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Mutational analysis of the HTLV-I *trans*-activator, Tax

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The gene coding for the *trans*-activating factor (Tax) of the human T-cell leukemia virus, type I (HTLV-I) was mutagenized in vitro using oligonucleotide-directed mutagenesis and recombinant DNA techniques. All except one of the mutagenized *tax* constructs failed to *trans*-activate the HTLV-I LTR in a eukaryotic test system. Moreover, negative Tax mutant Arg-39→Gly was found to be *trans*-dominant. This observation suggests that Tax contains distinct functional domains mediating different interactions of the protein in the process of *trans*-activation.

Trans-activation; *Oligonucleotide-directed* mutagenesis; *Trans*-dominant mutant; CAT assay; Human T-cell leukemia virus

1. INTRODUCTION

The human T-cell leukemia virus, type I (HTLV-I) encodes a 40-kDa *trans*-activating factor, termed Tax. This protein activates transcription of the HTLV-I provirus, but also a multitude of cellular genes, among them genes with regulatory functions like IL-2, IL-2R and c-*fos* [1–3]. Tax is regarded as the transforming agent of HTLV-I, leading to adult T-cell leukemia [4–6]. Beyond its intracellular function as a transcriptional regulator, extracellular Tax protein also affects lymphoid cells [7]. However, for a long period the mechanism of the protein's action remained poorly understood. Recent studies using in vitro mutagenesis suggest a multidomain structure for Tax [8,9]. In the present study, we subjected Tax to mutational analysis including single amino acid substitution in order to illuminate structural features underlying the function of Tax as a transcriptional *trans*-activator.

2. MATERIALS AND METHODS

2.1. *In vitro mutagenization of* tax

Mutagenization of *tax* (Fig. 1) was performed using pHTLV-tax, a plasmid containing the Tax-coding region under control of the HTLV-I LTR [5]. Deletion mutant 285–353 was constructed by cloning an amber stop oligonucleotide (5'-CTAGTCTAGACTAG-3') into a *Stu*I restriction site of pHTLV-tax. BLV/HTLV-I hybrid Tax was made by exchanging the *Nco*I-*Cla*I restriction fragment of HTLV-I *tax* coding for amino acids 1–59 for the sequence encoding amino acids 1–26 of BLV Tax. For oligonucleotide-directed mutagenesis, appropriate gene fragments were cloned into *E. coli* phage M13 mp18. Using *ung⁻ dut⁻ E. coli* strain CJ 236 for template preparation, efficiency of mutagenesis was 70–90% [10]. Point mutations were confirmed by DNA sequence analysis.

2.2. *Cell culture and transfection*

HeLa cells were grown in Dulbecco modified Eagle's medium supplemented with 5% fetal calf serum (Gibco). Cell transfection was done with DNA calcium phosphate coprecipitates in 60-mm Petri dishes. Carrier DNA was added to a final amount of 17 μg per dish. Cell extracts were prepared 48-h post-transfection [11].

2.4. *CAT assay and Western blot analysis*

For CAT assays, *tax* constructs were co-transfected with reporter plasmid pU3RCAT, carrying the chloramphenicol-acetyltransferase

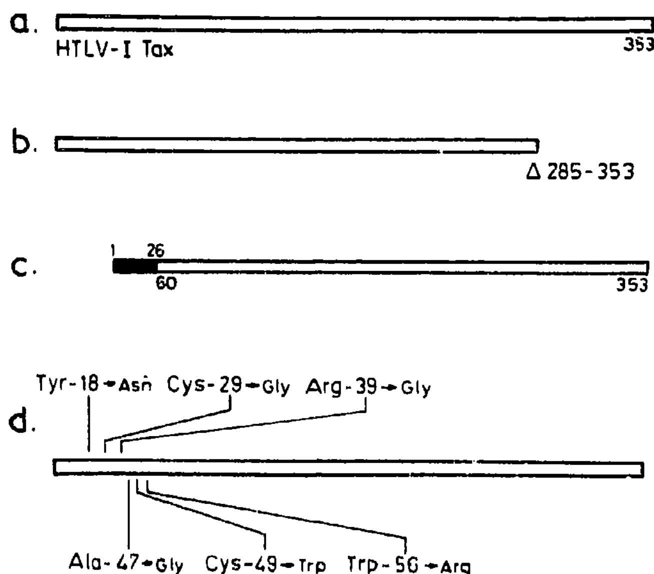

Fig. 1. In vitro mutagenization of HTLV-I Tax. (a) Wild-type Tax. (b) C-terminal deletion: an amber stop oligonucleotide (5'-CTAGTCTAGACTAG-3') was inserted into a *Stu*I site of *tax* at amino acid 285. (c) BLV/HTLV-I hybrid Tax contains amino acids 1–26 of BLV Tax (black area) and residues 60–353 of HTLV-I Tax (white area). (d) Point mutations introduced into the N-terminal region of Tax.

Abbreviations: BLV, bovine leukemia virus; CAT, chloramphenicol-acetyltransferase; CMV, cytomegalovirus; HTLV, human T-cell leukemia virus; LTR, long terminal repeat; Tax, *trans*-activating factor; *tax*, gene coding for Tax; TRE, Tax-responsive element

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gene under control of the HTLV-I LTR [12]. CAT assays were performed as described in [11].

For Western blots, HeLa cell extracts were separated by discontinuous SDS-PAGE on a 10% gel and transferred onto a Immobilon (Millipore) membrane. Immunoblots were carried out using the ProtoBlot Western blot system (Promega). Primary antibody from rabbit directed against the C terminus of Tax was kindly provided by Dr Peter Beimling [13].

3. RESULTS AND DISCUSSION

In the present study, we have mutagenized HTLV-I Tax using three approaches (Fig. 1). First we removed 20% of the protein's sequence from the C terminus, second we replaced 59 amino acids from the N terminus by the first 26 residues of BLV Tax, and third we performed site-directed, single amino acid substitutions at the N terminus. The ability of these mutants to *trans*-activate the HTLV-I LTR was tested in transient chloramphenicol-acetyltransferase (CAT) assays. The presence of inactive mutant Tax in cell extracts was confirmed by Western blot analysis.

Our first approach was to reveal whether the C terminus of Tax carries essential functions for *trans*-activation. Other authors found that minor deletions of up to 22 amino acids at the C terminus did not abrogate completely the protein's activity [9]. We deleted 69 amino acids from the C terminus by cloning an amber stop oligonucleotide into a *Stu*I restriction site of *tax*. In a transient co-transfection assay, this construct was found to be unable of *trans*-activating the HTLV-I LTR in HeLa cells (Fig. 2a). Therefore we suppose the region between residues 285 and 330 to be essential for *trans*-activation.

Tax is a potent transcriptional *trans*-activator found in HTLV-I, HTLV-II and BLV. A sequence comparison of *tax* products from different sources reveals that the N terminus is the most conserved region of the protein, indicating that this region supplies the structural basis for key functions of Tax. This notion is supported by our observation that exchange of the 59 N-terminal amino acid residues of HTLV-I Tax for the 26 N-terminal residues of BLV Tax results in a complete loss of its in vivo function (Fig. 2a). Moreover, single amino acid substitutions at distinct sites in the N ter-

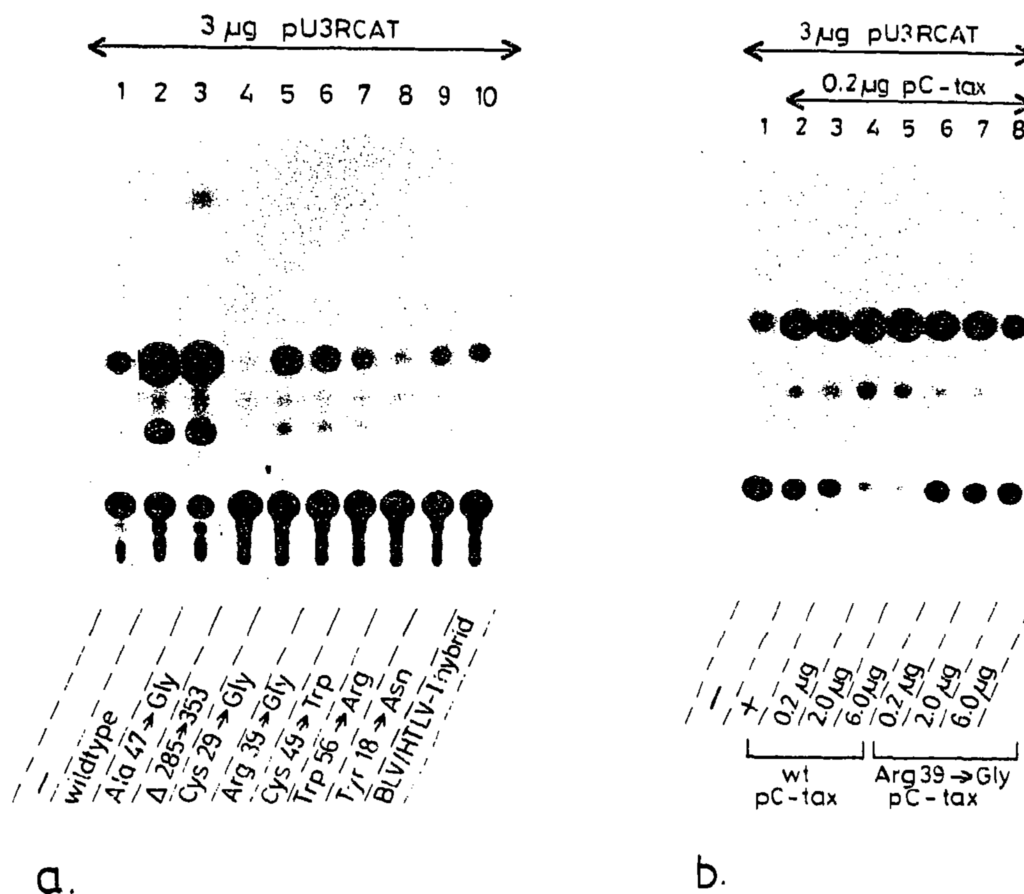

Fig. 2. CAT assay of mutagenized *tax* constructs. *tax* Constructs were co-transfected with reporter plasmid pU3RCAT into HeLa cells in a 60-mm Petri dish. Cells were collected 48 h post-transfection. (a) 2 μg of pHTLV-tax plasmid (*tax* under control of HTLV-I LTR) carrying the indicated mutation, were co-transfected with 3 μg pU3RCAT (lanes 3–10). Lane 2, 2 μg wild-type pHTLV-tax + 3 μg pU3RCAT (positive control). Lane 1, 3 μg pU3RCAT (negative control). (b) Wild-type and mutant pC-tax plasmid (*tax* under control of CMV promoter) were co-transfected with 3 μg pU3RCAT (lanes 1–8), in addition to 0.2 μg wild-type pC-tax (lanes 2–8).

minus had the same effect. We supposed residues Cys-29, Arg-39, Cys-49 and Trp-56 to be essential for *trans*-activity, as they are conserved in the same positions among HTLV-I, HTLV-II and BLV. We performed oligonucleotide-directed mutagenesis at these sites, substituting residue Cys-29 and Arg-39 for glycine, Cys-49 for tryptophane and Trp-56 for arginine. Any of these substitutions rendered the protein practically inactive. The same could be observed on substitution of Tyr-18 for asparagine (Fig. 2a). Several authors found that Tax does not show direct DNA binding activity. However, a series of cellular proteins could be shown to interact with Tax-responsive elements (TREs). Further to this, Marriott et al. demonstrated indirect DNA binding of Tax via protein–protein interaction with a 36 kDa cellular TRE binding factor [14]. Therefore, Tax is thought to form a complex with cellular transcription factors [14–19]. Beyond this, Tax has been shown to bind to a zinc affinity column [20]. Evidently, as any action of Tax seems to be mediated by intermolecular interactions, even the loss of a single functional group may inactivate the protein. However, exchange of Ala-47 for glycine had no detectable consequence (Fig. 2a). To make sure that reduced CAT activity is not the result of failure to express mutagenized *tax* constructs, presence of mutant Tax in extracts of transfected HeLa cells was confirmed by Western-blot analysis (data not shown).

In a further approach, we raised the question whether mutant *tax* product will show any interference with wild-type Tax, if the two proteins are expressed together in a eukaryotic in vivo test system. In a transient cotransfection assay, we introduced plasmids coding for mutant Arg-39→Gly and wild-type Tax together with the reporter plasmid pU3RCAT into HeLa cells. In order to avoid effects resulting from different amounts of TREs in the test system, we used tax constructs under control of CMV promoter for this CAT assay. Surprisingly, when given in excess mutant Tax seemed to diminish wild-type Tax activity (Fig. 2b). With the same amount of wild-type Tax, CAT activity was not decreased. Therefore, an unspecific effect of the added DNA cannot be the explanation for the drop in *trans*-activation caused by the mutant. We assume that the substitution Arg-39→Gly destroyed a necessary site for the function of Tax (e.g. a transcription factor binding site), but left other functionally essential regions of the protein intact. Thus, mutant Tax may be able to form inactive partial *trans*-activation complexes that compete with the active, wild-type Tax containing complex for binding sites in the target sequence or, alternatively, mutant Tax may compete with wild-type Tax for complex formation with a rate-limiting cellular transcription factor.

Further to mutant Arg-39→Gly, we have preliminary data suggesting also other Tax mutants to be *trans*-dominant. We feel that a further investigation of this effect may help to identify sequence motifs or domains of Tax carrying distinct functions. Beyond this, *trans*-dominant mutants may also be hopeful candidates for an anti-viral agent, particularly as Tax seems to be released from HTLV-I-infected cells and affects uninfected lymphocytes [7].

During our work on this project, Smith and Greene published a paper analyzing HTLV-I Tax by oligonucleotide-directed mutagenesis [9]. Their findings show good accordance with the results presented in this study.

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