Sequences within Fibrinogen and Intercellular Adhesion Molecule-1 (ICAM-1) Modulate Signals Required for Mitogenesis*

(Received for publication, January 19, 1999)

The interaction of fibrinogen (Fg) with intercellular adhesion molecule-1 (ICAM) on B-lymphoid Raji cells results in mitogenesis (Gardiner, E. E., and D'Souza, S. E. (1997) J. Biol. Chem. 272, 15474–15480). Incubation of Raji with Fg resulted in the increased tyrosine phosphorylation of the receptor-associated tyrosine kinase, pp60

\(^{src}\) and extracellular signal-regulated kinase-1 (ERK). The increase in ERK phosphorylation was blocked by a peptide with sequence matching ICAM-1 (8–22) and corresponded to a decrease in ERK-1 enzymatic activity. 100 µM amounts of Fg peptide γ(117–133) caused an increase in tyrosine phosphorylation of ERK-1. These results are consistent with our previous report wherein ICAM-1-(8–22) blocked Fg-induced mitogenesis and Fg-γ(117–133) induced proliferation in Raji. The specific inhibitor of MEK, PD98059 (25 µM), abrogated the increased phosphorylation of ERK-1 and blocked Raji mitogenesis by >50%. Inhibitors of pp60

\(^{src}\), geldanamycin (62 µM), and herbimycin A (2.5 µM) blocked >50% of Raji proliferation. These results indicate that the proliferation induced by Fg interactions with ICAM-1 is mediated in part by receptor-associated tyrosine kinases and ERK-1, and that the recognition sequences within Fg and ICAM-1 participate in the signaling process.

Intercellular adhesion molecule-1 (ICAM-1, CD54) is a highly glycosylated 90–110-kDa single chain protein that is up-regulated on endothelial cells (EC) by inflammatory cytokines such as interferon-γ, interleukin (IL)-1, IL-6, tumor necrosis factor α, and by phorbol esters. ICAM-1 belongs to a superfamily of immunoglobulin (Ig)-like proteins and is composed of five extracellular Ig-like domains, a single transmembrane domain, and a short cytoplasmic tail. The binding of leukocytes through integrins \(\alpha_4\beta_2\) and \(\alpha_m\beta_2\) to ICAM-1 on EC results in cellular interactions that regulate immune and inflammatory reactions (1). Fibrinogen (Fg), a plasma protein, has been reported to interact with ICAM-1. Fg bound to \(\alpha_m\beta_2\) on monocyte cells interact with ICAM-1 on EC resulting in cellular bridging between monocyteic cells and EC (2, 3). In addition, Fg has been shown to have a vasoconstrictive effect on the vessel wall through ICAM-1 interaction on EC (4). We have recently reported that an interaction between Fg and ICAM-1 on B-lymphoid Raji cells induces a proliferative response in Raji (5). Amino acid residues 8–22 within the first Ig domain of ICAM-1 and 117–133 within the γ chain of Fg have been demonstrated to be involved in cellular interactions (6, 7), including mitogenesis (5).

Apart from mitogenesis induced through the ligation of ICAM-1 by Fg, ICAM-1 ligation with integrins causes oxidative burst in neutrophils (8), and co-stimulation through ICAM-1 induces the release of IL-2 in B-cells (9). In addition, ICAM-1 modulates changes in intracellular Ca\(^{2+}\) in Burkitt’s lymphoma (10). These results suggest an active role for ICAM-1 in signal transduction. However, in recent reports the occupancy and cross-linking of ICAM-1 by anti-ICAM-1 antibodies has resulted in the phosphorylation of diverse intracellular proteins (11–13). The activation of certain members of the tyrosine kinase family of proteins including Lyn and Raf-1 was reported in a mouse B cell line (11). An increased tyrosine phosphorylation of cortactin was demonstrated in a rat brain endothelial cell line (12). Also, an increase in tyrosine phosphorylation of cdc2 kinase in peripheral blood T cells resulted in diminished cdc2 kinase activity (13). In these studies the cognitive natural ligands for ICAM-1 were provided by encephalitogenic T cells (12) and CDS activated T cells (11).

As Fg-ICAM-1 interactions mediated through recognition sequences in this ligand-receptor pair regulate growth in Raji, we sought to identify signals that may define the unique Fg-dependent proliferative function in this human B-cell line. The proliferative signals generated by activation or ligation of the prototypic growth factor receptors (platelet-derived growth factor and epidermal growth factor) result in the activation of the p21\(^{ras}\) signaling pathway. The cytoplasmic tails of the growth factor receptors possess intrinsic tyrosine kinase activity and become coupled to cytosolic mitogen-activated protein tyrosine kinases (MAPK) (14, 15). Studies utilizing either constitutively active or dominant negative mutants of kinases from the MAP kinase family establish a role for these kinases in cell proliferation, differentiation, and gene induction (16–18). In this study using soluble Fg to bind ICAM-1 on Raji, we have observed that the key proteins that are phosphorylated are pp60

\(^{src}\) and components of the MAP kinase cascade: p44 mitogen-activated protein kinase (MAPK) and p42 MAPK, also known as ERK-1 and ERK-2 (extracellular signal-regulated protein kinase-1 and -2). In addition, defined regions within Fg and ICAM-1 participate in the signaling process and inhibitors of pp60

\(^{src}\).
and MAPK kinase (MEK-1) block Raji mitogenesis induced through the binding of Fg to ICAM-1.

**MATERIALS AND METHODS**

**Reagents and Antibodies**—Transferin, bovine serum albumin (BSA), and dimethyl sulfoxide were purchased from Sigma. [methyl-3H]Thymidine was from Amersham Life Sciences (Arlington Heights, IL) and γ-32P]ATP was from NEN Life Science Products Inc. Myelin basic protein was from Upstate Biotechnology (Lake Placid, NY). Human fibronectin purified according to the method of Vuorio and Vaheri (19) was a gift from Dr. T. Ugarova (Cleveland Clinic Foundation, Cleveland, OH). PD98059, a specific inhibitor of MEK (20) and geldanamycin and herbimycin A, inhibitors of pp60src were purchased from Calbiochem. Each of these inhibitors was stored at a concentration of 10 mg/ml in 100% DMSO. Quots of cells (0.1 ml) were mixed with 0.1 ml of incubation medium Hanks' balanced salt solution containing 25 mM Hepes, pH 7.4, to an approximate cell concentration of 10⁵-10⁶ cells per ml. Aliquots of synthetic peptides ICAM-1-(8–22) or ICAM-1-(130–145) or the inhibitory compounds PD98059 or geldanamycin prior to the addition of cells. Cell suspensions were maintained at 22 °C for 10–60 min then 20 μl of 0.1 M sodium orthovanadate was added and cells were separated from medium by centrifugation (100 × g × 30 s). Cells were washed twice in Dulbecco's PBS containing 0.1% (v/v) trypsin/EDTA. Levels of protein in each cell lysate were estimated using the BCA kit (Pierce, Rockford, IL) and portions of each lysate containing equivalent amounts of protein (50–70 μg) were separated on 12.5% acrylamide/SDS gels and gels were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The membranes were soaked in a solution of 0.15 μM NaCl, 20 mM Tris/HCl, pH 7.5, containing 0.5% (w/v) Tween 20 (wash buffer) to which was added 5% (v/v) BSA for 1 h, rinsed in wash buffer, then incubated with anti-phosphotyrosine antibody PY20 for 4 h. Membranes were washed and probed with a goat anti-mouse IgG antibody conjugated with alkaline phosphatase. The bound antibody was detected using the ImmunoStar chemiluminescence kit (Bio-Rad).

**RESULTS**

**Occupancy of ICAM-1 by Fibrinogen Activates ERK-1**—and Fg-induced signal. Since phosphorylation of tyrosine residues within numerous intracellular proteins is an event key to the conveyance of a signal from the cell membrane to the nucleus, we appraised changes in levels of phosphorylation of intracellular proteins in Raji upon incubation with 200 nM Fg. This concentration of Fg was chosen as it consistently induced cell proliferation in Raji. Raji were incubated at 37 °C with Fg or transferrin (as control) for increasing periods of time. Cell suspensions were maintained at 22 °C for 10–60 min then 20 μl of 0.1 M sodium orthovanadate was added and cells were separated from medium by centrifugation (100 × g × 30 s). Cells were washed twice in Dulbecco's PBS containing 0.1% (v/v) trypsin/EDTA. Levels of protein in each cell lysate were estimated using the BCA kit (Pierce, Rockford, IL) and portions of each lysate containing equivalent amounts of protein (50–70 μg) were separated on 12.5% acrylamide/SDS gels and gels were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The membranes were soaked in a solution of 0.15 μM NaCl, 20 mM Tris/HCl, pH 7.5, containing 0.5% (w/v) Tween 20 (wash buffer) to which was added 5% (v/v) BSA for 1 h, rinsed in wash buffer, then incubated with anti-phosphotyrosine antibody PY20 for 4 h. Membranes were washed and probed with a goat anti-mouse IgG antibody conjugated with alkaline phosphatase. The bound antibody was detected using the ImmunoStar chemiluminescence kit (Bio-Rad).

**Cell Proliferation Assays**—ICAM-1-expressing lymphoblastoid Raji cells were obtained from ATCC (Rockville, MD). Raji were grown in RPMI 1640 (BioWhittaker, Walkersville, MD) containing 7.5% fetal bovine serum and 1.0 mM glutamine plus penicillin and streptomycin in incubators maintained at 37 °C and 5% CO₂. Proliferation assays were performed as described previously (5) with minor modifications. Raji were exchanged into serum-free medium 18 h prior to commencement of an experiment in order to achieve cellular quiescence. Cells were washed in ice-cold modified Dulbecco's medium (IMDM) (BioWhittaker) and counted with a hemocytometer. Aliquots of 0.2 ml of cells (4 × 10⁵ cells/ml) were mixed with 0.2 ml protein solutions diluted with IMDM and 4 μl of [3H]thymidine (1.0 μCi/μl). Some protein solutions contained reagents reported to inhibit known cell proliferation pathways. Cell suspensions (0.1 ml) were aliquoted into four replicate wells of a 96-well flat-bottomed plate (Becton Dickinson, Franklin Lakes, NJ) and plates were incubated at 37 °C and 5% CO₂ for 8 h. To measure [3H]thymidine uptake, the contents of each well were transferred to a 96-well plate with v-shaped wells. Cells were harvested by centrifugation (200 × g × 10 min) and washed twice in PBS then resuspended in 50 μl of PBS containing 1% (w/v) BSA. [3H]Thymidine that had been incorporated into cellular DNA was precipitated by addition of 0.1 ml of 20% (v/v) trichloroacetic acid for 12 h at 4 °C. Trichloroacetic acid pellets were dissolved in 0.2N NaOH containing 2% (v/v) SDS and assayed for radioactivity in a β-counter.

**Preparation of Fg and Fragments of Fg**—Fg was purified from fresh human plasma by cryoethanol precipitation (21, 22). Using electrophoretic conditions that allow the separation of fibrin from Fg monomer and subsequent visualization using Coomassie Blue R-250 (23), the isolated fibrinogen was estimated to compose greater than 95% Fg. Preparations of Fg were also analyzed for the presence of free fibrinopeptides A and B by elution on a Separ-Pak C₈₄ high performance liquid chromatography column using standard preparations of each of the fibrinopeptides (Sigma). At protein concentrations of at least 50-fold greater than those used in these experiments, amounts of fibrinopeptides A and B were below detectable levels.

**Synthetic Peptides**—Peptides with amino acid sequences corresponding to regions of ICAM-1 and Fg (6) were synthesized by the N-9-fluorenyl-methoxycarbonyl method on an Applied Biosystems ABI-66 instrument. Specific sequences were ICAM-1-(8–22), KVLPRGGSVLTVCS; ICAM-1-(130–145), REPAGPEAVTTTV; Fg-(γ117–133), NNGKTVNLEKQVAQLEA; Fg-(γ117–133), scrambled ALENADVQNVLKKICQN; and Fg-(γ124–133), LKEKVAQLEA. Peptides were cleaved from the resin and deprotected using crystalline phenol and thioanisole and then purified by high performance liquid chromatography.

**Measurement of Protein Tyrosine Phosphorylation in Raji**—Raji were maintained in serum-free medium for 18 h prior to the commencement of an experiment. Cells were washed twice in Dulbecco's PBS and once in ice-cold modified Dulbecco's medium containing 25 mM Hepes, pH 7.4, and resuspended in an incubation medium of Hanks' balanced salt solution containing 1.0 mM CaCl₂, 1.0 mM MgCl₂, and 25 mM Hepes, pH 7.4, to an approximate cell concentration of 10⁵-10⁶ cells per ml. Aliquots of cells (0.1 ml) were mixed with 0.1 ml of incubation medium alone or containing 200–400 μM amounts of Fg, fibronectin, transferrin, or BSA. Some cells were incubated for 10 min with either 100–500 μM amounts of synthetic peptides ICAM-1-(8–22) or ICAM-1-(130–145) or the

**Reaction of Raji with Fg Induces Increased Tyrosine Phosphorylation within Proteins**—Previous results from our laboratory have identified an increase in cellular proliferation in Raji upon incubation with nanomolar amounts of Fg (5). This 2–3-fold increase in proliferation was shown to be dependent in part on the association of Fg with ICAM-1 on the surface of Raji. As little is known about cell signaling events associated with occupancy of ICAM-1 by ligands we investigated the nature of the Fg-induced signal. Since phosphorylation of tyrosine residues within numerous intracellular proteins is an event key to the conveyance of a signal from the cell membrane to the nucleus, we appraised changes in levels of phosphorylation of intracellular proteins in Raji upon incubation with 200 nM Fg. This concentration of Fg was chosen as it consistently induced cell proliferation in Raji. Raji were incubated at 37 °C with Fg or transferrin (as control) for increasing periods of time. Cells were then rinsed with fresh medium and lysed in TBS containing 1% Nonidet P-40 and protease inhibitors. Lysates were clarified by centrifugation and equivalent amounts of protein from cell lysates were separated on 12.5% acrylamide/SDS gels,
Media Fibrinogen Fn

| kD  | 5    | 5  | 10 | 30  | 30 minutes |
|-----|------|----|----|-----|------------|
| 93  | -    | 93 | -  |     | -          |
| 56  | -    | -  | 56 | -   | -          |
| 43  | -    | -  | -  | 43  | -          |
| 28  | -    | -  | -  | -   | 28 -       |

Fig. 1. Increased tyrosine phosphorylation of intracellular proteins upon incubation of Raji with Fg. Raji were washed and resuspended in IMDM at a concentration of 5 × 10^6 cells per ml. Aliquots of cells (0.1 ml) were mixed with 0.1 ml of incubation medium alone or containing 200 nM Fg or fibronectin and incubated at 37 °C for 5–30 min. Cells were lysed, then equivalent amounts of protein were separated on 12.5% acrylamide/SDS gels, transferred to PVDF membranes, and the membranes were probed with an anti-phosphotyrosine antibody. Bound antibody was detected using alkaline phosphatase-conjugated goat anti-mouse IgG followed by enhanced chemiluminescence.

transferred to PVDF membranes, and probed with an anti-phosphotyrosine antibody. Fig. 1 shows an increase in phosphorylation of several proteins in lysates prepared from cells that were treated with 200 nM Fg when compared with control incubations containing fibronectin or media alone. The major proteins in which a specific increase in phosphorylation was observed were of approximate molecular masses 32, 40, and 56–65 kDa. The phosphorylation of these proteins was rapid and sustained up to 30 min. The 32-kDa protein became phosphorylated by 5 min and was dephosphorylated by 30 min. The 40-kDa protein and proteins migrating at 56–65 kDa became phosphorylated at 10 min and were sustained at the 30-min time point (Fig. 1, lane 4).

Increased Phosphorylation of ERK-1 and pp60src Induced by Fg—In order to identify specific proteins that demonstrated increased tyrosine phosphorylation as a result of treatment with Fg, Raji lysates were subjected to immunoprecipitation using antibodies specific for candidate proteins known to be phosphorylated during cellular proliferation events. Intracellular proteins ERK-1 and pp60src were isolated from cell lysates using monoclonal antibodies. The resultant immune complexes were captured on protein G-Sepharose beads. Proteins were eluted from the beads in SDS sample buffer then separated on SDS-acrylamide gels, transferred onto PVDF membranes. Membranes were probed with an anti-phosphotyrosine antibody. Fig. 2 shows an increased tyrosine phosphorylation of pp60src (panel A) and ERK-1 (panel B) in samples isolated from cell preparations that had been treated with 200 nM Fg but not control proteins. The lower portion of each panel demonstrate that equivalent amounts of each protein were isolated from the cell lysates. Under the assay conditions, we observed a marked increase in the phosphorylation of pp60src when cells were incubated in the presence of Fg compared with cells incubated in media lacking Fg (Fig. 2A, upper panel). The extent of ERK-1 phosphorylation was modest when Raji were incubated in the presence of Fg (Fig. 2B). The extent of phosphorylation in these two proteins could be a reflection of their relative abundance in Raji. More importantly, the inclusion of 20 μM PD98059 (a cell permeable inhibitor of MEK-1, the enzyme responsible for phosphorylating ERK-1) to Raji cultures blocked the increased phosphorylation of ERK-1 induced by 200 nM Fg. This result validates the specificity of ERK-1 activation in cells incubated with Fg.

The increased ERK-1 activity in cultures treated with Fg was further confirmed using a kinase assay. Samples of ERK-1 that had been purified from control or Fg-treated Raji lysates by immunoprecipitation were measured in an assay using [γ-32P]ATP as a phosphate donor and myelin basic protein as a kinase substrate. Assay tubes were incubated at 30 °C for 20 min before the addition of 3 × SDS gel loading buffer. Tubes were boiled and the contents were separated on 15% acrylamide/SDS gels and levels of incorporation of 32P were estimated by autoradiography and densitometry. Fig. 2 (panel C) illustrates an increase in levels of 32P associated with material corresponding to the molecular mass of myelin basic protein (18 kDa) in samples isolated from Fg-treated Raji lysates. These results indicate that phosphorylation of ERK-1 renders it enzymatically active in a manner that is dependent on the concentration of Fg. Densitometric measurements of the 32P-labeled bands revealed a 2.29-fold increase in incorporation of radioactivity into myelin basic protein by ERK-1 isolated from cells that had been treated with 20 μg/ml Fg, as compared with control cells treated with 20 μg/ml BSA. This fold increase in ERK-1 activity (Fig. 2C) parallels the extent of phosphorylation observed in Fig. 2B. An increased phosphorylation of tyrosine residues within both of these proteins is known to correspond to increased kinase activity of these proteins and correlates with known roles for these proteins in intracellular signaling cascades resulting in increased cellular proliferation.

Specific Inhibitors of Intracellular Kinases Block Proliferation of Raji induced by Fg—Geldanamycin (25, 26) and herbimycin A (27) are inhibitors of pp60src, PD98059 (20), a specific inhibitor of the upstream regulator of ERK-1 (MEK-1), has been previously used to block cell proliferation mediated through the MAP kinase pathway (17, 28). The above reagents were used to assess the role of these kinases in Fg-induced Raji proliferation. Fig. 3 shows that the inclusion of 62 nM geldanamycin (panel A), 2.5 μM herbimycin A (panel B), or 25 μM PD98059 (panel C) resulted in greater than 50% blockade of proliferation of Raji induced by 200 nM Fg. Higher concentrations of geldanamycin (1.0 μM), herbimycin A (>5.0 μM), or PD98059 (100 μM) completely abrogated the proliferation in Raji. Equivalent amounts of vehicle (dimethyl sulfoxide) alone did not influence the proliferation. These results indicate that the induction of proliferation in Raji generated by Fg requires the activation of the Src family and MAP kinase family of proteins, in a manner reminiscent of proliferation induced by growth hormones receptors.

A Peptide Corresponding to ICAM-1 Fragment-(8–22) Blocks Fg-induced Phosphorylation of ERK-1—The region ICAM-1-(8–22) has been shown to mediate Fg-binding (6) and block Fg-induced proliferation in Raji (5). To confirm a requirement for occupancy of ICAM-1 by Fg to generate increased phosphorylation of ERK-1, Fg was preincubated with peptides corresponding to ICAM-1 amino acid fragment-(8–22) or fragment-(130–145). Raji were incubated with 200 nM Fg/peptide mixtures at 37 °C for 10 min then cells were rinsed, lysed, and ERK-1 was isolated by immunoprecipitation as described. Immune complexes were eluted from protein G beads and separated on 12.5% acrylamide/SDS gels, transferred to PVDF membranes, and probed with an anti-phosphotyrosine antibody. Fig. 4 demonstrates that inclusion of 50 μM amounts of ICAM-1-(8–22) but not ICAM-1-(130–145) caused a reduction of the increased phosphorylation of ERK-1 in Raji, induced by 200 nM Fg. An analysis of ICAM-1-(8–22) and ICAM-1-(130–145) indicates that these two peptides are structurally comparable (5). Densitometric scanning of the upper panel of Fig. 4...
indicated a 26% reduction in the band intensity in incubations containing ICAM-1-(8–22) after correction for gel loading, compared with ERK-1 isolated from incubations treated with Fg alone. Inclusion of either ICAM-1-(8–22) or ICAM-1-(130–145) alone in incubations did not influence phosphorylation of ERK-1 (data not shown). In our proliferation assays we observe that 50–100 μM ICAM-1-(8–22) causes up to 60% reduction in Fg-induced proliferation of Raji (5). This indicates that association of Fg with ICAM-1 via the region spanning amino acids 8–22 is important for the generation of intracellular signals and increased tyrosine phosphorylation observed in ERK-1 upon treatment of Raji with Fg.

A Region of Fg Induces Increased Activity in ERK-1—A discrete region of Fg known to bind to ICAM-1 (7) is able to induce a proliferative response in Raji (5). We investigated whether this portion of Fg encompassing amino acids 117–133 of the γ chain could induce increased phosphorylation of ERK-1 in Raji. Raji were incubated with 100 μM amounts of either Fg peptides γ-(117–133) or γ-(117–133 scrambled) for 10 min at 37 °C, then cells were washed and lysed as described under “Materials and Methods.” ERK-1 was immunoprecipitated from equivalent amounts of protein from each lysate, and levels of tyrosine phosphorylation were analyzed by immunoblotting as described under “Materials and Methods.” Fig. 5 shows increased tyrosine phosphorylation of ERK-1 extracted from cell preparations treated with 100 μM amounts of Fg-γ-(117–133) but not Fg-γ-(117–133 scrambled), suggesting the interaction of this discrete region of Fg with ICAM-1 was sufficient to increase activity of ERK-1 in Raji. This result is in good agreement with previous data which demonstrated an increased cell proliferation in Raji that were incubated with 100 μM amounts of Fg-γ-(117–133) (5). Raji binding studies using soluble 125I-labeled Fg-γ-(117–133) produce a binding curve which suggests binding of Fg-γ-(117–133) to a single class of proteins on Raji (data not shown). Comparison of the primary structure of this peptide with amino acid sequences of other known peptide hormones reveal no homology. It remains to be determined whether the tertiary structure of this peptide resembles the structure of other peptides known to have a role in cell proliferation.

DISCUSSION

We have previously reported that Fg binding to ICAM-1 expressing B-lymphoid Raji cells results in the proliferation of...
these cells (5). In the present report, our results indicate that ligation of ICAM-1 by Fg, a dimeric molecule, induces the rapid activation of pp60Src and ERK-1. These kinases become specifically tyrosine phosphorylated within Raji in the presence of soluble 200 nM Fg. The immediate upstream regulator of ERK-1 is MAP kinase also known as MEK-1. A specific inhibitor of MEK-1, PD98059, blocked Fg induced cell proliferation in Raji and blocked the ERK-1 activation in Raji lysates (Figs. 2B and 3C). At similar concentrations PD98059 blocked growth activity induced by platelet-derived growth factor and epidermal growth factor (17). Concentrations of galanamycin and herbimycin A similar to those used in other studies to specifically inhibit pp60Src (25, 29, 30) were sufficient to block Raji proliferation. In our previous report, Fg recognition peptide ICAM-1-(8–22) blocked mitogenesis, while the ICAM-1 recognition peptide Fg-γ-(117–133) induced proliferation of Raji (5). In concordance with the above results, 50 μM ICAM-1-(8–22) blocked the hyperphosphorylation of ERK-1 induced through the interaction of Fg with Raji (Fig. 4). Fg-γ-(117–133) on the other hand through its interaction with ICAM-1 induced ERK-1 activation. The peptide results are an independent verification and extend the findings that suggest that the activation of the MAP kinase pathway is involved in B-cell proliferation induced through Fg interaction with ICAM-1. Therefore, Fg and its derivative Fg-γ-peptide-(117–133), utilize and transduce growth signal similar to that of bona fide growth factors (platelet-derived growth factor and epidermal growth factor), phorbol esters, and cytokines. In this context it is tempting to speculate that Fg binding to ICAM-1 may cause ICAM-1 dimerization or clustering (31), as is the case with platelet-derived growth factor and epidermal growth factor receptor upon ligand interaction. Nevertheless, this is the first report of Fg-induced ICAM-1 signaling, and more interestingly through a small discrete region Fg-γ-(117–133) that ligates ICAM-1.

The structure of ICAM-1 lacks the common tyrosine-containing motif required for the recognition of Src family kinases that has been identified in other receptors (14, 15). However, Lyn has been demonstrated to bind IL-3 and IL-5 receptors that lack the consensus Src family binding motifs (32). It is likely that ICAM-1 may transduce signals by association with adaptor proteins such as Grb 2, SOS, and Shc; some of these proteins are known to participate in signaling through integrins (33). Nevertheless, recently the Src-related protein tyrosine kinase Lyn, has been shown to be phosphorylated upon ICAM-1 ligation with monoclonal antibodies in murine B-cells (11). However, in this report a role for Lyn in mitogenesis was not investigated. Interestingly, in the rat brain endothelial cell line (RBE4), ligation of ICAM-1 with syngeneic encephalitogenic T cells induces tyrosine phosphorylation of pp60Src and its substrate cortactin, an 85-kDa actin-binding protein (12). Such cellular and cytoskeletal changes may facilitate the transmigration of lymphocytes in the brain. The physical association of Lyn is also highlighted (34). The interaction of Fg with integrin αMβ2 on monocytes cells augments the ability of these cells to adhere and transmigrate through the EC (2, 35).

In this context, future experiments will verify whether pp60Src and cortactin become phosphorylated, in contrast to ERK-1, in a distinct ICAM-1 ligation process.

There is now overwhelming evidence that the tyrosine phosphorylation of ERK-1 is involved in cell proliferation. However, recent reports now indicate that ERK-1/2 could also act as negative regulators of cell activation, in particular the integrin adhesion receptors (36, 37). ERK is also implicated in smooth muscle cell contraction (38). In a recent report (4), Fg induced contraction of saphenous vein endothelium in an ICAM-1-dependent manner. If the activation of ERK could be demonstrated in these cells through Fg-ICAM-1 interaction, the above findings may have a mechanistic explanation.

Fibrin and derivatives of Fg have been implicated in the
mitogenesis of endothelial cells (39, 40). Our results have now implicated intact Fg and a peptide within the \( \gamma \) chain of Fg (Fg-\( \gamma \)-(117–133)) in the activation of a classical MAP kinase pathway in proliferation of B-lymphoid cells through an adhesive receptor, ICAM-1. The Fg-ICAM-1 pathway has been proposed to play a role in inflammatory processes and in malignant cell growth (2, 5). Since compounds such as PD98059, herbimycin A, and geldanamycin, block proliferative signals and cell growth through Fg-ICAM-1 interaction, these reagents may help understand vascular diseases such as restenosis after angioplasty (17).

Acknowledgments—We thank Vicky Byers-Ward for expert technical assistance, Dr. Edward F. Plow for helpful discussions of the work, James Lang for help with digital imaging, and Jane Rein for assistance in preparation of the manuscript.

REFERENCES
1. Springer, T. A. (1990) Nature 346, 425–434
2. Languino, L. R., Plescia, J., Duperray, A., Brian, A. A., Plow, E. F., Gelotsky, J. E., and Altieri, D. C. (1995) Cell 73, 1423–1434
3. Altieri, D. C., Plescia, J., and Plow, E. F. (1993) J. Biol. Chem. 268, 1847–1853
4. Hicks, R. C. J., Golledge, J., Mir-Hasseine, R., and Powell, J. T. (1996) Nature 379, 818–820
5. Gardiner, E. E., and D'Souza, S. E. (1997) J. Biol. Chem. 272, 15474–15480
6. D'Souza, S. E., Byers-Ward, V. J., Gardiner, E. E., Wang, H., and Sung, S.-S. (1990) J. Biol. Chem. 271, 24270–24277
7. Altieri, D. C., Duperray, A., Plescia, J., Thornton, G. B., and Languino, L. R. (1995) J. Biol. Chem. 270, 696–699
8. Rothlein, R., Kishimoto, T. K., and Mainolfi, E. (1994) J. Immunol. 152, 2488–2495
9. Poudrier, J., and Owens, T. (1994) J. Exp. Med. 179, 1417–1427
10. van Horssen, M., Loman, S., Rijkers, G. T., Boom, S. E., and Bloem, A. C. (1995) Eur. J. Immunol. 25, 154–158
11. Holland, J., and Owens, T. (1997) J. Biol. Chem. 272, 9108–9112
12. Dierie-Truittmann, O., Chaverot, N., Cazaubon, S., Strosberg, A. D., and Couraud, P.-O. (1994) J. Biol. Chem. 269, 12536–12540
13. Chirathaworn, C., Tibbetts, S. A., Chan, M. A., and Benedict, S. H. (1995) Eur. J. Immunol. 25, 154–158
14. Cowley, S., Paterson, H., Kemp, P., and Marshall, C. J. (1994) Cell 77, 841–852
15. Leevers, S. J., Paterson, H. F., and Marshall, C. J. (1994) Nature 369, 411–414
16. Marshall, C. J. (1995) Cell 80, 179–185
17. Bornfeldt, K. E., Campbell, J. S., Koyama, H., Argast, G. M., Leslie, C. C., Raines, E. W., Krebs, E. G., and Ross, R. (1997) J. Clin. Invest. 100, 875–885
18. Xu, Q., Liu, Y., Gerospe, M., Udelsman, R., and Holbrook, N. J. (1996) J. Clin. Invest. 97, 508–514
19. Vuento, M., and Vahery, A. (1979) Biochem. J. 183, 331–337
20. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, A. R. (1995) Nature 379, 425–434

FIG. 4. ICAM-1-(8–22) blocks Fg-induced tyrosine phosphorylation of ERK-1 in Raji. Aliquots of 200 nM Fg were incubated at 37 °C with 50 μM amounts of peptides with sequences corresponding to ICAM-1-(8–22) or ICAM-1-(130–145) for 1 h. Fg/peptide mixtures were then incubated with 10^5 Raji at 37 °C for 10 min then cells were lysed and ERK-1 protein was isolated from cell lysates containing equivalent amounts of protein. Levels of tyrosine phosphorylation in immunoprecipitated ERK-1 was estimated by immunoblotting using an anti-phosphotyrosine antibody (upper panel) and measurement of relative band densities are indicated. Equivalent amounts of ERK-1 were confirmed by stripping immunoblots and reprobing with a rabbit anti-ERK-1 antibody (lower panel).

FIG. 5. A peptide from within the \( \gamma \) chain of Fg induces increased tyrosine phosphorylation of ERK-1. 5 x 10^5 Raji were incubated at 37 °C in IMDM containing either 200 nM Fg or 100 μM amounts of Fg peptides \( \gamma -(117–133) \) or \( \gamma -(117–133) \) scrambled for 10 min. Cells were lysed and a monoclonal antibody against ERK-1 was used to purify ERK-1 from lysates containing equivalent amounts of protein. Immunocomplexes were captured using protein G-Sepharose, washed, and eluted with 3 x SDS buffer then proteins were separated on SDS gels, transferred to PVDF membranes, and probed using an anti-phosphotyrosine antibody (upper panel). Bound antibodies were visualized using alkaline phosphatase-conjugated secondary antibody and enhanced chemiluminescence. Membranes were then stripped and reprobed with a rabbit anti-ERK-1 antibody to determine equal loading of ERK-1 (lower panel). Relative band densities are indicated.
Occupancy of ICAM-1 by Fibrinogen Activates ERK-1