Characterization of the Mr 65,000 Lymphokine-activated Killer Proteins Phosphorylated after Tumor Target Binding: Evidence That pp65a and pp65b Are Phosphorylated Forms of L-Plastin1,2

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ABSTRACT

Contact between lymphokine-activated killer (LAK) cells and natural killer-resistant tumor targets SK-Mel-1 (human melanoma) or Raji (human lymphoma) stimulates phosphorylation of two Mr 65,000 LAK proteins (pp65a and pp65b) with nearly identical isoelectric points. Phosphoamino acid analysis of pp65a and pp65b detected phosphorylation exclusively on serine residues. Phosphotyrosine could not be detected on either substrate after immunoblotting with an antiphosphotyrosine antibody, and herbimycin A treatment failed to inhibit pp65 phosphorylation induced by target contact. However, phorbol myristate acetate treatment alone induced LAK pp65a and pp65b phosphorylation, suggesting phosphorylation may be mediated by protein kinase C or a protein kinase C-regulated kinase. The molecular weight and isoelectric points of pp65a and pp65b are similar to that reported for the human actin-bundling protein L-plastin (L-fimbrin). On two-dimensional SDS-PAGE gel immunoblots, a peptide specific anti-L-plastin antiserum bound to pp65a and pp65b, suggesting that the phosphoproteins are similar or identical to L-plastin. In addition, two adjacent Mr 65,000 LAK proteins were also detected by the antiserum and may correspond to unphosphorylated forms of L-plastin. On the basis of known properties of phosphorylated L-plastin, it is hypothesized that pp65 phosphorylation in LAKs may regulate adhesion to tumor targets.

INTRODUCTION

Coincubation between human LAKs4 and the NK-resistant tumor targets SK-Mel-1 (human melanoma) or Raji (human lymphoma) was previously shown to stimulate phosphorylation of two Mr 65,000 LAK proteins (pp65a and pp65b) with nearly identical isoelectric points on 2-D PAGE gels (1). Increased phosphorylation of these substrates in LAKs was found to require target cell contact and did not occur in response to soluble factors generated by tumors. Induction of LAK pp65 phosphorylation correlated with target recognition because forced contact with normal peripheral blood lymphocyte targets did not lead to increased phosphorylation. In addition, cross-linking CD16, the receptor that triggers antibody-dependent cellular cytotoxicity (an alternate cytotytic pathway), also increased pp65a and pp65b phosphorylation. Therefore, prior correlative evidence suggested that pp65 phosphorylation might have a role in the cytolytic function of LAKs.

In the present report, the kinase pathways responsible for pp65 phosphorylation in LAKs are further characterized. Using a highly specific antiserum, pp65a and pp65b are tentatively identified as phosphorylated forms of the actin-bundling protein L-plastin (L-fimbrin). On the basis of the known biochemical and functional properties of L-plastin and its phosphorylated forms, a hypothesis to explain the role of pp65 phosphorylation in LAK-mediated cytotoxicity is proposed.

MATERIALS AND METHODS

Cells and Reagents. Cell cultures were grown in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin, streptomycin, 2 mM glutamine, and 20 mM HEPES. CD3-CD56+ LAKs were prepared as described previously. Essentially, enriched CD3-CD56+ LAKs were generated from peripheral blood mononuclear cells according to the method described by Perussia et al. (2). Contaminating T cells were removed by adherence to anti-CD3 Micro-Celllector flasks (Applied Immune Sciences, Santa Clara, CA). Resulting effectors were >90% CD3-CD56+ and >80% CD16+ as confirmed by FACS analysis using a panel of mAbs (Becton Dickinson, Mountain View, CA), and exhibited potent lytic activity when tested against NK-resistant targets. The Burkitt B-cell lymphoma line Raji and the nonadherent human melanoma cell line SK-Mel-1 (Mel-1) were obtained from American Type Culture Collection. Both tumors are NK resistant but LAK sensitive. The EBV-transformed lymphoma RPMI 8866 used as feeder cells was kindly provided by Dr. T. Whiteside (Pittsburgh, PA). All tumor cell lines were Mycoplasma free.

In Vivo Phosphorylation. In vivo phosphorylation experiments were carried out as described previously (1). Briefly, CD3-CD56+ LAKs were prelabeled with 0.75 mCi/ml 32Pi and 1 mCi total L-35S methionine for 2 h at 37°C in phosphate- and methionine-deficient DMEM supplemented with 20 mM HEPES, 2% dialyzed fetal bovine serum, and 100 units/ml diacylated recombinant IL-2. Postlabeled LAKs were centrifuged at room temperature with an equal number of unlabeled Raji targets or one-half as many Mel-1 targets and incubated for various times at 37°C. Before addition of ice-cold stop buffer containing PBS and phosphatase inhibitors. In experiments examining the effect of protein tyrosine kinase inhibition on pp65 phosphorylation, herbi mycin A was added at a final concentration of 10 μg/ml for the full duration of the labeling reaction. Herbimycin A was prepared in advance by dissolving in DMSO at 1.4 mg/ml and was stored at −80°C until used. The final concentration of DMSO was 0.7% in all drug incubations and controls.

Cell Lysis and 2-D PAGE. Cell pellets were lysed in “urea mix” (9.0 M urea-4% NP40-2% DTT; Ref. 3) containing 2% ampholytes (80% pH range, 3.5–10; 10%, 2.5–4.0; and 10%, 5–8) and the following phosphatase inhibitors: 1 mM sodium orthovanadate; 2 mM EDTA; 10 mM sodium PP1; and 10 mM sodium fluoride. Where applicable, presolubilized tumor targets were added to control samples containing LAKs alone so that final composition of proteins was the same for all samples. Complete solubilization was achieved by magnetic spin vanes stirred overnight at room temperature. Nucleic acids were removed by ultracentrifugation at 150,000 × g for 2 h, and samples were stored at −80°C. Proteins were resolved by 2-D PAGE using an ISO-DALT system as described previously (1). After fixation (10% HAc-30% methanol), gels were either treated with En3Hance (DuPont) for fluorography or neutralized in 30% ethanol before they were dried and exposed to Kodak X-Omat XAR-5 film. To detect phosphoproteins only, a sheet of colored paper (black, red, or blue) enclosed in a plastic sheet protector was placed between the gel and film to selectively block 35S emissions. Intergel comparisons were made after equal film exposure times.

Image Analysis and Protein Quantitation. Autoradiographs were digitized using a Compaq 386/20 running a General Purpose Image Digitizing

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4The abbreviations used are: LAK, lymphokine-activated killer; IL-2, interleukin 2; NK, natural killer; 2-D PAGE, two-dimensional SDS-PAGE; PMA, phorbol myristate acetate; mAb, monoclonal antibody; ECL, enhanced chemiluminescence; PI, isoelectric point; PKC, protein kinase C.
System (4) interfaced to a Megavision 1024 × M Digitizer/Display with a Texas Instruments 1230 × 1024 by 8-bit CCD black and white camera. The method of protein (spot) quantitation has been described previously in detail (1). The net integrated optical density (I. O. D.) for each spot was calculated by the formula:

\[ \text{I.O.D.}_s = [\text{T.O.D.}_s - (\text{Area}_s \times \rho)] \]

where T.O.D._s stands for total optical density of a spot, and Area_s equals the area of a spot in pixels and is the average background gray value per pixel in the vertical neighborhood of the spot. Levels of 35S-labeled β- and γ-actin were quantitated with a Betascope (Betagen, Waltham, MA) and used to compare differences in protein load between samples.

Immuno blotting. Antiphosphotyrosine mAb PY20 was obtained from Transduction Laboratories (Lexington, KY), and isoform specific rabbit anti-human L-plastin antiserum R325.4 (5) was generously provided by Dr. P. Matsudaira (Cambridge, MA). Proteins resolved by 1-D or 2-D PAGE were transferred to nitrocellulose filters using a Trans-Blot Semidry electrophoretic apparatus (Bio-Rad, Richmond, CA). For detection of phosphotyrosine, filters were blocked for 1.5 h at room temperature with PBS containing 1% BSA and 0.1% Tween 20. Filters were then incubated with a 1:9000 dilution of antiphosphotyrosine mAb PY20 in blocking buffer for 1.5 h, washed 4 times in PBS containing 0.1% Tween 20, and then incubated with horseradish peroxidase-conjugated goat antimouse secondary antibody (Transduction Laboratories) for 1 h at 1:4000 in blocking buffer. After additional washes, bound secondary antibody was detected via the nonradioactive ECL system (Amersham, Arlington Heights, IL) using the manufacturer’s instructions. For detection of L-plastin, filters were blocked with PBS containing 5% nonfat dry milk before incubation for 1.5 h with a 1:1000 dilution of rabbit anti-L-plastin antiserum R325.4 in antibody dilution buffer containing PBS, 2.5% dry milk, and 0.05% Tween 20. Washes were performed with PBS containing 0.05% Tween 20, and bound primary antibody was detected by a 1-h incubation with a 1:4000 dilution of horseradish peroxidase goat antirabbit serum (Transduction Laboratories) in antibody dilution buffer. Immune complexes were visualized using the ECL nonradioactive detection system as above. Radiolabeled proteins were visualized before and after immunodepletion by prolonged exposure of blots to film.

Phosphoamino Acid Analysis. Phosphoamino acid analysis was carried out using the method described by Cooper et al. (6). Essentially, spots of interest identified by autoradiography were excised from dried gels, rehydrated, and eluted overnight in 50 mM ammonium bicarbonate (pH 7.6) containing 0.1% SDS and 5% β-mercaptoethanol. Eluted proteins were precipitated with trichloroacetic acid, resolubilized, and partially hydrolyzed by boiling in 5.7 M HCl for 60 min. Free phosphoamino acids were resolved by two-dimensional thin-layer electrophoresis.

Immune Complex Kinase Assays. After incubation with herbimycin A or solvent, LAKs were washed three times in PBS and resuspended in ice-cold lysis buffer [25 mM Tris-140 mM NaCl-1% NP40 (pH 7.5)] containing 5 mM EDTA, 0.2 mM orthovanadate, 10 mM NaF, leupeptin, aprotinin, and phenylmethylsulfonyl fluoride. Lysates were then stored at −80°C for kinetic assays, lysates were thawed on ice and clarified by centrifugation at 10,000 × g for 15 min at 4°C. Immunoprecipitations were performed in duplicate by preclearing equal amounts of LAK protein (~100 µg) with normal rabbit serum supplemented with 0.2 mM orthovanadate and once with Tris-buffered saline (pH 7.5), and were resuspended in kinase reaction buffer containing 25 mM HEPES, 100 mM NaCl, 10 mM MnCl₂, and 5 mM MgCl₂ (pH 7.5). The kinase reaction was performed for 15 min at room temperature in 60 µl of kinase buffer containing 1 µM ATP, 10 µCi of [32P]ATP, and 6 µg of enolase. Reaction products were eluted from immune complexes by boiling in 3× Laemmli buffer (1× SSC = 6% SDS, 0.18 M Tris, 30% glycerol, 6% β-mercaptoethanol) and electrophoresed on 9% SDS-polyacrylamide gels. Gels were fixed (30% methanol-10% HAc) overnight, dried, and exposed to X-ray film for autoradiography. Phosphorylation levels of reaction products were quantitated directly from gels with a Betascope.

RESULTS

Herbimycin A Does not Block p65 phosphorylation. The ability of the tyrosine kinase-specific inhibitor herbimycin A to block LAK p65 phosphorylation induced by tumor target contact was examined. Pretreatment of LAKs with 10 µg/ml herbimycin A for 2.5 h did not inhibit the phosphorylation of pp65a or pp65b induced by contact with Raji tumor cells (Fig. 1). Although the area of spot pp65b appeared slightly larger on the gel from control LAKs exposed to Raji (Fig. 1A, lower panel) when compared to pp65b from LAKs pretreated with herbimycin A, no quantitative difference existed between these spots when the vertical backgrounds were subtracted after computer-assisted image analysis (Fig. 1C). To confirm that the kinase inhibitor was working properly, in vitro kinase assays were performed with anti-p56 lck immune complexes derived from LAKs pretreated with herbimycin A. Three reaction products corresponding to p60ck, p56ck, and enolase (Fig. 1D) were visible after in vitro kinase reactions performed with immunoprecipitates isolated from LAKs treated with DMSO alone. When normal rabbit serum was substituted for anti-p56ck in the immunoprecipitation, no bands were visible after the in vitro kinase assay, confirming the specificity of the reaction. Pretreatment of LAKs with 10 µg/ml herbimycin A before immunoprecipitation with anti-p56ck decreased the phosphorylation of all three in vitro reaction products by greater than 50%, as measured with a Betascope.

Phosphotyrosine Is Not Detected on pp65a or pp65b. Although the experiment performed with herbimycin A suggested that de novo p65 phosphorylation did not involve a tyrosine kinase pathway, it was still possible that pp65a or pp65b contained constitutively phosphorylated tyrosine residues. Immunoblotting was used to examine whether pp65a and pp65b contained phosphotyrosine. After stimulating 32P-labeled LAKs for 5 min with Raji, proteins resolved by 2-D PAGE were transferred to nitrocellulose, and phosphoproteins were detected by autoradiography (Fig. 2A). Proteins on the same blot reacting with an antiphosphotyrosine mAb PY20 (Fig. 2B) were then visualized using the ECL nonradioactive detection system and film exposure times less than 1 h. Before immunodetection, no phosphoproteins were visible by autoradiography with film exposure times under 1 h; thus, proteins detected by PY20 were easily distinguished from radiolabeled proteins. Both pp65a and pp65b were clearly visible by autoradiography (Fig. 2A), but phosphotyrosine was not detected on either substrate after a 10-min ECL exposure (Fig. 2B), or even upon longer ECL exposure times. The adjacent marker phosphoproteins “X,” “Y,” and “Z” were not apparent by autoradiography in Fig. 2A and were occasionally absent in other experiments as well, possibly because constitutive phosphorylation of these proteins during the labeling reaction was insufficient. Nevertheless, pp65a and pp65b were easily identified on the basis of molecular weight, pl, and induction by tumor contact. A number of proteins, including those visible in Fig. 2B, reacted with PY20 a mAb.

Phosphoamino Acid Analysis. Because herbimycin A did not prevent p65 phosphorylation, it seemed likely that the de novo phosphorylation was occurring either on Ser or Thr. Spots corresponding to pp65a and pp65b were excised from gels and subjected to phosphoamino acid analysis. Spot pp65b contained only phosphoserine (Fig. 3). Similarly, phosphorylation was detected exclusively on serine residues for pp65a as well (data not shown). Although we cannot completely exclude the possibility that other residues were phosphorylated and not detected due to limiting amounts of material, serine appears to be the major residue phosphorylated on pp65a and pp65b.

Phorbol Ester Induces p65 Phosphorylation. Data from phoshoamino acid analysis, immunodetection, and pharmacological studies using herbimycin A all suggested that a Ser kinase was responsible for phosphorylation induced by tumor target contact.
for p65 phosphorylation in LAKs. Consequently, the ability of PMA, a direct activator of PKC, to stimulate LAK p65 phosphorylation in the absence of tumor targets was examined. A 10-min treatment of LAKs with 100 ng/ml of PMA dramatically increased pp65a and pp65b phosphorylation when compared to treatment with solvent alone (Fig. 4). Levels of p65 phosphorylation after PMA treatment were determined to be at least 100-fold higher when compared to LAKs treated with solvent alone (Fig. 4C).

Both pp65a and pp65b React with an Antiserum Specific for L-Plastin. The apparent molecular weight, isoelectric point, and phosphorylation characteristics of pp65a and pp65b suggested these substrates might be identical to L-plastin, a Mr 65,000–68,000 actin-bundling protein that becomes phosphorylated in leukocytes after a variety of stimuli (7–10). Matsudaia et al. (5) described an antiserum highly specific for L-plastin that was generated against a synthetic peptide containing unique NH²-terminal sequences. The immunoreactivity of this antiserum is demonstrated in Fig. 5. Total cellular lysates from HeLa cells, heterogeneous 5-D LAK cultures, or enriched CD3-CD56+ LAKs were resolved by SDS PAGE on 1-D gels, transferred to nitrocellulose, and detected with anti-L-plastin antiserum. A single band with an apparent molecular weight of Mr 65,000 was detected in lysates derived from all three cell types. No bands were visible when control blots were reacted with normal rabbit serum alone (data not shown). To determine whether pp65a and pp65b reacted with anti-L-plastin antiserum, LAKs prelabeled with [35S]methionine and [32P]P1 were stimulated with Raji targets for 5 min, and solubilized proteins resolved by 2-D PAGE were transferred to nitrocellulose. Phosphoproteins from Raji-stimulated LAKs were detected...
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Fig. 2. Phosphotyrosine is not detected on pp65a or pp65b. LAKs prelabeled with [%32P]P were coincubated with Raji cells, and proteins resolved by 2-D PAGE were transferred to nitrocellulose. LAK phosphoproteins from the same blot were visualized first by autoradiography (A) and then by ECL after immunodetection with antiphosphotyrosine mAb PY20 (B). Spots a and b correspond to pp65a and pp65b, respectively. Gaussian positions corresponding to pp65a and pp65b on the immunoblot. Marker spots X, Y, and Z were not visible in the experiment shown. kD, molecular weight in thousands.

Fig. 3. Spot pp65b contains phosphosorine. [%35S]Labeled LAKs were coincubated with Raji cells for 5 min, and proteins were resolved by 2-D PAGE. Spots corresponding to pp65b were excised, eluted, and TCA precipitated. Subsequently, pp65b was hydrolyzed, and phosphoamino acids resolved by twodimensional thin layer electrophoresis were detected by a PhosphorImager. Positions of phosphoamino acid standards were visualized with ninhydrin. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

DISCUSSION

Previously, we reported that tumor-target binding induces phosphorylation of two Mr 65,000 proteins in LAKs (1), pp65a and pp65b. The apparent molecular weight, pf, and phosphorylation properties of these two LAK proteins suggested that they might be related to the actin-bundling protein L-plastin. In the present report, we tested whether pp65a or pp65b resolved by 2-D PAGE cross-reacted with a polyclonal rabbit antiserum specific for L-plastin. The antiserum reacted with both Mr 65,000 phosphoproteins, suggesting that they are similar or identical to L-plastin. In addition, two [%35S]-labeled proteins that migrated in the immediate vicinity of pp65a and pp65b were also detected by the antiserum and most likely represent unphosphorylated forms (or apoproteins) of L-plastin.

Because an allelic charge variant of L-plastin occurs frequently in humans (7, 8), pp65a and pp65b could represent distinct electrophoretic phosphoisoforms encoded by these alleles. The two unphosphorylated proteins that also reacted with anti-L-plastin antiserum would then be their corresponding apoproteins. In heterozygous individuals, the different electrophoretic isoforms of L-plastin focus as four closely spaced spots with slightly different pfS and nearly identical molecular weights when resolved by 2-D PAGE (8). The most acidic of these spots is the phosphorylated form of normal L-plastin, which is followed in order of increasing pf by the normal apoprotein, the phosphorylated variant protein, and the variant apoprotein. An identical pattern of alternating phosphorylation was also observed for the Mr 65,000 LAK proteins detected by the anti-L-plastin antiserum. Moreover, all four Mr 65,000 LAK proteins focus at the same positions relative to β- and γ-actin that have been observed for the four electrophoretic isoforms of L-plastin from heterozygous individuals on 2-D gels.

Although originally characterized as a Mr 66,000 molecule (9), molecular weights ranging from Mr 64,000 to 68,000 have been reported for normal L-plastin (8—12). The mobility of variant L-plastin appears slightly increased relative to normal L-plastin when resolved by 2-D PAGE (8). Consistent with this finding, pp65b and pp65b also had increased mobility relative to pp65a and pp65a in some experiments (i.e., Fig. 6). Thus, in addition to cross-reacting with a specific antiserum, pp65a and pp65b had the same appearance and
Phosphorylation of L-plastin occurs exclusively on Ser residues (11, 13, 14). In agreement with these findings, serine was the only residue found to be phosphorylated on pp65a and pp65b by phosphoamino acid analysis. Furthermore, tyrosine phosphorylation could not be detected on either pp65a or pp65b by immunoblotting. PMA alone was a potent stimulator of p65 phosphorylation in LAKs, suggesting that PKC or a PKC-regulated kinase might be responsible for the phosphorylation induced by tumor-target binding. A concomitant decrease in the relative amount of 35S-labeled p65a or pp65b on gels also occurred after PMA treatment, consistent with the hypothesis that these proteins are unphosphorylated forms of pp65a and pp65b, respectively. PMA is also known to stimulate L-plastin phosphorylation in leukocytes (13, 15), further strengthening the argument that pp65a and pp65b are identical to L-plastin. Moreover, the PTK inhibitor herbimycin A does not block phosphorylation of L-plastin induced by chemotactic factors in polymorphonuclear leukocytes (15). Similarly, herbimycin A did not inhibit LAK p65 phosphorylation induced by tumor targets. Thus, both L-plastin and p65 phosphorylation are similar in that protein tyrosine kinases are unlikely to be involved. Presently, the kinase responsible for plastin phosphorylation is unknown, but it is unlikely to be directly mediated by PKC because L-plastin is not a substrate for this kinase in vitro (15).

Increased phosphorylation of pp65a and pp65b in LAKs was shown previously to correlate with tumor-target binding and to occur after cross-linking FcγIIIa receptors that trigger an alternate antibody-dependent cytotoxic pathway expressed by LAKs (1). Thus, circumstantial evidence suggests that p65 phosphorylation may have a role in LAK-mediated cytotoxicity. Assuming that p65 is similar or identical to L-plastin, a possible role for pp65a and pp65b in LAK-mediated cytotoxicity can be postulated based on the known properties of plastins.

Fig. 4. PMA stimulates LAK p65 phosphorylation. Prolabeled LAKs were stimulated for 10 min at 37°C with either 0.1% DMSO solvent (A) or 100 ng/ml PMA (B), and proteins resolved by 2-D PAGE were detected as in Fig. 1. The same gel pair was exposed to film by two methods, producing autoradiographs of proteins labeled with 35S and/or 32P (top panels) or 32P alone (bottom panels). Spots labeled a and b correspond to pp65a and pp65b, respectively. Spots labeled α and β correspond to unphosphorylated proteins designated p65a and pp65b, respectively, which decreased in intensity after PMA treatment. kD, molecular weight in thousands.

Fig. 5. Detection of L-plastin in human LAKs by immunoblotting. Total cellular protein (0.5 × 10⁶ cell equivalents) from HeLa cells, 5-day-old heterogeneous LAK cultures, and highly purified CD3-CD56+ LAKs were resolved by 1-D SDS-PAGE, transferred to nitrocellulose, and detected with rabbit anti-L-plastin R325.2 antiserum (1:1000) as described in "Materials and Methods." kD, molecular weight in thousands.
Plastins (fimbrins) are a family of homologous genes that encode actin-bundling proteins (16). Although L-plastin was first identified as a protein unique to human cancer cells based on comparative 2-D gel analysis, it was later determined to be an abundant protein in freshly isolated T cells, NK cells, and other normal leukocytes (8). Currently, three genes encoding distinct isoforms of human plastin have been identified and are expressed in a somewhat tissue-specific manner (5, 17). L-plastin is expressed exclusively in leukocytes and is the only form known to become phosphorylated. The other two isoforms, T- and I-plastin, are not detected in leukocytes. All plastins have three functional domains consisting of two putative calcium-binding sites, a putative calmodulin-binding site, and two tandem actin-binding domains that probably permit actin cross-linking (5, 18, 19). In addition, all three plastin isoforms are known to bundle actin filaments in vitro (16).

A variety of agents are capable of stimulating L-plastin phosphorylation in leukocytes, including IL-1, IL-2, tumor necrosis factor, lipopolysaccharide, IL-8, chemotactic factors, and PMA (10–12, 20, 21). All of these factors are also capable of altering the adhesive properties of cells. Moreover, in adherent macrophages, most of the phosphorylated form of L-plastin is found in the insoluble cytoskeleton fraction (13), which consists primarily of proteins found in podosomes (points of tight contact between the plasma membrane and the substratum). On the basis of the macrophage data, Matsudaira (16) has proposed that L-plastin may have a role in cell adhesion. Cell adhesion is an important step in the killing process mediated by LAKs, as well as other cytotoxic lymphocytes. When viewed by scanning electron microscopy, LAKs appear attached to tumor cells through pseudopod-like extensions (22, 23). By transmission electron microscopy, LAKs appear bound to tumor targets via tight plasma membrane bonds, including numerous plasma membrane interdigitations (22). In addition, broad LAK cell processes are observed protruding into the cytoplasm of targets (23). Possibly, phosphorylation of L-plastin could regulate adhesion of LAKs to tumor targets.

Because staurosporine inhibits p65 phosphorylation without affecting the number of LAK-tumor target conjugates formed (1), it is unlikely that p65 phosphorylation is involved in initial adhesion of LAKs to tumor cells. However, binding of lymphocytes to target cells is probably a multistep process that involves signal transduction and several states of adhesion (24, 25). Models proposed for T-cell bind-
ing (24, 25) may explain the way LAKs interact with tumor cells. In such models, initial binding occurs through low-affinity interactions between relatively large integrins and their ligands, establishing points of contact that still permit lateral diffusion of smaller membrane proteins such as CD2 (or other signaling receptors) so that they find their perspective ligands. Once triggering molecules are engaged, second messenger generation can lead to phosphorylation of proteins such as LFA-1, which strengthen binding. Possibly, establishment of tight plasma membrane bonds, plasma membrane interdigitations, or protrusion of LAK cell processes into the cytoplasm of targets could depend on signal transduction and p65 phosphorylation. Examination of the ultrastructure of LAKs bound to tumor cells under conditions that inhibit p65 phosphorylation would help to clarify this possibility.

Phosphorylation of other actin-cross-linking proteins has been shown to inhibit their actin-bundling properties (18). A mechanism has been proposed (19) that might explain how phosphorylation of L-plastin could change its association with actin. It has been demonstrated in vitro that the ability of L-plastin to bundle actin filaments is inhibited by increasing concentrations of Ca$^{2+}$ (26). Because the serine residue phosphorylated on L-plastin resides in the first Ca$^{2+}$-binding domain (19), phosphorylation could affect affinity of L-plastin for Ca$^{2+}$ and influence the actin-bundling properties of plastin.

Currently, it is not known which LAK receptors are involved in p65 phosphorylation induced by tumor target binding. Although no antigen-specific receptors have been identified for LAKs, considerable evidence suggests that LAKs utilize some of the same general cell adhesion molecules used by NK and T cells when binding targets. The cell adhesion molecules LFA-1 and CD2 are present on the majority of LAKs, and antibodies to these molecules inhibit LAK killing (27, 28). Some of these adhesion molecules probably contribute to signal transduction as well (14, 29). In NK and T-cells, antibodies to CD2 reportedly induce phosphorylation of a Mr 67,000 protein that has also been tentatively identified as L-plastin (14), based on cross-reactivity and participation in cytolysis of L-plastin for Ca$^{2+}$ and influence the actin-bundling properties of plastin.

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REFERENCES

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