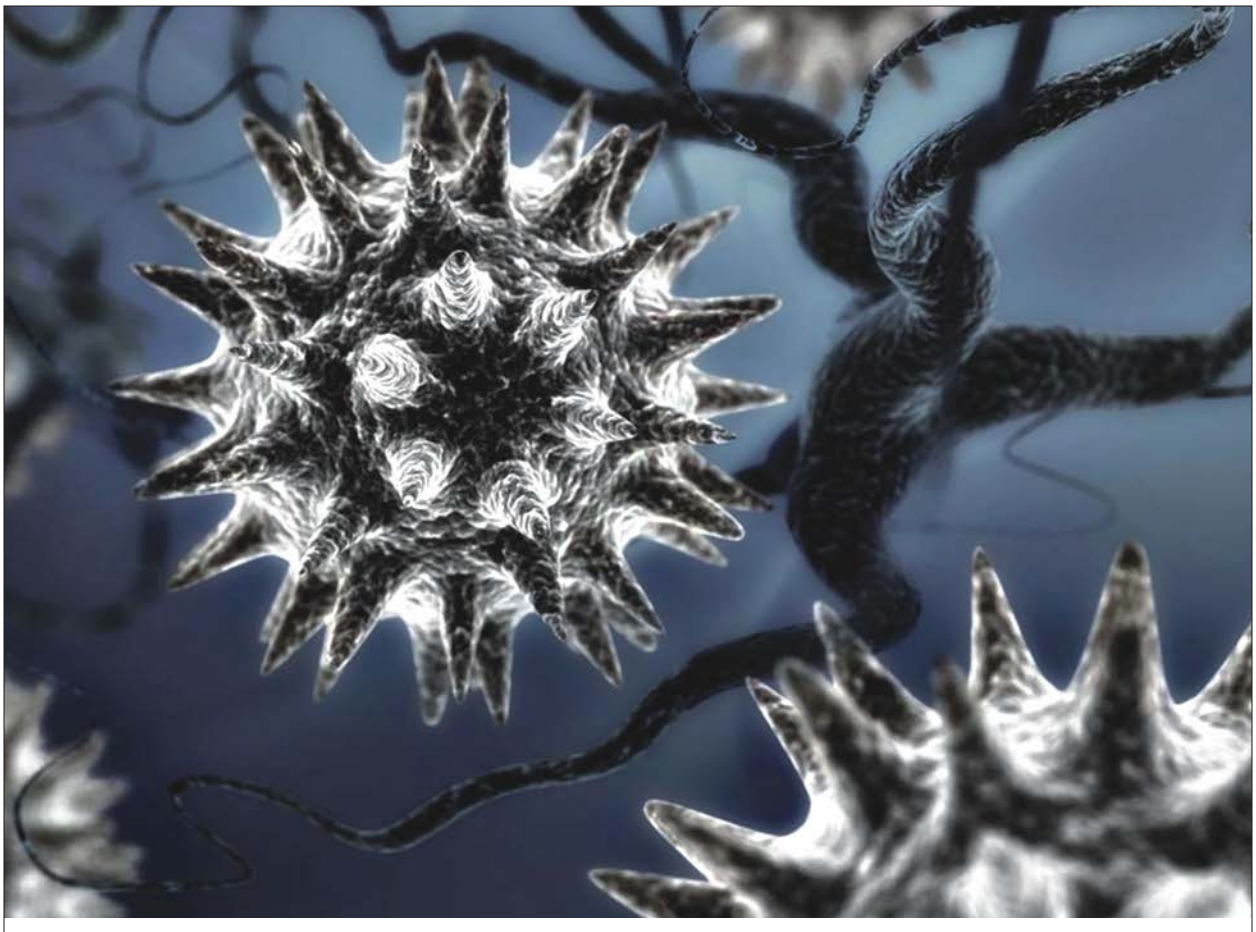

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Differential Phosphorylation of c-Cbl in Leukemogenic and Nonleukemogenic HTLV-I Cell Lines*

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Abstract: Two HTLV-I infected rabbit T cell lines differ in that one of them, RH/K34, causes leukemia-like disease, whereas the second, RH/K30, mediates asymptomatic infection. The two cell lines have minor differences in sequence of the integrated proviruses and in surface molecule expression, but the proto-oncogene product Vav is tyrosine phosphorylated (P-Tyr) in RH/K34 but not in RH/K30. Further examination of the cell lines revealed that the oncogene product c-Cbl is constitutively phosphorylated in RH/K34, but not in RH/K30. Anti-Cbl precipitates from RH/K34 contained Cbl along with an associated protein of 55-60 kDa of unknown identity which was revealed by anti P-Tyr. Activation of RH/K34 cells with pervanadate phosphates inhibitor enhances phosphorylation of Cbl and increases the number of associated molecules; similar treatment of RH/K30 induces phosphorylation of Cbl, but no molecular associations are seen. *In vitro* association of Cbl with other signaling molecules was stronger through SH-3 than SH-2 domains. Viral DNA clones corresponding to either RH/K30 or RH/K34 downregulated Cbl phosphorylation in a rabbit T-cell line upon transfection. These data indicate differential influence of HTLV-I on phosphorylation of oncogene products and raise questions concerning their role in pathogenesis.

Key words: HTLV-I, c-Cbl, oncogene, T-cell activation, phosphoprotein, ubiquitine ligases

Introduction

Human T Lymphotropic Virus-I (HTLV-I) is associated with various acute and chronic diseases, but the majority of infected individuals remain asymptomatic (Poiesz *et al*., 1980). HTLV-I readily infects rabbits and inoculation with certain infected cell lines leads to acutely fatal or smoldering disease that mimics human adult T cell leukemia/lymphoma (ATLL) and other diseases (Seto *et al*., 1987; Sawasdikosol *et al*., 1993; Zhao *et al*., 1993; Simpson *et al*., 1996). However, the majority of HTLV-I infected rabbit cell lines mediate chronic asymptomatic infection (Zhao *et al*., 1993). Detailed comparisons of infected cell lines with differences in pathogenic potential can provide insight into mechanisms by which HTLV-I infection results in diverse outcomes. Our studies of differences in HTLV-I pathogenicity involve comparisons of cell phenotype and provirus structures of two rabbit T cell lines, one (RH/K30) which mediates asymptomatic infection and another (RH/K34) which causes lethal experimental disease.

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Several reports show that HTLV-I transformed cells exhibit altered expression or activity of tyrosine kinases and suggest that constitutive activation of cells may play a role in pathogenesis (Yamanashi *et al*., 1989; Migone *et al*., 1995; Takemoto *et al*., 1997). We have investigated the profile of tyrosine phosphorylated proteins in the two cell lines and found that the proto-oncogene product Vav was expressed in both rabbit cell lines but was tyrosine phosphorylated only in the leukemogenic cell line (Mahana *et al*., 1998). The differential control of Vav phosphorylation was shown to be controlled by HTLV-I genes in the pX region. This finding, with the molecular weight of phosphorylated proteins, prompted examination of other oncogene products in the two cell lines. c-Cbl is the cellular homolog of V-Cbl, the transforming gene of the murine retrovirus cas-Ns1, which induces pre-B-cell lymphoma and leukemia upon inoculation in neonatal mice (Blake *et al*., 1991; Langdon, 1995). Cbl has several distinctive domains including a highly basic region, a leucine zipper, a RING finger motif, a large proline-rich domain and a ubiquitin ligase domain (Langdon, 1995; Liuy and Altman, 1999; Joazeiro *et al*., 1999). Cbl was demonstrated to be an early and dominant substrate of tyrosine phosphorylation in response to activation of cell surface receptors coupled to tyrosine kinases and forms a complex with SH3 and SH2 domain containing proteins (Panchamoorty *et al*., 1996; Chin *et al*., 1997; Barabe *et al*., 1998). Cbl was also described as a negative control of many molecules involved in signal transduction via an ubiquitine ligase activity (Rao *et al*., 2002a, 2002b; Kim *et al*., 2004).

Wild type Cbl is non-transforming even when overexpressed (Andoniou *et al*., 1994) but tyrosine phosphorylated Cbl promotes tumorogenesis and Cbl is a downstream target of the oncogene involved in Philadelphia chromosome-positive human leukemia Bcr-Abl and v-Abl (Jain *et al*., 1997). Cbl is constitutively phosphorylated on tyrosine in cells expressing oncogenically activated Abl, Src or Lck tyrosine kinases (Andoniou *et al*., 1994; Jain *et al*., 1997; Tanaka *et al*., 1995).

The present study examines differences in the expression and the phosphorylation of Cbl onco-protein in rabbit leukemogenic (RH/K34) and non leukemogenic (RH/K30) HTLV-I transformed cell lines and the role of the virus on this difference. The data indicate that the two cell lines differ in Cbl tyrosine phosphorylation and in associations of Cbl with other molecules containing P-Tyr.

Materials and Methods

Cell Lines, HTLV-1 Clone Construction, Cell Transfection and Treatment

The rabbit γδ T cell lines, RH/K30 and RH/K34, were obtained by transformation *in vitro* by HTLV-1 from irradiated human MT-2 T cell line (Sawasdikosol *et al*., 1993). RL-5 is a rabbit αβ T-cell line transformed *in vivo* with Herpes virus ateles (Daniel *et al*., 1974). K30p and K34p DNA molecular clones were constructed from RH/K30 and RH/K34 cells respectively and used to transfect RL-5 cells as described (Zhao *et al*., 1995). Sequence information for the HTLV-I clones K30p and K34p is available in GenBank with accession numbers L03561 and L03562, respectively. Cells were maintained in culture in RPMI complete medium containing 10% fetal calf sera (Intergen, Walkersville, MD), 2 mM L-Glutamine, 100 μ mL^{-1} Penicillin and 100 μg mL^{-1} Streptomycin. For pervanadate treatment, cells were incubated with 0.1 mM Na_3VO_4 and 0.1 mM H_2O_2 for 10 min at 37°C (Secrist *et al*., 1993).

Antibodies and Chemicals

Anti-phosphotyrosine monoclonal antibody (anti-PTyr 4G10) was from UBI (Lake Placid, NY). Anti-Cbl, C-Src, LCK, Lyn, Fyn, Sam-68, Phospholipase-C γ (PLCÇγ),

Phosphatidyl Inositol-3-kinase (PI3) antibodies and the Glutathione-S-tranferase (GST) molecules containing SH2 and SH3 domains were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti V-Src monoclonal antibody was from Oncogen (Cambridge, MA). Goat anti-Rabbit and anti-mouse horseradish peroxidase conjugated antibodies were from Bio-Rad (Richmond, CA). Protein A-sepharose, Triton X-100, PMSF, TLCK, Iodoacetamide, aprotinin and leupeptin were all from Sigma (St. Louis, MO). Immobilon P membrane from Millipore (Bedford, MA) and the enhanced chemiluminescence substrate ECL was from Amersham (Piscataway, NJ).

Immunoprecipitation and Western Blotting

Cell lines (10^7 cells mL^{-1}) were washed in PBS and treated with lysis buffer (10 mM Tris-HCl PH 7.5, 150 mM NaCl, 1% triton 100, 0.02% NaN_3, 1 mM PMSF, 10 mM TLCK, 5 mM Iodoacetamide, 2 mM aprotinin and 2 mM leupeptin). For Cbl immunoprecipitation, samples were precipitated as described (Mahana *et al*., 1988). Blots were developed either with anti-Cbl or anti-PTyr 4G10 antibodies and with corresponding peroxidase labeled goat anti-Ig antibodies, using the enhanced chemiluminescence substrate (ECL). For phosphotyrosine immunoprecipitations, lysates were cleared with normal mice sera and then reacted with agarose-conjugated 4G10 anti-PTyr Ab; washing and subsequent steps were as described above.

Precipitations with GST Containing SH2 and SH3 Domains

Cell lysates (10^7 cells) were precleared with GST-Glutathione-agarose conjugate, then precipitated using agarose-immobilized GST containing different SH2 (Grb2, Ras-Gap, PLCg1, SH-PTP2, LCK, Fyn and PI3) or SH3 (Grb2 N and C, Ras-Gap, Lck, Fyn and PLCg1) domains. Reaction mixtures were washed as previously described and separated by SDS-page (8% gel), transferred onto immobilon P membranes and developed with rabbit anti-Cbl Ab and peroxidase labeled goat anti rabbit Ig.

Results

HTLV-I transformed cells from human ATLL patients are constitutively activated in certain tyrosine kinase pathways (Migone *et al*., 1995; Takemoto *et al*., 1997). In the rabbit model of HTLV-I infection studied here, the proto-oncogene product Vav was activated in the leukemogenic rabbit cell line RH/K34, but not in the non leukemogenic RH/K30 cells. Vav phosphorylation was shown to be controlled by virus genes in the pX region (Mahana *et al*., 1998). In an extension of these studies Cbl was examined because of its molecular weight which can be contained into the phosphorylated proteins from the two cell lines and its reported association with Vav (Marengere *et al*., 1997). Cell lysates from RH/K30 and RH/K34 cell lines were immunoprecipitated using a rabbit polyclonal anti-Cbl and blotted with either anti-PTyr or anti-Cbl antibodies. Results in Fig. 1A show that Cbl is tyrosine phosphorylated in both cell lines but only in RH/K34 it was associated with another molecule of 55-60 kD. The second molecule is revealed on the blot by anti-PTyr but does not react with anti-Cbl.

Upon repeating the analyses after two weeks and at all subsequent times, it was found that the RH/K30 (non-leukogenic) cell line, maintained under normal conditions of culture, expressed normal levels of Cbl, however, it was not phosphorylated. By contrast, Cbl phosphorylation in RH/K34 persisted throughout the approximately one year period of observation (Fig. 1B). Activation of the cell lines by pervanadate for 10 min increased Cbl phosphorylation in RH/K34 and gave rise to a

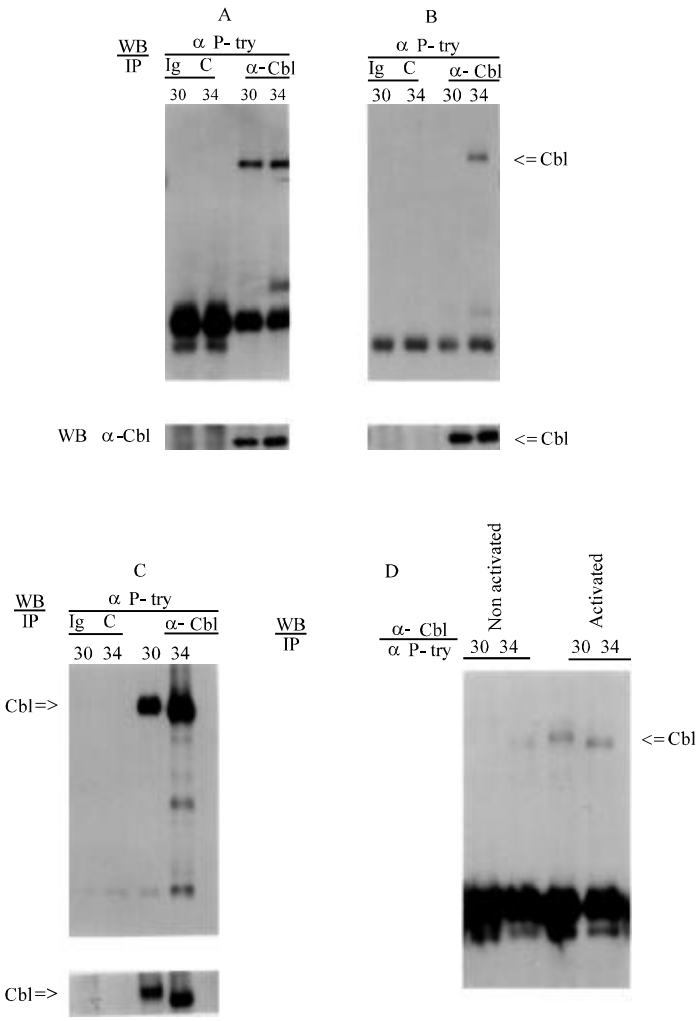

Fig. 1: Phosphorylation of Cbl in HTLV-I transformed cells

A) Freshly cultured rabbit HTLV-I transformed T cell lines RH/K30 and RH/K34 (10^7 cells) were washed in PBS and treated with lysis buffer. Lysates were precleared with normal rabbit serum and immunoprecipitated either with normal rabbit Ig (Ig C) or with anti-Cbl antibodies (α- Cbl). Samples were mixed with 100 μL of Laemmli sample buffer and 10 μL aliquots were separated by SDS-PAGE on a 8% gel and transferred onto immobilon P membrane and blots were developed with anti-phosphotyrosine antibody 4G10 and peroxidase labeled goat anti mouse antibodies, using the enhanced chemiluminescence substrate

B) The same RH/K30 and RH/K34 cells (10^7 cells) were maintained in culture for two weeks, washed, lysed, precleared, immunoprecipitated and treated as in A

C) RH/K30 and RH/K34 cell lines activated with 0.1 mM sodium pervanadate (Na_3VO_4) and 0.1 mM H_2O_2 for 10 min at 37°C, washed and lysed in lysis buffer. Precleared cell lysates were immunoprecipitated and blotted as in A)

D) Pervanadate activated and non-activated RH/K30 and RH/K34 cells. Lysates were precleared with normal mouse serum and agarose conjugated protein A and immunoprecipitated with agarose conjugated anti-PTyr 4G10 Ab. 10 μL aliquots were separated by SDS-PAGE on a 8% gel and transferred onto immobilon P membrane and blotted with rabbit anti-Cbl

number of other phosphorylated molecules which coprecipitated with Cbl. By contrast, pervanadate treatment of RH/K30 induces the phosphorylation of Cbl with a little shift in its molecular weight, but consistently, no associated molecules are observed (Fig. 1C). These results were confirmed by precipitations of cell lysates from nonactivated and pervanadate-activated RH/K30 and RH/K34 cells using anti-PTyr 4G10. This antibody precipitated Cbl from RH/K34 prior to activation and from both cell lines after activation (Fig. 1D).

The molecule associated with Cbl in RH/K34 was a 55-60 kD tyrosine phosphorylated protein of unknown identity. The possibility that it is a product of Cbl degradation is minimized by the fact that it did not react on the blot with the polyclonal anti-Cbl and by its presence at similar levels in the presence or absence of protease inhibitors (result not shown). Cbl is reported to associate with a number of molecules involved in signal transduction (Langdon, 1995; Liuy and Altman, 1998; Panchamoorthy *et al*., 1996; Chin *et al*., 1997) and based on these reports it was tried to immunoprecipitate Cbl with antibodies against c-src, Fyn, Lck, Lyn, v-src, sam68, PI3, Vav and PLCÇγ. Cbl was not precipitated, in our experimental conditions with antibodies directed against any of these molecules nor were any of the known intermediates precipitated by anti-Cbl. (Data not shown).

We then investigated the ability of Cbl from RH/K30 and RH/K34 to associate with other signaling molecules. Cell lysates were incubated with immobilized GST molecules containing SH2 domains from Grb2, Ras-Gap, PLCγ1, SH-PTP2, LCK, Fyn and PI3 and with SH3 domains from Grb2, Ras-Gap, PLCg1, LCK and Fyn. Results show that none of the SH2 domains precipitated Cbl from either cell line prior to activation. Following activation of RH/K34, Cbl was precipitated only with Fyn SH2 (Fig . 2A). In contrast to the rarity of SH2 interactions, several strong associations of Cbl with SH3. The phosphorylation of Cbl in other cell lines and the possibility that the HTLV-I virus influences this phosphorylation was investigated. Two additional cell lines were tested, first was the HTLV-I infected domain were observed. Cbl was precipitated by SH3 domains from Grb-2 N, Fyn, PLCγ and to a

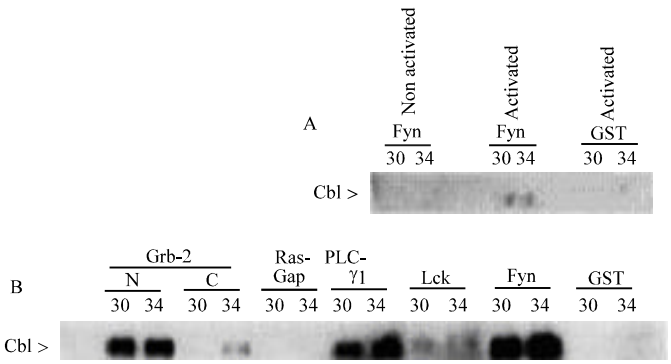

Fig. 2: *In vitro* association of Cbl with SH2 and SH3 domains from molecules involved in signal transduction

A) Pervanadate activated and non-activated RH/K30 and RH/K34 cell lysates (10^7 cells) were precleared with GST-Glutathione-agarose conjugate, then precipitated using agarose immobilized GST containing SH2 domains corresponding to Fyn. Precipitates were separated on SDS-PAGE (8% gel), transferred onto immobilon P membrane and developed with rabbit anti-Cbl Ab and peroxidase labeled goat anti-rabbit Ig

B) Precleared RH/K30 and RH/K34 cell lysates were precipitated using agarose immobilized GST containing different SH3 domains corresponding to Grb2 N and C, Ras-Gap, PLCγ1, Lck, Fyn and GST and processed as in A

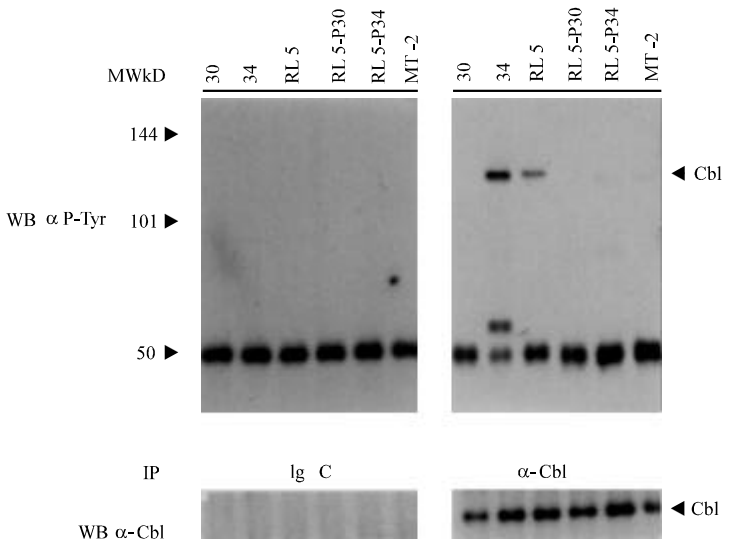

Fig. 3: Phosphorylation of Cbl in other cell lines and modulation of this phosphorylation in RL-5 cells by transfection with HTLV-I DNA clones. Precleared cell lysates (10^7 cells) from RH/K30, RH/K34, RL-5 and RL-5 transfected with HTLV-1 molecular clone K30p (RL5-p30) or K34p (RL5-p34) and human cell line MT-2 were immunoprecipitated with anti-Cbl Ab, transferred onto immobilon P membrane and immunoblotted with anti-PTyr antibody. Transfected cells were shown to produce HTLV-I p19 gag protein. Shown below are duplicate samples immunoblotted with anti-Cbl

lesser extent Lck in both cell lines. Grb-2C was positive only for RH/K34 and Ras-Gap was negative for both lines (Fig. 2B). The phosphorylation Cbl in other cell lines and the possibility that the HTLV-I virus influences this phosphorylation was invented. Two additional cell lines were tested, first was the HTLV-I infected human cell line MT-2 which was used to derive the rabbit cell lines and the second was the rabbit T cell line RL-5. Lysates from these cell lines were precipitated by anti c-Cbl antibody and checked for the presence and the tyrosine phosphorylation status of Cbl. Cbl is phosphorylated in Herpes rabbit transformed cell line RL-5, but not in MT-2, although the protein is present in both lines (Fig. 3).

The role of HTLV-I on Cbl activation was tested by tranfection of RL-5 cells with the HTLV-I DNA clones corresponding to RH/K30 and RH/K34 (p30 and p34, respectively). Virus was produced by RL-5 following transfection with both virus DNA; lysates from these transfectants were tested for Cbl. Although Cbl was expressed at levels comparable to non transfected controls (Fig. 3), it was no longer phosphorylated in cells transfected with either viral clone. While it is clear that viral infection has a striking negative effect, identity of the viral gene (or genes) responsible for this effect in RL-5 cells was not learned from this experiment because both clones produce the same result, despite their differences in sequence.

Discussion

Cbl is a modular docking protein that provides binding sites for numerous signaling proteins and promotes their involvement with activated tyrosine kinases (Langdon, 1995;

Liu *et al*., 1998). In addition, Cbl becomes highly phosphorylated on tyrosine residues upon activation by a number of mitogens and or by engagement of certain surface receptors (Liuy *et al*., 1998). Recently, Cbl has been shown to target tyrosine receptors for degradation by the activation of ubiquitin ligases allowing Cbl to negatively control the activated tyrosine kinases (Joazeiro *et al*., 1999). In this study, we analyzed the functional status of the onco-protein c-Cbl in two HTLV-I rabbit cell lines RH/K30 and RH/K34, which differ in their pathogenic potential. RH/K30 produces higher levels of virus and induces asymptomatic infection, while RH/K34 induces a lethal ATLL-like disease characterized by thymic atrophy (Zhao *et al*., 1993; Simpson *et al*., 1996). We found that Cbl is constitutively phosphorylated in RH/34 and associated with another, yet unidentified molecule of 55-60 kD. By contrast, Cbl was phosphorylated in RH/K30 sporadically and phosphorylation was lost under normal culture conditions. A major difference between the two cell lines was in the response to activation by the tyrosine phosphatase inhibitor, pervanadate, which mimics activation via the T-Cell receptor (Secrist *et al*., 1993). This activation enhances significantly the phosphorylation of Cbl and other associated molecules in RH/K34. However, in RH/K30 only c-Cbl was altered by the treatment. These results suggest that RH/K30 differs from RH/K34 in the tyrosine signaling cascade of the molecules downstream of c-Cbl.

The low level of Cbl interactions with SH2 domains corresponding to Grb2, Ras-Gap, PLCγ1, SH-PTP2, LCK, Fyn and PI3 in both cell lines before activation indicates that phosphorylated Cbl, is unable to interact by this domain with other molecules in our model. The result is normal for the not phosphorylated Cbl from RH/K30 but still ambiguous for phosphorylated Cbl in RH/K34. After activation, SH2 association was found only with Fyn SH2 in RH/K34. It is possible that the unidentified molecule associated with Cbl in RH/K34 cells inhibits interaction but this molecule is not observed in RH/K30. The large amount of Cbl precipitated by the SH3 domains and more specifically SH3 corresponding to Fyn and Grb-2 suggests that the interactions of Cbl with the other molecules involved in signal transduction are stronger via prolin region and SH3 than the PTB and SH2 domains. This was supported by the facts that only SH3 domain of Nck and Hck were able to interact with Cbl (Wunderlich *et al*., 1999; Scholz *et al*., 2000) and the pivotal role Lck SH3 domain in its interaction and degradation by Cbl (Rao *et al*., 2002). Moreover, the amounts of Cbl pulled down by SH3 domains in the leukemogenic cell line which has a phosphorylated Cbl were higher than those from the non leukemogenic cell line having non phosphorylated Cbl ; this may reflect the role of phosphorylated tyrosine on the other activities of Cbl. Difference between our results and other concerning the interaction of Cbl with other molecules involved in signal transduction via SH2 and SH3 domains may be due to the difference between the systems used in each study jurkat cell versus HTLV-I transformed cells (Donovan *et al*., 1994).

The presences of the yet unidentified Cbl associated molecule in the RH/K34 cell line at the level of 60 kD my be the product of degraded molecules in this cell by activated Cbl ubiquitin ligase activity. Although the profile of c-Cbl phosphorylation and the presence of the, associated molecule is specific for RH/K34, it was not possible to link this difference to a specific virus gene. The molecular clones from either of the two cell lines down regulated Cbl phosphorylation in RL-5. This result is unexpected in light of the constitutive activation of Cbl in RH/K34 and is not consistent with the differential effect of the identical clones on Vav phosphorylation in RL-5 cells (Mahana *et al*., 1998).

Information regarding the physiological role of the Sli/Cbl family of proteins has emerged from genetic studies in *C. elegans* which identified Sli-1 as a negative regulatory element of the *let*23 signaling pathway (Jongeward *et al*., 1995). An inhibitory role for mammalian Cbl was also suggested by experiments showing that overexpression of Cbl downregulates the allergic response in

mast cells activated via the Fc receptor (Ota and Samelson, 1997). This role was attributed to the ubiquitination activity of Cbl and the degradation of different tyrosine kinases (Miyake *et al*., 1998; Andoniou *et al*., 2000; Rao *et al*., 2002; Kaabeche *et al*., 2004; Kim *et al*., 2004). In contrast to these studies, Cbl was shown to be positive regulator for many transduction pathways; such in Src signaling pathway leading to bone resorption (Tanaka *et al*., 1996), in the activation of the adhesion activity and the spreading of malignant cells (Feshchenko *et al*., 1999; Teckchandani *et al*., 2001), in the increase of the adherence of lippolysaccharide-stimulated macrophages (Scholz *et al*., 2000) and during the activation of osteoclasts and mature dendritic cells by TNF related activation-induced cytokine (Arron *et al*., 2001).

Initial interest in c-Cbl was sparked by reports that Cbl can interact with Vav, the GDP/GTP exchange factor for the Rac-1 GTPase, in different systems (Marengere *et al*., 1997) and that a member of the Cbl family, cbl-b, negatively regulates Vav activity (Bustelo *et al*., 1997). In our system, Vav and Cbl are both activated in the leukemogenic cell line RH/K34 which promote their interaction, (although no interaction between them could be detected). This with the potential association of Cbl in vitro with Grb2 and Fyn which can enhance the catalytic activity of guanine nucleotide exchanger SOS suggest that the Ras and Ras-like pathways and mitogen-activated protein kinase cascades may be constitutively activated through the association of signaling molecules with HTLV-I proteins. Consistent with this, a member of the mitogen-activated protein kinase family, c-Jun was constitutively activated in HTLV-I infected cells (Xu *et al*., 1996).

Based in both roles attributed for Cbl, we just can suggest that if Cbl has a negative regulatory role, it's phosphorylation in the leukemogenic RH/K34 reflect the necessity to control the hyper activation statue of this cell line. By contrast, if Cbl has a positive role on the adhesion and the spreading of malignant cells, the role may be used by RH/K34 cell line to increase its capacity to adhere and invade organs in the infected rabbit resulting a dangerous situation as mentioned in the our experimental system. In fact, our tow cell lines have a different profile regarding their adhesion behavior, RH/K34 has an activated adhesion profile comparing to the non leukemogenic cell line RH/K30 (data not shown).

The functional significance of the phosphorylation of Cbl in HTLV-I pathogenicity remains to be determined although Cbl has been implicated in tumorogenesis; Phosphorylated Cbl may play a role in promoting Abl-mediated tumorogenesis (Jain *et al*., 1997). Cbl was found to be tyrosine phosphorylated in a tumor specific manner in human cancer tissues (Kamei *et al*., 2000). And the role of phosphorylated Cbl on the ubiquitination activity specifically with the increase of the importance of the ubiquitination and deubiquitination machinery in the oncogenic potential of human tumor viruses (Shackelford and Pagano, 2004).

Although there is no direct link between the leukemogenic potential of RH/K34 and the phosphorylation of Cbl, the constitutive phosphorylation of Cbl may be a marker of HTLV-I leukemogenesis. The results here show a clear distinction in activation levels of molecules involved in lymphoid cell signaling pathways between the two rabbit cell lines. This finding supports results suggesting that RH/K34 is highly activated as compared to RH/K30 which mediates asymptomatic infection. The role of the virus in control of oncoprotein activation provides a possible explanation for the variable pathogenicity of this retrovirus.

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