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Protooncogene-encoded protein kinases in interleukin-2 signal transduction

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Protooncogenes are the normal forms of cellular genes that when altered in their expression or coding sequences can contribute to neoplastic transformation. As these genes often are important for normal cellular growth control, we explored the possibility that protein kinases encoded by particular protooncogenes could participate in signal transduction pathways regulated by the T cell growth factor, interleukin-2 (IL-2). In this review we summarize our findings to date regarding Raf-1, a serine/threonine-specific kinase that becomes phosphorylated on tyrosine residues and enzymatically activated in response to IL-2 stimulation. In addition, we describe our investigations of Lck and Lyn, two closely related protein tyrosine kinases of the src gene family that physically associate with the IL-2 receptor complex and whose activities are regulated by IL-2 in at least some T cells and B cells, respectively.

Key words: interleukin-2 / Raf-1 / Lck / Lyn / proto-oncogene

INTERLEUKIN-2 (IL-2) is thought to be a principal regulator of *in vivo* immune responses. The biological activities documented for this lymphokine range from control of lymhocyte proliferation and differentiation to enhancement of immune cell-mediated cytolysis of tumors and virus-infected cells.[1-3] The human gene for IL-2 has been molecularly cloned and sequenced, and its recombinant product is beginning to find clinical uses.[4] The side-effects of *in vivo* IL-2 administration are severe, however, drastically limiting its applications. Clearly, a greater understanding of the mechanisms of IL-2 action is needed if we are to effectively and safely exploit the potential of this lymphokine for treating human diseases.

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The IL-2 receptor

Three polypeptides have been identified that associate non-convalently to form high-affinity receptors for IL-2($K_d = 10^{-11}$ M). These are: (a) the 50-55 kDa α-chain which by itself has only low affinity for IL-2 ($K_d = 10^{-8}$ M) and appears to be insufficient for biological activity; (b) the 70-75 kDa β-chain; and (c) the p64 γ-chain which together with the β-chain forms an intermediate affinity binding site for IL-2 ($K_d = 10^{-9}$ M) that appears to be capable of mediating many of the biological actions of IL-2, including IL-2 internalization, cellular proliferation, and NK cell-mediated tumorilysis.[5-8] Unfortunately, despite molecular cloning of cDNAs for all three of these receptor subunits and extensive biochemical characterization of their encoded proteins, few clues have been provided that might suggest the mechanism by which the IL-2 receptor (IL-2R) complex transmits its signals to the interior of cells. Unlike many other growth factor receptors that possess ligand-dependent, tyrosine-specific kinase activity, for example, none of the IL-2-receptor subunits has intrinsic kinase activity. The observations that IL-2 induces rapid phosphorylation of several intracellular proteins in lymphocytes and that pharmacological inhibitors of protein tyrosine kinases (PTKs) can suppress many of the effects of IL-2, however, have suggested that IL-2-mediated regulation of PTKs represents a critical event in the signal transduction mechanisms whereby this lymphokine controls the actions of immune cells.[9-12]

Protooncogenes as mediators of the intracellular actions of IL-2

In considering candidates for the IL-2-regulated PTK, as well as other downstream participants in

IL-2 signal transduction pathways, our laboratory has turned to protooncogenes and their encoded proteins. Our reasoning was that protooncogenes, with their clear precedence for being capable of regulating cellular growth and differentiation, represented excellent candidates for potential key intracellular regulators of IL-2's signals. Our initial studies, much of which were performed in collaboration with Dr Peter Nowell at the University of Pennsylvania, focused on delineating which of the various protooncogenes were expressed in normal IL-2-responsive T cells, and defining some of the details of the mechanisms responsible for regulating their expression (reviewed in ref 13). For the most part, the regulation of the expression of these genes correlated with their encoded proteins. For example, expression of protooncogenes that encode nuclear proteins which are currently believed to function as transcription factors (e.g. c-*myc*, c-*myb*) was absent in resting T cells but was induced by stimulation with IL-2. In contrast, protooncogenes that encoded protein kinases (e.g. c-*abl*, c-*raf-1*, *lck*, *fyn*, *yes*) were expressed even in unstimulated T cells and the expression of these protooncogenes either did not increase after treatment with IL-2 or only rose modestly, with the exception of *pim*-1 whose pattern of expression was more like *myc* and *myb*. Given the biochemical evidence at the time that unknown protein kinases were involved in IL-2 signal transduction, we then focused our attention on kinase-encoding protooncogenes that were expressed in T cells even in the absence of IL-2 stimulation. Our reasoning was that the kinases encoded by these genes would be present in the lymphocytes even before IL-2 binding, and thus poised to receive activation signals from the IL-2-receptor complex or to participate in signal transduction pathways regulated by this lymphokine. As a first attempt to pinpoint which of these protooncogene-encoded kinases might be involved in IL-2 signalling, we began gene transfer experiments using an IL-2-dependent murine T cell line CTLL-2, searching for oncogenes that could abrogate or reduce the lymphokine dependence of these cytolytic T cells.

Gene transfer investigations of the Raf-1 kinase

Our first success with stable transfer of expression constructs into IL-2-dependent CTLL-2 cells occurred with *raf-1*. This gene encodes a 72 kDa serine/threonine-specific kinase that is located primarily in the cytosol. The Raf-1 kinase has a two domain structure, where a NH_2-terminal regulatory domain is proposed to fold over onto the C-terminal catalytic domain, thus suppressing the activity of the kinase and preventing it from interacting with substrates. Upon phosphorylation at critical sites, the kinase is speculated to assume a more open conformation that allows the catalytic domain access to appropriate substrates (reviewed in ref 14).

For gene transfer experiments, we made use of recombinant retroviruses that contained either a full-length normal human *raf-1* cDNA or a version where a large portion of the sequences encoding the NH_2-regulatory domain was deleted, thus resulting in a kinase with constitutively high levels of activity. When retroviruses encoding normal or truncated Raf-1 were stably introduced into CTLL-2 cells, we discovered that the cells producing the truncated Raf-1 were capable of undergoing approximately one round of replication in the absence of IL-2, as opposed to cells containing only the normal Raf-1 kinase whose growth was strictly dependent on IL-2 (see Figure 1).

Investigations of Raf-1 kinase phosphorylation and regulation in CTLL-2 cells

At about the time we obtained these data suggesting a role for Raf-1 in the regulation of IL-2-dependent T cell growth, it was discovered that the Raf-1 kinase becomes phosphorylated in response to stimulation of murine fibroblasts by various growth factors and furthermore, that certain oncoproteins such as pp60-v-src and p21-Ras activate pathways leading to growth factor-independent phosphorylation and enzymatic activation of the Raf-1 kinase.[15] We therefore investigated the possibility that IL-2 regulated the phosphorylation and activity of the Raf-1 kinase in CTLL-2 cells.[16]

Stimulation of CTLL-2 cells with IL-2 resulted in rapid and transient phosphorylation of Raf-1. IL-2-mediated increases in the incorporation of $^{32}PO_4$ into the RAF-1 protein were detectable within 3-5 min after addition of IL-2 to cultures of CTLL-2 cells, reached maximal levels at 10-20 min, and then declined to baseline within 1-3 h. Furthermore, these increases in the activity of the Raf-1 kinase were dependent on the concentration of IL-2 used, and correlated well with IL-2-induced

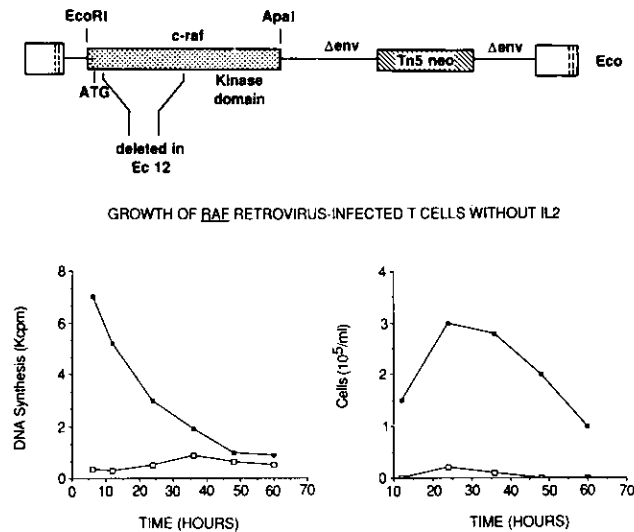

Figure 1. Activated Raf-1 reduces IL-2-dependence of CTLL-2 cells. CTLL-2 cells were stably infected with recombinant retroviruses encoding either the normal full-length human Raf-1 kinase (open squares) or a truncated version of Raf-1 that lacks portions of the regulatory domain resulting in a kinase with constitutively high activity (closed squares). T cells were deprived of IL-2 and their relative rates of DNA synthesis (measured by 3H-thymidine incorporation into DNA) and growth were determined at various times thereafter. Data represent the mean of three determinations.

proliferation of these T cells.[16] In CTLL-2 cells the IL-2-mediated elevations in Raf-1 kinase activity occurred without an alteration in the levels of p72-Raf-1 protein,[16] but in primary human T cells IL-2 was reported to also stimulate increases in raf-1 gene expression.[17]

IL-2 induces phosphorylation of Raf-1 on tyrosines

Though somewhat controversial,[18] studies of the regulation of Raf-1 kinase phosphorylation in murine fibroblasts have suggested that Raf-1 activity can be up-regulated through at least two mechanisms: one that is associated with tyrosine phosphorylation of Raf-1, and the other which involves strictly serine and threonine phosphorylation.[19] We therefore examined the issue of tyrosine phosphorylation of Raf-1 in IL-2-stimulated CTLL-2 cells. Based on immunoprecipitation and immunoblotting experiments using anti-phosphotyrosine (PY)-specific antibodies, we determined that Raf-1 becomes inducibly phosphorylated on tyrosines in IL-2-stimulated CTLL-2 cells. Furthermore, about half of the Raf-1 molecules in these cells appeared to undergo this post-translational modification, based on their ability to be immunoprecipitated with anti-PY antibodies. This surprisingly high stoichiometry of tyrosine phosphorylation was confirmed by two-dimensional phosphoamino acid analysis, demonstrating that nearly half of the $^{32}PO_4$ incorporated into Raf-1 after stimulation of CTLL-2 cells with IL-2 occurred on tyrosines.[16]

Tyrosine phosphorylation regulates the activity of Raf-1 kinase in CTLL-2

Having demonstrated that IL-2 stimulates phosphorylation of the Raf-1 kinase on tyrosine residues in CTLL-2 cells, we next wished to determine the functional significance of this post-translational modification for regulation of Raf-1 kinase activity. For these experiments, which were performed in collaboration with Dr Nick Tonks of the Cold Spring Harbor Laboratories, Raf-1 was immunoprecipitated from IL-2-stimulated CTLL-2 cells and phosphate was selectively removed from tyrosines by treatment with tyrosine-specific phosphatases, either CD45 or T cell protein tyrosine phosphatase (TCPTP). The *in vitro* kinase activity of Raf-1 was then measured.

Treatment of Raf-1 with either of these phosphatases reduced Raf-1 kinase activity by an average

of 68%, consistent with the data described above where about half of the Raf-1 protein in IL-2-stimulated CTLL-2 cells was found to be immunoprecipitable with anti-PY antibodies. This effect was completely blocked by the phosphatase inhibitor Na-orthovanadate, demonstrating the specificity of the results. Two-dimensional phosphoamino acid analysis confirmed the selective removal of phosphate from tyrosines, and not from serines or threonines. The immunoreactivity of Raf-1 with anti-phosphotyrosine antibodies was also completely abolished by treatment with CD45 in the absence but not in the presence of Na-orthovanadate.[20]

Taken together, these findings indicate that IL-2-induced phosphorylation of Raf-1 on tyrosines plays a critical role in upregulating the enzymatic activity of this serine/threonine-specific kinase in CTLL-2 cells. As such, the regulation of Raf-1 kinase activity by IL-2-inducible tyrosine phosphorylation provides a model system in which to study a question of fundamental importance for cell biology: namely, how do PTKs pass information on to serine/threonine-specific kinases? It should be borne in mind, however, that because CTLL-2 cells differ from normal T cells in their ability to remain constantly responsive to IL-2 in the absence of periodic stimulation with antigen, the high stoichiometry of tyrosine phosphorylation of Raf-1 observed in CTLL-2 cells many represent a special circumstance. It is well known that the Raf-1 kinase can also be activated through signal transduction pathways that do not involve tyrosine phosphorylation. For example, stimulation of certain T cell hybridomas through the T cell antigen receptor (TCR) complex has been reported to induce phosphorylation and activation of Raf-1 through a protein kinase-C-dependent mechanism that involves no detectable tyrosine phosphorylation.[29] In fact, when the effects on Raf-1 phosphorylation of a wide variety of growth factors are considered, on balance it appears that serine/threonine phosphorylation rather than tyrosine phosphorylation represents the predominant post-translational modification associated with activation of this enzyme (reviewed in ref 22). Even in cases where tyrosine phosphorylation of Raf-1 does occur, such as in IL-2 stimulated CTLL-2 cells, we cannot exclude the possibility that a combination of serine/threonine and tyrosine phosphorylation events are necessary to achieve maximal elevations in the specific activity of this enzyme. In this regard, treatment of Raf-1 with tyrosine-specific phosphatases removed essentially all detectable phosphates from tyrosines but did not completely abolish kinase activity, suggesting a role either for an upstream IL-2-regulable serine/threonine kinase or for Raf-1 autophosphorylation in addition to an upstream PTK. Current efforts, therefore, are being directed at delineation of the tyrosine- and serine/threonine-specific kinases responsible for phosphorylating and activating Raf-1 in IL-2-stimulated T cells.

In addition to regulating the enzymatic activity of Raf-1, tyrosine phosphorylation may also control the proteins with which this serine/threonine-specific kinase interacts. Maslinski *et al*, for example, reported that a small portion of the Raf-1 in T cells is physically associated with the p75-IL-2Rβ-chain and that IL-2-stimulation results in dissociation of Raf-1 from the receptor and translocation to the cytosol. This dissociation and translocation is blocked by the PTK inhibitor genistein, implying regulation by an IL-2-responsive PTK.[23]

A search for PTKs involved in IL-2 signal transduction: IL-2 specifically regulates the activity of p56-Lck

Our discovery that IL-2 induces phosphorylation of Raf-1 on tyrosines led to a search for IL-2-regulated PTKs. Since we and others had previously observed constitutive phosphorylation and activation of Raf-1 in v-*src*-transformed fibroblasts,[15,24] we initiated a systematic evaluation of the Src-like PTKs in IL-2-dependent T cells. Of the known Src-like PTKs, we detected only p56-Lck, p59-Fyn, and small amounts of p62-Yes in T cells. Of these, IL-2 was found by us and others to induce increases in the enzymatic activity only of p56-Lck.[25-27] IL-2-induced increases in Lck occurred rapidly in all T cell clones and lines tested, becoming detectable within seconds of IL-2 binding, reaching peak levels within 5 to 10 min and then declining to baseline typically within 30 to 60 min. These kinetics imply that Lck is a proximal participant in an IL-2-regulated signal transduction cascade. Consistent with this notion, p56-Lck has been reported to physically associate with the IL-2Rβ-chain, suggesting that this member of the Src family of PTKs may receive activation signals directly from the IL-2R complex.[28]

Figure 2A shows an example of some of our results where p56-Lck was co-immunoprecipitated with the β-chain of the IL-2R complex in YT-cells, an NK-like leukemia line which was employed for these experiments because of its high levels of p75-IL-2Rβ.

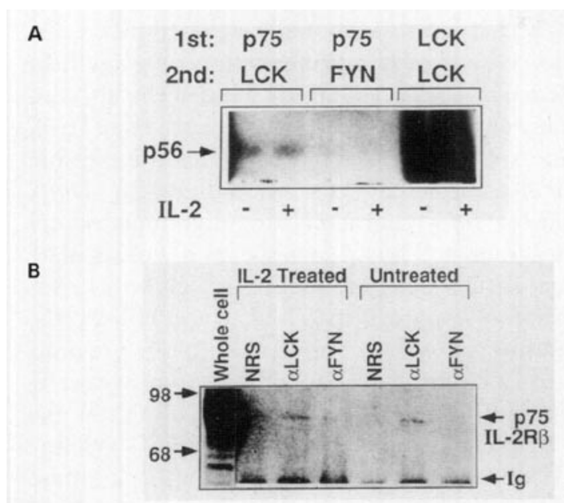

Figure 2. Lck associates with the p75-β-chain of the IL-2R complex. In A, lysates were prepared using YT cells before (−) or 10 min after (+) stimulation with IL-2, and subjected to immunoprecipitation using a monoclonal antibody specific for p75-IL-2Rβ or an antiserum specific for p56-Lck (first immunoprecipitation), essentially as described previously.[37] The resulting immune complexes were incubated in a reaction buffer containing ^{32}P-γ-ATP to allow for PTK autophosphorylation. Afterwards, the immune complexes were dissociated in a solution containing 1% SDS, and a second immunoprecipitation was subsequently performed using antisera specific for either p56-Lck or p59-Fyn, as indicated. Note that a few percent of the total Lck kinase activity in YT cells was present in the immune complexes prepared with the anti-p75-β antibody, whereas essentially no p59-Fyn was found in association with this IL-2R subunit. In B, immunoprecipitates where prepared using YT cell lysates and antisera specific for either Lck or Fyn. Normal rabbit serum (NRS) was also included as a negative control. The resulting immune complexes, as well as a whole cell lysate prepared from an equivalent number of cells, were subjected to SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose filters for incubation with an anti-p75-IL-2Rβ antibody followed by a ^{125}I-labeled secondary antibody. Note that a few percent of the total p75-IL-2Rβ can be found in the samples where immunoprecipitations were preformed using anti-Lck antibody, but not NRS or anti-Fyn antiserum. (Ig refers to the heavy-chain of the antibodies used for immunoprecipitations.)

Figure 2B shows the converse experiment, where antibodies specific for Lck were used to co-immunoprecipitate p75-IL-2R-β and then the receptor was detected by immunoblotting using a monoclonal antibody specific for the β-chain. In both experiments, note that the association of Lck and IL-2Rβ exists even in the absence of IL-2 stimulation, indicating that at least some of the p56-Lck in the cell appears to be pre-associated with the IL-2R complex and thus ready to receive activation signals upon IL-2 binding. Though the stiochiometry of Lck and IL-2Rβ-association appears to be low (1-5%), it is possible that many of the kinase-receptor complexes do not survive *in vitro* isolation thus resulting in an underestimation of their abundance in intact cells.

At present the functional significance of the association of p56-Lck with the IL-2R complex remains unknown. Hatakeyama *et al* mapped the regions within Lck and IL-2Rβ necessary for association using a deletional approach and expression in COS cells. For Lck the involved region is the catalytic domain of the kinase; for p75-IL-2Rβ the important region was localized to a cytosolic segment that is rich in acidic residues and that also contains the putative tyrosine phosphorylation sites (Tyr 355 and Tyr 358) that appear to be the primary targets of p56-Lck, at least *in vitro*.[28] Thus, Lck's association with the IL-2R complex may merely represent a kinase-substrate interaction. Alternatively, the association of Lck with the receptor could provide a mechanism for activating this kinase in an IL-2-regulable manner. In this regard, some models for Lck kinase activation via CD4 or CD8 suggest that ligand-induced receptor dimerization or clustering may bring associated Lck molecules into close proximity, allowing them to *trans*-phosphorylate each other at critical tyrosines such as Tyr 394 that up-regulate Lck kinase activity (reviewed in ref 29). By analogy to Lck's association with CD4 and CD8, IL-2 potentially could induce IL-2R dimerization or clustering and thus result in Lck activation through a mechanism not unlike that proposed for activation of the kinase domains of classical PTK growth factor receptors such as those that bind PDGF or FGF.[30,31] Still another possibility is that the interaction of Lck with the IL-2R complex plays a role in bringing Lck into contact with some IL-2-regulated PTK or tyrosine phosphatase that up-regulates the activity of Lck either by phosphorylating the kinase at tyrosines that elevate its activity (Tyr 394) or dephosphorylating it at tyrosines that depress the enzyme (Tyr 505). Phosphopeptide mapping and mutagenesis studies are currently underway to discriminate among some of these possibilities.

Investigations of the function of Lck in IL-2-dependent T cells

As a first attempt to explore the functional significance of IL-2-mediated increases in Lck kinase activity in T cells, we stably introduced Lck-encoding

expression plasmids into the IL-2-dependent T cell lines CTLL-2 (cytolytic) and HT-2 (helper). For these experiments, constructs containing cDNAs for either the normal Lck kinase or for a mutant version of this PTK with constitutively high levels of enzymatic activity [Lck(505Tyr->Phe)] were employed. T cells stably transfected with these plasmids contained 5- to 10-fold elevations in their relative levels of Lck kinase activity.[27] Moreover, these high levels of Lck kinase activity were accompanied by marked increases in the levels of tyrosine phosphorylation of several proteins, particularly where the Lck(Y505F) mutant was concerned. We also observed that T cells expressing the activated form of Lck contained constitutively high levels of phosphatidylinositol-3'-kinase (PI3K) activity and tyrosine phosphorylation of the 85 kDa subunit of PI3K, unlike T cells that contain normal p56-Lck where PI3K activity and tyrosine phosphorylation of p85 are IL-2-inducible.[32,33] Figure 3 shows an example of PI3K assay data obtained using normal and Lck (Y505F)-containing HT-2 cells. In contrast to the normal kinase, activated p56-Lck (Y505F) was also constitutively associated with PI3K, in that the two could be co-immunoprecipitated from cells even in the absence of prior IL-2 stimulation.[32]

Despite the high levels of Lck kinase activity achieved through this gene transfer approach and the biochemical evidence confirming that this

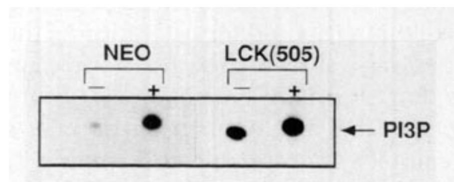

Figure 3. Activated Lck kinase induces IL-2-independent increases in the levels of PI3K activity in HT-2 cells. A plasmid conferring resistance to the neomycin analog G418 was introduced alone or in combination with an expression plasmid encoding p56-Lck[Y505F] into the IL-2-dependent T cell line HT-2. Stably transfected cells were obtained by selection in G418 and their relative levels of PI3K activity were measured by an assay involving immunoprecipitation with anti-PY antibodies followed by incubation with ^{32}P-γ-ATP and phosphatidylinositol substrate. Reaction products were analyzed by thin-layer chromatography as described previously.[33] Note that the control cells (NEO) contain little anti-PY immunoprecipitable PI3K activity when deprived of IL-2 (–) for 4 h. A 10 min stimulation with IL-2 (+) induces PI3K activity. In contrast, HT-2 cells containing p56-Lck[Y505F] have constitutively high levels of PI3K activity, irrespective of IL-2 stimulation.

kinase's activity was functionally elevated in T cells, no enhancement of cell proliferation or growth in the absence of IL-2 was found. These cells also did not display enhanced survival when deprived of IL-2, and underwent apoptosis with kinetics comparable to control-transfected cells, as defined by DNA degradation into characteristic oligonucleosomal length fragments. Thus, Lck appears not to regulate pathways sufficient for inducing cell growth or for suppression of apoptosis in CTLL-2 and HT-2 cells. These findings are also consistent with our previous studies of Lck performed in collaboration with Drs Daniela Santoli and Rosemary O'Connor at the Wistar Institute using a human leukemia T cell line where IL-2-mediated elevations in p56-Lck kinase activity were dissociated from lymphokine-regulated cell growth in that IL-4 blocked IL-2-induced proliferation without affecting IL-2-stimulated increases in Lck activity.[26]

In the absence of a demonstrable effect of Lck on T cell growth or survival, we next turned our attention to immune cell effector functions. Transfected CTLL-2 cells containing elevated levels of Lck kinase activity were initially tested for cytolytic activity against a variety of tumor targets. Our particular subline of CTLL-2 cells was able to lyse Yac-1, Jurkat, and RL1 tumor cells, through an MHC-independent mechanism that is probably similar to that used by some NK-cells and LAK-cells. Tumor cell killing by the untransfected control CTLL-2 cells was entirely dependent on IL-2. In contrast, CTLL-2 cells with elevated Lck kinase activity killed these same tumor targets without requirement for IL-2, and did so with 5- to 10-times greater efficiency than control CTLL-2 cells. Further evidence that p56-Lck plays an important role in cytolytic effector function comes from studies performed in collaboration with Robert Abraham, Larry Karnitz and colleagues at the Mayo Clinic who discovered a subclone of CTLL-2 cells that fails to express *lck*. These Lck-deficient CTLL-2 cells behave similar to wild-type cells with respect to IL-2-induced proliferation, but are incapable of killing in an antibody-redirected lysis assay. Transfection of *lck*-expression plasmids into these T cells restored killing activity, thus establishing the importance of Lck for this immune cell effector function.[34] The mechanisms by which Lck controls the cytolytic activity of killer T cells are now being explored, and additional investigations of the effects of this kinase on helper T cells are underway. Furthermore, we are attempting to exploit the ability of *lck* gene transfer to markedly

enhance cytolytic effector function in a gene therapy model of adoptive immunotherapy for cancer.

Flexibility in coupling of Src-like kinases to IL-2 signalling pathways

Any model for IL-2-signal transduction that places p56-Lck in a pivotal role must ultimately deal with the problem that not all IL-2-responsive cells express the *lck* gene. Many B lymphocytes, for example, undergo proliferation and/or differentiation when stimulated with IL-2 but lack p56-Lck.[2,35] As a first attempt to explore this issue, we employed a subclone of the pro-B cell line BAF that had been stably transfected with a human IL-2Rβ expression plasmid and that was provided to us by Drs M. Hatakeyama and T. Taniguchi of the University of Osaka.[36] Of the eight Src-like PTKs that were known at that time, these B lineage cells were found to contain only p53/56-Lyn, p59-Fyn and p62-Yes. Of these, IL-2 specifically stimulated increases only in the activity of the Lyn kinase. Furthermore, p53/56-Lyn could be co-immunoprecipitated with p75-IL-2Rβ using lysates prepared from these pro-B cells.[37] Thus, the Lyn kinase appears to fill the void in these cells that lack p56-Lck.

It is of interest that of the known Src-like kinases, Lyn is the one that is most homologous to Lck in its predicted amino acid sequence.[38] This implies that similarities in the structures of the Lck and Lyn kinases allow them to interact with and become regulated by the IL-2R complex in T and B cells, respectively. When considered within the context of our discovery that Lck regulates the cytolytic effector function of killer T cells, these data also raise the interesting possibility that variations in the repertoire of *src*-family genes expressed in cells may allow for lineage-specific (T versus B cell) responses to IL-2. For example, IL-2 increases the cytolytic activity of $CD8^+$ T cells and NK cells which are known to express *lck* but not *lyn*, whereas IL-2 stimulation can lead to increases in antibody production by B cells which have been shown to express *lyn* but not *lck*. Though these findings regarding Lyn regulation in IL-2-responsive B cells need to be confirmed in other B cell lines, conceivably differences in the substrates of p56-Lck in T cells and of p53/56-Lyn in B cells could account for at least some of these lineage-specific responses to IL-2. Further investigations of the regulation and function of the Lyn kinase in IL-2-stimulated B cells are currently underway in our laboratory, but based on our knowledge thus far about Lck in T cells we would predict that Lyn might participate in some B cell effector functions such as antibody production or antigen presentation, as opposed to cell growth regulation.

Future questions and challenges

By following a hunch that protooncogenes would be involved in IL-2 signal transduction mechanisms, we have discovered that at least two protooncogene-encoded kinases, Raf-1 and Lck, are regulated by IL-2 in T cells. Furthermore, we have begun to delineate some of the functions of these proteins with regards to T cell growth and immune cell effector functions. Figure 4 diagrammatically summarizes our findings to date. As discussed above, Raf-1 appears to be involved in a signalling pathway involved in cell growth in IL-2-dependent T cells. Recent data derived from IL-3-dependent cells suggest that part of the effect of Raf-1 on growth may actually be due to enhanced cell survival by suppression of apoptosis, as well as increased rates of mitogenesis.[39]

In contrast to Raf-1, the Lck kinase regulates the cytolytic killing function of T cells through unknown mechanisms. One of the challenges for the future will be to dissect the molecular events that account for the regulation of cytolytic activity by p56-Lck and to attempt to exploit this information for improved immunotherapy for cancer and viral diseases. Thus far, we have determined that activated Lck kinase physically interacts with PI3K and leads to marked increases in the activity of PI3K.[32] It remains to be determined, however, whether the phospholipids generated by this enzyme or their metabolites directly or indirectly contribute to IL-2-mediated enhancement of cytolytic killing. At this point, we still do not know whether Lck is the upstream PTK that is responsible for phosphorylating and activating Raf-1 in T cells. The gene transfer experiments discussed above, as well as preliminary data obtained thus far (not shown) suggest that there may not be a direct connection between these two kinases. It should be noted, however, that Lck has been found to inducibly associate with Raf-1 in the setting of anti-CD4 antibody stimulation of T cell clones, and that this interaction of Lck with Raf-1 coincided with tyrosine phosphorylation of the latter.[40]

Another question that begs to be answered concerns the ability of Lck and Lyn to interact with more than

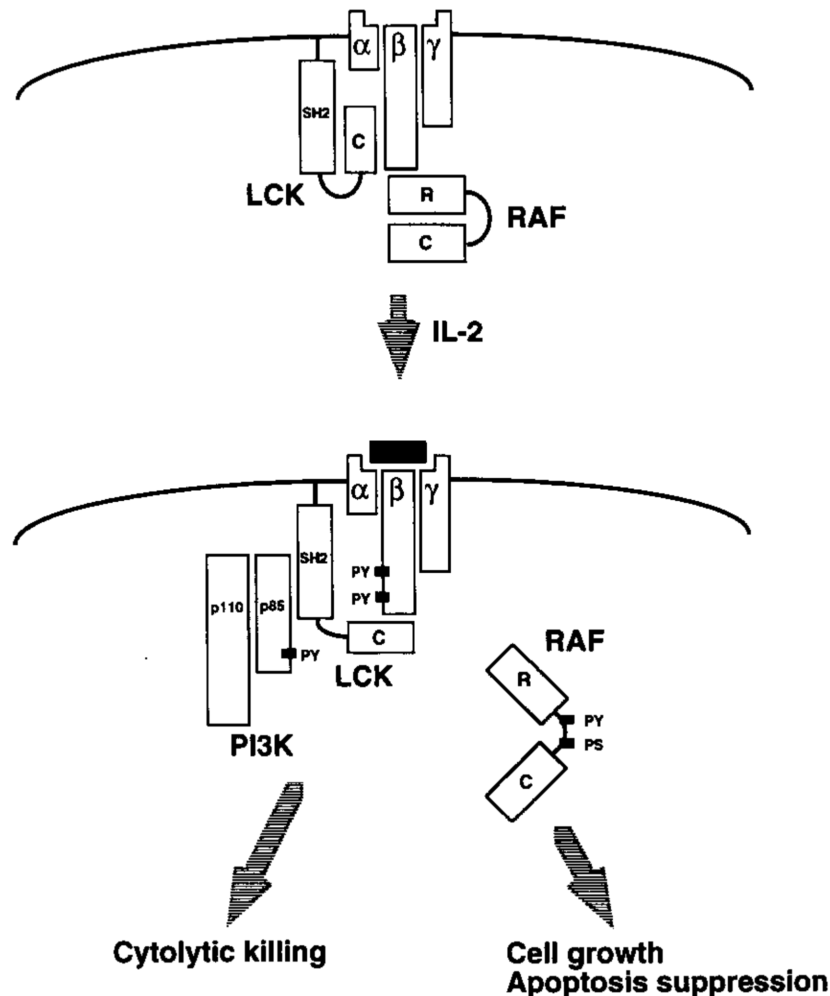

Figure 4. Involvement of Raf-1 and Lck in IL-2 signal transduction. The diagram illustrates some of the events believed to occur prior to (top) and following (bottom) binding of IL-2 to its high-affinity receptors in T cells. The diagram is highly over-simplified for purposes of clarity of presentation. Before binding of IL-2, both Lck (Lyn in B cells) and Raf-1 can be found in association with the IL-2R complex, particularly with the p75-IL-2Rβ-chain. With regards to Lck, the data available to date indicate that the interaction with the β-chain occurs via the catalytic domain (C) of Lck and not through its regulatory domains which include the Src-homology-2 (SH2) region. The details of Raf's interaction with the β-chain have not been defined. After binding of IL-2, Lck becomes activated but remains in association with the IL-2R complex. Tyrosine phosphorylation occurs on the β-chain as well as on the 85 kDa subunit of PI3K, which can be found in association with Lck in IL-2-stimulated T cells.[32] The phosphorylation by Lck of p85 on tyrosines [PY] is associated with increased activity of the 110 kDa catalytic subunit of PI3K seen in IL-2-stimulated T cells. Lck controls a pathway that regulates cytolytic effector function, at least in killer T cells. Raf-1 becomes phosphorylated on both tyrosines (PY) and serines (PS) after IL-2 stimulation, resulting in dissociation of Raf-1 from the IL-2R complex. The hyperphosphorylated form of Raf-1 is hypothesized to assume an open conformation where its regulatory domain (R) becomes dislodged from the catalytic domain (C) thus allowing the kinase to interact with substrates. Various gene transfer data suggest that Raf-1 participates in a pathway leading to enhanced growth factor-mediated mitogenesis and contributing signals for the suppression of apoptosis.

one receptor in T and B cells, respectively. In this regard, Lck has been shown to physically associate with the cytoplasmic domains of CD4 and CD8 in T cells and in this context appears to be able to enhance T cell antigen receptor (TCR)-initiated signals that lead to IL-2 production.[41,42] In B cells, Lyn as well as other Src-like PTKs can be co-immunoprecipitated with proteins within the surface immunoglobulin complex, and stimulation with antigen rapidly increases the specific activity of Lyn and these other kinases.[43,44] What then are the molecular mechanisms that allow for this flexibility in the interaction of Lck and Lyn with various receptors in lymphocytes and how does their interaction with specific receptors affect function? With regards to the latter, one obvious possibility that comes to mind is that association of Lck and Lyn with certain plasma membrane receptors helps to bring these kinases into proximity with certain key substrates, and thus imparts at least some specificity to the responses.

Still another issue that requires attention is the functional significance of the interaction of Lck and Raf-1 with the IL-2R complex. Do these kinase-receptor associations provide a mechanism for IL-2-inducible activation of Lck and Raf, and if so how? For example, is the interaction of the kinase with the receptor sufficient, or are other upstream regulators such as unknown kinases or phosphatases involved? A related question that has yet to be adequately addressed is whether Lck or Raf-1 phosphorylate components of the IL-2R complex *in vivo*, and if so what the functional consequences of this post-translational modification are. The ultimate hope for the future is that with further experimental investigations of these and other questions, our understanding of the molecular mechanisms of IL-2 signal transduction will be delineated in sufficient detail that new approaches can be developed for improved treatment of human diseases.

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