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Lethal neurotoxicity in mice of the basic domains of HIV and SIV Rev proteins

Study of these regions by circular dichroism

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We have recently reported a basic domain-mediated neurotoxic activity of HIV-1 Tat [1991, J. Virol. 65, 961–965]. Here we have tested the neurotoxicity in vivo of several Rev-related synthetic peptides and found that only those mimicking the basic regions of Rev from HIV-1, HIV-2 and SIV were lethal to mice. In contrast, the homologous domain of HTLV-1 Rex was found to be inactive for lethal activity. Analysis of the tropism of these peptides for phospholipids has demonstrated a direct interaction of the basic domain-containing peptides, except Rex, with acidic – but not neutral – phospholipids. As determined by circular dichroism, a possible correlation between the conformation of the basic regions and the toxicity is discussed.

Retrovirus; *Trans*-activator; Basic domain; Synthetic peptide; Neurotoxicity; Circular dichroism

1. INTRODUCTION

The human immunodeficiency virus (HIV), the causative agent of acquired immune deficiency syndrome (AIDS) [1], is characterized by a genome containing at least 10 separate open reading frames. Two of these, referred to as *rev* (formerly *art* or *trs*) [2] and *tat* genes [3], encode *trans*-acting nuclear regulatory proteins whose functional expression is essential for viral replication in vitro [4,5]. The HIV-1 Rev and Tat *trans*-activators are 116 and 86 residue proteins, respectively, and are translated from overlapping reading frames encoded in two exons in the *env* region of the HIV genome. While the Tat protein dramatically increases the steady state levels of viral mRNAs and seems to function both transcriptionally and posttranscriptionally [6], the *trans*-activator Rev acts at the post-transcriptional level and induces the cytoplasmic expression of the unspliced *gag-pol* mRNA and the singly spliced *env* mRNA that encode the structural proteins [2,4]. At the same time, Rev also modulates the level and quality of HIV regulatory gene expression [7]. The Rev-mediated effect depends on a *cis*-acting element located in the *env* region of HIV-1, named Rev-responsive element (RRE) [8,9]. Unexpectedly, it has been reported that HIV-1 Rev can be functionally substituted by the nuclear human T-cell leukemia virus type 1 (HTLV-1) Rex protein (189 amino-acid residues), although these phosphoproteins are distantly related and lack amino-acid similarity [10,11]. Furthermore, computer-generated predictions of secondary structure reveal no significant similarities between both *trans*-activators.

In a recent study, we have reported a basic domain-mediated neurotoxic activity of Tat from HIV-1 [12]. Interestingly, functional Rev proteins from HIV-1 and related primate lentiviruses HIV-2 and SIV [13], as well as Rex from HTLV-1, contain an unusual highly basic region. As for Tat, mutational analyses suggest that the arginine-rich region is essential for Rev *trans*-acting function and is important for nuclear translocation [7,14]. Here we have investigated with synthetic peptides whether the basic domains of these proteins were able to exhibit comparable neurotoxicity. In addition, the basic domain-mimicking peptides have been tested for a potential interaction with phospholipids using the monomolecular film technique [15], and their secondary structures in polar and nonpolar solvents were assessed by circular dichroism analysis.

2. MATERIALS AND METHODS

2.1. *Peptide synthesis*

The peptides covering the HIV-1 Rev sequence were purchased from Neosystem (Strasbourg, France). Chemical synthesis of other peptides was performed by the solid-phase method in the laboratory

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[16,17]. Stepwise elongation of the peptide chains was carried out semi-automatically (synthesizer NPS 4000, Neosystem) on 4-(oxymethyl)-phenylacetamidomethyl polystyrene resin (0.5 mmol) using *t*-butyloxycarbonyl (Boc)/benzyl chemistry. Side-chain protecting groups used for trifunctional Boc-amino acids were of the HF labile type, except for tryptophan (formyl). The coupling method was a formation of Boc-amino acid hydroxybenzotriazole active esters (2 mmol) in *N*-methylpyrrolidone. After full assembly was completed, deformylation of tryptophan-containing peptides was achieved on the resin by treatment in darkness with 1 M piperidine in dimethylformamide (2 h, 0°C). Final deprotection was achieved by anhydrous hydrogen fluoride treatment, HF/*p*-cresol/ethanedithiol (85:10:5, vol/vol), for 1 h at −5°C. The synthetic products were highly purified (>98%) by C_{18} reverse-phase medium pressure liquid chromatography and were characterized by C_{18} analytical HPLC and amino acid analysis after hydrolysis under vacuum (6 N HCl, 1 h, 150°C).

2.2. *Specific neurotoxicity of synthetic peptides in mice*

Determination of the 50% lethal dose (LD_{50}) was done by intracerebroventricular injection (5 μl) of peptide solutions in water containing 0.1% (wt/vol) bovine serum albumin and 0.9% (wt/vol) sodium chloride (eight 20-g C_{57}/BL_6 mice per dose).

2.3. *Monomolecular film technique*

Surface pressure (π) was measured with a platinium plate tensiometer according to the Wilhelmy method [15]. A cylindrical teflon trough (6.3-cm diameter × 1.4 cm) was used in a temperature-controlled (25°C) chamber for constant surface experiments and enabled surface pressure variations to be determined ($\Delta\pi$). The phospholipid was first dissolved in chloroform at a concentration of 2 mg/ml and was then gently deposited at the air–water interface with a microsyringe in order to obtain a lipid monolayer. For the aqueous phase, the buffer used was 100 mM NaCl, 21 mM $CaCl_2$, 1 mM EDTA, 10 mM TrisHCl, pH 8.

2.4. *Circular dichroism analysis*

Low ultraviolet circular spectroscopies were recorded on a Jobin-Yvon spectrophotometer, (Longjumeau, France). The instrument was calibrated with (+)-10-camphorsulfonic acid. The spectra were performed at a temperature of 25°C by using a 0.5-mm pathlength cell, with a 2-s time constant and a scan rate of 0.5 nm/s. The peptide concentration in the solutions, based on the absorption spectra, was of 500 μg/ml. The spectra were cumulated 5-fold in water or water–TFE and 3-fold in TFE, and automatically averaged.

Table I

Specific neurotoxicity of the Rev and Rex-related peptides in mice. The synthetic peptides were tested in vivo for their neurotoxicity by determining the 50% lethal dose (LD_{50}) in mice. The peptides including the basic domain are underlined. The LD_{50} is expressed in both micrograms and nanomoles (in parentheses) of inoculated peptides. Peptides were considered inactive when neither neurotoxic symptoms nor lethal effects were observed after injection of 200 μg. About thirty various length (M_r=1500–8000) control peptides, either derived from HIV proteins or unrelated to HIV, were found to be inactive (data not shown). These control peptides include highly basic compounds: fragment 1–34 of human spleen H_1b histone, full-length histone from calf thymus or its fragments 1–25 or 21–38 from H_2b and 1–29 from H_4, peptide 1419–1444 from *M. genitalium* adhesin, and poly-L-lysine (M_r = 3800). Proteolytic digestions of active peptides were controlled by Edman sequencing.

Peptide		LD_{50} (μg)
HIV-1 Bru.	Rev 1–16	Inactive
"	Rev 9–26	"
"	Rev 18–30	"
"	Rev 31–44	"
"	Rev 34–51	57 (22.3)
"	Rev 34–51 after tryptic digestion	Inactive
"	Rev 37–50	72 (34.9)
"	Rev 37–50 after tryptic digestion	Inactive
"	Rev 52–64	"
"	Rev 75–88	"
"	Rev 87–100	"
"	Rev 96–110	"
"	Rev 102–116	"
HIV-2 Rod.	Rev 34–49	35 (15.1)
"	Rev 34–49 after tryptic digestion	Inactive
SIV mm142.	Rev 34–49	33 (14.1)
"	Rev 34–49 after tryptic digestion	Inactive
SIV Agm.	Rex 23–45	46 (16.0)
"	Rex 23–45 after tryptic digestion	Inactive
HTLV-I.	Rex 1–17	not lethal (toxic symptoms)

3. RESULTS

We have tested by intracerebroventricular injections in mice a set of 11 synthetic peptides covering almost the full sequence of HIV-1 Rev. We found that only the basic domain-containing peptides Rev 34–51 and Rev 37–50 were able to exhibit a lethal neurotoxicity with LD_{50} of 57 and 72 μg, respectively (Table I). The peptides including the basic sequences of Rev from HIV-2 Rod, SIV mm142 and SIV Agm (Fig. 1) showed similar activity (LD_{50} of 35, 33 and 46 μg). In contrast, the homologous region of HTLV-I Rex was found to be non-lethal in mice at the tested dose of 200 μg, in spite of significant behavioral neurotoxic symptoms that could be detected from a 10-μg dose and which totally disappeared within 1 h post-inoculation. As shown in Table I, the tryptic digestion of active Rev peptides resulted in complete loss of neurotoxicity. The specificity of this activity was also demonstrated by the unability of highly basic control peptides, such as poly-L-lysine, to induce neurotoxic symptoms and lethality in mice (data not shown).

The possible interaction of the basic domain-containing Rev and Rex peptides with specific phospholipids was studied by using the monomolecular film technique.

HIV-1 Bru, Rev 34–51: **TRQARRNRRRRWRERQRQ**

HIV-2 Rod, Rev 34–49: **SQRRNRRRRWKQRWRQ**

SIV mm142, Rev 34–49: **NQRRQKRRRWRQRWQQ**

SIV Agm, Rev 23–45: **YPPSGEGTARQRR-RARRRWRQQQ**

HTLV-I, Rex 1–17: **MPKTRRRPRRSQRKRPP**

Fig. 1. Primary structures, in the one-letter code, of the basic domain-containing Rev and Rex peptides.

Table II

Specificity of interactions between the basic domain-containing Rev and Rex peptides and phospholipids using the monomolecular film technique. The peptides were tested on either neutral (dilaurylphosphatidylcholine, DLPC) or negatively charged (dilaurylphosphatidylserine, DLPS) phospholipid monolayers. The surface pressure variations ($\Delta\pi$) were measured in the presence of peptides (3 μM) in contact with DLPC or DLPS monolayers at initial film pressures π of 10.90 and 10.60 dynes/cm, respectively. The surface activity of peptides at the air–water interface is also reported.

Peptides	$\Delta\pi$ [air water] (dynes/cm)	$\Delta\pi$ [DLPC]	$\Delta\pi$ [DLPS]
HIV-1 Bru, Rev 34–51	0.06	0.00	3.63
HIV-2 Rod, Rev 34–49	0.37	0.00	6.13
SIV mm142, Rev 34–49	0.38	0.00	5.38
SIV Agm, Rev 23–45	0.25	0.00	2.50
HTLV-1, Rex 1–17	0.00	0.00	0.00

Results expressed in Table II show that the surface activity of Rev peptides became much greater in the presence of DLPS monolayer ($\Delta\pi$ [DLPS] from 2.50 to 6.13 dynes/cm) than that observed at the air–water interface ($\Delta\pi$ [air–water] from 0.06 to 0.38). This indicates the capacity of toxic Rev peptides to penetrate negatively charged DLPS, but not neutral DLPC ($\Delta\pi$ [DLPC] = 0) monolayers. The Rex peptide was unable to penetrate both types of phospholipid monolayers ($\Delta\pi$ [DLPS] or [DLPC] = 0).

Finally, secondary structures of the peptides solubilized in water, water–TFE or TFE solvents, were approached by circular dichroism (CD) analysis (Fig. 2). The CD data show that active Rev peptides possess comparable organized structures in each solvent, which are mainly β-turns/β-sheets (water) and α-helices/β-

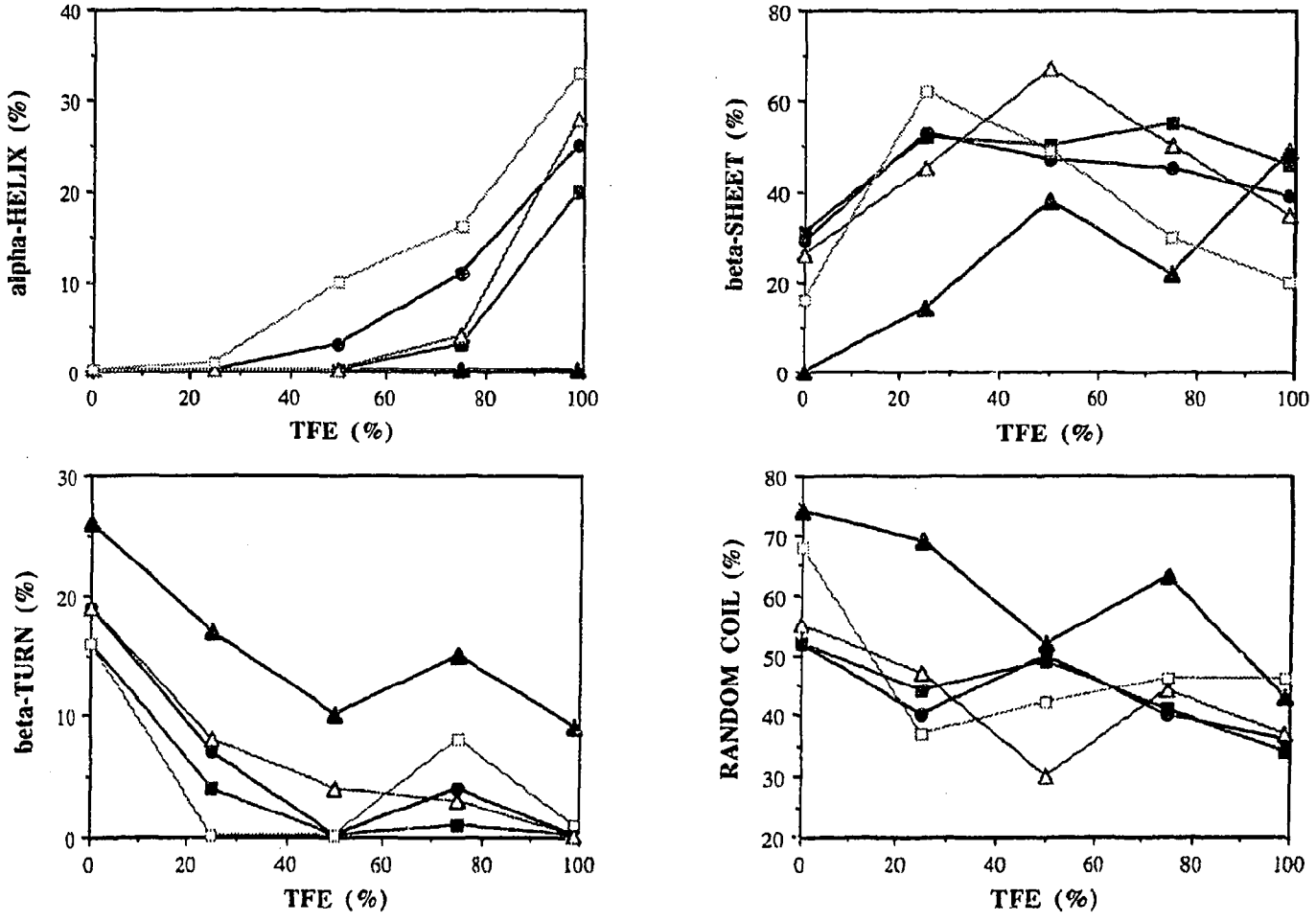

Fig. 2. Circular dichroism (CD) analysis of the basic domain-containing Rev and Rex peptides. The secondary structures estimation of the peptides from CD data was determined according to the method described by Chang et al. [22]. For each peptide, % secondary structures is reported in function of % non-polar TFE solvent. The peptides tested are HTLV-1 Rex 1–17 (▲), HIV-1 Bru Rev 34–51 (●), HIV-2 Rod Rev 34–49 (■), SIV mm142 Rev 34–49 (▲), and SIV Agm Rev 23–45 (□).

sheets (TFE), while the Rex peptide was either partially structured in β-turns (water) or β-turns/β-sheets (TFE).

4. DISCUSSION

We have reported a neurotoxic activity of HIV-1 Tat [12], which was shown to be associated with the presence of the conserved basic region from 49 to 57 (i.e. sequence RKKRRQRRR). The *trans*-activators Rev from HIV-1, HIV-2 and SIV, as well as Rex from HTLV-1, also possess highly basic domains which are presumed to contain nuclear–nucleolar targeting signals and/or RNA binding sites [11]. Although the basic sequences of Rev proteins are not closely related to that of HIV-1 Tat, except consensus motif RR[Q,N][R,K]RR, we have investigated with synthetic peptides whether these target regions were able to exhibit similar toxic activity. First, the neurotoxicity of eleven synthetic peptides covering almost the full sequence of HIV-1 Rev was tested and second, that of the basic regions of HIV-2 and SIV Rev, and HTLV-1 Rex. The activity of peptides was studied in vivo by intracerebroventricular injection in mice. Only the basic domain-containing Rev peptides were lethal to mice with clinical effects resembling the behavioral symptoms induced by some snake toxins such as cardiotoxins. These post-inoculation effects are apathy followed by preliminary muscular tremors, convulsions, wasting, and spastic paralysis just before death (15 min to 2 h). Moreover, the LD_{50} of active Rev peptides were in the same range as those obtained with these snake toxins [18], or with a synthetic peptide mimicking the Tat basic region from 49 to 57 [12]. This neurotoxic activity is specific, since a number of control peptides, including highly basic compounds, were inactive at the dose of 200 μg injected in mice. Further, a tryptic cleavage of the arginine-rich Rev peptides resulted in a complete loss of neurotoxicity. Although the full-length Rev proteins are not available to be tested in vivo, the results suggest the whole HIV and SIV Rev to be neurotoxic.

Since interactions with specific phospholipids are thought to be responsible for pharmacological activities of some snake toxins, such as basic cardiotoxins [15], we have analyzed the tropism of active peptides for DLPS and DLPC using the monomolecular film technique. This approach permits to demonstrate a possible direct interaction of the Rev basic regions with only negatively charged DLPS, indicating the capacity of these sequences to bind to specific components of the membrane lipid bilayer. Similar interaction with DLPS, but not DLPC, was previously obtained with cardiotoxins, or with the HIV-1 Tat basic domain from 49 to 57 which was further reported to be responsible for the binding of Tat to cell membranes. Interestingly, the peptide mimicking the HTLV-1 Rex basic region from 1 to 17 was unable to penetrate these phospholipid films in spite of its highly basic characteristics (net electrical charge of +9 at neutral pH) and the presence of partial sequence homology with the toxic basic region of Tat [19]. This result could be correlated with the lack of lethal activity found for the Rex peptide. May the distinct activity of this peptide, as compared to other Rev basic domains, be related to a conformational particularism?

In a recent report, CD was used to examine the conformation of full-length HIV-1 Rev [20]. The protein was estimated to contain approximately 50% α-helices, 25% β-sheets and 25% random coil when either bound or unbound to its target RRE sequence. It is worth noting that interaction of Rev with the RRE sequence is presumably mediated by the basic domain [21]. Here, CD was used to compare the conformations of the Rev and Rex peptides solubilized in polar (water) and non-polar (TFE) solvents. The CD data show that toxic basic regions of Rev proteins exhibit comparable secondary structures in each solvent, which are β-turns/β-sheets in water and α-helices/β-sheets in TFE. Similar structures are obtained with the HIV-1 Tat basic domain (data not shown). In contrast, the Rex peptide was structured exclusively in β-turns in water and β-turns/β-sheets in TFE. The Rex peptide differs from Rev peptides by the presence of a cluster of proline residues at specific positions 2, 8, 16 and 27 close to or within its basic domain. The cluster of proline residues favors formation of β-turns which are indeed predominant in water (26%), while they prevent formation of α-helices even in TFE which strongly stabilizes this conformation. Considering that TFE mimicks the nonpolar environment of the membrane lipid bilayer, one can suppose the conformation of a given peptide in TFE to approach that adopted in the close neighborhood. Among the peptides tested, only the Rex peptide appears to be unable to form α-helical structure in TFE. The lack of lethal activity of this peptide could thus be relied on its unability to form such a structure. Therefore, we speculate that the lethal neurotoxicity of active peptides depends on their structuration into α-helical conformation during interaction with the membrane lipid bilayer.

However, more investigations are required to study both the validity of this hypothesis and the physiological relevance of the potential Rev-related neurotoxicity, particularly in the neurological disorders associated with retroviral infection.

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