Inhibition of interleukin-2-mediated DNA synthesis in activated human T-lymphoblasts by okadaic acid is accompanied by hyperphosphorylation of *lck*

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We have previously shown that, during interleukin-2-driven G₁/S transition in activated human T-lymphoblasts, a restricted group of cellular proteins become tyrosine phosphorylated *de novo*, and that p56^{lck} is the major active tyrosine kinase at this stage of the cell cycle. We now report that okadaic acid, a potent and specific inhibitor of protein phosphatases type 1 and type 2A, inhibits S-phase entry, and that this occurs with the simultaneous disappearance of a 56 kDa tyrosine phosphoprotein. We show that this protein is the *lck* tyrosine kinase and that okadaic acid stimulates a mobility shift to 59 and 64 kDa

forms. These two forms of *lck* were found to have decreased autocatalytic activity, as judged by immune-complex kinase assays. Two-dimensional phosphopeptide mapping and phosphoamino-acid analyses revealed that, in the presence of okadaic acid, *lck* becomes phosphorylated mainly on serine and to a lesser extent threonine, and that phosphorylation occurs at novel sites. These results show that the kinase activity of *lck* is at least partly regulated by protein phosphatases, and suggest a role for *lck* in directing growth-factor-mediated DNA synthesis during T-cell proliferation.

INTRODUCTION

Occupation of the high-affinity interleukin-2 (IL-2) receptor (IL-2R) by its cognate ligand in activated human T-lymphoblasts results in cell-cycle entry and proliferation [1]. During the first few minutes of receptor occupation, many cellular proteins become tyrosine phosphorylated, due to activation of cytoplasmic non-receptor tyrosine kinases, including *lck* [2]. Although the mechanism of *lck* activation is not fully understood, physical association with the cytoplasmic domain of the IL-2R β -chain has been suggested to elicit a conformational change leading to activation [3,4]. After IL-2R stimulation, *lck* itself becomes phosphorylated, resulting in the characteristic division into 56 and 59 kDa isoforms [4]. Recent studies by Winkler et al. [5] have shown that phosphorylation of serine-42 in the N-terminus of *lck* by protein kinase C does not cause a gel shift, whereas phosphorylation of serine-59 by MAP2 (mitogen-activated protein 2) kinase causes a mobility shift to 59 kDa. When examined in immune-complex kinase assays, the activity of p56^{lck} increases significantly after IL-2R stimulation, whereas p59^{lck} appears to lack autocatalytic activity [6]. It is therefore possible that, whereas activation of the MAP2 kinase pathway results in inactivation of *lck*, activation of protein kinase C provides a positive stimulus. In addition to regulation by serine phosphorylation, *lck*, like all members of the *src* family of tyrosine kinases, is normally maintained in an inactive state by phosphorylation at a conserved C-terminal tyrosine residue [7,8]. Mutation of this site results in a constitutively active form of *lck* capable of cell transformation [9]. Furthermore, *lck* was one of several tyrosine kinases found to be constitutively active in mice lacking the C-terminal *src* kinase (*csk*) gene [10]. These studies collectively illustrate that regulation of *lck* is essential for normal T-lymphoblast growth, and that regulation is mediated by the co-ordinate activities of an array of protein kinases and phosphatases.

Although early signalling events via the IL-2R have been extensively studied, little is known about the role, if any, of tyrosine kinases in cell-cycle progression through G₁ and into S phase. We have previously shown that the 56 and 59 kDa isoforms of *lck* persist throughout G₁ and into S phase, that serine phosphorylation of *lck de novo* increases during G₁/S, and that an associated increase in activity of p56^{lck} is accompanied by increased tyrosine phosphorylation of certain cellular proteins [6]. It is known that lymphocytes contain the four major classes of serine/threonine phosphatases [11], and it seems likely that one or more of these must be involved in the regulation of *lck* activity during the cell cycle. Protein phosphatases have been implicated in regulating progression from G₁ to S-phase. For example, the activity of protein phosphatase type 2A (PP2A) is modulated by binding to the small transforming protein of SV40 [12], PP2A stimulates SV40 large-T dependent DNA synthesis *in vitro* [13], and physical association of the SV40 small tumour antigen with PP2A stimulates the MAP kinase pathway and induces cell proliferation [14]. In mouse fibroblasts the RB protein maintains cells in G₁ by inhibiting transcription of growth-stimulating genes and is active only in its dephosphorylated state, suggesting that protein phosphatases play a key role in S-phase entry [15].

Recently, it has become possible to study the roles of PP1 and PP2A by using the potent and specific inhibitor okadaic acid (OA), a polyether fatty acid isolated from marine sponges, which has the ability to traverse the cell membrane and is therefore useful in experiments involving intact cells [16]. Although OA is generally considered a tumour-promoting agent, it has been shown to have tumour-suppressor activity [17], to block DNA synthesis in primary T-lymphocytes following mitogenic activation [18], and to inhibit growth-factor-mediated DNA synthesis in murine fibroblasts [15]. However, the mechanism of action of OA in this context is unknown. In this study we show

Abbreviations used: IL-2, interleukin-2; IL-2R, IL-2 receptor; MAP kinase, mitogen-activated protein kinase; PP, protein phosphatase; P_{Ser}, phosphoserine; P_{Tyr}, phosphotyrosine; P_{Thr}, phosphothreonine; OA, okadaic acid.

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that OA-mediated G_1/S block in activated human T-lymphoblasts is accompanied by decreased autocatalytic activity and hyperphosphorylation of *lck*.

EXPERIMENTAL

Materials

Human T-lymphoblasts isolated from peripheral blood were generously provided by Dr. Doreen Cantrell (ICRF, London) on a weekly basis. Purified recombinant human phosphatase type 1 (PP1 γ) [19,20] and bovine skeletal-muscle type 2A catalytic subunit (PP2Ac) were generously given by the groups of Professor Philip Cohen and Dr. Patricia Cohen (University of Dundee). Antisera to *lck* were kindly provided by Dr. Sigmund Fischer (ICGM, Paris) and Dr. Steve Ley (NIMR, London). Recombinant human IL-2 was obtained from Eurocetus, phorbol 12,13-dibutyrate was from Sigma, and OA and nor-okadaone were from Gibco-BRL. [3H]Thymidine and [^{32}P]P_i (8500–9120 Ci/mmol) were supplied by DuPont-NEN, and [γ - ^{32}P]ATP (5000 Ci/mmol) was from Amersham. All other reagents were of analytical grade.

Cell culture

Human T-lymphoblasts were expanded in culture in RPMI 1640 containing 10% (v/v) foetal-calf serum (Life Technologies) plus 20 ng/ml recombinant IL-2 at 37 °C in a humidified atmosphere of 5% CO₂ in air. For cell-cycle studies, cells were synchronized as described elsewhere [1]. Briefly, cells cultured in IL-2 were washed out of growth factor for 24 h and then treated with 50 ng/ml phorbol 12,13-dibutyrate for 2 h, and washed again to remove remaining phorbol 12,13-dibutyrate. Cells were then maintained in IL-2-free medium for a further 20 h before addition of IL-2 (20 ng/ml) to stimulate entry into the cell cycle.

Cells were washed after 6 or 7 h (see Figure legends for details), then returned to medium in the presence of IL-2, IL-2 + OA or IL-2 + nor-okadaone (negative control) as described in the Figure legends. Cells were maintained in these conditions for up to 4 h further. At appropriate time points, samples of cells were removed for determination of DNA synthesis by [3H]thymidine uptake as described previously [6]. Immunoprecipitation with polyclonal *lck* antibody, immune-complex kinase assays, metabolic labelling and two-dimensional phosphoamino-acid analysis were as described previously [6].

Immune-complex phosphatase assays

lck immunoprecipitates were collected from cells metabolically labelled with ^{32}P (see above), washed twice in 10 mM Tris/HCl/150 mM NaCl, pH 7.4, and then treated with purified PP1 or PP2A (~10 units per assay) at 30 °C for 30 min in 50 mM Tris/HCl (pH 7.0), 1 mM MnCl₂, 0.1% (v/v) mercaptoethanol, 0.1 mM EGTA and 1 mg/ml BSA in a final volume of 50 μ l. The reaction was terminated by the addition of SDS/PAGE sample buffer; proteins were resolved by SDS/PAGE, transferred to Immobilon-P by Western blotting and finally autoradiographed for 24 h at -70 °C. In control experiments, phosphatases were replaced by an equal volume of water.

Two-dimensional phosphoamino-acid analysis

lck immunoprecipitates were prepared as described from ^{32}P -labelled cells. After SDS/PAGE and Western blotting, *lck* protein bands were subject to two-dimensional phosphoamino-acid analysis as described previously [6]. Data were analysed quantitatively by using a Fuji Phosphorimager with MacBas software.

Two-dimensional phosphopeptide mapping

T-cells were metabolically labelled with ^{32}P as described above in the presence of IL-2 with either nor-okadaone (1 μ M or OA (1 μ M). Immunoprecipitates of *lck* were subjected to SDS/PAGE, transferred to Immobilon P and autoradiographed. The 56, 59 and 64 kDa bands were excised and digested with trypsin using 10 μ l of tosylphenylalanylchloromethane (TPCK)-trypsin (1 mg/ml) overnight at 37 °C. A further 10 μ l of TPCK-trypsin was added, and the digests were incubated for 3 h. The samples were briefly centrifuged, and the supernatants containing liberated peptides were retained, mixed with 100 μ l of 0.05 M NH₄HCO₃ and added to freshly prepared PA (900 μ l of formic acid, 100 μ l of 30% hydrogen peroxide). Insoluble peptides were repeatedly washed and dried, and then eventually resuspended in 10 μ l of pH 1.9 electrophoresis buffer (50 ml of 88% formic acid, 156 ml of acetic acid, 1794 ml of double-distilled water), spotted on TLC plates (loading 300 c.p.m. per sample) and run in the first dimension in a Hunter thin-layer electrophoresis unit HTLE 7000 (CBS Scientific) for 1 h at 1000 V and in the second dimension in a tank containing 187.5 ml of butan-1-ol, 125 ml of pyridine, 37.5 ml of acetic acid and 150 ml of distilled water for 16 h. Plates were dried and autoradiographed.

RESULTS

OA dose-dependently inhibits IL-2-mediated DNA synthesis and induces a mobility shift in *lck*

Activated human T-lymphoblasts were quiesced and synchronized by IL-2 deprivation and then stimulated to enter the cell cycle by addition of IL-2. To investigate the effect of inhibition of PP1 and PP2A on S-phase entry, OA was added to cultures containing IL-2 after 7 h and [3H]thymidine incorporation was measured at 10 h (see Figure 1 for the results of a

Figure 1 OA inhibits IL-2-mediated DNA synthesis

Activated T-lymphoblasts (IL-2R⁺) were quiesced by IL-2 deprivation and then incubated with IL-2, either alone or in the presence of nor-okadaone (NO) or OA as indicated. NO and OA were added to cultures at 7 h in the presence of IL-2, and DNA synthesis was measured at 0, 7 and 10 h as incorporation of [3H]thymidine (shown in d.p.m.), as judged by liquid-scintillation counting of radioactivity in triplicate samples.

Figure 2 Dose-response analysis of OA on DNA synthesis and *lck*

Activated human T-lymphoblasts were quiesced and then stimulated with IL-2 for 7 h, at which time OA was added to final concentrations of 0–1 μ M as indicated. DNA synthesis was measured as incorporation of [3 H]thymidine (as in Figure 1) at 10 h and is expressed as percentage of maximum. The lower panel shows a Western blot of *lck* immunoprecipitated from cell lysates prepared at 10 h, resolved by SDS/PAGE, transferred to Immobilon-P and probed for *lck* as described in the Experimental section. Detection was by enhanced chemiluminescence. The p56 and p59 forms of *lck* are indicated; lane C shows a peptide-pre-adsorbed antibody control.

typical experiment). In control experiments, an inactive methylated analogue of OA, nor-okadaone, was added at the same concentration at the same time. OA was observed to inhibit DNA synthesis completely, even when added after the onset of [3 H]thymidine incorporation. Nor-okadaone had no effect on the rate of DNA synthesis. This was a highly reproducible observation, although the time of start of DNA synthesis varied between 6 and 9 h.

Given that inhibition of DNA synthesis was obtained with 1 μ M OA (based on the findings of Amaral et al. [24]), we next examined the dose-sensitivity to OA in the range 1 nM to 1 μ M (Figure 2). Measurement of [3 H]thymidine uptake after 10 h stimulation with IL-2, during which time OA was added 3 h before assay, revealed that at 1 and 10 nM there was no decrease in incorporation of 3 H, but at 100 nM OA, DNA synthesis was partially inhibited, and at 1 μ M, DNA synthesis was totally blocked. Since we had previously shown that *lck* might play a role in G_1/S transition, we investigated the expression of *lck* protein isoforms in response to the same range of OA concentrations. *lck* was immunoprecipitated from cell lysates prepared from the same cultures used for DNA-synthesis studies, and detected by immunoblotting after SDS/PAGE and electrophoretic transfer to Immobilon-P. In cells which received only IL-2, *lck* appeared as the characteristic polypeptide doublet of 56

Figure 3 Analysis of autocatalytic activity and phosphorylation of *lck* de novo in response to OA

Activated T-lymphoblasts were quiesced and then stimulated as described in the legend to Figure 2. Cell lysates (3×10^7 cell equivalents) were prepared from quiescent cells, or after 6 or 10 h stimulation with IL-2, nor-okadaone or OA as indicated. For kinase assays, immune complexes formed using polyclonal anti-*lck* sera on Protein A-Sepharose beads were incubated with [γ - 32 P]ATP and then analysed by SDS/PAGE, Western blotting and autoradiography. In metabolic labelling studies, [32 P]P_i was added to cells 4 h before lysis and *lck* immunoprecipitates were resolved by SDS/PAGE, transferred to Immobilon-P and analysed by autoradiography. The positions of p56^{lck}, p59^{lck} and p64^{lck} are indicated.

and 59 kDa (p56^{lck} and p59^{lck}). At very low concentrations of OA (1 nM and 10 nM) there appeared to be a slight decrease in the intensity of p59^{lck}, but at 100 nM OA there was a decrease in p56^{lck} and a concomitant increase in p59^{lck}, which was extended at 1 μ M OA to the complete disappearance of p56^{lck}, with all protein in the 59 kDa form. These results show that loss of p56^{lck} occurs at the same concentrations of OA that inhibit IL-2-mediated DNA synthesis.

Hyperphosphorylation and inactivation of *lck* by OA

Since we had shown (Figure 2) that OA caused a mobility shift in *lck* from 56 to 59 kDa, we next examined the effects of OA on phosphorylation and activity of *lck*. T-lymphoblasts were cultured in the presence of 32 P, IL-2 and either nor-okadaone or OA, and *lck* was immunoprecipitated after 10 h (Figure 3). Consistent with our previous observations [6], the 56 kDa form of *lck* became phosphorylated in unstimulated cells, whereas both the 56 and 59 kDa forms became phosphorylated after stimulation with IL-2. However, in the presence of both IL-2 and OA, there was no detectable p56^{lck}, the p59^{lck} appeared more intensely labelled, and a 64 kDa form of *lck* was also present. In immune-complex kinase assays the 56 kDa form of *lck* showed increased autocatalytic activity after stimulation with IL-2, whereas in the presence of OA the 59 kDa form of *lck* exhibited little autocatalytic activity, comparable with that observed in unstimulated cells. These data suggest that increased phosphorylation of *lck* stimulated by OA causes a decrease in autocatalytic activity.

OA promotes serine and threonine phosphorylation of *lck*

Having shown that S-phase block induced by OA is accompanied by increased phosphorylation and decreased activity of *lck*, we further investigated the effect of OA on *lck* by phosphoamino-acid analysis (Figure 4). Although autoradiography showed phosphoserine (PSer) to be the major component of all samples, analysis of the results using a Phosphorimager revealed the presence of low levels of both phosphothreonine (PThr) and phosphotyrosine (PTyr) in some samples, allowing the quantitative calculation of phosphoamino-acid ratios. After 10 h

Figure 4 Two-dimensional phosphoamino-acid analysis of *lck*

T-lymphoblasts were metabolically labelled with [32 P]P_i as shown in Figure 3 before immunoprecipitation of *lck*. Protein bands corresponding to the 56 and 59 kDa forms of *lck* observed with IL-2 and nor-okadaone, and the 59 and 64 kDa forms observed with OA (examples shown in Figure 3), were excised from the blotting membrane and subjected to total acid hydrolysis as described in the Experimental section. Hydrolysates were resolved first at pH 1.9 and then at pH 3.5 as indicated before being revealed by autoradiography. The relative positions of PSer, PThr and PTyr are shown diagrammatically, as well as free phosphate (P_i) and partially digested peptides. The origin is marked with a cross. Phosphoamino-acid spots were also analysed quantitatively by using a Phosphorimager. This revealed PThr and PTyr levels undetected by autoradiography, and allowed phosphoamino-acid ratios to be determined as described in the text.

stimulation with IL-2, the ratio of PSer:PTyr for p56^{lck} (in the presence or absence of nor-okadaone) was 6:1, whereas the PSer:PTyr ratio for the 59 kDa protein was 12:1. There was no detectable PThr. In the presence of both IL-2 and OA the PSer:PTyr ratio for p59^{lck} was 18:1, with virtually identical amounts of PTyr and PThr. For the 64 kDa form of *lck* generated in the presence of OA, the PSer:PTyr ratio was approx. 17:1 and the PSer:PThr ratio was 5:1. These results show that OA stimulates a marked increase in the relative level of serine phosphorylation of *lck* with respect to tyrosine phosphorylation. OA also stimulated threonine phosphorylation of both p59^{lck} and p64^{lck} at similar levels to PTyr for the former and significantly higher levels for the latter. These data suggest that loss of autocatalytic activity of p56^{lck} induced by OA is linked primarily to increased serine phosphorylation, and possibly to a lesser extent threonine phosphorylation.

PP1 and PP2A reverse the effects of OA on *lck*

To establish whether the effects of OA on *lck* were due to inhibition of cellular PP1 and PP2A, we examined the sensitivity to purified phosphatases of *lck* immunoprecipitated from 32 P-labelled cells in an assay *in vitro* (Figure 5). The results showed that the two phosphatases were almost equally effective in dephosphorylating 32 P-labelled *lck* in an immune-complex phosphatase assay. However, in both cases there was greater residual *lck* phosphorylation in the samples isolated from cells treated with OA than from cells treated with nor-okadaone or IL-2 alone. Indeed, when the same membrane was investigated by immunoblotting, PP1 and PP2A converted virtually all the *lck* into the 56 kDa form, with the exception of the OA-treated samples, which persisted as a doublet of 56 and 59 kDa when treated with PP1. Although PP2A appeared slightly more effective in dephosphorylating *lck*, this is probably due to differences in catalytic activity of the purified proteins. These data confirm that hyperphosphorylation of *lck* caused by OA can be reversed by treatment with purified PP1 and PP2A.

Figure 5 Sensitivity of 32 P-labelled *lck* to treatment with PP1 and PP2A

T-lymphoblasts were stimulated with IL-2 in the presence or absence of nor-okadaone and OA as indicated. [32 P]P_i, nor-okadaone and OA were added to cultures after 6 h, and lysates ($\sim 3 \times 10^7$ cell equivalents) were prepared after a further 4 h. Immune complexes of *lck* were washed and then incubated with purified PP1 or PP2A as shown, then resolved by SDS/PAGE, transferred to Immobilon P and autoradiographed (upper panel). The same membrane was then immunoblotted for *lck* as described in Figure 2, and bands were revealed by using enhanced chemiluminescence (lower panel). The p56 and p59 forms of *lck* are indicated.

OA stimulates phosphorylation of *lck* at novel sites

To investigate further the possibility that OA might down-regulate *lck* activity by promoting phosphorylation at important, possibly novel, regulatory sites, *lck* was immunoprecipitated from 32 P-labelled cells after 10 h of stimulation with IL-2 (the final 3 h in the presence of radioisotope and either nor-okadaone or OA) and subjected to two-dimensional phosphopeptide analy-

Figure 6 Two-dimensional phosphopeptide analysis of *lck*

T-lymphoblasts were stimulated with IL-2 for 10 h; [^{32}P]P_i and either OA (1 μM) or nor-okadaone (1 μM) were added for the last 4 h. Cells were lysed, and *lck* was immunoprecipitated, resolved by SDS/PAGE and transferred to Immobilon-P. After autoradiography, *lck* bands were excised and digested with trypsin as described in the Experimental section. Tryptic phosphopeptides were subjected to electrophoresis in the first dimension (left to right) and ascending chromatography in the second (bottom to top). Major spots are numbered 1 to 11; spots indicated by arrows highlight differences between phosphopeptide profiles as discussed in the text. The origin is marked with a cross.

sis after digestion with trypsin (Figure 6). The simplest pattern of phosphopeptides was yielded by p56^{lck}, numbers 2, 4, 6 and 8 representing the major fragments. This pattern differed from that of p59^{lck} (in the presence of nor-okadaone), partly in the relative amounts of each phosphopeptide, but also in the presence of fragments 1 and 5 and another indicated in Figure 6 by an arrow.

Comparison of the 59 kDa forms of *lck* isolated from cells cultured in the presence of OA or nor-okadaone revealed that, despite having the same mobility on SDS/PAGE, these two proteins exhibited subtle differences in their phosphopeptide profiles. Fragment 3, and another indicated by an arrow (lower right corner, Figure 6) were present in the sample isolated from OA-treated cells. The p64^{lck} also isolated from these cells

displayed several unique fragments (all indicated by arrows). The other major difference was the substantial increase in intensity of phosphopeptide 11. From these tryptic phosphopeptide maps, it can be concluded that OA stimulates phosphorylation of *lck* both at novel sites and at increased levels at sites normally phosphorylated in response to IL-2.

DISCUSSION

The cell division cycle is regulated at three key checkpoints, G₁/S (initiation of DNA synthesis), G₂/M (entry into mitosis) and M to G₁ (exit from mitosis). The molecular events which enable cells to progress through the cell cycle are controlled in part by the co-

ordinated activity of protein kinases and phosphatases [21]. The involvement of protein tyrosine kinases in early signalling events is well established, and several non-receptor kinases, including *lck* and *fyn*, have been shown to act as critical signalling molecules which regulate lymphocyte development and proliferation [22]. We have previously shown that protein tyrosine phosphorylation *de novo* occurs as T-lymphoblasts progress from G₁ to S-phase in the presence of IL-2, and that *lck* is a major active tyrosine kinase during this stage of the cell cycle [6]. In this study we have shown that OA, a potent and specific inhibitor of PP1 and PP2A, both down-regulates the activity of *lck* and blocks S-phase entry in the presence of IL-2.

Examination of the dose-sensitivity to OA revealed that 1 μ M OA was required to block S-phase entry completely when added for 3 h. Although this is higher than concentrations normally used to inhibit PP1 and PP2A in cultured cells, the use of primary T-cells combined with the relatively short incubation time means that non-specific or cytotoxic effects are highly unlikely, consistent with findings of other workers [23,24]. Interestingly, the dose-sensitivity to okadaic acid associated with S-phase block was accompanied by a parallel change in the mobility of *lck* on SDS/PAGE, manifested as a gradual loss of p56^{lck} with the simultaneous appearance of p59^{lck}, the latter form of which has markedly decreased autocatalytic activity in immune-complex kinase assays. Amaral et al. [24] have reported that OA induced MAP kinase activity in a similar dose-dependent fashion in Jurkat cells, with an optimal response achieved at a concentration of 1 μ M. Given that phosphorylation of serine-59 of *lck* by MAP kinase causes a mobility shift to the less active 59 kDa isoform [5], it is possible that the mobility shift observed in Figure 2 is a consequence of activation of MAP kinase by OA. The observation that OA stimulates an alteration of the phosphorylation status of *lck* and subsequent change in autocatalytic activity of the tyrosine kinase (Figure 3) suggests that maintenance of kinase activity is dependent on the activity of protein phosphatases. Inhibition of PP1 and PP2A therefore results in hyperphosphorylation of *lck*, loss of the 56 kDa isoform and appearance of weakly active 59 and 64 kDa forms: whether this is a direct consequence of phosphatase inhibition, or a secondary effect perhaps due to activation of serine/threonine kinases, is unclear. Our data suggest not only that serine/threonine phosphatases may regulate *lck* tyrosine kinase activity, but also that this regulation is linked to growth-factor-mediated S-phase entry.

To address this question, we examined the sensitivity of *lck* from OA-treated cells to purified PP1 and PP2A. In an immune-complex phosphatase assay *in vitro*, both PP1 and PP2A effectively dephosphorylated *lck*. These observations suggest that the effects of OA on *lck* may indeed be a direct consequence of phosphatase inactivation. It is already well established that the tyrosine kinase activity of *lck* is normally suppressed *in vivo* by phosphorylation at tyrosine-505 [25]. Our results show that *lck* activity can also be suppressed by increased serine (and perhaps threonine) phosphorylation mediated by OA. To characterize the effects of OA on *lck* phosphorylation further, we performed phosphoamino-acid and tryptic-phosphopeptide analyses. The most striking effect of OA was the relative increase in serine phosphorylation and the presence of threonine phosphorylation, particularly on p59^{lck}. Although the p64^{lck} had a similarly high P_{Ser}:P_{Tyr} ratio, this form also contained a significant amount of P_{Thr}. The serine-phosphorylation results are in keeping with the findings of Luo and Sefton [26], who showed that phorbol ester stimulated phosphorylation of *lck*, yielding forms of approx. 56, 60 and 64 kDa. These authors also showed the presence of some P_{Thr}. In tryptic-phosphopeptide mapping experiments,

p64^{lck} revealed several fragments not present in either p59^{lck} (generated in the presence or absence of OA) or p56^{lck}. Equally significant was the increased phosphorylation of p64^{lck} (and to a lesser extent p59^{lck}) at a site (phosphopeptide 11) normally phosphorylated in IL-2-stimulated T-cells. Either this site or potentially novel sites could be important in down-regulating the activity of *lck*. It has also been reported that phorbol esters are able to induce distinct phosphorylated sites in the SH2 domain of p56^{lck} which may mediate interaction with other proteins or with other internal p56^{lck} domains [27]. This supports the suggestion that changes in *lck* phosphorylation observed in response to OA may compromise protein interactions, which in turn lead to failure to enter S-phase.

It is known that phosphatases play an important role in cell-cycle control; for example, PP2A modulates the activity of cyclin-dependent kinases [28]. In addition, the major enzyme activity in *Xenopus* egg extract that inactivated and dephosphorylated cdc25 was inhibited by OA [29], and a type 2A phosphatase has been implicated in the regulation of p34^{cdc2} histone H1 kinase [30]. Ishida et al. [31] demonstrated that OA could induce cell-cycle arrest in T-lymphocytes at either G₁/S or G₂/M, depending on the dose used. These authors showed that higher concentrations of OA (> 500 nM) could inhibit proliferation within 3 h, consistent with the findings reported in the present study. Our results show that, during G₁/S transition in activated human T-lymphoblasts, *lck* is a major target for OA, a known and established inhibitor of PP1 and PP2A, and that decreased activity of *lck* coincides with failure to enter DNA synthesis. A priority for future studies will be to define the phosphatase(s) involved in the regulation of *lck* activity and establish further the identity of proteins associated with *lck* at this stage of the cell cycle.

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