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Ectophosphorylation of CD36 Regulates Cytoadherence of *Plasmodium falciparum* to Microvascular Endothelium under Flow Conditions

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The adhesion of *Plasmodium falciparum*-infected erythrocytes (IRBCs) to human dermal microvascular endothelial cells (HDMECs) under flow conditions is regulated by a Src family kinase- and alkaline phosphatase (AP)-dependent mechanism. In this study, we showed that the target of the phosphatase activity is the ectodomain of CD36 at threonine-92 (Thr^{92}). Mouse fibroblasts (NIH 3T3 cells) transfected with wild-type CD36 or a mutant protein in which Thr^{92} was substituted by Ala supported the rolling and adhesion of IRBCs. However, while the Src family kinase inhibitors PP1 and PP2 and the specific AP inhibitor levamisole significantly reduced IRBC adhesion to wild-type CD36 transfectants as with HDMECs, the inhibitors had no effect on IRBC adhesion to the mutant cells. Using a phosphospecific antibody directed at a 12-amino-acid peptide spanning Thr^{92}, we demonstrated directly that CD36 was constitutively phosphorylated and could be dephosphorylated by exogenous AP. Endothelial CD36 was likewise constitutively phosphorylated. The phosphospecific antibody inhibited IRBC adhesion to HDMECs that could be reversed by preincubating the antibody with the phosphorylated but not the nonphosphorylated peptide. Pretreatment of HDMECs with AP abrogated the effect of PP1 on IRBC adhesion. Collectively, these results are consistent with a critical role for CD36 dephosphorylation through Src family kinase activation in regulating IRBC adhesion to vascular endothelium.

The scavenger receptor CD36 expressed on erythroblasts, platelets, monocytes/macrophages, dendritic cells, microvascular endothelial cells, striated muscle cells, adipocytes, and mammary epithelial cells is increasingly recognized as a signaling molecule and/or coreceptor for diverse ligands that are implicated in the pathogenesis of major inflammatory diseases. Specifically, CD36 has been shown to elicit a proinflammatory response in microglial cells in the brain (3, 22) and macrophages in an atheroma (21) through its interaction with fibrillar β-amyloid. In binding to CD36, β-amyloid inhibits CD36-mediated clearance of oxidized lipoproteins and thus promotes accumulation of lipid peroxidases and accelerated atherogenesis (19). Evidence is also emerging that CD36 acts as a coreceptor for some but not all bacterial ligands for Toll-like receptor 2 activation (17). As a result, CD36-deficient mice were hypersusceptible to the gram-positive microbe *Staphylococcus aureus*.

In *Plasmodium falciparum* malaria, CD36 has long been considered a major contributor to pathogenesis by acting as a vascular receptor for the adhesion of infected erythrocytes (IRBCs) (16). The deleterious effect of cytoadherence has been attributed to impairment of microcirculatory blood flow, with subsequent tissue hypoxia and organ dysfunction. There is strong clinical evidence to support this mechanical mechanism, such as the demonstration of lower-than-expected oxygen tension and reduced perfusion pressure in the cerebral circulation of patients with cerebral malaria (23, 28). IRBC adhesion may also contribute to pathology through modulation of endothelial cell function. Indeed, we have previously shown that the binding of a 179-amino-acid peptide representing the minimal binding domain of the parasite cytoadherent ligand *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) to CD36 induces intracellular signaling (32). One of the immediate effects of the activation of signaling events appears to be an enhancement of IRBC adhesion under flow conditions that is Src family kinase dependent and can be abrogated by the specific alkaline phosphatase (AP) inhibitor levamisole. These results led us to hypothesize that the phosphorylation state of the ectodomain of endothelial CD36 may be critical for optimal IRBC adhesion. We proposed that CD36 is constitutively phosphorylated. Upon initial IRBC adhesion and Src family kinase activation, CD36 becomes dephosphorylated through the activation of an ecto-AP that is expressed on the surface of endothelial cells. Dephosphorylated CD36 binds to IRBCs with higher affinity than phosphorylated CD36. This proposed mechanism would mimic the interaction of CD36 with its natural ligands thrombospondin 1(TSP-1) and collagen, in that

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phosphorylated CD36 binds collagen but acts as a low-affinity receptor for TSP-1 (2). Initial CD36–TSP-1 interaction induces platelet degranulation with the release of acid phosphatases that dephosphorylate threonine-92 (Thr^{92}) in the ectodomain of CD36, resulting in higher binding affinity for TSP-1.

In the present study, we provide molecular, biochemical, and functional evidence to support a critical role for CD36 ectodomain phosphorylation at Thr^{92} in regulating IRBC adhesion to CD36 under flow conditions.

MATERIALS AND METHODS

Tissue culture and other reagents. Unless otherwise stated, tissue culture and PCR reagents were obtained from Invitrogen Canada, Inc. (Burlington, Ontario, Canada) and chemical reagents were from Sigma-Aldrich Co. (St. Louis, Mo.). The Src family kinase selective inhibitors PP1 and PP2 and the inactive analog PP3 were purchased from BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA. Protease inhibitors were from Calbiochem (EMB BioSciences, Inc., La Jolla, CA). Calf intestine AP was purchased from New England Biolabs, Toronto, Ontario, Canada. Enhanced chemiluminescence substrate (ECL) was purchased from Amersham Pharmacia Biotech, Piscataway, NJ.

Parasites. Cryopreserved parasite isolates from adult Thai patients with well-documented *P. falciparum* malaria were thawed and studied during their first cycle in culture as described previously (26). The collection of specimens was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. Informed consent was obtained from all participating patients.

Antibodies. The anti-CD36 monoclonal antibody (MAb) OKM5 was a kind gift of Ortho Diagnostics, Raritan, NJ. MAb FA6-152 was purchased from Immunotech (Montreal, Quebec, Canada). Polyclonal anti-CD36 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Cayman Chemical Co. (Ann Arbor, Michigan). Fluorescein-labeled secondary antibodies for flow cytometry were purchased from Becton Dickinson (San Jose, CA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA), and Pierce Biotechnology (Rockford, IL). A phosphospecific CD36 antibody (Ab) to the peptide KQRG PYT*YRVRF, where the asterisk is the phosphorylated Thr^{92}, was raised in sheep at the MRC Protein Phosphorylation Unit, School of Life Sciences, Division of Cell Signaling, University of Dundee, Scotland, United Kingdom. The immunogen was conjugated to keyhole limpet hemocyanin and bovine serum albumin. The antiserum was affinity purified on CH-Sepharose 4B to which the phosphorylated peptide had been covalently coupled. The bound fraction was eluted. Specificity of the antibody was confirmed by dot blot analysis and enzyme-linked immunosorbent assay with the phosphorylated and nonphosphorylated peptides as the capture antigen.

Endothelial cell culture. Human dermal microvascular endothelial cells (HDMECs) were harvested from discarded neonatal human foreskins by using 0.5 mg/ml type IA collagenase (Boehringer Mannheim Biochemicals, Indianapolis, IN) as described previously (31). The protocol was approved by the Conjoint Health Ethics Board of the University of Calgary. The cells were maintained in endothelial basal medium (BioWhittaker, Walkerville, MD) with supplements provided by the manufacturer. Experiments were performed with cells from passages 1 to 5, on which adhesion molecule expression was shown to be stable.

CD36 transfectants. The mouse fibroblast cell line NIH 3T3 (ATCC CRL-1658) was used to produce stable transfectants expressing human CD36. The cDNA of full-length human CD36 cloned in the plasmid pJEF14 was a kind gift of John Elliott, University of Alberta, Canada. 3T3 cells were cotransfected with a plasmid expressing the puromycin resistance gene, pBabe, by the FuGene 6 method according to the manufacturer's instructions (Roche Diagnostics, Laval, Quebec, Canada). The transfected cells (clone 1-10) were screened for surface expression of CD36 by flow cytometry using the MAb OKM5. Transfectants expressing a mutant CD36 in which threonine at position 92 was substituted by alanine (clone 22-2-F1) were similarly produced.

Site-directed mutagenesis. A mutant plasmid, CD36ala92, was generated from HDMEC cDNA, using a protocol modified from Asch et al. (2), by overlap extension PCR with the following primer pairs (with the single mismatched nucleotides shown in bold): 5′GCTCTAGACCACCATGGGCTGTGACCG3′ and GAACTCTGTACG**C**ATAAGGACCTC for the N-terminal portion, and GAGGTCCTTAT**G**CGTACAGAGTTC and CCGGAATTCTTATTTTATTG TTTTCGATC for the C-terminal portion. PCR was performed with Proofstart DNA polymerase (QIAGEN, Inc., Mississauga, Ontario, Canada) by using the following reaction conditions: 95° for 5 min; 30 cycles of 95° for 30 s, 58° for 1 min, and 72° for 4 min; followed by 72° for 7 min. The full-length product was cloned into the T/A cloning vector pDrive (QIAGEN). Clones were verified by restriction mapping and DNA sequencing. The mutant CD36 insert was subcloned into pJFE14 for expression in NIH 3T3 cells.

Flow cytometry. Cells were stained for flow cytometric analysis by using standard methods. The MAb OKM5 at 5 μg/ml was used to detect surface expression of CD36. Mouse immunoglobulin G (IgG) was used as a control. For detection of CD36 phosphorylation, cells were stained with the phosphospecific Ab at 10 μg/ml, with sheep IgG as a control. To determine the effect of AP on phospho-CD36 expression, 3×10^5 to 4×10^5 cells were treated with 50 U of AP in 100 μl of phosphate-buffered saline, pH 7.4, for 15 min at 37°C before staining. The activity of the enzyme under these experimental conditions was confirmed using the standard substrate *p*-nitrophenol phosphate.

Parallel plate flow chamber assay. IRBC-endothelial cell interactions at fluid shear stresses approximating those in the microvasculature were studied using a parallel plate flow chamber as described previously (31). In previous studies, we established that infusion of a 1% IRBC suspension over endothelial cell monolayers at 1 dyne/cm^2 allowed us to optimally visualize the adhesive interactions in real time.

Immunoprecipitation and Western blotting. Immunoprecipitation of CD36 from transfectants and HDMECs was performed as described previously (18). Cells were lysed directly in 100-mm tissue culture dishes in 1 ml of modified RIPA buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 1% deoxycholic acid, 0.1% sodium dodecyl sulfate, phosphatase inhibitors [1 mM Na_3VO_4, 25 mM β-glycerophosphate, 100 mM NaF], and protease inhibitors [10 μg/ml leupeptin, 10 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride]). Supernatant from the lysate was incubated with 5 μg of the anti-CD36 MAb FA6-152 at 4°C for 3 h, after which 30 μl of 50% protein G and 30 μl of 50% protein A beads (Sigma) were added. The mixture was incubated at 4°C for a further 2 h. The beads were washed two times with 1 ml of Triton-only buffer at 4°C, followed by two times with 1 ml of HNTG buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) at 4°C. The beads were resuspended in 2× Laemmli's sample buffer and boiled for 10 min at 90°C. The supernatant was separated on 7.5% sodium dodecyl sulfate-polyacrylamide gels. Following electrophoresis, the proteins were transferred to nitrocellulose membranes (Schleicher & Schuell BioSciences, Inc., Keene, NH). In control experiments, mouse IgG (Santa Cruz) was used instead of MAb FA6-152 for immunoprecipitation.

In experiments to detect the effect of exogenous AP on CD36 phosphorylation, washed beads were resuspended in 2 ml of AP buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9) and divided into two aliquots. AP (100 U) was added to one aliquot. Both tubes were incubated at 37°C for 1 h. At the end of the incubation, beads were washed twice with HNTG buffer and processed for Western blotting as described above.

For the detection of CD36, membranes were blocked in Tris-buffered saline containing 0.1% Nonidet P-40, 0.1% Tween 20, and 5% skim milk for 1 h. Membranes were then probed with a polyclonal anti-CD36 antibody (Santa Cruz) at a 1:200 dilution for 2 h, followed by a goat anti-rabbit-HRP secondary antibody diluted 1:10,000 for 1 h at room temperature. The membranes were washed and developed using ECL (Pierce).

For the detection of phosphospecific CD36, membranes were blocked in 10% bovine serum albumin in Tris-buffered saline (25 mM Tris-HCl, 500 mM NaCl) with 0.1% Tween 20. Membranes were incubated overnight at 4°C in the primary antibody (10 μg/ml) and 2 μg/ml of the nonphosphorylated peptide. Membranes were washed and then incubated with donkey anti-sheep-HRP secondary antibody diluted 1:5,000 for 1 h at room temperature. After being washed for 1 h, membranes were developed as described above.

Statistical analysis. All data are presented as means ± standard errors of the means. Data between control and test groups, from studying the same clinical parasite isolate on endothelial monolayers from the same skin preparation and passage subjected to different treatments, were compared by Student's *t* test for paired samples. A P value of <0.05 compared to the control was considered statistically significant.

RESULTS

CD36 expressing murine fibroblasts as a model of ectodomain phosphorylation. In the original report implicating a critical role for CD36 ectodomain phosphorylation in differential ligand binding, it was demonstrated that phosphorylation occurred solely on Thr^{92} (2). In order to directly assess the role of this phos-

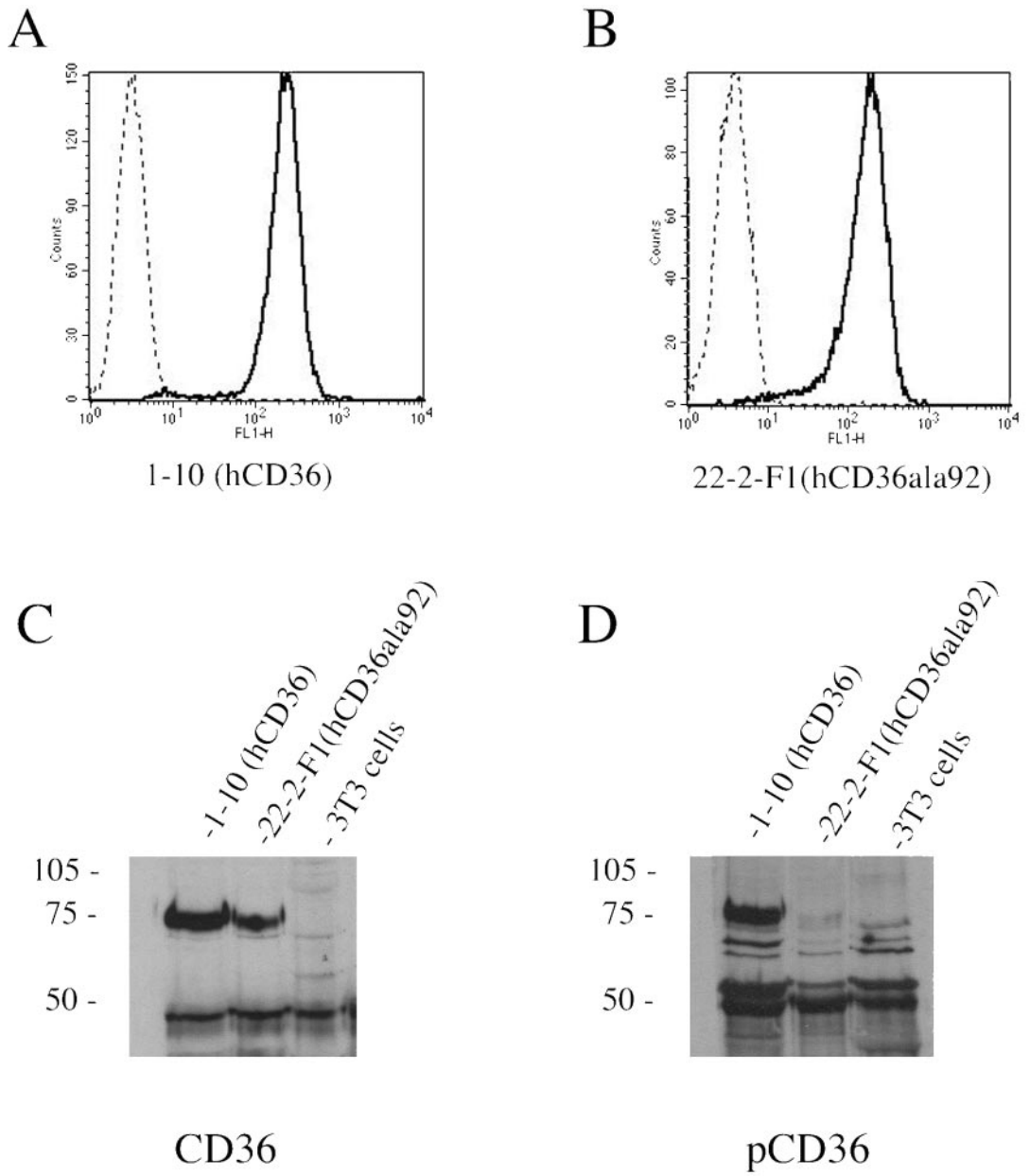

FIG. 1. Flow cytometric and Western blot analyses of NIH 3T3 transfectants. Cells expressing (A) wild-type human CD36 (hCD36) (clone 1-10) or (B) mutant protein CD36ala92 (clone 22-2-F1) were stained with MAb OKM5 (5 μg/ml) for flow cytometric analysis. Dashed curves represent cells stained with secondary antibody alone. FL1-H, fluorescent intensity at 530 nm. For Western blot analysis, CD36 was immunoprecipitated from 1-10, 22-2-F1, and untransfected 3T3 cells. After electrophoresis, the blots were first probed with (D) a phosphospecific Ab (pCD36) (10 μg/ml) raised to a 12-amino-acid peptide flanking Thr^{92} and then reprobed with (C) a polyclonal anti-CD36 Ab (1 μg/ml).

phorylation site on IRBC cytoadherence, we established murine fibroblasts (NIH 3T3) that expressed wild-type and mutant CD36 (CD36ala92). Both types of transfectants expressed human CD36 on the cell surface that was detectable by MAb OKM5 by flow cytometry with comparable mean fluorescent intensities (Fig. 1A and B), while no human CD36 was detected with untransfected 3T3 cells (data not shown).

CD36 protein from wild-type and mutant cells was studied further by Western blot analysis using a phosphospecific antibody that detected only the phosphorylated but not the dephosphorylated 12-amino-acid peptide flanking Thr^{92}. In both immunoprecipitation and lipid raft preparations (data not shown), wild-type CD36 appeared as a major band at 75 kDa as detected by a polyclonal anti-CD36 antibody (Fig. 1C and D). Wild-type CD36 could also be detected by the phosphospecific anti-CD36 antibody. In contrast, the mutant protein was recognized by the anti-CD36 antibody, but was not detected by the phosphospecific antibody. These results demonstrate both expression levels of CD36 in the transfectants and specificity for the phosphospecific Ab we have generated.

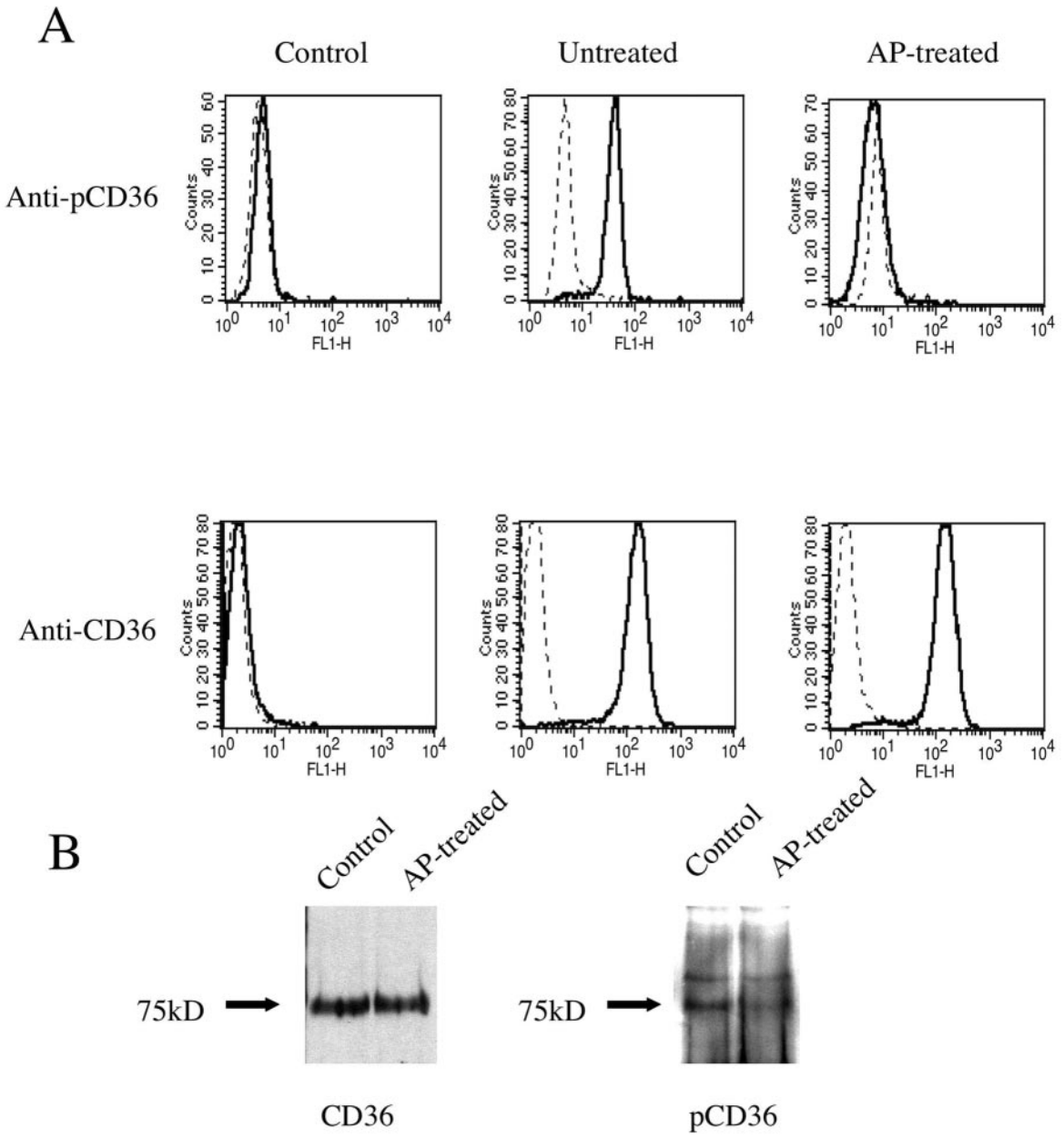

FIG. 2. Effect of exogenous AP on CD36 phosphorylation in transfectants expressing wild-type CD36. (A) For flow cytometry, untreated and AP-treated cells were stained with OKM5 (5 μg/ml) and the phosphospecific Ab (pCD36) (10 μg/ml). Normal mouse and sheep IgG were used as controls. Dashed curves represent cells stained with secondary antibody alone. FL1-H, Fluorescent intensity at 530 nm. (B) For Western blotting, untreated and AP-treated immunoprecipitates were first probed with a phosphospecific Ab (pCD36) and then reprobed with a polyclonal anti-CD36 Ab.

Cell surface CD36 was phosphorylated and could be dephosphorylated by AP. Flow cytometry and Western blotting were further employed to study the effect of exogenous AP on CD36 phosphorylation on wild-type transfectants. 1-10 cells were 100% positive when stained with the phosphospecific antibody (Fig. 2A). Control cells stained with sheep IgG were negative. Cells treated with AP prior to staining did not show any surface staining with the phosphospecific antibody, but the enzyme did not affect the staining with OKM5. The effect of exogenous AP on CD36 phosphorylation was also demonstrated by Western blotting (Fig. 2B). Immunoprecipitates from 1-10 cell lysates that were AP treated were positive for CD36 but were not detected when probed with the phosphospecific Ab. The results shown are representative of four experiments.

IRBC adhesion to wild-type but not mutant transfectants was Src family kinase and alkaline phosphatase dependent. We have previously demonstrated that the adhesion of IRBCs to HDMECs was inhibited by the Src family kinase inhibitor PP1 and the specific AP inhibitor levamisole (32). To determine if transfected NIH 3T3 cells expressing wild-type and mutant

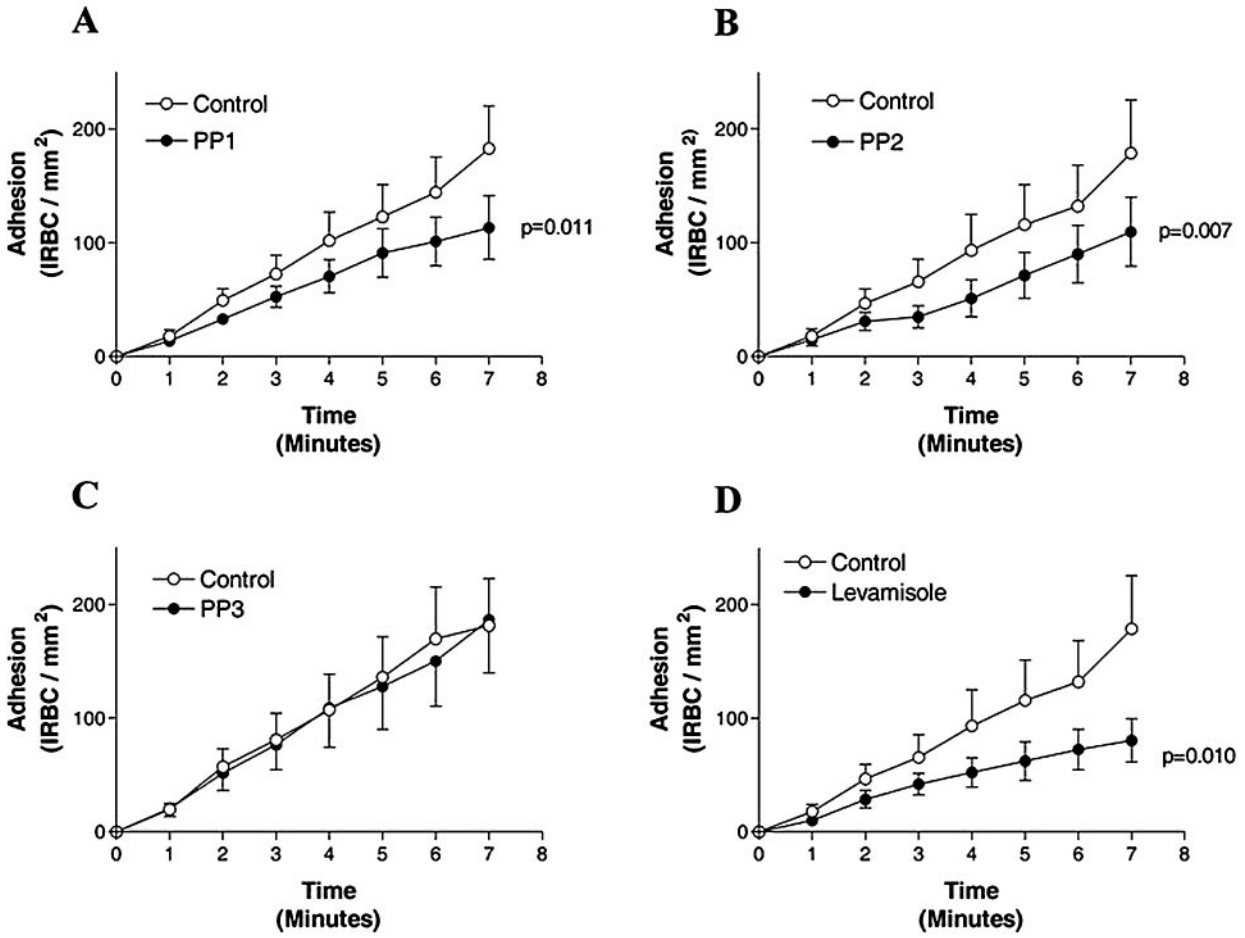

FIG. 3. Effects of the Src family kinase inhibitors PP1 and PP2 and the specific AP inhibitor levamisole on IRBC adhesion to NIH 3T3 transfectants expressing wild-type CD36 under flow conditions. Confluent monolayers of transfectants were pretreated with 10 μM of (A) PP1, (B) PP2, (C) the inactive analog PP3, or (D) levamisole, 1 mM, for 30 min at 37°C. IRBCs were infused at 1 dyne/cm^2 ($n = 9$ for PP1 and $n = 6$ for PP2, PP3, and levamisole). The total numbers of adherent cells at the end of the 7-min infusion for inhibitors versus controls, respectively, were (A) 105 ± 21 versus 184 ± 37 IRBCs/mm^2, (B) 110 ± 30 versus 179 ± 31 IRBCs/mm^2, (C) 159 ± 43 versus 179 ± 31, and (D) 81 ± 19 versus 179 ± 31 IRBCs/mm^2. *P* values shown are for paired comparison of each treatment to the corresponding controls.

CD36 behave in a manner similar to primary HDMECs, flow experiments were performed with transfectant monolayers pretreated with the inhibitors. IRBCs rolled and adhered to both the wild-type and the mutant transfectants but did not interact with untransfected 3T3 cells. Adhesion to the wild-type transfectants was partially inhibited by the Src family kinase inhibitors PP1 ($P = 0.011$, $n = 9$), PP2 ($P = 0.007$, $n = 6$), and levamisole ($P = 0.010$, $n = 6$) (Fig. 3). The inactive analog PP3 had no effect on IRBC adhesion to the transfectants. The statistical analysis took into account the difference in the numbers of adherent IRBCs to untreated and treated monolayers at every minute throughout the infusion. When the differences at each time point were analyzed, statistical significance was reached in every case after 4 to 5 min.

In contrast to the above results, neither the Src family kinase inhibitors nor levamisole had any significant effect on the adhesion of IRBCs to monolayers of mutant cells throughout the infusion (Fig. 4).

Endothelial CD36 was constitutively phosphorylated. The above results with the ectopically expressing fibroblasts support the hypothesis that Src family kinases and an ecto-AP work in concert to promote IRBC firm adhesion under physiological shear stress by dephosphorylating CD36 at Thr^{92}. For a similar process to occur on primary human microvascular endothelium, CD36 on these cells needs to be present on the cell surface in its phosphorylated state. Western blot analysis of CD36 immunoprecipitated from lysates of HDMECs was performed. A protein band at approximately 85 kDa was observed and confirmed to be human CD36 by matrix-assisted laser desorption ionization mass spectroscopy (Fig. 5A). The same protein was also detected by the phosphospecific antibody. No protein band was detected by either antibody when control mouse IgG was used for immunoprecipitation (data not shown).

Phosphospecific antibody inhibits IRBC adhesion to HDMECs. To determine if the phosphospecific antibody could affect IRBC adhesion, we studied IRBC adhesion to HDMECs by a flow chamber assay in which endothelial monolayers were pretreated with 10 μg/ml of the phosphospecific antibody at 37°C for 30 min. We observed that the phosphospecific antibody inhibited IRBC adhesion by approximately 50% ($P = 0.024$,

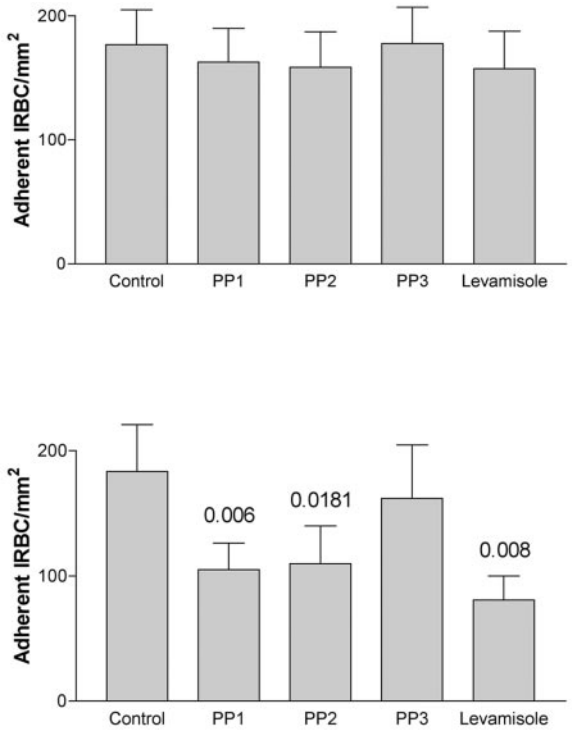

FIG. 4. Effects of the Src family kinase inhibitors PP1 and PP2 and the specific AP inhibitor levamisole on IRBC adhesion under flow conditions to (top) 3T3 transfectants expressing mutant CD36 (CD36ala92) or (bottom) transfectants expressing wild-type CD36. Bars represent the total numbers of adherent IRBCs at the end of the 7-minute infusion (control, 177 ± 28 IRBCs/mm^2; PP1, 163 ± 27 IRBCs/mm^2; PP2, 160 ± 29 IRBCs/mm^2; PP3, 178 ± 29 IRBCs/mm^2; and levamisole, 157 ± 30 IRBCs/mm^2; $n = 6$ [$n = 9$ for PP1, bottom panel]). P values shown are for paired comparison of each treatment to the corresponding control.

$n = 7$) (Fig. 5B). The inhibitory effect could be abrogated by preincubating the antibody with 2 mg/ml of the phosphorylated peptide ($P = 0.446$, $n = 4$) (Fig. 5C) but not the nonphosphorylated peptide ($P = 0.011$, $n = 4$) (Fig. 5D).

To exclude steric hindrance by the phosphospecific antibody as a major mechanism of the observed inhibition, we preincubated monolayers with an anti-CD36 polyclonal Ab raised to residues 99 to 112 of human CD36 (Cayman Chemical Co.), just adjacent to the phosphorylation site. This antibody had no effect on IRBC adhesion (data not shown).

PP1 had no effect on IRBC adhesion to HDMECs pretreated with exogenous AP. We have previously shown that exogenous AP could reverse the inhibitory effect of the Src family kinase inhibitor PP1 on IRBC adhesion to HDMEC monolayers (32). To further substantiate a link between phosphotyrosine kinase activity and CD36 dephosphorylation, monolayers were pretreated with 200 U of AP in 1 ml of Hanks balanced salt solution, pH 7.4, for 15 min at 37°C, after which 10 μM PP1 was added for a further 30 min. The results indicate that in the presence of AP, Src family kinase inhibition had no effect on IRBC adhesion (control, 137 ± 18 IRBCs/mm^2; PP1, 54 ± 7 IRBCs/mm^2; and AP plus PP1, 128 ± 23 IRBCs/mm^2; $n = 5$).

Effect of alkaline phosphatase inhibitors on IRBC adhesion to HDMECs. Our hypothesis for endothelial CD36 regulation of IRBC adhesion involves the activity of a membrane-bound AP. AP expressed on endothelial cells as a glycosylphosphatidyl inositol-anchored protein is the liver, bone, and kidney (LBK) isoenzyme (11), the activity of which can be inhibited by phenylimidazothiazoles such as (−)-*p*-bromotetramisole and levamisole [(−)-tetramisole]. As the enzyme inhibition is stereospecific, the D-enantiomers of bromotetramisole and levamisole would be expected to have no effect on IRBC adhesion. Therefore, the use of these compounds should clarify whether the dephosphorylation of the CD36 ectodomain is mediated by an AP or by other phosphatases, such as protein phosphatase type 1 (PP1), PP2A, PP2B, and PP2C, whose inhibition is nonstereospecific. Endothelial monolayers were preincubated with 100 μM to 1 mM (−)-*p*-tetramisole (levamisole), a racemic mixture of (−)-*p*-tetramisole and (+)-*p*-tetramisole, (−)-*p*-bromotetramisole, and (+)-*p*-bromotetramisole before being used in flow chamber assays. The results in Table 1 showed that levamisole and (−)-*p*-bromotetramisole, but not (+)-*p*-bromotetramisole, inhibited IRBC adhesion by approximately 50%. As expected, the racemic mixture of (−)-*p*-tetramisole and (+)-*p*-tetramisole was seen to have approximately half the effect of levamisole. Theophylline, another potent AP inhibitor, was also found to be effective in suppressing IRBC adhesion.

DISCUSSION

We have previously reported on pharmacological and functional data that are suggestive of a role for intracellular signaling and subsequent dephosphorylation of ectodomain CD36 in mediating firm adhesion of IRBCs to human microvascular endothelium under flow conditions (32). In this study, we used two experimental systems to further delineate several key steps in this proposed mechanism. First, a model for IRBC-CD36 interaction was established with NIH 3T3 transfectants expressing human CD36. These cells supported the rolling and adhesion of IRBCs under flow conditions, a process that was enhanced by the activation of Src family kinases and the participation of an ecto-AP, just as shown with primary human microvascular endothelial cells. Using an antibody raised to a phosphorylated peptide flanking Thr^{92}, we found that the ectodomain of CD36 on these transfectants was constitutively phosphorylated and that it became dephosphorylated upon treatment with exogenous AP. When murine fibroblast transfectants that expressed alanine in the phosphorylation site were established, the mutant CD36 was no longer recognized by the phosphospecific antibody. Interestingly, IRBC adhesion to the mutant cells was comparable to what was observed with cells expressing wild-type CD36, which suggests that dephosphorylation of CD36 on wild-type cells occurs rapidly on initial contact with interacting IRBCs. However, unlike with wild-type cells, IRBC adhesion to the mutants could not be modulated by Src family kinase or AP inhibitors.

Our second experimental model consisted of HDMECs that constitutively expressed CD36. We found that the ectodomain of CD36 on HDMECs was phosphorylated as in the transfectants, consistent with the preservation of a PKC-dependent targeting sequence, RGPYTYRVRFLA, for Thr^{92} phosphorylation on the endothelial protein (29). Furthermore, the phosphospecific antibody inhibited IRBC adhesion to HDMECs to a similar degree as Src family kinase or AP inhibitors. As the IRBC binding site on CD36 (residues 145 to 171) (4) is distinct

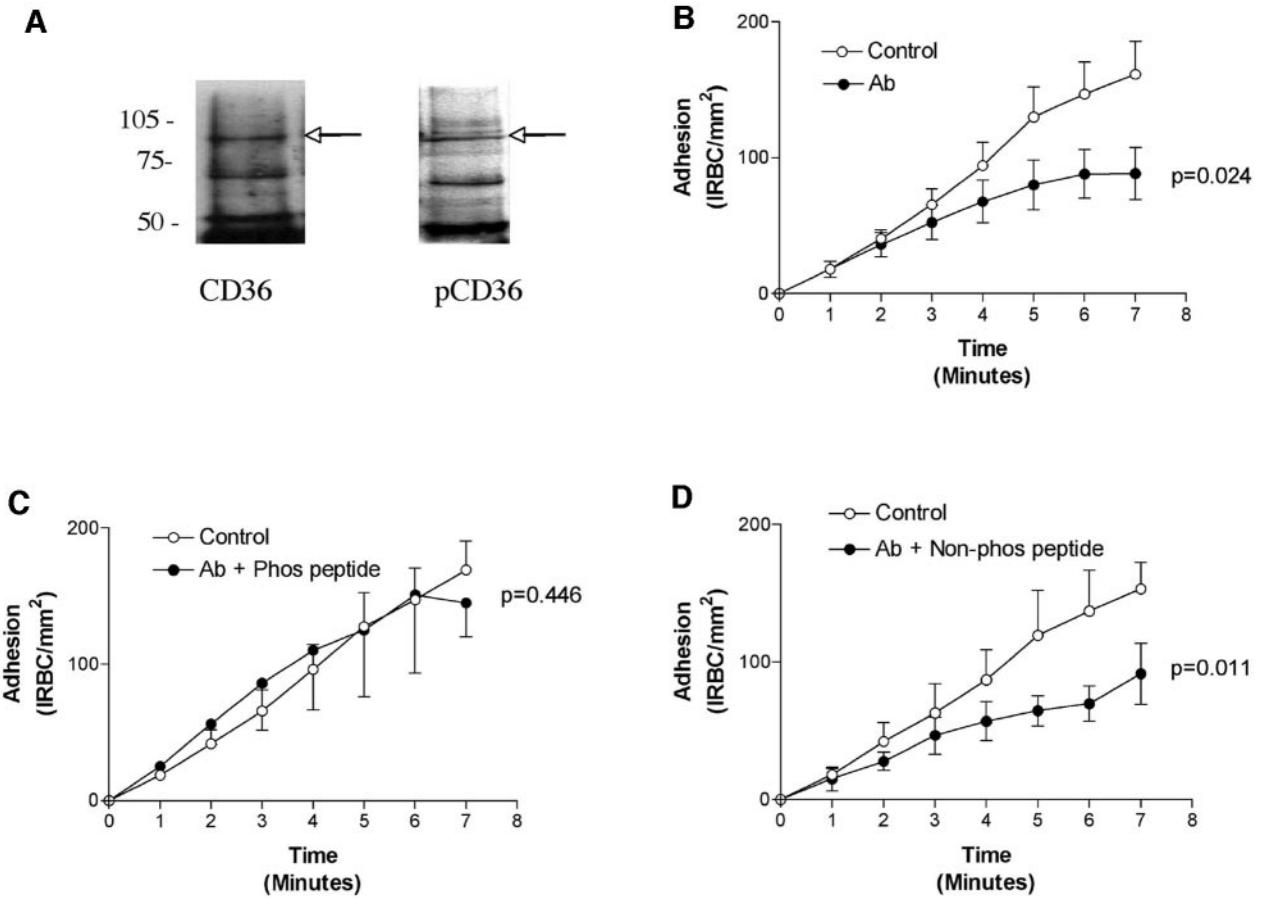

FIG. 5. Phosphospecific Ab and HDMECs. (A) Western blot analysis of CD36 immunoprecipitated from HDMECs. The blots were first probed with a phosphospecific CD36 Ab (pCD36) and then reprobed with a polyclonal anti-CD36 Ab. (B to D) Effect of the phosphospecific Ab on IRBC adhesion to HDMECs under flow conditions. Confluent monolayers were pretreated for 30 min at 37°C with (B) Ab at 10 μg/ml (n = 7), (C) Ab and 2 μg of phosphorylated (Phos) peptide (n = 4), or (D) Ab and 2 μg of nonphosphorylated (Non-phos) peptide (n = 4). The total numbers of adherent cells at the end of the 7-min infusion for inhibitors versus controls, respectively, were (B) 88 ± 19 versus 161 ± 24 IRBC/mm^2, (C) 145 ± 25 versus 169 ± 21 IRBCs/mm^2, and (D) 92 ± 22 versus 153 ± 19 IRBCs/mm^2.

from the epitope to which the phosphospecific antibody was raised, competitive inhibition by the phosphospecific antibody appears unlikely. The antibody might protect Thr^{92} from the effects of AP or it may cause a conformational change in CD36 that reduces IRBC adhesion. These possibilities need to be investigated further. There is some suggestion that CD36 folding as revealed by conformational epitope expression might be essential for the binding of IRBCs (13). Consistent with our previous finding that exogenous AP could reverse the effect of PP1 on IRBC adhesion to HDMECs (32), the Src family kinase inhibitor had no effect on IRBC adhesion to monolayers that were pretreated with exogenous AP. Collectively, the results with the transfectants and HDMECs further substantiate a link between CD36 dephosphorylation and optimal IRBC adhesion through a Src family kinase-dependent mechanism.

To distinguish the involvement of ecto-AP activity from other ectophosphatase activities in the regulation of IRBC adhesion, experiments with different phosphatase inhibitors were performed. Only those inhibitors that inhibit LBK alkaline phosphatases stereospecifically, i.e., levamisole and its brominated derivative, (−)-*p*-bromotetramisole, were shown to be effective. The inhibitory effects were concentration dependent

TABLE 1. Effects of levamisole and other AP inhibitors on IRBC adhesion to microvascular endothelium

Amt of compound	% Adhesion (± SEM)[a] with:				
	(−)-*p*-Tetramisole (levamisole) (n = 5)	(−)-*p*-Bromotetramisole (n = 4)	(+)-*p*-Bromotetramisole (n = 4)	(+)- and (−)-*p*-Tetramisole (n = 4)	Theophylline (n = 5)
100 μM	81 ± 6	81 ± 8	102 ± 10	96 ± 11	84 ± 7
500 μM	48 ± 7	67 ± 6	111 ± 2	92 ± 7	65 ± 4
1 mM	49 ± 5	49 ± 8	95 ± 6	50 ± 10	44 ± 4

[a] Compared to controls.

and corresponded to the activities of these compounds on the dephosphorylation of phosphothreonine at neutral pH in a biochemical assay (B. J. Berger and M. Ho, unpublished data). AP anchored to the plasma membrane acts as an enzyme to regulate extracellular concentrations of some phosphate compounds, such as pyridoxal-5′-phosphate, and the phosphorylation status of cell surface proteins. Ecto-AP activity is increased in some types of malignant cells (7) and appears to be involved in insulin transport in the brain (6), cell differentiation (26), and wound repair (11). In experimentally induced wounds, AP activity is increased by interleukin-6 and other members of this family of proinflammatory cytokines, particularly oncostatin M. Interestingly, interleukin-6 is markedly elevated in severe falciparum malaria, and the levels are positively correlated with disease severity (8).

Phosphorylation/dephosphorylation is now known to regulate many physiological processes. Consensus protein kinase phosphorylation sites are present in the extracellular domains of the majority of functionally important surface proteins, and ectoprotein kinases are present on the outer surface of a wide variety of cells (9). These ectoenzymes can phosphorylate both extracellular (soluble) and cell surface proteins, including adhesion molecules. Thus, they might play an important role in the regulation of ligand binding, signal transduction, and cell-cell interactions. A good example is the cystic fibrosis transmembrane conductance regulator chloride channels that are regulated by phosphorylation and dephosphorylation at multiple sites (5). Phosphorylation by protein kinase A provides the primary stimulus for channel activation, while dephosphorylation is mediated by a membrane-associated phosphatase (probably PP2C). Similarly, the ectodomains of the T-cell receptor αβ chains are phosphorylated intracellularly but are subsequently modified by ectoprotein phosphatase(s) and ectokinase(s) (1). Such modification may change T-cell cognate interactions by affecting antigen binding affinity (1) and effector functions (25). In the case of CD36, the identity of the protein kinase that phosphorylates Thr^{92} is unknown, as is whether CD36 is phosphorylated on this site by an intracellular kinase or an ectokinase. There is some evidence to support the presence on the surface of platelets of a cyclic AMP-dependent ectokinase that phosphorylates CD36 (15). Ectodomain phosphorylation of CD36 is also believed to regulate initial palmitate uptake by human platelets (14). However, it is equally likely that the phosphorylation event is mediated by an intracellular kinase that phosphorylates this site during the transport of CD36 to the cell surface. This would imply that when this ectophosphorylation site is dephosphorylated by the ectophosphatase this regulatory event would be irreversible, whereas the ectokinase model would suggest that this event could be reversible.

In addition to the experimental results presented above, there is preliminary clinical evidence that the inhibition of AP activity is associated with reduced IRBC adhesion. In a pilot study of quinine sulfate with or without levamisole administered to patients with uncomplicated malaria in western Thailand, patients who received both drugs had a significantly higher percentage of trophozoite/schizont-infected erythrocytes in the peripheral circulation than those who were treated with quinine and placebo (A. M. Dorndorp, N. J. White, and M. Ho, unpublished data). In other words, IRBCs that would normally have adhered and sequestered were remaining in the circulation where they could be cleared by the spleen. Although direct measurement of the correlation between cytoadherence and tissue oxygenation has not been performed, a relatively small change in tissue oxygenation, i.e., ~15%, can result in significant symptomatic improvement in patients with sickle cell disease (24). The AP inhibitory effect of theophylline is of particular interest, as this is a fully licensed drug routinely used for the relief of bronchospasm. Its toxicity is lower than that of levamisole, and it can be given in multiple doses.

While antiadhesive therapy directed at CD36 is likely to ameliorate some of the complications of severe falciparum malaria, e.g., malarial adult respiratory syndrome, its potential efficacy in reducing cerebral sequestration remains to be determined. The recent demonstration that cerebral pathology in *Plasmodium berghei* ANKA infection in mice is not linked to cytoadherence to CD36 (10) does not negate a role for the molecule in the human disease, as the pathogenic role of proinflammatory cytokines with mononuclear cell infiltrate in this murine malaria model is well established (12). In studies of human postmortem brain tissues, CD36 has been found by immunohistochemical staining to be sparsely expressed on cerebral microvasculature (27). However, adhesion molecules can and do function at site densities that are not detectable by standard methods. The best example of this is P-selectin, 10 to 20 molecules/mm^2 of which are sufficient for mediating the critical initial step in leukocyte recruitment to sites of inflammation (30). In addition, a low level of CD36 expression might well be sufficient to mediate cytoadherence through synergistic interactions with other adhesion molecules by IRBCs (20, 31). From a broader perspective, our findings on the modulation of a receptor molecule by *P. falciparum* represent a novel mechanism of host-parasite interaction that might be employed by other pathogens.

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