Available online at www.sciencedirect.com
SCIENCE DIRECT®
PERGAMON
Leukemia Research 28 (2004) 71–82
Leukemia Research
www.elsevier.com/locate/leukres

Down-regulation of SHP1 and up-regulation of negative regulators of JAK/STAT signaling in HTLV-1 transformed cell lines and freshly transformed human peripheral blood CD4$^+$ T-Cells

Jihua Cheng [a,b], Dongsheng Zhang [a,b], Changhong Zhou [a,b], Wayne A. Marasco [a,b,*]

[a] *Department of Cancer Immunology & AIDS, Dana-Farber Cancer Institute, Harvard Medical School, 44 Binney Street, Boston, MA 02115, USA*
[b] *Department of Medicine, Harvard Medical School, 44 Binney Street, Boston, MA 02115, USA*

Received 17 February 2003; accepted 21 May 2003

Abstract

Adult T-cell leukemia (ATL) is an aggressive malignancy that is associated with human T-cell lymphotropic virus I (HTLV-I) infection. HTLV-I transformed T-cell lines and fresh ATL cells are characterized by constitutive activation of the interleukin-2 receptor (IL-2R) signaling pathway however, the mechanism(s) responsible for constitutive IL-2R activation are unknown. To further examine the cause of this signaling pathway deregulation, we measured mRNA and protein expression levels by real-time PCR and Western blots, respectively, of four negative regulators of the IL-2R signaling pathway including src homology 2 (SH2)-containing phosphatase (SHP1), cytokine-inducible (CIS) SH2-containing protein, suppressor of cytokine signaling-1 (SOCS1) and protein inhibitor of activated signal transducer and activator of transcription 3 (STAT3) (PIAS3) in six HTLV-1 negative and seven HTLV-1 positive T-cell leukemia lines. The activation status of the JAK/STAT pathway was also examined. SHP1 mRNA and protein expression levels were selectively down regulated in all HTLV-1-infected transformed cell lines, while CIS, SOCS1, and PIAS3 protein expression were markedly but variably upregulated and the cells showed evidence of constitutive STAT3 activation. In acutely HTLV-1 infected primary CD4$^+$ T-cells there was a gradual loss of SHP1 expression over 10 weeks in culture which correlated with progression from immortalization to transformation and loss of IL-2 dependence for growth. Two transformed cell lines that were established following HTLV-1 infection showed loss of SHP1 expression and overexpression of CIS, SOCS1, PIAS3. However, this overexpression was not adequate to block constitutive activation of the JAK/STAT pathway. Thus, multiple levels of IL-2 receptor signal deregulation are found in HTLV-1 transformed cells, which may be a result of early loss of SHP1 expression.

Keywords: Signal transduction; Adult T-cell leukemia; Retrovirus; Cancer; IL-2 receptor

1. Introduction

Human T-cell lymphotropic virus I (HTLV-I) is the etiological agent for adult T-cell leukemia (ATL) and HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [1]. Deregulation of IL-2 receptor (IL-2R) signaling appears to play an important role in the events that lead to immortalization and oncogenic transformation of CD4$^+$ T-cells by HTLV-1. HTLV-1-infected peripheral blood T-cells exhibit an initial phase of interleukin-2 (IL-2)-dependent growth; over time, by an unknown mechanism the cells become IL-2 independent [2]. Typically the transformed cell lines display activation of the JAK/signal transducer and activator of transcription (STAT) signaling pathway with increased constitutive phosphorylation of the IL-2R complex, including IL-2Rβ, the Janus kinases JAK1 and JAK3 and the signal transducers and activators of transcription, STAT3 and STAT5 [2–4].

Many studies have focused on the mechanisms by which IL-2 exerts its actions and how activation of the IL-2R is controlled [5]. Recently, several control mechanisms for the negative regulation of cytokine signaling have been elucidated. These include protein degradation [6–8], the activation of phosphatases such as SHP1 [6,9], a recently discovered family of small src-homology 2 (SH2)-containing proteins including cytokine-inducible (CIS SH2-containing protein) [10], and seven SOCS proteins (SOCS1–7), suppressor of cytokine signaling [11–14], and protein inhibitor of activated STAT (PIAS) proteins including PIAS1 and PIAS3 [15,16]. Each of these negative regulators has a distinct mode of action [17,18]. For example, SHP1 is present constitutively in cells, and as such is able to downregulate signaling immediately when receptor/kinase complexes are

* Corresponding author. Tel.: +1-617-632-2153; fax: +1-617-632-3889.
E-mail address: wayne_marasco@dfci.harvard.edu (W.A. Marasco).

0145-2126/$ – see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0145-2126(03)00158-9

activated. In particular, IL-2 induces association of SHP1 with the IL-2R complex, and once SHP1 is recruited to the activated receptor it is able to decrease tyrosine phosphorylation of IL-2Rβ and the associated tyrosine kinases JAK1 and JAK3 [19]. CIS is induced in hematopoietic cells within 30 min of stimulation by IL-2 and other cytokines [10,20]. CIS interacts with the A region of IL-2Rβ which also mediates the association of IL-2Rβ with Lck and JAK3 and inhibits functions associated with both of these kinases: Lck-mediated phosphorylation of IL-2Rβ and IL-2-mediated JAK3 activation and subsequent activation of STAT5 [20]. Like CIS, SOCS1 is also strongly induced by IL-2, can associate with IL-2Rβ through the A region of IL-2Rβ and can potently inhibit IL-2-induced STAT5 function [21]. However, the association of SOCS1 with IL-2Rβ is not essential for its inhibitory activity. More likely, it is the direct binding of SOCS1 with both JAK1 and JAK3 and the resulting inhibition of JAK1 tyrosine phosphorylation and kinase activity that is responsible for its negative regulatory effects [21]. SOCS3 is also strongly induced and in parallel phosphorylated by IL-2 stimulation, is associated with JAK1 and can inhibit JAK1 phosphorylation and kinase activity and markedly reduce IL-2-induced tyrosine phosphorylation of STAT5b [22]. PIAS3 can inhibit IL-6 induced STAT3-dependent transcriptional activation however, the role of PIAS3 in IL-2 signaling has not been reported. The fact that PIAS3 appears to be constitutively expressed suggests that the PIAS proteins may act as a basal-level barrier to STAT protein action, rather than as cytokine-inducible negative feedback effectors [18]. Finally, the deubiquitinating enzyme DUB-2, which is induced in response to IL-2 has been reported to markedly prolong IL-2-induced STAT5 phosphorylation even after growth factor withdraw [8].

In two recent reports, SHP1 expression was found to be greatly decreased or undetectable in several IL-2-independent HTLV-1 transformed T-cell lines that exhibit constitutive JAK/STAT activation [19,23]. In addition, in HTLV-1 infected T-cells, down-regulation of SHP1 was also found to correlate with the acquisition of IL-2 independence. However, these reports did not evaluate the expression levels of other negative regulators of cytokine signaling or the expression levels of SHP1 at early times following HTLV-1-infection of $CD4^+$ T-cells. We therefore undertook this study to evaluate the levels of mRNA and protein expression of several negative regulators of IL-2 signaling in acutely and chronically HTLV-1-infected transformed cell lines as well as the activation status of the JAK/STAT signaling pathway. We observed that not only were SHP1 levels low to undetectable but the levels of inducible (CIS and SOCS1) and constitutive (PIAS3) negative regulators were markedly but variably elevated. We also found that there was constitutive activation of the JAK/STAT pathway despite overexpression of these downstream negative regulators. Furthermore, a time course of HTLV-1 infection of human peripheral blood $CD4^+$ T-cells established that the loss of SHP1 expression, acquisition of IL-2 independence, overexpression of CIS, SOCS1 and PIAS3 and constitutive activation of the JAK/STAT pathway occur early in the transformation process.

2. Methods and materials

2.1. Cell lines and culture conditions

The six HTLV-1 negative cell lines (obtained from ATCC) used in this study are the following: HUT78—human cutaneous T-cell lymphoma cell line [24]; SupT1—non-Hodgkin's T-cell lymphoma line [25]; Molt4 Clone8—acute lymphoblastic leukemia line [26]; CEM-T4—human T-lymphoblastoid line [27]; Jurkat Clone E6-1 [28]; COS-7—African green monkey kidney fibroblast-like cell line transformed with SV40 [29]. The seven HTLV-1 positive cell lines included: HUT102—[30]; MT2—in vitro transformed and continuous producer of HTLV-1 virions [31]; MT4—derived from ATL patient [32]; C8166-45—[33]; OS-P_2—in vitro producer of HTLV-1 virions [34]; C10/MJ—[35] and SP—derived from ATL patient [36]. All studies were performed on cell lines during the first three in vitro passages.

HUT78, SupT1, Molt4 Clone8, CEM-T4, Jurkat E6-1, HUT102, MT2, MT4, C8166-45, OS-P_2 and C10/MJ cell lines were cultured in RPMI-1640 with 2 mM L-glutamine plus 10% fetal bovine serum. COS-7 cells were cultured in Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate and 4.5 g/l glucose plus 10% fetal bovine serum (FBS). OS-P_2 cells were cultured in RPMI-1640 plus 15% FBS. SP was cultured in RPMI-1640 with 2.0 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin plus 10% FBS, 100 U/ml purified human IL-2 (Chiron Co., NDC 53905-991-01). Cells were maintained at between 1×10^5 and 1×10^6 cells/ml.

2.2. Purification and infection of human $CD4^+$ T-lymphocytes

$CD4^+$ T-cells were purified from peripheral blood using RosetteSep $CD4^+$ T-Cell Enrichment Cocktail (StemCell Technologies Inc., 15062) according to the manufacturers instructions. Briefly, 50 μl RosetteSep cocktail was added per ml of blood, mixed well and then incubated for 20 min at RT. The samples were diluted with an equal volume of PBS + 2% FBS and mixed gently. The diluted sample was layered on top of Ficoll–Paque without mixing and then centrifuged at room temperature for 20 min at 1200 g with the brake off. The enriched cells were removed from the density medium: plasma interface and then washed with PBS + 2% FBS two times. The cells were resuspended in 3 ml ammonium chloride for 3 min to lyse RBCs followed by washing three times with 20 ml 10% FBS/AIM-V medium, centrifugation at 1200 rpm for 10 min and resuspension of the cells

in 10% FBS/AIM-V medium (GibcoBRL, 12055) prior to final cell counting.

2.3. Immortalization and transformation of fresh CD4$^+$ T-cells by co-culture with HTLV-1-producing MT2 cells

Purified CD4$^+$ T-cells were infected with HTLV-1 by co-culture. 1×10^5 CD4$^+$ T-cells were placed in each well of a six-well plate containing a Millicell-CM Culture Plate Insert (Millipore Co., PICM03050) with 1×10^4 MT2 cells in the insert. The cells are cultured in 1.5 ml AIM-V media with 15% FBS and 100 U/ml IL-2. Aliquots of CD4$^+$ cells were collected every 2 weeks and the MT2 cells were replaced with fresh cells when they reached a density of 1×10^6 cell/ml. The co-culture was continued for 12 weeks. Frozen samples of the harvested CD4$^+$ T-cells were defrosted and used to detect the expression of SHP1 by Western blot.

After 12 weeks of co-culture, IL-2 was tapered down and was finally discontinued in four more weeks. Sub-clones were established over a 2-week period by limiting dilution seeding of 1 cell/ml RPMI-1640 plus 15% FBS in 24-well plates. Two IL-2 independent clones, B1 and C5 were established. FACS using FITC-conjugated HLA-A2, B8 and BW4 antibodies (One Lambda Inc., Canoga Park, CA, USA, FH0037, FH0536A, FH0007, respectively) confirmed that B1 and C5 had different HLA phenotypes compared to MT2 (data not shown).

2.4. Cell activation and stimulation

Fresh CD4$^+$ cells and the transformed B1 and C5 clones, as well as MT2 cell lines were maintained in RPMI-1640/10%FBS. Before stimulation, wash the cells with 1XPBS, then cultured for 72 h in RPMI-1640/10%FBS and phytohemagglutinin (PHA 1 μg/ml, Sigma, St. Louis, MO L9132) and rested overnight in RPMI-1640/10%FBS. Cells were washed and resuspended in fresh medium containing 1% FBS for 4 h, and then resuspended in RPMI-1640/10% FBS for 1 h before treatment with 200 IU/ml IL-2. Cells are maintained in IL-2 stimulation for 30 min, then washed prior to preparing cell lysates.

Whole cell lysates were prepared from fresh and two transformed CD4$^+$ clones (B1 and C5) and MT2 cells by using RIPA lysis buffer. Proteins were loaded on 10% polyacrylamide gels and transferred to a polyvinylidene difluoride membrane. Blots were incubated with the appropriate concentrations of primary and HRP-labeled secondary antibodies, and then were visualized using the enhanced chemiluminescence detection kit (ECL; Pierce, Rockford, IL 1856136).

2.5. Real-time PCR analysis of the negative regulators

All RT-PCR reagents were purchased from PE/Applied Biosystems, Foster City, CA except were otherwise mentioned. Total RNA was extracted from cultured cells with Trizol reagent (GibcoBRL, Gaithersburg, MD). One-step real-time RT-PCR was performed using TaqMan Gold RT-PCR Kit according to the manufacturers protocol. Fifty nanograms of total RNA from each cell was used as template. The primers and probes sequences were as follows: SHP1: 91 bp; Forward: GGAGTCGGAGTACGGGAACAT, Reverse: ATCCTCCTTGTGTTTGGACGA, Probe: CCCCAGCCATGAAGAATGCCCA; PIAS3: 80 bp; Forward: TTACCCAAACCCATGGCTCTA, Reverse: GTCTGTTTGGCCGAAGGGA, Probe: TCTTCACATCCATTGTCTCTTCATGTCTATTCCA; SOCS1: 109 bp; Forward: CCCTGGTTGTTGTAGCAGCTT, Reverse: CAACCCCTGGTTTGTGCAA, Probe: TGAACTCGCACCTCCTACCTCTTCATGTTTAC. The GAPDH probe (TaqMan GAPDH control reagents, 402869) is labeled with JOE (6-carboxy-4,5-dichloro-2,7-dimethoxyfluorescein) reporter dye on the 5′ end and measured at 554 nm wavelength. SHP1, PIAS3 and SOCS1 are labeled with FAM (6-carboxyfluorescein) reporter dye on 5′ end and measured at 518 nm wavelength. Both reporter dyes are quenched by TAMRA on 3′ end (6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine). PCR reaction conditions were as follows: all samples were performed in triplicates. Thermal cycler parameters included 48 °C for 30, 95 °C for 10 min and 40 cycles involving denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min (GAPDH); 52 °C for 1 min and 60 °C for 1 min (SHP1 and PIAS3); 55 °C for 2 min (SOCS1).

2.6. Western blot analysis

Cells were washed twice with 1 × PBS, and then solubilized in 500 μl RIPA buffer (50 mM Tris, pH 7.8, DTT 1 mM, $MgCl_2$ 10 mM, KCl 70 mM, NP-40 0.5%, and SDS 0.1%) plus protein inhibitor cocktail tablets (Roche Diagnostics GmbH, 1873580). The cell lysate was incubated on ice for 30 min followed by centrifugation. The insoluble material was disgarded and the supernatants were stored at −20 °C. Samples of cell lysate (0.5–1.0) × 10^6 cell equivalent were loaded on a 10% Tris–glycine SDS-polyacrylamide gel and resolved by applying voltage of 8 V/cm to the stacking gel and 15 V/cm to the resolving gel, respectively. For Western blots, transfer of the protein from SDS-polyacrylamide gel to a polyvinylidene difluoride (NEN Research products, NEF-1002) membrane was performed at 100 V constant voltage for 1 h at 4 °C. Antibodies used were as follows: From Santa Cruz: SHP1 (mouse monoclonal, sc-7289), CIS (goat polyclonal, sc-1528), SOCS1 (goat polyclonal, sc-7005), PIAS3 (goat polyclonal, sc-8154), actin (mouse monoclonal, sc-8432), actin (goat polyclonal, sc-1615), JAK1 (rabbit polyclonal, sc-7228), JAK3 (rabbit polyclonal, sc-513), β2-microtubulin (rabbit polyclonal, sc-9104). HRP-labeled goat anti-mouse IgG (sc-2005), HRP-labeled bovine anti-goat IgG (sc-2701), and HRP-labeled goat anti-rabbit IgG (sc-2301). From Cell Signaling Technology:

STAT3 antibody (rabbit polyclonal, #9132), phosphorylated STAT3 (rabbit polyclonal, no. 9131), phosphorylated STAT5 (rabbit polyclonal, 9351S). Rabbit anti-tax antiserum was obtained from the NIH AIDS Research and Reference Program (cat. #712). Western blot results were quantified by using NIH image 1.62f software.

3. Results

3.1. Analyses of mRNA and protein expression levels of negative regulators of JAK/STAT pathway in HTLV-1 negative and positive cell lines

Quantitative real-time RT-PCR and Western blot analyses were used to assess the mRNA and protein expression levels of several negative regulators of the JAK/STAT signaling pathway, respectively. In total, 12 T-cell leukemia cell lines were evaluated; five were HTLV-1 negative (HUT78, SupT1, Molt4 Clone8, CEM-T4, Jurkat E6-1) and seven were HTLV-1 positive cell lines (MT2, MT4, C8166-45, OS-P_2, C10/MJ and SP). Of the latter, only the MT2 and OSP_2 cell lines produce infectious HTLV-1 viruses [31,32,37,38]. COS-7 cells, an African green monkey kidney fibroblast-like cell line transformed with SV40 was also included in these studies as a control however, their relative mRNA and protein expression levels of the different negative regulators of JAK/STAT signaling pathway were not used in the comparative analyses with the T-cell leukemia cell lines. To quantify transcripts of the SHP1, PIAS3 and SOCS1 genes, we monitored the housekeeping protein GADPH mRNA transcripts as the quantitative control and each sample was normalized on the basis of its GADPH transcript content. We also normalized protein expression levels of SHP1, CIS, SOCS1 and PIAS3 against β-actin to ensure that equal amount of protein were loaded on the SDS-PAGE gel. A summary of the results of the mRNA and protein expression analyses are provided in Tables 1 and 2, respectively. Two ranking systems are shown in each table that provide both the numeric rank of each cell line among the 12 T-cell leukemia cell lines that were examined and the quartile rank of each cell line among the 12 cell lines.

3.1.1. SHP1 mRNA and protein expression levels

As can be seen in Table 1, there is considerable variation of SHP1 mRNA levels among the different cell lines tested. In particular, for the HTLV-1 negative T-cell leukemia lines there was a 26-fold range in relative SHP1 mRNA levels between the highest Molt4 Clone8 and lowest HUT78 cells. For the HTLV-1 positive cell lines, the range was markedly broader (>1.5×10^6-fold). In addition, five of seven (71%) HTLV-1 positive cell lines (HUT102, MT2, MT4, OS-P_2 and SP) expressed the lowest relative levels of SHP1 mRNA. For OS-P_2 and SP cells, only very low levels of SHP1 mRNA were detectable. To determine if mutations in the promoter region of the SHP1 gene were responsible for loss of mRNA expression, genomic DNA was prepared from MT2 and OS-P_2 cells and PCR/DNA sequencing of the promoter region was performed however, no mutations in the promoter regions were found.

Fig. 1 (panel A) and Table 2 shows the results of Western blot analysis for SHP1 protein expression. As can be seen, SHP1 protein was detected in all five of the HTLV-1 cell lines although the protein levels were lower in HUT78 and Molt4 Clone8 cells. In contrast, there is a marked reduction of full length SHP1 protein in six of seven (86%)

Table 1
Real-time RNA PCR of SHP1, SOCS1 and PIAS3 in HTLV-1 negative and positive cell lines

Cell line	SHP1			SOCS1			PIAS3		
	Relative quantity mRNA	No. rank[a]	Rank (%)[b]	Relative quantity mRNA	No. rank	Rank (%)	Relative quantity mRNA	No. rank	Rank (%)
Control									
COS-7	2.13×10^3			1.89×10^4			5.94×10^5		
HTLV-1-neg									
HUT78	1.22×10^4	8	+	2.72×10^2	10	+	1.70×10^5	7	+
SupT1	5.24×10^4	5	+	1.78×10^4	5	+	7.84×10^5	4	+
Molt4 Clone8	3.19×10^5	1	++++	6.76×10^3	8	+	3.27×10^6	1	++++
CEM-T4	5.18×10^4	6	+	2.30×10^4	4	+	2.91×10^5	5	+
Jurkat E6-1	6.07×10^4	3	+	4.07×10^3	9	+	1.72×10^3	6	+
HTLV-1-pos									
HUT102	6.82×10^3	9	+	4.64×10^4	3	+	2.83×10^4	9	+
MT2	1.35×10^3	10	+	5.41×10^4	2	++	1.44×10^6	2	++
MT4	2.88×10^4	7	+	6.77×10^3	7	+	4.00×10^3	10	+
C8166-45	5.38×10^4	4	+	1.36×10^4	6	+	5.03×10^4	8	+
OS-P_2	0.73×10^0	11	+	1.52×10^1	11	+	7.03×10^0	12	+
C10/MJ	1.26×10^5	2	++	2.16×10^5	1	++++	9.58×10^5	3	++
SP	0.08×10^0	12	+	1.52×10^0	12	+	2.04×10^1	11	+

[a] No. rank—numeric ranking from the highest (1) to lowest (12) for the 12 lines tested.
[b] Rank (%) is a quartile ranking system—(+) 0–25%; (++) 26–50%; (+++) 51–75%; (++++) 76–100%.

Table 2
Relative protein expression levels of SHP1, PIAS3, SOCS1 and CIS in HTLV-1 negative and positive cell lines

Cell line	SHP1			CIS			SOCS1			PIAS3		
	Relative[a] units (RU)	No. rank[b]	Rank (%)[c]	Relative units (RU)	No. rank	Rank (%)	Relative units (RU)	No. rank	Rank (%)	Relative units (RU)	No. rank	Rank (%)
Control												
COS-7	0.14			0.02			1.79			0.71		
HTLV-1-neg												
HUT78	0.29	6	++	0.06	10	+	0.11	(8)	+	0.05	11	+
SupT1	0.76	3	++++	0.10	8	+	0.04	12	+	0.12	(9)	+
Molt4 Clone8	0.24	8	++	0.14	7	+	0.08	10	+	0.19	7	+
CEM-T4	0.78	2	++++	0.08	9	+	0.17	6	+	0.12	(9)	+
Jurkat E6-1	0.82	1	++++	0.03	12	+	0.05	11	+	0.03	12	+
HTLV-1-pos												
HUT102	0.27	7	++	1.15	2	++++	0.52	4	+++	0.66	3	+++
MT2	0.14	9	+	0.40	5	++	0.51	5	+++	0.49	4	++
MT4	0.12	(10)	+	0.28	6	+	0.58	2	+++	0.41	5	++
C8166-45	0.71	4	++++	0.05	11	+	0.80	1	++++	0.38	6	++
OS-P_2	0.30	5	++	1.43	1	++++	0.39	6	++	0.78	2	+++
C10/MJ	0.12	(10)	+	0.67	3	++	0.11	(8)	+	0.18	8	+
SP	0.06	12	+	0.59	4	++	0.57	3	+++	1.07	1	++++

[a] Western blot results were quantified using NIH image 1.62s software.
[b] No. rank—numeric ranking from the highest (1) to lowest (12) for the 12 lines tested.
[c] Rank (%) is a quartile ranking system—(+) 0–25%; (++) 26–50%; (+++) 51–75%; (++++) 76–100%.

HTLV-1 cell lines. Only C8166-45 cells express a high level of full-length SHP1 however, an additional smaller molecular weight protein (circa 50 kD) is also seen in this cell line and in HUT102 and OS-P_2 cells. To determine if this smaller molecular weight protein was an alternatively spliced form of SHP1 or a proteolytic cleavage protein, we performed RT-PCR on these three cell lines with primers located in the first and last coding exons of SHP1 to amplify the gene product from mRNA. The results demonstrated that only full length PCR products of the SHP1 gene were obtained (data not shown). Since the anti-SHP1 Mab that was used for this Western blot is directed against a region in the carboxy-terminus of SHP1, we assume that this 50 kD protein is not an alternatively spliced isoform of SHP1 but rather a proteolytically cleaved form of native SHP1, with the cleavage site, based on its molecular weight, occurring in the second SH2 domain of SHP1.

3.1.2. CIS protein expression levels

Protein expression but not mRNA expression was studied for CIS since this work began later in our investigations. The Western blot analyses of CIS (and SOCS1 and PIAS3 see below) showed remarkably different results compared to SHP1. As can be seen in Fig. 1 (panel B) and Table 2, with the exception of C8166-45 cells, six of seven (86%) HTLV-1 positive cell lines overexpressed CIS compared to the HTLV-1 negative cell lines. Interestingly, C8166-45 cells for which CIS protein expression was undetectable, expressed a high level of SHP1.

3.1.3. SOCS1 mRNA and protein expression levels

The mRNA levels of SOCS1 showed the lowest variation of mRNA levels of any of the three negative regulators that were examined in the five HTLV-1 negative cell lines (Table 1). There was an overall 85-fold variation with CEM-T4 cells expressing the highest levels and HUT78 cells expressing the lowest levels. For the HTLV-1 positive cell lines, the range was again broader (>1,40,000-fold). The C10/MJ cells showed the highest levels of SOCS1 mRNA and as with SHP1 mRNA the OS-P_2 and SP cells again showed the lowest levels. Only three of seven (43%) HTLV-1 positive cell lines (MT4, OS-P_2 and SP) were among the cell lines that expressed the lowest relative levels of SOCS1 mRNA. The Western blots results for SOCS1 protein expression are shown in Fig. 1 (panel C). As can be seen, six of seven (86%) HTLV-1 positive cell lines also overexpressed SOCS1 compared to the HTLV-1 negative cell lines. Only MT2 cells and C10/MJ cells did not express comparably high levels of SOCS1.

3.1.4. PIAS3 mRNA and protein expression levels

The variation of PIAS3 mRNA for the five HTLV-1 negative cell lines was 1900-fold with Molt4 Clone8 cells as in the case of SHP1 mRNA, again expressing the highest levels but with Jurkat E6-1 cells expressing the lowest levels. For the HTLV-1 positive cell lines, the range was much broader (>2,00,000-fold). In addition, five of seven (71%) HTLV-1 positive cell lines (HUT102, MT4, C8166-45, OS-P_2 and SP) were among the cell lines that expressed the lowest

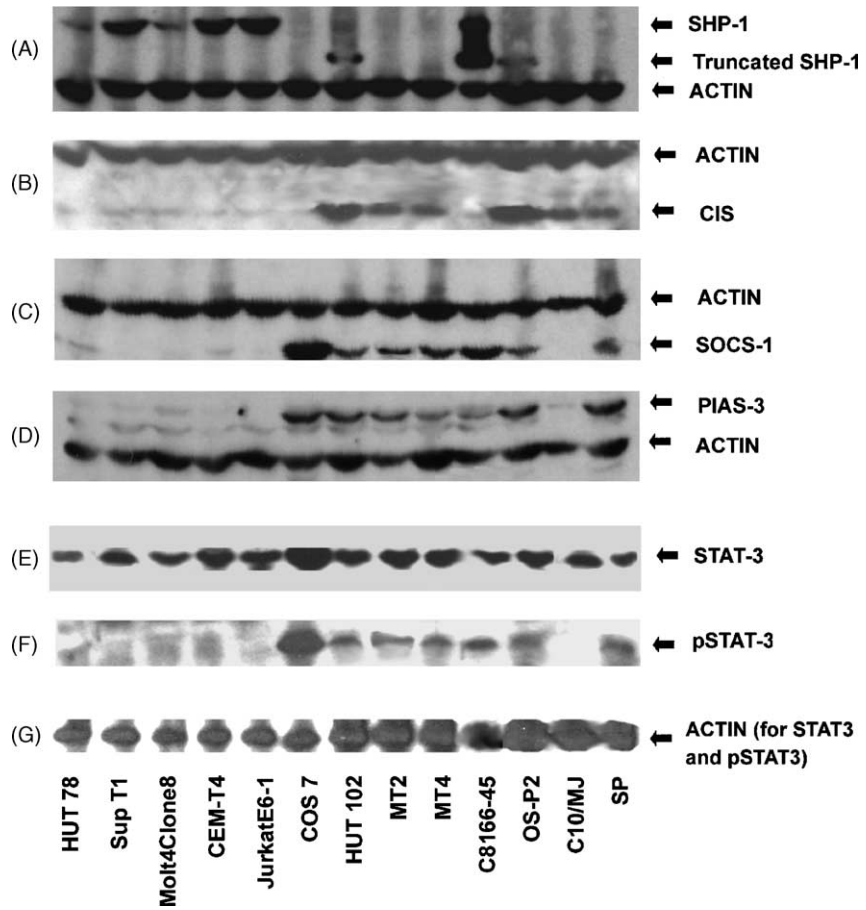


Fig. 1. Western blot analysis of negative regulators and STAT3 and STAT5. Samples of cell lysate (0.5–1.0) $\times 10^6$ cell equivalent were loaded on a 10% Tris–glycine SDS-polyacrylamide gel and then for Western blotting transferred to a polyvinylidene difluoride (NEN) membrane. Antibodies were used at the following dilutions. Panel A—primary antibodies: anti-SHP1 Mab (Santa Cruz, sc-7289) [1:266] and mouse anti-human actin (Santa Cruz, sc-8432) [1:2000]; secondary antibody: HRP-labeled goat anti-mouse IgG (Santa Cruz, sc-2005) [1:500]. Panel B—primary antibodies: goat anti-CIS (Santa Cruz, sc-1528) [1:1000] and goat anti-human actin (Santa Cruz, sc-1615) [1:6000]; secondary antibody: HRP-labeled bovine anti-goat IgG (Santa Cruz, sc-2701) [1:3000]. Panel C—primary antibodies: goat anti-SOCS1 (sc-7005) [1:2000] and goat anti-human actin (sc-8432) [1:6000]; secondary antibody: HRP-labeled bovine anti-goat IgG (Santa Cruz, sc-2701) [1:3000]. Panel D—primary antibodies: goat anti-PIAS3 (Santa Cruz, sc-8154) [1:1000] and goat anti-human actin (Santa Cruz, sc-8432) [1:6000]; secondary antibody: HRP-labeled bovine anti-goat IgG (Santa Cruz, sc-2701) [1:3000]. Panel E—primary antibody: rabbit anti-human STAT3 antibody (phosphorylation-state independent): Cell Signaling Technology (#9132) [1:1000]; secondary antibody: goat anti-rabbit IgG (Santa Cruz, sc-2301) [1:2000]. Panel F—primary antibody: rabbit anti-human phospho-STAT3 (Ser727) (phosphorylation-state dependent): Cell Signaling Technology (#9134S) [1:2000]; secondary antibody: goat anti-rabbit IgG (Santa Cruz, sc-2301) [1:2000]. Panel G—internal control for STAT3 and pSTAT3, primary antibody: goat anti-human actin (Santa Cruz, sc-1615) [1:6000]; secondary antibody: HRP-labeled bovine anti-goat IgG (Santa Cruz, sc-2701) [1:3000].

relative levels of PIAS3 mRNA. For OS-P_2 and SP cells, only very low levels of PIAS3 mRNA were detectable, a similar observation that was seen for these two cell lines with SHP1 and SOCS1 mRNA. The Western blot analysis of PIAS3 is shown in Fig. 1 (panel D). As can be seen, there is overexpression of PIAS3 in six of seven (86%) HTLV-1 positive cell lines as compared to the HTLV-1 negative cell lines. Only C10/MJ cells did not express comparably high levels of PIAS3.

3.1.5. Summary of Western blot and mRNA results for the negative regulators

As can be seen in Table 2, there is a reciprocal relationship between low levels of SHP1 and high levels of CIS, SOCS1 and PIAS3 protein expression in the HTLV-1 positive cell lines. The opposite findings are seen with the HTLV-1 negative cell lines which all express high levels of SHP1 and low levels of CIS, SOCS1 and PIAS3. However, there are three exceptions to this generalization: (1) HTLV-1 positive C8166-45 cells express high levels of SHP1 and very low levels of CIS which is more in agreement with the pattern seen in HTLV-1 negative cells although the C8166-45 cells differ from the HTLV-1 negative cells in that they also express high levels of SOCS1 and PIAS3; and (2) C10/MJ cells express very low levels of SHP1, PIAS3 and SOCS1 but high levels of CIS. COS-7 cells also show a different pattern with low to undetectable SHP1 and CIS.

A linear regression analysis was performed to compare and correlate the real-time PCR and Western blot results in Tables 1 and 2. The correlation coefficient for SHP1 mRNA and Western blot expression levels was 0.91 whereas the correlation coefficients for SOCS1 and PIAS3 were low at 0.50 and 0.26, respectively. Two specific differences between the two data sets are notable. HTLV-1 positive C10/MJ cells ranked among the highest of the 12 T-cell leukemia cell lines tested for mRNA levels of SHP1 (2/12), SOCS1 (1/12) and PIAS3 (3/12) yet had very low to undetectable SHP1, SOCS1 or PIAS3 protein on Western blot (Fig. 1, panels A, C and D) suggesting perhaps accelerated proteasome-mediated protein degradation. HTLV-1 positive OS-P_2 and SP cells had amongst the lowest mRNA levels for SOCS1 and PIAS3 (11 and 12/12, and 12 and 11/12), respectively, while the protein expression levels of SOCS1 and PIAS3 were moderately high.

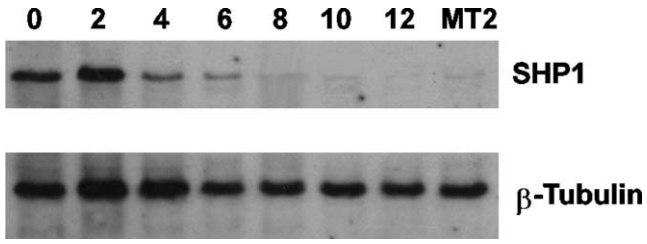
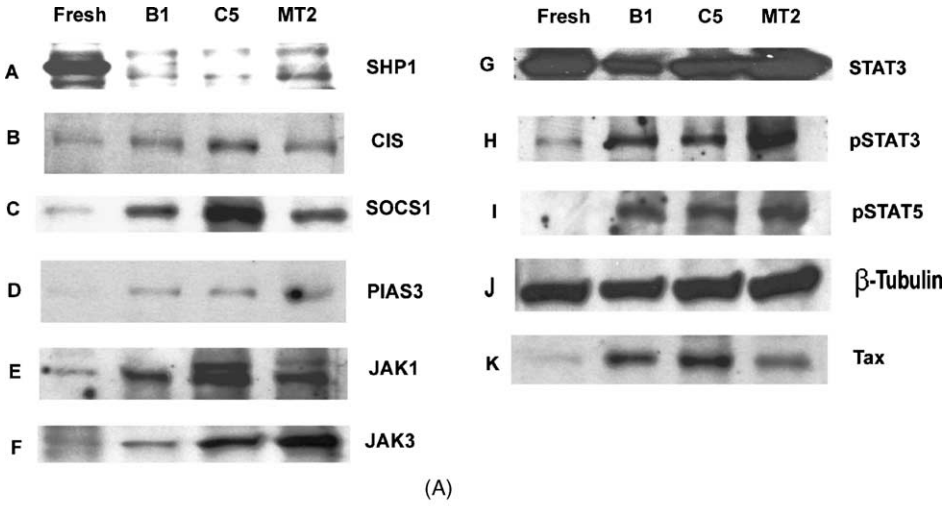


Fig. 2. Western blot analysis of SHP1 expression over time following HTLV-1 infection of $CD4^+$ peripheral blood T-cells. Aliquots of $CD4^+$ cells were collected every 2 weeks and the co-culture was continued for 12 weeks. For Western blots, the cell lysates [$(0.5–1.0) \times 10^6$ cell equivalent] were transferred from a 10% Tris–glycine SDS-polyacrylamide gel to a polyvinylidene difluoride membrane, followed by mouse anti-SHP1 Mab (sc-7289) [1:266] as primary antibody and HRP-labeled goat anti-mouse IgG (sc-2005) [1:500] as secondary antibody or rabbit anti-β-tubulin (sc-9104) [1:1000] and HRP-labeled goat anti-rabbit IgG (sc-2301) as secondary antibody [1:2000] as described in Fig. 1.



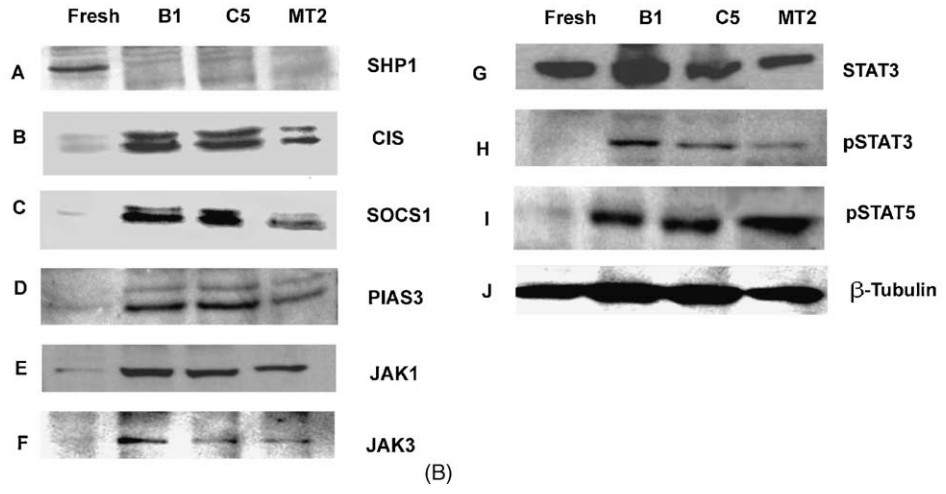


Fig. 3. Expression negative regulators and JAK/STAT proteins in freshly isolated $CD4^+$ T-cells, transformed B1 and C5 clones and MT2 cells. Cells were either unstimulated (A) or stimulated for 72 h with 1 μg/ml PHA followed by washing and stimulation with 200 U/ml IL-2 for 30 min (B). Panel A, SHP1; panel B, CIS; panel C, SOCS1; panel D, PIAS3; panel E, JAK1 (Santa Cruz, sc-7228) [1:1000]; panel F, JAK3 (Santa Cruz, sc-513) [1:1000]; panel G, STAT3; panel H, pSTAT3; panel I, pSTAT5 (Cell Signaling Technology, #9351S) [1:1000]; panel J, β-tubulin (Santa Cruz, sc-9104) [1:1000]; and panel K, rabbit-anti-tax (NIH AIDS Research and Reference Program, cat. #712) [1:1000]. Western blot analysis was performed as described in Fig. 1.

3.1.6. Activation of signal transducer and activator of transcription (STAT) proteins

Phosphorylated STAT proteins are found to be associated with phosphorylated JAK proteins in cells triggered by cytokines, e.g. IL-2 [39], IFN-γ [40], IL-6 [41]. In HTLV-1 transformed cell lines and uncultured leukemic cells from ATL patients, co-immunoprecipation of STAT5 with JAK3 and constitutive activation of STAT proteins has been reported [2,3]. We examined the phosphorylation status of STAT3 in the HTLV-1 transformed cells. As can be seen in Fig. 2 (panels E and F), STAT3 was found to be constitutively activated in six of seven HTLV-1 cell lines with the exception of C10/MJ cells.

3.2. Loss of SHP1 expression over time for in vitro HTLV-1-Infected CD4$^+$ human peripheral blood T-cells

Purified human peripheral blood CD4$^+$ T-cells were infected with HTLV-1 by co-culture with MT2 cells contained with a Millicell-CM Culture Plate Insert. The cells are cultured in AIM-V media with 15% FBS and 100 U/ml interleukin-2. Aliquots of CD4$^+$ cells were collected every 2 weeks and the MT2 cells were replaced with fresh cells when they reached a density of 1×10^6 cell/ml. The co-culture was continued for 12 weeks. Serial frozen samples of the harvested CD4$^+$ T-cells were used to detect the expression of SHP1 by Western blot. As can be seen in Fig. 2, beginning at circa 4–6 weeks post-infection there is a gradual loss of SHP1 over time and by 10 weeks SHP1 protein expression is nearly undetectable. Expression of the control protein, β-tubulin did not change over time. Concominant with this loss of SHP1 expression was the acquisition of IL-2 independence and the ability of the bulk populations of cells to grow continuously in the absence of exogenous IL-2 (data not shown).

At 12 weeks post-infection, two IL-2 independent subclones were established by limiting dilution. FACS analysis demonstrated that both clones were CD4$^+$, CD8$^-$ and CD25$^+$ (data not shown) and Western blot analysis confirmed that both clones expressed HTLV-1 tax protein (Fig. 3A, panel K). An analysis of negative regulator protein expression and activation status of the JAK/STAT pathway was also performed. As shown in Fig. 3 (panel A), in the absence of IL-2 stimulation there is no detectable expression of SHP1 in subclones B1, C5 or in MT2 cells as compared to fresh CD4$^+$ T-cells. Under the same conditions, SOCS1 was markedly overexpressed (panel C) whereas CIS and PIAS3 were also expressed but to a lesser degree (panels B and D). Analysis of the JAK/STAT pathway demonstrated that there was also overexpression of JAK1 and JAK3 in the B1 and C5 subclones and MT2 cells (panels E and F) as well as activation of STAT3 (panel H) and STAT5 (panel I). Moreover, stimulation with PHA/IL-2 did not result in detectable expression of SHP1 in subclones B1, C5 or in MT2 cells as compared to fresh CD4$^+$ T-cells. Noteworthy is that stimulation of PHA activated CD4$^+$ T-cells with IL-2 for 10–15 min results in transient phosphorylation of JAK/STAT molecules whereas if treatment is extended for longer periods of time (e.g. 30 min) as used in our study, this effect is not seen [42–45]. Therefore as shown is Fig. 3B, this 30 min treatment interval with IL-2 can be used to distinguish between normal signal transduction for fresh CD4$^+$ T-cells and the dysregulated signal transduction seen in the HTLV-1 transformed cell lines.

4. Discussion

The results of this study demonstrate that SHP1 expression is uniformly downregulated in HTLV-1 transformed cell lines at both the mRNA and protein level compared to non-HTLV-1 transformed leukemia T-cell lines. In addition, variable patterns of upregulation of downstream inducible (CIS and SOCS1) and constitutive (PIAS3) negative regulators of JAK/STAT signaling were also observed. However, the increase in CIS, SOCS1 and PIAS3 expression was not sufficient to overcome loss of signal regulation since constitutive activation of JAK/STAT signal pathway was observed. In addition, in acutely HTLV-1 infected primary CD4$^+$ T-cells there was a gradual loss of SHP1 expression over 10 weeks in culture which correlated with progression from immortalization to transformation and loss of IL-2 dependence for growth. Whether this gradual loss of SHP1 expression represents the temporal silencing of the SHP1 gene over time or is due to the outgrowth of a small number of HTLV-1 immortalized/transformed cells in the cultures, remains to be determined. Direct cell-to-cell is known to be required for efficient HTLV-1 infection [46] and the efficiency of infection with the transwell infection scheme that was used in our studies was not investigated. Two transformed cell lines that were established following HTLV-1 infection showed loss of SHP1 expression, overexpression of CIS, SOCS1, PIAS3 and evidence of constitutive activation of the JAK/STAT pathway including STAT3 and STAT5. Importantly, stimulation of these cell lines with PHA/IL-2 did not overcome the loss of SHP1 expression thereby providing additional evidence that SHP1 gene silencing had occurred.

The pattern of negative regulator expression seen in the HTLV-1 positive cell lines was not uniform. Five of the HTLV-1 positive cell lines (HUT102, MT2, MT4, OS-P_2 and SP) showed a pattern of low to nearly undetectable SHP1 and overexpression of CIS, SOCS1 and PIAS3 with constitutive activation of STAT3. Overexpression of CIS, SOCS1 and PIAS3 is likely a compensatory result of loss of SHP1 expression which works earlier in the signaling cascade. It is therefore surprising that overexpression of these three downstream negative regulators was not adequate to inhibit STAT3 activation. These observations raise the possibility that other signaling pathways are also deregulated in these transformed cells at a level sufficient to overcome negative regulation and allow STAT3 activation. Within this group, HUT102 and OS-P_2 cells were also notable for having a

truncated form of SHP1 protein, probably resulting from intracellular proteolytic cleavage in its SH2c domain. Whether the truncated SHP1 can function as a dominant-negative mutant by direct substrate binding is not known although JAK2 and EpoR binding activity has been reported to be predominantly associated with the SH2n domain of SHP1 [47].

A different pattern of negative regulators seen in C8166-45 cells which showed high levels of full length and truncated SHP1 and low levels of CIS, a pattern that is similar to HTLV-1 negative cell lines except for the additional presence of truncated SHP1. However, in contrast to HTLV-1 negative cell lines, there is also overexpression of SOCS1, PIAS3 and constitutive activation of STAT3. Therefore for this cell line, upregulation of SOCS1, PIAS3 and constitutive activation of STAT3 occurred in the absence of SHP1 down-regulation. How CIS protein expression could be low in this cell line is puzzling particularly because of the presence of STAT response elements in the promoter region of CIS and the concomitant activation of STAT3 in these cells [48].

C10/MJ cells had high levels of SHP1, SOCS1 and PIAS3 mRNA and very low levels of SHP1, SOCS1 and PIAS3 protein. However, this cell line also had high levels of CIS protein expression and was the only HTLV-1 transformed cell line that had undetectable levels of phosphorylated STAT3. The marked discrepancy between mRNA and protein expression levels of these three negative regulators suggests either a generalized post-transcriptional block and/or accelerated proteasome-mediated protein degradation. The high levels of CIS protein alone could be sufficient to block activation of STAT3.

In this study we observed that the correlation coefficients for SHP1 mRNA and protein expression levels were high whereas the correlation coefficients for SOCS1 and PIAS3 mRNA and protein expression levels were low (Tables 1 and 2). This latter observation was most notable for the OS-P_2 and SP cells. The reasons for these discrepancies may be related, in part, to inhibition of proteasome degradation of SOCS1 and PIAS3 since higher protein expression levels of SOCS1 and PIAS3 were detected than would be expected from the mRNA levels. The deubiquitinating enzyme DUB-2 has been shown to be constitutively upregulated in one HTLV-1 transformed cell line and can prolong CIS expression [8]. Whether DUB-2 or other proteins that inhibit proteasome-mediated degradation are involved in the overexpression of these negative regulatory proteins will require further evaluation.

Loss of SHP1 expression has been recently reported in various other types of T-cell lymphomas including aggressive types of non-Hodgkin's T-cell lymphomas, Hodgkin's lymphoma with a T-cell phenotype and NK/T lymphomas and others [49,50]. These findings suggest that lack of the SHP1 protein expression is frequent in T-cell lymphoma cells regardless of their HTLV-1 status. In order to determine if the decreased expression of SHP1 in the HTLV-1 cell lines was due to mutations in the promoter region of SHP1, the promoter region of MT2 and OS-P_2 cells was subjected to PCR DNA sequencing however, no mutations were found (data not shown). In agreement with these results, the loss of SHP1 expression in cases of T-cell lymphoma has been shown to result from a transcriptional block of the SHP1 gene because of extensive methylation of its promoter and not from mutations in the SHP1 gene-coding, splice-junction or promoter regions [44]. Interestingly, cell lines derived from one patient showed only a mild decrease in expression of SHP1 protein early in disease and a total lack of SHP1 expression at a more advanced stage of the T-cell lymphoma. This finding suggests that loss of SHP1 protein expression may be progressive and lymphoma-stage dependent and that an epigenetic mechanism may be involved [49,50]. Oka et al. [50] reported findings showing that the more aggressive malignancies were completely negative for SHP1 expression suggesting that there is a correlation between loss of SHP1 expression and aggressiveness.

Current evidence supports the view that SHP1 is a member of a growing list of candidate tumor-suppressor genes that are silenced in certain cancers by promoter hypermethylation [49,51]. Although the mechanism(s) of methyation-mediated gene inactivation are incompletely understood, much progress has been made in elucidating the molecular mechanisms that underlie the interplay between DNA methylation and histone acetylation in gene silencing [52,53]. Proteins that bind to methyl-CpG (MeCP1 and MeCP2) and also associate with various chromatin-remodeling complexes have been shown to act as transcriptional repressors in vitro [54–57]. Recent identification of the family of DNA methyltransferases capable of de novo methlyation and CpG demethylase indicates that an imbalance between these two types of enzymes with the opposite function may play a role in carcinogenesis [58–61]. How this imbalance occurs in HTLV-1 transformed cells and in other T-cell and NK/T-cell and Hodgkin's lymphoma's to effect SHP1 expression and whether restoration of SHP1 expression can reverse these biochemical and phenotypic changes that are associated with this transformation will be an important area of future investigation. The recent report that treatment of HTLV-1 transformed cell lines with an inhibitor of JAK3/STAT5 signaling failed to inhibit cell proliferation suggests that this pathway has become functionally redundant in these transformed cells [62] and that any effect of SHP1 replacement on cell proliferation may be mediated through other signaling pathways that are also coupled to SHP1 expression.

In summary, these results demonstrate that loss of SHP1 expression is a central feature of HTLV-1 transformed cells lines. Upregulation of two inducible (CIS and SOCS1) and a constitutive (PIAS3) negative regulator with variable patterns and constitutive activation of JAK/STAT signal pathway was also observed. In acutely HTLV-1 infected primary $CD4^+$ T-cells there was a gradual loss of SHP1 expression over 10 weeks in culture which correlated with progression from immortalization to transformation and loss of IL-2

dependence for growth. Concominant with loss of SHP1 expression was overexpression of CIS, SOCS1, PIAS3 and evidence of constitutive activation of the JAK/STAT. However, a direct causal relationship between loss of SHP1 expression, upregulation of downstream negative regulators and constitutive activation of the JAK/STAT signaling pathway has not been established in our studies. Interestingly, several studies that have examined gene expression profiles in HTLV-1-infected and immortalized or transformed cell lines or in Jurkat cells induced to express HTLV-1 Tax did not report on the transcription profiles of these four negative regulators of JAK/STAT signaling pathway [63–66]. Thus, although in vitro infection of $CD4^+$ T-cells with HTLV-1 may provide an ideal model system to apply a systems biology approach to examine the entire transcriptome of the infected cells during the progression from cell activation to immortalization to transformation, a technical approach that does not rely on the analysis of a finite set of genes but that can interrogate all expressed genes is required [67,68]. In this way, the biochemical events that surround loss of SHP1 expression could be examined in their entirety.

Acknowledgements

This work was supported by the National Foundation for Cancer Research (W.A.M.), and by a joint Dana-Farber Cancer Institute-Beth-Israel Deaconess Medical Center and Children's Hospital Center for AIDS Research (CFAR) Grant. The authors also wish to acknowledge the NIH AIDS Research and Reference Reagent Program for their invaluable supply of cell lines and other materials for this project.

References

[1] Yamano Y, Nagai M, Brennan M, Mora CA, Soldan SS, Tomaru U, et al. Correlation of human T-cell lymphotropic virus type 1 (HTLV-1) mRNA with proviral DNA load, virus-specific CD8(+) T cells, and disease severity in HTLV-1-associated myelopathy (HAM/TSP). Blood 2002;99:88–94.

[2] Migone TS, Lin JX, Cereseto A, Mulloy JC, O'Shea JJ, Franchini G, et al. Constitutively activated Jak-STAT pathway in T cells transformed with HTLV-1. Science 1995;269:79–81.

[3] Takemoto S, Mulloy JC, Cereseto A, Migone TS, Patel BK, Matsuoka M, et al. Proliferation of adult T cell leukemia/lymphoma cells is associated with the constitutive activation of JAK/STAT proteins. Proc Natl Acad Sci USA 1997;94:13897–902.

[4] Xu X, Kang SH, Heidenreich O, Okerholm M, O'Shea JJ, Nerenberg MI. Constitutive activation of different Jak tyrosine kinases in human T cell leukemia virus type 1 (HTLV-1) tax protein or virus-transformed cells. J Clin Invest 1995;96:1548–55.

[5] Lin JX, Leonard WJ. Signaling from the IL-2-receptor to the nucleus. Cytokine Growth Factor Rev 1997;8:313–22.

[6] Neel BG. Role of phosphotases in lymphocyte activation. Curr Opin Immunol 1997;9:405–20.

[7] Aman MJ, Leonard WJ. Cytokine signaling: cytokine-inducible signaling inhibitors. Curr Biol 1997;7:784–8.

[8] Migone T-S, Humbert M, Rascle A, Sanden D, D'Andrea A, Johnston JA. The deubiquitinating enzyme DUB-2 prolongs cytokine-induced signal transducers and activators of transcription activation and suppresses apoptosis following cytokine withdrawal. Blood 2001;98:1935–41.

[9] Damen JE, Liu L, Rosten P, Humphries RK, Jefferson AB, Majerus PW, et al. The 145-kDa protein induced to associate with Shc by multiple cytokines is an inositol tetraphosphate and phosphatidylinositol 3,4,5-triphosphate 5-phosphatase. Proc Natl Acad Sci USA 1996;93:1689–93.

[10] Yoshimura A, Ohkubo T, Kiguchi T, Jenkins NA, Gilber DJ, Copeland NG, et al. A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoietin receptors. EMBO J 1995;14:2816–26.

[11] Starr R, Willson TA, Viney EM, Murray LJ, Rayner JR, Jenkins BJ, et al. A family of cytokine-inducible inhibitors of signaling. Nature 1997;66:917–24.

[12] Endo TA, Masuhara M, Yokouchi M, Suzuki R, Sakamoto H, Mitsui K, et al. A new protein containing an SH2 domain that inhibits JAK kinases. Nature 1997;387:921–4.

[13] Naka T, Narazaki M, Hirata M, Matsumoto T, Minamoto S, Aono A, et al. Structure and function of a new STAT-induced STAT inhibitor. Nature 1997;387:924–9.

[14] Hilton DJ, Richardson RT, Alexander WS, Viney EM, Willson TA, Sprigg NS, et al. Twenty proteins containing a C-terminal SOCS box form five structural classes. Proc Natl Acad Sci USA 1998;95:114–9.

[15] Chung CD, Liao J, Liu B, Rao X, Jay P, Berta P, et al. Specific inhibition of STAT3 signal transduction by PIAS3. Science 1997;278:1803–5.

[16] Liu B, Liao J, Rao X, Kushner SA, Chung CD, Chang DD. Inhibition of STAT1-mediated gene activation by PIAS3. Proc Natl Acad Sci USA 1998;95:10626–31.

[17] Starr R, Hilton DJ. Negative regulation of the JAK/STAT pathway. BioEssays 1999;21:47–52.

[18] Kile BT, Nicola NA, Alexander WS. Negative regulators of cytokine signaling. Int J Hematol 2001;73:292–8.

[19] Migone TS, Cacalano NA, Taylor N, Yi T, Waldmann TA, Johnston JA. Recruitment of SH2-containing protein tyrosine phosphatase SHP-1 to the interleukin 2 receptor; loss of SHP-1 expression in human T-lymphotropic virus type I-transformed T cells. Proc Natl Acad Sci USA 1998;95:3845–50.

[20] Aman MJ, Migone T-S, Sasaki A, Ascherman DP, Zhu M-h, Soldaini E, et al. CIS associates with the interleukin-2 receptor β chain and inhibits interleukin-2-dependent signaling. J Biol Chem 1999;274:30266–72.

[21] Sporri B, Kovanen PE, Sasaki A, Yoshimura A, Leonard WJ. JAB/SOCS1/SSI-1 is an interleukin-2-induced inhibitor of IL-2 signaling. Blood 2001;97:221–6.

[22] Cohney SJ, Sanden D, Cacalano NA, Yoshimura A, Mui A, Migone TS, et al. SOCS-3 is tyrosine phosphorylated in response to interleukin-2 and suppresses STAT5 phosphorylation and lymphocyte proliferation. Mol Cell Biol 1999;19:4980–8.

[23] Zhang Q, Lee B, Korecka M, Li G, Weyland C, Eck S, et al. Differences in phosphorylation of the IL-2R associated JAK/STAT proteins between HTLV-I(+), IL-2-independent and IL-2-dependent cell lines and uncultured leukemic cells from patients with adult T-cell lymphoma/leukemia. Leuk Res 1999;23:373–84.

[24] Gazdar AF, Carney DN, Bunn PA, Russell EK, Jaffe ES, Schechter GP, et al. Mitogen requirements for the in vitro propagation of cutaneous T-cell lymphomas. Blood 1980;55:409–17.

[25] Smith SD, Shatsky M, Cohen PS, Warnke R, Link MP, Glader BE. Monoclonal antibody and enzymatic profiles of human malignant T-lymphoid cells and derived cell lines. Cancer Res 1984;44:5657–60.

[26] Daniel MD, Li Y, Naidu YM, Durda PJ, Schmidt DK, Troup CD, et al. Simian immunodeficiency virus from African green monkeys. J Virol 1988;62:4123–8.

[27] Foley GE, Lazarus H, Farber S, Uzman BG, Boone BA, McCarthy RE. Continuous culture of human lymphoblasts from peripheral blood of a child with acute leukemia. Cancer 1965;18:522–9.
[28] Weiss A, Wiskocil RL, Stobo JD. The role of T3 surface molecules in the activation of human T cells: a two-stimulus requirement for IL 2 production reflects events occurring at a pre-translational level. J Immunol 1984;133:123–8.
[29] Gluzman Y. SV40-transformed simian cells support the replication of early SV40 mutants. Cell 1981;23:175–82.
[30] Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. Proc Natl Acad Sci USA 1980;77:7415–9.
[31] Harada S, Koyanagi Y, Yamamoto N. Infection of HTLV-III/LAV in HTLV-I-carrying cells MT-2 and MT-4 and application in a plaque assay. Science 1985;229:563–6.
[32] Larder BA, Darby G, Richman DD. HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. Science 1989;243:1731–4.
[33] Salahuddin SZ, Markham PD, Wong-Staal F, Franchini G, Kalyanaraman VS, Gallo RC. Restricted expression of human T-cell leukemia—lymphoma virus (HTLV) in transformed human umbilical cord blood lymphocytes. Virology 1983;129:51–64.
[34] McKendall RR, Oas J, Lairmore MD. HTLV-I-associated myelopathy endemic in Texas-born residents and isolation of virus from CSF cells. Neurology 1991;41:831–6.
[35] Popovic M, Lange-Wantzin G, Sarin PS, Mann D, Gallo RC. Transformation of human umbilical cord blood T cells by human T-cell leukemia/lymphoma virus. Proc Natl Acad Sci USA 1983;80: 5402–6.
[36] Rowe T, Dezzutti C, Guenthner PC, Lam L, Hodge T, Lairmore MD, et al. Characterization of a HTLV-I-infected cell line derived from a patient with adult T-cell leukemia with stable co-expression of CD4 and CD8. Leuk Res 1995;19:621–8.
[37] Haertle T, Carrera CJ, Wasson DB, Sowers LC, Richman DD, Carson DA. Metabolism and anti-human immunodefficiency virus-1 activity of 2-halo-2′,3′-dideoxyadenosine derivatives. J Biol Chem 1988;263:5870–5.
[38] Pauwels R, De Clercq E, Desmyter J, Balzarini J, Goubau P, Herdewijn P, et al. Sensitive and rapid assay on MT-4 cells for detection of antiviral compounds against the AIDS virus. J Virol Methods 1987;16:171–85.
[39] Leonard WJ, O'Shea JJ. JAKs and STATs: biological implications. Annu Rev Immunol 1998;16:293–322.
[40] Schindler C, Darnell JE. Trascriptional responses to polypeptide ligands: the JAK-STAT pathway. Annu Rev Biochem 1995;64: 621–51.
[41] Darnell Jr JE. Reflections on STAT3, STAT5 and STAT6 as fat STATs. Proc Natl Acad Sci USA 1996;93:6221–4.
[42] Matikainen S, Sareneva T, Ronni T, Lehtonen A, Koskinen PJ, Julkunen I. Interferon-alpha activated multiple STAT proteins and upregulates proliferation-associated IL-2R alpha, c-myc and pim-1 genes in human T cells. Blood 1999;93:1980–91.
[43] Brunn GJ, Falls EL, Nilson AE, Abraham RT. Protein-tyrosine kinase-dependent activation of STAT transcription factors in interleukin-2 or interleukin-4 stimulated T lymphocytes. J Biol Chem 1995;270:11628–35.
[44] Kirken RA, Malabarba MG, Xu J, DaSilva L, Erwin RA, Liu S, et al. Two discrete regions of interleukin-2 (IL-2) receptor beta independently mediate IL-2 activation of a PD98059/rapamycin.wortmannin-insensitive Stat5a/b serine kinase. J Biol Chem 1997;272:15459–65.
[45] Frank DA, Robertson MJ, Bonni A, Ritz J, Greenberg ME. Interleukin-2 signaling involves the phosphorylation of Stat proteins. Proc Natl Acad Sci USA 1995;92:7779–83.
[46] Igakura T, Stinchcombe JC, Goon PKC, Taylor GP, Weber JN, Griffiiiths GM, et al. Spread of HTLV-1 between lymphocytes by virus-induced polarization of the cytoskeleton. Science 2003;299:1713–6.
[47] Jiao H, Berrada K, Yang W, Tabrizi M, Platanias LC, Yi T. Direct association with and dephosphorylation of JAK2 kinase by the SH2-domain containing protein tyrosine phosphatase SHP-1. Mol Cell Biol 1996;16:6985–92.
[48] Matsumoto A, Masuhara M, Mitsui K, Yakouchi M, Ohtsubo M, Missawa H, et al. CIS, a cytokine inducible SH2 protein, is a target of the JAK-STAT5 pathway and modulates STAT5 activation. Blood 1997;89:3148–54.
[49] Zhang Q, Raghunath PN, Vonderheid E, Odum N, Wasik MA. Lack of phosphotyrosine phosphatase SHP-1 expression in malignant T-cell lymphoma cells results from methylation of the SHP-1 promoter. Am J Pathol 2000;157:1137–46.
[50] Oka T, Yoshino T, Hayashi K, Ohara N, Oka T, Jin ZS, et al. Reduction of hematopoietic cell-specific tyrosine phosphatase SHP-1 gene expression in natural killer cell lymphoma and various types of lymphomas/leukemias: combination analysis with cDNA expression array and tissue microarray. Am J Pathol 2001;159: 1495–505.
[51] Wu C, Sun M, Liu L, Zhou GW. The function of the protein tyrosine phosphatase SHP-1 in cancer. Gene 2003;306:1–12.
[52] Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev: Gen 2002;3:415–28.
[53] Jenuwein T, Allis CD. Translating the histone code. Science 2001;293:1074–80.
[54] Bird AP, Wolfe AP. Methylation-induced repression—belts, braces, and chromatin. Cell 1999;99:451–4.
[55] Jones PL, Veenstra GJ, Wade PA, Vermaak D, Kass SU, Landsberger N, et al. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat Genet 1998;19:187–91.
[56] Ng HH, Zhang Y, Hendrich B, Johnson CA, Turner BM, Erdjument-Bromage H, et al. MBD2 is a transcriptional repressor belonging to the MeCP21 histone deacetylase complex. Nat Genet 1999;23:58–61.
[57] Wade PA, Gegonne A, Jones PL, Ballestar E, Aubry F, Wolffe AP. Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. Nat Genet 1999;23:62–6.
[58] Okano M, Xie S, Li E. Dnmt2 is not required for de novo and maintenance methylation of viral DNA in embryonic stem cells. Nucleic Acids Res 1998;26:2536–40.
[59] Bhattacharya SK, Ramchandani S, Cervoni N, Szyf M. A mammalian protein with specific demethylase activity for mCpG DNA. Nature 1999;397:579–83.
[60] Robertson KD, Uzvolgyi E, Liang G, Talmadge C, Sumegi J, Gonzales FA, et al. The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. Nucleic Acids Res 1999;27:2291–8.
[61] De Marzo AM, Marchi VL, Yang ES, Veeraswamy R, Lin X, Nelson WG. Abnormal regulation of DNA methyltransferase expression during colorectal carcinogenesis. Cancer Res 1999;59:3855–60.
[62] Kirken RA, Erwin RA, Wang L, Wang Y, Rui H, Farrar WL. Functional uncoupling of the Janus Kinase 3-Stat5 pathway in malignant growth of human T cell leukemia virus type 1-transformed human T cells. J Immunol 2000;165:5097–104.
[63] Harhaj EW, Good L, Xiao G, Sun SC. Gene expression profiles in HTLV-I-immortalized T cells; deregulated expression of genes involved in apoptosis regulation. Oncogene 1999;18:1341–9.
[64] De La Fuente C, Deng L, Santiago F, Arce L, Wang L, Kashanchi F. Gene expression array of HTLV type 1-infected T cells: up-regulation of transcription factors and cell cycle genes. AIDS Res Hum Retroviruses 2000;16:1695–700.
[65] Ng PW, Iha H, Iwanaga Y, Bittner M, Chen Y, Jiang Y, et al. Genome-wide expression changes induced by HTLV-1 tax: evidence for MLK-3 mixed lineage kinase involvement in Tax-mediated NF-κB activation. Oncogene 2001;20:4484–96.

[66] Pise-Masison CA, Radonovich M, Mahieux R, Chatterjee P, Whiteford C, Duvall J, et al. Transcription profile of cells infected with human T-cell leukemia virus type I compared with activated lymphocytes. Cancer Res 2002;62:3562–71.
[67] Brenner S, Johnson M, Bridgham J, Golda G, Lloyd D, Johnson D, et al. Gene expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays. Nat Biotech 2000;18: 630–4.
[68] Brenner S, Williams S, Vermass EH, Storck T, Moon K, McCollum C, et al. In vitro cloning of complex mixtures of DNA on microbeads: physical separation of differentially expressed cDNAs. Proc Natl Acad Sci USA 2000;97:1665–70.