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The SH2-Containing Inositol-5′-Phosphatase Enhances LFA-1-Mediated Cell Adhesion and Defines Two Signaling Pathways for LFA-1 Activation[1]

Jose A. Rey-Ladino,[2]* Michael Huber,* Ling Liu,† Jacqueline E. Damen,* Gerald Krystal,*‡ and Fumio Takei[2]*‡

The inside-out signaling involved in the activation of LFA-1-mediated cell adhesion is still poorly understood. Here we examined the role of the SH2-containing inositol phosphatase (SHIP), a major negative regulator of intracellular signaling, in this process. Wild-type SHIP and a phosphatase-deficient mutant SHIP were overexpressed in the murine myeloid cell line, DA-ER, and the effects on LFA-1-mediated cell adhesion to ICAM-1 (CD54) were tested. Overexpression of wild-type SHIP significantly enhanced cell adhesion to immobilized ICAM-1, and PMA, IL-3, or erythropoietin further augmented this adhesion. In contrast, phosphatase dead SHIP had no enhancing effects. Furthermore, PMA-induced activation of LFA-1 on DA-ER cells overexpressing wild-type SHIP was dependent on protein kinase C but independent of mitogen-activated protein kinase activation, whereas cytokine-induced activation was independent of protein kinase C and mitogen-activated protein kinase activation but required phosphatidylinositol-3 kinase activation. These results suggest that SHIP may regulate two distinct inside-out signaling pathways and that the phosphatase activity of SHIP is essential for both of them. ***The Journal of Immunology,*** **1999, 162: 5792–5799.**

Leukocyte function-associated Ag-1 (LFA-1) is a member of the integrin superfamily (1, 2). Interaction of LFA-1 with its main ligand, ICAM-1 (CD54), is important for a variety of adhesion-dependent functions of leukocytes (3, 4). LFA-1 expressed on resting cells in general does not strongly interact with ICAM-1. However, cross-linking of CD3 (5), CD2 (6), B cell surface Ig (7), CD44 (8), MHC class II (9), or CD45 (10) with specific mAbs or stimulation of cells with PMA results in a rapid enhancement of LFA-1-mediated cell adhesion to ICAM-1. This activation of LFA-1 by "inside-out signaling" does not require increased expression of LFA-1 on the cell surface or de novo protein synthesis (5, 6). Although the inside-out signaling pathways by which LFA-1-mediated cell adhesion is regulated are largely unknown, they have been associated with increases in phosphoinositide hydrolysis.

The SH2-containing inositol phosphatase (SHIP)[3] plays a major role as a negative regulator of intracellular signal transduction (11). It selectively hydrolyses the 5′ phosphate from phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P_3) (12–14) and inositol-1,3,4,5-tetraphosphate (I-1,3,4,5-P_4) and regulates calcium influx into various cell types (15–17). Therefore, SHIP may play a role in LFA-1 activation by regulating phosphatidylinositol levels.

In this study, we examined the role of SHIP in the activation of LFA-1 by testing the effects of overexpressing wild-type (WT) and phosphatase dead SHIP in a hemopoietic cell line on LFA-1-mediated cell adhesion to ICAM-1. The results show that overexpression of a phosphatase active, but not inactive, SHIP enhances the activation of LFA-1 in both resting and stimulated cells. Furthermore, PMA-induced activation of LFA-1 on cells overexpressing SHIP is regulated by protein kinase C (PKC), whereas erythropoietin (Epo)- or IL-3-induced activation of LFA-1 involves a phosphatidylinositol-3 kinase (PI-3K) regulated pathway.

Materials and Methods

Reagents

Rat hybridomas producing anti-murine CD18 (TIB213; FD441.8) and anti-rat Igκ (TIB169; RG11/39.4) were obtained from the American Type Culture Collection (Manassas, VA). Purification of these mAbs as well as FITC conjugation of RG11/39.4 mAb have been described (18). Anti-phospho-mitogen-activated protein kinase (MAPK) Abs were obtained from New England Biolabs (Mississauga, Ontario, Canada), anti-Erk-1-CT Abs were obtained from Upstate Biotechnology (Lake Placid, NY), and anti-Raf-1 Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The generation of the N-terminal-specific anti-SHIP Ab was described (19). The anti-hemaglutinin (HA) mAb was obtained from Babco (Richmond, Ca). HRP-conjugated secondary Abs were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). The production of murine IL-3 and murine Epo was described previously (20, 21). Protein-grade Nonidet P-40 was obtained from Calbiochem (La Jolla, CA). Aminoalkyl bisindolylmaleimide (compound 3) was purchased from Calbiochem, and PD98059 was obtained from Biomol (Plymouth Meeting, PA). Wortmannin and PMA were purchased from Sigma (St. Louis, MO), and

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[3] Abbreviations used in this paper: SHIP, SH2-containing inositol phosphatase; WT, wild type; D675-SHIP, mutant containing a substitution of aspartic acid with glycine at position 675 of SHIP; PI-3,4,5-P_3, phosphatidylinositol-3,4,5-trisphosphate; I-1,3,4,5-P_4, inositol-1,3,4,5-tetraphosphate; PI-3,4-P_2, phosphatidylinositol-3,4-biphosphate; I-1,3,4-P_3, inositol-1,3,4-trisphosphate; I-1,4,5-P_3, inositol-1,4,5-trisphosphate; CH-1, cytohesin-1; Epo, erythropoietin; PKC, protein kinase C; PI-3K, phosphatidylinositol-3 kinase; MAPK, mitogen activated protein kinase; HA, hemaglutinin; BCECF-AM, 2′,7′-bis-(2-carboxyethyl)-(and-6)-carboxyfluo-rescein, acetoxymeyhyl ester; PH, pleckstrin homology.

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the fluorescence dye 2′,7′-bis-(2-carboxyethyl)-(and-6)-carboxyfluo-rescein, acetoxymeyhyl ester (BCECF-AM) was obtained from Molecular Probes (Eugene, OR). The enhanced chemiluminescence Western blotting reagents were obtained from Pierce (Rockford, IL).

Generation of DA-ER cells overexpressing SHIP

The murine myeloid line DA-ER was generated by retrovirally introducing the Epo receptor into the IL-3-dependent murine myeloid line DA-3 (22). The generation of HA-tagged SHIP constructs and the retroviral-mediated gene transfer of these constructs into DA-ER cells have been described previously (19). The mutant SHIP (D675G-SHIP) retroviral construct, defective in its phosphatase domain due to a substitution of a critical aspartic acid with glycine at position 675 (23), was generated by PCR-site directed mutagenesis (Quick change site-directed mutagenesis kit; Stratagene, La Jolla, CA). Primers used were: 5′-CCGTCCTGGTGCG$^{A\rightarrow G}$CCGAGTCC TCTGGAAGT-3′ and 5′-CTTCCAGAGGACTCGG$^{T\rightarrow C}$CGCACCAGG ACGGC-3′, with arrows indicating the nucleotide substitutions. The PCR product was subcloned into the MSCVPac viral vector and then used to infect DA-ER cells in the same manner as that used for the WT-SHIP. Cells infected with the viral vector alone were used as controls. Infected cells were selected in RPMI 1640 medium containing 10% FCS, IL-3 (5 ng/ml), and 2 μg/ml puromycin.

Flow cytometric analysis

DA-ER cells (0.5×10^6) were stained with 5 μg/ml rat mAb (FD441.8), recognizing the CD18 chain of murine LFA-1, for 30 min on ice. After washing with HBSS containing 2% FCS, the cells were incubated with 5 μg/ml FITC-conjugated anti-rat Igκ (TIB169) mAb for 30 min on ice. Finally, cells were washed with HBSS containing 2% FCS and 0.1% sodium azide. Stained cells were analyzed by a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Stimulation of cells and cell adhesion assay

Cells were cytokine deprived for 4 h at 37°C in RPMI 1640 containing 0.5% BSA and then stimulated with PMA for 25 min at 37°C or with IL-3 (400 ng/ml) or Epo (50 U/ml) for 10 min at 37°C as described previously (19). For adhesion assays using inhibitors, cells were pretreated for 30 min at 37°C with either compound 3 (5–20 μM) or chelerythrine (24), PD98059 (25–50 μM) (25), or wortmannin (50–100 nM) (26) as described. Cells were then incubated with medium alone or stimulated with either PMA, IL-3, or Epo as indicated above. Cells were also treated with $MnCl_2$ (5 mM) in HBSS containing 2% FCS for 25 min at 37°C before the binding assays. Unstimulated or treated cells were then labeled with 1 μg/ml of the fluorescent dye, BCECF-AM, in HBSS containing 2% FCS at 37°C for 10 min. After washing, cells were resuspended in HBSS containing 2% FCS, and adhesion to immobilized ICAM-1 was assayed as described previously (27). The degree of cell adhesion was quantitated using a cytofluor 2300 microplate reader (Millipore, Bedford, MA) and was expressed as the percentage of the fluorescence remaining in the wells after washing away unbound cells.

Immunoprecipitation and Western blotting

DA-ER cells infected with either vector alone, HA-tagged WT-SHIP, or HA-tagged mutant D675G-SHIP were analyzed for SHIP expression by Western analysis of total cell lysates using anti-HA mAbs as described previously (19). Total cell lysates were also used to assess the phosphorylation status of Erk-1, Erk-2, and Raf-1 and were prepared by lysing $1–2 \times 10^7$ cells/ml at 4°C with 0.5% Nonidet P-40 in phosphorylation solubilization buffer, (50 mM HEPES, pH 7.4, 100 mM NaF, 10 mM sodium pyrophosphate, 2 mM Na_3Vo_4, 4 mM EDTA, 2 mM PMSF, 10 μg/ml leupeptin, and 2 μg/ml aprotinin). Following centrifugation, supernatants (total cell lysates) were either subjected directly to SDS-PAGE or to immunoprecipitation with anti-SHIP Abs and then SDS-PAGE. Western analysis was conducted with anti-phosphotyrosine (4G10; Upstate Biotechnology), anti-phospho-MAPK, anti-Raf-1, anti-MAPK, or anti-SHIP Abs as described previously (19).

Results

LFA-1 expression on DA-ER cells

To determine whether SHIP could influence LFA-1 activation, the murine IL-3-dependent cell line DA-ER (see *Materials and Methods*) was infected with either HA-tagged WT-SHIP, HA-tagged mutant SHIP with a nonfunctional phosphatase (D675G-SHIP) (22), or with the retroviral vector alone. Puromycin-resistantclones were expanded, and those expressing similar levels of WT and mutant SHIP, as assessed by Western blot analysis using anti-HA and anti-SHIP Abs, were selected for further study (Fig. 1*A*, *upper panel*). The chosen clones expressed ~2-fold higher levels of total SHIP (i.e., HA-SHIP plus endogenous SHIP) than that present in control cell lines infected with vector alone, as determined by immunoprecipitation and Western blotting with anti-SHIP Abs (Fig. 1*A*, *lower panel*). This relatively low level of exogenous expression is consistent with that obtained previously, both in our laboratory (19) and in others (16), and most likely reflects SHIP's reported negative effects on proliferation/survival (16, 19). Nonetheless, even this slight increase in total SHIP protein has been shown previously to inhibit cell proliferation and increase apoptosis (16, 19). FACS analysis showed the same level of cell surface LFA-1 on all the DA-ER cell lines tested (Fig. 1*B*), indicating that overexpression of SHIP had no effect on cell surface expression of LFA-1.

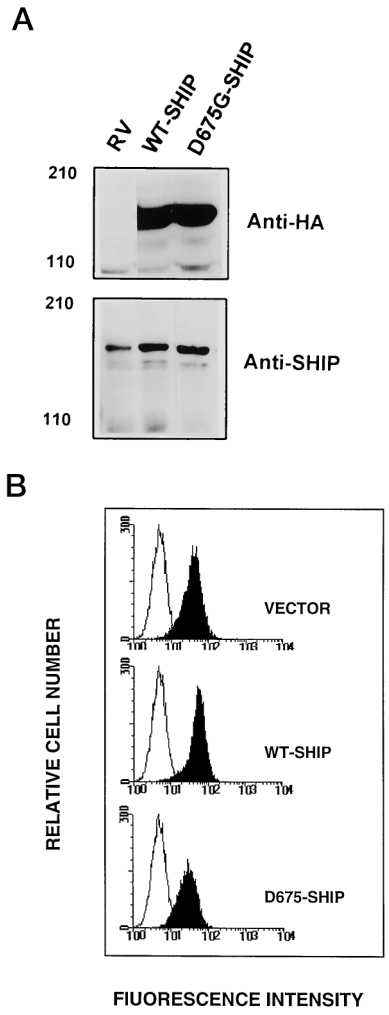

FIGURE 1. Expression of SHIP and LFA-1 in DA-ER cells. *A*, Equal amounts of total cell lysates from DA-ER cells expressing vector alone (RV), HA-tagged WT-SHIP, or HA-tagged D675G-SHIP were subjected to Western analysis using an anti-HA-mAb (*upper panel*). In the *lower panel*, SHIP was immunoprecipitated from DA-ER cells and analyzed by immunoblotting using anti-SHIP Abs. Molecular weight markers are shown in the left margin. *B*, Expression levels of LFA-1 were determined by indirect immunofluorescence using an anti-LFA-1 mAb (FD441.8) and FITC-conjugated secondary Ab.

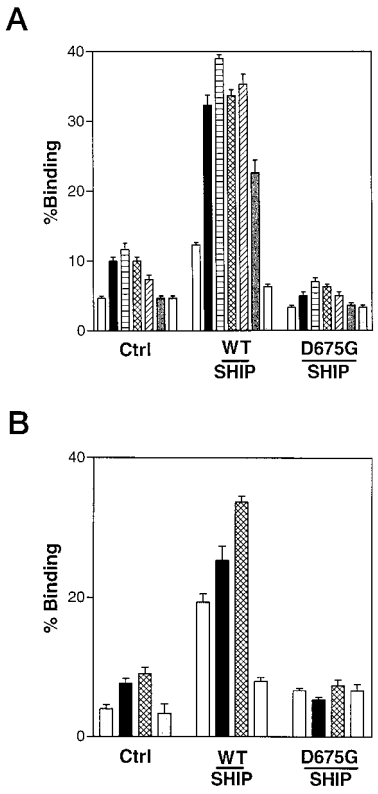

FIGURE 2. Adhesion to ICAM-1 of DA-ER cells overexpressing WT-SHIP and D675G-SHIP. *A*, Cells were cytokine starved as described in *Materials and Methods*. They were then unstimulated (open bars), or treated for 25 min at 37°C with either 150, 100, 50, 25, or 12 ng/ml PMA (black bars, bars with horizontal lines, cross-hatched bars, bars with diagonal lines and dark gray bars, respectively) or with 50 ng/ml PMA plus anti-LFA-1 (light gray bars). *B*, Cytokine starved cells were incubated for 10 min with either medium alone (open bars), 50 U/ml Epo (black bars), 400 ng/ml IL-3 (cross-hatched bars), or 400 ng/ml IL-3 plus 10 μg/ml anti-LFA-1 (gray bars). Cells were labeled with BCECF-AM and added to microwells coated with soluble ICAM-1. After removing unbound cells, the fluorescence emission of bound cells was determined. The results are expressed as the mean of triplicate measurements for each condition ± SEM and are representative of four independent experiments. Ctrl, control cells expressing retroviral vector alone.

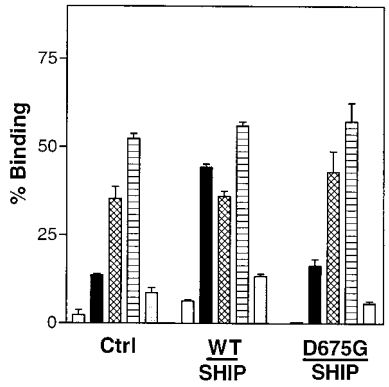

FIGURE 3. Effect of Mn^{2+} on adhesion to ICAM-1 of DA-ER cells overexpressing WT-SHIP and D675G-SHIP. Cytokine-starved cells were unstimulated (open bars), treated for 25 min at 37°*C* with 50 ng/ml PMA (black bars), 5 mM $MnCl_2$ (cross-hatched bars), 50 ng/ml PMA plus 5 mM $MnCl_2$ (bars with horizontal lines), or a combination of 50 ng/ml PMA plus 5 mM $MnCl_2$ plus anti-LFA-1 (gray bars). Assays were performed as in Fig. 2. Results are the mean of triplicate determinations ± SEM and are representative of three independent experiments. Ctrl, control cells expressing retroviral vector alone.

Effect of SHIP overexpression on LFA-1-mediated cell adhesion to ICAM-1

DA-ER cells overexpressing SHIP were tested for LFA-1-mediated adhesion to ICAM-1. The degree of cell adhesion to ICAM-1 was determined in an in vitro binding assay in which cell adhesion to purified ICAM-1, immobilized in microtiter wells, was quantitated. Control DA-ER cells infected with vector alone was found to bind to ICAM-1 at low levels, i.e., ~4% (Fig. 2*A*). PMA stimulation increased this cell adhesion in a concentration-dependent manner to a maximum of ~12%. Resting DA-ER cells overexpressing WT-SHIP bound to ICAM-1 at significantly higher levels (~3- to 4-fold) than did resting control cells. Moreover, upon PMA stimulation a significant increase in adhesion was consistently observed with these cells. Depending on the PMA concentration, the level of adhesion was ~3- to 4-fold higher than that observed with PMA-stimulated control cells (Fig. 2*A*). Importantly, the addition of anti-LFA-1 mAb (FD441.8) abrogated PMA-stimulated (50 ng/ml) adhesion of all three cell types (Fig. 2*A*), whereas isotype-matched control Abs had no effect (data not shown), indicating that adhesion in all cases was mediated specifically by LFA-1. Anti-LFA-1 mAb had no significant effect on resting cells. Thus, the overexpression of SHIP enhanced LFA-1-mediated cell adhesion of resting or PMA-stimulated cells to ICAM-1, and this effect appeared to be dependent upon a functional phosphatase domain within SHIP. Similar results were obtained with three different clones of each cell type (data not shown).

DA-ER cells express endogenous IL-3 receptors and exogenously added, biologically active Epo receptors (22). Therefore, we tested whether LFA-1 on DA-ER cells could be activated by stimulating these cells with Epo or IL-3, and whether overexpression of SHIP affected inside-out signaling through Epo or IL-3 receptor-mediated signaling pathways. Specifically, DA-ER cells, which were cytokine deprived in serum-free medium, were treated with Epo or IL-3 and tested for adhesion to ICAM-1. In control DA-ER cells, Epo and IL-3 modestly enhanced the LFA-1-mediated adhesion of cells to ICAM-1 (Fig. 2*B*). However, in DA-ER cells overexpressing WT-SHIP, Epo and IL-3 substantially increased adherence to ICAM-1. Importantly, the adhesion of DA-ER cells expressing the mutant D675G-SHIP was not enhanced by Epo or IL-3 stimulation (Fig. 2*B*), suggesting that overexpression of SHIP enhanced not only PMA- but also cytokine-mediated LFA-1 activation, and the phosphatase activity of SHIP was critical for these effects. As in the case with PMA stimulation, the addition of anti-LFA-1 mAb (FD441.8) abrogated Epo- and IL-3-induced binding, and similar results were obtained with three different clones of each cell type.

Effects of Mn^{2+} on LFA-1-mediated binding to ICAM-1

To test whether SHIP overexpression was altering inside-out signaling or directly affecting LFA-1 binding to ICAM-1, we tested the three cell types for their ability to bind to ICAM-1 in the presence of Mn^{2+}. Mn^{2+} treatment converts low-avidity LFA-1 into an activated state independent of inside-out signaling (28, 29). This is thought to be caused by Mn^{2+} directly binding to and altering the conformation of LFA-1. As expected, treatment of DA-ER cell lines with Mn^{2+} greatly enhanced LFA-1-mediated cell adhesion to ICAM-1, and the levels of cell adhesion achieved were the same with the three cell types (Fig. 3). Interestingly, cell adhesion was further enhanced by a combination of PMA and

Mn^{2+} (Fig. 3). These results show that the extracellular domain of LFA-1 was not altered in cells overexpressing SHIP. Thus, the effects of SHIP overexpresssion are most likely due to altered inside-out signaling.

PMA activates LFA-1 in SHIP-overexpressing cells via PKC

To gain a better understanding of the mechanism(s) of SHIP-mediated LFA-1 activation, cells overexpressing WT-SHIP were treated with two widely used inhibitors of PKC, bisindolylmaleimide (compound 3) and chelerythrine (24, 30). The cells were then stimulated with PMA and tested for binding to ICAM-1. As shown in Fig. 4*A*, pretreatment with compound 3 markedly inhibited PMA-induced binding to ICAM-1, even at concentrations as low as 5 μM. Surprisingly, though, pretreatment with chelerythrine had very little effect (Fig. 4*B*). Serendipitously, shortly after we obtained these rather confusing results, Lee et al. reported that chelerythrine was in fact not a PKC inhibitor in human leukemia (HL-60) or mouse 308 cells (31). To test whether this was also the case in our system, we monitored PMA-stimulated PKC activation in WT-SHIP-expressing DA-ER cells, using MAPK phosphorylation as a readout, in the presence and absence of compound 3 and chelerythrine. As can be seen in Fig. 4*C*, *panel A*, PMA, as expected, stimulated a dramatic increase in the phosphorylation of both Erk-1 and Erk-2 (*lane 2*). Pretreatment with compound 3 markedly inhibited this PMA-induced phosphorylation (*lane 3*), while chelerythrine was without effect (*lane 4*). Reprobing this blot with anti-MAPK Abs confirmed this result because MAPK phosphorylation leads to a slower migration (band shift) in SDS-gels (Fig. 4*C*, *panel B*). Because the phosphorylation of MAPK via PKC has been shown to be mediated by Raf-1 (32), we also examined the effect of PMA, with or without compound 3 or chelerythrine pretreatment, on Raf-1 phosphorylation. As can be seen in Fig. 4*C*, *panel C*, PMA stimulated the phosphorylation of Raf-1, indicated by the band shift, and compound 3 but not chelerythrine prevented this shift. In Fig. 4*C*, *panel D*, a reprobing of the blots shown in *panels A*, *B*, and *C* with anti-SHIP Abs to confirm equal loading is shown. Thus, our results are consistent with those of Lee et al. and suggest, contrary to previous reports (30), that chelerythrine is not a PKC-specific inhibitor. More importantly, our results show that PMA-stimulated activation of LFA-1 in SHIP-overexpressing DA-ER cells is mediated by a PKC-induced pathway.

Activation of LFA-1 by IL-3 and Epo does not require PKC

To test PKC's role in LFA-1-mediated adherence to ICAM-1 under more physiological conditions, the effect of PKC inhibition on IL-3- and Epo-induced adhesion to ICAM-1 was examined. Specifically, DA-ER cells overexpressing WT-SHIP were pretreated with compound 3 and stimulated either with IL-3 or Epo before analysis of cell adhesion to ICAM-1. As shown in Fig. 5*A*, treatment with compound 3 had no significant effect on cytokine-mediated LFA-1 activation. To confirm that Epo and IL-3, which have been shown to stimulate PKC in other hemopoietic cell lines (33) actually stimulated PKC in DA-ER cells, total cell lysates were subjected to Western analysis with anti-phospho-MAPK Abs. Both Erk-1 and Erk-2 were indeed highly phosphorylated following the addition of these cytokines (Fig. 5*B*, *panel A*, *lanes 2* and *4*). This is consistent with previous reports in other hemopoietic cell lines (33). However, paralleling the lack of effect of compound 3 on cell adhesion, little reduction of MAPK phosphorylation was observed in cells treated with a combination of compound 3 and IL-3 or compound 3 and Epo (Fig. 5*B*, *panel A*, *lanes 3* and *5*, respectively). Reprobing this phospho-MAPK blot with anti-Erk-1 showed a clear band shift of the MAPKs Erk-1 and Erk-2 in cells stimulated with IL-3 or Epo and confirmed equal loading (Fig. 5*B*,

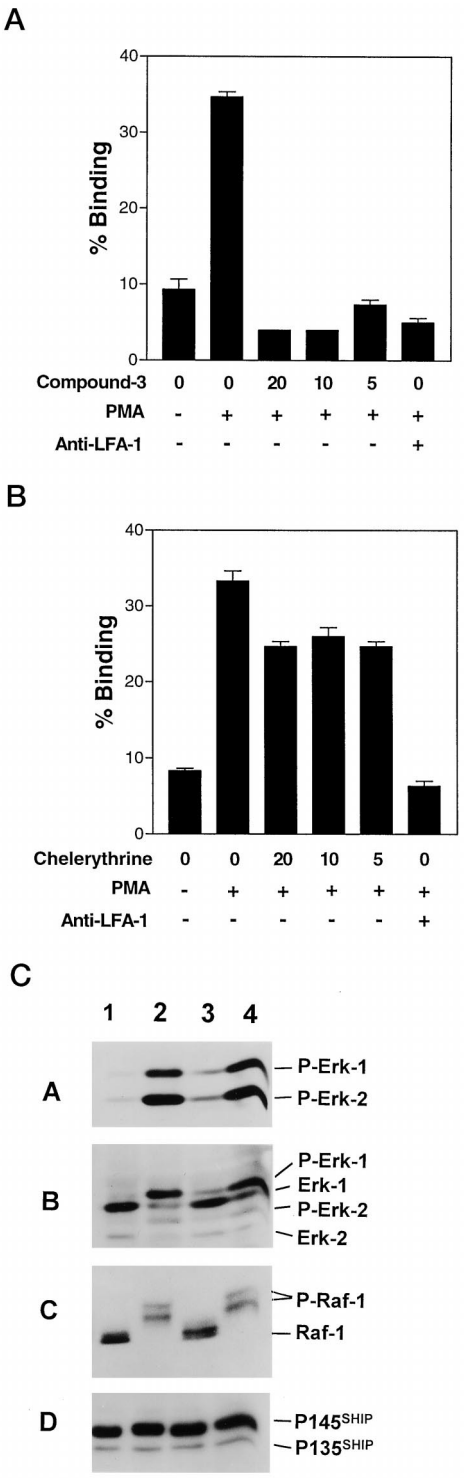

FIGURE 4. Effect of PKC inhibitors on PMA-induced activation of LFA-1. *A*, Cells overexpressing WT-SHIP were cytokine starved and pretreated with the indicated concentrations (μM) of compound 3 for 30 min at 37°C. They were then incubated with (+) or without (−) 50 ng/ml PMA for 25 min at 37°C then tested for ICAM-1 binding in the presence (+) or absence (−) of 10 μg/ml anti-LFA-1 mAb as in Fig. 2. *B*, Cytokine-starved cells were preincubated with the indicated concentration (μM) of chelerythrine, stimulated with PMA, and tested for ICAM-1 binding as in *A*. *C*, Cytokine-starved cells were untreated (*lane 1*), stimulated with 50 ng/ml PMA for 25 min (*lane 2*), or pretreated with 10 μM (*lane 3*) or 1 μM (*lane 4*) compound 3 for 30 min before stimulation with PMA (50 ng/ml) for 25 min. All incubations were at 37°C. Total cell lysates were subjected to Western analysis using Abs against phospho-MAPK (*panel A*), MAPK (*panel B*), Raf-1 (*panel C*), and SHIP (*panel D*).

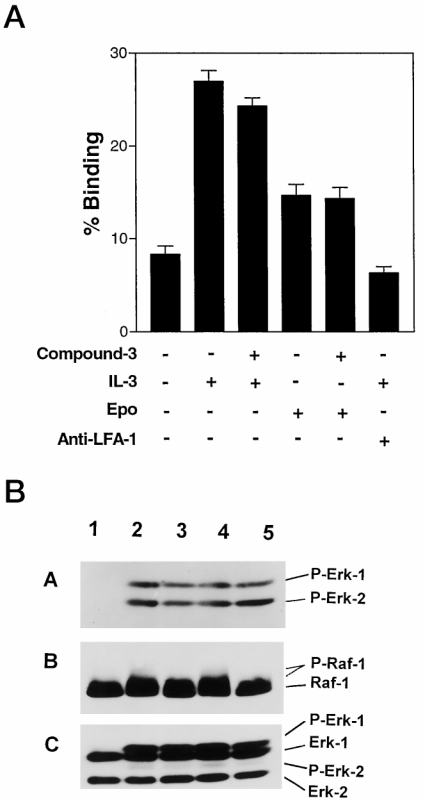

FIGURE 5. Effect of PKC inhibition on cytokine-induced activation of LFA-1. *A*, DA-ER cells overexpressing WT-SHIP were cytokine starved, preincubated with (+) or without (−) 10 μM compound 3 as in Fig. 4, and either unstimulated (−) or stimulated (+) with 400 ng/ml IL-3 or 50 U/ml Epo. Cells were then tested for ICAM-1 binding in the presence (+) or absence (−) of 10 μg/ml anti-LFA-1. *B*, Cytokine-starved cells were untreated (*lane 1*), stimulated with 400 ng/ml IL-3 or 50 U/ml Epo (*lanes 2* and *4*, respectively), or pretreated with 10 μM compound 3 and stimulated with either 400 ng/ml IL-3 or 50 U/ml Epo (*lanes 3* and *5*, respectively). Total cell lysates were subjected to Western analysis using Abs against phospho-MAPK (*panel A*), Raf-1 (*panel B*), and MAPK (*panel C*).

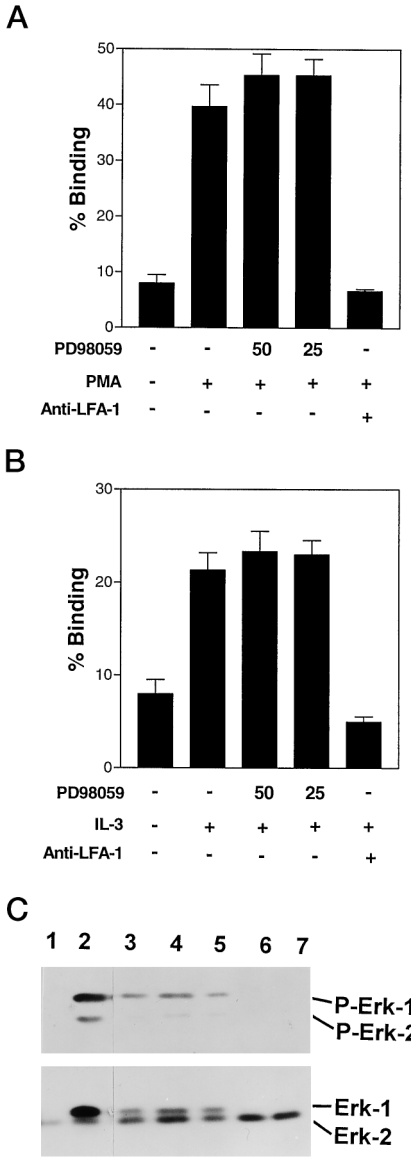

FIGURE 6. Effect of MAPK inhibition on LFA-1 activation. *A*, DA-ER cells overexpressing WT-SHIP were cytokine starved and preincubated with the indicated concentrations (μM) of PD98059 for 30 min. They were then incubated with (+) or without (−) 50 ng/ml PMA for 25 min and tested for adhesion to ICAM-1 in the presence (+) or absence (−) of 10 μg/ml anti-LFA-1. All incubations were at 37°C. *B*, Cells were cytokine starved, pretreated with PD98059 as in *A*, and incubated with (+) or without (−) 400 ng/ml IL-3 (black bars). The treated cells were tested for adhesion to ICAM-1. *C*, Cytokine-starved cells were untreated (*lane 1*), stimulated with PMA without compound 3 pretreatment (*lane 2*), pretreated with 50 or 25 ng/ml compound 3 and stimulated with PMA (*lane 3* and *4*, respectively), stimulated with IL-3 without compound 3 pretreatment (*lane 5*), or pretreated with 50 or 25 μM compound 3 and stimulated with IL-3 (*lane 6* and *7*, respectively) as in *A* and *B*. Total cell lysates from equivalent amounts of cells were subjected to Western analysis using Abs against phospho-MAPK (*upper panel*) or MAPK (*lower panel*).

panel C, *lanes 2* and *4*, respectively). However, in cells treated with compound 3 and IL-3 or compound 3 and Epo, there was both a reduced Raf-1 bandshift (Fig. 5*B*, *panel B*, *lanes 3* and *5*) and a reduced band shift of Erk-2 (Fig. 5*B*, *panel C*, *lanes 3* and *5*), indicating that PKC does contribute slightly to Epo- and IL-3-induced MAPK activation. Nonetheless, these results indicate that IL-3 and Epo stimulate MAPK phosphorylation in SHIP-overexpressing DA-ER cells primarily through pathways other than those activated by PKC. A likely possibility is the Ras pathway because both Epo and IL-3 have been shown to activate Ras (34, 35).

Activation of LFA-1 on SHIP-overexpressing DA-ER cells is MAPK independent

Because PMA, IL-3, and Epo activated LFA-1 and also induced MAPK phosphorylation, we examined whether MAPK is involved in the activation of LFA-1. To test this, SHIP-overexpressing DA-ER cells were preincubated with the MAPK pathway inhibitor, PD98059, which specifically inhibits the immediate upstream threonine/tyrosine kinase MAPK/extracellular regulated kinase (24), and then stimulated with PMA or IL-3, and the cells were assessed for ICAM-1 binding. As can be seen in Fig. 6, *A* and *B*, PD98059 treatment before PMA or IL-3 did not significantly reduce ICAM-1 binding, suggesting no involvement of MAPK in this process. Immunoblotting with anti-phospho-MAPK Abs showed that while both Erk-1 and Erk-2 were phosphorylated upon PMA or IL-3 stimulation (Fig. 6*C*, *lanes 2* and *5*, respectively), PD98059 at concentrations of either 50 μM or 25 μM followed by stimulation with PMA reduced both Erk-1 and Erk-2 phosphorylation (Fig. 6*C*, *upper panel*, *lanes 3* and *4*). Similarly, treatment of

cells with either 50 μM or 25 μM of PD98059 before IL-3 stimulation completely inhibited MAPK phosphorylation (Fig. 6*C*, *upper panel*, *lanes 6* and *7*, respectively), indicating that the concentrations of inhibitor used were sufficient to block MAPK activation. These results were further supported by reblotting with anti-Erk-1 mAb that also served as a control for protein loading (Fig. 6*C*, *lower panel*). A band shift was observed when cells were stimulated with PMA (Fig. 6*C*, *lower panel*, *lane 2*), and this was markedly reduced in cells preincubated with either 50 μM or 25 μM PD98059 (Fig. 6*C*, *lower panel*, *lanes 3* and *4*). Stimulation of cells with IL-3 also induced a band shift of MAPK (Fig. 6*C*, *lower panel*, *lane 5*) which was completely inhibited by PD98050 (Fig. 6*C*, *lower panel*, *lanes 6* and *7*). These results indicate that LFA-1 activation in cells overexpressing SHIP is independent of MAPK activation.

Epo and IL-3, but not PMA, activate LFA-1 in SHIP-overexpressing DA-ER cells via PI-3K

Because enhancement of cell adhesion by SHIP overexpression was dependent on its catalytic activity (Fig. 2, *A* and *B*), and because we and others have shown previously that one of its catalytic activities is to hydrolyse PI-3K-generated PI-3,4,5-P_3 (12–14), we next examined the role of PI-3K in LFA-1-mediated cell adhesion. Specifically, cells were incubated with the PI-3K inhibitor, wortmannin, and then stimulated with PMA, IL-3, or Epo and tested for their ability to bind to ICAM-1. Interestingly, while preincubation of cells with wortmannin followed by stimulation with PMA did not significantly affect binding (Fig. 7*A*), the treatment with wortmannin before stimulation with IL-3 or Epo profoundly inhibited cell binding (Figs. 7, *B* and *C*, respectively). This demonstrates that PI-3K activation plays an important role in cytokine-mediated but not phorbol ester-mediated LFA-1 activation.

Discussion

In this study, we have investigated whether SHIP plays a role in PMA- or cytokine-mediated LFA-1 activation by overexpressing both WT and phosphatase dead forms of SHIP in DA-ER cells. Our results show that 1) overexpression of WT-SHIP in unstimulated DA-ER cells increases LFA-1-mediated cell adhesion to ICAM-1, and this adhesion is further augmented by the addition of PMA, IL-3, or Epo; 2) SHIP requires a functional 5′-phosphatase domain for these effects, and overexpression of a phosphatase dead form actually leads to a slight inhibition of LFA-1-mediated adhesion to ICAM-1; 3) SHIP overexpression most likely enhances adhesion via its effect on inside-out signaling because its overexpression has no effect on the external activation of LFA-1 by Mn^{2+}; 4) LFA-1 activation on cells overexpressing WT-SHIP does not involve activation of Erk-1 and Erk-2; and 5) LFA-1 activation in response to PMA in SHIP-overexpressing cells is via its effects on a PKC-stimulated pathway, while LFA-1 activation in response to Epo and IL-3 occurs via its effects on a PI-3K-stimulated pathway.

Previous studies have shown that WT-SHIP overexpression profoundly alters cell functions. For example, ectopic expression of SHIP in FDC-P1 cells significantly reduces M-CSF-dependent cell growth (16), while in DA-ER cells it leads to a faster rate of apoptosis at high cell densities (19). Of interest, in both FDC-P1 and DA-ER cells, exogenous SHIP expression, regardless of the promoter used, never exceeds twice the level of endogenous SHIP (16, 19), suggesting that high levels of SHIP may be deleterious to survival/proliferation.

Importantly, in the current study, overexpressing the phosphatase dead form of SHIP, even at the low levels we were capable of achieving, appears to reduce adhesion of the cells to levels lower than control cells. This is consistent with it having a dominant negative effect on endogenous SHIP and suggests that endogenous SHIP is involved in LFA-1 activation. Utilization of this phosphatase dead form of SHIP has also made clear that the catalytic activity of SHIP is critical to its enhancement of both cytokine- and PMA-induced LFA-1 activation. As mentioned earlier, SHIP has

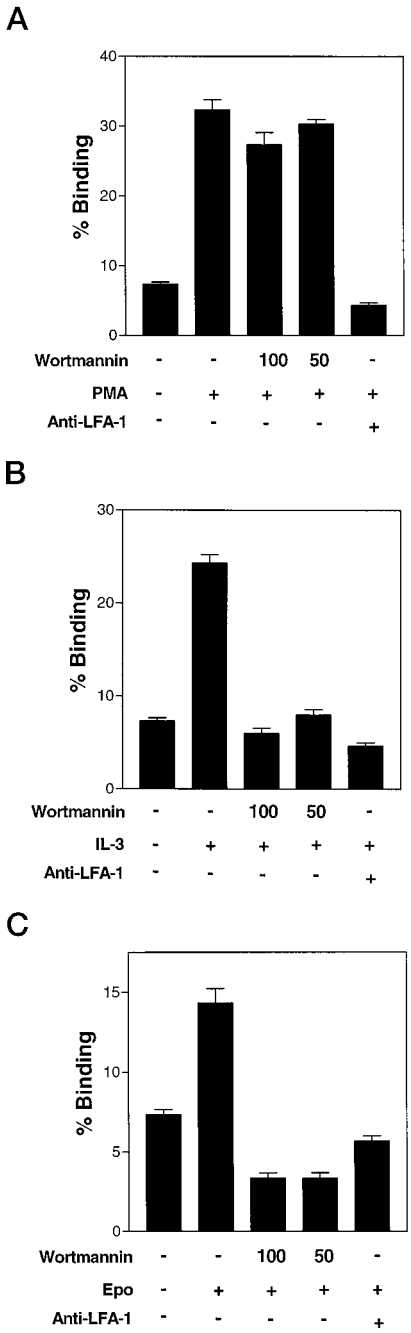

FIGURE 7. Role of PI-3K in LFA-1 activation. *A*, DA-ER cells overexpressing WT-SHIP were cytokine starved, pretreated with the indicated concentrations (nM) of wortmannin for 30 min, incubated with (+) or without (−) PMA, and tested for ICAM-1 binding in the presence (+) and absence (−) of 10 μg/ml anti-LFA-1. All incubations were at 37°C. *B*, Cells were pretreated with wortmannin, stimulated, and tested for ICAM-1 binding as in *A*. IL-3 (400 ng/ml) was used to stimulate cells in place of PMA. *C*, Cells were pretreated with wortmannin, stimulated, and tested for ICAM-1 binding as in *A*. Epo (50 U/ml) was used in place of PMA to stimulate the cells.

been shown in vitro to have two catalytic activities. It hydrolyzes both PI-3,4,5-P_3 (which is generated by cell surface receptor activation of PI-3K) to phosphatidylinositol-3,4-biphosphate (PI-3,4-P_2) and I-1,3,4,5-P_4 (which is generated by an inositol-1,4,5-trisphosphate (I-1,4,5-P_3)-specific PI-3K) to inositol-1,3,4-trisphosphate (I-1,3,4-P_3) (12).

Our results showing that Epo- and IL-3-induced activation of LFA-1 can be inhibited by wortmannin suggests that SHIP's enhancement of this effect is most likely mediated by hydrolyzing PI-3,4,5-P_3 to PI-3,4-P_2. This in turn suggests that LFA-1 is activated by reducing PI-3,4,5-P_3 levels and/or by increasing PI-3,4-P_2 levels. SHIP overexpression is expected to reduce the intracellular level of PI-3,4,5-P_3 by hydrolyzing it to PI-3,4-P_2. In fact, recent studies in our laboratory comparing PI-3,4,5-P_3 levels in bone marrow-derived mast cells from $SHIP^{+/+}$ and $SHIP^{-/-}$ mice indicates that this is the case.[4] PI-3,4,5-P_3 has been shown to play an important role in many signaling events, including actin polymerization (36), regulation of PI-3K association with tyrosine-phosphorylated proteins (37), activation of PKC isoforms (38), increases in intracellular calcium (13–15, 39) and cell survival and proliferation (40). Activation of PI-3K, which generates PI-3,4,5-P_3 (40) has been reported to induce LFA-1 activation in T cells triggered by IL-2 (41). However, recent data suggest that activation of PI-3K may also inhibit LFA-1-mediated T cell adhesion. Specifically, the engagement of CD4 either by MHC class II-mimicking peptides, anti-CD4 mAb, or the HIV component gp160 down-regulates LFA-1-mediated T cell adhesion, and PI-3K is required for this effect (42). Therefore, the role of PI-3K and its product PI-3,4,5-P_3 on the regulation of LFA-1 remains to be fully resolved. Nevertheless, our results suggest that, at least in DA-ER cells, a reduction of PI-3,4,5-P_3 and a concomitant increase in PI-3,4-P_2 may be responsible for LFA-1 activation. In this regard, PI-3,4-P_2 has been shown to be a more potent activator of calcium-independent PKC isoforms than is PI-3,4,5-P_3 (38), and perhaps a compound 3-insensitive isoform plays a role in Epo- and IL-3-mediated activation of LFA-1.

It is generally thought that the cytoplasmic domains of LFA-1 are important for its activation, because truncations of these domains render LFA-1 unresponsive to activating signals (43, 44). Furthermore, transfection of the cytoplasmic domain of the CD18 subunit of LFA-1 into LFA-1^+ cells exerts a dominant negative effect on LFA-1-mediated cell adhesion (45). These observations suggest that the cytoplasmic domains of LFA-1 may interact with signaling molecules that regulate LFA-1 activation. One such candidate signaling molecule is cytohesin-1 (CH-1), because it has been shown to associate with the cytoplasmic domain of CD18 (46). CH-1 contains both a SEC7 domain in its amino terminus that binds to the cytoplasmic portion of CD18 and a pleckstrin homology (PH) domain in its carboxyl terminus that targets it to the plasma membrane by binding PI-3,4,5-P_3 (47, 48). In Jurkat cells transfected with full length CH-1 or with the CH-1 SEC7 domain, LFA-1 is constitutively activated. Conversely, transfection of cells with only the CH-1 PH domain inhibits binding of LFA-1 to ICAM-1 (46, 48). Interestingly, one might predict that overexpression of WT-SHIP would lead to the depletion of PI-3,4,5-P_3 and a reduced binding of CH-1 to LFA-1. However, the affinity of CH-1's PH domain for PI-3,4-P_2 has not been investigated and, as has been reported for the PH domain of PKB/Akt (49), it may be stronger than for PI-3,4,5-P_3. There might also be a CH-1-independent, PI-3,4-P_2-dependent pathway that has still to be defined.

PMA activation of PKC (50) and Rho (51) has been correlated with increased activation of LFA-1 (51). However, the PKC isoforms activated and the intracellular mechanisms involved in this PMA-induced activation of LFA-1 have not been elucidated. Nevertheless, our results clearly show that overexpression of SHIP strongly enhances PMA-induced LFA-1 activation. How SHIP modulates this PKC pathway is still an open question. However, SHIP requires its catalytic activity to enhance PMA-stimulated adhesion and yet it is not inhibited by wortmannin. We tentatively conclude from this that SHIP's ability to hydrolyze I-1,3,4,5-P_4 to I-1,3,4-P_3 might be critical here. This is somewhat at odds with the notion that I-1,3,4,5-P_4 may be capable, at least in platelets, of inducing extracellular Ca^{2+} influx by opening a Ca^{2+} channel (52). One might predict that SHIP would limit this influx by hydrolyzing this putative second messenger to I-1,3,4-P_3. This is important because an influx of extracellular Ca^{2+} has been shown to play an important role in LFA-1 activation (53). For example, phorbol ester-mediated T cell adhesion to ICAM-1 or activation of LFA-1 by TCR cross-linking has been shown to be Ca^{2+} dependent (52, 54). Furthermore, Ca^{2+} mobilization has been shown to increase LFA-1 avidity by clustering the LFA-1 molecules on the cell surface and modifying the cytoskeleton (55, 56). While this appears in conflict with our results, nothing is as yet known about the possible function of I-1,3,4-P_3, and it might be a more potent stimulator of calcium influx than I-1,3,4,5-P_4.

In summary, we have shown that overexpression of SHIP markedly enhances LFA-1-mediated adhesion to ICAM-1. We are currently exploring the role of endogenous SHIP in this process.

[4] M. P. Scheid, M. Huber, Y. Duronio, and G. Krystal. Loss of SHIP leads to elevated PI-3, 4,5-P_3 reduced PI-3, 4-P_2 and increased PKB activation in steel factor stimulated primary mast cells. *Submitted for publication.*

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