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Review

The human T-cell leukemia virus type 1 p13^{II} protein: effects on mitochondrial function and cell growth

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Abstract

p13^{II} of human T-cell leukemia virus type 1 (HTLV-1) is an 87-amino-acid protein that is targeted to the inner mitochondrial membrane. p13^{II} alters mitochondrial membrane permeability, producing a rapid, membrane potential-dependent influx of K^+. These changes result in increased mitochondrial matrix volume and fragmentation and may lead to depolarization and alterations in mitochondrial Ca^{2+} uptake/retention capacity. At the cellular level, p13^{II} has been found to interfere with cell proliferation and transformation and to promote apoptosis induced by ceramide and Fas ligand. Assays carried out in T cells (the major targets of HTLV-1 infection *in vivo*) demonstrate that p13^{II}-mediated sensitization to Fas ligand-induced apoptosis can be blocked by an inhibitor of Ras farnesylation, thus implicating Ras signaling as a downstream target of p13^{II} function.

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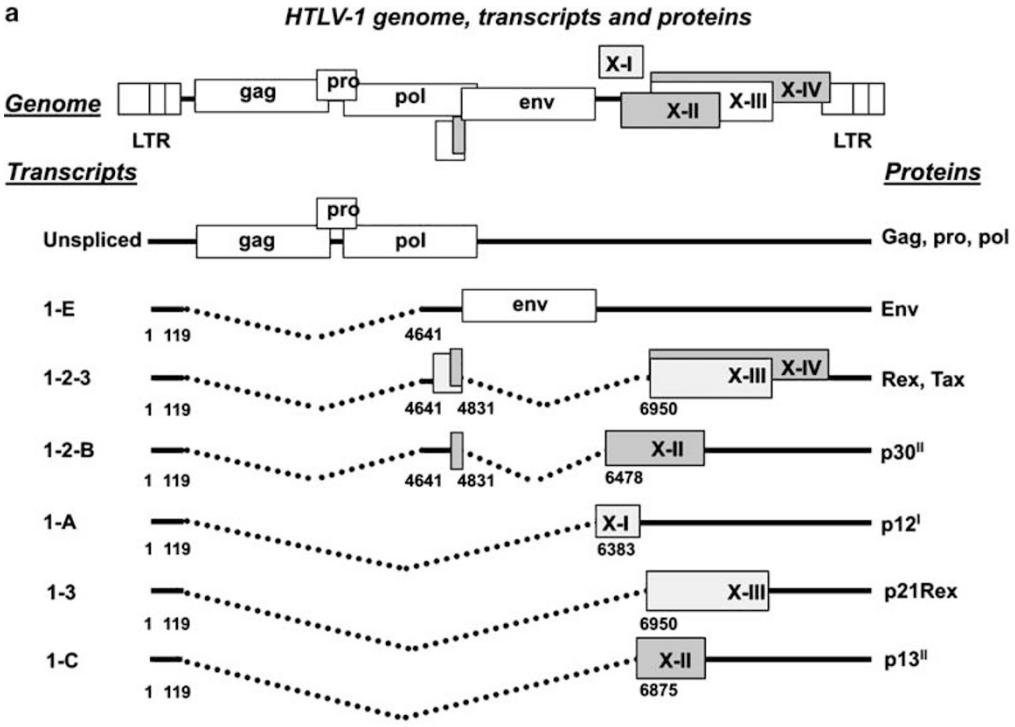
Abbreviations: ATLL, adult T-cell leukemia/lymphoma; CREB, cyclic AMP-responsive element binding protein; $\Delta\psi$, inner mitochondrial membrane potential; FasL, Fas ligand; FPPS, farnesyl pyrophosphate synthase; HAM/TSP, HTLV-associated myelopathy/tropical spastic paraparesis; HTLV-1, human T-cell leukemia virus type 1; IL-2, interleukin 2; MTS, mitochondrial targeting sequence; NLS, nuclear localization signal; ORF, open reading frame; PBMC, peripheral blood mononuclear cells; PTP, permeability transition pore; REF, rat embryo fibroblasts; RLM, rat liver mitochondria; RT-PCR, reverse transcription/polymerase chain reaction; SDS, sodium dodecyl sulfate

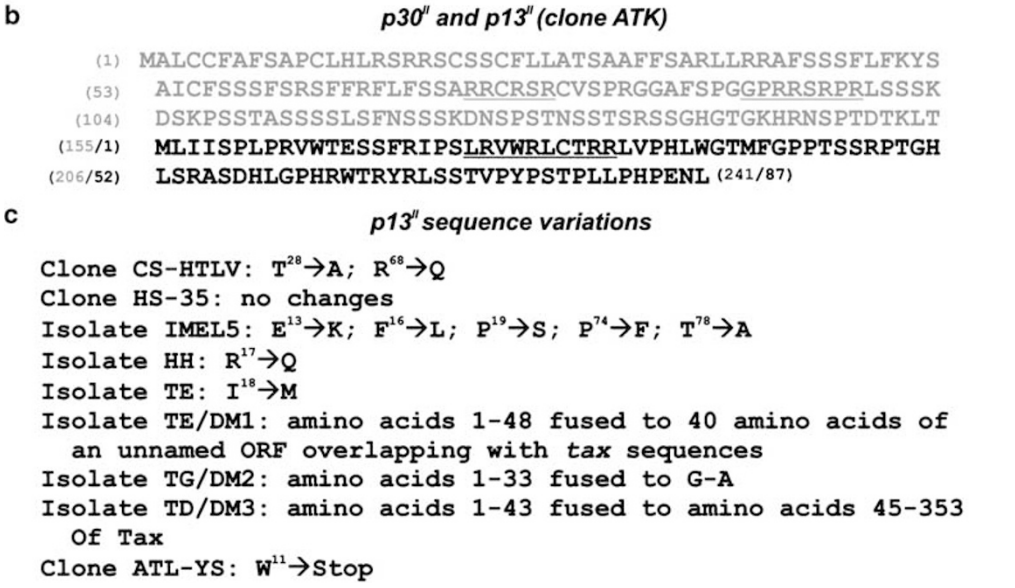
Introduction

Human T-cell leukemia virus type 1 (HTLV-1), the first human retrovirus identified, is the causative agent of an aggressive neoplasia of mature CD4 + T cells referred to as adult T-cell leukemia/lymphoma (ATLL), as well as a progressive demyelinating neurodegenerative disease termed HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP). The virus is transmitted through exchange of blood, semen, and breast milk, mainly by transfer of infected T lymphocytes (the principal targets of infection) rather than by free virus particles. It is estimated to infect 15–25 million people worldwide, with higher prevalence in southwestern Japan, Sub-Saharan Africa, the Caribbean basin, and among Amerindians. While most infected individuals remain asymptomatic, about 5% eventually develop HAM/TSP or ATLL after a latency period of several years (HAM/TSP) to decades (ATLL). HTLV-1 infection is also associated with several other disorders, including infectious dermatitis, arthritis, and uveitis (for recent reviews on HTLV-1 pathogenesis, see Franchini *et al.*[1] and Matsuoka[2]).

HTLV-1 is a member of the deltaretrovirus genus within the Retroviridae family of RNA viruses. It is classified as a 'complex' retrovirus, as its genome contains at least five extra open reading frames (ORFs) in addition to the gag, pol, pro, and env genes common to all retroviruses. The extra ORFs, designated with the prefix 'X', are found in a 3′ portion of the viral genome termed the 'X' region; they are expressed from alternatively spliced mRNAs (Figure 1a). The x-III and x-IV ORFs code for a post-transcriptional regulatory protein named Rex and a transcriptional transactivator named Tax, respectively, both of which are required for completion of the viral life cycle. The products of the x-I ORF (p12^{I}) and the x-II ORF (p30^{II} and p13^{II}) are referred to as accessory proteins (reviewed in Green and Chen[10] and Albrecht and Lairmore[11]).

Despite over 20 years of study, the molecular events responsible for the development of ATLL and HAM/TSP remain to be thoroughly understood. HTLV-1 does not code for an oncogene or integrate promoter sequences near a specific cellular gene, properties that define acutely and chronically transforming retroviruses, respectively. Infection of peripheral blood mononuclear cells (PBMC) with HTLV-1 yields interleukin 2 (IL-2)-dependent immortalized T cells, some of which progress to a fully transformed phenotype (i.e. grow in the absence of exogenous IL-2) after months to years in culture. The viral protein Tax plays a critical role in this process through its activation of the viral promoter and ability to influence the expression and function of a bewildering number of cellular genes involved in signal transduction, cell growth and apoptosis (reviewed in Franchini *et al.*[1]). While Tax is able to immortalize T cells *in vitro*, other undefined viral





c

p13^{II} sequence variations

Clone CS-HTLV: T^{28}→A; R^{68}→Q
Clone HS-35: no changes
Isolate IMEL5: E^{13}→K; F^{16}→L; P^{19}→S; P^{74}→F; T^{78}→A
Isolate HH: R^{17}→Q
Isolate TE: I^{18}→M
Isolate TE/DM1: amino acids 1-48 fused to 40 amino acids of an unnamed ORF overlapping with *tax* sequences
Isolate TG/DM2: amino acids 1-33 fused to G-A
Isolate TD/DM3: amino acids 1-43 fused to amino acids 45-353 Of Tax
Clone ATL-YS: W^{11}→Stop

Figure 1 Expression of the x-II ORF of HTLV-1. (**a**) HTLV-1 genome, ORFs, transcripts, and protein products are shown. The exon boundaries are indicated below each transcript, with nucleotide numbering starting at the first nucleotide in the R region of the LTR. (**b**) Protein sequences of p30^{II} (expressed from mRNA 1-2-B; in grey letters) and p13^{II} (expressed from mRNA 1-C; in black letters) coded by HTLV-1 molecular clone ATK are shown. The p13^{II} sequence spans nucleotides 6936–7197 and corresponds to the C-terminal 87 amino acids of p30^{II}. The bipartite NLS of p30^{II} and the MTS of p13^{II} are underlined. (**c**) Differences in the p13^{II} amino-acid sequence of the indicated viruses compared to ATK p13^{II} (see **b**) are shown. ATK and ATL-YS are proviruses cloned directly from peripheral blood lymphocytes of Japanese ATLL patients.[3,4] CS-HTLV is a full-length, infectious provirus derived from a North American ATLL patient.[5] HS-35 is a molecular clone obtained from an infected patient of Caribbean origin.[6] IMEL5 is an HTLV-1 isolate from a healthy Melanesian Solomon Islander.[7] Isolates HH, TE, TG and TD are from healthy carriers (HH) and HAM/TSP patients;[8,9] changes are based on published analyses of the portion of exon 3 (the second Tax/Rex exon) overlapping with exon C

and host factors appear to be necessary for the fully transformed phenotype associated with ATLL cells; a multi-faceted process of transformation is consistent with the low prevalence and long latency period associated with ATLL development. HAM/TSP patients exhibit higher proviral DNA loads in their peripheral blood cells and cerebral spinal fluid compared to ATLL patients and asymptomatic carriers. They also have high antibody titers to viral proteins, elevated levels of inflammatory cytokines, high numbers of activated T cells, and a high frequency of Tax-specific cytotoxic T lymphocytes,

suggesting that the immune response might contribute to the attack of myelin-producing cells and neuron destruction characteristic of this disease (reviewed in Barmak *et al*.[12]).

Unclear aspects of HTLV-1 replication and pathogenesis are being investigated from a number of directions, including studies of the viral accessory proteins. This review describes our current knowledge of p13^{II}, an 87-amino acid, 13-kDa accessory protein that is targeted to mitochondria.

Expression and Mitochondrial Targeting of p13^{II}

p13^{II} expression

p13^{II} is encoded in the x-II ORF. As shown in Figure 1b, this ORF is expressed from two distinct mRNAs that produce two proteins. The larger protein, named p30^{II}, is 241 amino acids in length and is produced from a doubly spliced mRNA that places the initiator codon of Tax in frame with x-II sequences.[13] p30^{II} is a nuclear/nucleolar protein[13,14] that contains a bipartite, arginine-rich nuclear localization signal (NLS) spanning residues 71–98.[15] p30^{II} influences transcription of cyclic AMP-responsive element binding protein (CREB)-responsive promoters, including the HTLV-1 promoter,[16,17] and acts as a post-transcriptional negative modulator of expression of the doubly spliced viral mRNA coding for Tax and Rex.[18,19] p13^{II} corresponds to the carboxy-terminal 87 amino acids of p30^{II} and is produced from a singly spliced mRNA that lacks the Tax initiator codon.[20,21] p13^{II} also lacks the NLS sequence of p30^{II}, and is predominantly mitochondrial,[22] although it can occasionally be detected in the nucleus,[14] especially when expressed at high levels (our unpublished observations). Mitochondrial accumulation of p13^{II} has been documented in a variety of cell types, including T cells, the natural target of HTLV-1 infection (Figure 2).

The singly spliced p13^{II} mRNA was initially identified in a reverse transcription/polymerase chain reaction (RT-PCR)-based analysis of infected cell lines and uncultured PBMC from HTLV-1-infected individuals. In this study, the p13^{II} mRNA was detected in two out of three IL-2-dependent HTLV-1-infected cell lines, 4/4 IL-2-independent infected cell lines, and in 6/10 ATLL patients, but in 0/3 healthy HTLV-1 carriers.[21] These observations suggest that the expression of p13^{II} might be more prominent during the course of disease development than in asymptomatic infection. This could reflect higher expression levels in individual cells or increased numbers of infected cells at later stages of disease, possibilities that could be explored by measuring viral DNA loads *versus* p13^{II} mRNA. Results of RNase protection assays carried out using cell lines derived from ATLL and HAM/TSP patients suggested that the p13^{II} mRNA may be expressed at relatively high levels.[23] Further studies of the expression of p13^{II} relative to other viral transcripts would greatly benefit by the more quantitative reverse transcription-real-time PCR technique, which would also be useful for determining the kinetics and levels of p13^{II} mRNA expression during natural infection and in different disease states.

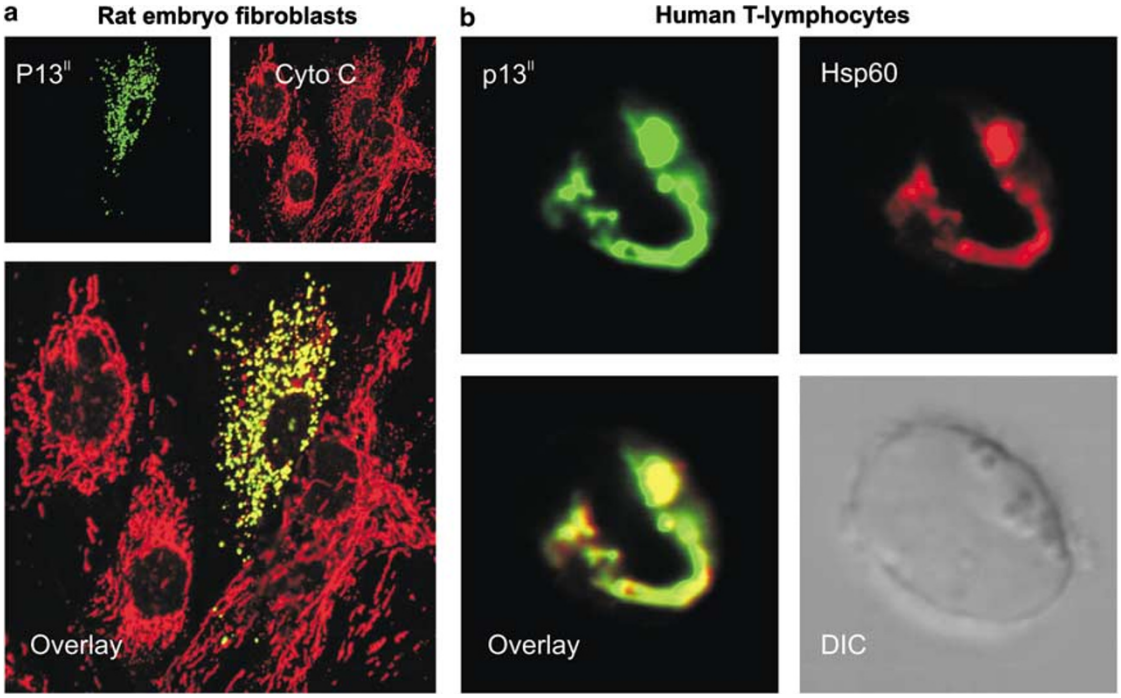


Figure 2 Mitochondrial localization of p13^{II}. (**a**) Primary rat embryo fibroblasts transfected with the p13^{II} expression plasmid pSGp13^{II} using Fugene 6 (Roche) are shown; (**b**) A human T-cell (Jurkat TetOn cell line; Clontech) transfected with the p13^{II} expression plasmid pcDNAp13^{II} using MegaFectin (Q.Biogene) are shown. After formaldehyde-fixation and permeabilization with Nonidet P40, the cells were analyzed by indirect immunofluorescence using a rabbit antibody recognizing p13^{II} and Alexa 488-conjugated anti-rabbit secondary antibody (Molecular Probes); to visualize mitochondria, the cells were counterstained with mouse anticytochrome *c* antibody (Pharmingen; **a**) or goat anti-Hsp60 antibody (Santa Cruz; **b**) and appropriate Alexa 546 red-conjugated secondary antibodies (Molecular Probes). In addition to verifying colocalization of 13^{II} with the mitochondrial marker proteins, (**a**) shows the dramatic changes in mitochondrial morphology induced by the protein

Expression of p13II at the protein level during infection and disease also remains to be established. Initial attempts to verify whether the x-II proteins are produced in HTLV-1-infected cells were discouraging, as immunoprecipitations carried out to identify x-II gene products in chronically infected cell lines failed to detect p30II or p13II, and antibodies against x-II sequences were not detected in a panel of sera from ATLL and HAM/TSP patients.[24] However, evidence for production of the x-II proteins *in vivo* was subsequently provided by the identification of antibodies recognizing p30II in a patient with HAM/TSP[25] and the detection of CTL directed against ORF x-II-encoded epitopes in HTLV-1-infected asymptomatic carriers, ATLL patients, and HAM/TSP patients, with one epitope specific for p30II and another common to p30II and p13II.[26] The inability to detect the x-II proteins in infected cells containing their mRNAs could be explained by limitations in the sensitivity of the antibodies used in these assays. In alternative, their expression might be restricted to specific stages in the viral life cycle and/or cellular states.

As shown in Figure 1b, the p13II ATG is positioned just 5′ to the splice acceptor used to generate exon 3, which contains the bulk of the *tax* and *rex* coding sequences; the virus thus exploits all three reading frames of the same sequence to produce p13II, Tax and Rex. Consistent with the generally low genetic variability of the HTLV-1 genome, the p13II amino-acid sequence appears to be highly conserved even among viruses of distant geographical origin (e.g. ATK, from Japan; CS-HTLV-1, from North America; HS-35, from the Caribbean; and IMEL5, from Melanasia; see Figure 1c). On the other hand, evidence indicates that the *tax* gene is subjected to immune pressure resulting in mutations and deletions during the natural course of infection.[8] Interestingly, some of the sequence variations affecting Tax would also introduce changes in the p13II sequence, resulting in single amino-acid substitutions, C-terminal truncations, and fusion proteins (see isolates HH, TE, TG, and TD; Figure 1c). In addition, analysis of HTLV-1 proviral sequences cloned from freshly isolated ATLL cells revealed the presence of a premature stop codon in the x-II ORF that would truncate 13II and p30II after residues 10 and 164, respectively.[4] It would be useful to build on these studies by determining whether disruption of the x-II ORF can be detected from the onset of infection or correlates with a specific disease stage.

HTLV-1 molecular clones constructed to contain mutations that disrupt either p30II or both p30II and p13II are able to produce infectious virus and immortalize human T cells in tissue culture, indicating that they are not essential for replication or immortalization *in vitro*.[27,28] However, subsequent *in vivo* studies of rabbits inoculated with T-cell lines immortalized by the wild-type HTLV-1 virus, a p30II/p13II double knockout, or a p30II knockout verified the importance of the x-II products for viral propagation *in vivo*. Both mutant viruses were found to be less infectious, raised a poor immune response and produced substantially lower proviral loads compared to the wild-type control, with the p30II knockout tending to revert to the wild-type sequence.[29,30] A mutant virus with a selective mutation ablating p13II expression was recently constructed and is being characterized (MD Lairmore, unpublished).

Mitochondrial targeting of p13II

The mitochondrial targeting sequence (MTS) of p13II was identified through analyses of p13II-green fluorescent fusion proteins lacking amino-terminal sequences.[22] While a deletion mutant lacking the first 18 residues exhibited mitochondrial targeting, a mutant lacking the first 31 amino acids showed a diffuse pattern throughout the cell, indicating that p13II's MTS lies between residues 19 and 31. A tag consisting of residues 21–30 was sufficient to relocalize GFP to mitochondria, thus identifying it as an MTS.[22] Amino-terminal positioning of the MTS appears to be important for it to function efficiently, given that neither p30II nor an N-terminal truncation mutant of p30II that lacks the NLS and initiates 56 residues before p13II's ATG is able to accumulate in mitochondria.[15]

p13II's MTS sequence (LRVWRLCTRR) includes four arginines that are predicted to form a positively charged face within an α-helix, thereby imparting amphipathic properties to this region.[22] Circular dichroism spectroscopy of a synthetic peptide spanning residues 9–41 (i.e. including the MTS) revealed that it folds into an α-helix upon exposure to membrane-mimetic solutions containing phospholipids or sodium dodecyl sulfate (SDS) micelles.[31] As expected, a peptide in which the four arginines were substituted with four prolines, which are known to disrupt α-helical folding, failed to adopt an α-helical conformation, while substitution of the arginines with glutamines or alanines and leucines did not interfere with folding.[31] Surprisingly, none of these substitutions had a substantial effect on the ability of p13II to accumulate in mitochondria (see below).[31]

Mitochondria are complex organelles bounded by two highly specialized membranes that define four mitochondrial subcompartments – outer membrane, intermembrane space, inner membrane, and matrix. Immunoelectron microscopy and fractionation techniques based on differential sensitivity to extraction with digitonin and sodium carbonate demonstrated that p13II is an integral membrane protein and accumulates mainly in the inner mitochondrial membrane.[31]

Import of nuclear-encoded proteins into mitochondria involves interactions with specific chaperones and translocases in the outer and inner membranes, and proceeds through different pathways depending on the nature of the protein's targeting sequence and structure (reviewed in Wiedemann *et al.* [32]). Some inner membrane proteins contain an N-terminal positively charged, cleavable presequence and a hydrophobic sequence that stops the protein in the inner membrane. Most inner membrane proteins with multiple membrane-spanning domains lack a presequence and generally remain intact after import. Membrane proteins encoded in the mitochondrial genome and a subset of nuclear-encoded mitochondrial proteins contain a cleavable N-terminal presequence that directs them into the matrix prior to insertion into the inner membrane (reviewed in Stuart[33]). Although p13II's MTS lies close to its N-terminus and is positively charged, it does not appear to be cleaved.[22] In addition, the observations made for the arginine substitution mutants described above indicate that the p13II MTS does not require the presence of these positively charged residues and works independently of its ability to fold into an α-helix.[31] These properties do not

appear to fit well with any of the inner membrane protein import pathways described above and suggest that the p13II MTS has peculiar sequence-structure requirements that might direct mitochondrial import through an alternative mechanism.

Effects of p13II at the Mitochondrial Level

Mitochondria are the site of a variety of essential processes including energy production and conservation, lipid metabolism, and control of apoptosis, redox potential, and intracellular calcium homeostasis. To gain insight into the mechanism of function of p13II at the mitochondrial level, current studies are aimed at examining its effects on mitochondrial morphology, ion permeability, and inner mitochondrial membrane potential ($\Delta\psi$), as well as its interaction with membranes, including the potential for multimerization and channel formation.

Effects of p13II on mitochondrial morphology

Examination of p13II-expressing cells by immunofluorescence showed that its accumulation in mitochondria disrupts the mitochondrial network into isolated clusters of round-shaped, apparently swollen mitochondria, some of which form ring-like structures (see Figure 2a and Ciminale *et al.*[22]). Electron microscopy confirmed morphological changes in mitochondria expressing p13II, including swelling and fragmentation of the cristae, with mitochondria exhibiting more prominent alterations often located in close proximity to cisternae of the endoplasmic reticulum,[31] suggesting a link between microdomains of high Ca^{2+} concentration and mitochondrial swelling. Interestingly, although mutants in which the arginines are replaced with glutamines, prolines or alanines and leucines retain mitochondrial targeting, they produce little or no mitochondrial fragmentation/swelling, indicating that the arginines of the amphipathic α-helical domain are essential for these effects.[31]

Effects of p13II on isolated mitochondria

In vitro assays revealed that a synthetic peptide spanning residues 9–41 ($p13^{9-41}$) is able to induce energy-dependent swelling of isolated rat liver mitochondria (RLM). This effect was not observed in sucrose-based media and depended on K^+ concentration and $\Delta\psi$. Additional assays showed that swelling also occurs (at a reduced rate) in the presence of other small cations such as Na^+, tetramethylammonium, and choline, but not Tris or larger cations, and that it depends on the presence of phosphate as a counter ion.

In addition to the swelling effect, $p13^{9-41}$ was found to induce mitochondrial depolarization and to alter the Ca^{2+} retention capacity of mitochondria. Importantly, all of these effects are evident when the peptide is used in the low micromolar range and require the presence of the critical arginine residues in the amphipathic α-helical domain,[31] suggesting a direct link between changes in mitochondrial permeability and the morphological alterations observed in the context of intact cells. More recent analyses of full-length synthetic p13II revealed that it is 10- to 15-fold more potent than $p13^{9-41}$ in inducing K^+-dependent permeability changes (Ciminale *et al.*, manuscript in preparation).

Mitochondrial swelling and altered permeability often reflect opening of the permeability transition pore (PTP), a phenomenon that can in turn lead to apoptosis. However, the observation that $p13^{9-41}$-induced swelling is not affected by the PTP inhibitor cyclosporin A suggests that PTP opening is not directly involved. However, p13II's ability to depolarize mitochondria is likely to lower the threshold for PTP opening and thus increase a cell's sensitivity to apoptosis induced by agents that work through the PTP, including Ca^{2+} (Figure 3); p13II-induced changes in the Ca^{2+} retention capacity of mitochondria could also have an impact on important signaling pathways controlled by this cation. In line with this hypothesis, cells expressing p13II show increased sensitivity to ceramide-induced apoptosis (Silic-Benussi *et al.*[34]; see below) as well as enhanced Ca^{2+}-dependent phosphorylation of the CREB transcription factor.[34]

Interaction of p13II with membranes – multimerization/channel formation

One of the many questions left unanswered by the studies carried out thus far is whether p13II's effects on mitochondrial permeability result from its interaction with an endogenous mitochondrial channel or an autonomous channel-forming activity. Investigations for binding partners of p13II have not provided any evidence for an interaction with endogenous channels; yeast 2-hybrid screens and pull-down assays indicate that p13II binds to a protein of the nucleoside monophosphate kinase superfamily, actin-binding protein 280, and farnesyl pyrophosphate synthase (FPPS).[35,36]

On the other hand, the following properties of p13II are suggestive of channel-forming activity: its insertion into membranes, presence of an amphipathic α-helix, and association into high-order, SDS-resistant complexes (D'Agostino *et al.*[31] and manuscript in preparation). These features are found in proteins that assemble into transmembrane oligomeric α-helical bundles such as glycophorin A and phospholamban (reviewed in Arkin[37]), and the viroporins, a family of small viral proteins that contain transmembrane amphipathic α-helical domains whose multimerization in the context of membranes results in the formation of channel-like structures. Two interesting examples of viroporins are the M2 protein of influenza A virus and p7 of hepatitis C virus, which, like HTLV-1, is a human tumor virus. M2, a 96-amino-acid integral membrane protein, is considered to be a prototype viroporin. Membrane insertion of M2 is mediated through a 19-amino-acid amphipathic α-helical domain. In the context of endosomal membranes, M2 assembles into homotetramers with proton channel activity and lowers endosomal pH, favoring uncoating, and nuclear import of the viral genome; these activities are inhibited by the anti-influenza drug amantadine (reviewed in Fischer and Sansom[38] and Gonzalez and Carrasco[39]). p7 is a 63-amino-acid protein that promotes both entry and release of viral particles. p7 forms a hexameric cation channel whose activity is also inhibited by amantadine.[40–42] Although, like p13II, p7 is mainly localized to

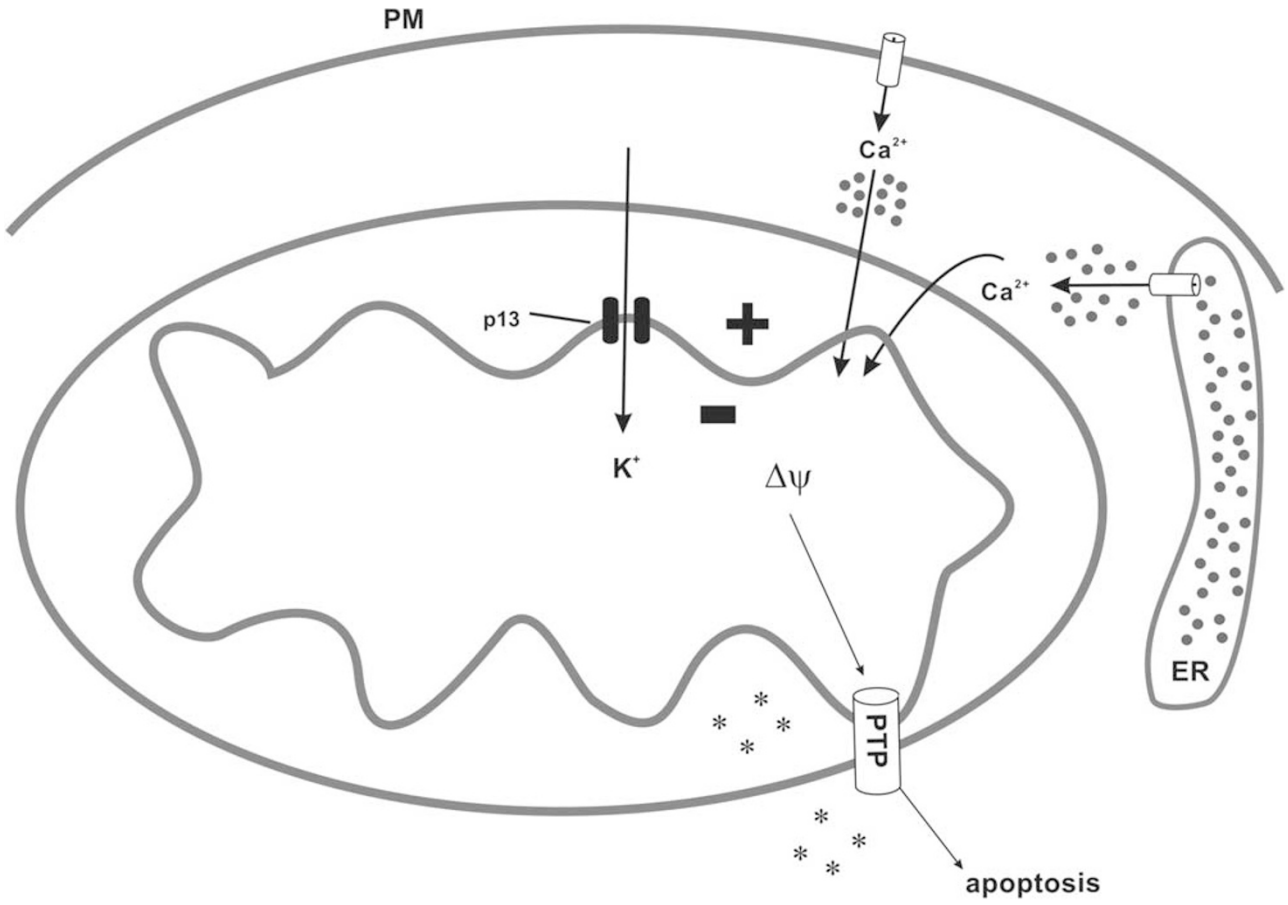


Figure 3 Working model of p13^{II}'s mechanism of action at the mitochondrial level. Insertion of p13^{II} into the inner mitochondrial membrane either directly or indirectly induces a rapid potential-dependent K^+ current in mitochondria. This influx of positive charges may lead to mitochondrial depolarization which, in turn, may (i) increase sensitivity to apoptosis by lowering the threshold for PTP opening; and (ii) alter the Ca^{2+} uptake capacity of mitochondria, thus changing intracellular Ca^{2+} homeostasis and signaling. PM, plasma membrane; ER, endoplasmic reticulum; $\Delta\psi$, inner mitochondrial membrane potential; PTP, permeability transition pore; grey dots indicate Ca^{2+}, asterisks indicate proapoptotic factors contained in the mitochondrial intermembrane space, for example cytochrome *c*, apoptosis-inducing factor, SMAC/Diablo

mitochondria,[41] its effects on mitochondrial morphology and function have not been investigated. Another small viral protein with channel forming-properties is human immunodeficiency virus type 1 Vpr, a 96-amino acid, multifunctional protein that is detected in mature viral particles, the nucleus, and mitochondria. Vpr is able to form cation-selective-channels in phospholipid bilayers, a property that involves an arginine-rich C-terminal portion of the protein that is predicted to fold into a membrane-spanning α-helix.[43] In the context of mitochondria, Vpr induces depolarization and triggers release of proapoptotic proteins.[44] Vpr-induced cell death is dependent on both ANT and VDAC, suggesting involvement of the PTP[44] (reviewed in Boya *et al*.[45]). In artificial membranes, a Vpr peptide that includes the α-helix is able to associate with ANT, resulting in the formation of large conductance channels.[46] In addition to inducing apoptosis *in vitro*,[47–49] Vpr exerts antitumor effects in immunocompetent mice, probably through modulation of the immune response.[50] Vpr also mediates nuclear targeting of the viral genome following reverse transcription, induces cell cycle arrest at G2/M, and activates a number of viral and cellular promoters (reviewed in Kino and Pavlakis[51]).

The Impact of p13^{II} on Cell Growth and Death

In addition to p13^{II}, several other proteins coded by tumor viruses are targeted to mitochondria and have an impact on mitochondrial morphology and functions including ion permeability and $\Delta\psi$. Importantly, some of these proteins also have documented effects on cell death and oncogenic transformation (Table 1). As described below, we are only beginning to unravel the effects of p13^{II} on cell turnover.

HeLa cell lines expressing p13^{II} either transiently or stably do not appear to be more prone to spontaneous apoptosis. However, increased apoptosis is evident upon treatment of p13^{II}-expressing HeLa cell lines with C2-ceramide,[34] a proapoptotic agent that acts by triggering opening of the PTP.[66–70] Enhancement of ceramide-induced apoptosis by p13^{II} could be caused by p13^{II}-mediated changes in $\Delta\psi$ and/or mitochondrial Ca^{2+} permeability, two well-defined powerful effectors that act by lowering the threshold for PTP opening and are observed upon exposure of isolated mitochondria to p13^{II}.

To extend these observations to T cells, subsequent studies tested the effects of p13^{II} in the T-cell line Jurkat

Table 1 Comparison of p13II with other tumor virus proteins that disrupt mitochondrial morphology and function

Protein	Effects on mitochondrial morphology	Effects on mitochondrial function	Effects on cell growth and death	Role in viral lifecycle
HTLV-1 p13II	Fragmentation, swelling[22]	Increased permeability to small cations; depolarization (ion channel activity?)[31]	Antiproliferative and antitumor effects; promotes apoptosis induced by C2-ceramide (Silic-Benussi *et al*.[34]; Hiraragi *et al*., submitted)	X-II ORF required for maintaining high proviral loads *in vivo*[30]
BLV G4	Fragmentation, swelling[52]	Unknown	Cooperates with Ha-Ras in REF transformation;[53] required for development of BLV-associated neoplasia[53]	Required for maintaining high proviral loads *in vivo*[54]
HBV HBx	Perinuclear clustering[55,56]	Interaction with VDAC3;[57] depolarization;[57] reduced Ca^{2+} uptake;[58] increased ROS and lipid peroxide;[59] decreased levels of enzymes involved in oxidative phosphorylation and electron transport[59]	Cell system dependent: can either suppress or promote oncogenic transformation, cell growth, and apoptosis (reviewed in D'Agostino *et al*.[60])	Required for productive infection *in vivo* (woodchuck model)[61]
HPV E1^E4	Perinuclear clustering[62]	Depolarization[62]	Proapoptotic;[62] arrests cells in G2[63,64]	Required for productive infection *in vivo* (cottontail rabbit model)[65]

BLV, bovine leukemia virus; HBV, hepatitis B virus; HPV, human papillomavirus; HTLV-1, human T-cell leukemia virus type 1; ORF, open reading frame; ROS, reactive oxygen species; VDAC3, voltage-dependent ion channel 3

(Hiraragi *et al*., submitted). Annexin V staining assays confirmed that Jurkat T cells expressing p13II show increased sensitivity to apoptosis induced by C2-ceramide or Fas ligand (FasL). These two signals are connected in the same pathway and lead to the release of proapoptotic factors from the mitochondrial intermembrane space: Fas-FasL engagement on T cells results in activation of endogenous acidic sphingomyelinase, which leads to ceramide accumulation and apoptosis (Figure 4).[71]

FasL- and ceramide-induced apoptosis in lymphocytes is controlled by the Ras signal transduction pathway, as indicated by the fact that pharmacological inhibition of Ras prenylation with a farnesyl transferase inhibitor interferes with Ras-mediated apoptosis in Jurkat T cells.[72] In fact, farnesylation is a critical step for Ras's association with the plasma membrane and functional activation. Consistent with this model, we observed that Jurkat T cells transiently transfected with a plasmid expressing Ha-Ras undergo apoptosis when exposed to FasL, and can be protected from this apoptotic stimulus by the addition of the farnesyl transferase inhibitor B581 (Hiraragi *et al*., submitted). Likewise, preincubation of p13II-expressing Jurkat T cells with B581 results in marked, dose-dependent protection against apoptosis induced by FasL (Hiraragi *et al*., submitted). Taken together, these results indicate that Ras is an important modulator of apoptosis in p13II-expressing Jurkat T cells and reveal a potential new mechanism of HTLV-1-induced alteration of lymphocyte survival that could be used as a target for intervention against viral-induced cell transformation[73] (see Figure 4).

Upon FasL exposure, Ras can translocate to mitochondria and bind to Bcl-2, resulting in interference with Bcl-2's antiapoptotic function.[72] Studies carried out using a murine T-cell line indicate that mitochondrial association of different Ras isoforms is differentially regulated by IL-2.[74] The control of Ras localization by IL-2 suggests that p13II might play a unique role in lymphocyte responses to apoptotic stimuli during the progression of HTLV-1-mediated T-cell transformation, a process that is intimately linked to the switch from IL-2-dependent to IL-2-independent growth.[1,75]

The p13II-Ras signaling connection is further substantiated by studies which revealed interesting analogies and differences between p13II and the G4 protein of bovine leukemia virus, an oncogenic deltaretrovirus of cattle that is related to HTLV-1.[76] Oncogenic conversion assays showed that G4 cooperates with H-Ras to transform primary rat embryo fibroblasts (REF).[53] In contrast, p13II was found to inhibit transformation of REF by c-Myc plus H-Ras, significantly reducing both tumor incidence and growth rate. This antitumor effect was confirmed in additional experiments carried out using HeLa-derived cell lines stably expressing p13II, which produced significantly fewer, more slowly growing tumors compared to control lines.[34]

Like p13II, G4 interacts with FPPS,[36] an enzyme involved in the biosynthesis of isoprenoid-derived molecules (reviewed in Liang *et al*.[77]). Among their many functions, these molecules are transferred by prenyl transferases to a large number of key proteins controlling cell growth, differentiation, vesicle trafficking, and cytoskeletal dynamics (reviewed in Roskoski Jr[78]). As mentioned above, one very noteworthy substrate of this pathway is Ras, whose activity requires prenylation.[79] The fact that G4's oncogenic properties and ability to interact with FPPS map to the same region of the protein support the hypothesis that G4 might promote transformation by altering FPPS-dependent prenylation of key regulatory proteins such as Ras. The apparently opposite effects on transformation exerted by G4 and p13II suggest that they might exert a distinct control on FPPS function and Ras signaling. In alternative, the contrasting properties documented for p13II and G4 may reflect variations in their expression levels and other subtle differences in the experimental systems employed.

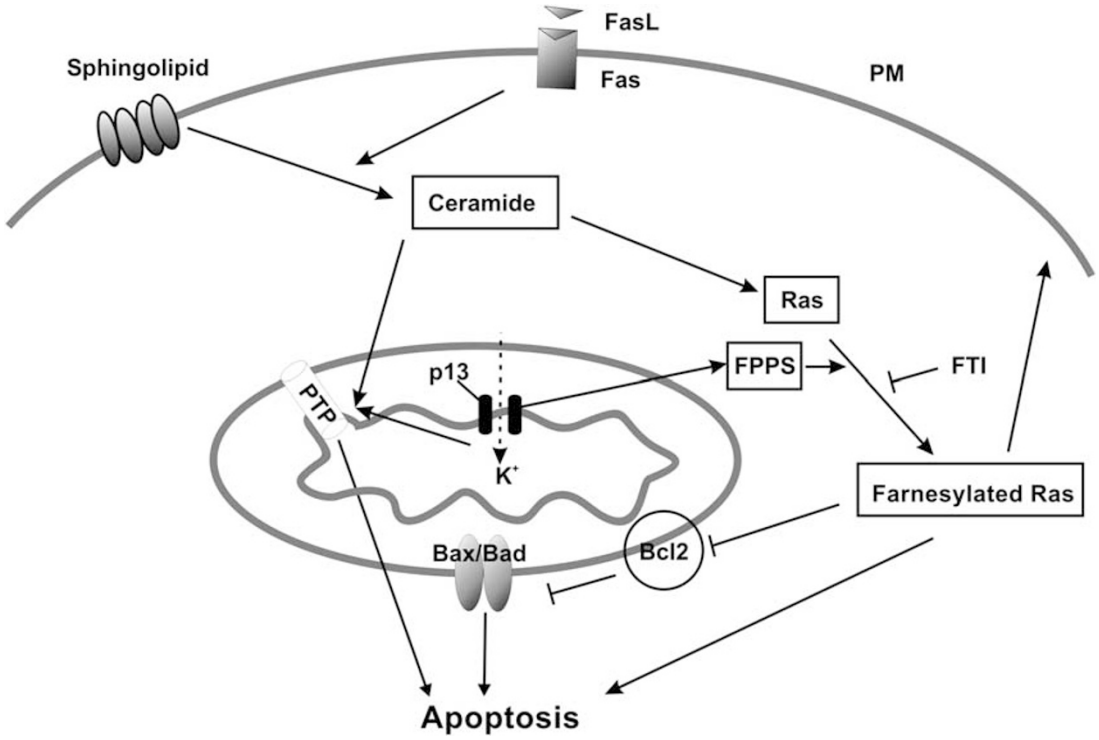


Figure 4 Possible effects of p13^{II} on apoptosis and Ras function. The Fas and Fas-ligand (FasL) interaction results in activation of sphingomyelinase and generation of ceramide, which leads to PTP sensitization and is a known activator of Ras. Ras function is dependent on its post-translational farnesylation, mediated by farnesyl transferase (FTase). The farnesyl substrate group (farnesyl pyrophosphate) is synthesized through the mevalonate–squalene pathway by farnesyl pyrophosphate synthetase (FPPS), a cellular binding partner of p13^{II}. p13^{II} might affect these pathways by enhancing PTP sensitization and/or by controlling Ras farnesylation/function through its binding to FPPS. FTI, farnesyl transferase inhibitor

Although p13^{II} has a clear enhancing effect on C2-ceramide-induced apoptosis in the experimental systems tested so far, its influence of cell growth may in fact be more complex based on recent studies which revealed that ceramide and its metabolites sphingosine and sphingosine 1-phospate can exert multiple controls on several T-cell signaling pathways, including T-cell receptor (TCR)-mediated activation and activation-induced cell death, TCR surface expression, Ca^{2+}-mediated apoptosis, cell cycle progression, and proliferation in response to IL-2 (reviewed in Adam *et al*.[71]). These different effects are largely dependent on the activation status of the cell and on the concentration of the sphingolipids, which would thus act as a 'rheostat' that ultimately controls the balance between T-cell proliferation and death.[71,80,81]

It is thus not surprising that, in addition to their increased sensitivity to C2-ceramide and FasL, Jurkat T cells expressing p13^{II} exhibit reduced proliferation rates compared to control cells, especially as they reach high cell densities.[34] This property could be indicative of the reaction of p13^{II} expressing cells to stress conditions (e.g. reduced availability of metabolic substrates and/or growth factor deprivation) associated with high density culture.

The apoptosis-sensitizing and antiproliferative effects of p13^{II} could be thought of as a safeguard that limits the oncogenic potential of HTLV-1 in general and of the Tax transactivator in particular, resulting in enhanced long-term coexistence with its host, a hallmark of the natural history of HTLV-1 infection. Disruption of p13^{II} expression/function may favor termination of this benign coexistence and promote development of ATLL. This hypothesis is supported by the above-mentioned description of a premature stop codon in p13^{II} in a molecular clone derived from primary, uncultured ATLL cells (clone ATL-YS; see Figure 1c and Chou *et al*.[4]), and could be verified by thorough analyses of p13^{II}'s sequence and expression in other freshly isolated ATLL samples. On the other hand, p13^{II}'s effects might be important to optimize viral spread by reaching an 'ideal compromise' between expansion of the infected cell population, persistent infection, and prolonged survival of the host, a possibility that is suggested by studies in animal models indicating the importance of the x-II ORF for efficient viral propagation *in vivo*.[30] p13^{II}'s resemblance to viroporins suggests that one of its major tasks might indeed be to control viral production/transmission, a possibility that we are testing using the p13^{II} knockout virus mentioned above. Such a role would open up the possibility of interfering with p13^{II}'s activities by treatment with viroporin inhibitors modeled from amantadine.

Although the data collected so far have mainly been interpreted in the framework of ATLL and cell transformation, it is possible that the effects of p13^{II} on mitochondria and cell survival might also be relevant in the context of HAM/TSP. The detection of HTLV-1 in the astrocytes of a patient suffering from both HAM/TSP and HIV-associated dementia[82] and in cells of the microglia/macrophage lineage in a rat model of HAM/TSP[83] suggests a direct role for the virus in triggering

a toxic environment for neurons. The established contribution of mitochondrial dysfunction to a variety of pathologies, including neurodegenerative diseases,[84] suggests that p13II might indeed have an impact on the development of HAM/TSP.

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