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Inducible Knockout of the Interleukin-2 Receptor α Chain: Expression of the High-Affinity IL-2 Receptor Is Not Required for the *in Vitro* Growth of HTLV-I-Transformed Cell Lines

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Adult T cell leukemia (ATL) is an aggressive malignancy that is associated with HTLV-I infection and characterized by constitutive expression of the high-affinity interleukin-2 receptor. The α subunit of the high-affinity receptor (IL-2Rα), which is normally present only on activated T cells, is specifically upregulated by HTLV-I and constitutively expressed on fresh leukemic cells from ATL patients as well as cell lines transformed by HTLV-I *in vitro.* Here we directly address the functional significance of IL-2Rα expression in HTLV-I transformed cell lines by using an endoplasmic reticulum-targeted single-chain antibody to inhibit the cell surface expression of IL-2Rα. Using constitutive and tetracycline-repressible systems to express the ER-targeted antibody against IL-2Rα, we have reduced cell surface expression of IL-2Rα by more that 2 logs of mean fluorescence intensity to virtually undetectable levels in the IL-2-independent HTLV-I-transformed cell lines C8166-45 and HUT102. No toxicity was associated with the intracellular retention of IL-2Rα, and the growth rate of the IL-2Rα-negative cells was in each case comparable to that of the parental cell line. We conclude that cell surface expression of IL-2Rα is dispensable for the *in vitro* growth of these HTLV-I-transformed cells.

Key Words: Adult T cell leukemia; Tac; single-chain antibody; intracellular antibody; intrabody; cytokine receptors.

INTRODUCTION

Adult T cell leukemia (ATL) is an aggressive and rapidly fatal T cell malignancy associated with HTLV-I infection (Uchiyama *et al.,* 1977). About 1% of virus carriers develop the disease and there is currently no effective treatment. The molecular basis of T cell transformation by HTLV-I is poorly understood but does not appear to involve any of the classical oncogenic mechanisms (oncogene transduction, insertional mutation, or *cis*-activation of cellular oncogenes) that are employed by murine and avian retroviruses (Cann *et al.,* 1990). The Tax protein of HTLV-I is thought to play a role in the early stages of leukemogenesis, as it activates a variety of genes involved in cellular growth (Gitlin and Shimotohno, 1993; Franchini, 1995) and is the only viral gene required to immortalize primary T cells *in vitro* (Akagi *et al.,* 1993; Grassman *et al.,* 1992). However, the expression of *tax* and other viral genes is generally undetectable in freshly isolated leukemic (ATL) cells, suggesting that growth of the end-stage leukemic cell is driven by an alternative, *tax*-independent, mechanism (Franchini *et al.,* 1984; Tendler *et al.,* 1990).

One of the phenotypic hallmarks of leukemic (ATL) cells and T cells immortalized by HTLV-I *in vitro* is the constitutive upregulation of IL-2Rα, the α subunit of the high-affinity interleukin-2 receptor (Hattori *et al.,* 1981; Popovic *et al.,* 1983; Waldmann *et al.,* 1984). The high affinity IL-2 receptor (K_d 10^{-11} *M*) is a trimer composed of α, β, and γ chains (Minami *et al.,* 1993). The β and γ chains (IL-2Rβ and γ_c), which are critical for IL-2 signal transduction, are present on both resting and activated T cells and together form a functional receptor with lower affinity for IL-2 ($K_d 10^{-9}$ *M*). In contrast, the IL-2Rα chain — and hence the high-affinity receptor — is expressed only transiently following antigenic stimulation of the cell (Smith and Cantrell, 1985). The constitutive expression of IL-2Rα by HTLV-I-transformed cells has led to the hypothesis that constitutive signaling by the IL-2 receptor may be a key factor in the growth of HTLV-I-transformed cells. A simple autocrine IL-2 mechanism can be ruled out in most cases, as a majority of fresh ATL cells and many HTLV-I-immortalized cell lines neither produce nor respond to IL-2 (Tendler *et al.,* 1990; Arya *et al.,* 1984; Kodaka *et al.,* 1989; Uchiyama *et al.,* 1985; Maeda *et al.,* 1985). An alternative hypothesis of ligand (IL-2)-independent signaling by an aberrant or constitutively activated IL-2 receptor has therefore been proposed (Yodoi *et al.,* 1983). While formally untested, indirect support for this model has come from reports documenting the constitutive phosphorylation of IL-2Rβ in HUT102 cells, and constitutive activation of the IL-2Rγ-associated tyrosine kinase Jak3 in several IL-2-independent HTLV-I-trans-

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formed cell lines (Sharon *et al.,* 1989; Migone *et al.,* 1995; Xu *et al.,* 1995).

A direct way to investigate the significance of IL-2Rα upregulation in HTLV-I-transformed cells would be to examine the fate of cells in which the expression of this receptor subunit has been abolished. This could be achieved by homozygous mutation of the IL-2Rα genes or by using antisense RNA to prevent translation of the IL-2Rα mRNA. An alternative strategy employed here involves the use of intracellular antibodies (termed "intrabodies") which are synthesized by the cell and targeted to the endoplasmic reticulum (ER). ER-targeted intrabodies have proved to be highly effective at capturing specific proteins as they enter the ER and preventing their transport to the cell surface (Richardson and Marasco, 1995). When combined with an inducible expression system, ER-targeted intrabodies represent a powerful and reversible way to downregulate plasma membrane or secreted proteins. We have previously described a single-chain antibody (sFvTac) directed against the external domain of IL-2Rα and shown that intracellular expression of this intrabody leads to complete inhibition of cell surface IL-2Rα expression in stimulated Jurkat cells (Richardson *et al.,* 1995). Here we have used the sFvTac intrabody to study the consequences of IL-2Rα downregulation in HTLV-I-transformed cells.

METHODS

Cell culture

The cell lines PE501 and PA317 were grown in Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum and antibiotics and maintained under HAT selection. The T cell lines Jurkat, Kit225, C8166-45, and HUT102 (Hori *et al.,* 1987; Salahuddin *et al.,* 1983; Miyoshi *et al.,* 1981) were grown in RPMI-1640 with 10% fetal bovine serum and antibiotics. Kit225 medium was supplemented with 100 units/ml recombinant human interleukin-7. Tetracycline hydrochloride was added at 1 μg/ml for suppression of tTA activity.

Plasmid constructions

To construct LNITA, the tTA gene was PCR amplified from pUHD15-1 (Grossen and Bujard, 1992) and cloned downstream of the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) in pCITE, purchased from Novagen (Madison, WI). The nucleotides surrounding the tTA initiation codon (underlined) were modified to create an *Nco*I site (ATATGTCT → CCATGGCT), which allowed precise positioning of the tTA initiation codon at the natural EMCV polyprotein start site. The changes result in a serine to alanine substitution at position two. The IRES and tTA gene were PCR-amplified from pCITE and inserted into the murine retroviral vector LNCX as an *Xho*I–*Bam*HI fragment, replacing the CMV promoter. To construct the 10-3Tac vector, the sFvTac gene was cloned into the pUHD10-3 (Grossen and Bujard, 1992) vector as a *Hin*dIII–*Xba*I fragment. A hygromycin selectable marker from the vector p3′SS (Stratagene, La Jolla, CA) was inserted into the vector at a second *Hin*dIII site. sFvTac is a single-chain antibody derivative of the anti-Tac monoclonal antibody which binds to the extracellular domain of IL-2Rα (Uchiyama *et al.,* 1981). The version of sFvTac used here has a C-terminal ER retention signal (amino acids K-D-E-L) and was designated sFvTacKDEL in a previous publication (Richardson *et al.,* 1995).

Construction of stably transfected cell lines

Generation of producer cell lines. The retroviral vector LNITA was introduced into the ecotropic packaging cell line PE501 by calcium phosphate transfection. Forty-eight hours later, supernatant from these cells was used to infect PA317 cells in the presence of 8 μg/ml polybrene. G418 selection was applied at 600 μg/ml to the PA317 cells 24 hr postinfection for the selection of stable cell lines.

Generation of tTA-expressing T cell lines. A 0.45-μm filtered supernatant from bulk or cloned LNITA producer lines was used to infect 1×10^6 target cells. G418 selection was applied 24 hr later at 800 μg/ml. Two weeks later, single-cell clones were obtained by seeding the G418-resistant cells at 0.3 cell per well of a 96-well plate in the presence of 25% conditioned medium. G418 selection and subsequent cloning were performed in the absence of tetracycline to ensure the tTA-tolerance of selected cells. Individual clones were screened for tTA activity by transient transfection with a phCMV*-1CAT reporter plasmid. Transfected cells were cultured for 72 hr in the presence or absence of tetracycline and then harvested and analyzed for CAT activity. Clones demonstrating a low baseline transcription from the phCMV*-1 promoter and high inducibility on withdrawal of tetracycline were used for further studies.

Introduction of 10-3Tac into stable tTA cell lines. The 10-3Tac vector was linearized at an *Xmn*I site within the β-lactamase gene and introduced into T cell lines by electroporation. Hygromycin selection was applied after 48 hr at 250 to 400 μg/ml and resistant cells were cloned as described above. Both selection and cloning were performed in the presence of tetracycline.

Introduction of the sFvTac gene using a bicistronic HIV-1 based retroviral vector. Constitutive, high level expression of the sFvTac gene was achieved by stably transducing cell lines with an HIV-1 vector, in which the selectable marker and gene of interest are translated from the same mRNA by means of an internal ribosome entry site (submitted). Vector particles were produced in

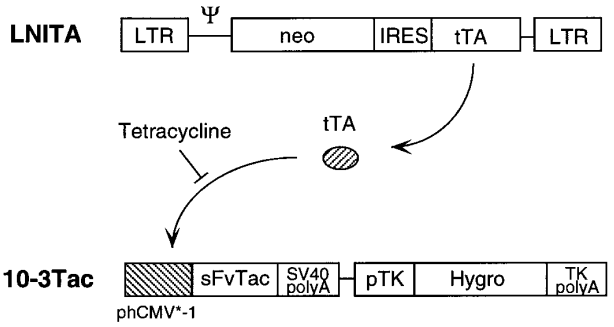

FIG. 1. LNITA and 10-3Tac vectors used to achieve tetracycline-repressible expression of the sFvTac gene.

COS-1 cells as described elsewhere (Richardson and Lever, 1995) and vector-containing cells were selected in the presence of puromycin (0.5 μg/ml).

Flow cytometry

Cells were stained indirectly with anti-Tac or 7G7/B6 monoclonal antibodies followed by FITC-conjugated goat anti-mouse IgG and analyzed by flow cytometry.

Metabolic labeling of cells and immunoprecipitation

For metabolic labeling, 3×10^6 cells were washed with PBS and incubated for 6 hr with or without tetracycline in 3 ml cysteine-free RPMI 1640 containing 10% FBS and 100 μCi [^{35}S]cysteine. Cells were lysed in RIPA buffer and cleared lysates incubated with mAb 7G7/B6 coupled to protein A–Sepharose beads for immunoprecipitation of IL-2Rα. Precipitation of sFvTac was then performed using polyclonal rabbit anti-mouse IgG from Sigma (St. Louis, MO) bound to protein A–Sepharose beads. Samples were washed five times with lysis buffer then run on a 10% SDS–PAGE gel which was fixed and treated with Enhance from DuPont-NEN (Boston, MA) prior to autoradiography.

Tritiated-thymidine incorporation assay

Kit225 cells were washed three times with PBS then incubated in cytokine-free medium for 96 hr in the presence or absence of tetracycline. The cytokine-starved cells were then washed again and plated in triplicate (with or without tetracycline) at 1×10^4 cells per well of a 96-well plate. IL-2 or IL-7 were added at doses ranging from 0.1 to 1000 units/ml and 48 hr later, wells were pulsed with 1 μCi [^{3}H]thymidine. Cells were harvested 18 hr later and [^{3}H]thymidine incorporation was measured by liquid scintillation counting.

RESULTS

Construction of cell lines expressing a tetracycline-repressible transcriptional activator (tTA)

As a first step toward achieving inducible expression of the sFvTac gene, the hybrid transactivator, tTA, was stably introduced into several T cell lines using the murine retroviral vector LNITA (Fig 1). The tTA protein consists of the DNA-binding domain of the *Escherichia coli* tet repressor fused to the activation domain of the herpes simplex virus transactivator VP16 and activates transcription from a minimal promoter (designated phCMV*-1), which contains multiple binding sites for the tet repressor (Grossen and Bujard, 1992). The tTA protein is unable to interact with the promoter in the presence of tetracycline; hence, transcriptional activation can only occur in the absence of tetracycline. tTA-expressing clones displaying a normal morphology and growth rate were identified as described under Materials and Methods.

Tetracycline-repressible knockout of IL-2Rα in the T cell line Kit225

The ability of the tetracycline-repressible system to downregulate a highly abundant receptor was first established using the cell line Kit 225 (Hori *et al.,* 1987). Kit225 is a cytokine-dependent, HTLV-I-negative cell line derived from a chronic T cell leukemia, which expresses high levels of IL-2Rα and can be maintained using either IL-2 or IL-7. Kit225 cells doubly transfected with the LNITA and 10-3Tac vectors showed a reduction in cell surface expression of IL-2Rα by more than 2 logs of mean fluorescence intensity when tetracycline was removed (Fig 2). Immunoprecipitation studies showed full induction of sFvTac expression within 24 hr of tetracycline withdrawal. No new IL-2Rα was expressed at the cell surface from this time, as judged by the absence of the mature p55 in cell lysates (Fig 2a). Instead, the cells accumulated a 40-kDa molecule (p40) which we have previously shown to be an endoglycosidase-H sensitive precursor of IL-2Rα trapped in a pre-Golgi compartment (Richardson *et al.,* 1995). As the 7G7/B6 mAb used for immunoprecipitation of IL-2Rα does not recognize the same epitope as anti-Tac, the sFvTac intrabody can be seen coprecipitating with p40 (lanes 8–12).

Despite the rapid shut off in IL-2Rα maturation and transport, significant levels of IL-2Rα persisted at the cell surface for up to 72 hr following tetracycline withdrawal (Fig 2b). This probably reflects the long half-life of IL-2Rα molecules already present at the time of sFvTac induction, as previous studies have shown IL-2Rα to be recycled to the cell surface following endocytosis, resulting in a half-life of greater than 24 hr (Hemar *et al.,* 1995). The cell surface expression of IL-2Rβ and γ_c on the IL-2Rα-negative cells was confirmed by FACS analysis (data not shown).

Downregulation of IL-2Rα is accompanied by loss of IL-2 responsiveness

As IL-2Rβ and γ_c expression is preserved on the IL-2Rα-negative Kit225 cells maintained in the absence of

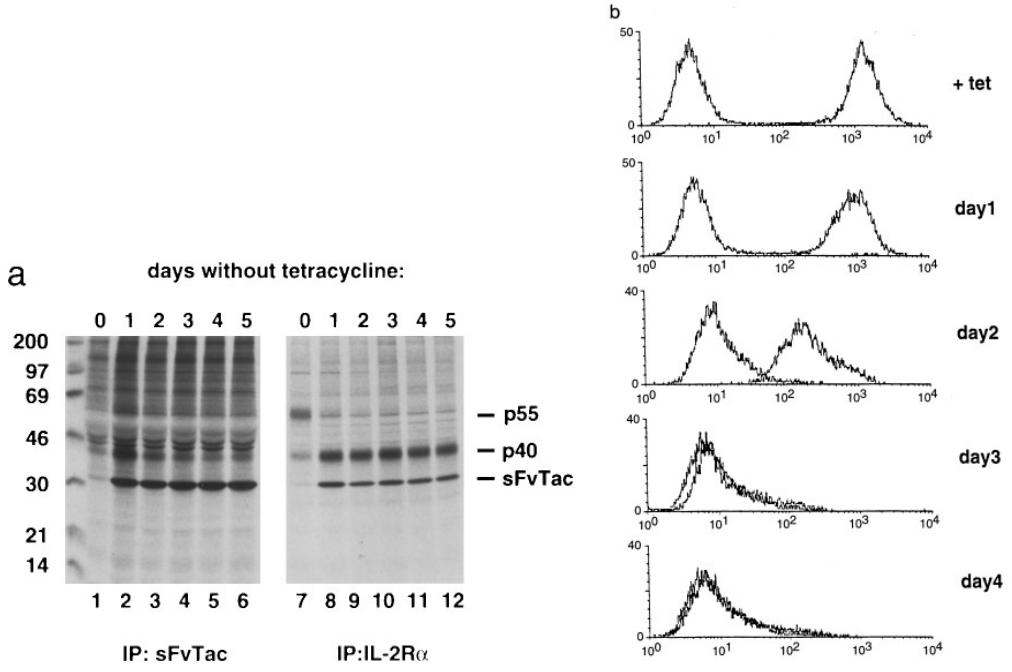

FIG. 2. Tetracycline-repressible expression of sFvTac in Kit225 cells. (a) Immunoprecipitation of IL-2Rα and sFvTac from cells grown in the presence (lanes 1, 7) or absence (lanes 2–6, 8–12) of tetracycline for 1–5 days. IL-2Rα was precipitated with mAb 7G7/B6 and sFvTac with rabbit anti-mouse IgG. (b) Flow cytometric analysis of Kit225 cells stained with anti-Tac (right curve) or with secondary antibody alone (left curve). Cells were analyzed 1–4 days after tetracycline withdrawal, as indicated. Top panel shows cells maintained in the presence tetracycline.

tetracycline, these cells would be expected to express intermediate-affinity (β/γ) IL-2 receptors but not the high-affinity ($\alpha\beta\gamma$) receptor. A [^{3}H]thymidine incorporation assay was used to evaluate the IL-2-responsiveness of Kit225 cells grown in the presence or absence of tetracycline. As shown in Fig. 3, between 10^2- and 10^3-fold higher concentrations of IL-2 were required to generate an equivalent proliferative response in IL-2Rα-negative cells, whereas the response of these cells to IL-7 was not affected. This is consistent with the higher IL-2 concentration required to signal through the β/γ receptor and indicates that the ER-retention of IL-2Rα molecules does not interfere with the cell surface transport or function of IL-2Rβ and γ_c. The IL-2 responsiveness of Kit225 cells transfected with the LNITA vector alone was unaffected by the presence or absence of tetracycline, confirming that expression of the tTA gene does not affect IL-2R signaling (data not shown).

Downregulation of IL-2Rα in HTLV-I transformed cell lines

To examine the consequences of IL-2Rα downregulation in HTLV-I-transformed cells, the sFvTac gene was stably introduced into the cell lines C8166-45 and HUT102 under the control of tetracycline repressible or constitutive promoters. For constitutive expression, a HIV-1-based retroviral vector (HVTac) was used which we have shown to provide high level expression of sFvTac in >90% of transduced cells (submitted). The HTLV-I-negative Jurkat cell line, in which IL-2Rα expression can be induced by stimulation with PHA and PMA, was also transduced with the HVTac vector. Flow cytometric analysis of the transduced cells after staining with anti-Tac monoclonal antibody indicated that IL-2Rα surface expression intensity could be reduced by more than 2 logs of mean fluorescence intensity to a level undetectable by flow cytometric analysis in both C8166-45 and HUT102 cells as well as the control Jurkat cells (Fig. 4). Use of the tetracycline-repressible system in fact proved unnecessary as both HTLV-I-infected cell lines were found to tolerate long-term, constitutive downregulation of IL-2Rα without any adverse effect on cell growth. The doubling times of both parent and IL-2Rα-negative C8166-45 cells was 26 hr, and parent and IL-2Rα-negative HUT102 cells doubled every 18–20 hr (Fig. 5). To eliminate the possibility that IL-2Rα was present at the cell surface but masked by sFvTac (which might be co-transported to the cell surface or released by dead cells), the cells were stained with the monoclonal antibody 7G7/B6, which recognizes a different and noncompeting IL-2Rα epitope (Rubin *et al.,* 1985). The lack of staining with 7G7/B6 confirmed that IL-2Rα was indeed not present at the cell surface (Fig. 4 and data not shown). Immunoprecipitation studies further confirmed the absence of mature p55 and the accumulation of p40 in the sFvTac-expressing cells (Fig. 6, lane 2). As the growth rate of

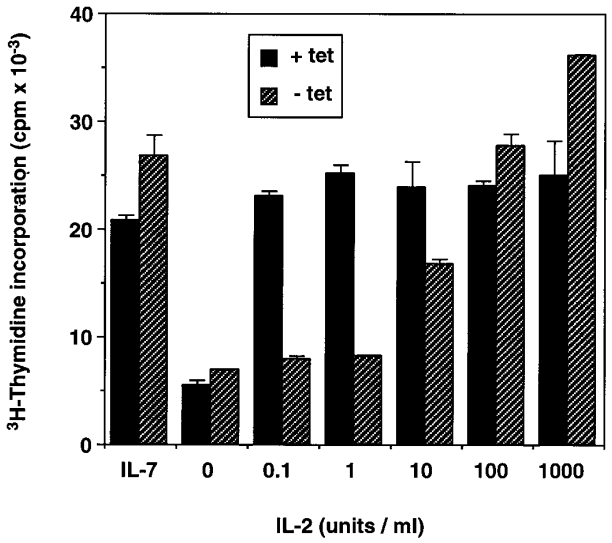

FIG. 3. IL-2-induced proliferation of 10-3Tac-transfected Kit225 cells maintained in the presence or absence of tetracycline.

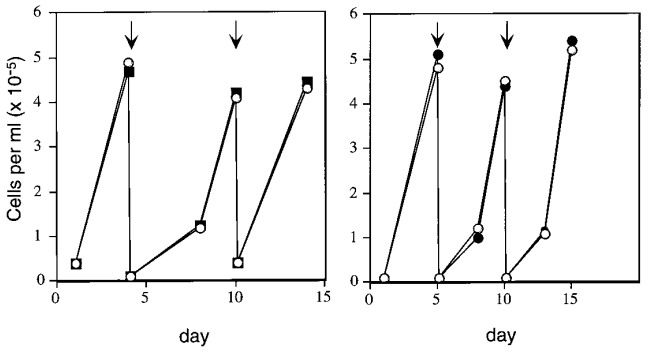

FIG. 5. Growth rate studies for parental and sFvTac-expressing C8166-45 cells (left panel) and HUT102 cells (right panel). Open symbol, parental cell line; closed symbol, sFvTac-expressing line. Arrows indicate days on which cells were split and seeded at 1 or 4 $\times$ 10^4 cells/ml.

the IL-2Rα-negative cells was in each case comparable with that of the parental cell line, we conclude that cell surface expression of IL-2Rα is dispensable for the *in vitro* growth of these HTLV-I-transformed cells. These studies do not rule out the possibility that immature IL-2Rα (p40) molecules retained in the ER may somehow function to promote cell growth. This seems unlikely, however, in view of the fact that this receptor subunit is required for efficient ligand (IL-2) binding but does not appear to play a role in downstream signaling events (Minami *et al.,* 1993).

DISCUSSION

Single-chain antibodies synthesized by the cell and targeted to the relevant cellular compartment have been used to interfere in a highly specific manner with diverse biological processes ranging from growth factor signaling to the replication and assembly of the human immunodeficiency virus (HIV-1) (Richardson and Marasco, 1995). Our demonstration here that IL-2Rα surface expression can be reduced to a level undetectable by flow cytometric analysis in three T cell leukemia lines which normally express >200,000 molecules per cell indicates the extraordinary power of this technology to downregulate even highly abundant cell surface receptors. When coupled with an inducible or repressible expression sys-

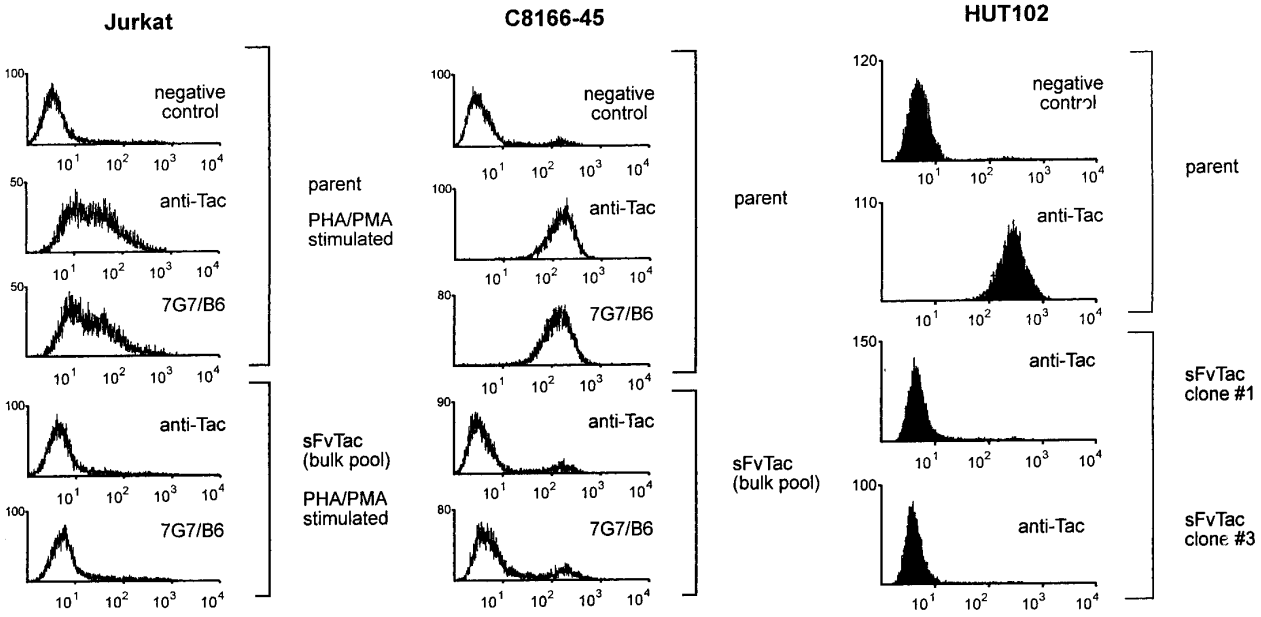

FIG. 4. Flow cytometric analysis of Jurkat, C8166-45, and HUT102 cells stably transduced with the HIV-1-based retrovirus vector, HVTac. Parental or transduced cells were stained with anti-Tac, 7G7/B6, or a control antibody, as indicated.

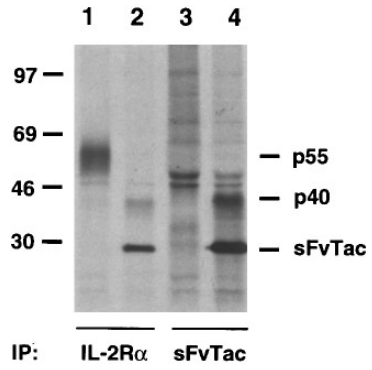

FIG. 6. Immunoprecipitation of IL-2Rα and sFvTac from C8166-45 cells (lanes 1 and 3) or C8166-45 cells stably transduced with HVTac (lanes 2 and 4). IL-2Rα was precipitated with mAb 7G7/B6 and sFvTac with rabbit anti-mouse IgG.

tem, intracellular antibodies represent a highly versatile tool for the manipulation of cellular signaling pathways.

The finding that IL-2Rα is not required for the *in vitro* growth of HTLV-I-transformed cell lines poses two important questions. First, what is the purpose of IL-2Rα upregulation on these cells and second, what signals are driving the proliferation of leukemic cells in ATL? The functional significance of IL-2Rα upregulation has been inferred from the fact that multiple mechanisms exist to maintain high level IL-2Rα expression in HTLV-I-transformed cells. In cells productively infected by HTLV-I, the viral proteins Tax and Rex induce the expression of IL-2Rα by enhancing both the synthesis and stability of IL-2Rα mRNA (Siekevitz *et al.,* 1987; Kanamori *et al.,* 1990). In leukemic cells, where the HTLV-I provirus is transcriptionally silent, the mechanism of IL-2Rα upregulation is less well understood but may be related to the production of ATL-derived factor (ADF), the human homolog of thioredoxin. Originally identified as an IL-2Rα-inducing factor released by ATL cells, ADF is a redox-regulator which appears to indirectly stimulate the IL-2Rα promoter (Teshigawara *et al.,* 1985; Yodoi and Uchiyama, 1992).

The finding that IL-2Rα is dispensable for the growth of HTLV-I-transformed lines *in vitro* suggests that IL-2Rα is not serving a function related to cell growth. It is possible that IL-2Rα expression is an irrelevant consequence of Tax or ADF activity in immortalized lines and ATL cells, respectively, but perhaps a more likely explanation is that the high-affinity IL-2R was required for IL-2-mediated growth at an earlier stage in the leukemogenic or immortalization process. Several observations suggest that loss of IL-2 dependence may be a relatively late event in leukemogenesis, that is separable from the process of immortalization. First, in a small number of ATL patients with chronic or acute disease, the leukemic cells retain the capacity to respond to IL-2 and may be representative of an earlier-stage cell (Maeda *et al.,* 1985, 1987; Arima, 1986). Second, a progression from IL-2 dependence to independence has been observed in many cell lines immortalized by HTLV-1 *in vitro* (Popovic *et al.,* 1983; Hoshino *et al.,* 1983; Yssel *et al.,* 1989; Inatsuki *et al.,* 1989).

While data presented here suggests that expression of the IL-2Rα subunit is not required for the growth of HTLV-I-immortalized cells, the role of $\beta\gamma$ receptor signaling in the growth of both these and fresh ATL cells is an important area that requires further investigation. Two recent reports suggest that the switch to IL-2 independence in HTLV-I-immortalized lines may be associated with the constitutive activation of Jak3, a tyrosine kinase associated with the IL-2 receptor (Migone *et al.,* 1995; Xu *et al.,* 1995). Normally activated upon IL-2 binding, it appears that the Jak3 can be activated by alternative mechanisms in certain cells, resulting in the constitutive activation of Stat factors. In murine pre-B lymphocytes transformed by the v-*abl* oncogene, the abl kinase itself may be responsible for Jak3 phosphorylation (Danial *et al.,* 1995). The mechanism of Jak3 activation in HTLV-I-transformed cells has yet to be determined but may perhaps involve the action of p12^{I}, an HTLV-I gene product of unknown function which is reported to interact with both IL-2Rβ and γ_c (Mulloy *et al.,* 1996). The constitutive activation of Jak-Stat signaling pathways has recently been demonstrated in other human leukemias and an inhibitor of Jak2 was found to inhibit the *in vitro* proliferation of acute lymphoblastic leukemia cells (Meydan *et al.,* 1996; Gouilleux-Gruart *et al.,* 1996). In light of the apparent link between Jak-Stat activation and cell proliferation in other leukemias, the $\beta\gamma$ receptor and associated signaling pathways may represent a strategic therapeutic target in adult T cell leukemia. The development of intracellular antibodies directed against IL-2Rβ and γ_c should permit direct experimental testing of this hypothesis.

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