The EMBO Journal vol.9 no.13 pp.4243-4248, 1990

Mutations introduced along the HTLV-I envelope gene result in a non-functional protein: a basis for envelope conservation?

Claudine Pique, Thomas Tursz and Marie-Christine Dokhelar

CNRS UA 1156, Institut G. Roussy, Rue C. Desmoulins, 94805 Villejuif, France

Communicated by A.Burny

The envelope protein of the human T-cell leukemia virus type I (HTLV-I) is highly conserved among the isolates sequenced so far, as opposed to what is observed for the human immunodeficiency virus (HIV) envelope. By linker insertion scanning, we have produced 33 random mutations along the HTLV-I envelope gene, cloned into a eukaryotic expression vector. The resulting envelope products were analysed by immunoprecipitation and syncytia formation after transfection into COS-1 cells. We show here that 25 out of 33 mutations result in a non-functional envelope product as assessed by the lack of ability to form syncytia. In the majority of these mutants, the processing of the envelope gp61 precursor into the mature gp45 and gp20 proteins was affected. We propose that conformational constraints for processing and fusion abilities tend to limit the variability of the HTLV-I envelope. In three mutants, processing was observed but no syncytia were formed. These mutations might affect regions important for HTLV-I envelope functions, such as the receptor binding region.

Key words: envelope protein/HTLV-I/syncytia formation

Introduction

The human T-cell leukemia virus type I (HTLV-I) is a retrovirus responsible for adult T-cell leukemia (ATL) (Poiesz *et al.*, 1980; Yoshida *et al.*, 1982). It has also been associated with a neurological disease, the tropical spastic paraparesis (TSP) (Gessain *et al.*, 1985) also called HTLV-I associated myelopathy (Osame *et al.*, 1986). HTLV-I isolates obtained from either TSP or ATL show a remarkable conservation of the amino acid sequence of the envelope protein (Daenke *et al.*, 1990; Seiki *et al.*, 1983; Tsujimoto *et al.*, 1988; Malik *et al.*, 1988). However, a very high mutation rate has been observed in retroviruses (Gojobori and Yokoyama, 1985). Comparing the sequences of nine retroviruses to establish evolutionary relationship, McClure *et al.* (1988) could show that the various gene products are changing at different rates, the reverse transcriptase being the least, and the envelope proteins the most different from one virus to another. When distinct isolates of the human immunodeficiency virus type 1 (HIV-1) are compared, as much as 20% differences in the envelope amino acid sequences can be observed (Hahn *et al.*, 1985; Alizon *et al.*, 1986; Starcich *et al.*, 1986; Willey *et al.*, 1986; Srinivasan *et al.*, 1987; Gurgo *et al.*, 1988). As proteins exposed at the viral surface, the envelopes are believed to exhibit very rapid evolutionary changes under selective pressure for evading host defence systems. In this regard, the high degree of conservation of HTLV-I envelopes is remarkable.

The synthesis of the envelope gene products of HTLV-I is essentially similar to that of other retroviruses (Dickson *et al.*, 1984): a precursor protein (gp61) is synthesized on the endoplasmic reticulum, it then becomes glycosylated in the Golgi apparatus, proteolytically cleaved and expressed at the host cell surface (Lee *et al.*, 1984; Schneider *et al.*, 1984; Hattori *et al.*, 1984). By analogy with what is known for HIV-1 (Kowalski *et al.*, 1987; Lasky *et al.*, 1987) or animal retroviruses (Weiss *et al.*, 1982; Haseltine *et al.*, 1985), the larger mature product of the HTLV-I envelope, gp45, is believed to be responsible for the binding to a still unidentified receptor, which gene is encoded on human chromosome 17 (Sommerfelt *et al.*, 1988). The gp45 is bound to the smaller protein, gp20 which would ensure anchorage of the envelope protein at the membrane. Gp20 would also be responsible for fusion between the virus and the target cell membrane, as well as for syncytia formation between infected and receptor bearing cells (Hoshino *et al.*, 1983; Nagy *et al.*, 1983). Analysis of the HTLV-I envelope protein sequence indeed shows that the putative NH_2-terminal part of the gp20 bears a 29 amino acid long hydrophobic stretch which has the characteristics of a fusion peptide (Weiss *et al.*, 1982). The anchorage domain would correspond to a hydrophobic sequence located near the carboxyl terminus of the gp20, before a short intracytoplasmic region (see Figure 2A).

In this study, we have analysed the effects of random mutations introduced along the HTLV-I envelope (*env*) gene on the proteolytic cleavage of the gp61 precursor glycoprotein and on syncytia formation. We have found that most of the mutations studied highly affect the processing of the envelope and shown that the uncleaved gp61 is not able to induce syncytia formation in the absence of detectable gp45 and gp20. We discuss the relevance of this phenomenon to the observed high degree of conservation of the envelope among various HTLV-I strains.

Results

Mutagenesis of the HTLV-I envelope gene

In order to analyse the effect of mutations on HTLV-I envelope mediated functions, 33 linker insertion mutants of the HTLV-I *env* gene were constructed at random sites. As no infectious HTLV-I provirus is available, the envelope gene had previously been cloned into a eukaryotic expression vector, HTE-1 (Dokhélar *et al.*, 1989). The mutations resulted into the insertion of three to four amino acids into the envelope protein. They were named HTE-'X', X referring to the position of the last wild type amino acid before the insertion (counting from the Met corresponding to the initiation codon of the envelope). The predicted amino acid

Table I. Effect of HTLV-I envelope mutations located near the tryptic-like cleavage site on gp61 precursor cleavage and syncytia formation

Plasmid name	Amino acid sequence at the cleavage site[a]	gp61 Cleavage	Syncytia formation
HTE-1[b]	LGSRSRR*AV	+++	+
HTE-307	LGGRSSSRSRR*AV	+	−
HTE-308	LGSQFRSRSRR*AV	+++	+
HTE-309	LGSRSGSRSRR*AV	+++	+

[a]The consensus cleavage sequence is underlined, *: cleavage site between gp45 and gp20.
[b]Wild-type envelope expressor plasmid.

Table II. HTLV-I envelope mutations and their effect on envelope precursor cleavage and syncytia formation

Plasmid name	Amino acid sequence wt[a]	mutant[b]	gp61 Cleavage	Syncytia formation
Wild-type			+++	+
HTE-24	PSC	PREDLPC	+	+
HTE-25	PSC	PSRDRDC	+	+
HTE-27	CTL	CGRSSTL	−	−
HTE-37	YHS	YHGRSSS	−	−
HTE-59	ADQ	AQQDLPQ	−	−
HTE-74	SSY	SREDLPY	−	−
HTE-75	SSY	SSRDRDY	−	−
HTE-166	VDA	VDRSVDA	−	−
HTE-195	SNL	SNRSANL	+	−
HTE-200	LEP	LGRSSEP	+	−
HTE-205	IPW	IPCRSAW	+	−
HTE-206	PWK	PWGFPWK	−	−
HTE-215	QLT	QFRDRET	−	−
HTE-216	QLT	QLEDLPT	−	−
HTE-229	IDR	IADLR	−	−
HTE-282A	QAI	QGKIFPI	−	−
HTE-282B	QAI	QVAIATI	−	−
HTE-290	CHN	CHRSGHN	−	−
HTE-313	AVP	AEDLP	−	−
HTE-327	GAG	GIPMGAG	−	−
HTE-328	AGV	AAQACV	−	−
HTE-329	GVA	GKIFLA	+	−
HTE-343	SGK	SGRSSSGK	+	−
HTE-381	RGL	RGEDLPL	−	−
HTE-382	LDL	LEEDLPL	−	−
HTE-383	LDL	LDRDRDL	+	−
HTE-441A	EAL	EGKIFPL	++	+
HTE-441B	EAL	EGLIFPL	++	+
HTE-466A	QLR	QGRSSLR	+++	+
HTE-466B	QLR	QSRSRLR	+++	+

[a]The amino acid sequence of the wild-type envelope is given using the one letter amino acid code.
[b]The amino acids inserted in the mutant are underlined.

sequences of the envelope products expressed by the mutants are compared with the wild-type sequence in Tables I and II.

As the envelope-mediated fusion is known to be dependent upon a correct processing in other viruses, three different mutations were introduced near the potential tryptic-like cleavage site (Arg-Ser-Arg-Arg) which starts at position 309 (detailed in Table I). The three resulting mutants conserve the tryptic-like sequence and the −1 position relative to this site, but in a modified surrounding. The other 30 mutations constructed are listed in Table II. In some cases, two different linkers were introduced at the same enzymatic site to check whether introducing different amino acids resulted in similar phenotypes (HTE-282A and B, HTE-441A and B, HTE-466A and B). These mutated constructions were transfected into COS-1 cells, and compared with the plasmid expressing wild-type envelope glycoproteins (HTE-1). As a negative control, a non-sense codon was introduced into the *env* frame (HTE-24stop), at position 24 of the envelope protein immediately after the presumed signal peptide sequence. The effects of the envelope mutations on envelope glycoprotein expression and processing, and on syncytia formation were analysed.

Expression and processing of the mutant envelope glycoproteins

After transfection of the mutant constructions into COS-1 cells, immunoprecipitation of radiolabelled lysates was performed using ATL and TSP patients sera (gifts from Dr A.Lever, London and Dr F.Denis, Limoges). Both the cell supernatants and cell lysates were analysed. The results of representative radioimmunoprecipitations are shown in Figure 1. During the labelling period, wild-type envelope precursor gp61 was cleaved into the mature products gp45 and gp20 (Figure 1, lanes 3, 9 and 15). As previously reported (Dokhélar *et al.*, 1989), the *env* products obtained from the wild-type construction were comparable with those obtained from the C91/PL HTLV-I infected cell line. The envelope precursor gp61 could be detected in all 33 mutants tested (Figure 1, Table I and data not shown). None of the mutations appeared to drastically disrupt the glycosylation process of the envelope precursor, since the precursor's mobility in SDS−polyacrylamide gels was virtually the same for all the mutants and similar to the wild-type gp61 (Figure 1).

Among the three mutations performed near the tryptic-like cleavage site (HTE-307, HTE-308 and HTE-309), one resulted into a reduction of cleavage (Figure 1, lane 23) whereas the other two did not affect the cleavage (Figure 1, lane 22 and data not shown). These results show that the direct surrounding of the tryptic site can be modified without loss of cleavage.

Four out of 30 mutations located away from the tryptic site on the linear envelope sequence had no detectable or low effect (Table II and Figure 1) on the envelope glycoprotein synthesis or processing as compared with the wild-type envelope (Figure 1, lanes 28, 29, 30 and data not shown). These mutations are located in the carboxy-terminal part of the envelope, just before (HTE-441A and HTE-441B) the transmembrane region or right after it (HTE-466A and HTE-466B) (Figure 2, white circles). Surprisingly 26 mutations resulted in a complete (18 mutants) or partial (8 mutants) loss of cleavability (Table II). In these mutants, the amount of precursor gp61 protein obtained was comparable with the wild-type (Figure 1), whereas the gp45 and gp20 were barely or not detected. The lack of detection of mature products was not due to a defect of binding to the lentil/lectin in the immunoprecipitation experimental process (see Materials and methods), as no gp45 or gp20 could be detected in the transfected cell lysates prior to lectin

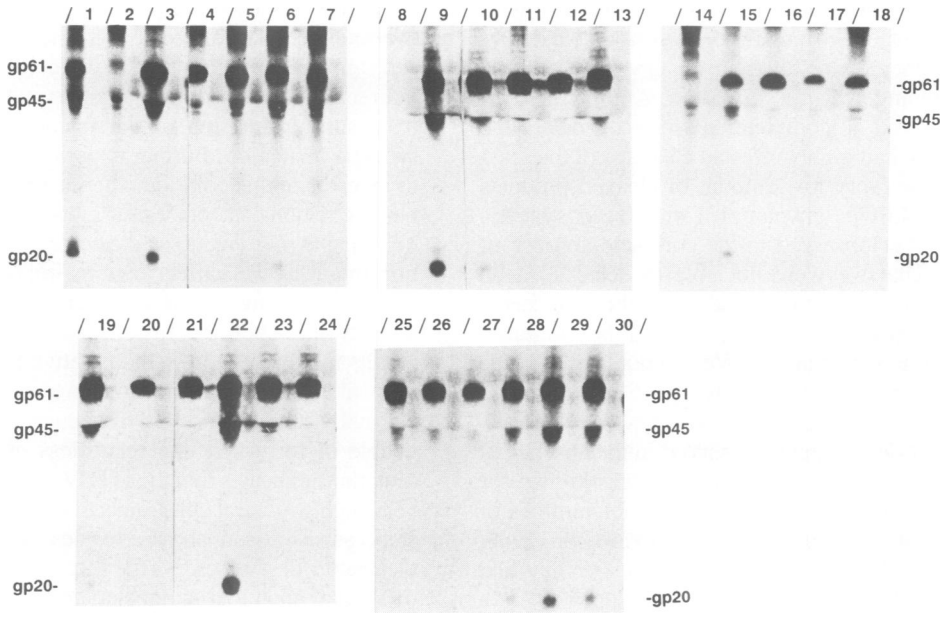
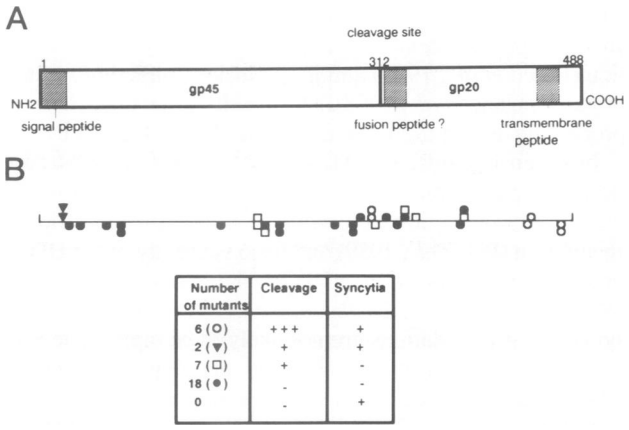


Fig. 1. Immunoprecipitation of the envelope proteins expressed by the different envelope mutants. For each mutant, the left and right lanes correspond to immunoprecipitation of transfected cell lysates, and cell supernatant, respectively. Lane 1: C91/PL HTLV-1 infected cells; lanes 2, 8, 14: HTE-24stop (negative control); lanes 3, 9, 15: HTE-1 (wild-type envelope); lane 4: HTE-37; lane 5: HTE-75; lane 6: HTE-195; lane 7: HTE-200; lane 10: HTE-24; lane 11: HTE-27; lane 12: HTE-74; lane 13: HTE-205; lane 16: HTE-59; lane 17: HTE-206; lane 18: HTE-215; lane 19: HTE-329; lane 20: HTE-216; lane 21: HTE 282A; lane 22: HTE-309; lane 23: HTE-307; lane 24: HTE-313; lane 25: HTE-343; lane 26: HTE-381; lane 27: HTE-382; lane 28: HTE-441A; lane 29: HTE-466A; lane 30: HTE-441B.

treatment (data not shown). No envelope protein could be detected in the cell supernatant, ruling out the possibility of a shedding of the envelope mature proteins (Figure 1, right lane for each mutant). When two different insertions were made at the same site (Table II: HTE-282A and HTE-282B; HTE-441A and HTE-441B; HTE-466A and HTE-466B), both mutants exhibited the same phenotype (cleavage for HTE-441 and HTE-466, absence of cleavage for HTE-282).

Effect of the mutations on syncytia formation

Cells transfected with the different constructions were cocultivated for 24 h with rat XC cells, and syncytia formation was scored after coloration of the plates. Cells transfected with the wild-type envelope construction routinely formed 50 syncytia per 2×10^5 transfected cells. The effect of the envelope mutations on syncytia formation was analysed. As shown in Table I and Table II and summarized in Figure 2, 25 out of 33 mutants were not able to form syncytia. In the absence of detectable processing, syncytia formation was never observed (Figure 2). Syncytia formation (> 20 nuclei) was detected only with some of the mutants that allowed a partial or a normal cleavage of the gp61 precursor into gp45 and gp20. When observed, syncytia were usually similar to the wild-type syncytia, both in terms of size and of number of syncytia per transfected cell, even when the ratio of precursor to cleaved products was higher than in the wild-type (see for instance HTE-24 in Table II and Figure 1). This probably indicates that the syncytia assay is much more sensitive for detection of mature products than the immunoprecipitation. Finally, seven mutants exhibited a clearly detectable amount of cleaved products, but were not able to induce syncytia formation (Figure 2). These mutants are HTE-195, 200, 205, 307, 329, 343 and 383. Note that processing is hardly seen for HTE-200 in Figure 1, but was clear on the original gel.



Number of mutants	Cleavage	Syncytia
6 (○)	+++	+
2 (▼)	+	+
7 (□)	+	-
18 (●)	-	-
0	-	+

Fig. 2. Schematic representation of the HTLV-I envelope protein, and summary of the envelope mutants phenotype. **A.** The representation of the HTLV-I envelope protein is based upon the deduced amino acid sequence (Seiki *et al.*, 1983). **B.** Schematic representation of the different mutations, showing their localization in the different envelope regions and their phenotype.

These mutations might affect directly the receptor binding region (HTE-195, HTE-200, HTE-205, see discussion below). They might also indirectly affect binding via conformational effects, or act on a subsequent step of the fusion process (HTE-307, HTE-329, HTE-343, HTE-383) required for syncytia formation. This is obviously the case for HTE-329, which is mutated in the hydrophobic stretch in the N-terminal part of gp20, thus emphasizing that this is indeed the fusion peptide.

Discussion

In this study, we have introduced mutations into the HTLV-I envelope gene and analysed their effects on envelope-

mediated functions. Results have been summarized in Figure 2. Surprisingly, out of 33 mutations performed randomly along the *env* gene, 25 resulted in an envelope product incapable of inducing syncytia formation. Among these 25 mutants, 18 showed a complete absence of detectable cleavage and seven had highly affected cleavage of the gp61 precursor envelope (very low amount of cleaved products compared with wild-type envelope). Conversely, syncytia formation was never observed in the complete absence of gp61 cleavage. That we were not able to detect cleaved products was not due to gp45 shedding in the transfected cell supernatant, since no envelope could be immunoprecipitated in the supernatant. We cannot exclude the possibility, however, that the mutated gp45 are degraded very rapidly as compared with the wild type.

Syncytia formation was never observed in the absence of gp61 cleavage, which is reminiscent of what is known for other viruses. Not only retroviruses, but other families of enveloped viruses such as paramyxoviruses (Morrison, 1988; Paterson *et al.*, 1989) and orthomyxoviruses (Wiley and Skehel, 1987) synthesize their envelope glycoprotein as a precursor product. In these viruses, cleavage of the immature protein is required for efficient infectivity of the virus (Homma and Ohuchi, 1973; Nagai *et al.*, 1976; Choppin and Scheid, 1978), probably because the glycoprotein cleavage is necessary for the exposition of its fusion domain (Hsu and Choppin, 1981), and the subsequent disassembly of the virus and finally delivery into the cytoplasm. Similarly, in the HIV-1 virus, it has been shown that mutations of the cleavage site which prevent the normal processing of the envelope glycoprotein also result in a loss of syncytia formation (Freed *et al.*, 1989) and in the lack of infectivity of the virus (McCune *et al.*, 1988). Our results show that a correct processing is required for fusion in HTLV-I as well.

In seven mutants, syncytia formation was not observed despite a partial precursor cleavage. Glycosylations of the envelope proteins are known to be important for efficient maturation (Pal *et al.*, 1989) and fusion capacity of the HIV-1 envelope (Gruters *et al.*, 1987; Matthews *et al.*, 1987; Walker *et al.*, 1987; Dewar *et al.*, 1989). In our mutants however, glycosylations are not likely to be highly affected, since the mobility of the mutated envelope products in SDS–polyacrylamide gels remains similar to that of the wild-type products. Partial cleavage *per se* cannot explain the absence of syncytia formation either, since some mutants exhibiting lower amounts of gp45 and gp20 than the wild-type are still able to form syncytia (see for instance HTE-24 and HTE-25 in Table II). It is believed that subsequent to the precursor cleavage step, syncytia formation further requires a correct binding to the virus receptor, followed by folding of the envelope leading to exposure of the fusion peptide and finally to membrane fusion. Perturbation of any of these steps is likely to disturb syncytia formation. Among the seven mutations which do not lead to syncytia formation despite significant processing of the gp61, those located in the gp45 protein (HTE-307 in Table I, and HTE-195, HTE-200 and HTE-205, Table II) might affect the receptor binding region. When HTLV-I envelope protein is aligned to murine or feline retroviral envelopes, analogy to the attachment site for cell recognition is found in a region located between amino acids 138 and 202 of the HTLV-I envelope (Haseltine *et al.*, 1985). This is in agreement with the assumption that mutations HTE-195, HTE-200 and HTE-205 (insertions at position 195, 200 and 205 respectively) might disturb receptor binding, thus preventing fusion and syncytia formation. These three mutations might as well indirectly affect binding via conformational effects, or act on a subsequent step of the fusion process required for syncytia formation. Further experiments are under progress to investigate this point, and delineate the HTLV-I receptor binding region. The other four mutants (HTE-307, 329, 343, 383) displaying precursor cleavage but not syncytia formation might as well affect regions important for the protein folding implicated in the receptor binding or the fusion, or directly influence the fusion process. HTE-329 mutation results in an insertion in the putative fusion peptide, and is very likely to prevent the fusion process directly.

Finally, the very high frequency of envelope mutants unable to form syncytia regardless of the position of the mutation, indicates that the HTLV-I envelope protein shows very high structural constraints to be functional. The limiting step, as discussed above, resides in the gp61 precursor cleavage. In viruses, variability might be considered as depending upon four parameters: (i) the mutation rate, linked to the amount of reverse transcriptase errors in retroviruses, (ii) the virus load and rate of replication, (iii) the selective pressure applied by the immune system, (iv) constraints to retain function. Comparing nine different retroviruses, McClure *et al.* (1988) showed that enzymatic proteins (such as reverse transcriptase) are very conserved, probably because their function is prevalent, whereas external proteins (envelopes) being more exposed are more variable. Comparison of various HIV-1 strains fits well with this model. In HIV-1, the high rate of variability observed among different envelopes is usually considered as reflecting a high rate of errors of the reverse transcriptase (Preston *et al.*, 1988; Roberts *et al.*, 1988; Weber and Grosse, 1989) and protein 'adaptation' to evade host immunity. In this virus, constraints to retain envelope functions among mutants are probably not as restrictive as in HTLV-I. In consequence, HIV-1 viruses bearing mutant envelopes are not eliminated (at least when variable regions are concerned), whereas HTLV-I seem to be. Some sub-regions of the envelope protein are, however, conserved among HIV-1, HIV-2 and STLV-III viral stains. When random mutations were introduced into the conserved region, precursor cleavage was usually not affected, but syncytia formation was very sensitive to mutations (Kowalski *et al.*, 1987). It would be interesting to perform random mutations into HIV-1 highly variable regions. Finally, in influenza A viruses it has been proposed (Gorman *et al.*, 1990), that, for a given protein, variation occurs very fast when the virus is not adapted to the host, but very slowly in host-adapted viruses (the more ancient ones). We propose that the conservation of the HTLV-I envelope is based upon its conformational constraints for being functional, which might reflect an efficient adaptation to its host.

Materials and methods

DNA mutagenesis and molecular cloning

The HTLV-I envelope expressor plasmid (HTE-1) used in this study has been described elsewhere (Dokhélar *et al.*, 1989). Briefly, HTE-1 contains an HTLV-I promoter and all the viral sequences corresponding to *env*, as well as *tax* and *rex* of HTLV-I. The plasmids encoding the mutant envelope proteins were made by complete or partial digestion of HTE-1 with restriction

enzymes (*Alu*I, *Bam*HI, *Dpn*I, *Fok*I, *Hpa*I, *Kpn*I, *Mnl*I, *Nco*I, *Pvu*II, *Rsa*I, *Sal*I, *Stu*I and *Taq*I) to obtain linearized DNA, followed by creation of blunt ends using the Klenow fragment of DNA polymerase I or the T4 DNA polymerase, if necessary. Synthetic linkers appropriately sized to maintain frame (8, 10 or 12 bp *Eco*RI, *Bgl*II or *Pvu*I linker) were then ligated. After digestion of the synthetic linker with the appropriate restriction enzyme, the singly cut DNA was isolated on a low melting point agarose gel, ligated and transformed in *Escherichia coli*. The position of the linker insert was determined by restriction enzyme digestion and confirmed by direct DNA sequencing, using the Sequenase kit as recommended by the supplier (United States Biochemical Corp., Cleveland, OH).

Cell lines

The cells were maintained at 37°C in a 5% CO_2 atmosphere. The COS-1 cells are SV40-transformed African green monkey cells (Gluzman, 1981), they were obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The Rous sarcoma virus transformed XC cells (Svoboda *et al.*, 1963) were a gift from Dr S.Gisaelbrecht (INSERM U152, Hôpital Cochin, Paris). They were grown in minimum essential medium supplemented with 10% fetal calf serum. The C91/PL cell line is an HTLV-I infected cell line (Popovic *et al.*, 1983). C91/PL cells were grown in RPMI 1640 supplemented with 10% fetal calf serum.

Transfection procedure

Plasmid DNA (3 μg) was transfected into 0.5 × 10^6 COS-1 cells according to the procedure described by Cullen (1987), using DEAE–dextran and chloroquine. 48 h following transfection, cells were labelled for immunoprecipitation or used for syncytia formation assay.

Detection of the envelope proteins by radioimmunoprecipitation

The transfected cells were labelled as previously described (Dokhélar *et al.*, 1989), using 3 ml of cysteine-free RPMI containing 100 μCi/ml of [^{35}S]cysteine (Amersham, France) and 10% dialysed fetal calf serum. After overnight labelling of the cells, the cell culture medium was kept to look for envelope protein shed in the cell supernatant. Cells were washed once and lysed in 1 ml of 0.02 M Tris–HCl pH 8.0, 0.12 M NaCl containing 0.2 mM phenylmethylsulphonylfluoride, aprotinin (5 μg/ml), 0.2 mM EGTA, 0.2 mM NaF, 0.2% sodium deoxycholate, and 0.5% Nonidet P-40. The lysates were then cleared for 1 h at 80 000 *g*. Cleared lysates or cell supernatants were incubated for 4 h with lentil/lectin–sepharose (Pharmacia) at 4°C. The lectin bound proteins were eluted twice with 0.4 ml of 0.2 M α-methyl-mannoside, and immunoprecipitations performed using a serum from a TSP patient (a generous gift of Dr Lever, London) as already described (Dokhelar *et al.*, 1989).

Syncytia formation assay

The XC cells were used as indicator cells. COS-1 cells transfected with the mutant plasmid DNA were trypsinized 48 h post-transfection, and counted. 2 × 10^5 transfected cells were added to 10^5 XC indicator cells, in total volume of 1 ml in 24 wells flat bottom microplates. 24 h later, after removal of the culture medium, the cell monolayer was washed with phosphate buffer saline, fixed in 100% methanol and stained using a Giemsa blue solution. Syncytia containing > 20 nuclei were then counted under an inverted microscope.

Acknowledgements

We want to thank Professor W.A.Haseltine and Dr M.Kowalski for helpful discussions, Drs A.Lever (London, UK) and F.Denis (Limoges, France) for providing us with patients' sera, Drs S.Chouaïb, A.Harel and M.Lipinski for critical reading of the manuscript. This work was supported by a grant (No. 958916) from the Association Nationale pour la Recherche sur le SIDA (ANRS, Paris, France), and by the Association pour la Recherche sur le Cancer (ARC/CCCR, Villejuif, France/USA No. 6523).

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Received on July 19, 1990; revised on September 20, 1990