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p21^{ras} contributes to HIV-1 activation in T-cells

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Activation of T-cells infected by HIV-1 results in activation of long terminal repeat (LTR)-dependent viral transcription and ultimately the production of infectious virus. Although full T-cell activation requires a complex series of intracellular signals, including protein kinase C activation, calcium mobilisation, and less-well defined lymphokine-induced signals, the HIV-1 LTR can be activated by subsets of these signals. We have studied the interaction of these signals in the human lymphoma line, Jurkat, in activation of the HIV-1 LTR. The HIV promoter was induced by IL-1 and phorbol ester activation of PKC but not by a calcium ionophore. The constitutively active form of Ha-ras could replace phorbol ester stimulation of the HIV promoter and of a synthetic promoter containing NFκB binding sites.

HIV-1; Interleukin; Signal transduction; Cyclosporin A

1. INTRODUCTION

Human immunodeficiency virus 1 (HIV-1) is a lymphotropic retrovirus which is believed to be the causative agent of acquired immune deficiency syndrome (AIDS) [1,2]. One of the characteristics of HIV-1 infection is a very long latency period between infection, as evidenced by seroconversion, and onset of clinical disease. The virus apparently lies dormant in infected cells until some stimulus activates viral expression [3,4]. Peripheral blood lymphocytes from infected individuals produce little or no virus, however, viral expression can be activated, and infectious virus recovered, after treatment with T-cell-activating agents, such as PMA and PHA [5,6]. Treatment of HIV-1-infected Jurkat cells with T-cell mitogens results in activation of the viral promoter contained in the long terminal repeat (LTR), and subsequent expression of viral functions [7,8]. The viral transactivating protein, tat, then cooperates with host proteins to maintain a high level of proviral transcription [9,10]. Thus a primary event in the transition from the asymptomatic phase to outright disease may be the activation of host cell transcription factors capable of inducing LTR-driven transcription.

T-Cell activation is a complex process triggered by T-cell antigen receptor (TCR) recognition of specific antigen in the context of the appropriate histocompatibility. TCR stimulation, plus other ancillary signals usually delivered by the antigen presenting cell, results in a series of events involving the release of lymphokines, proliferation and maturation to a fully differentiated immunocompetent cell. A key event in this process is the induction of the lymphokine, IL-2, and its cell surface receptor, which results in autocrine proliferation, and signals a point of commitment after which the activating stimuli may be removed without interrupting normal differentiation (reviewed in [11,12]). Several intracellular signalling pathways, including mobilization of both monovalent and divalent ions [13,14] and activation of several protein kinases [15–17], are involved in T-cell activation, however, activation of protein kinase C (PKC) [18–20] and mobilization of calcium by the TCR [21–24] have been shown to be necessary. These two signals in combination with a less-well defined but independent signal, which can be delivered by the lymphokine IL-1 [25], are sufficient to induce full IL-2 and IL-2R expression.

We have studied the induction of HIV LTR-driven transcription by T-cell-activating stimuli using an LTR reporter gene construct transfected into the human lymphoma line, Jurkat. It has previously been reported that IL-1 does not activate the LTR in Jurkat cells [6,26]. We now show that this is due to the lack of functional IL-1 receptors on the surface of these cells. Treatment of Jurkat cells co-transfected with the LTR reporter construct, and a construct capable of expressing IL-1 receptors, results in activation of the LTR. We have compared the ability of PKC activation, calcium mobilisation and IL-1 stimulation to activate the LTR, and show that the LTR responds to subsets of the signals required for IL-2 induction. In addition, we have shown that the ras protein, which is activated on TCR triggering [27], plays a role in LTR activation, and that this effect

Abbreviations: LTR, long terminal repeat; PKC, protein kinase C; CAT, acetyl chloramphenicol transferase; CsA, cyclosporin A.

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is likely to be mediated by the NFκB transcription factor.

2. MATERIALS AND METHODS

2.1. *Reagents*

Recombinant human IL-1β from *E. coli* [28] was used at 1 ng/ml. PMA (Sigma) and ionophore, A23187 (Boehringer Mannheim), were dissolved in dimethyl sulfoxide at 100 μg/ml and 10 mg/ml, respectively. PHA (Wellcome Diagnostics, Dartford, UK) was dissolved in phosphate-buffered saline at 1 mg/ml. Cyclosporin A (Sandoz, East Hanover, NJ) was dissolved in ethanol at 10 mg/ml. For protein determination the kit, BCA, from Pierce was used. Acetyl coenzyme A (Boehringer) and [^{14}C]chloramphenicol (Amersham International) were used for chloramphenicol acetyl transferase (CAT) assays, as described by Gorman et al. [29]. [^{3}H]Acetyl coenzyme A (Du Pont, New England Nuclear) and chloramphenicol, dissolved in ethanol at 50 mM, were used for CAT assays as described [30]. Restriction enzymes, Klenow polymerase and T4 DNA ligase (Boehringer) were used according to the manufacturer's instructions.

2.2. *Plasmids*

An approximately 1.7 kb fragment from the *Eco*RV site in the pUC18 polylinker to the *Hin*dIII site at map position 1.71 of the HIV-1 LTR was excised from the plasmid, pNL4-3 [31], and subcloned upstream of the CAT gene in the plasmid, IL2-CAT [32], digested with *Hin*dIII and *Sma*I to remove the IL-2 promoter fragment.

pSVT7 is an expression vector containing a polylinker downstream of the SV40 early promoter [33]. pSVT7/IL-1R has been described elsewhere [34].

The ras expression plasmid was constructed by insertion of the 1,400 bp *Cla*I/*Dra*I restriction fragment containing the MLV LTR from pDOL^{-} [35] into the *Hin*dIII site into the plasmid pT24C3 [36] after filling of the overhanging ends with Klenow polymerase. The NFκB-containing plasmid was described as PRDII [37].

2.3. *Cell culture, transfections and CAT assays*

The human lymphoma line, Jurkat, was maintained in RPMI supplemented with 2 mM L-glutamine, 20 mM HEPES, pH 7.9, 12.5 μM 2-mercaptoethanol and 10% heat-inactivated (56°C for 90 min) fetal calf serum (Seromed, Berlin). The medium used for transfections included 200 U/ml penicillin (Farmitalia, Italy). Transfections were carried out using a modification of the DEAE-dextran procedure, as described [20], scaled down to one tenth with 1 μg of the plasmid LTR/CAT per sample (1×10^{6} Jurkat cells). To avoid error due to variation in transfection efficiency, comparisons of stimuli or inhibitors were carried out on identical aliquots of single pools of transfected cells. Cells were allowed to recover for 24 h before activation. EGTA or CsA were added when required 30 min before stimulation. After incubation for 8–16 h cells were collected by centrifugation, washed in TBS, resuspended in 0.25 M TRIS hydrochloride (pH 7.5) and extracted by freeze–thawing. Equal amounts of proteins, determined according to a modification of the method described by Lowry [38], were used for CAT assays. CAT enzyme activity was assayed using both [^{14}C]chloramphenicol, according to Gorman et al. [29], and [^{3}H]acetyl coenzyme A, according to Eastman [30].

3. RESULTS AND DISCUSSION

Activation of the T-cell antigen receptor with the non-specific ligand, PHA, results in a stimulation of protein kinase C activity and an increase in intracellular free calcium concentrations [11,12]. These signals can be mimicked by phorbol myristate acetate (PMA), which activates OKC [16], and calcium ionophores [39]. Fig. 1 shows the results of CAT assays of extracts of Jurkat cells transfected with a plasmid construct, containing the bacterial gene coding for chloramphenicol acetyl transferase under the control of the HIV-1 LTR (LTR/CAT), after treatment with T-cell mitogens. Treatment with either PMA or PHA resulted in an increase in CAT activity (Fig. 1, upper panel, lanes 2 and 3) of between 10–20-fold in several experiments, as determined by laser densitometry of autoradiograms or ^{3}H incorporation into chloramphenicol. A combination of PMA and PHA induced LTR/CAT only slightly more than the individual stimuli (lane 8).

Treatment with the calcium ionophore, A23187, resulted in a decrease in the constitutive activity (lane 5) and treatment with PMA plus A23187 resulted in less CAT activity than PMA alone. We believe that the reduction in activity was due to slight toxicity of the ionophore after long treatments, since the quantity of protein in the cell extracts was always less than in extracts after no treatment or treatment with any of the other stimuli. PMA/A23187 treatment of Jurkat cells transfected with either an IL-2 promoter/CAT construct or an SV40 promoter/CAT construct resulted in substantial increases in CAT activity [24], even when the protein content of the extracts was slightly reduced.

We conclude that, unlike the IL-2 promoter, the HIV LTR is not activated by increases in intracellular free calcium. The LTR, however, responds to either PHA or PMA alone, whereas the IL-2 promoter requires a combination of these treatments or combinations of either of these agents with IL-1 [24].

The LTR promoter/CAT construct did not respond to IL-1 either alone or in combination with the other mitogens (Fig. 1, upper panel, lanes 4, 6 and 7). This is due to a lack of sufficient cell surface IL-1 receptors in Jurkat cells [24]. Co-transfection of the LTR construct with a construct capable of expressing type-I IL-1 receptors resulted in a response to IL-1. The lower panel of Fig. 1 shows the results of CAT assays of Jurkat cells co-transfected with the LTR/CAT construct and the IL-1R construct treated with different stimuli. In the experiment shown IL-1 alone resulted in an approximately 10-fold increase in CAT activity compared with 13-fold by PHA and 11-fold by PMA (Fig. 1, lower panel, lanes 2, 3 and 4). IL-1 in combination with PHA or PMA resulted in induction approximately equal to the sum of the individual treatments (Fig. 1, lower panel, lanes 6 and 7). Note that the absolute level and relative induction of CAT activity varied between experiments, at least in part due to variations in transfection efficiency. Comparisons in any single experiment were however made on aliquots of a single pool of transfected cells.

Thus the HIV-1 LTR responds, like the IL-2 promoter, to PMA, PHA and IL-1 but does not respond to increases in calcium induced by the calcium ionophore. Activation of IL-2 expression by PHA is, how-

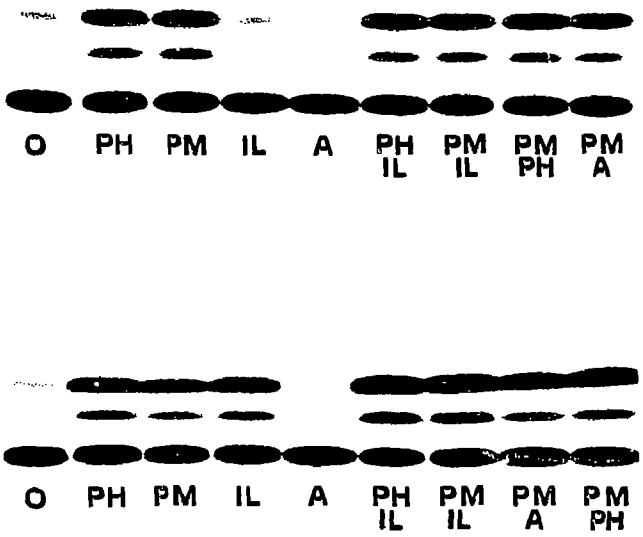


Fig. 1. Activation of the HIV LTR in Jurkat cells. CAT assays of Jurkat cells co-transfected with the plasmid LTR/CAT and either the control plasmid, pSVT7 (upper panel), or the same plasmid containing the cDNA for the human IL-1 receptor (lower panel). The samples were activated as shown below each lane. PH=PHA (2 μg/ml); PM=PMA (10 ng/ml); IL=IL-1β (1 ng/ml); A=A23187 (1 μg/ml).

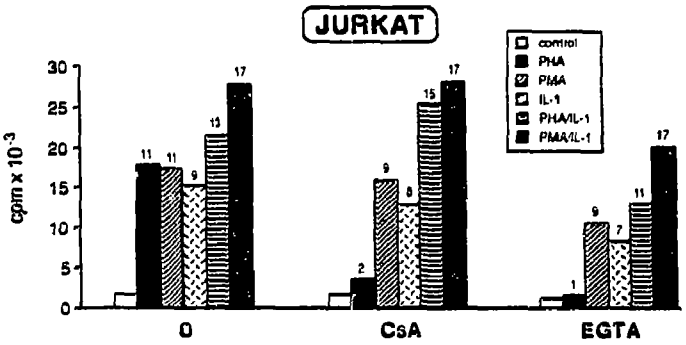


Fig. 2. LTR activation in the presence of EGTA and cyclosporin. CAT assays of Jurkat cells transfected with the plasmid, LTR/CAT, and activated in the presence of 500 ng/ml cyclosporin A or 3 mM EGTA. PHA, 2 μg/ml; PMA, 10 ng/ml; IL-1β, 1 ng/ml. Samples were assayed for CAT activity using [^{3}H]acetyl-CoA, and counted in the linear range of the reaction. The numbers on each column express the -fold stimulation of activated samples to the control, non-activated sample for each group.

ever, dependent on calcium [24]. To investigate whether activation of the LTR by PHA involves calcium we have treated Jurkat cells, after co-transfection with the LTR/CAT construct and the IL-1R construct, with the various stimuli in the presence of EGTA. As can be seen from the results shown in Fig. 2 only the PHA induction is substantially abrogated in the presence of EGTA. CAT activity in extracts of cells treated with EGTA showed a slight general reduction, which we believe is due to toxicity of long incubations in EGTA, however, the relative inductions, compared to the LTR constitutive activity, varied little for treatments with PMA or IL-1. As with the IL-2 promoter calcium was required for PHA activation of the LTR. A similar specific reduction of the PHA-induced signal was observed when the cells were activated in the presence of the immunosuppressive drug, Cyclosporin A (Fig. 2), which blocks calcium-dependent pathways of gene induction in these cells [24].

It has recently been reported that TCR triggering by PHA results in an increase in the active GTP bound form of p21$^{Ha\text{-}ras}$ [27], and that a constitutively active form of p21$^{Ha\text{-}ras}$ contibutes to IL-2 gene activation in a murine T-cell line [40]. To investigate a possible role for ras proteins in HIV LTR activation we have co-transfected Jurkat cells with the LTR/CAT construct and a construct which codes for the T24 oncogenic form of p21$^{Ha\text{-}ras}$. The results of CAT assays of cell extracts after various treatments are shown in Fig. 3. In the presence of T24 constitutive CAT activity was clearly increased. Comparison of the CAT activity in the presence or absence of T24 by laser densitometry of the autoradiographs from several experiments revealed that T24 resulted in a 2–3-fold induction of the LTR. The presence of T24 had little effect on CAT activity in extracts of cells treated with PMA. In the experiment shown the PMA-induced CAT activity was slightly reduced in cells expressing T24. Expression of T24, however, resulted in an increase in IL-1-induced LTR/CAT activity in cells co-transfected with the IL-1R gene (between 30–60% in different experiments). Thus T24 does not increase PMA activation but augments both constitutive and IL-1-induced CAT activity.

On the basis of its synergism with IL-1 and A23187, Baldari et al. [40] have suggested that activated p21^{ras} can replace the signal delivered by PMA in activation of the IL-2 promoter in EL4 cells. Our results are consistent with this hypothesis since T24 did not augment the CAT activity achieved with saturating doses of PMA but did augment the IL-1 signal.

The HIV enhancer contains two copies of a sequence capable of binding the NFκB transcription factor, and these sequences are involved in LTR inducability [26].

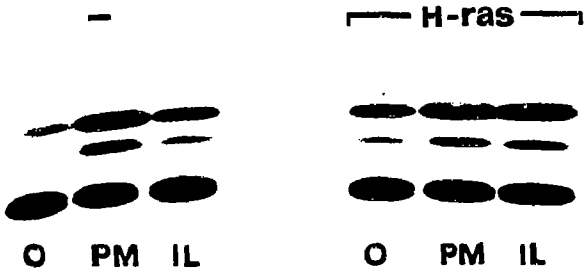


Fig. 3. Oncogenic p21^{ras} contributes to HIV LTR activation. CAT assays of Jurkat cells co-transfected with the plasmid, LTR/CAT, and either the control plasmid, pDOL$^-$ (left), or the corresponding construct encoding the T24 oncogenic form of Ha-ras (right). The samples were activated as shown above each lane. PM=PMA 10 ng/ml; IL=IL-1β 1 ng/ml.



Fig. 4. NFκB is activated by oncogenic $p21^{ras}$. CAT assays of Jurkat cells co-transfected with the plasmid, NFκB/CAT, and either the control plasmid, pDOL⁻ (left), or the corresponding construct encoding the T24 oncogenic form of Ha-ras (right). The samples were activated as shown above each lane. PM=PMA 10 ng/ml; IL=IL-1β 1 ng/ml.

We have asked whether NFκB may be involved in ras-mediated HIV induction in Jurkat cells as it appears to be in IL-2 induction in EL4 cells. We have co-transfected Jurkat cells with the T24 construct and a construct containing the CAT gene under the control of a synthetic promoter containing several copies of the NFκB binding site. In the absence of induction or T24 no CAT activity was detected (Fig. 4). Co-transfection with T24 resulted in detectable CAT activity in the absence of stimuli. As with the LTR CAT, T24 had no significant effect on PMA induction, but IL-1 induction was increased by between 40–90% in several experiments. Thus the NFκB promoter responded to T24 and stimulation with PMA and IL-1 in a manner similar to that of the HIV LTR.

In conclusion the HIV LTR can be activated by signals which, alone, are not normally sufficient for full T-cell activation. Although induction, mediated by the TCR, is dependent on calcium; artificially increasing intracellular free calcium using a calcium ionophore does not increase constitutive or PMA-induced expression. As with the IL-2 promoter, T24 ras can at least in part replace the PMA signal, and the transcription factor NFκB is likely to be involved. Clearly the HIV LTR has evolved to take advantage of important T-cell transcription mechanisms in a fashion which is less regulated than the IL-2 promoter.

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