20. C. Chang *et al.*, *Plant Cell* **4**, 1263 (1992); C. M. Tobias, B. Howlett, J. B. Nasrallah, *Plant Physiol.* **99**, 284 (1992).
21. J. C. Walker and R. Zheng, *Nature* **345**, 743 (1990).
22. J. C. Stein, B. Howlett, D. C. Boyes, M. E. Nasrallah, J. B. Nasrallah, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8816 (1991).
23. T. I. Bonner *et al.*, *Nucleic Acids Res.* **14**, 1009 (1986); G. E. Mark, R. J. MacIntyre, M. E. Digan, L. Ambrosio, N. Perrimon, *Mol. Cell. Biol.* **7**, 2134 (1987).
24. S. H. Hulbert and R. W. Michelmore, *Theor. Appl. Genet.* **70**, 520 (1985); J. L. Bennetzen and S. H. Hulbert, *Plant Mol. Biol.* **20**, 575 (1992); M. J. Dickinson, D. A. Jones, J. D. G. Jones, *Mol. Plant-Microbe Interact.* **6**, 341 (1993).
25. D. W. Gabriel and B. G. Rolfe, *Annu. Rev. Phytopathol.* **28**, 365 (1990).
26. A. M. Schultz, L. E. Henderson, S. Oroszlan, E. A. Garber, H. Hanafusa, *Science* **227**, 427 (1985); J. E. Buss, M. P. Kamps, K. Gould, B. M. Sefton, *J. Virol.* **58**, 468 (1986); R. J. A. Grand, *Biochem. J.* **258**, 625 (1989).
27. L. A. Feig, *Science* **260**, 767 (1993); J. Marx, *ibid.*, p. 1588.
28. G. Felix, D. G. Grosskopf, M. Regenass, T. Boller, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8831 (1991); E. E. Farmer, T. D. Moloshok, M. J. Saxton, C. A. Ryan, *J. Biol. Chem.* **266**, 3140 (1991); V. Raz and R. Fluhr, *Plant Cell* **5**, 523 (1993).
29. C. L. Gough, S. Genin, C. Zischek, C. A. Boucher, *Mol. Plant-Microbe Interact.* **5**, 384 (1992); S. Fenselau, I. Balbo, U. Bonas, *ibid.*, p. 390; H.-C. Huang, S. Y. He, D. W. Bauer, A. Collmer, *J. Bacteriol.* **174**, 6878 (1992).
30. J. B. Bliska, J. E. Galan, S. F. Falkow, *Cell* **73**, 903 (1993); K. Guan and J. E. Dixon, *Science* **249**, 553 (1990); J. Bliska, K. Guan, J. E. Dixon, S. Falkow, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1187 (1991).
31. G. Chu, D. Vollrath, R. W. Davis, *Science* **234**, 1582 (1986).
32. Genomic DNA was isolated as described in (*8*). Hybridization was as described in [R. Bernatzky and S. D. Tanksley, *Genetics* **112**, 887 (1986)] with the use of random-hexamer ^{32}P-labeled [A. P. Feinberg and B. Vogelstein, *Anal. Biochem.* **132**, 6 (1983)] PCR product (1 × 10^6 to 2 × 10^6 cpm/ml of buffer) amplified from the cDNA clone. The filter was washed to 0.5× standard saline citrate at 65°C and exposed to x-ray film for 24 hours.
33. J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor, Cold Spring Harbor, NY, ed. 2, 1989), chap. 7.
34. Genomic DNA was isolated from the following plants and digested with Eco RI (amount of DNA loaded on gel is indicated): tomato (Rio Grande–PtoR, 3 μg); potato (M200, hybrid: *Solanum tuberosum*/*S. berthaultii*, 5 μg); potato (B11B, *S. berthaultii*, 5 μg); tobacco (Samsun, 5 μg); *Arabidopsis thaliana* (Col-0, 1 μg); bean (*Phaseolus acutifolius*, G40178, 3 μg); bean (*P. acutifolius*, PI310800, 3 μg); soybean (*Glycine max*, Centennial, 4 μg); pea (*Pisum sativum*, Sparkle, 10 μg); rice (*Oryza sativa*, IRAT, 3 μg); maize (*Zea mays*, RI24, 15 μg); barley (*Hordeum vulgare*, SE16, 15 μg); wheat (*Triticum aestivum*, R-4, 15 μg); sugarcane (*Saccharum spontaneum*, SES208, 10 μg).
35. Leaves were inoculated as in (*16*). At each time point, nine 0.63-cm^2 leaf disks were taken from Rio Grande–PtoR, Moneymaker, and backcross progeny plants with or without pPTC8. The nine disks were randomly divided into sets of three and the disks were macerated in 10 mM $MgCl_2$. The density of bacterial populations was determined by the plating of serial dilutions and the counting of colony-forming units on King's medium B supplemented with rifampicin (20 μg/ml) and kanamycin (25 μg/ml) [E. O. King, N. K. Ward, D. E. Raney, *J. Lab. Clin. Med.* **44**, 301 (1954)].
36. Abbreviations for the amino acids are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
37. We thank N. Van Eck, J. Lindell, L. Miller, and G. X. Yu for technical assistance; J. Santini and W. Nyquist for statistical consultation; M. Thomashow for helpful discussion; L. Walling for the PT11; and P. Ronald and B. Staskawicz for the T1 and T1(pPTE6). Supported, in part, by U.S. Department of Agriculture grant 91-37300-6565 (S.D.T., M.W.G., G.B.M.), Cornell Plant Science Center (R.S.), Binational Agricultural Research and Development grant IS-1822-90C (S.D.T.), National Science Foundation (NSF) Plant Biology Fellowship grant DMB-89-05997 (G.B.M.), and NSF grant MCB-92-22531 (G.B.M.).

13 July 1993; accepted 23 September 1993

Analysis of CD36 Binding Domains: Ligand Specificity Controlled by Dephosphorylation of an Ectodomain

Adam S. Asch,* Isaac Liu, Frederick M. Briccetti, John W. Barnwell, Frank Kwakye-Berko, Ayotunde Dokun, Jeffrey Goldberger, Mona Pernambuco

The protein CD36 is a membrane receptor for thrombospondin (TSP), malaria-infected erythrocytes, and collagen. Three functional sequences were identified within a single disulfide loop of CD36: one that mediates TSP binding (amino acids 87 to 99) and two that support malarial cytoadhesion (amino acids 8 to 21 and 97 to 110). One of these peptides (p87-99) is a consensus protein kinase C (PKC) phosphorylation site. Dephosphorylation of constitutively phosphorylated CD36 in resting platelets and a megakaryocytic cell line led to the loss of collagen adhesion and platelet reactivity to collagen, with a reciprocal increase in TSP binding. PKC-mediated phosphorylation of this ectodomain resulted in a loss of TSP binding and the reciprocal acquisition of collagen binding. In site-directed mutagenesis studies, when the threonine phosphorylation site was changed to alanine, CD36 was expressed in a dephosphorylated state and bound to TSP constitutively.

Regulation of membrane receptor function may be mediated by translocation of the receptor to the cell surface or by association or dissociation of the receptor from regulatory molecules. Although posttranslational modifications such as glycosylation or phosphorylation regulate many protein interactions, the rapid regulation of these characteristics has been confined to events that occur within the membrane or on the cytoplasmic side of the receptor. CD36 is an 88-kD membrane glycoprotein present on platelets, monocytes, erythroid precursors, endothelial cells, and several tumor cell lines and is a receptor for the adhesive platelet and extracellular matrix protein TSP (*1*–*6*), *Plasmodium falciparum*–infected erythrocytes (*7*–*9*), and collagen (*10*). CD36 is one of several TSP binding sites on platelets, and purified CD36 binds TSP (*1*–*6*). In addition, CD36 acts as a receptor that is critical for the cytoadherence of *P. falciparum*–infected erythrocytes to endothelium (*7*–*9*). Both TSP binding and malarial cytoadhesion are inhibited by the same monoclonal antibody (*1*, *11*), suggesting that the domains that mediate these events may be related. CD36 is also one of several reported collagen receptors (*12*).

Random CD36 domains were expressed in a recombinant expression system, and we screened the library by probing with biotinylated purified TSP, a biotinylated peptide (CSVTCG) (*13*) corresponding to a CD36 binding domain within TSP (*2*), and ^{51}Cr-labeled malaria-infected erythrocytes (*14*). Positively hybridizing clones were subjected to secondary or tertiary rounds of screening and then sequenced. Analysis of the clones identified overlapping consensus sequences as candidate binding domains. Peptides corresponding to the predicted amino acid sequence of these regions were synthesized and examined for TSP binding and malarial cytoadhesion (*15*).

The domains of CD36 that are responsible for TSP binding and for the cytoadhesion of *P. falciparum*–infected erythrocytes were identified by random domain library screening (RANDOLS) (Fig. 1A) and confirmed by means of synthetic peptides (Fig. 1B). Three functional sequences were identified within a single putative disulfide loop of CD36 defined by Cys^7 and Cys^{243}: one that mediates TSP binding (amino acids 87 to 99) and two that support malarial cytoadhesion (amino acids 8 to 21 and 97 to 110). The malarial binding domain, residues 97 to 110 that we have identified, overlaps p93-110 identified as a binding site

A. S. Asch, I. Liu, F. M. Briccetti, A. Dokun, J. Goldberger, M. Pernambuco, Division of Hematology-Oncology, Cornell University Medical College, New York, NY 10021.
J. W. Barnwell and F. Kwakye-Berko, Department of Molecular Parasitology, New York University Medical School, New York, NY 10010.
*To whom correspondence should be addressed.

(16) for the monoclonal antibody (mAb) to CD36, OKM5, which inhibits malarial cytoadhesion and TSP binding (1, 11, 17).

The TSP binding sequence identified by RANDOLS (RGPYTYRVRFLA) is of interest because it is a PKC consensus site as defined by the computer algorithm PROSITE (18). This raised the possibility that CD36 function might be regulated by phosphorylation of the receptor at the threonine in this sequence during CD36 synthesis and translocation to the cell surface. To explore this possibility, we coated microtiter wells with CD36 or a peptide corresponding to amino acids 87 to 99, then treated with PKC, and assayed for binding to purified TSP (19). The binding of TSP to the peptide was inhibited by 60% after phosphorylation, but a peptide in which alanine was substituted for threonine (RGPYAYRVFLA) was not phosphorylated and showed no inhibition of TSP binding with PKC. Similar results were obtained with purified CD36. Incorporation of ^{32}P into CD36 after incubation with [γ-^{32}P]adenosine triphosphate (ATP) and PKC was confirmed by autoradiography. The results of TSP binding to purified CD36 revealed that phosphorylation was associated with a 60% decrease in the amount of TSP bound; TSP binding was restored after incubation with a phosphatase-containing platelet fraction. Purified acid phosphatase (0.25 U/ml) restored TSP binding equally well (20).

Dephosphorylation of this extracellular domain of CD36 might occur with release of platelet acid phosphatase when the platelet is activated. According to this model, CD36 is posttranslationally modified so that p87-99 is expressed phosphorylated in the resting state and therefore not competent to bind TSP. Upon platelet activation and degranulation, released phosphatases dephosphorylate CD36 and alter its affinity for TSP. This might explain the variation in function that has been observed in CD36-transfected cells. In particular, a PKC-like activity may be responsible for constitutive phosphorylation of CD36 and lack of TSP binding in CD36-transfected COS cells (21).

A phosphorylated CD36 molecule was expressed by CD36-transfected COS cells, but treatment with the PKC inhibitors staurosporine or H7 blocked phosphorylation of expressed CD36 (Fig. 2A). Both H7 and, to a lesser extent, staurosporine resulted in the expression of CD36 that bound TSP in a specific manner; acid phosphatase treatment also increased TSP binding by the cells, confirming the extracellular location of the phosphorylated domain (Fig. 2A) (22).

To demonstrate that CD36 is synthesized by the megakaryocyte and expressed phosphorylated in resting platelets, we studied phosphorylation of CD36 in the human megakaryocytic cell line, CRL 9792 (23). A band at 88 kD and lower bands that correspond to previously demonstrated coprecipitating protein tyrosine kinases (24) were observed in immunoprecipitates of ^{32}P-labeled cells (Fig. 2B). Treatment of the metabolically labeled cells with crude platelet acid phosphatase or purified acid phosphatase resulted in the loss of CD36-associated surface label. This dephosphorylation was also associated with an increase in TSP binding (Fig. 2B), suggesting that megakaryocytes synthesize CD36 in a phosphorylated state and that with platelet stimulation and release of acid phosphatase, the ectodomain of CD36 is dephosphorylated.

Examination of the functional correlates of these findings led to some unexpected observations. Platelet aggregation was examined in gel-filtered platelets treated with acid phosphatase (0.25 U/ml). Aggregation to collagen at 4 μg/ml was blocked by acid phosphatase treatment, but not to collagen at 20 μg/ml or to adenosine diphosphate (ADP) (Fig. 3), raising the possibility that dephosphorylation of CD36 plays a role in signal transduction mediated by collagen.

The adhesion of ^{51}Cr-labeled resting platelets or resting acid phosphatase–treated platelets (RAPTP) to plastic microtiter wells coated with collagen types I, III, or IV or purified TSP was also examined. Adhesion of resting platelets to type I collagen was observed as described (10), and CD36-dependent adhesion was observed with type IV collagen but not type III collagen. Dephosphorylation of platelet CD36 by acid phosphatase treatment blocked CD36-dependent adhesion to collagen types I and IV. In contrast, adhesion of resting platelets to TSP was increased by dephosphorylation (Table 1).

To explore the possibility that CD36 specificity for the ligands collagen or TSP is regulated by its state of phosphorylation, we phosphorylated purified platelet CD36 and examined it for its ability to bind TSP and collagen (Table 1). Immunoisolated CD36 bound TSP but not type IV collagen; con-

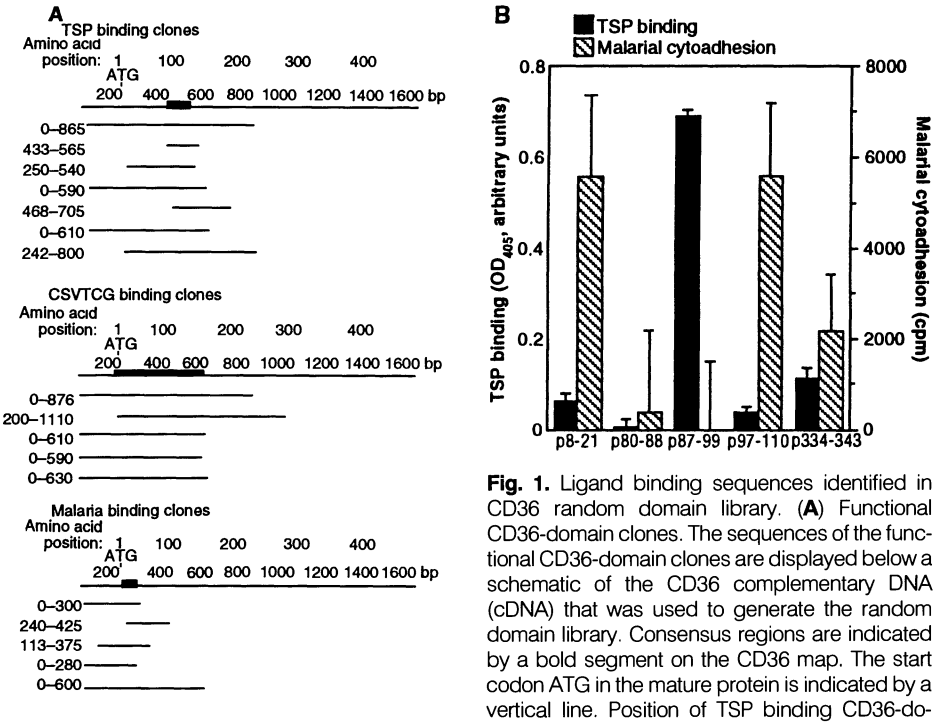

Fig. 1. Ligand binding sequences identified in CD36 random domain library. (**A**) Functional CD36-domain clones. The sequences of the functional CD36-domain clones are displayed below a schematic of the CD36 complementary DNA (cDNA) that was used to generate the random domain library. Consensus regions are indicated by a bold segment on the CD36 map. The start codon ATG in the mature protein is indicated by a vertical line. Position of TSP binding CD36-domain clones: The sequences of the CD36-related TSP binding clones are aligned with the complete CD36 cDNA. The seven clones have in common a single consensus 70-bp region (bps 470 to 540) corresponding to amino acids 86 to 109 in the mature protein. Position of CSVTCG-albumin binding CD36-domain clones: Five positive clones shared a single consensus of 390 bp (bps 200 to 590) corresponding to translated amino acids 1 to 126 in the mature protein. The region identified by this sequence included the TSP binding domain identified by TSP screening of the library and is consistent with data showing that CSVTCG binds to CD36 and inhibits the binding of TSP to CD36 (2). Position of *P. falciparum*–parasitized erythrocyte binding CD36-domain clones: Five positive clones coded for CD36 segments that shared a single consensus of 40 bp (bps 240 to 280) corresponding to amino acids 10 to 23. [For methods, see (14).] (**B**) Ligand binding peptides. The binding of TSP to immobilized peptides is shown by the solid bars (OD_{405}, optical density at 405 nm). Data are the means of six replicates ± SEM. The adhesion of *P. falciparum*–infected erythrocytes is shown by the cross-hatched bars. Labeled uninfected erythrocytes did not bind. Data are the means of 10 replicates ± SEM from a representative experiment (cpm, counts per minute). [For methods, see (15).]

versely, PKC-treated CD36 bound type IV collagen but not TSP. In control studies, malaria-infected erythrocytes bound equally well to phosphorylated and unphosphorylated CD36. We tested the hypothesis that both TSP and collagen binding are mediated by the same domain, with dephosphorylation acting as a switch to direct binding specificity from collagen to TSP. The phosphorylated peptide (p87-99–PO_4) bound TSP less well than did p87-99; conversely, p87-99–PO_4 supported the binding of collagen to purified CD36, whereas the dephosphorylated peptide bound collagen less well (Table 1).

The site of phosphorylation was identified and the orientation of phosphorylated CD36 was defined by phosphoamino acid analysis of the phosphorylated extracellular CD36 domain. Flow cytometry with a polyclonal antibody specific for p87-99 (anti–p87-99), which also recognized intact CD36, confirmed that this antibody recognizes an extracellular domain in intact resting platelets. To examine the state of phosphorylation of this region (residues 87-99) we metabolically labeled CD36-transfected COS cells with ^{32}P and treated the cell monolayers with trypsin to proteolyze the extracellular region. An autoradiograph of immunoprecipitates obtained with anti–p87-99 revealed partial proteolysis of phosphorylated CD36 and the absence of phosphorylated immunoprecipitated proteins from supernatants derived from either trypsin-treated control transfectants or CD36 transfectants that were not treated with trypsin. The smallest phosphorylated band corresponding to a molecular weight of 800 to 3000 was excised from the gel and subjected to phosphoamino acid analysis by acid hydrolysis and two-dimensional electrophoresis. Phosphothreonine, but not phosphoserine or phosphotyrosine, was detected in this fragment (*25*).

To confirm the site of phosphorylation in CD36, we generated a variant CD36 by site-directed mutagenesis in which Thr^{92} was changed to Ala (CD36ala92). The state of phosphorylation of immunoprecipitated CD36 and TSP binding by the transfected monolayers were examined. The wild type, but not CD36ala92, was expressed phosphorylated in COS cells (Fig. 4); CD36ala92 bound TSP (2699 ± 559 ng per well) constitutively compared with no specific binding to wild-type CD36 (*26*).

The mechanisms by which receptors change affinity or specificity for extracellular ligands remain largely undefined. Platelets, as well as inflammatory cells such as monocytes, rely on the ability to rapidly modulate the interaction of cell surface adhesion receptors with their ligands in order to effect a functional change such as platelet aggregation. Although agonist receptors are the principal mediators of platelet activation, outside-to-inside signal transduction, manifest by intracellular phosphorylation events in response to adhesion receptor occupancy, also occur (*27*). CD36 is a platelet membrane protein that is regulated in such a fashion: On resting platelets CD36 is not a functional TSP receptor, but after platelet activation, it undergoes transformation and becomes one of several competent TSP binding sites. CD36 on the resting platelet surface is also capable of binding collagen, and there is evidence that it may be involved in some signal transduction events: thus, information is transmitted across the membrane in both directions. Our data suggest that CD36 ligand specificity is directed by the extracellular release or activation of serine or threonine phosphatases in response to a principal platelet agonist and that signaling may be affected by dephosphorylation of CD36 or other molecules. Regulation of the ligand-binding function or ligand specificity of membrane receptors by dephosphorylation of an ectodomain has not been reported (*28*).

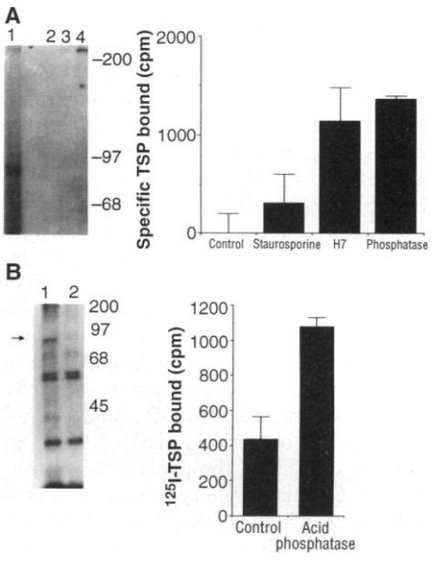

Fig. 2. (**A**) (left panel) Phosphorylation of CD36 expressed in transfected COS cells. Autoradiograph of CD36 mAb immunoprecipitates from $^{32}PO_4$-labeled CD36-transfected (lanes 1, 3, and 4) or control transfectants (lane 2). Monolayers were treated with the PKC inhibitors H7 (100 μM, lane 3) or staurosporine (1 μM, lane 4). (Right panel) Specific TSP binding to CD36-transfected COS cells. The binding of ^{125}I-TSP to COS cell monolayers was examined after CD36 transfection and subsequent addition of staurosporine, H7 (to inhibit the phosphorylation of expressed CD36), or acid phosphatase (to dephosphorylate the extracellular domain). [For methods, see (*22*).] (**B**) (Left panel) Acid phosphatase treatment of the megakaryocyte-like CRL 9792 cell line. Autoradiograph of CD36 mAb immunoprecipitates from $^{32}PO_4$-labeled CRL 9792 cells (lane 1) or the same cells after treatment with acid phosphatase (lane 2). [For methods, see (*23*).] (Right panel) Binding of ^{125}I-TSP to CRL 9792 cells. The binding of TSP to control or phosphatase-treated CRL 9792 cells (*23*) was examined by incubation of the cells (10^6 per milliliter) in 200-μl aliquots with ^{125}I-TSP at a concentration of 100 μg/ml (specific activity 1.2×10^3 cpm/μg) for 1 hour at 4°C. The cells were spun through silicone oil and the bound ^{125}I was counted.

Table 1. Reciprocal regulation of CD36 ligand binding function. For measurement of platelet adhesion, gel-filtered platelets were ^{51}C labeled (*10*) and incubated with buffer or acid phosphatase (0.25 U/ml) for 1 hour at 37°C. The platelets were resuspended in tris-buffered saline, and their adhesion to 96-well microtiter plates coated with fibrillar purified collagen types I, III, or IV (1 μg/ml, Hoechst) or TSP was measured essentially as described (*10*). Binding in the presence of anti-CD36 (2 μg/ml) (*7*) was subtracted from the total binding to obtain CD36-dependent adhesion, and the results were normalized to the maximal binding observed. To examine the regulation of collagen and TSP binding to purified CD36 and p87-99, we incubated purified collagen (type IV) or TSP in wells containing purified control or phosphorylated platelet CD36. CD36 was purified from platelets by immunoaffinity chromatography (*1*) or by the method of McGregor *et al.* (*5*) and was coated on 96-well mictrotiter plates at a concentration of 10 μg/ml. Before incubation with purified biotinylated TSP or collagen (Hoechst), the immobilized CD36 was incubated with PKC (UBI) and ATP (10 mM) or ATP alone for 4 hours at 22°C. Incorporation of $^{32}PO_4$ was examined as described (*32*). After the wells were washed, the binding of purified TSP to CD36 was measured by ELISA as described above.

Platelet adhesion (Percent of maximum)			TSP and collagen binding (Percent of maximum)		
Substrate	Control	Phosphatase -treated	Substrate	TSP	Collagen
TSP	0 ± 10	100 ± 4	CD36	100 ± 6.5	17.5 ± 13.4
Collagen I	72.5 ± 6	0 ± 6	CD36-PO_4	30 ± 10.7	100 ± 16.5
Collagen III	0 ± 9	0 ± 19	p87-99	100 ± 5.6	61.6 ± 16.5
Collagen IV	81.5 ± 12	21 ± 8	p87-99-PO_4	4 ± 0.4	100 ± 8.3

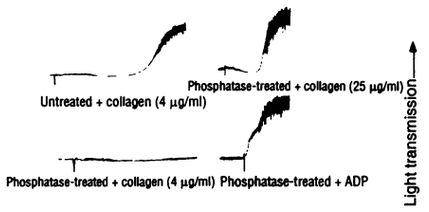

Fig. 3. Acid phosphatase treatment of normal platelets abolishes response to low-dosage collagen. Platelet aggregation studies were done with a Payton aggregometer. Acid phosphatase (0.25 U/ml final concentration, Sigma) in citrate buffer or buffer alone was added to platelet-rich plasma prepared in acid citrate dextrose and incubated at 37°C for 1.5 hours or for 5 min in the presence of NEM, PMSF, benzamidine, and leupeptin. Aggregometry was measured in response to collagen (4 μg/ml or 25 μg/ml), ADP (5 μM), or A23187 (1 μM).

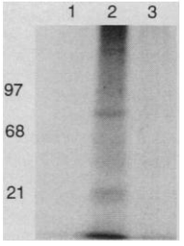

Fig. 4. Substitution of Ala for Thr at position 92 leads to expression of an unphosphorylated CD36. Site-directed mutagenesis was done with polymerase chain reaction to generate a substitution of Ala for Thr^{92} (CD36ala92). Shown is an autoradiograph of an immunoprecipitation of ^{32}P-labeled COS cell monolayers obtained with a CD36 mAb either from cells transfected with CD36ala92 (lane 1) or wild-type CD36 (lane 2) or from mock transfectants (lane 3). Molecular sizes are indicated to the left in kilodaltons. [For methods, see (*26*).]

The loss, after acid phosphatase treatment, of platelet reactivity to low-dosage but not high-dosage collagen or ADP, along with evidence that CD36-dependent adhesion of platelets to collagen types I and IV is blocked by surface dephosphorylation, provides strong evidence that the CD36-mediated collagen interaction reported previously (*10*) is regulated by dephosphorylation. Indeed, the binding of both TSP and collagen are mediated, at least in part, by the same CD36 domain, with ligand specificity determined by the state of phosphorylation (*29, 30*). The extent to which modulation of cellular receptor function in other systems is influenced by the state of phosphorylation of ectodomains or by the activity of extracellular phosphatases needs to be explored.

REFERENCES AND NOTES

1. A. S. Asch, J. Barnwell, R. L. Silverstien, R. L. Nachman, *J. Clin. Invest.* **79**, 1054 (1987).
2. A. S. Asch, S. Silbiger, E. Heimer, R. L. Nachman, *Biochem. Biophys. Res. Commun.* **182**, 1208 (1992).
3. N. Kieffer, A. T. Nurden, M. Hasitz, M. Titeux, J. Breton-Gorius, *Biochim. Biophys. Acta* **967**, 408 (1988).
4. C. Legrand, D. Pidard, P. Beiso, D. Tenza, L. Edelman, *Platelets* **2**, 99 (1991).
5. J. L. McGregor *et al.*, *J. Biol. Chem.* **264**, 501 (1989).
6. F. C. Stomski, J. S. Gani, R. C. Bates, G. F. Burns, *Exp. Cell Res.* **198**, 85 (1992).
7. J. W. Barnwell *et al.*, *J. Clin. Invest.* **84**, 765 (1989).
8. C. F. Ockenhouse, N. N. Tandon, C. Magowan, G. A. Jamieson, J. D. Chulay, *Science* **243**, 1469 (1989).
9. P. Oquendo, E. Hundt, J. Lawler, B. Seed, *Cell* **58**, 95 (1989).
10. N. N. Tandon, U. Kralisz, G. A. Jamieson, *J. Biol. Chem.* **264**, 7576 (1989).
11. J. M. Barnwell, C. F. Ockenhouse, D. M. Knowles III, *J. Immunol.* **135**, 3494 (1985).
12. Immunoinhibition data suggest that initial attachment and platelet reactivity to collagen is in part mediated by CD36 (*10*), whereas the spreading of platelets on immobilized collagen appears to depend on integrin-mediated (GPIa and GPIIa) interactions [H. K. Nieuwenhuis, K. S. Sakariassen, W. P. Houdijk, P. F. Nievelstein, J. J. Sixma, *Blood* **68**, 692 (1986); H. K. Nieuwenhuis, J. W. Akkerman, W. P. Houdijk, J. J. Sixma, *Nature* **318**, 470 (1985); B. Kehrel *et al.*, *Blood* **71**, 1074 (1988); (*30*)].
13. Abbreviations for the amino acid residues are the following: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
14. We purified a 1.7-kb Xba I fragment corresponding to the entire extracellular domain of CD36 and then sonicated it with a Heat Systems Sonicator model W200R (Ultrasonics Inc.) equipped with a microtip that generated a pulsed output for 3 min to produce random fragments of 200 to 600 base pairs (bps). The fragments were repaired with T4 polymerase and Klenow, ligated to Lambda Zap II (Stratagene) with Eco RI linkers, and packaged with Gigapack II packaging extract (Stratagene). The random library was expressed in XL1-blue bacteria with isopropyl-β-D-thiogalactopyranoside (IPTG) induction. We screened plaque lifts using (i) biotinylated TSP (100 μg/ml) or biotinylated CSVTCG-albumin conjugates (1 mg/ml) and analysis with an avidin-biotin detection system (Bio-Rad) or (ii) ^{51}Cr-labeled malaria-parasitized erythrocytes from *Aotus* monkeys (*7*) and analysis by autoradiography. Positive clones were purified by secondary and tertiary rounds of screenings. Inserts were sequenced with Sequenase (Stratagene) from the Lambda Zap II bluescript plasmid that was purified by cotransfection of XL1-blue bacteria with phage and helper virus (Stratagene). We identified 28 positive plaques in the first round of screening with biotinylated TSP and subjected the plaques to secondary or tertiary rounds of screening that yielded 16 positive clones. Of these, nine contained inserts that were not in frame with β-galactosidase or were in the wrong orientation to have resulted in a fusion protein related to CD36. The remaining seven coded for expressed fusion proteins. We identified 22 positive primary plaques from 10^5 clones in the first round of screening with biotinylated CSVTCG-albumin and then subjected them to secondary and tertiary screening and then sequencing which yielded five positives that were in frame. To identify the CD36 domain that is responsible for malarial cytoadhesion, we screened the CD36 random domain library with *P. falciparum*–infected erythrocytes from *Aotus* monkeys that were labeled with ^{51}Cr. Erythrocytes parasitized by *P. falciparum* (CD36-binding strains FVO, IC5, or CD36-nonbinding 2B3) were cultured to the late trophozoite stage at 6 to 10% parasitemia as described (*7*) from isolates derived from infected *Aotus* monkeys or continuously cultured in vitro in human O^+ erythrocytes. The malaria parasites were cultured in standard RPMI tissue culture medium with 10% human serum overnight, and from late ring stages, trophozoite-infected cells were incubated with ^{51}Cr for plaque screening. Five positive clones were identified by autoradiography after secondary and tertiary library screening and shared a single consensus corresponding to amino acids 240 to 280. In preliminary studies, screening with collagen was found unfeasible because of the high reactivity of the collagen used with nonrecombinant plaques.
15. Peptides corresponding to CD36 domains were synthesized by fluorenyl methoxycarbonyl (FMOC) chemistry with a Milligen Biosearch 9600 automated peptide synthesizer [B. Merrifield, *Science* **232**, 341 (1986)] and analyzed by thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC). After purification, peptides were conjugated to albumin as described [Y. N. Danilov and R. L. Juliano, *Exp. Cell Res.* **182**, 186 (1989)] and coated in 96-well microtiter plates. After they were washed three times with tris-buffered saline, the wells were passivated with 1% albumin and probed with purified TSP, prepared as described (*1*), that was biotinylated by incubation with sulfo-NHS-biotin (Pierce) and dialyzed against 20 mM tris and 150 mM NaCl overnight. After 2 hours of incubation, the wells were washed free of unbound protein and probed with an avidin alkaline phosphatase detection system (Bio-Rad). Control wells coated with albumin were used to determine nonspecific reactivity of the detection system. Peptides corresponding to the consensus region as well as control peptides were conjugated to bovine serum albumin and used to coat 96-well microtiter plates, and their ability to support TSP binding was determined by enzyme-linked immunosorbent assay (ELISA) [expressed as optical density (OD) at 405 nm × 10^4]. *Plasmodium falciparum*–parasitized erythrocytes (CD36-binding strains FVO, IC5, or CD36-nonbinding 2B3) were cultured as described (*14*) and labeled with ^{35}S-methionine (50 μCi/ml) for binding assays. Metabolically labeled infected erythrocytes were allowed to adhere to wells coated with CD36 peptide–albumin conjugates. After three washes, the adherent cells were removed with an LKB cell harvester, immobilized on filtration membrane, and counted with an LKM betaplate reader. We also examined adhesion of parasitized erythrocytes to purified CD36 and CD36-transfected cells in the presence of peptide-albumin conjugates to assess the ability of peptides to block cytoadhesion. Peptides corresponding to amino acids 8 to 21 and 97 to 110 also inhibited the adhesion of infected erythrocytes to purified CD36 by 90% and 45%, respectively, whereas control peptide conjugates (including a scrambled peptide of amino acids 8 to 21, AIGLAAGVGIALVV) did not (*31*).
16. L. L. K. Leung, L. Wei-Xing, J. L. McGregor, G. Albrecht, R. J. Howard, *J. Biol. Chem.* **267**, 18244 (1992).
17. Domains responsible for mediating the interaction of CD36 with TSP and malaria are distinct but contained within the region of amino acids 1 to 126 that mediated binding of the peptide CSVTCG. The hypothesis that the sequence CSVTCG plays a role in malarial cytoadhesion has little experimental support because the peptide CSVTCG has had no effect in cytoadhesion assays. The function of this domain as a cell adhesive motif [K. A. Rich, F. W. George IV, J. L. Law, W. J. Martin, *Science* **249**, 1574 (1990); C. A. Prater, J. Platkin, D. Jaye, W. A. Frazier, *J. Cell Biol.* **112**, 1031 (1991)] associated with sporozoites [C. Cerami *et al.*, *Cell* **70**, 1021 (1992)] raises the possibility that CD36, or sequences homologous to the binding regions identified, may be involved in mediating sporozoite interactions with certain cell types.
18. J. R. Woodgett, K. L. Gould, T. Hunter, *Eur. J. Biochem.* **161**, 177 (1986).
19. We confirmed phosphorylation of the peptide by examining the incorporation of ^{32}P after incubation with [γ-^{32}P]ATP and PKC. TLC confirmed the incorporation of ^{32}P into 70% of the ninhy-

drin-stainable peptide. Compared with phosphorylation by PKC, other kinases, including casein kinases I and II and PKA, were less efficient.

20. Crude platelet releaseate for experiments on the effect of phosphatase was prepared from washed platelets in acid citrate dextrose with 5 mM EDTA by sonication of the platelets and centrifugation at 10,000*g* to remove debris or by stimulation of platelet release in the same buffer with the ionophore A23187 (2 μM).
21. TSP binding by CD36 appears to be regulated, in that resting platelets that express from 1.5×10^4 to 2.5×10^4 CD36 molecules bind only a few thousand TSP molecules, but with platelet activation and alpha granule release, TSP binding increases to about 2.5×10^5 molecules per platelet. Similar regulation of function is displayed by CD36 expressed in transfected cell lines where TSP binding appears to be regulated by cell-specific factors present in some but not all cell types (*2, 9*).
22. COS cells were transfected with CD36 in the CDM8 plasmid as described (*9*). After transfection, cells were exposed to fresh medium or to the PKC inhibitors staurosporine (1 μM) or H7 (100 μM) in the presence or absence of [^{32}P]orthophosphate (100 μCi/ml) (DuPont Biotechnology Systems). At 48 hours post-transfection, the expression of CD36 was monitored by fluorescein isothiocynate (FITC) fluorescence with CD36 mAb. The binding of ^{125}I-labeled TSP to CD36- or control-transfected COS cell monolayers was measured as previously described with TSP (100 μg/ml). Monolayers labeled with ^{32}P were solubilized in phosphate-buffered saline (PBS) containing 2% Triton-X 100, and CD36 was immunoprecipitated with CD36 mAb and examined by autoradiography as described (*9*). In control studies, CD36-transfected cells were treated with acid phosphatase (0.25 U/ml) for 5 min in citrate buffer (pH 5) with 10 μM *N*-ethylmaleimide (NEM), 100 μM Phe·Pro·Arg-chloromethyl ketone, 0.4 μM phenylmethysulfonyl fluoride (PMSF), 10 μM benzamidine, and leupeptin (0.1 μg/ml), and TSP binding was measured by ELISA as previously described. Specific binding to the monolayers was obtained by subtraction of the binding observed in the appropriate (untreated, H-7, or staurosporine-treated) control transfectants (plasmid alone) from the binding measured in CD36-transfected cells (untreated, H7-, or staurosporine-treated). The expression of CD36 by these cells as measured by fluorescence was unaffected by H7 or staurosporine.
23. The megakaryocytic cell line, CRL 9792 [S. M. Greenberg, D. S. Rosenthal, T. A. Greeley, R. Tantravahi, R. I. Handin, *Blood* **72**, 1968 (1988)], was obtained from American Type Culture Collection (ATCC) and maintained in minimum Eagle's medium (MEM) with 10% fetal calf serum. Cells were labeled overnight with [^{32}P]orthophosphate (100 μCi/ml), washed three times in tris-buffered saline, and then incubated in buffer containing purified bovine milk acid phosphatase (0.25 U/ml, Sigma) in 100 mM sodium citrate buffer (pH 5), or buffer alone, for 2 hours at 37°C or for 5 min in the presence of NEM, PMSF, benzamidine, and leupeptin. In some studies purified PP1 and PP2a phosphatases (UBI) were used at a concentration of 0.1 U/ml in the presence or absence of 4 μM microcystin as a control (GIBCO). The cells were washed and resuspended in tris-buffered saline containing 2 mM $CaCl_2$. An aliquot of cells was solubilized, immunoprecipitated with CD36 mAb as previously described, and analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.
24. M.-M. Huang, J. B. Bolen, J. W. Barnwell, S. J. Shattil, J. S. Brugge, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 7844 (1991).
25. An antibody specific to p87-99 was raised in rabbits with an albumin-peptide conjugate immunogen as described (*2*). Reactivity was assayed by ELISA against purified peptide or intact platelet CD36 as well as to control peptides and proteins. The antibody was reactive against CD36 in ELISA studies to a titer of 1:10,000. Antibody was purified by affinity separation with protein G–Sepharose (Pharmacia). Flow cytometry was done on an EPICS Profile II cytometer (Coulter). CD36- and control-transfected COS cells were labeled with [^{32}P]orthophosphate for 24 hours, as described above, washed free of medium, and then incubated for 40 min at 37°C with trypsin-EDTA (GIBCO) to proteolyze extracellular proteins. The supernatants from trypsin-treated CD36- and control-transfected COS cells, as well as supernatant from CD36 transfectants not treated with trypsin, were first incubated with protein G–Sepharose that was coated with nonimmune immunoglobulin G (IgG) to preabsorb any nonspecific precipitating proteins. Protein G–Sepharose coated with anti–p87-99 was used to immunoprecipitate from the supernatants, and the beads were washed three times before SDS-PAGE. After SDS-PAGE and autoradiography, the region corresponding to molecular sizes of 800 to 3000 was excised from the gel and subjected to acid hydrolysis and 2D phosphoamino acid analysis, as described [N. Feuerstein and H. L. Cooper, *J. Biol. Chem.* **258**, 10786 (1983)], with buffer containing 88% formic acid:glacial acetic acid:water (50:156:1794 v/v) (pH 1.9) in the first dimension and buffer containing pyridine:glacial acetic acid:water (10:100:1890 v/v) (pH 3.5) for the second dimension. The migration of standard phosphoamino acids was determined by ninhydrin treatment of the plate. Also, a truncation mutation of CD36, which lacked the COOH-terminal hydrophobic region, was constructed and expressed in COS cells. A mutant CD36 lacking the COOH-terminal transmembrane domain was obtained by the digestion of CD36 with Nsp I (Boehringer Mannheim) at 10 U per microgram of DNA for 2 hours at 37°C to produce deletions at positions 1495–1605, 1605–1732, and 1495–1732, which were isolated by agarose gel electrophoresis and glass milk (Geneclean, Bio101) and then religated in CDM8. A mutation corresponding to the deletion of bps 1495 to 1605 was used in transfection studies. COS cell monolayers were transfected with this mutant or with intact CD36 and labeled for 24 hours with [^{32}P]orthophosphate, as described. Post-culture supernatants were harvested 48 hours after transfection, and immunoreactive CD36 was precipitated with CD36-bound CNBr-Sepharose–bound anti-CD36. After ^{32}P metabolic labeling, a soluble form of phosphorylated CD36 was recovered from post-culture medium by immunoprecipitation (*31*). These experiments confirm the extracellular orientation of the labeled domain (because proteolytic cleavage or export would not occur if this were an intracellular domain).
26. CD36ala92 was generated by PCR essentially as described [S. N. Ho, H. D. Hunt, R. M. Horton, J. K. Pullen, L. R. Pease, *Gene* **77**, 51 (1989)] with the following primer pairs: 5′ to 3′, CCCAAGCTTGGGCTGTGACTCATCAGTTCC and 3′ to 5′, CTCCAGGAATA**C**GCATGTCTCAAG; and 5′ to 3′, GAGGTCCTTAT**G**CGTACAGAGTTC and 3′ to 5′, CAGATCGGTGACTAGTAGGCGAGCTCGCC. The purpose was to amplify cDNA from wild-type CD36 containing a mutation of G for A leading to a substitution of Ala for Thr at position 92 (substitutions in bold). Intermediate products from the first two reactions were mixed at a 1:1 ratio and used as templates to amplify a full-length mutagenized transcript with the external primers. The full-length inserts were ligated into a TA cloning vector (Invitrogen) and then cloned into the pcDNA expression vector (Invitrogen). The mutagenized sequence was confirmed by dideoxy sequencing. COS cells were transfected and labeled with ^{32}P as described above. Expression of CD36 and CD36ala92 was monitored with CD36 mAb and a FITC-labeled antibody, mAb 8A6, to mouse as a secondary antibody (*7*) and was equivalent in wild-type and mutant CD36–transfected monolayers. Labeling with [^{32}P]orthophosphate and immunoprecipitation were done as described above. The binding of ^{125}I-TSP (specific activity 2400 cpm/μg) to the monolayers was done on ice for 90 min in 24-well culture plates (Costar). After incubation, the wells were washed and the monolayers were solubilized in 2% SDS to quantitate the bound TSP. (Binding to mock-transfected cells was used to define nonspecific binding. Specific binding is defined as total binding minus nonspecific binding.)
27. J. E. Ferrell and G. S. Martin, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2234 (1989); A. Golden, J. S. Brugge, S. J. Shattil, *J. Cell Biol.* **111**, 3117 (1990).
28. In the case of platelets, the release of phosphatases has been noted for more than 25 years [A. J. Marcus, D. Zucker Franklin, L. B. Safier, H. L. Ullman, *J. Clin. Invest.* **45**, 14 (1966)], but little if any function has been attributed to these events. The identity of the platelet phosphatase responsible for the dephosphorylation of platelet CD36 is not certain. Platelets contain both PP1 and PP2a phosphatases as well as another 41-kD phosphatase [F. Erdodi, C. Csortos, L. Sparks, A. Muranyi, P. Gergely, *Arch. Biochem. Biophys.* **298**, 682 (1992)] with serine and threonine specificity. The latter may be identical to a recently described protein activity on the surface of activated platelets [U. P. Naik, E. Kornecki, Y. H. Ehrlich, *Biochim. Biophys. Acta* **1092**, 256 (1991)]. Microcystin, a cell-impermeable inhibitor specific for PP1 and PP2a phosphatases, inhibits platelet aggregation in response to ionophore as well as blocks PP2a-mediated dephosphorylation of CRL 9792 cell membrane proteins including CD36 (*31*).
29. It is of interest that type III collagen, which interacts with platelets through a different collagen domain, does not support CD36-dependent adhesion and presumably interacts with platelets by means of other collagen receptors [L. F. Morton, C. M. Fitzsimmons, J. Rauterberg, M. J. Barnes, *Biochem. J.* **248**, 483 (1987)].
30. T. J. Kunicki *et al.*, *J. Biol. Chem.* **263**, 4516 (1988).
31. A. Asch *et al.*, unpublished results.
32. To examine the incorporation of phosphate into purified CD36, we did the same studies in fluid phase with [γ-^{32}P]ATP (Du Pont Biotechnology Systems) as a source of phosphate. After incubation, the reaction mixture was analyzed by SDS-PAGE and autoradiography. For studies on phosphorylation of p87-99, purified peptide was coupled to COOH surface covalent binding 96-well plates (Costar) with 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride (50 mM, Pierce) to activate the plate surface. The immobilized peptides were first incubated with PKC (50 U/ml, UBI) with ATP (100 mM) or with ATP alone, as a source of phosphate, in buffer (20 mM tris, pH 6.5, containing 5 mM $MgCl_2$ and 5 mM $CaCl_2$) before TSP or collagen binding. To examine the phosphorylation of the peptide, we did the same studies in fluid phase. We incubated peptide with purified PKC (50 U/ml, UBI) in fluid phase using [γ-^{32}P]ATP (Du Pont Biotechnology Systems) as a source of phosphate (4.8×10^5 cpm/nM) in 20 mM tris (pH 6.5) containing 5 mM $MgCl_2$ and 5 mM $CaCl_2$. The reaction mixture was analyzed by TLC with JT Baker Silica IB2 plates and ethyl acetate:pyridine :acetic acid:water (20:20:6:11 v/v) as eluant. We detected peptides on the TLC plates by ninhydrin (0.2% in ethanol), excised the ninhydrin-stainable region of the plate, and quantitated ^{32}P incorporation with an LKB scintillation counter.
33. Supported by NIH funds HL44389, HL02541, and HL18828. We thank H. Uhara (Mt. Sinai Hospital, New York) for automated DNA sequencing. We thank B. Seed (Massachusetts General Hospital) for the CD36-containing plasmid; P. Bornstein, D. Hajjar, R. Nachman, and S. Wright for reviewing the manuscript; and D. Brautigan for helpful discussion.

6 August 1993; accepted 4 October 1993