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Increased protein tyrosine-phosphorylation in primary T-cells transduced with Tax1 of Human T-cell leukemia virus type I

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Abstract Protein tyrosine-phosphorylation in primary human T-cells transduced with Tax1 of Human T-cell leukemia virus type I was investigated. In comparison with control T-cells, the level of protein tyrosine-phosphorylation after stimulation with anti-CD3 antibody increased significantly in Tax1-transduced T-cells. This enhancement in tyrosine-phosphorylation possibly accounted for the augmented proliferative response of these cells, which has been reported previously [J. Virol. 67 (1993) 1211–1217].

Key words: Human T-cell leukemia virus type I; Tax1; Cdc2; Tyrosine phosphorylation; *src*-family tyrosine kinase; Lyn

1. Introduction

Human T-cell leukemia virus type I (HTLV-I), the causative agent of adult T-cell leukemia (ATL) and tropical spastic paraparesis (TSP)/HTLV-I-associated myelopathy(HAM), transforms normal T-cells in vitro [1]. Tax1, a transcriptional *trans*-activator of this virus, is supposed to be responsible for this process [2]. Transcriptional activation of cellular genes, such as IL-2 and IL-2Rα, by Tax1 is suspected to lead to T-cell transformation [3,4].

Previously, we established Tax1-transduced primary human $CD4^+$ T-cells by using a retroviral vector system [5]. In comparison with the same subset of T-cells transduced with a control vector, we found that Tax1-transduced T-cells showed a greatly increased proliferative response to antigenic stimulation through T-cell receptor(stimulation with anti-CD3 antibody), whereas no significant levels of proliferation could be observed in both cell types without stimulation. This proliferation was mediated mainly by an IL-2 independent pathway because IL-2 mRNA was barely detectable, and also because antibodies against IL-2 receptors had little inhibitory effect [6].

To elucidate the mechanism of this hyper-responsiveness, here we investigated protein tyrosine-phosphorylation and found that, compared to control T-cells, Tax1-transduced T-cells exhibited enhanced level of protein tyrosine-phosphorylation in response to anti-CD3 stimulation both at early (1–2 min) and late (24–48 h) time points after stimulation.

2. Materials and methods

2.1. Cells and cell culture

Primary human T-cells derived from peripheral blood of a healthy donor were infected with a Tax1-expressing retroviral vector, DGL-Tax1, or a control vector, DGL, followed by selection with G418. Detailed experimental procedure for establishment of these retroviral vector infected cells was described previously [5,6]. Over 99% of both infectants were CD3 positive, and over 95% were CD4 positive [6]. These cells were maintained in AIM-V medium(GIBCO) supplemented with 10% fetal calf serum(FCS), recombinant IL-2 (Takeda) at 10 ng/ml, and 0.05 mM 2-mercaptoethanol. Cells at 5 to 6 months post-infection were used throughout this work. During this culturing period, both DGL-Tax1-infectants and DGL-infectants could be maintained in the same way in the IL-2 containing medium.

2.2. Antibodies

Monoclonal anti-CD3 antibody UCHT-1 was purchased from IMMUNOTECH. Purified rabbit polyclonal antibody specific for phosphotyrosine was purchased from ZYMED. Monoclonal anti-phosphotyrosine antibody PT66 was purchased from SIGMA. Both the polyclonal and monoclonal antibodies gave substantially same results. Monoclonal anti-Cdc2 antibody was purchased from Santa Cruz Biotechnology,Inc. Purified rabbit polyclonal anti-Cdk2 antibody and purified rabbit polyclonal anti-FYN antibody were purchased from UBI. Monoclonal anti-LYN antibody Lyn9 [7] was kind gift of Drs. Yamamoto and Yamanashi (University of Tokyo). Rabbit polyclonal anti-ZAP70 antibody [8] was kind gift of Drs. Iwashima and Weiss (University of California San Francisco). A polyclonal antibody to LCK was raised in rabbit by immunization with LCK protein expressed in *Escherichia coli*.

2.3. Immunoblotting

Immunoblotting was performed essentially as described [9]. Briefly, 5×10^5 cells were pelleted and lysed in 100 μl of 2 × SDS-PAGE sample buffer containing 1 mM Na_3VO_4. 10 μl of each samples was fractionated on 10% SDS-PAGE, and the proteins were electrophoretically transferred to Immobilon (Millipore). After blocking with 3% BSA in TBS (10 mM Tris-HCl, pH 7.4, 140 mM NaCl) overnight at room temperature, the membranes were incubated with appropriate primary antibodies in TBS for 1 h. The membranes were then washed extensively with TBS containing 0.1% Tween 20, incubated for 1 h at room temperature with secondary antibodies, washed and developed with the Amersham ECL chemiluminescence reagent as directed by the manufacturer.

2.4. Immunoprecipitation

1×10^7 cells were pelleted and lysed in 400 μl of 1% NP40 lysis buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol, 2.5 mM EDTA, 2 mM Na_3VO_4, 50 mM NaF, 20 mM $Na_4P_2O_7$, 1 mM PMSF, 10 μg/ml leupeptin, 1% aprotinin at 4°C for 20 min. Insoluble material was removed by centrifugation at 4°C for 20 min at 10,000 × *g*. Cell lysates were incubated with antibodies for 2 h at 4°C, and then with protein G-Agarose for 1 h at 4°C. Immunoprecipitates were washed three times in lysis buffer, and then suspended in 2 × SDS-PAGE sample buffer containing 1 mM Na_3VO_4 and analyzed by SDS-PAGE.

2.5. Affinity purification of $p34^{cdc2}$

Cell lysates, similarly prepared as in immunoprecipitation, were mixed with 20 μl of agarose beads conjugated with $p13^{suc1}$, a well-known $p34^{cdc2}$ binding protein [10,11] (Oncogene Science) for 3 h at 4°C. Precipitate (affinity complex with $p13^{suc1}$-beads)was used as affinity purified $p34^{cdc2}$, and the supernatant was used as pre-cleared lysate.

2.6. Stimulation with anti-CD3 antibody

Before the stimulation, cells were cultured for 24 h in AIM-V medium

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in the absence of FCS and IL-2 to obtain cells in the resting state. Stimulation with anti-CD3 antibody within 60 min was carried out as follows. 2×10^6 cells were suspended in 100 μl of HEPES-buffered saline containing 20 mM HEPES, pH 7.2, 140 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 1 mM $CaCl_2$, incubated with 10 μg/ml anti-CD3 antibody (UCHT-1) for 10 min on ice, followed by addition of 80 μg/ml anti-mouse IgG for cross-linking, and incubated at 37°C for various time periods. Stimulation over 60 min was carried out by culturing the cells in anti-CD3 antibody (UCHT-1)-coated plates as described previously [6]. 2×10^6 cells were suspended in 2 ml of AIM-V medium containing 10% FCS, and cultured in UCHT-1-coated plates for various time periods.

3. Results and discussion

Tax1-transduced $CD4^+$ T-cell population, PBL/DGL-Tax1, showed much higher (about 10 times) proliferative response to anti-CD3 antibody stimulation than the same subset of T-cells transduced with a control vector, PBL/DGL [6]. Since there was no quantitative difference in CD3 antigen expression between the two cell types [6], we assumed that the difference in responsiveness originated in the process of signal transduction following stimulation with anti-CD3 antibody. To gain insight

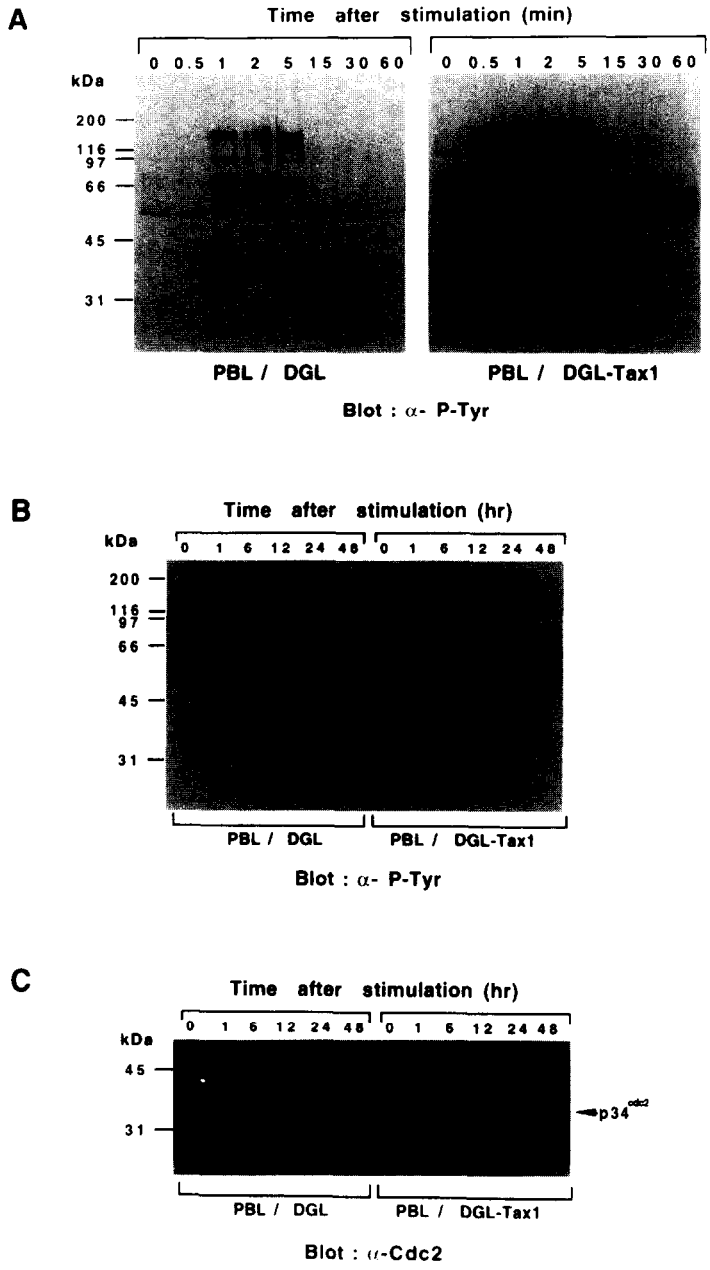

Fig. 1. Immunoblot analysis of T-cells stimulated with anti-CD3 antibody. (A) Short-term kinetics of protein tyrosine-phosphorylation. (B) Long-term kinetics of protein tyrosine-phosphorylation. Both cell types were stimulated with anti-CD3 antibody (UCHT-1), and subjected to immunoblot analysis using rabbit polyclonal anti-phosphotyrosine antibody (ZYMED) as described in section 2. Arrow indicates the position of the 64 kDa protein. (C) Immunoblot analysis for $p34^{cdc2}$. The same filter as in (B) was subjected to immunoblot analysis with anti-Cdc2 monoclonal antibody (Santa Cruz Biotechnology, Inc.). Arrow indicates the position of the $p34^{cdc2}$.

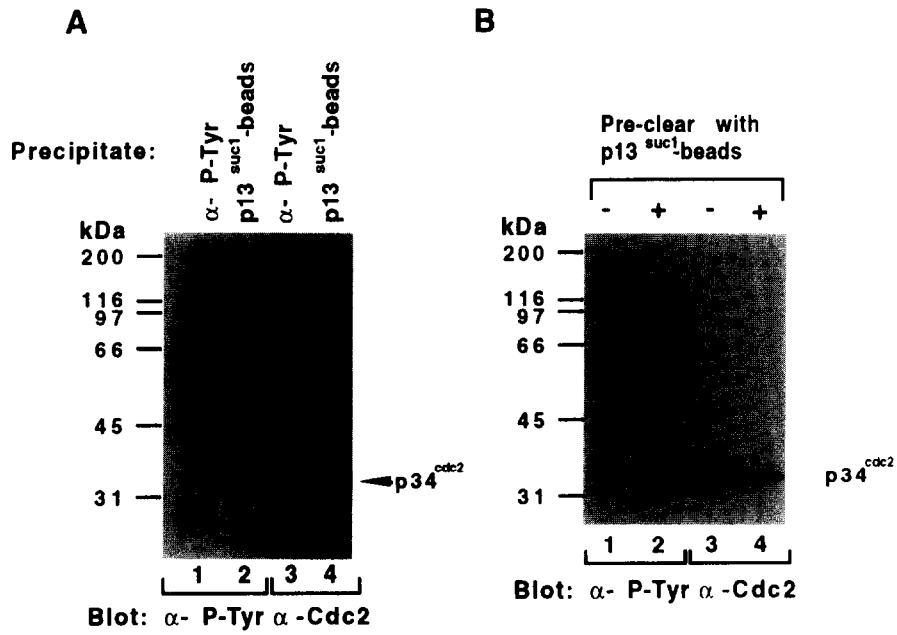

Fig. 2. Identification of the 34 kDa tyrosine-phosphoryrated protein as $p34^{cdc2}$. (A) From the lysate of PBL/DGL-Tax1 cells at 48 h after anti-CD3 antibody stimulation, tyrosine phosphorylated proteins were immunoprecipitated and $p34^{cdc2}$ was affinity purified as described in section 2. Precipitates (lanes 1 and 3: immune complex with anti-phosphotyrosine antibody, lanes 2 and 4: affinity complex with $p13^{suc1}$-beads)were subjected to immunoblot analysis using anti-phosphotyrosine polyclonal antibody(lanes 1 and 2) or anti-Cdc2 monoclonal antibody (lanes 3 and 4). (B) Lysates from PBL/DGL-Tax1 cells at 48 h after anti-CD3 antibody stimulation were pre-cleared of $p34^{cdc2}$ as described in section 2. Lysates corresponding to 5×10^4 cells, before (−) and after (+) pre-clear, were subjected to immunoblot analysis using anti-phosphotyrosine antibody (lanes 1 and 2) or anti-Cdc2 antibody (lanes 3 and 4).

into the mechanism of this hyper-responsiveness, we first investigated protein tyrosine-phosphorylation, which is known to be one of the earliest response to the stimulation with anti-CD3 antibody [12,13]. Both infectants were stimulated with anti-CD3 antibody, and the state of protein tyrosine-phosphorylation was examined by the immunoblotting with the anti-phosphotyrosine antibody. During the first hour after stimulation, as shown in Fig. 1A, numerous tyrosine-phosphorylated proteins were detected transiently in both cell types, which is consistent with previous reports [14–16]. PBL/DGL-Tax1 showed markedly increased tyrosine-phosphorylation after the stimulation. At 30 s after the stimulation, PBL/DGL-Tax1 showed a significant level of tyrosine-phosphorylation. In both cell types, tyrosine-phosphorylation reached a maximum between 1 and 2 min and returned to the level of unstimulated condition by 15 min. The difference was quantitative rather than qualitative, because the patterns of tyrosine-phosphorylated proteins were almost same in both cell types, with the exception of a faint band at 64 kDa protein (indicated by an arrow in Fig. 1A) that was detected only in PBL/DGL-Tax1. This 64 kDa protein could be seen even in time 0, and there was no significant change during this early stimulation period.

In PBL/DGL-Tax1, a marked enhancement of tyrosine-phosphorylation was also seen much later after stimulation (Fig. 1B), however, the pattern of tyrosine-phosphorylated proteins was different from that of early response. Gradual increases in tyrosine-phosphorylated proteins at 64 kDa and around 55 kDa were observed. Tyrosine-phosphorylation of these proteins was also seen in PBL/DGL, but the levels of phosphotyrosine were very low. Because tyrosine-phosphorylation of these proteins remained at a constant low level throughout the early time-course (Fig. 1A), these proteins seemed to be proteins phosphorylated by tyrosine kinases activated in the late stage of signal transduction. At 48 h after stimulation, tyrosine-phosphorylated 34 kDa protein was detected only in PBL/DGL-Tax1 (Fig. 1B). Following serum stimulation of quiescent NIH3T3 cells, $p34^{cdc2}$ is reported to be synthesized and tyrosine-phosphorylated in the S and G2 phases of the cell cycle [17]. From the molecular weight as well as the time-course of appearance, it is suggested that the tyrosine-phosphoryrated 34 kDa protein observed in PBL/DGL-Tax1 at 48 h after stimulation was $p34^{cdc2}$. We tested this possibility by affinity purification of the tyrosine-phosphorylated proteins and $p34^{cdc2}$ from the lysate of PBL/DGL-Tax1 at 48 h after stimulation; we used anti-phosphotyrosine antibody [18], and $p13^{suc1}$ (a well-known $p34^{cdc2}$ binding protein [10,11])-agarose beads. Precipitates with the anti-phosphotyrosine antibody contained a 34 kDa protein that was recognized by anti-$p34^{cdc2}$ antibody (Fig. 2A, lane 3). Likewise, $p13^{suc1}$-agarose beads precipitated a 34 kDa protein that was recognized both by anti-$p34^{cdc2}$ antibody and anti-phosphotyrosine antibody (Fig. 2A, lanes 4,2). Moreover, after pre-clear of $p34^{cdc2}$ by treating with $p13^{suc1}$-beads, the 34 kDa protein-signal in the lysate was selectively reduced in immunoblotting with anti-phosphotyrosine antibody(Fig. 2B, lanes 1,2). Since $p33^{cdk2}$ is also known to be tyrosine-phosphorylated and to bind $p13^{suc1}$ [19], we performed immunoblot analysis with anti-$p33^{cdk2}$ for the precipitates with $p13^{suc1}$-agarose beads and found that it contained only a small amount of $p33^{cdk2}$ (data not shown). These results indicated that the majority of tyrosine-phosphorylated 34 kDa protein detected in PBL/DGL-Tax1 at 48 h after stimulation was $p34^{cdc2}$. Immunoblot analysis with anti-$p34^{cdc2}$ antibody revealed that the difference between PBL/DGL and PBL/DGL-

Tax1 in tyrosine-phosphorylation of 34 kDa protein, as shown in Fig. 1B, reflected a difference in the actual amount of $p34^{cdc2}$ protein (Fig. 1C), and presumably reflected the difference in the number of cells entering S phase.

The same pattern of tyrosine-phosphoryrated proteins as in PBL/DGL-Tax1 at 48 h after stimulation could be detected in HTLV-I immortalized human $CD4^+$ helper T-cell clone, KN6-HT [20] (Fig. 3). Considering a plethora of evidence demonstrating the importance of tyrosine-phosphorylated proteins in growth signal transduction pathway [21–24], identification of other unknown tyrosine-phosphorylated proteins such as 64 kDa and 55 kDa proteins observed in PBL/DGL-Tax1 and KN6-HT, and the clarification of their functions should provide important insights into the mechanisms of the aberrant growth responses in these cells. For this purpose, we tested our cells with a panel of antibodies against well-known tyrosine- phosphorylated proteins whose molecular weights were reported to be similar to those of our unidentified proteins. Immunoprecipitates with anti-phosphotyrosine antibody were probed with antibodies against several kinds of *src*-family tyrosine kinase, CD5 [25,26], GAP associated p62 [27], Shc [28], IL-2Rγ [29]. None of these antibodies reacted positively with our unidentified tyrosine-phosphorylated proteins, although we confirmed that these antibodies could recognize their respective antigens in the cell lysates derived from PBL/DGL-Tax1 or KN6-HT. Identification of the 64 kDa and 55 kDa proteins is still under way.

To determine the kinase that was responsible for the elevated tyrosine-phosphoryration, expression of several kinds of *src*-family tyrosine kinase were compared between PBL/DGL and PBL/DGL-Tax1. We analyzed Fyn, Lck, and Lyn, because Fyn and Lck are known to be expressed in T-cells [30] and Lyn is

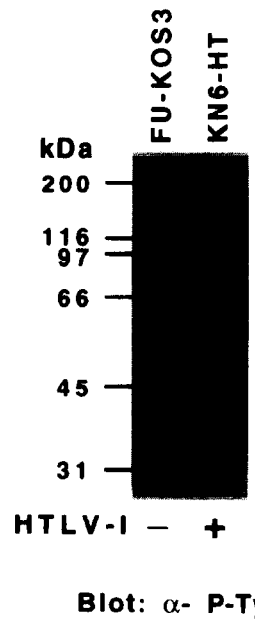

Fig. 3. Tyrosine-phosphorylated proteins in HTLV-I infected and uninfected human $CD4^+$ helper T-cell clone. An HTLV-I infected human $CD4^+$ helper T-cell clone, KN6-HT [20], and an uninfected same subset of the T-cell clone, FU-KOS3 (provided by Dr. Yasukawa, Ehime University), were maintained as described previously [20]. Whole cell lysates of these T-cell clones were prepared, and subjected to immunoblot analysis using anti-phosphotyrosine antibody. –, HTLV-I uninfected; +, HTLV-I infected.

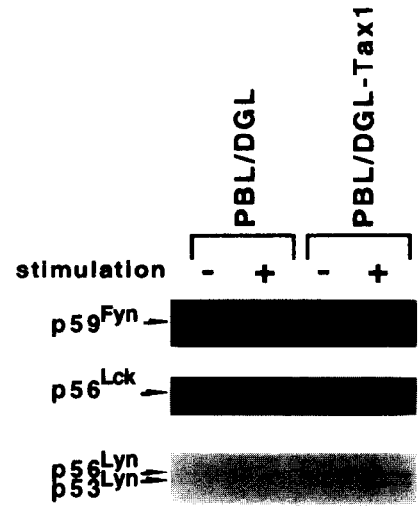

Fig. 4. Immunoblot analysis for *src*-family tyrosine kinases. Whole cell lysates were prepared before(-) or 48h after(+) stimulation with anti-CD3 antibody, and subjected to immunoblot analysis using anti-Fyn polyclonal antibody or anti-Lck polyclonal antibody or anti-Lyn monoclonal antibody, Lyn9.

reported to be expressed in HTLV-I infected cells [7]. Immunoblot analyses of lysates from both cell types, either before or 48 h after stimulation, were performed against each specific antibody (Fig. 4). The Fyn expression level was almost the same in both cell types and there was no significant difference seen between the cells before and after the stimulation. Lck was also expressed similarly in both cell types, but its expression level decreased after stimulation. Although Lyn was expressed at a very low level in both cell types before stimulation, its expression was upregulated specifically in PBL/DGL-Tax1 at 48 h after stimulation. We confirmed that this increase in Lyn protein accompanied an increase in its mRNA by Northern blot analysis(data not shown). This result suggests that Lyn plays some role in the elevated tyrosine-phosphoryration of PBL/DGL-Tax1 in the late stage of stimulation. In this context, it is noteworthy that Lyn is reported to be activated and to associate with $p34^{cdc2}$ in irradiated HL-60 cells [31]. On the contrary, we could not detect any significant differences in expression and activity of the above *src*-family tyrosine kinases or in ZAP70 immediately after stimulation(data not shown). The precise mechanism of this upregulation of tyrosine-phosphorylation remains to be resolved. But considering the function of Tax1 as a transcriptional activator, a likely possibility is that Tax1 *trans*-activates genes encoding some as yet unidentified tyrosine kinase(s); this is, in fact, the case for Lyn [32]. Alternatively, genes for so called adapter proteins containing the SH2 or SH3 domain, which facilitate the recruitment of substrates by activated tyrosine kinases [23], may be *trans*-activated by Tax1. These unidentified genes may have enhancer motifs such as CRE, NFkb or SRE which are well-known Tax1-responsive enhancers [2]. Or, as is supposed in the case of Lyn [32], Tax1 may stimulate the expression of these unidentified genes through interaction with a more general factor such as a transcriptional initiator.

The elevated protein tyrosine-phosphorylation, we have described here, possibly accounts for the increased proliferative response of Tax1-transduced T-cells to the stimulation with anti-CD3 antibody [6]. And it is suggested that Tax1 can sensi-

tize T-cells to mitogenic signals by augmenting protein tyrosine-phosphorylation.

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