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THE HUMAN T-CELL LEUKEMIA VIRUS REX PROTEIN

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Abstract

A critical step in the life cycle of complex retroviruses, including HTLV-1 and HTLV-2 is the ability of these viruses to adopt a mechanism by which the genome-length unspliced mRNA as well as the partially spliced mRNAs are exported from the nucleus instead of being subjected to splicing or degradation. In HTLV, this is accomplished through the expression of the viral Rex, which recognizes a specific response element on the incompletely spliced mRNAs, stabilizes them, inhibits their splicing, and utilizes the CRM1-dependent cellular pathway for transporting them from the nucleus to the cytoplasm. Rex itself is regulated by phosphorylation, which implies that proper activation of the protein in response to certain cellular cues is an important tool for the virus to ensure that specific viral gene expression is allowed only when the host cell can provide the best conditions for virion production. Having such a critical role in HTLV life cycle, Rex is indispensable for efficient viral replication, infection and spread. Indeed, Rex is considered to regulate the switch between the latent and productive phases of the HTLV life cycle. Without a functional Rex, the virus would still produce regulatory and some accessory gene products; however, structural and enzymatic post-transcriptional gene expression would be severely repressed, essentially leading to non-productive viral replication. More detailed understanding of the exact molecular mechanism of action of Rex will thus allow for better design of therapeutic drugs against Rex function and ultimately HTLV replication. Herein we summarize the progress made towards understanding Rex function and its role in the HTLV life cycle.

Keywords

Human T-cell leukemia virus (HTLV); Rex; nucleo-cytoplasmic mRNA export; Review

2. INTRODUCTION

Human T-cell leukemia virus type-1 (HTLV-1) and type-2 (HTLV-2) are complex retroviruses that infect and transform T-lymphocytes (1). Despite the fact that the two viruses share 66% nucleotide sequence homology and almost identical genome structure (1), they have distinct pathogenic properties. HTLV-1 is the causative agent of adult T-cell leukemia (ATL), a

malignant transformation of CD4⁺ T lymphocytes (2), and HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP), a chronic progressive neurological disease (3). Although HTLV-2 was initially isolated from a patient with a rare variant of hairy T-cell leukemia (4-5), its disease association is less clear (6). However, there have been a few reports of HTLV-2 association with a neurodegenerative disease (7,8). In order to understand the differences in pathology of these related viruses a molecular understanding of their life cycle and protein functions is of critical importance.

A requirement for successful replication of all retroviruses is their ability to override the nuclear retention of intron-containing mRNAs, resulting in their efficient export to the cytoplasm. Like other complex retroviruses (9-12), HTLV-1 and HTLV-2 (HTLV) have evolved to encode a protein, termed Rex, which facilitates the expression of viral unspliced and singly spliced mRNAs. Rex is a post-transcriptional regulator that specifically binds viral mRNA and utilizes host machinery to actively export these mRNA species from the nucleus to the cytoplasm (13-15). Since Rex has been shown to be essential for HTLV replication (13,16,17), the full understanding of its function and regulation remains a critical objective in HTLV research that could yield new strategies for therapeutic intervention.

3. HTLV LIFE CYCLE AND GENE EXPRESSION

Human T-cell leukemia virus belongs to the deltaretrovirus family of complex retroviruses. Upon viral infection of a susceptible host cell, the viral RNA genome is reverse transcribed into DNA that is imported into the nucleus and randomly integrated into the cellular genome (18,19). The integrated provirus utilizes promoter elements in the 5'-long terminal repeat (LTR) to drive transcription. Three imperfect 21-base pair repeats in the U3 region of the LTR constitute the response elements for the HTLV transcriptional activator Tax (20,21). Tax-mediated transcription leads to the efficient production of multiple mRNA species that are expressed at different levels in infected cells (22-24) (see Figure 1). Thus, like other complex retroviruses, HTLV expresses multiple gene products from a relatively small 9 kb genome by utilizing strategies such as alternative mRNA splicing, frame shifting, polycistronic translation, and protease-mediated cleavage of large viral proteins into smaller polypeptides with specific function.

Alternative splicing of HTLV mRNA provides the provirus with a complex array of gene products. These mRNAs are grouped into three major classes: the unspliced full length mRNA, the singly spliced mRNAs, and the doubly spliced mRNAs (Figure 1). The unspliced mRNA serves as genomic RNA to be packaged into virions or is used as a template for translation of essential structural and enzymatic proteins such as Gag, Pol and Pro. Recognition of the multiple splice donor and acceptor sites contained in the unspliced mRNA, leads to its splicing into ~4.3 kb singly spliced mRNA coding for the Envelope protein and other singly and doubly spliced mRNAs that encode regulatory and accessory proteins (19).

Their compact genomes and complexity of gene expression present HTLV and other retroviruses with a problem that they have evolved to surmount. Both the full length mRNA and the Env-encoding singly spliced mRNA contain introns and hence are recognized by the cell as premRNAs. By default these pre-mRNAs are highly unlikely to be efficiently transported to the cytoplasm because, once they are recognized, the pre-mRNAs are retained in the nucleus until they are completely spliced or degraded. If such default processes were the only fate for these viral mRNA species, the virus would ultimately fail to produce structural and enzymatic proteins or express cytoplasmic, genomic RNA required for packaging.

Simple retroviruses exploit cellular machinery that recognizes specific elements in their incompletely spliced mRNAs, which in turn inhibits splicing and facilitates the nucleo-

cytoplasmic transport of the intron-containing mRNAs. Mason-Pfizer monkey virus (M-PMV), simian retrovirus type-1 (SRV-1) and type-2 (SRV-2), and mouse intracisternal A-particle (IAP) all contain highly structured RNA segments termed constitutive transport elements (CTEs) that are recognized by host cell factors responsible for their transport to the cytoplasm (25-29). At least for M-PMV and SRV-1, the host factor is TAP (30). In addition to relying completely on cellular factors for export of unspliced and partially spliced mRNA, CTEs are constitutively active and lack a regulatory control mechanism, thus leading to a constitutively productive replication program and hence no post transcriptional regulation of viral latency.

Complex retroviruses, on the other hand, encode a specific regulatory protein that act in trans to actively transport intron-containing mRNAs into the cytoplasm. The Rev protein of human immunodeficiency virus type-1 (HIV-1) is a prototype posttranscriptional regulator (reviewed in reference 31). HTLV encode an analogous protein, Rex, from a doubly spliced mRNA that also expresses Tax. Using internally deleted proviruses and complementation assays, it has been shown that although Tax alone can activate transcription, the accumulation of full-length mRNA is absolutely dependent on Rex (32,33). The accumulation of the singly spliced Env mRNA, which still contains a functional intron, is also dependent on Rex (13).

4. REX FUNCTION

4.1. Role of Rex in viral RNA export

Since the accumulation of viral structural proteins is dependent on Rex, and Rex itself is generated from completely spliced mRNA, the virus has a biphasic life cycle: an early Rex-independent phase and a late Rex-dependent phase. Early during infection, when insufficient Rex protein is being made, most of the viral mRNAs are doubly spliced, due to default splicing by the host cellular machinery. Accumulation of sufficient levels of Rex results in the expression of incompletely spliced mRNA in the cytoplasm, leading to the production of structural and enzymatic gene products and assembly of virus particles. Therefore, Rex is considered to be a positive regulator that controls the switch between early, latent and late, productive infection.

The ability of Rex to function depends on several characteristics: (a) its ability to recognize and bind a responsive element on specific mRNA species in the nucleus prior to their splicing, (b) its ability to utilize defined cellular pathways to export the incompletely spliced mRNA cargo, and (c) its potential to be regulated through specific domains in order to ensure that the protein functions best when it is needed. Below, we will discuss in detail each characteristic, its contribution to Rex function, and significance to the virus life cycle. Mapping of functional domains of Rex and their regulation may ultimately provide us with therapeutic tools to disrupt Rex function and HTLV replication and pathogenesis.

First, it is important to note that the unspliced and incompletely spliced viral mRNAs have essential features that render them Rex responsive. In addition to containing a unique Rex response element (RxRE), these mRNAs have cis-acting repressive sequences (CRS) that retain and stabilize the unspliced mRNA in the nucleus to ensure the availability of sufficient amount of Rex substrate (34,35). The fact that unspliced pre-mRNA in the nucleus is unstable and is targeted for processing (splicing to completion followed by export) or degradation (36,37) gives the combination of CRS and RxRE a unique role in providing suboptimal conditions for mRNA splicing efficiency and hence is a prerequisite for the ability of Rex to activate cytoplasmic transport. Drawing from the similarity between HIV-1 Rev and HTLV Rex, one can assume that the absence of these elements corresponds with rapid splicing which depletes the nuclear pool of Rex substrates. On the other hand, if these elements were to render splicing too inefficient, the mRNA would be recognized by the cell as intronless mRNA that

would be constitutively exported to the cytoplasm in a Rex independent manner (38-40). Thus, the working model for these elements is that the CRS retains the unspliced mRNA in the nucleus until Rex binds to the RxRE, overrides the repressive effects of the CRS, and exports the mRNA to the cytoplasm (34).

There are two suggested models for the mechanism by which Rex induces cytoplasmic expression of unspliced mRNA. The first model proposes that Rex actively transports the unspliced mRNA to the cytoplasm where it is translated. Here, Rex would directly bind to the RxRE on the mRNA, override the nuclear retention signals, and carry this mRNA cargo through the nuclear pore to the cytoplasm. The second model suggests that Rex actively inhibits splicing of mRNA by stripping it of splicing factors (34,41,42). Once the mRNA is free of splicing factors the cellular machinery would recognize it as a processed mRNA and export it efficiently to the cytoplasm. Although there is some evidence for both models, the first is supported by a greater amount of published work.

A less investigated possibility is that Rex may also increase the translational efficiency of Rex-responsive mRNA. Kusuhara *et al.* have shown that Rex-2 increases the levels of incompletely spliced mRNA in the cytoplasm by 7 to 9 fold, while Gag protein production increases by 130 fold (15). These results are consistent with HIV-1 Rev data (43,44), and suggest that Rex has an effect on translation efficiency. Additional support for this hypothesis comes from studies that show an association between Rex-1 and translational initiation factor 5A (eIF-5A) (45, 46). Despite some evidence for this mechanistic effect of Rex, the primary function of Rex remains the nucleo-cytoplasmic export of unspliced and partially spliced viral mRNA.

4.2. Rex Proteins: Structure, subcellular localization, and functional domains

The 189 amino acid Rex-1 and 170 amino acid Rex-2 proteins share 60% homology at the amino acid level. When analyzed by SDS-PAGE, Rex-1 has an apparent size of 27 kDa, and Rex-2 is detected as two major bands of 24 and 26 kDa¹. The two isoforms of Rex-2, p24^{rex} and p26^{rex}, have the same amino acid backbone and differ by a post-translational modification, specifically a conformational change induced by serine phosphorylation (47,48). HTLV-1 and HTLV-2 also produce truncated forms of Rex from alternatively spliced mRNAs. These proteins, named p21^{rex-1} and p22/20^{rex-2}, lack N-terminal sequences of Rex responsible for nuclear import and RNA binding, and interfere with Rex localization and function (49-51).

Both Rex-1 and Rex-2 are phosphoproteins that localize to nucleus, nucleoli, and nucleolar speckles in transiently transfected cells as well as HTLV infected cell lines (23,50,52-54). Studies using a recombinant baculovirus expressing Rex-2 in SF-9 cells, as well as cell fractionation analysis following transfection of human lymphoid cells, have shown that the p24^{rex} isoform is predominantly cytoplasmic, while the p26^{rex} isoform is nuclear/nucleolar (55). Despite the nuclear and nucleolar localization of Rex at steady state, studies have shown that Rex is a shuttling protein (54,56). Thus, Rex localization is in dynamic equilibrium between the nucleus and the cytoplasm. In fact, this property is essential for Rex to exert its function as a nucleo-cytoplasmic mRNA export facilitator. There is no definitive evidence for the role of the nucleolar localization of Rex in its function. Using *in situ* hybridization, it was shown that env mRNA can be detected in the nucleoli of cells cotransfected with Rex (57). In addition, several cellular mRNAs such as c-myc, n-myc and myo-D can be detected in nucleoli (58). Recently, the nucleolus has been considered to be a site for mRNA processing prior to export (59,60). Thus, a likely hypothesis is that Rex interacts with its target mRNA at the site where the mRNA is to be processed. Studies of HIV-1 Rev suggest that the nucleolus might also serve as a storage compartment that ensures preservation of Rev and Rex in the cell (61).

Mutational analyses allowed the assignment of biochemical and functional properties to discrete protein domains of Rex (Figure 2). Both Rex-1 and Rex-2 have homologous nuclear

localization signals (NLS), RNA binding domains (RBD), multimerization domains, and an activation domain which encompasses the nuclear export signal (NES) (48,62-66). In addition, a unique C-terminal domain has been described for Rex-2 that is a target for serine phosphorylation and may also contribute to efficient nucleocytoplasmic shuttling (54).

The amino terminal 19 residues of Rex contain an arginine rich cluster that serves as both an NLS and RBD. Upon fusion to heterologous proteins such as β -galactosidase, the arginine rich motif of Rex, is sufficient to impose nuclear import of the chimeric protein, thus establishing it as an NLS (49,67,68). Hammes *et al.* showed that substitution of all 7 arginines in the NLS of Rex-1 with positively charged lysine residues does not affect nuclear and/or nucleolar localization. This suggests that the mere presence of a stretch of positive charges within this domain is sufficient for nuclear localization (66). However, mutating certain arginine residues in the NLS alters the nucleolar but not the nuclear localization of Rex. Several studies indicate that targeting of Rex to its correct subcellular compartment is critical for its proper function (69,70).

The same stretch of arginine residues also serves as an RBD for the R_xRE and is similar to domains found in a diverse group of proteins including HIV Rev (31), HIV Tat (71), and RNAase P (72). The RBD of Rex binds specifically to a cis-acting regulatory element in viral mRNAs called the Rex responsive element (R_xRE) (73,74). The specific binding of Rex to its R_xRE is a critical step in its transactivation of mRNA export by allowing the mRNA to overcome nuclear retention due to the inhibitory CRS (75). The CRS is located in the 5'LTR in HTLV-1. In HTLV-2, it is located in the R/U5 region, within the 5' R_xRE and downstream of the splice donor site (34,75). Another CRS in HTLV overlaps with the 3'R_xRE.

Although the R_xRE harbors a single high affinity binding site for Rex, it has been suggested that multiple Rex proteins bind to a single mRNA molecule prior to export via cooperative protein-protein and protein-RNA interactions (76,77). There are two regions in both Rex-1 and Rex-2 that serve as multimerization domains (54,64,78). The multimerization domains map to amino acids 57-66 and 106-124 in Rex-1 (79,80) and approximately residues 60-70 and 120-130 in Rex-2 (54). Mutations in the multimerization domains render Rex nonfunctional. In fact, multimerization mutants behave as transdominants and hinder wild type Rex when cotransfected in transient assays, suggesting that the ability of Rex to assemble into high order complexes on the mRNA is critical for its function (54,78). However, there are some conflicting reports on the role of multimerization in Rex function. First, Rex-1 has been shown to form stable homo-oligomers in the absence of R_xRE *in vivo* using a mammalian-two hybrid assay (46). Furthermore, studies by Heger *et al.* indicated that despite the critical role for multimerization in Rex function, mutants defective in multimerization were still efficient in nucleocytoplasmic shuttling, suggesting that multimerization of Rex protein on its target mRNA does not play a direct role in export (80).

The activation domain (AD) of Rex was originally identified as the minimal region that could functionally replace the HIV Rev activation domain in cis (65). It maps to residues 79-99 in Rex-1 (63,81) and residues 81 to 94 in Rex-2 (54). Subsequent studies showed that the sequence responsible for nuclear export of Rex, NES, is in fact its minimal effector domain (56,82). The NES in Rex is a leucine rich motif involved in protein-protein interactions that are critical for its function (31). First identified in HIV-1 Rev, this type of NES is found in many cellular and viral proteins with shuttling properties including visna virus Rev (83,84), adenovirus E4 34-kDa (85), RanBP1 (86), TAP (87), and I κ B α (88). The consensus leucine-rich NES is **LX₂₋₃ λ X₂₋₃LXL/I** (with X being any amino acid and λ being an amino acid with a bulky hydrophobic side chain) with the core tetramer being **LXLX** (81,82). As described later, the most critical protein-protein interaction that occurs at the Rex AD/NES is with chromosome region maintenance interacting protein 1 (CRM1)/exportin1 (89,90). Other proteins that

interact with AD/NES include eIF-5A (46), and human nucleoporin-like protein (hRIP/Rab) (79).

4.3. The Rex Responsive Element

In the mRNA, the Rex-1 responsive element (RxRE-1) is a stem loop structure of 205 nucleotides that is found in the 3' LTR, but has subsequently been shown to be present, at least in part, in the 5' LTR (91,92). The Rex-2 responsive element (RxRE-2) is 226 nucleotides and is located in the 5'LTR and maps to the region in the R/U5 that also contains the CRS (34,35, 93,94). Mutational analyses indicate that the actual Rex binding subdomains in the ~200 nucleotide RxRE are relatively short. A stretch of 43 nucleotides constitutes the high affinity Rex-1 binding motif (73,74), whereas two short motifs (nucleotides 46-168 and 145-205) in RxRE-2 are sufficient for maximal Rex-2 binding (55). Studies showing that only a short sequence or a single stem loop structure is sufficient to mediate Rex function (74,95) raises the question of the significance of the complex and relatively large stem loop structure formed by the RxRE. It is likely that the free energy from a longer secondary structure ensures proper folding into a stable secondary structure with a high affinity binding site. Another explanation is that additional stem loops form secondary and less efficient binding sites for Rex, or allow binding of cellular factors involved in Rex response. Also, the ability of the RxRE to fold into stem loop structure brings the polyadenylation signal (AAUAAA) into close proximity to the GU rich polyadenylation site (62,96), giving the full RxRE a novel role in ensuring efficient polyadenylation of viral mRNA. Substitution and deletion studies have demonstrated that proper folding of RxRE is required to provide a docking site for Rex, and hence, is essential for its *in vivo* function (16,97).

Unlike HIV-1, all HTLV transcripts contain the RxRE. However, it is still unclear how Rex preferentially regulates the unspliced and incompletely spliced versus the fully spliced mRNAs. One likely explanation is that Rex binds to viral mRNA prior to the commitment of splicing factors. On the other hand, Rex could actively inhibit the splicing machinery prior to facilitating mRNA export (14,98). Indeed, Rex has been shown to bind pre-mRNA splicing factors such as SF2/ASF and inhibit mRNA splicing *in vitro* (42,99,100). In addition, studies by Seiki *et al* and King *et al* provide evidence that the RxRE acts as a negative element in the absence of Rex (101,102).

Finally, more recent reports indicated that certain cellular mRNA-binding proteins could bind to the RxRE. One such factor is hnRNP A1, which binds to RxRE-1 and interferes with Rex function (103). One study suggests that hnRNP A1 competes with Rex-1 for binding to RxRE. The consequence of this competition might be impairment of Rex function in cells over-expressing hnRNP A1. Indeed, Rex-1 exhibits impaired function in Jurkat lymphoblastoid T-cells (104) due to interference from hnRNP A1, whereas HTLV-1 infected cell lines such as MT-2 and HUT-102 have low levels of this protein (103).

4.4. Interaction of Rex with host cellular factors

Several studies have identified cellular proteins that interact with Rex and either augment its function or inhibit its activity. Of particular interest are proteins that interact with the AD/NES, given the essential role of this domain in Rex function. Among the proteins that interact with NES, CRM1 (89,90), eIF-5A (46), and hRIP/Rab (79) have been studied in greatest detail. The most functionally significant interaction is with CRM1, which bridges Rex to several cellular proteins at the nuclear pore complex as well as the Ran family of proteins. Using chemical crosslinking studies, it has been shown that while CRM1 and eIF-5A bind directly to the Rex NES, other proteins such as hRIP/Rab are brought to the NES via their association with CRM1 (105).

CRM1, also called exportin 1, is a ~112 kDa nucleoprotein which is a member of the importin- β family of transport receptors. The role of CRM1 in nuclear transport was first identified using the antibiotic leptomycin B (LMB) (106), which specifically inhibits the exit of Rev and Rex from the nucleus (107). CRM1 localizes to the nucleoplasm, nucleolus, and NPC, and associates specifically with CAN/Nup214; this association as well as NPC localization can be inhibited by the FG-repeat domain of CAN/Nup214 (90,108,109). Since the importin- β family had been shown to be involved in mRNA export (110), and CRM1 itself can shuttle between the nucleus and cytoplasm (111), it was hypothesized that CRM1 could be a transport receptor for leucine rich NES-containing proteins. Indeed, CRM1 can directly interact with Rex-1 and Rex-2 and mediate their function *in vivo* (112,113). At the molecular level, CRM1, through its interaction with a wide range of proteins, can mediate the export of leucine-rich NES-containing proteins. CRM1 can interact with GTP-bound Ran, but only in the presence of the NES (109,111,114). Since Ran-GTP is predominantly a nuclear protein, it is assumed that the formation of Rex NES/CRM1/Ran-GTP complex can only occur in the nucleus. CRM1 is then thought to extensively interact with members of the NPC to facilitate the export of this assembled complex (115-118). Studies using LMB have confirmed the essential role of CRM1 in the export of NES-containing proteins (109,119). Interestingly, the overexpression of CRM1 partially rescues the ability of Rex mutants to multimerize indicating that CRM1 facilitates Rex multimerization (113).

A role for eIF-5A in mRNA export was first indicated in studies of HIV-1 Rev (120), and was subsequently implicated in Rex-mediated mRNA transport (46). Studies using glutathione S-transferase (GST)-fusion proteins containing the NES of HTLV-1 Rex showed that eIF-5A inhibitors selectively block the nucleo-cytoplasmic export of Rex NES and indicated a role for eIF-5A upstream of CRM1 (119). Although it was initially thought that eIF-5A is only involved in translation initiation, this small protein (~19 kDa) is pleiotropic and functions in cell proliferation, gametogenesis, senescence and apoptosis (121-123).

Yeast two-hybrid screens revealed another protein that interacts with the NES of Rev and Rex, termed Rev/Rex interacting protein or Rev/Rex activation domain binding protein (RIP/Rab) (79). This protein is classified as a nucleoporin because it contains FG-repeat sequences and is homologous to the FG-nucleoporin Nup153p/CAN1. The ability of Rex to interact with RIP/Rab was utilized to identify the leucine rich NES (81). In yeast, RIP/Rab is essential for Rev nucleoplasmic as well as nuclear pore complex (NPC) localization. However, in yeast strains that are deficient in RIP/Rab, Rev is still partially functional (40,124). A recent study of the role of RIP/Rab in Rev function reveals that it promotes the release of Rev/RRE complexes from the perinuclear region (125). Based on the similarity between Rex and Rev, it is reasonable to conclude that RIP/Rab plays a similar role in Rex function. Yeast two-hybrid screens have identified a number of FG-repeat containing proteins that interact with Rev and Rex (126). Interestingly, it was later shown that the interaction between the NES and these FG-repeat containing proteins is lost in yeast strains lacking the export receptor CRM1/exportin 1, indicating that the latter is important for bridging Rex/Rev and the nucleoporins (105).

4.5. Mechanism of Rex function and the Rex transport cycle

The mechanism of Rex function involves several discrete steps that must occur in a sequential order for proper RxRE-dependent export of intron-containing mRNA. As shown in Figure 3, the proposed model reflects the transport cycle of Rex. A prerequisite for proper Rex activity is the localization of newly synthesized Rex to the nucleus/nucleolus. The transport cycle itself involves: (a) binding of Rex to the RxRE present in incompletely spliced mRNA, resulting in the protection of this mRNA from splicing and/or degradation; (b) Rex multimerization; (c) formation of an RNA/Rex/CRM1/Ran-GTP complex; (d) interaction of the complexes with NPC and exit from the nucleus; (e) hydrolysis of Ran-GTP to Ran-GDP, resulting in

dissociation of the complex and subsequent release of the cargo mRNA; and (f) return of Rex to the nucleus to start another cycle.

As mentioned earlier, the nascent Rex protein is actively imported into the nucleus through the NPC. Passage of proteins through the NPC is strictly selective and involves bridging factors such as the import/transport receptors that bind to the FG-repeat containing nucleoporins resulting in the transport of their cargo through the NPC (127-129). Rex is able to localize to the nucleus due to the arginine-rich NLS at its N-terminus. Transport receptors that recognize and import NLS-containing proteins are termed importins (130). Importin- β heterodimerizes with importin- α and enters the nucleus with cargo protein (NLS-containing protein) (131). Classically, importin- α is the subunit that directly binds to the prototype NLS (lysine-rich or basic). In contrast, the Rex NLS is arginine-rich and hence does not interact with importin- α . Indeed, the results of digitonin-permeabilized nuclear import assays demonstrated that Rex does not require importin- α for its import and that importin- β alone is sufficient for this purpose (132). Due to the sequence homology between the Rex NLS and the importin- α -binding domain on importin- β , it is suggested that Rex NLS binds directly to importin- β without the adaptor protein, importin- α (31). The nuclear Ran-GTP binds to the N-terminus of importin- β once it reaches the inner side of the nuclear membrane and possibly as early as the nuclear pore basket, resulting in the release of Rex into the nucleus (133,134). The newly formed Ran-GTP/importin- β complex is then transported back into the cytoplasm, where the GTP on Ran is hydrolyzed into GDP, leading to the dissociation of the complex. This makes importin- β available for another round of import, whereas Ran-GDP is translocated into the nucleus, where it is converted back to Ran-GTP (135). Some earlier reports indicated that other molecules are also involved in Rex import into the nucleus such as B23 (136) and p32 or its murine homologue YL2 (137). Using affinity column chromatography with Rex-1 NLS, it has been shown that the NLS specifically binds to B23 from the nuclear extract of a variety of cell lines (Jurkat, HUT 102, Molt-4, Hela) (136). However, later studies failed to confirm co-localization of B23 and Rex proteins (53). YL2 has been shown to interact with Rex NLS using a yeast two-hybrid screen (137). YL2 also copurifies with SF2/ASF, a factor involved in alternative splicing (138). The exact role of these factors in Rex function or nuclear import is still not clearly understood.

Once Rex is released from importin- β , the RNA-binding domain, which overlaps with the NLS, becomes available for binding to the RxRE. As stated earlier, several events could explain the mechanism of Rex function at the molecular level. First, binding of Rex increases the stability of mRNA, which might in turn facilitate its cytoplasmic transport (15). Alternatively, Rex could bind to the mRNA and inhibit the cellular splicing machinery (14,42,98,137).

Rex likely binds with high affinity to its primary site in the RxRE, and then additional molecules of Rex are bound to secondary sites due to cooperative protein-protein and protein-RNA interactions. The multimerization of Rex on its target mRNA has been shown by several groups to be essential for its function (54,64,78,113). It should be noted that multimerization deficient mutants have been shown to be able to export RxRE-containing mRNA with the same efficiency as wild type protein as long as there is an intact NES (80,139). Thus, although multimerization of Rex is important for function, it is not essential for mRNA export. It is likely that multiple Rex molecules are required to effectively counteract the nuclear retention signals on intron-containing mRNA.

Rex-dependent mRNA export proceeds once a stable Rex/RxRE/CRM1 complex is assembled. CRM1 interacts directly with Rex and its cargo mRNA and facilitates their transport through the nuclear pore complex through a series of protein-protein interactions with the FG-repeat-containing nucleoporins (80,90,109,113,140). The complex formed between CRM1, Rex and the mRNA is then recognized and bound by Ran-GTP (114). This last association leads to the

final steps needed to exit the nuclear pore (141). Other proteins that play a role in the export of Rex have been identified. Ran binding protein (RanBP3) contains FxFG and FG repeats and is proposed to interact with NPC (115,116,142). Since RanBP3 is a non-shuttling protein (117), it is suggested that this protein is essential for the steps leading to the migration of the transport complex to the nuclear face of the NPC, but is released upon exit to the cytoplasm. As mentioned earlier, eIF-5A and RIP/Rab may also play a role in Rex-mediated mRNA export. More recently, another nuclear protein termed Src-associated protein in mitosis (Sam) 68 has been reported to augment Rex/RxRE and Rev/RRE function 143,144. The synergistic effects of Sam 68 on Rex/RxRE function appear to be LMB independent, suggesting that Sam 68 augments Rex function by utilizing a pathway that is distinct from that of CRM1 (143). The Rex transport cycle ends in the cytoplasm upon the release of cargo mRNA. Cytoplasmic RanGTP and RanBP bind to Ran-GTP and catalyze the hydrolysis of GTP to GDP. This hydrolysis step likely causes the dissociation of the complex and the subsequent release of the mRNA. Free Rex and CRM1 proteins shuttle back to the nucleus to start another cycle, whereas the mRNA can be used for translation of enzymatic and structural viral proteins or ultimately used for packaging into virions.

4.6. Role of phosphorylation in the regulation of Rex

Due to the critical role of Rex in the viral life cycle, it is unlikely that such a protein would lack regulation. Posttranslational modification by phosphorylation is one of the most common mechanisms of protein regulation. It has been well documented that a common consequence of phosphorylation is a charge-induced conformational change in the protein's 3-dimensional structure, leading to different interactions with distinct partners and ultimately function. Examples of conformational changes causing a mobility shift that is detectable by SDS-PAGE include Fos (145), Myc (146), the polyomavirus large T-antigen (147), and adenovirus E1A protein (148). In most cases, the shift results from phosphorylation of one or more residues. As mentioned earlier, HTLV-2 Rex migrates as two isoforms in SDS-PAGE, p24 and p26 (149). These forms differ in their level of phosphorylation (47). There is no such distinction for phosphorylated versus unphosphorylated Rex-1 (150).

Early reports indicated that Rex-1 and Rex-2 are phosphoproteins and that phosphorylation is essential for their function. For example, treatment of HTLV-1 infected cells with the protein kinase C inhibitor H-7 results in a decreased level of Rex-mediated accumulation of viral unspliced mRNA (151), suggesting that phosphorylation is important for Rex-1 function; a subsequent study showed that Rex-1 is phosphorylated on serines 70 and 177 and threonine 174 (150). In HTLV-2, the two forms of Rex-2 are detected in virus-infected cells as well as cells transfected with Rex expression plasmids (47,55,149,152,153). Using immunofluorescence techniques, it was shown that p24 predominantly localizes to the cytoplasm whereas p26 has nuclear/nucleolar localization (50,54), suggesting that the active form of Rex-2 is the phosphorylated one. Indeed, it was shown that phosphorylation of Rex-2 enhances its ability to bind to RxRE-containing mRNA (154). In addition, the phosphorylated form of Rex-2 has been implicated in the inhibition of mRNA splicing (42).

A more detailed mutational analysis of Rex-2 targeting all serines and threonines revealed that phosphorylation of two key serines in the C-terminus (S151, S153) is critical for Rex-2 function. Replacement of these serines with alanine relocalizes Rex-2 to the cytoplasm and renders it functionally inactive, whereas aspartic acid substitutions lock the protein in a constitutively active form that localizes mainly to the nucleolus (48). Several pieces of evidence suggest that Rex-2 is also phosphorylated on other residues, leading to the hypothesis that additional phosphorylation sites are required to stabilize the active form of Rex-2. A subsequent study suggested that the C-terminus of Rex-2, including the important phosphorylation sites S151 and S153, is involved in nucleocytoplasmic shuttling of the protein, thus providing a

better molecular understanding of the influence of phosphorylation on Rex function (54). The implication of phosphorylation and the critical role of this event on the function of Rex is that HTLV gene expression is regulated at multiple levels by cellular factors. Such regulation might be essential for the virus to better adapt by responding to regulatory signals of the cells it infects, providing HTLV with an additional level of replication control.

4.7. The Role of Rex in cellular transformation, viral spread, and tropism

At the molecular level, the basic role of Rex is to positively regulate the cytoplasmic levels of viral genomic mRNA and the expression of the structural and enzymatic gene products that are essential for production of viral progeny (1). Therefore, it is proposed that Rex plays a critical role in the transition from the early, latent phase to the late, productive phase of HTLV infection and hence is required for efficient viral spread. Despite the critical role of Rex for efficient viral replication, studies have shown that low, but detectable levels of HTLV p19Gag and p24Gag are produced from T-cells transfected with HTLV-1 and HTLV-2 proviruses that are Rex-deficient (15,17). These reports bring into question the absolute requirement of Rex for HTLV replication. It is thus important to understand the contribution of Rex to HTLV outside its function in replication.

The pleiotropic protein Tax is the main transcriptional activator of HTLV genes, and its role in cellular immortalization/transformation has been very well established (155-157). The role of Rex in this process has been addressed in a study that utilized a Rex-deficient HTLV-1 proviral clone and characterized the role of Rex in cellular immortalization/transformation, viral replication, spread, and persistence in inoculated rabbits (17). This report confirmed that Rex is not absolutely required for structural protein expression, as the production of p19Gag was still detectable, albeit at much lower levels compared to the wild type virus (116-fold less). Using an *in vitro* system in which irradiated producer cells were cocultured with peripheral blood mononuclear cells (PBMCs), it was shown that Rex-deficient viruses were able to sustain IL-2-dependent, long-term growth of T-cells, suggesting that Rex is dispensable for cell-mediated infection/spread of HTLV as well as immortalization of primary T lymphocytes. However, when inoculated into immune competent rabbits, the Rex-deficient provirus failed to infect, spread, persist or induce a detectable immune response, indicating that Rex is important for the establishment of viral infection and persistence *in vivo* (17).

Although HTLV-1 and HTLV-2 can infect a variety of cell types *in vitro*, they only show transforming and/or pathogenic activity in T lymphocytes (158-161). However, the tropism of these two viruses in T cells is distinct. While HTLV-1 has a preferential tropism for CD4⁺ T cells in both asymptomatic patients and those with neurological disease (162), the *in vivo* tropism of HTLV-2 is less clear. Some reports indicate that it can be detected in both CD4⁺ CD8⁺ T cells, with greater viral burden in CD8⁺ T-cells (162-165). *In vitro*, HTLV-2 has a preferential tropism for CD8⁺ T cells (166,167). Given the critical role of the post-transcriptional regulator Rex in viral gene expression and replication, it was proposed that Rex might be the genetic viral determinant responsible for the transformation tropism of HTLV-1 and HTLV-2. A study in which Tax and Rex ORFs were exchanged between the two viruses indicated that despite the altered Tax and Rex transactivation activities in the new recombinant viruses, both recombinants were able to replicate, infect and immortalize T lymphocytes. Thus, although Tax and Rex are critical for efficient replication and cellular transformation, neither protein confers the distinct transformation tropism of HTLV-1 and HTLV-2 (167).

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Figure 1.

Genome organization of HTLV-1 and HTLV-2 and their unspliced (U), singly spliced (S) and doubly spliced (D) mRNAs. A. HTLV-1 expresses 8 major mRNA species. The genomic unspliced mRNA encodes the Gag, Pol and Pro proteins. Four singly spliced mRNA species are the result of splicing of exon 1 (nt 1-119) to unique splice acceptors at positions 4641 (Env), 6383 (p12), 6950 (p21^{rex}), and 6875 (p13). The three doubly spliced mRNAs include exon 1, exon 2 (4641-4831) and a third exon that starts at 6950 (Tax/Rex), 6478 (p30), or 6383 (p27, a putative Rex-p12 hybrid). Exons are designated by their positions in the viral mRNA. B. The similar HTLV-2 genome expresses at least 7 mRNAs. In addition to the unspliced species, three singly spliced mRNAs contain exon 1 (nt 1-134) linked to splice acceptor sites at 4729 (Env), 6629 (p28, p22/p20), or 6899 (p28, p22/p20). The major doubly spliced mRNA encodes Tax/Rex and contains exon 1 and exon 2 (nt 4729-4868) linked to a splice acceptor at position 6899. The other doubly spliced mRNAs contain exon 1 and 2 linked to a splice acceptor at 6629 or 6491; their protein products are less characterized. In both panels, the positions of the RxRE and CRS are indicated by horizontal bars; nucleotide numbering starts at the beginning of the R region.

Figure 2.

Domain structure of the HTLV-1 and HTLV-2 Rex. The functional domains or regions of the 189 aa Rex-1 and 170 aa Rex-2 proteins are depicted in shaded boxes. The RNA binding domain (RBD) and nuclear localization signal (NLS) are located within the first 19 amino acids in the N-terminus. The Core activation domain (AD), which encompasses the nuclear export signal (NES), lies between residues 79 and 99 in Rex-1 and 81-94 in Rex-2, respectively. Two multimerization domains span amino acids 57-66 and 106-124 in Rex-1, whereas residues 57-71 and 124-132 constitute the multimerization domains in Rex-2. A unique C-terminal domain has been described for Rex-2 spanning residues 144 to 164 that is important for efficient function and includes key phosphorylation sites (Ser 151 and Ser 153). Mutations in this region are also impaired for efficient nucleo-cytoplasmic shuttling.

Figure 3.

The Rex-dependent export of viral mRNA. Shortly after transcription the RxRE-containing mRNA is recognized and bound by Rex through its RBD (a). Rex multimerization and CRM1 binding generates a complex (b) that is recognized by Ran-GTP (c). The passage of RNA/Rex/CRM1/Ran-GTP complex through the nuclear pore depends on extensive protein-protein interactions between CRM1, the FG-repeat domains of nucleoporins, and other factors (d). Once in the cytoplasm, Ran-GTP is hydrolyzed into Ran-GDP, causing the dissociation of the complex (e). The free mRNA is now available for either translation or packaging into virions. The other components of the complex shuttle back into the nucleus (f) to start another cycle. Rex itself utilizes importin- β for nuclear import.