

Constitutive and Stimulus-induced Phosphorylation of CD11/CD18 Leukocyte Adhesion Molecules

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Abstract. The leukocyte CD11/CD18 adhesion molecules ($\beta 2$ integrins) are a family of three heterodimeric glycoproteins each with a distinct α subunit (CD11a, b, or c) and a common β subunit (CD18). CD11/CD18 mediate crucial leukocyte adhesion functions such as chemotaxis, phagocytosis, adhesion to endothelium, aggregation, and cell-mediated cytotoxicity. The enhanced cell adhesion observed upon activation of leukocytes is associated with increased surface membrane expression of CD11/CD18, as well as a qualitative up-regulation of CD11/CD18 functions. To elucidate the nature of the qualitative modifications that occur, we examined the phosphorylation status of these molecules in resting human leukocytes and upon activation with PMA or with the chemotactic peptide F-met-leu-phe (FMLP). In unstimulated cells, all three CD11 subunits were found to be constitutively phosphorylated. In contrast, phosphorylation of the common CD18 subunit was minimal. PMA induced rapid

and sustained phosphorylation of CD18 that occurred at high stoichiometry, but had only minimal effects on phosphorylation of the associated CD11 subunits. FMLP also induced rapid phosphorylation of CD18, but the effect was of short duration. FMLP-induced phosphorylation of CD18 was not related to its Ca^{++} -mobilizing effect, as CD18 phosphorylation was not observed upon treatment of leukocytes with the Ca^{++} ionophore, ionomycin. Phosphoamino acid analysis of CD11/CD18 in PMA- or FMLP-treated monocytes revealed a predominance of phosphoserine residues in all CD11/CD18 subunits. A small component of phosphothreonine was present in CD11c and CD18 and a minor component of phosphotyrosine was also detected in CD18. We conclude that phosphorylation of CD18 upon leukocyte activation may regulate the adhesion functions mediated by the CD11/CD18 family of molecules.

THE leukocyte differentiation antigens CD11/CD18 are a family of three related glycoprotein heterodimers implicated in a variety of leukocyte adhesion processes (Dana and Arnaout, 1988). CD11/CD18 glycoproteins are members of a larger family of $\alpha\beta$ heterodimeric adhesion molecules known as integrins (Hynes, 1987). These include the fibronectin receptor/VLA antigens ($\beta 1$ integrin subfamily), CD11/CD18 ($\beta 2$ integrin subfamily), and the vitronectin and gpIIb/IIIa receptors ($\beta 3$ integrin subfamily). Each member of the CD11/CD18 family is a heterodimer consisting of a distinct α subunit (CD11a, b, and c, 180, 155, and 140 kD, respectively) non-covalently associated with an identical 94-kD common β subunit (CD18). CD11a/CD18 (LFA-1) is expressed on all leukocytes, and serves as a general adhesion molecule involved in leukocyte homotypic and heterotypic interactions (Patarroyo et al., 1985; Howard et al., 1986; Rothlein and Springer, 1986; Arnaout et al., 1988a). CD11a/

CD18 plays a particularly important role in vitro in the proliferation of T cells to antigens and mitogens (Berzins et al., 1988; Schwartz et al., 1989), in the interaction of cytotoxic T cells and NK cells with their target cells (Davignon et al., 1981; Schmidt et al., 1985), in the activation of B cells and their differentiation into immunoglobulin-secreting cells (Howard et al., 1986; Fisher et al., 1986), and in leukocyte-endothelium adhesion (Mentzer et al., 1986; Arnaout et al., 1988a). CD11a/CD18 adhesion functions are in part mediated by CD54, an endogenous ligand with wide cell distribution (Dustin et al., 1986; Simmons et al., 1988). CD11b/CD18 (Mol) and CD11c/CD18 (p150/95) are selectively expressed on granulocytes, monocytes, and NK cells. Both promote a variety of leukocyte adhesive interactions such as phagocytosis, chemotaxis, aggregation, adhesion to endothelium, and binding to the complement C3 fragment iC3b (Arnaout et al., 1983, 1985, 1988a; Micklem and Sim, 1985; Dana et al., 1986; Keizer et al., 1987). The importance of CD11/CD18 antigens in the adhesive functions of leukocytes has been highlighted by the identification of a human immunodeficiency disease characterized by recurrent severe bacterial infections in which leukocytes are deficient

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in CD11/CD18 surface membrane expression. The adhesion-dependent functions displayed by the leukocytes of these patients, including chemotaxis, leukoaggregation, iC3b binding, adhesion to endothelium, and phagocytosis are all uniformly defective (Dana and Arnaout, 1988).

The adhesive properties of CD11/CD18 are significantly enhanced after leukocyte activation with a variety of inflammatory mediators (Arnaout et al., 1984; Berger et al., 1984; Patarroyo et al., 1985; Rothlein and Springer, 1986). In polymorphonuclear leukocytes, granulocytes (PMNs)¹ and monocytes, cell activation is also associated with increased expression of CD11b,c/CD18 on the cell surface (Arnaout et al., 1984; Berger et al., 1984; Freyer et al., 1988), suggesting one mechanism for stimulus-induced enhancement of leukocyte adhesion. There is also evidence that qualitative changes in these molecules are crucial for mediating adhesion upon cellular activation. Treatment of lymphocytes with the protein kinase C (PKC) activator PMA led to rapid induction of CD11a/CD18-dependent aggregation without an apparent increase in CD11a/CD18 surface expression (Patarroyo et al., 1985; Rothlein and Springer, 1986). Treatment of PMN with PMA resulted in the sequential activation and deactivation of CD11b/CD18 adhesive functions in a manner that could not be accounted for by quantitative alterations in the receptor's surface membrane expression (Wright and Meyer, 1986). Similarly, phagocytosis of iC3b-coated sheep erythrocytes by monocytes required stimulation of these cells with tumor-promoting phorbol esters (Wright and Silverstein, 1982). Other studies have demonstrated that the adhesive functions of CD11b/CD18 in PMN can be dissociated from stimulus-induced increase in surface membrane expression of this heterodimer (Buyon et al., 1988; Vedder and Harlan, 1988). Similarly, treatment of monocytes with ADP resulted in exposure of new epitopes on CD11b/CD18 and its acquiring of new functional specificities without a quantitative increase in its surface membrane expression (Altieri and Edington, 1988). These studies suggest that CD11/CD18 adhesive functions can be qualitatively altered upon transmembrane signaling, and support a role for PKC-induced phosphorylation event(s) in regulating these functions.

One potential target for PKC-mediated phosphorylation is CD11/CD18 itself. Examination of published cDNA-derived amino acid sequences of human CD11a (Larson et al., 1989), CD11b (Arnaout et al., 1988b), CD11c (Corbi et al., 1987), and CD18 (Kishimoto et al., 1987; Law et al., 1987) reveals available serine, threonine, and tyrosine residues in various combinations in the cytoplasmic domains of all four proteins (Fig. 1). These residues may serve as putative phosphorylation sites for serine/threonine-specific protein kinases, including PKC, as well as for tyrosine-specific protein kinases. Interestingly, the cytoplasmic domain of CD18 contains multiple basic amino acids distributed on both sides of serine residues, a feature characteristic of potent PKC substrate peptides (House, 1987). Previous studies aimed at looking for evidence of CD11/CD18 phosphorylation upon PKC activation by phorbol esters arrived at conflicting conclusions. While one study found that PMA induced phosphorylation of CD18 (Hara and Fu, 1986), two other studies failed to sup-

port that conclusion (Changelian and Fearon, 1986; Skubitz et al., 1988). In the present study, we examined the phosphorylation status of all three CD11/CD18 heterodimers in human monocytes and PMN, both at rest and after stimulation with PMA or F-met-leu-phe (FMLP), both of which enhance CD11/CD18-mediated adhesion functions in these cells. We found that all three α subunits were constitutively phosphorylated and that PMA or FMLP induced a major increase in phosphorylation of CD18 that was dose and time dependent. Both stimuli induced a small increase in the intrinsic phosphorylation of CD11. Stimulus-induced phosphorylation of CD18 may play an important role in mediating the various functions dependent on this group of leukocyte adhesion molecules.

Materials and Methods

Reagents

The following murine mAbs recognizing the various subunits of CD11/CD18 were used: mAb TSI/22 directed against CD11a (Mentzer et al., 1986); mAb 44a (IgG 2a), directed against CD11b (Todd et al., 1981; Dana et al., 1984); mAb L29 (IgG 1), directed against CD11c (Arnaout et al., 1988a); and mAb 10F12 (IgG 1), directed against CD18 (Rosemarin et al., 1989). PMA and ionomycin were obtained from Calbiochem-Behring Corp. (La Jolla, CA). FMLP, crystalline BSA (globulin free), leupeptin, pepstatin, chymostatin, antipain, iodoacetic acid, Hepes, PMSF, and phosphoamino acids were from Sigma Chemical Co. (St. Louis, MO). Protein A-Sepharose was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Rabbit anti-mouse IgG (RAMIG) was obtained from Pel-Freez Biologicals (Rogers, AR). ³²P-orthophosphate, HCl-free, and ¹²⁵I carrier-free were from New England Nuclear (Boston, MA).

Cells

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy individuals by leukopheresis and were depleted of contaminating red blood cells and PMN by centrifugation over Ficoll-Hypaque. Monocytes were isolated by adhering PBMCs to petri dishes as described (Todd et al., 1981). The purified monocyte preparations were usually >90% positive for CD11b/CD18 by immunofluorescence. Human PMNs were purified from peripheral blood by centrifugation over Ficoll-Hypaque and hypotonic lysis as described (Dana et al., 1984).

³²P Radiolabeling, Immunoprecipitation, and Gel Electrophoresis

Cells were washed three times in a phosphate-free medium containing 30 mM Hepes, 110 mM NaCl, 110 mM glucose, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 2 mg/ml BSA, pH 7.4. Cells were resuspended at 2.5×10^7 cells/ml in the same buffer and incubated for 1 h at 37°C, pelleted, resuspended as above, and incubated in the presence of 1 mCi/ml ³²P-orthophosphate for 3 h at 37°C. Thereafter, cells were washed, resuspended in fresh phosphate-free buffer at 37°C, and stimulated with the desired agent for the indicated times. Cell activation was terminated by pelleting the cells for 10 s in a microcentrifuge (8,000 g), and resuspending them in ice-cold lysis buffer containing 20 mM Tris-HCl, 250 mM NaCl, 50 mM NaH₂PO₄, 50 mM NaF, 25 mM Na₄P₂O₇, 10 mM iodoacetic acid, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 1 mM Na₃VO₄, 1% NP-40, and 1 mg/ml each of leupeptin, pepstatin, chymostatin, and antipain at pH 7.4 (lysis buffer). Cell lysates were spun down at 15,000 g for 30 min at 4°C, and supernatants were precleared by incubating them with 25 μ l of packed protein A-Sepharose sequentially precoated with RAMIG followed by normal mouse serum. CD11/CD18 antigens were thereafter precipitated by incubating the pre-cleared lysates for 4 h with 25 μ l of protein A-Sepharose sequentially coated with RAMIG and the respective anti-CD11a, b, c, or CD18 mAb. Sepharose beads were then pelleted and washed five times with BSA-free lysis buffer. Immunoprecipitates were solubilized with 25 μ l of an SDS sample buffer containing 2% SDS, 10% glycerol, 100 mM Tris-HCl, pH 6.8, 0.1 M 2-mercaptoethanol, and 0.02% bromophenol blue. Samples were electrophoresed on polyacrylamide gels using the system of Laemmli (1970). Gels were

1. *Abbreviations used in this paper:* [Ca⁺⁺]_i, intracellular free Ca⁺⁺ concentration; DAG, diacylglycerol; FMLP, F-met-leu-phe; PBMC, peripheral blood mononuclear cell; PKC, protein kinase C; PMN, polymorphonuclear leukocyte granulocyte; RAMIG, rabbit anti-mouse IgG.

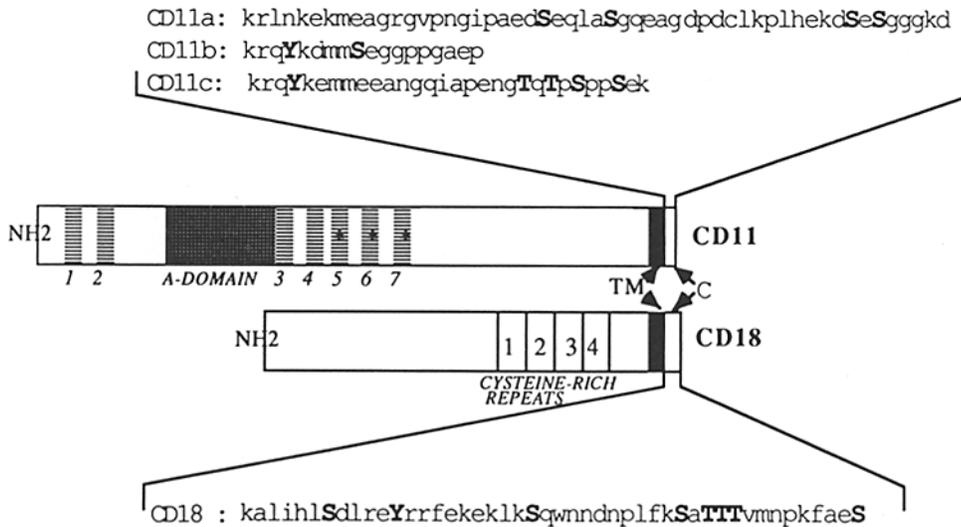


Figure 1. Schematic diagram showing the overall structure of CD11/CD18 and the amino acid sequences of the cytoplasmic end of each subunit (derived from published data). The larger CD11 subunits are homologous and contain seven tandem repeats (*horizontal bars*), three of which contain putative metal-binding sites (*), and an adhesion-related domain (*A-domain*) with homologies to several collagen-binding proteins (Arnaout et al., 1988b; Pytela, 1988). The smaller common CD18 subunit contains a unique cysteine-rich motif repeated four times. *TM*, transmembrane region. *C*, cytoplasmic end. The potential phosphorylation sites corresponding to serine (S), threonine (T), and tyrosine (Y) residues are in bold letters.

dried and exposed to x-ray film (XAR-5; Eastman Kodak Co., Rochester, NY) with intensifying screen (Dupont Co., Wilmington, DE). The relative degree of phosphorylation in the labeled bands of interest was determined by scanning the radiograms under conditions of linear exposure using a densitometer (Ultrosan 2; LKB Instruments, Inc., Gaithersburg, MD) and correcting for band width.

Phosphoamino Acid Analysis

Phosphoamino acid analysis was carried out as described (Avruch et al., 1982). Immunoprecipitates from PMA- or FMLP-treated ^{32}P -labeled monocytes were electrophoresed on polyacrylamide gels as described above. Gels were dried and subjected to autoradiography. Regions in the gel corresponding to CD11a, b, c, and CD18 were excised, rehydrated, and extracted in 1% SDS overnight at 37°C. BSA was added to each extract (0.5 mg/ml), and proteins were precipitated with 5 vol of acetone at -20°C. After two washes, the pellets were dried and subjected to acid hydrolysis (6 N HCl, 2 h, 110°C). Each hydrolysate was separately subjected to two-dimensional thin layer electrophoresis on cellulose. Electrophoresis in the first dimension was done in formic acid/acetic acid/H₂O (25:87:888) buffer, pH 3.5, for 4 h at 800 V; electrophoresis in the second dimension was performed in pyridine/acetic acid/H₂O (1:10:89) buffer for 2 h at 800 V. Added phosphoamino acid standards were visualized by spraying the plates with ninhydrin, while radiolabeled phosphoamino acids were visualized by autoradiography as described above.

Stoichiometry of Phosphorylation

Aliquots each consisting of 5×10^7 washed monocytes were resuspended in 0.5 ml of ice-cold PBS containing 1 mM MgCl₂ and 100 μg lactoperoxidase (Sigma Chemical Co.), and surface labeled by addition of 1 mCi of carrier-free ^{125}I (New England Nuclear), followed by six additions of 10 μl of 1:1,000 dilution of hydrogen peroxide (37% solution) over a 30-min period. Reaction was stopped by addition of PBS, containing 1 mM sodium iodide and saturating amounts of tyrosine. After two washes, cell aliquots (in Hanks' buffer, pH 7.4) were either exposed to 250 ng/ml PMA or to buffer alone (as a control) for 10 min at 37°C. Cells were then washed, solubilized in lysis buffer, and immunoprecipitated with anti-CD11b mAb as described above. Immunoprecipitates from one PMA-treated sample were eluted from the immunoadsorbant by boiling in 1% SDS and the eluate was either mock-treated or treated with bacterial alkaline phosphatase (molecular biology grade, Boehringer Mannheim Biochemicals, Indianapolis, IN) exactly as described (Krangel, 1987). Eluates were analyzed by two-dimensional gel electrophoresis (first dimension, IEF, using equal volumes

of pH 3.5-10 and pH 5-7 ampholines [O'Farrell et al., 1977]; second dimension, 7.5% PAGE [Laemmli, 1970]). Radioactive species were identified by autoradiography of the dried gels using film (XAR-5; Eastman Kodak Co.) with an intensifying screen. Quantitation of relative band intensity was performed by densitometric scanning of the radiograms under conditions of linear exposure using a densitometer (Ultrosan 2; LKB Instruments).

Results

Phosphorylation of CD11/CD18 Antigens in Human Monocytes

Under basal conditions, immunoprecipitation of ^{32}P -labeled monocytes using mAbs directed against CD11a, b, c, or CD18 subunits followed by SDS-PAGE revealed that all three α subunits were constitutively phosphorylated (Figs. 2 and 3 A). Little or no phosphorylation of the common CD18 subunit was seen under these conditions. Treatment of monocytes with PMA at 250 ng/ml for 1-15 min resulted in a dramatic phosphorylation of CD18 with only small changes in the phosphorylation status of the associated α subunits (Figs. 2 and 3 A).

Kinetics of PMA-induced Phosphorylation of CD18

The time course and concentration dependence of PMA-induced phosphorylation of CD18 were examined using CD11b-associated CD18 as a prototype. Treatment of monocytes with PMA at a concentration of 250 ng/ml resulted in rapid phosphorylation of CD18 (Fig. 3 A). CD18 was nearly maximally phosphorylated by 1 min after PMA addition and remained so for the entire study period of 30 min. Constitutive phosphorylation of CD11b on the other hand showed only a small rise over this time period (Fig. 3 A). PMA-induced CD18 phosphorylation was also concentration dependent being notable at 10 ng/ml of PMA, the lowest concentration tested, and plateaued at ~ 100 ng/ml (Fig. 3 B).

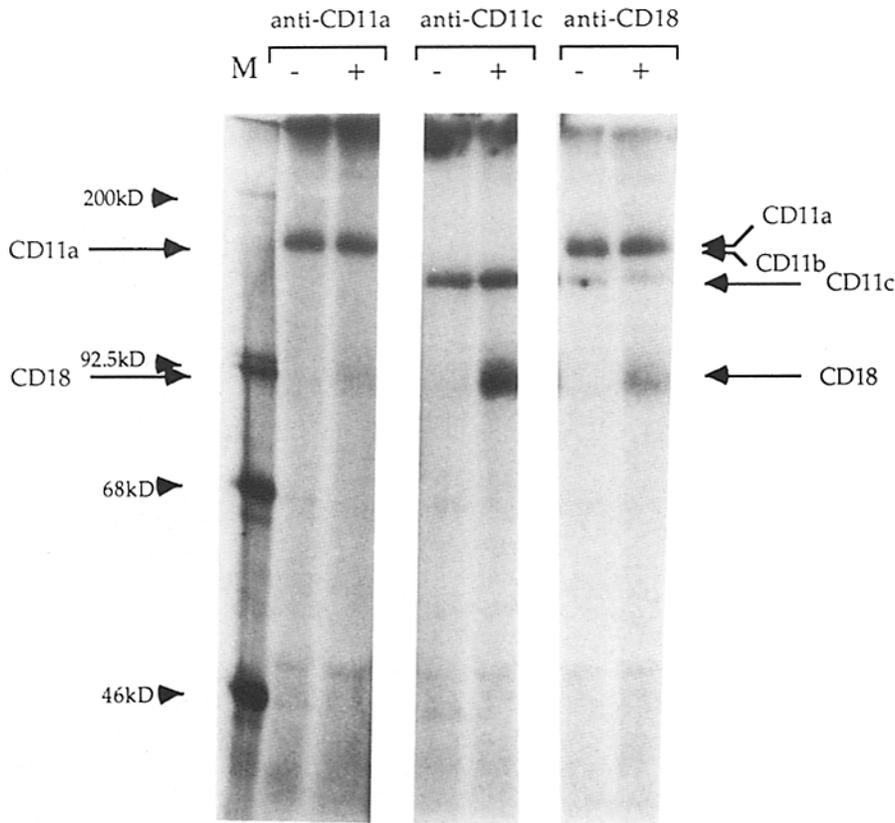


Figure 2. Autoradiograph of a 7.5% SDS-PAGE showing the phosphorylation status of CD11/CD18 antigens in ^{32}P -loaded monocytes before (-) and after (+) stimulation with PMA (250 ng/ml, 15 min, 37°C). The subunit-specific mAbs TS1/22 (anti-CD11a), L29 (anti-CD11c), and 10F12 (anti-CD18) used to immunoprecipitate the respective antigens are indicated. None of the radiolabeled bands precipitated with anti-CD11/CD18 mAbs were seen when a control nonreactive mAb was used. *M* represents the following molecular mass markers (arrowheads): myosin (200 kD), phosphorylase b (92.5 kD), BSA (68 kD), and ovalbumin (46 kD).

Stoichiometry of PMA-induced CD11/CD18 Phosphorylation

To determine the percentage of CD18 species that is phosphorylated after PMA treatment, CD11b/CD18 immunoprecipitates from PMA-activated or resting surface-iodinated monocytes were analyzed by two-dimensional IEF-PAGE. Resting cells displayed two major ^{125}I -labeled CD18 bands (apparent molecular mass, 94 kD) (Fig. 4 A). The more basic species was predominant (74% of total). CD11b (apparent molecular mass, 155 kD), migrated as a broad band (Fig. 4 A). Upon PMA treatment, the CD18 acidic species became predominant (73% of total CD18-related radioactivity) (Fig. 4 B). No significant change in CD11b was observed after PMA activation by this analysis (Fig. 4, A and B). Alkaline phosphatase treatment of a sample from PMA-induced cells resulted in a 70% drop in the radioactivity associated with the acidic form of CD18 and a 49% increase in that associated with the basic form (not shown), indicating that the charge differences observed in CD18 after PMA treatment are predominantly due to phosphorylation. Alkaline phosphatase treatment similarly resulted in a shift of the broad CD11b-associated band to a more basic form (data not shown).

Phosphorylation of CD18 by FMLP

The chemotactic peptide FMLP, like PMA, enhances CD11/CD18-mediated adhesion of phagocytic cells to various targets (Arnaout et al., 1984; Dana et al., 1986), although its adhesion-promoting effects tend to be more transient (Boxer et al., 1979). We therefore examined the effect of FMLP treatment of monocytes on the phosphorylation status of CD11/CD18 antigens using CD11b/CD18 as a prototype.

Treatment of monocytes with FMLP at 10^{-7} M for 30 s induced phosphorylation of CD18 without significantly altering intrinsic CD11b phosphorylation (Fig. 5). FMLP-induced CD18 phosphorylation was transient (Fig. 5), peaking at 30 s, the earliest time point examined, and was almost completely reversed by 5 min after stimulation.

FMLP acts via its surface receptor to induce phospholipase C activation resulting in inositol trisphosphate and diacylglycerol (DAG) accumulation and leading respectively to Ca^{++} mobilization and PKC activation (Becker, 1986; Berridge, 1987). We found the FMLP-induced CD18 phosphorylation was not a direct consequence of its increasing the intracellular free Ca^{++} concentration ($[\text{Ca}^{++}]_i$) in treated cells. This was evidenced by the failure of the Ca^{++} ionophore, ionomycin to induce phosphorylation of CD18 in monocytes when used at a concentration of 50 nM (Fig. 6). At this concentration, we found that ionomycin induces an increase in $[\text{Ca}^{++}]_i$ comparable to or even exceeding that observed with FMLP at 10^{-6} M without significantly affecting phospholipase C activity (our unpublished observations). These data suggest that FMLP-induced phosphorylation of CD18 is predominantly mediated by intracellular accumulation of DAG, a major physiologic PKC activator (Bell 1986; Berridge, 1987).

Constitutive and Stimulus-induced Phosphorylation of CD11/CD18 in Human Granulocytes

To determine if similar changes in CD11/CD18 phosphorylation also occur in circulating PMN, we examined the phosphorylation status of CD11/CD18 antigens in human PMN both at rest and upon activation with PMA or FMLP. Fig.

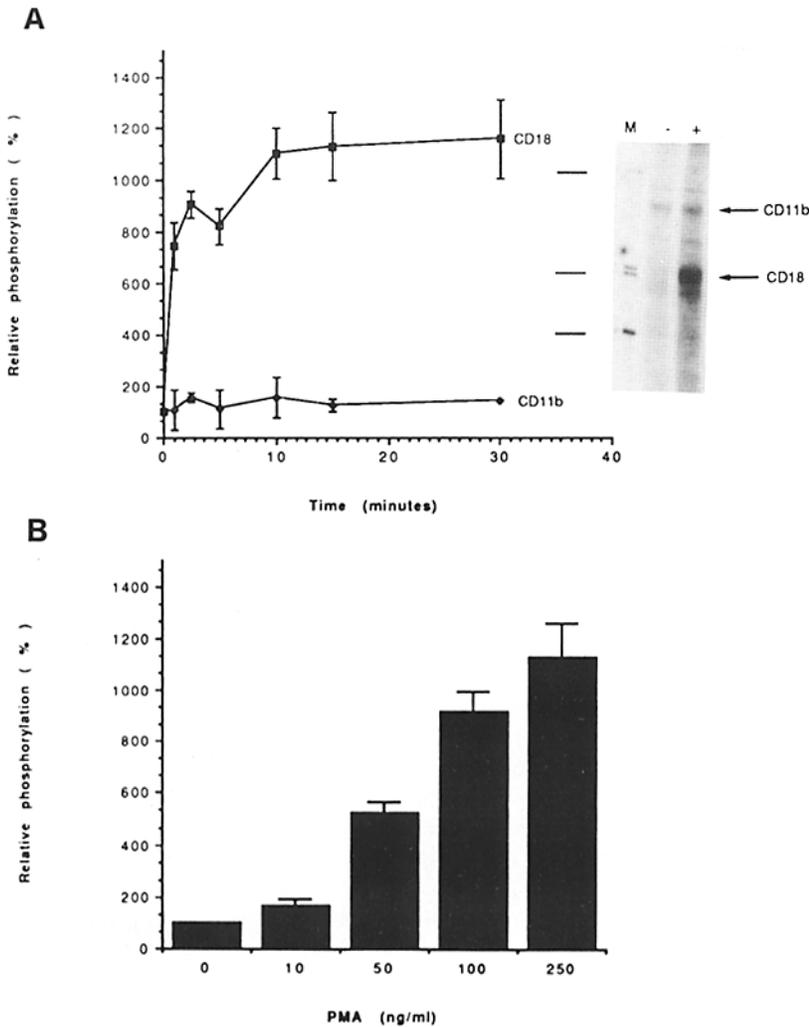


Figure 3. PMA-induced phosphorylation of CD11b/CD18 in human monocytes. In this figure and subsequent figures, the anti-CD11b mAb, 44, was used for immunoprecipitations. (A) Time course of PMA (250 ng/ml)-induced phosphorylation of CD11b/CD18. Each time point represents the mean \pm SD of two separate experiments. Changes in CD11/CD18 phosphorylation were quantitated by densitometric scanning of autoradiographs. In this and subsequent figures, a baseline value of 100 was assigned for CD18 and [32 P]-CD11b. In the case of CD18, this value was derived by scanning the area of the gel (in the uninduced control sample) corresponding to that which contained stimulus-induced [32 P]-CD18. (Inset) Autoradiograph of a 7.5% SDS-polyacrylamide gel showing CD18-induced phosphorylation after 1 min of monocyte exposure to PMA (250 ng/ml, 37°C). - and + indicate CD11b/CD18 immunoprecipitates in the absence or presence of PMA, respectively. The positions of the molecular mass markers myosin (200 kD), phosphorylase b (92.5 kD), and BSA (68 kD) are indicated. (B) Histogram showing the degree of phosphorylation of CD18 (y-axis) upon treatment of 32 P-loaded monocytes with increasing concentrations of PMA (x-axis). CD18-induced phosphorylation reached a plateau at \sim 100 ng/ml of PMA. Each histogram represents mean \pm SD of two independent experiments.

7 demonstrates that, in 32 P-loaded PMN, CD11b is constitutively phosphorylated with minimal phosphorylation of CD18. Treatment of PMN with PMA at 30 ng/ml induced a dramatic rise in phosphorylation of CD18 which peaked 30 min after addition of PMA (Fig. 7 A). FMLP, at 10^{-7} M, also induced phosphorylation of CD18 which peaked at 30 s, the earliest time point studied, and declined by 5 min after FMLP addition (Fig. 7 B). In contrast to the marked increase in CD18 phosphorylation, only a modest 1.6-fold rise in CD11b phosphorylation was seen after PMA treatment (Fig. 7 A). Basal as well as PMA- or FMLP-induced phosphorylation of CD11a/CD18 and CD11c/CD18 in PMN followed a similar pattern to that observed for these antigens in monocytes (data not shown).

Phosphoamino Acid Analysis of Phosphorylated CD11/CD18 Species

To identify the amino acid(s) phosphorylated in CD11/CD18 subunits in response to PMA or FMLP, regions encompassing each of the phosphorylated subunits were excised from polyacrylamide gels, eluted, precipitated, and subjected to acid hydrolysis and two-dimensional thin layer electrophoresis. The amounts of 32 P subjected to thin layer electrophore-

sis were directly proportional to the counts detected in the gel slices before elution (data not shown). Both PMA- and FMLP-induced phosphorylation of CD18 in monocytes was largely due to [32 P]phosphoserine, with small amounts contributed by [32 P]phosphothreonine and [32 P]phosphotyrosine (Fig. 8 A). Phosphorylation of CD11a was solely due to [32 P]phosphoserine (Fig. 8 B), in agreement with the presence of serine as the only amino acid target for phosphorylation in the cytoplasmic end of CD11a (Fig. 1). Only [32 P]phosphoserine contributed to CD11b phosphorylation under these conditions (Fig. 8 B), while an abundance of [32 P]phosphoserine and traces of [32 P]phosphothreonine were detected in CD11c (Fig. 8 B). No [32 P]phosphotyrosine residues were detected in CD11b or CD11c.

Discussion

In this study, we determined the phosphorylation status of the various subunits of CD11/CD18 antigens in resting and activated human monocytes and PMN. All three CD11 subunits were found to be constitutively phosphorylated. The basal phosphorylation level of CD11 subunits increased only marginally (1.0–1.6-fold) upon treatment of monocytes with

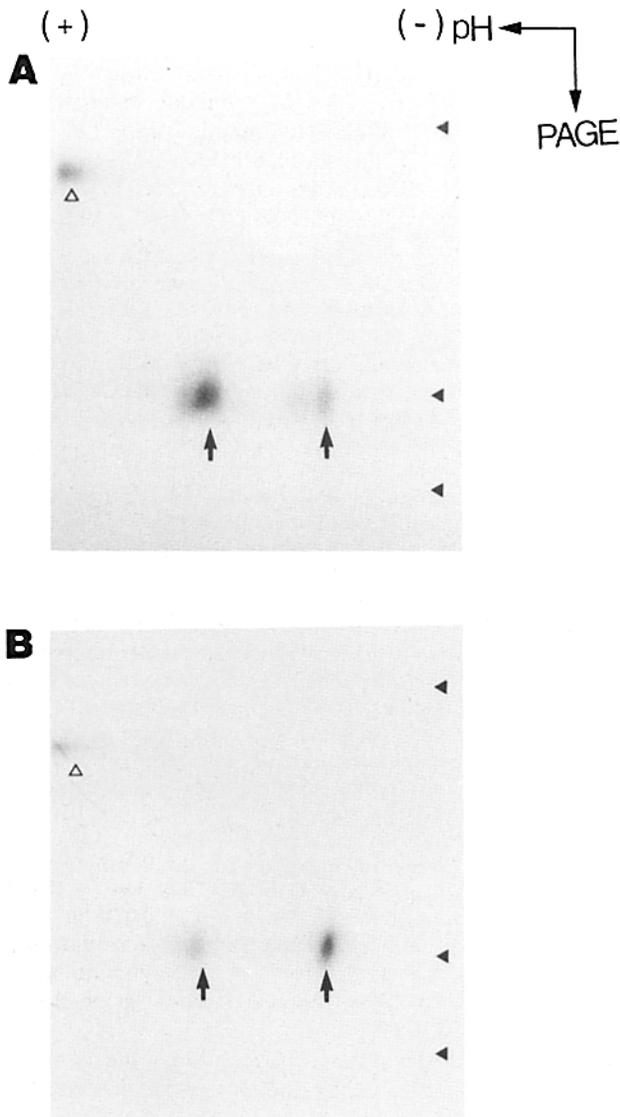


Figure 4. Stoichiometry of phosphorylation of surface-expressed CD11b and CD18. Untreated (resting) (A) or PMA-treated (B) surface-iodinated monocytes were detergent lysed and immunoprecipitated with anti-CD11b mAb. Immunoprecipitates were analyzed by two-dimensional gel electrophoresis (IEF, in the first dimension; PAGE, in the second dimension) followed by autoradiography. - and + indicate the acidic and basic ends of the isofocusing gel, respectively. Arrowheads indicate the position of the molecular mass markers myosin (200 kD), phosphorylase b (92.5 kD), and BSA (68 kD). The position of the two major CD18 bands (arrows) as well as that of the broad CD11b band (open arrowhead) are indicated. Densitometric scanning showed that the proportion of the basic and acidic forms of CD18 shown in A were 74 and 26%, respectively. After PMA treatment (B), the proportion became 27 and 73% for the basic and acidic forms, respectively.

PMA, FMLP, or the Ca^{++} ionophore ionomycin. In contrast, little or no constitutive phosphorylation of the common CD18 subunit was observed in $[^{32}P]$ -labeled resting leukocytes. Cell activation with the adhesion-promoting stimuli PMA or FMLP led to a marked phosphorylation of CD18 that was dose and time dependent and predominantly due to $[^{32}P]$ phosphoserine with small contributions by $[^{32}P]$ phos-

phothreonine and $[^{32}P]$ phosphotyrosine. CD11 phosphorylation was either totally (CD11a,b) or mostly (CD11c) due to $[^{32}P]$ phosphoserine.

PMA induced a significant shift in the isofocusing profile of CD18 (Fig. 4). Under baseline conditions, CD18 isofocused as one major ($\sim 75\%$) basic species and one minor ($\sim 25\%$) more acidic species (Fig. 4 A). Activation of monocytes with PMA resulted in a shift in the relative intensity of these two CD18 species with the bulk of radioactivity ($\sim 75\%$) now associating with the more acidic form (Fig. 4 B). Previous studies have shown variable amounts of these two forms by IEF-PAGE analysis and attributed this heterogeneity to sialic acid content (there are six potential N-glycosylation sites in CD18) (Sanchez-Madrid et al., 1983; Hildreth and August, 1985). The present studies identify phosphorylation as a major factor in the IEF-PAGE profile of CD18 and also indicate that PMA-induced phosphorylation of CD18 occurs with a relatively high stoichiometry.

Receptor-mediated activation of protein kinases is a key mechanism by which extracellular ligands regulate cellular functions. In particular, activation of two pathways of phosphorylation, one mediated by PKC and the other by Ca^{++} /calmodulin-dependent kinases has emerged as a mechanism common to many activators of leukocytes such as FMLP (Gerard et al., 1986; Hayakawa et al., 1986; Wright and Hoffman, 1987). Activation of these diverse protein kinases occurs through receptor-mediated accumulation of the second messengers inositol triphosphate and DAG. Inositol triphosphate induces an increase in $[Ca^{++}]$, resulting in the activation of multiple Ca^{++} -dependent pathways including Ca^{++} /calmodulin-dependent kinases, while DAG is an important physiologic activator of PKC (Bell, 1986; Berridge, 1987). Phorbol esters, such as PMA, are poorly hydrolyzable analogues of DAG that induce sustained activation of PKC

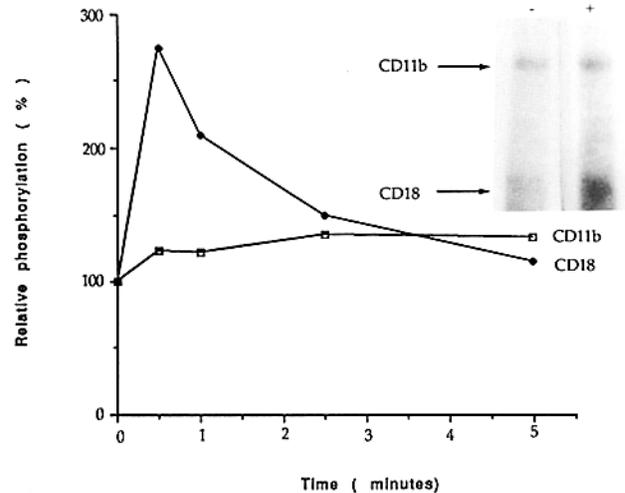


Figure 5. Effect of FMLP (10^{-7} M) on the kinetics of CD11b/CD18 phosphorylation in human monocytes. FMLP induced a rapid phosphorylation of CD18 which peaked at 30 s and progressively declined to almost baseline levels by 5 min. Changes in CD11b phosphorylation were minimal. This experiment is representative of two independent experiments. (Inset) Autoradiograph of an SDS-polyacrylamide gel (7.5%) showing the phosphorylation status of CD11b/CD18 in ^{32}P -loaded monocytes in the absence (-) or presence (+) of FMLP (10^{-7} M, 0.5 min, $37^{\circ}C$).

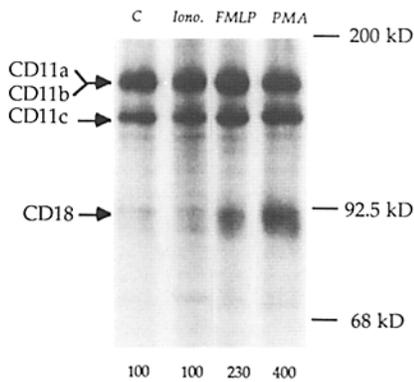


Figure 6. Comparison of CD18 phosphorylation status in response to ionomycin (50 nM), FMLP (10^{-7} M), and PMA (250 ng/ml). 32 P-labeled monocytes were untreated (C), or exposed to ionomycin (Iono), FMLP, or PMA for 30 s at 37°C. CD11/CD18 were immunoprecipitated from detergent-solubilized cells using the anti-CD18 mAb 10F12, and electrophoresed on a 10% polyacrylamide gel in SDS. The arabic numerals at the bottom of each lane reflect the levels of CD18 phosphorylation (quantitated by densitometry) in response to various stimuli, with the baseline value assigned an arbitrary unit of 100. PMA and FMLP increased CD18 phosphorylation by 4- and 2.3-fold, respectively, above baseline levels. No significant change in CD18 phosphorylation was detected in response to ionomycin. Similar results were observed in two other experiments.

(Nishizuka, 1984). The induction of CD18 phosphorylation by PMA suggests that this antigen is a substrate of PKC. FMLP-induced phosphorylation, on the other hand, may equally result from the action of Ca^{++} /calmodulin-dependent kinases that have been activated by the elevated $[Ca^{++}]_i$ as well as by PKC. To differentiate between these two possibilities, we examined the influence of an isolated increase in $[Ca^{++}]_i$, independent of PKC activation, on the phosphorylation of CD11/CD18. Treatment of monocytes with ionomy-

cin at 50 nM had no effect on the phosphorylation status of CD11b or CD18 (Fig. 6). At this concentration, ionomycin induced an increase in $[Ca^{++}]_i$ equal to or exceeding that noted with FMLP (at 10^{-6} M) without significantly influencing the activity of phospholipase C (data not shown; Preiss et al., 1987). This suggests that FMLP-mediated phosphorylation of CD18 is not primarily related to the activation of Ca^{++} /calmodulin-dependent kinases but is more likely due to DAG-mediated activation of PKC.

The magnitude and the kinetics of PMA-induced phosphorylation of CD18 differed from those observed for FMLP. At optimal concentrations, PMA-induced phosphorylation was intense and sustained (Figs. 3 and 7A) while that of FMLP was less intense and more transient in nature (Figs. 5 and 7B). The greater intensity of PMA-induced phosphorylation of CD18 relative to that of FMLP may relate to the greater potency of PMA in activating PKC as compared with DAG (Nishizuka, 1984). The rapid decline in FMLP-induced phosphorylation of CD18 may relate to the transient nature of DAG accumulation in FMLP-treated cells and/or activation of phosphatases. The intracellular accumulation of DAG may be rapidly terminated by a process of receptor uncoupling from phospholipase C (Allen et al., 1988).

While stimulation of monocytes with PMA or FMLP resulted in the predominant phosphorylation of CD18 on serine/threonine residues, consistent with a direct PKC effect, it also resulted in the appearance of a minor component of tyrosine phosphorylation (5–10%). Activation of PKC has been reported to secondarily activate some tyrosine kinases (Gilmore and Martin, 1983), and it is possible that this is the mechanism underlying the observed $[^{32}P]$ phosphotyrosine in CD18 from activated cells. The nature of the responsible tyrosine kinase(s) and the role, if any, that tyrosine phosphorylation plays in CD18 function remains to be determined.

PMA- or FMLP-induced phosphorylation of the common CD18 subunit followed similar kinetics to those observed for

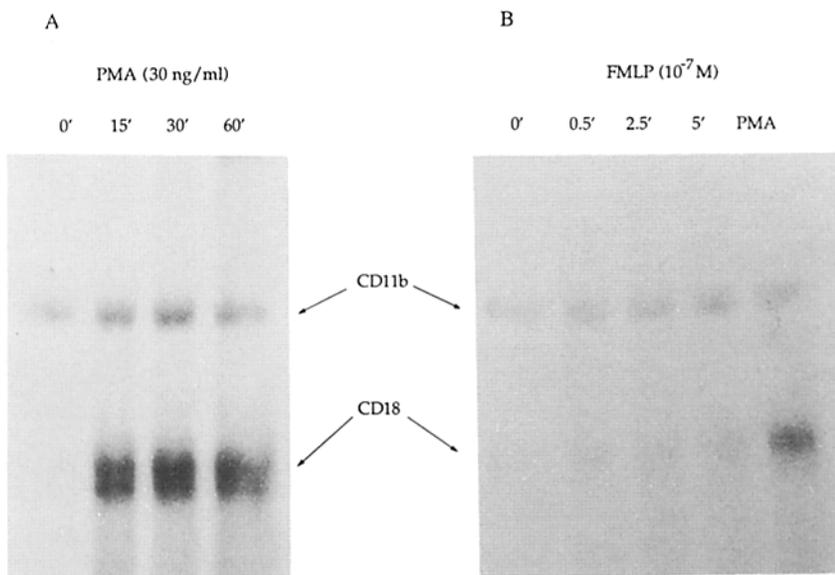


Figure 7. PMA- and FMLP-induced CD11b/CD18 phosphorylation in human PMN. (A) Autoradiograph of CD11b/CD18 immunoprecipitated from ^{32}P -loaded PMN that were either left untreated (0 time point) or treated with PMA at 30 ng/ml for 15–60 min. Immunoprecipitates were electrophoresed on 7.5% polyacrylamide gels according to Laemmli (1970). Densitometric scanning of $[^{32}P]$ CD18 and $[^{32}P]$ CD11b at 15, 30, and 60 min (compared to a baseline value of 100 for each subunit) were as follows: $[^{32}P]$ CD18: 1016, 1302, 1347; $[^{32}P]$ CD11b: 162, 161, 148. (B) A similar analysis showing the phosphorylation status of CD11b/CD18 in PMN before (0 time point) or after (0.5–5 min) exposure to FMLP (10^{-7} M). Densitometric scanning of $[^{32}P]$ CD18 and $[^{32}P]$ CD11b at 0.5, 2.5, and 5 min (assuming baseline values of 100) were as follows: $[^{32}P]$ CD18: 203, 181, 178; $[^{32}P]$ CD11b: 95, 112, 114. CD18 phosphorylation induced by PMA (30 ng/ml, 5 min) is shown for comparison.

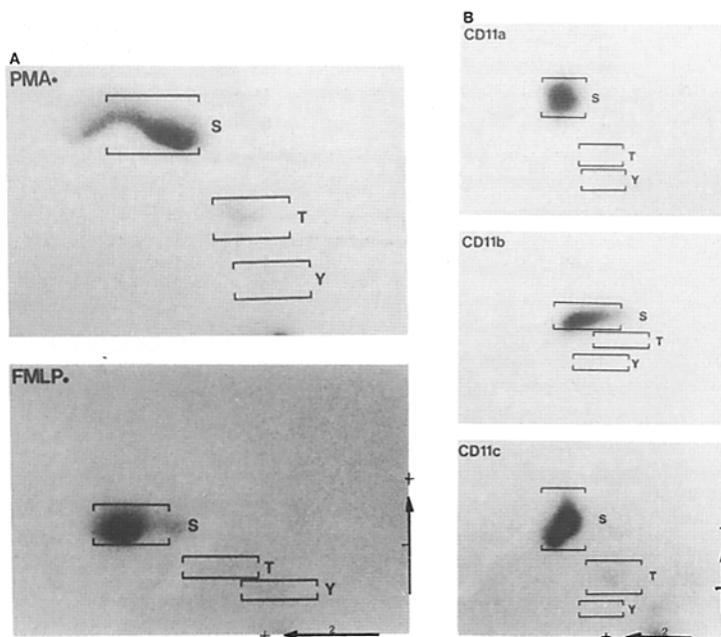


Figure 8 Phosphoamino acid analysis of PMA- and FMLP-induced CD11/CD18 phosphorylation. (A) Phosphoamino acids in PMA- or FMLP-phosphorylated CD18. Each CD18 hydrolysate (see Materials and Methods) was subjected separately to thin layer electrophoresis in pH 3.5 buffer (first dimension, 1), then electrophoresed in pH 1.9 buffer (second dimension, 2). + indicates direction of the anode in each case. After detecting the unlabeled carrier phosphoamino acids (with ninhydrin), cellulose filters were autoradiographed and the photographs (shown here) cropped to demonstrate the regions of interest. Trace amounts of [32 P]phosphothreonine and [32 P]phosphotyrosine were detected in both PMA- and FMLP-induced phosphorylated CD18. Treatment of 32 P-loaded monocytes with PMA (250 ng/ml) and FMLP (10^{-6} M) were carried out for 15 min and 30 s, respectively, at 37°C . (B) A similar analysis showing 32 P-labeled amino acids in CD11 subunits from 32 P-labeled monocytes activated with PMA (250 ng/ml, 15 min, 37°C). [32 P]Phosphoserine was present in all CD11 subunits. No [32 P]phosphothreonine or [32 P]phosphotyrosine was detected in CD11a or CD11b. Trace amounts of [32 P]phosphothreonine were detected in CD11c.

enhanced cell adhesion induced by these same stimuli. In particular, the onset of FMLP-induced CD18 phosphorylation was maximal in 30 s, and decreased by 5 min, a time course which closely parallels FMLP-induced homotypic or heterotypic adhesion in PMN (Boxer et al., 1979; O'Flaherty et al., 1979; Smith et al., 1979). Relatively little alterations in CD11b phosphorylation were observed under these conditions (Figs. 5 and 7 B). It is tempting to speculate, therefore, that the dramatic increase in CD11/CD18-dependent adhesion of activated leukocytes is mediated in part by stimulus-induced CD18 phosphorylation. The high stoichiometry of phosphorylation of surface-expressed CD18 (Fig. 4) is consistent with an important physiologic role for phosphorylation in receptor function. Phosphorylation could, for example, explain, at least in part, the qualitative upregulation in CD11/CD18 function which occurs in activated leukocytes or in cytoplasts (Wright and Silverstein, 1982; Patarroyo et al., 1985; Vedder et al., 1988; Buyon et al., 1988). It could also be a mechanism for the observed dissociation between increased CD11/CD18 expression on the cell surface and reduced cell adhesion in normal leukocytes and in certain myeloid cell lines (Arnaout et al., 1985; Dana et al., 1987). The mechanism by which CD18 phosphorylation would promote CD11/CD18 adhesion-dependent functions is unclear. Like other integrins, CD11/CD18 may provide a transmembrane link between the extracellular environment and cytoskeleton. It is conceivable that the phosphorylation status of CD18 could regulate receptor-cytoskeleton or perhaps receptor-ligand interactions. In other integrins, it has been suggested that their phosphorylation status may regulate their adhesive functions (Hirst et al., 1986; Dahl and Grable, 1989). In PMN, the ability of CD11b/CD18 to bind to its ligand, iC3b, temporally correlated with the extent of ligand-independent receptor aggregation (clustering) induced by PMA (Detmers et al., 1987). It has also been shown that the CD11b/CD18 receptor acquires the ability to recognize coagulation factor X in activated but not resting monocytes (Altieri et al., 1988).

It is also possible that phosphorylation may play a role in receptor internalization as was shown in several other receptor systems (Klausner et al., 1984; Begiunot et al., 1985). The contribution of the intrinsic state of CD11 phosphorylation and its variable increase upon stimulation to such interactions remains to be determined. Despite this temporal association, establishing a direct cause and effect relationship between stimulus-induced CD18 phosphorylation and enhanced cell adhesion would require additional studies on the functional properties of CD18 mutagenized in its cytoplasmic serine, threonine, or tyrosine residues. This approach may also be helpful in delineating a possible role of intrinsic CD11 phosphorylation in the regulation of CD11/CD18-mediated leukocyte adhesion.

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