Several potential CTL epitopes can be identified in the Rex protein sequence which conform to HLA-restricted peptide motifs (Ramensee et al., 1995), including one (HLA-B27 motif Rex aa 5–15) within the RNA-binding and nuclear localization domain of Rex. However, there is no colocalization of potential epitopes with mutations which lead to loss of function.

In the calculation of Dn/Ds ratio for the tax/rex overlapping sequence in the Rex translation frame, most but not all of the ratios were lower (less pressure for amino acid change) than the same sequences analyzed in the Tax translation frame, with no difference observed between the healthy seropositive patients and the TSP patients (Niewiesk et al., 1996). This suggests that the virus can afford to mutate in order to escape detection at the expense of lost or reduced activity of Tax, but that escape mutations which abolish Rex activity are not tolerated.

All of the sequence variants found in patient samples and reported here were commonly found in isolates from the viral swarm. The frequency of deletion mutants was between 1/37 to 3/17 isolates sequenced per TSP patient sample, and in one patient increased over a 10-month period during which disease progression was rapid (patient TE; see Niewiesk et al., 1996). Deletions are a common event in retroviral transcription and frequently occur between short direct repeats (Pusinelli et al., 1991). The fact that deletion mutants are found more frequently in symptomatic patients (5/5 TSP vs 1/4 healthy carriers) is notable, but may result from the higher rate of HTLV-1 replication in these patients than in healthy carriers. All of the deleted Rex mutants were unable to dimerize, which suggests loss of function as higher order com-

FIG. 2. Rex mutants export RXRE-containing mRNA from the nucleus. (a) 1% agarose gel analysis of RT-PCR reactions of cytoplasmic mRNA from Cos7 cells transfected with pgTAT-LTR/R* and pJFE-Rex. Lane 1, PCR reaction of mRNA without reverse transcription; lane 2, cells transfected with pgTAT-LTR/R* only; lane 3, cells transfected with pgTAT-LTR/R* and pJFE-wtRex; lanes 4–8, cells transfected with pgTAT-LTR/R* and pJFE-Rex PM1–5. DNA markers are HaeIII-digested øX174 DNA (New England Biolabs). Spliced RT-PCR amplification product is shown as 251-bp fragment; unspliced RT-PCR amplification product is shown as 421-bp fragment. (b) RT-PCR analysis of cytoplasmic mRNA from stable Rex-expressing CHO cells (lane 1) or normal CHO cells (lane 2) transfected with pgTAT-LTR/R*.

and the tat mRNA of 251 bp was detected in the cytoplasm (Fig. 2a, lane 2). In the presence of Rex, unspliced tat mRNA (421 bp) was detected in the cytoplasm, although some spliced product was also present (lane 3). Presence of spliced and unspliced tat mRNA, following cotransfection of Cos7 cells with PM1-5 and pgTat-LTR/ R* is shown in lanes 4–8. All PMs promoted export of unspliced tat mRNA from the nucleus into the cytoplasm of transfected cells with approximately equal efficiency to wtRex. Control PCR reactions from mRNA omitting the reverse transcriptase step, did not amplify a 421-bp tat-specific product, indicating that the cytoplasmic mRNA reactions were not contaminated with pgTat-LTR/R* plasmid DNA (Fig. 2a, lane 1). In all cases, we were unable to show the exclusive nuclear export of unspliced tat mRNA in the presence of Rex. This was also the case in a CHO cell line showing stable expression of Rex (R. Smith, unpublished; Fig. 2b).
plexes of Rex bound to the RxRE are thought to be required for export of mRNA (Ahmed et al., 1990). This, and the fact that all lack the Rab-binding motif (Bogerd et al., 1996), makes it unlikely that they act as negative inhibitors of wtRex. Although these sequences were detected as mRNA transcripts, we have no evidence that they are translated into proteins. DM1, 2, 4, and 5 all lack the epitope for anti-Rex antibody recognition and were therefore undetectable by Western blot. We were also unable to detect DM3 protein, using an antibody directed to an epitope in the C-terminus of Tax (data not shown), which might also suggest rapid degradation in the cytoplasm. The presence of these “null” deletion mutants may be more indicative of inaccurate transcription than positive selection for Tax/Rex variants, although undoubtedly, most would also evade CTL recognition if translated in the Tax coding frame.

Of the five single amino acid changes found in Rex (PM1-5) in our panel of patient samples and thought to have arisen due to CTL pressure on Tax (Niewiesk et al., 1995), all retain wt function. For comparison, of the nine Tax amino acid changes within CTL epitope sequences and identified in the same panel of patients, six abolished all transactivation function, and the remaining three were partially inactive (Niewiesk et al., 1995). The PM3 mutation V→M results in the translation of an N-terminal truncated form of Rex. This protein, which retains the Rab-binding activation domain, could act as an inhibitor of wtRex function by competing for nuclear shuttling cofactors (Kubota et al., 1996), although no evidence was found of this in the export of tat mRNA. Three other Rex mutations lie outside known CTL epitope regions of Tax and have not been tested individually (P132L, P149L, and P169L). They are within the C-terminal region of Rex for which no function has been defined and which can be deleted without loss (Hope et al., 1991). The R100Q mutation, which is outside any known CTL epitope is fully functional. R9 and R10 have been shown to be the least important of the charged residues in the RNA binding domain of Rex. A protein containing double lysine substitutions at R9 and R10 retained the ability to export RXRE mRNA from the nucleus (Hammes et al., 1993).

These data support the view that CTL recognition of Tax does not also select for dysfunctional mutants of Rex. Sequences containing point mutations outside regions known to be targeted by CTL also maintain Rex function. The only nonfunctional Rex coding sequences identified in patient isolates were deletion mutants which lack all or most of the Rex functional domains; these had no constitutive or inhibitory function.

In a persistent infection, viral protein expression and virion production may be low but not absent. However, it is essential that all the requirements for rapid reactivation of the infection are in place if needed. The worst strategy for the virus would be to perpetuate sequences encoding partially active or inhibitory proteins. Completely defective rex genes in a minority of proviruses would be less devastating to the infection because they would not be replicated. The sequence variants reported here result either in Rex proteins with normal function or ones with complete loss of function. We suggest that an absolute requirement for Rex function might constrain the degree of sequence variation in the tax/rex coding region and therefore the number of CTL escape variants within the Tax protein. If there were no such constraint, more effective evasion by Tax of the immune response might be the result. Although Tax is not the only target for CTL (Parker et al., 1994), it is the immunodominant CTL antigen of HTLV-1 and is chronically expressed in infected cells. Also, the profound effects of Tax on a number of constitutive cellular activation pathways may contribute to the pathogenesis of HTLV-1 disease (Daenke et al., 1994). We propose that the requirement for Rex function in HTLV-1 infection limits the capacity for mutation-led evasion of immune recognition. This suggests that a functional Rex protein is particularly important in the persistence of HTLV-1 infection.

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