Lymphocyte Function-associated Antigen-1-mediated T Cell Adhesion Is Impaired by Low Molecular Weight Phosphotyrosine Phosphatase-dependent Inhibition of FAK Activity*

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Protein tyrosine phosphorylation is one of the earliest signaling events detected in response to lymphocyte function-associated antigen-1 (LFA-1) engagement during lymphocyte adhesion. In particular, the focal adhesion kinase p125FAK, involved in the modulation and rearrangement of the actin cytoskeleton, seems to be a crucial mediator of LFA-1 signaling. Herein, we investigate the role of a FAK tyrosine phosphatase, namely low molecular weight phosphotyrosine phosphatase (LMW-PTP), in the modulation of LFA-1-mediated T cell adhesion. Overexpression of LMW-PTP in Jurkat cells revealed an impairment of LFA-1-dependent cell-cell adhesion upon T cell receptor (TCR) stimulation. Moreover, in these conditions LMW-PTP causes FAK dephosphorylation, thus preventing the activation of FAK downstream pathways. Our results also demonstrated that, upon antigen stimulation, LMW-PTP-dependent FAK inhibition is associated to a strong reduction of LFA-1 and TCR co-clustering toward a single region of T cell surface, thus causing an impairment of receptor activity by preventing changes in their avidity state. Because co-localization of both LFA-1 and TCR is an essential event during encounters of T cells with antigen-presenting cells and immunological synapse (IS) formation, we suggest an intriguing role of LMW-PTP in IS establishment and stabilization through the negative control of FAK activity and, in turn, of cell surface receptor redistribution.

Lymphocytes have a dual function that requires them to circulate in a nonadhesive form through blood and lymph and become adherent when they have to interact with endothelial cells for transmigration into sites of inflammation or infection, to stabilize cell-cell contact with antigen-presenting cells (APCs)‡ for the establishment of a proper immune response, or to act as effector cells to lyse their targets (1). The integrin family of cell surface receptors plays a key role in mediating these cellular events. In particular, the main receptor responsible for the regulation of T cell adhesion is the β2 integrin LFA-1 (lymphocyte function-associated antigen-1 or CD11a/CD18), which mediates adhesive phenomena through interaction with its counter-receptors, intercellular adhesion molecule-1 (ICAM-1 or CD54), ICAM-2 (CD102), or ICAM-3 (CD50) (2, 3). To avoid an improper activation of LFA-1, its functional state is tightly regulated (4). LFA-1 is indeed not constitutively active on T cells but becomes firmly able to bind its ligands after the engagement of particular membrane receptors, such as the antigen-specific T cell receptor (TCR) and CD2, or by means of cytokine and chemokine stimulation (5, 6). Growing evidence demonstrates that such stimuli result in the generation of poorly understood intracellular signals that lead to an increased avidity of LFA-1 as a result of an enhanced clustering at the cell surface. How the signal transduction pathways involved in this “inside-out” signaling modify the adhesive state of LFA-1 is not yet clarified. However, compelling evidence indicates that cytoskeleton rearrangement is an event intimately involved in the control of the LFA-1 functional state. Indeed, under resting conditions, LFA-1 is kept inactive by means of tight interactions of its cytoplasmic region with the actin cytoskeletal network below the plasma membrane (7, 8). The disruption of these cytoskeleton restraint transiently releases LFA-1 molecules from their inhibitory connections and leads to the formation of integrin clusters with an increased ligand binding activity.

The signaling pathways triggered by ligand engagement of LFA-1 have not been so far well elucidated. However, a variety of intracellular signals arising from the switch on of the receptor have been characterized, including phosphorylation of phospholipase Cγ, protein kinase C activation, mobilization of intracellular Ca2+, and activation of several tyrosine kinases, such as the Syk kinase ZAP-70 and the Src family kinases, both responsible for the triggering of a cascade of tyrosine phosphorylation events (9–12). Recently, it has been demonstrated that a key step of LFA-1 signaling is the tyrosine phosphorylation and activation of the focal adhesion kinase p125FAK, which in turn is responsible for the actin cytoskeleton reorganization (13–15). Fak is a nonreceptor tyrosine kinase that co-localizes with integrins to focal adhesion (16, 17). FAK autophosphorylation at Tyr-397 is an essential step of kinase activation, because this phosphorylated residue constitutes a docking site for the SH2-dependent binding of the p60Src kinase (18). The FAK/Src signaling complex leads to an

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‡ The abbreviations used are: APCs, antigen-presenting cells; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; FCS, fetal calf serum; HUVECs, human umbilical vein endothelial cells; ICAM, intercellular adhesion molecule; IS, immunological synapse; LFA-1, lymphocyte function-associated antigen-1; LMW-PTP, low molecular weight phosphotyrosine phosphatase; TCR, T cell receptor; (m)Abs, (monoclonal) antibodies; SMAC, supramolecular activating complex; PBS, phosphate-buffered saline.

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increase of FAK catalytic activity and to Src-mediated phosphotyrosine phosphorylation of additional tyrosines on FAK (19). These phosphotyrosines act as specific docking sites for several signaling proteins, including Fyn, Csk, Grb2, and PI3K, and for cytoskeletal proteins such as paxillin and tensin, which are also potential targets for FAK phosphorylation (20). It is very likely that the role of FAK during LFA-1 signaling might be related to its ability to bind and activate a number of these signaling and cytoskeletal proteins and, thereby, orchestrating cytoskeleton remodeling upon the receptor engagement.

Recent findings propose a further role for FAK in regulating changes in cell morphology such as T cell polarization (21, 22). Lymphocyte polarization is a key feature of T cells observed during chemokine-directed transendothelial migration as well as following the interaction with APCs (23). In particular, it is well documented that T cell recognition of APCs is accomplished by the dynamic redistribution of several cell surface receptors toward the cell-cell interface, forming a specialized junction referred to as the immunological synapse (IS) (24, 25). Recent evidence indicates that the remodeling of actin cytoskeleton is intimately involved in T cell/APC conjugate formation (26). Indeed, both the actin cytoskeleton and the actin-based myosin motors contribute toward an early receptors reorientation and concentration (“capping”) into the cell-cell interface (27).

Because tyrosine phosphorylation is a critical event in integrin-mediated signal transduction, we investigated the role of a phosphotyrosine phosphatase, namely low molecular weight phosphotyrosine phosphatase (LMW-PTP) in the modulation of T cell adhesion. LMW-PTP is an enzyme widely distributed in mammalian tissues (28), which is able to control cell adhesion through the regulation of the cytoskeleton reassembly. In particular, Rigacci et al. (29) have recently demonstrated that, upon β3 integrin engagement, LMW-PTP is able to move toward the cytoskeleton, where it physically associates and dephosphorylates FAK, thus leading to an impairment of focal contacts formation and to an increase of cell motility.

Experimental evidence recently obtained in T cells indicates that LMW-PTP is also involved in the signaling triggered by antigen stimulation (30, 31). In particular, LMW-PTP plays a positive role in TCR signaling by preferentially dephosphorylating the negative regulatory Tyr-292 of ZAP-70, thereby inducing a relevant increase of kinase activity (32).

Herein, we show that the overexpression of LMW-PTP in Jurkat cells causes an impairment of the LFA-1/ICAM-1-dependent T-cell adhesion. This effect is very likely mediated by the LMW-PTP-dependent control of FAK activity. In fact, we demonstrate that upon antigen stimulation the phosphatase induces a dramatic decrease of FAK functional state, through the association and dephosphorylation of the kinase. Moreover, the LMW-PTP-dependent down-regulation of FAK activity leads to a remarkable reduction of both LFA-1 and TCR clustering toward a single region of T cell surface. According to these findings, we propose (i) a novel role for FAK as a crucial mediator of receptors relocalization and (ii) a possible involvement of LMW-PTP in the regulation of T cell-cell adhesion, through the control of TCR and LFA-1 redistribution.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Unless specified, all reagents were obtained from Sigma. The stimulatory anti-CD3 monoclonal antibodies (mAbs) were purified from OKT3 hybridoma culture medium. The blocking monoclonal Abs (mAbs) specific for LFA-1α (mouse IgG2a anti-CD11a, clone R7.1), ICAM-1 (mouse IgG2a, MAB2146Z), and ICAM-3 (mouse IgG1, clone CBR-IC3/1) were from Chemicon. FAK polyclonal Abs (C-20) were from Santa Cruz Biotechnologies. The anti-phospho-Src (Tyr-416) and phospho-ERK Abs were from Cell Signaling. The anti-LFA-1 (C-17) and anti-CD3 (CD3-ζ, 6B10.2) Abs for immunofluorescence analysis were from Santa Cruz Biotechnologies.

Cell Cultures and Transfection—Jurkat E6.1 cells were purchased from the European Collection of Cell Culture and routinely cultured in RPMI supplemented with 10% fetal calf serum (FCS) in 5% CO2 humidified atmosphere. 10 μg of pB人家Wm-wt-LMW-PTP or pB人家WmC-CD3 LMW-PTP were transfected in Jurkat cells by electroporation (32). Mock-transfected cell lines were obtained by transfecting 2 μg of pB人家WmCneo alone. Stable transfected clonal cell lines were isolated by selection with G418 (1 mg/ml). All the experiments were performed on clones expressing comparable levels of the protein and estimated to be about ten times the level of the endogenous protein.

Jurkat immortal umbilical vein endothelial cells (HUVECs) were obtained from BioWhittaker and cultured according to BioWhittaker instructions. Hybridoma OKT3-producing Abs against CD3 were purchased from the American Type Culture Collection and cultured in Iscove's modified Dulbecco's medium (BioWhittaker) supplemented with 2 mM glutamine and 20% FCS.

T Cell Aggregation Assay—Homotypic aggregation of Jurkat cells was induced by promoting LFA-1 activation by means of TCR stimulation. Briefly, T cells were washed once in RPMI and resuspended in fresh medium at a concentration of 5 × 10⁶ cells/ml. Cells were then stimulated for 20 min at 4 °C with 10 μg/ml anti-CD3 Abs, followed by a 1-h incubation at 37 °C in the presence of rabbit anti-mouse Abs (25 μg/ml) to block FAK phosphorylation. Jurkat cells suspended in complete medium were stimulated with anti-CD3 Abs as previously described, added to HUVEC monolayers (2.5 × 10⁶ cells in 500 μl/well), and incubated with target cells. Unbound cells were removed by aspiration, and the wells washed with PBS. Adherent cells were then solubilized in 0.2 N NaOH, and radioactivity was determined by liquid scintillation counting.

Immunoprecipitation and Western Blot Analysis—Jurkat cells (5 × 10⁶ cells/ml) were cross-linked with anti-CD3 mAbs as previously described, with or without pretreatment with anti-LFA-1-blocking mAbs. The stimulation was terminated by harvesting and solubilizing the cells for 15 min in 0.5 ml of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM NaF, 1% Triton X-100, 2 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). For each Western blot experiment, 25 μg of total proteins was used. For immunoprecipitation analysis, each sample (1 mg/ml) was immunoprecipitated with 2 μg of anti-FAK Abs. Immunocomplexes were then separated by SDS-PAGE and transferred onto nitrocellulose. Immunoblots were incubated in 3% bovine serum albumin, 10 mA Tris-HCl, pH 7.5, 1 mA EDTA, and 0.1% Tween 20, for 1 h at room temperature, probed with specific antibodies, and developed with the Enhanced Chemi-Luminescence kit. Densitometric analysis of the results was performed with Chemidoc QuantiCount Analysis Software (Bio-Rad).

Immunofluorescence Microscopy Analysis—Stably transfected Jurkat cells were washed once in RPMI, resuspended in fresh medium at a concentration of 5 × 10⁵ cells/ml, and allowed to adhere to 20 μg/ml poly-l-lysine-coated coverslips. Cells were then incubated for 20 min at 4 °C with 10 μg/ml anti-CD3 Abs, followed by a 1-h incubation at 37 °C with rabbit anti-mouse Abs (25 μg/ml), in the absence or presence of 10 μg/ml anti-LFA-1-blocking mAbs. Cells were then washed once with PBS, fixed in 3% paraformaldehyde for 20 min at 4 °C, permethylated with three washes with TBST (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Triton X-100) and finally blocked with 5.5% horse serum in TBST for 1 h at room temperature. Cells were incubated overnight at 4 °C with anti-LFA-1 and anti-CD3 Abs diluted 1:100 in TBS (50 mM Tris-HCl pH 7.4, 150 mM NaCl). Cells were then washed once with TBST and once with TBST plus 0.1% bovine serum albumin and incubated for 1 h at room temperature with fluorescein or rhodamine-conjugated secondary Abs in TBST with 3% bovine serum albumin. After extensive washes in TBST, cells were mounted with glycerol plastine and ob-
FIG. 1. LMW-PTP affects LFA-1-mediated cell-cell adhesion. A, wt-LMW-PTP-, C12S-LMW-PTP-, and mock-transfected Jurkat cells were plated at a density of $5 \times 10^5$ cells/ml in fresh medium and grown for 24 h. Cells were then treated (right panels) or not (left panels) with 10 μg/ml anti-LFA-1-blocking mAbs for 30 min, before taking photographs with a phase-contrast microscope. This experiment is representative of three others with similar results. Bar, 100 μm. B, wt-LMW-PTP-, C12S-LMW-PTP-, and mock-transfected Jurkat cells were washed once in RPMI and resuspended in fresh culture medium at a concentration of $5 \times 10^5$ cells/ml. Cells were then incubated for 20 min at 4 °C with 10 μg/ml anti-CD3 mAbs (OKT3) and then crosslinked with 25 μg/ml rabbit anti-mouse Abs for 1 h at 37 °C. Photographs were taken under a phase-contrast microscope. The result is representative of three independent experiments. Bar, 100 μm.
served under a laser scanning confocal microscope (Bio-Rad MRC 1024 Es, Hercules, CA). Superimposition analyses were performed with Confocal Assistant version 4.02.

RESULTS

LMW-PTP Is Involved in the Inhibition of T Cells Homotypic Aggregation—The signaling pathways starting from LFA-1 engagement are not yet very well understood. However, the central role of tyrosine phosphorylation events triggered by several tyrosine kinases upon integrin activation is largely demonstrated (9). In this context, we investigated the role of LMW-PTP in the modulation of the signal transduction arising from LFA-1 activation and in the control of its functional state. Jurkat cells were transfected with both the active form of the enzyme (wt-LMW-PTP) and the dominant negative mutant (C12S-LMW-PTP). The dominant negative form of LMW-PTP, carrying the substitution of the catalytic cysteine 12 with a

FIG. 2. ICAM-1-dependent cell adhesion is impaired by LMW-PTP overexpression. wt-LMW-PTP-, C12S-LMW-PTP-, and mock-transfected Jurkat cells were resuspended in complete culture medium at a concentration of 5 × 10⁵ cells/ml. Following a 20-min incubation at 4 °C with 10 μg/ml anti-CD3 mAbs, cells were cross-linked with 25 μg/ml rabbit anti-mouse Abs for 1 h at 37 °C, with or without pretreatment with 10 μg/ml anti-ICAM-1 or anti-ICAM-3-blocking mAbs. Photographs were taken under a phase-contrast microscope. The result is representative of at least three independent experiments. Bar, 80 μm.

FIG. 3. T cell adhesion to TNFα-activated HUVEC monolayers is negatively affected by LMW-PTP. 6 × 10⁵ wt-LMW-PTP-, C12S-LMW-PTP-, and mock-transfected cells/ml labeled overnight with [2-3H]glycerol were incubated in complete medium for 15 and 60 min with HUVEC monolayers activated by means of a 4-h incubation with TNFα (10 ng/ml) (see “Experimental Procedures” for detail). After washing with PBS, adherent cells were solubilized in NaOH, and radioactivity was evaluated by liquid scintillation counting. The values are normalized on the basis of the radioactivity incorporated by each clone during the overnight labeling. Results represent the average of three independent experiments.
serine residue, is still able to bind the substrate, but it is catalytically inactive. The observation of the stable transfected cells reveals that wt-LMW-PTP- and C12S-LMW-PTP-expressing cells displayed important changes in their adhesive properties with respect to mock-transfected ones. In particular, as shown in Fig. 1A (left panels), overexpression of wt-LMW-PTP causes an impairment in the ability of T cells to establish cell-cell contacts even in resting condition. On the contrary, the inactive mutant C12S-LMW-PTP induces the opposite effect, showing a remarkable propensity to homotypic aggregation, even in the absence of any specific stimulation. The analysis of several different wt-LMW-PTP- and C12S-LMW-PTP-transfected cell clones for their phenotypic properties led to the same results, thus excluding the clonal specificity of the observed phenomenon. The treatment with anti-LFA-1-specific blocking mAbs allowed us to confirm the involvement of β2 integrin engagement in the observed phenomenon. In fact, administration of the anti-LFA-1 mAbs completely abrogates the ability of all the transfected cell lines to undergo homotypic aggregation (Fig. 1A, right panels). The evaluation of the total amount of LFA-1 receptors exposed on T cell surface indicates that similar levels are expressed on all the tested cells, ruling out the hypothesis that an altered LFA-1 expression could be responsible for the different ability to establish cell-cell contacts (data not shown).

It is well known that activation of TCR constitutes one of the strongest activatory signals for lymphocytes adhesion. TCR ligation indeed initiates a poorly understood process of "inside-out" signaling that ultimately results in the conversion of the integrin receptor LFA-1 from the inactive to its active state and the improvement of its ligand binding ability (5, 33). We observed that, upon TCR stimulation, achieved by means of anti-CD3 mAb prior to TCR activation, totally abrogated the ability of all the tested cells to aggregate. On the contrary, the anti-ICAM-3 mAb was completely ineffective. These results suggest that LMW-PTP specifically affects ICAM-1-mediated cell adhesion.

ICAM-1 engagement is involved not only in the establishment of T cell-cell adhesion but also plays a central role in mediating the interaction of lymphocytes to endothelial cells (34). Integrin binding to ICAM-1 and to other molecules belonging to the Ig superfamily as well, known as vascular cell adhesion molecule and mucosal addressin cell adhesion molecule, provides a firm attachment of lymphocytes to the endothelium and facilitates the following transendothelial migration (35). To further confirm the involvement of LMW-PTP in the specific regulation of ICAM-1-mediated cell adhesion, we performed an adhesion assay of the stable transfected T cells to a monolayer of TNF-α activated human umbilical vein endothelial cells (HUVECs). Cytokine treatment is indeed well known to enhance ICAM-1 expression on endothelial cells, increasing their adhesiveness for blood lymphocytes (36). [3H]Glycerol-labeled wt-LMW-PTP and C12S-LMW-PTP Jurkat cells were stimulated by CD3 cross-linking, to induce the conversion of LFA-1 molecules to their adhesive state, and then plated for different times on a monolayer of TNF-α-pretreated HUVECs (Fig. 3). The results indicated that wt-LMW-PTP overexpression significantly decreases lymphocyte adhesion to endothelial cells. On the contrary, C12S-LMW-PTP-expressing cells exhibited the opposite effect, showing an increased ability to interact with the HUVEC monolayer. The differences in the adhesive properties of the analyzed cells were evident already after 15 min and increased at least up to 1 h. Altogether, these data are consistent with a role of LMW-PTP in the specific regulation of both T cell-T cell and T cell-endothelium LFA-1/ICAM-1-dependent adhesion.

**Fig. 4.** The tyrosine phosphorylation of a 120- to 130-kDa protein is dependent on LMW-PTP activity. 5 × 10⁵ wt-LMW-PTP-, C12S-LMW-PTP-, and mock-transfected cells/ml were cross-linked with 10 µg/ml anti-CD3 mAbs for 1 h as previously described, with or without a 30-min pretreatment with anti-LFA-1-blocking mAbs (10 µg/ml). The stimulation was terminated by solubilizing 3 × 10⁵ cells for 15 min in 0.5 ml of lysis buffer. 25 µg of total proteins for each sample were separated by a 10% SDS-PAGE and a Western blot with anti-phosphotyrosine mAbs (clone 4G10) was performed. The results are representative of three independent experiments.

LMW-PTP Specifically Affects LFA-1 Binding to Its Ligand ICAM-1—Following its conversion to the active state, the β2 integrin LFA-1 is able to interact with distinct members of the ICAM superfamily, although ICAM-1 and ICAM-3 are the main mediators of lymphocyte homotypic aggregation (2). To understand which specific couple of ligand-receptor binding was affected by the regulatory action of LMW-PTP, we analyzed the effect of the treatment with anti-ICAM-1 and anti-ICAM-3 blocking mAbs on the homotypic aggregation of stable transfected Jurkat cells (Fig. 2). Pretreatment of the different cell lines with 10 µg/ml anti-ICAM-1 mAb prior to TCR activation, totally abrogated the ability of all the tested cells to aggregate. On the contrary, the anti-ICAM-3 mAb was completely ineffective. These results suggest that LMW-PTP specifically affects ICAM-1-mediated cell adhesion.

Monitoring the cellular adhesion assay, we can observe that ICAM-1 engagement is involved not only in the establishment of T cell-cell adhesion but also plays a central role in mediating the interaction of lymphocytes to endothelial cells (34). Integrin binding to ICAM-1 and to other molecules belonging to the Ig superfamily as well, known as vascular cell adhesion molecule and mucosal addressin cell adhesion molecule, provides a firm attachment of lymphocytes to the endothelium and facilitates the following transendothelial migration (35). To further confirm the involvement of LMW-PTP in the specific regulation of ICAM-1-mediated cell adhesion, we performed an adhesion assay of the stable transfected T cells to a monolayer of TNF-α activated human umbilical vein endothelial cells (HUVECs). Cytokine treatment is indeed well known to enhance ICAM-1 expression on endothelial cells, increasing their adhesiveness for blood lymphocytes (36). [3H]Glycerol-labeled wt-LMW-PTP and C12S-LMW-PTP Jurkat cells were stimulated by CD3 cross-linking, to induce the conversion of LFA-1 molecules to their adhesive state, and then plated for different times on a monolayer of TNF-α-pretreated HUVECs (Fig. 3). The results indicated that wt-LMW-PTP overexpression significantly decreases lymphocyte adhesion to endothelial cells. On the contrary, C12S-LMW-PTP-expressing cells exhibited the opposite effect, showing an increased ability to interact with the HUVEC monolayer. The differences in the adhesive properties of the analyzed cells were evident already after 15 min and increased at least up to 1 h. Altogether, these data are consistent with a role of LMW-PTP in the specific regulation of both T cell-T cell and T cell-endothelium LFA-1/ICAM-1-dependent adhesion.
LMW-PTP Controls FAK Tyrosine Phosphorylation under Pro-aggregating Conditions—The results presented in the previous sections clearly indicated the involvement of LMW-PTP in the regulation of signaling starting from LFA-1 engagement. In an attempt to identify the specific target of this phosphatase during lymphocyte adhesion, we analyzed the tyrosine phosphorylation pattern in whole extracts of wt-LMW-PTP and C12S-LMW-PTP transfected T cells, under pro-aggregating conditions induced by TCR stimulation (Fig. 4). Interestingly, we observed a marked difference in the tyrosine phosphorylation of a main band, ranging from 120 to 130 kDa. Moreover, the phosphorylation of this protein was found dependent on LFA-1 ligation, because the prevention of integrin activation by means of blocking antibodies pretreatment almost completely abrogated this phosphorylation. We hypothesized that this 120- to 130-kDa protein may correspond to the tyrosine kinase p125FAK, for two main reasons. First, FAK is one of the major substrates of integrin-dependent tyrosine phosphorylation,
and, in particular, it is considered a relevant intermediate of the signal transduction starting from LFA-1 engagement by the ICAMs family (13). Second, FAK has been recently identified as a target for LMW-PTP activity during the \( /H_9252 \) integrin-mediated adhesion of NIH3T3 cells to fibronectin (29). To determine whether the 120–130 kDa protein was actually p125FAK, FAK immunoprecipitates from lysates of TCR-activated wt-LMW-PTP and C12S-LMW-PTP Jurkat cells were blotted with anti-phosphotyrosine mAbs. The blot was then reprobed with anti-FAK Abs for normalization (Fig. 5A). The results clearly showed an almost complete inhibition of FAK tyrosine phosphorylation in wt-LMW-PTP-expressing cells with respect to control cells. As expected, the LMW-PTP inactive mutant exhibited instead a hyperphosphorylation of the kinase, thereby suggesting a clear involvement of LMW-PTP in the regulation of FAK tyrosine phosphorylation. Hence, because FAK activity is reported to be tightly related to its tyrosine phosphorylation (20, 37), LMW-PTP is very likely involved in the inhibition of the kinase activity.

To verify whether FAK tyrosine phosphorylation actually depends on LFA-1 activation, \( /H_9262 \beta_2 \) integrin engagement and the consequent T cell aggregation were prevented by means of an anti-LFA-1-blocking mAb pretreatment before TCR stimulation. FAK phosphorylation was then assayed as described above (Fig. 5B). As expected, anti-LFA-1 mAb pretreatment abrogated FAK phosphorylation, resulting in the disappearance of any difference among the transfected cell lines. However, the total amount of FAK in cell lysates did not show any variation, indicating that the inhibition of LFA-1 engagement did not affect the protein expression but only its tyrosine phosphorylation.

Additional experiments were performed to assess the existence of a physical association between LMW-PTP and FAK (Fig. 6). Homotypic lymphocytes aggregation was stimulated by TCR cross-linking. FAK was then immunoprecipitated from lysates and an anti-LMW-PTP Western blot was performed. The same samples were also probed with anti-FAK Abs to confirm that the kinase was immunoprecipitated to a similar extent in all samples. The results clearly revealed the presence of LMW-PTP in the FAK immunocomplexes. The stronger association of the two proteins in C12S-LMW-PTP-expressing cells with respect to those expressing the active form of the enzyme was expected, due to the inability of the dominant negative mutant to hydrolyze the substrate, which makes the FAK/LMW-PTP interaction more stable. Taken together, these data indicated that LMW-PTP is involved in the control of FAK tyrosine phosphorylation during the LFA-1-mediated T cells adhesion, suggesting a role of the phosphatase in the negative regulation of FAK-dependent downstream effects.

**LMW-PTP-dependent FAK Dephosphorylation Is Associated with the Inhibition of Molecular Pathways Downstream FAK**
**Activation**—The major site of FAK phosphorylation is Tyr-397, which results in the SH2-dependent recruitment of the Src tyrosine kinase. The FAK/Src complex formation is then responsible for the Src-dependent phosphorylation of multiple tyrosines on FAK, resulting in the increase of FAK kinase activity and in the recruitment of several signaling and structural proteins (38). One of the major signaling pathways downstream the Src-mediated FAK phosphorylation and activation is the triggering of the mitogen-activated protein kinase cascade, which depends on FAK phosphorylation on Tyr-925 and the consequent recruitment of Grb2. To assess whether the LMW-PTP-dependent dephosphorylation of FAK leads to the regulation of both Src and ERK activity during TCR-induced T cell adhesion, we analyzed the activation patterns of both p60Src and p42/p44ERKs. wt-LMW-PTP and C12S-LMW-PTP cells were stimulated with anti-CD3 mAbs and allowed to undergo homotypic aggregation. The activation of Src and ERKs was then assayed by probing the whole cell lysates with anti-phospho-Src (Tyr-416) and anti-Src Abs (for normalization) with anti-phospho-ERK and anti-ERK Abs. Results were quantified by densitometric analysis, and the normalized values, indicating the activation state of Src and ERKs, respectively, were plotted. Both the reported experiments are representative of three others with similar results.

**LMW-PTP Negatively Affects LFA-1 Activation, Blocking Its Cell Surface Clustering**—Our findings are consistent with a role of LMW-PTP in the negative regulation of LFA-1/ICAM-1-dependent lymphocyte adhesion. The conversion of LFA-1 into a functional state and the consequent improvement of its ligand binding ability are mainly achieved by the induction of receptor clustering on the cell surface (8). We hypothesized that LMW-PTP may have a role in the control of β2 integrin avidity,
participating to the regulation of LFA-1 mobility and cluster formation. To prove this hypothesis, we investigated by confocal microscopy analysis the cell surface distribution of LFA-1 molecules in TCR-stimulated Jurkat cells overexpressing either wt-LMW-PTP or C12S-LMW-PTP. Interestingly, we observed a very different pattern of LFA-1 distribution between these cell lines (Fig. 8, left panels). In particular, the overexpression of the active LMW-PTP inhibited the LFA-1 clustering triggered by TCR engagement, leading to a more diffuse localization of integrin receptors on the plasma membrane with respect to that observed in control cells. This effect was reverted and emphasized by the expression of the inactive mutant of the enzyme. C12S-LMW-PTP Jurkat cells indeed exhibited a relevant increase in receptors clustering, in agreement with the greater ability of these cells to establish cellular aggregates. To better estimate the differences in LFA-1 clustering among the tested cells, we analyzed the percentage of cells exhibiting the clustered structure. The results indicate that clusters are evident in 90% of C12S-LMW-PTP-expressing cells, whereas this value is dramatically reduced in wt-LMW-PTP-transfected cells (15%) with respect to control ones (35%). Interestingly, pretreatment of T cells with anti-LFA-1-blocking mAbs before TCR-induced LFA-1 activation, totally prevented integrin clustering in all tested cells, indicating that this phenomenon depends on LFA-1 engagement (Fig. 8, right panels).

The specificity of the immunostaining was confirmed by the absence of any signal in cells labeled with secondary antibodies only (data not shown). It is remarkable to note that in mock-transfected cells and, to a greater extent, in C12S-LMW-PTP-expressing T cells, integrin receptors labeled by the anti-LFA-1 immunostaining were not dispersed throughout the plasma membrane, but they were rather restricted to a single zone at one pole of the cell. A similar segregation of LFA-1 receptors has been well characterized during the interaction between T cells and APCs. In fact, it has been extensively demonstrated that T cell recognition of APC is accompanied by the redistribution of several cell surface receptors toward the cell-cell interface and the formation of a specialized junction known as IS (24, 39). High resolution confocal microscopy analysis allowed us to demonstrate that IS is constituted by a central zone, where TCR and surface accessory molecules such as CD4, CD2, and CD28 segregate, and an external ring-like region, where adhesion molecules accumulate, that appears particularly enriched for the integrin LFA-1 (40). To check whether the single region evidenced by the anti-LFA-1 immunofluorescence may resemble the IS structure, we analyzed the distribution of activated TCRs in wt-LMW-PTP- and C12S-LMW-PTP-expressing cells by means of anti-CD3
immunofluorescence staining (Fig. 9). In mock-transfected cells upon antigen stimulation, LFA-1 (green fluorescence, panel A) and TCR (red fluorescence, panel B) exhibited a similar pattern of cell surface distribution, because they were concentrated in a distinct area of the plasma membrane, a process called “capping” (41, 42). TCR clustering, as well as LFA-1 redistribution, appeared to be influenced by LMW-PTP overexpression. This phenomenon is indeed inhibited in cells expressing the wt-LMW-PTP, where LFA-1 exhibits a ring-like positivity at cell periphery, while TCR capping is reduced. On the other hand, the redistribution of both the receptors appears to be rescued and greatly enhanced by the expression of the inactive mutant of LMW-PTP. The superimposition of the two distinct images (Fig. 9C), obtained for each cell line with anti-LFA-1 or anti-CD3 mAbs, indicates a clear overlapping of the two signals (yellow fluorescence), demonstrating that upon antigen stimulation TCR and LFA-1 co-localize toward a single capping region at one pole of the cell.

Taken together, our findings suggest that the LMW-PTP-dependent inhibition of LFA-1 clustering may be responsible for the prevention of LFA-1 from achieving an active state, thus accounting for the observed down-regulation of the LFA-1/ICAM-1-dependent lymphocyte adhesion. The concomitant impairment of the polarization of activated TCRs, observed in wt-LMW-PTP-expressing cells, also suggests a role for the phosphatase in the regulation of LFA-1 and TCR co-clustering, an essential step for the IS formation and cell-cell contact stabilization.

**DISCUSSION**

The results reported here lead to three major conclusions: 1) LMW-PTP is involved in the negative regulation of LFA-1/ICAM-1-mediated cell-cell adhesion, by preventing LFA-1 clustering and activation; 2) the phosphatase is able to dephosphorylate and inhibit FAK activity, most likely preventing, in this way, the cytoskeleton remodeling essential to allow LFA-1 redistribution; and 3) LMW-PTP negatively affects the activated TCR capping and co-localization with LFA-1, suggesting a possible role of the phosphatase in the regulation of IS formation and stability during T cell/APC encountering.
The $\beta_2$ integrin LFA-1 plays a crucial role in mediating adhesive processes of T cells. The LFA-1 functional state has to be kept under a tight control to avoid uncontrolled aggregation of circulating cells and clogging of the vessels, as well as lymphocytes improper adhesion to endothelial cells. Although the general events leading to LFA-1 activation have been characterized quite well (4), the mechanisms involved in the negative regulation of $\beta_2$ integrin activity are still largely unknown. However, given the relevant function of tyrosine phosphorylation during integrin-mediated T cell adhesion (9), phosphotyrosine phosphatases may be good candidates for the downregulation of this phenomenon. In particular, our interest focused on the possible involvement of LMW-PTP in the control of integrin signaling. The results we obtained showed that wt-LMW-PTP induces a strong impairment of LFA-1/ICAM-1-dependent T cell-cell adhesion (Figs. 1 and 2). Furthermore, we demonstrated that T cells adhesion to endothelial cells, which is mainly mediated by the interaction between LFA-1 and ICAM-1, is also negatively affected by LMW-PTP (Fig. 3). It is well documented that a firm attachment to the endothelium is essential for transmigration of T cells and establishment of a proper immune response to infection or inflammation (35). However, because an improper adhesion to vessel walls could be responsible for the development of pathological conditions, LMW-PTP may represent an important regulatory mechanism by preventing T cell adhesion to endothelial cells in the absence of specific stimuli.

In the present study we also demonstrated that p125FAK tyrosine phosphorylation is severely affected by LMW-PTP overexpression (Figs. 4 and 5A) because of a physical association between the two proteins (Fig. 6). The prevention of LFA-1 engagement and, in turn, of cell-cell aggregation by means of blocking antibodies pretreatment, negatively affects FAK phosphorylation, thus confirming that (i) FAK is a key mediator of integrin signal transduction and (ii) LMW-PTP association with FAK and the consequent regulation of the kinase activity requires LFA-1 engagement, an essential event to promote FAK phosphorylation (Fig. 5B). Because FAK tyrosine phosphorylation controls its catalytic activity and association with other signaling molecules, dephosphorylation of specific tyrosine residues on FAK is potentially a very important mechanism for the regulation of its functional state and for the activation of its downstream molecular pathways. Herein, in agreement with previous data (29), we showed that LMW-PTP activity on FAK may be directed toward two main sites of tyrosine phosphorylation, namely Tyr-397 and Tyr-925, which are responsible for the recruitment of Src and Grb2, respectively. Accordingly, we found that upon LFA-1 engagement both p60$^{Src}$ and p42/44 ERK are negatively affected by LMW-PTP (Fig. 7). It is well known that Src activation is crucial for many of FAK functions, because Src directly phosphorylates many tyrosine residues on FAK, thereby enhancing FAK activity and promoting the recruitment and phosphorylation of several structural and signaling intermediates (18). Thus LMW-PTP, by specifically dephosphorylating Tyr-397 of FAK, may prevent Src from...
interacting with FAK, thus interfering with the improvement of FAK catalytic activity and the right outcome of FAK-dependent biological effects.

Several studies provided evidence that LFA-1 activation is mainly mediated by its redistribution and organization into clusters. In addition, many reports indicate that cytoskeleton is

Fig. 10. Model explaining the proposed role of LMW-PTP in the FAK-mediated inhibition of LFA-1 and TCR reorganization into clusters. A, in resting conditions LFA-1 is kept into the inactive state by means of tight interactions with the cytoskeleton scaffold. These inhibitory connections force LFA-1 to retain a dispersed location on T cell surface, preventing the achievement of a high avidity conformation. B, upon TCR stimulation, LFA-1 is converted into an adhesive state and becomes able to bind its ligand ICAM-1. Signaling starting from the engaged LFA-1 leads to FAK phosphorylation and to the activation of its downstream effectors. This is very likely to be responsible for the transient release of LFA-1 from its inhibitory linkages and for receptor polarization. In this context, LMW-PTP may interfere with such a process through its negative regulation of FAK activity, thereby preventing receptors organization into clusters and their consequent activation (C).
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intimately involved in this process (43). In particular, the association of LFA-1 with cytoskeletal proteins represents an obstacle to its free mobility on the plasma membrane, whereas the breakdown of this inhibitory tethering allows LFA-1 diffusion and clustering and ultimately promotes an enhanced adhesion to ICAM-1 (44, 45). In this context, we propose that the inhibitory effect of LMW-PTP on T cell-cell adhesion may be related to an impairment of integrin clustering and activation, likely due to the LMW-PTP-dependent FAK inhibition. In keeping with our hypothesis, recent data obtained by Rodriguez-Fernandez et al. (14) suggest the involvement of FAK in mediating LFA-1 polarization, because the kinase has been shown to rapidly activate and co-localize with LFA-1, following the integrin engagement by ICAM-1. In agreement with this view, our results indicated that LFA-1 redistribution is severely affected by LMW-PTP. In fact, in LMW-PTP-expressing cells, despite the induction of integrin activation by means of TCR stimulation, LFA-1 receptors maintain a diffuse localization throughout the plasma membrane (Fig. 8). It is noteworthy that cell surface distribution of TCR in activated T cells closely resembles that of LFA-1 (Fig. 9, A and B). It is well known that the antigen stimulation of TCRs results in their accumulation into a single region of T cell surface, a well-characterized phenomenon commonly referred to as “capping.” We demonstrated that LMW-PTP blocks clustering of activated TCRs, inducing their maintenance in a dispersed location throughout the T cell surface (Fig. 9B). In addition, we reveal a strict overlapping of the region where both LFA-1 and TCR are segregated (Fig. 9C). Therefore, taken together, our findings clearly demonstrate that following antigen stimulation LFA-1 and TCR co-localize into a single cluster and that LMW-PTP negatively affects the redistribution of both the engaged receptors toward the capping area.

The co-localization of TCR and LFA-1 is an event that has been described during T cell/APC junction formation and IS establishment (24, 39). It is well documented that IS organization is accompanied by a redistribution, of several cell surface receptors, which promotes the formation of the so-called supramolecular activating complex (SMAC). This structure includes a central core mainly constituted by activated TCRs (c-SMAC) and a surrounding ring (p-SMAC) consisting of LFA-1/ICAM-1 ligand pairs and other adhesive receptors (26, 27). Although the function of IS is not yet very clear, this specialized structure seems to provide a mechanism for T cell-APC contact stabilization, thus allowing a sustained signaling from the engaged TCR and a full T cell activation. In this way, we advance the hypothesis that LMW-PTP may participate to the regulation of T cell/APC junction formation and stabilization, by exerting a tight control on receptors redistribution and IS assembly.

Bottini et al. (30) have recently proposed the involvement of LMW-PTP in TCR signal transduction, as a positive regulator of ZAP-70 kinase activity. Although these data seem in apparent disagreement with our observations, we emphasize that LMW-PTP may act on ZAP-70 and FAK in two distinct temporal phases. LMW-PTP may indeed enhance TCR signaling in the early phases of stimulation by acting on ZAP-70 and, later on, reduce cytoskeleton rearrangements by acting on FAK. Hence, it is likely that LMW-PTP participates first in the improvement of TCR activation and, in a second phase, in the regulation of the movement of the membrane-associated signaling molecules.

In summary, on the basis of our findings we propose a novel role for FAK as a key mediator of T cell cytoskeleton reorganization (Fig. 10). In particular, we suggest that upon TCR engagement and the following improvement of LFA-1 clustering and ligand binding ability, FAK is rapidly phosphorylated and activated. According to our data, this event seems to be a crucial step for LFA-1 signaling. FAK activation indeed plays a central role in promoting a dynamic reorganization of the cytoskeletal scaffold, an essential event for orchestrating TCR and LFA-1 redistribution, and in achieving their active conformation. In this context, LMW-PTP exerts an intriguing role as a negative regulator of FAK activity (Fig. 10B). In fact, LMW-PTP binds and dephosphorylates FAK, thus preventing FAK activation and the consequent activation of its downstream pathways. As a consequence, LMW-PTP leads to an impairment of cytoskeleton remodeling, thus interfering with receptors organization into clusters (Fig. 10C). In this way, LMW-PTP prevents the conversion of LFA-1 into a functional state, thereby impairing the LFA-1/ICAM-1-dependent T cell-cell adhesion.

Our findings also demonstrate that LMW-PTP prevents the co-localization of both LFA-1 and TCR toward a single region that closely resembles the IS structure. On the basis of this indication, we suggest a role for LMW-PTP in the regulation of the IS formation and stability and in the consequent modulation of sustaining of the immune response. In conclusion, we propose a novel circuitry for the regulation of the IS establishment during T cell/APC interaction, involving a tight control of cytoskeleton-associated proteins phosphorylation and dephosphorylation.

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Lymphocyte Function-associated Antigen-1-mediated T Cell Adhesion Is Impaired by Low Molecular Weight Phosphotyrosine Phosphatase-dependent Inhibition of FAK Activity

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