ERK 1/2- and JNKs-dependent Synthesis of Interleukins 6 and 8 by Fibroblast-like Synoviocytes Stimulated with Protein I/II, a Modulin from Oral Streptococci, Requires Focal Adhesion Kinase*

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Protein I/II, a pathogen-associated molecular pattern from oral streptococci, is a potent inducer of interleukin-6 (IL-6) and IL-8 synthesis and release from fibroblast-like synoviocytes (FLSs), cells that are critically involved in joint inflammation. This synthesis implicates ERK 1/2 and JNKs as well as AP-1-binding activity and nuclear translocation of NF-κB. The mechanisms by which protein I/II activates MAPKs remain, however, elusive. Because focal adhesion kinase (FAK) was proposed to play a role in signaling to MAPKs, we examined its ability to contribute to the MAPKs-dependent synthesis of IL-6 and IL-8 in response to protein I/II. We used FAK+/− fibroblasts as well as FAK+/- fibroblasts and FLSs transfected with FRNK, a dominant negative form of FAK. The results demonstrate that IL-6 and IL-8 release in response to protein I/II was strongly inhibited in both protein I/II-stimulated FAK+/− and FRNK-transfected cells. Cytochalasin D, which inhibits protein I/II-induced phosphorylation of FAK (Tyr-397), had no effect either on activation of ERK 1/2 and JNKs or on IL-6 and IL-8 release. Taken together, these results indicate that IL-6 and IL-8 release by protein I/II-activated FLSs is regulated by FAK independently of Tyr-397 phosphorylation.

Integrins are a family of heterodimeric transmembrane proteins that bind a variety of ligands, including proteins of the extracellular matrix, intercellular adhesion molecules, plasma proteins, and complement factors (1). Furthermore, integrins act as receptors for many pathogenic bacteria or viruses and are presently referred to as pattern recognition receptors as they recognize molecular structures called pathogen-associated molecular patterns (PAMPs) in pathogens. For example, α9β1 integrin is the known receptor of several bacterial PAMPs such as invasin of Yersinia pseudotuberculosis (2), fimbrillin A of Porphyromonas gingivalis (3), ipa proteins of Shigella spp. (4), filamentous hemagglutinin of Bordetella pertussis (5), and protein I/II of Streptococcus mutans (6). In addition, several other observations have demonstrated that integrin recognition of fibronectin promotes adhesion and internalization of bacteria expressing fibronectin-binding proteins, for example, protein M1 of Streptococcus pyogenes (7) or fibronectin-binding protein A of Staphylococcus aureus (8).

Because integrins are known to control cellular processes as diverse as proliferation, differentiation, apoptosis, and cell migration, it is likely that their interactions with pathogens will have an important impact on host cell responses as well as on microbial pathogenesis. There are many outside-in signaling pathways that have been identified downstream from integrins, notably, the MAPKs pathway, which converts extracellular stimuli to intracellular signals and which is central to many cellular functions. MAPKs belong to one of the major pathways transmitting signals to early genes implicated in the regulation of cytokine responses. Numerous data demonstrate that pro-inflammatory cytokine synthesis in response to bacteria or bacterial components (e.g. lipopolysaccharide, polysides, lipoteichoic acids, and proteins), after binding to their cognate receptors on different eukaryotic cells, is controlled by the MAPKs pathway and that this synthesis may play an important role in innate immunity as well as in various inflammatory disorders (9, 10). Using protein I/II, a PAMP from oral streptococci, we reported previously that interaction of this cell wall component with fibroblast-like synoviocytes (FLSs), cells that are critically involved in rheumatoid arthritis-associated joint inflammation, triggers the production and release of inflammatory mediators such as IL-6 and IL-8 (11, 12). This cytokine synthesis involves ERK 1/2 and JNKs as well as AP-1 binding and nuclear translocation of NF-κB (13).

However, the mechanisms by which integrins initiate the MAPKs pathway are generally not fully understood. There is increasing evidence that FAK is critical in linking integrins to this pathway insofar as FAK, which colocalizes with integrins in focal adhesions, is associated with different signaling, adaptor, or structural proteins, including Src family protein-tyrosine kinases, phospholipase C-γ, PI 3-kinase, p130Cas, Shc, Grb2, and paxillin. Several mechanisms can be used by FAK to...
activate ERK 1/2. For example, autophosphorylation of FAK at Tyr-397 generates a binding site for Src family protein-tyrosine kinases (14), and Src-mediated phosphorylation of FAK Tyr-925 allows the binding of the SH2 domain of Grb2 and the formation of a Grb2/Sos complex, which activates the Ras/MAPKs cascade. In addition, interaction with Src leads to the phosphorylation of FAK Tyr-576 and Tyr-777 and full kinase activity. The Ras/MAPKs pathway can also be activated by recruitment and phosphorylation of p130Cas, which promotes the binding of the adaptor proteins Crk, Nck, and Sos1 (15–19).

Many integrins use more than one mechanism to activate the ERK pathway, and some of them seem to be independent of FAK and cell-specific. One group has provided evidence that caveolin-1 and the adaptor protein Shc play a role in relaying signals from integrins to ERK in primary cells, but FAK-Src complexes might control the temporal response of ERK initiated by Shc in B-Raf-expressing cells (20–22). In addition, FAK, independently of tyrosine phosphorylation and kinase activity, was proposed to regulate integrin-dependent activation of JNKs by a mechanism involving paxillin and the small GTP-binding proteins of the Rho family (23). Another linkage was suggested, occurring through association of FAK with Src and p130Cas and the recruitment of Crk and Dock 180 (24). Recent findings have also demonstrated that FAK coordinates MAPKs signaling, following costimulation of integrins and growth factors receptors for EGF or platelet-derived growth factor, through interactions mediated by FAK-C-terminal and -N-terminal domain connections to the respective transmembrane receptors (25, 26).

Based on these observations, it can be hypothesized that FAK may participate in various biochemical routes linking integrins to MAPKs cascades. This work was thus undertaken to examine the ability of FAK to contribute to signaling events leading to ERK 1/2- and JNKs-dependent synthesis of pro-inflammatory cytokines, in response to protein I/II. Our results indicate that FAK is critical for IL-6 and IL-8 release by protein I/II-activated FLSs but that Tyr-397, the major site of autophosphorylation that promotes the assembly of a number of signaling complexes, is not essential for this process.

EXPERIMENTAL PROCEDURES

Material—Cell culture media (RPMI 1640 and M199), fetal calf serum (FCS), penicillin, streptomycin, amphotericin B, Tøg DNA polymerase, dNTPs, Moloney murine leukemia virus, RNase inhibitor, IL-6, and β-actin primers were from Invitrogen (Cergy-Pontoise, France). Cell culture media had an endotoxin content that never exceeded 0.04 ng/ml, as tested by the Limulus chromogenic assay. Polynyxin B, Tri reagent, type XI collagenase, woundsman, cytochalasin B, and Genetin were obtained from Sigma (Saint-Quentin-Fallavier, France). Protease inhibitor mixture was from Roche Applied Science (Meylan, France). Hexanucleotide mix was from Roche Applied Science (Mannheim, Germany). The enzyme immunoassay kits for human IL-8 (Mannheim, Germany). The enzyme immunoassay kits for human IL-8 (Mannheim, Germany). The enzyme immunoassay kits for human IL-8 (Mannheim, Germany). The enzyme immunoassay kits for human IL-8 (Mannheim, Germany). The enzyme immunoassay kits for human IL-8.

Cell Culture and Transfections—Human FLSs were isolated from RA synovial tissues from three different patients, at the time of knee joint arthroscopic synovec-omy as described previously (28). The diagnoses conformed to the revised criteria of the American College of Rheumatology (29). Briefly, tissues were minced, digested with 1 mg/ml collagenase (in serum-free RPMI 1640 for 3 h at 37 °C, centrifuged 1,500 × g, 10 min, 4 °C), and resuspended in M199-RPMI 1640 (1:1) containing 2 mM l-glutamine, penicillin (100 IU/ml), streptomycin (100 μg/ml), amphotericin B (0.25 μg/ml), and 20% heat-inactivated FCS (complete medium). After overnight culture, non-adherent cells were removed, and adherent cells were cultured in complete medium. At confluence, cells were trypsinized and precipitated in 75-cm2 flasks in complete medium containing 10% heat-inactivated FCS. Between the third and the tenth passages, during which time cultures were a homogeneous population of fibroblastic cells, negative for CD16 as determined by fluorescence-activated cell sorting analysis, cells (5 × 10^5 cells per well) were grown to confluence in 96-well plates (10–10 days). Cells were deprived of serum for 24 h, before addition of the appropriate stimuli, and diluted in serum-free RPMI 1640 with antibiotics. Cell number and cell viability were examined by the MTT test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test) as described elsewhere (30).

FAK+/+ and FAK−/− primary mouse embryo fibroblasts were a generous gift from Dr. Dusko Ilic (Department of Medicine, University of California, San Francisco). Cells were cultured in Dulbecco’s modified Eagle’s medium containing penicillin (100 IU/ml), streptomycin (100 μg/ml), amphotericin B (0.25 μg/ml), β-mercaptoethanol (0.1 mM), non-essential amino acids (1%), sodium pyruvate (1%), and 10% heat-inactivated FCS. FAK+/+ cells were transfected with FRNK-YCam (27) by the calcium phosphate method. Briefly, 15 μg of plasmidic DNA in 1 ml of BES-buffered saline (borate-buffered saline, pH 6.95, containing 2.5 mM of CaCl_2) was added to 5 × 10^5 cells for 2 h at 37 °C (3% CO_2). Following transfection, cells were rinsed and then cultured in complete Dulbecco’s modified Eagle’s medium containing Geneticin (1.5 mg/ml) for 2 weeks. The antibiotic-resistant cells were then pooled and used for further analysis. Green fluorescent protein (GFP) was used to determine transfection efficiency. Transient transfection of FLSs was performed using the Nucleofector™ kit. 2 μg of plasmidic DNA was added to 5 × 10^4 FLSs suspended in 100 μl of human dermal fibroblast Nucleofector™ solution. The program U-23 was selected for a high density of transfection according to the manufacturer’s instructions. Cells were then plated in 96-well plates (5 × 10^4 cells per well) and serum-starved for 24 h before activation experiments.

Purification of Protein I/II—Recombinant protein I/II of S. mutans OMZ 175 was purified from pHbar-1-transformed Escherichia coli cell extract by gel filtration and immunoaffinity chromatography as previously described (31). The purity of the protein was checked by SDS-PAGE after staining with Coomassie Blue. Protein I/II migrated as a single band having an apparent molecular mass of 195 kDa.

Activation of Cells—FLSs were preincubated with 100 μl of various inhibitors diluted in serum-free RPMI 1640 with antibiotics: for 40 min at 37 °C with cytochalasin D (0.5, 1 and 2 μM), for 1 h at 37 °C with anti-integrin β1 chain mAbs (5, 20, and 40 μg/ml), with wortmannin (50, 100, and 200 μM), and then incubated with 100 μl of serum-free RPMI 1640 containing protein I/II (125 pt final concentration). Protein I/II (125 pt, 200 μl) was also preincubated for 2 h at 4 °C with purified αβ2 integrins (1, 5, and 10 μg/ml) and then used to stimulate FLSs. After a 24 h incubation period, culture supernatants were harvested and used to estimate IL-6 and IL-8 release by a heterologous two-site sandwich ELISA as previously described (32). To confirm that the observed effects were due to possible lipopolysaccharide contamination, the experiments were performed in presence of polymyxin B (2 μg/ml).

Detection of IL-6-mRNA—Total RNA was extracted from 10^6 FAK+/+ or FAK−/− cells activated with 125 pM protein I/II for 1 h, using 1 ml of Tri reagent and reverse-transcribed for 45 min at 42 °C. 100 ng of total RNA was mixed with 5 μl of Moloney murine leukemia virus, 10 units of M-MLV reverse transcriptase, 10 μl of random hexameric primers, and 50 μl of heptadecane as substrate in a 50 μl reaction volume. The PCR fragments were then separated on 1.5% agarose gels and visualized with ethidium bromide. The specific primers for IL-6 and β-actin were selected based on published mouse IL-6 and β-actin sequences.
actin cDNA sequences. The oligonucleotide primers used were for IL-6: 5'-TCCTCTCTGCGAAGACT-3' and 5'-GAGGACTCTCTTATGT-3', and for β-actin: 5'-ATGGATGACGATATCGCT-3' and 5'-GAGGACTCTCTTATGT-3'.

Western Blot Detection of Tyrosine Phosphorylation—106 cells were incubated for various times in 100 μl of serum-free RPMI 1640 supplemented with antibiotics with or without 125 μM of protein I/II, in the presence or absence of cytochalasin D (1 μM). After incubation, cells were centrifuged (130 × g for 10 min at 4 °C), and the pellets were suspended for 20 min in 100 μl of ice-cold lysis buffer (1% Triton X-100, 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1 mM sodium orthovanadate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors) (33). The Triton X-100-soluble proteins were separated by centrifugation (14,000 × g for 10 min at 4 °C), and the supernatant was subjected to SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. Membranes were blocked using 1% bovine serum albumin in TBS (20 mM Tris, pH 7.5, 150 mM NaCl) for 1 h at 25 °C. The blots were then incubated with various antibodies: anti-FAK (pY397), anti-Shc (pY317), anti-active ERK 1/2, and anti-active JNKs in TBS-Tween (0.1% Tween 20) for 2 h at 25 °C, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG polyclonal antibodies (1 h at 25 °C) and detected by enhanced chemiluminescence according to the manufacturer's instructions. To confirm the presence of equal amounts of FAK, ERK 1/2, JNK, and Shc proteins, bound antibodies were removed from the membrane by incubation in 62.5 mM Tris, pH 6.7, 100 mM β-mercaptoethanol, 2% SDS for 30 min at 50 °C and probed again with either anti-FAK, anti-ERK 1/2, anti-JNKs, or anti-Shc polyclonal antibodies.

FAK Tyrosine Kinase Assay—4 × 106 cells were incubated for 15 min in serum-free RPMI 1640 supplemented with antibiotics with or without 125 μM of protein I/II, in the presence or absence of cytochalasin D (1 μM). Cells activated for 15 min with fibronectin (10 μg/ml) were used as control. Cells were lysed as described above, and FAK was immunoprecipitated with anti-FAK polyclonal antibodies. Protein A-Sepharose CL-4B (50%, 100 μl) was then added for 90 min at 4 °C. After centrifugation (10,000 × g, 1 min, 4 °C), the pellet was used for a non-radioactive tyrosine kinase assay according to the manufacturer's instructions. FAK kinase activity was assessed using protein-tyrosine kinase standards included in the assay kit.

RESULTS

Exposure of FLSs to Protein I/II Causes Phosphorylation of FAK—Because previous observations from our laboratory indicated that, in endothelial cells, protein I/II interactions with α1β1 integrins increased the tyrosine phosphorylation of FAK and lead to IL-8 secretion (6), we first investigated the role of α1β1 integrins in the FLSs activation process. The results show that addition of either increasing amounts of purified α1β1 integrins or anti-integrin β1 chain antibodies induced inhibition of protein I/II-stimulated IL-6 and IL-8 release (Fig. 1). An inhibition of cytokine release of about 80% was obtained with 10 μg/ml α1β1 integrins and 45% with 40 μg/ml anti-integrin β1 chain antibodies (p < 0.05). As observed in endothelial cells, this suggests that α1β1 integrins are implicated in the activation process leading to IL-6 and IL-8 release from protein I/II-activated FLSs.

We next examined the capacity of protein I/II to stimulate phosphorylation of FAK in FLSs. Cell lysates were analyzed directly by blotting with specific anti-FAK (pY397) antibodies. Stimulation of FLSs with protein I/II for various times (1, 5, 15, 30, and 60 min) resulted in an increased amount of phosphorylated FAK (Fig. 2), which was detectable within 5 min and remained elevated for at least 30 min. Fibronectin was used as a positive control. These results demonstrate that interaction of protein I/II with FLSs induces phosphorylation of FAK at Tyr-397.

Protein I/II-induced Signaling to MAPKs and IL-6 and IL-8 Release Occurs via an FAK-dependent Pathway—Recently, Neff et al. (13) studied the eventual role of bacterial components such as protein I/II in promoting joint inflammation and reported that NF-κB and the MAPKs/AP-1 pathways are both involved in IL-6 and IL-8 release from FLSs stimulated with protein I/II. Thus, we next asked whether FAK could participate in the signaling events leading to IL-6- and IL-8 release from activated cells via the MAPKs pathway. In one set of studies and as preliminary experiments, we used FAK−/+ and FAK−/− primary mouse embryo fibroblasts. FAK−/+ and FAK−/− cells were incubated with protein I/II (125 μM) for 30 min, and then immunoblotting experiments were performed.
As shown in Fig. 3A, stimulation of FAK+/+ fibroblasts with protein I/II increased FAK as well as ERK 1/2 tyrosine phosphorylation, however, protein I/II failed to stimulate tyrosine phosphorylation of ERK 1/2 in FAK−/− cells. These results raise the possibility that FAK participates in MAPKs activation in protein I/II-stimulated cells. We thus explored the cytokine response of FAK−/− fibroblasts stimulated with protein I/II (125 pM) for 20 h at 37 °C before lysis. Cells lysates were then analyzed by Western blotting with phosphospecific antibodies to FAK (pFAKY397) or ERK 1/2 (pERK 1/2). Control cells were incubated for the same time without protein I/II. The same membranes were stripped and reprobed with antibodies to total FAK or ERK 1/2. The results shown are representative of three identical experiments.

To further demonstrate the requirement of FAK in cytokine release from protein I/II-activated fibroblasts, the cytokine response of FLSs transiently transfected with FRNK was examined. FLSs transfected with a GFP-expressing vector were used as controls. Wild-type FLSs and transfected FLSs were then incubated with 125 pM protein I/II for 20 h at 37 °C. As seen in Fig. 4, overexpression of FRNK inhibited significantly IL-6 and IL-8 release from protein I/II-activated FLSs.

In a complementary approach, we have also evaluated the contribution of the adaptor protein Shc to this pathway. It is known that a subset of integrins, including α₅β₁, activates the Ras/ERK pathway by a mechanism implicating the membrane protein caveolin and tyrosine phosphorylation of Shc by the tyrosine kinase Fyn (20). This last event is necessary and sufficient to activate MAPKs as demonstrated by results from dominant negative studies and from mouse embryos deficient in Shc (18). Two tyrosine phosphorylation sites have been identified on Shc: Tyr-239/240, which has been linked to c-Myc activation and Tyr-317, which appears to be critical for MAPKs activation in response to integrins and growth factor receptors. Western blotting analysis using specific anti-Shc (pY317) antibodies showed that two isoforms of Shc, p46 and p52, were constitutively phosphorylated in control FLSs and that protein I/II did not increase the level of Shc phosphorylation (Fig. 5A). EGF was used as a positive control. To further demonstrate...
that Shc is not involved in cytokine synthesis, FLSs were transiently transfected with a dominant negative version of Shc (pRk5-Shc-Y317F), in which the tyrosine residue that is phosphorylated and binds Grb2 is replaced by a phenylalanine. FLSs transfected with a GFP-expressing vector were used to control transfection. Wild-type and transfected cells were then incubated with 125 pM protein I/II for 20 h at 37 °C. Overexpression of a dominant negative version of Shc had no effect on IL-6 and IL-8 release from protein I/II-activated FLSs, as compared with activated wild-type FLSs, indicating that IL-6 and IL-8 release from protein I/II-activated FLSs does not require integrin-mediated Shc (Y317) signaling. Taken together, these results indicate that FAK plays a predominant role in protein I/II-induced IL-6 and IL-8 release from activated FLSs.

**FAK Tyr-397 Phosphorylation Is Not Implicated in Either Signaling to MAPKs or Release of IL-6 and IL-8**—To further study the mechanisms by which FAK is required for protein I/II-induced IL-6 and IL-8 release, we used cytochalasin D, which has been shown to prevent FAK Tyr-397 phosphorylation by disrupting the actin cytoskeleton (35–37). A number of FAK-signaling events are dependent on FAK phosphorylation at Tyr-397. Immunoblotting with anti-FAK (pY397) polyclonal antibodies revealed that 1 µM cytochalasin D, a concentration that neither affects cell viability nor basal phosphorylation levels (data not shown), suppresses protein I/II-induced phosphorylation of FAK at Tyr-397 (Fig. 6A). By contrast, at this concentration, cytochalasin D had no effect on protein I/II-stimulated phosphorylation of ERK 1/2 and JNKs (Fig. 6, B and C). These results indicate that cytochalasin D does not suppress signaling to ERK 1/2 and JNKs in protein I/II-activated FLSs, suggesting that phosphorylation of FAK at Tyr-397 is not required for MAPKs signaling in protein I/II-stimulated FLSs. To confirm that IL-6 and IL-8 release, which is known to be an ERK 1/2- and JNKs-mediated event (13), is not dependent on phosphorylation of FAK at Tyr-397, FLSs were first...
Interestingly, we also found that PI 3-kinase, IL-8 Release—

suggest that this integrin-mediated IL-6 and IL-8 synthesis
Tyr-397 phosphorylation. Furthermore, these observations
protein I/II-activated FLSs proceeds independently of FAK

From these findings, we conclude that IL-6 and IL-8 release by
secretion of IL-6 and IL-8 was not affected by cytochalasin D.

I/II for 20 h at 37 °C. As shown in Fig. 6, pretreatment of cells with 1
μM cytochalasin D, a concentration that totally inhibits FAK
Tyr-397 phosphorylation, did not significantly affect protein
I/II-induced FAK kinase activity (Fig. 8). Based on these
results, we suggest that protein I/II activates FAK by a mecha-
nism that does not involve phosphorylation at Tyr-397.

DISCUSSION

Most of the data published to date suggest that FAK is
implicated in physiological pathways that regulate motility
and possibly also cell growth and/or survival in response
to engagement of integrins and/or stimulation of
growth factor receptors. In addition, FAK has been shown to be
involved in adhesion and internalization of pathogenic bacteria
for example, the invasin-mediated uptake of Y. pseudotuberculosis (38) and the invasion of brain endothelial cells by E. coli
(39). Presently, little is known on the eventual participation of FAK in pro-inflammatory responses induced by PAMPs-pattern
recognition receptors interactions. We previously demonstra-
ted that interaction of protein I/II with αβ2 integrins ac-
vitates the MAPKs cascades and that activation of this
pathway (6) accounts for secretion of IL-6 and IL-8. We thus
close to probe the role of FAK in cytokine synthesis in response
to integrin engagement.

In agreement with our previous results showing that protein
I/II enhanced tyrosine phosphorylation of FAK in endothelial
cells (6), we found that in FLSs, protein I/II caused tyrosine
phosphorylation of FAK at Tyr-397, the major phosphoryla-
tion site of FAK. Moreover, using FAK−/− fibroblasts, we found
that protein I/II did not stimulate either tyrosine phosphoryl-
ation of ERK 1/2 or IL-6 synthesis in these cells. FAK−/−
fibroblasts are considered as an excellent tool to test the role of
FAK despite the fact that they express elevated levels of pro-
ceine- rich tyrosine kinase 2, which may function in a compensa-
tory manner in the absence of FAK (40). The inability of
FAK−/− cells to respond to protein I/II suggests that FAK is
critical to IL-6 synthesis. Moreover, cytokine release is not
rescued by the presence of Pyk 2 in this model. These findings
are fully consistent with studies from several groups showing
that FAK plays a major role in mediating signal to MAPKs and
to downstream targets in response to various stimuli. For ex-
ample, FAK is involved in the MAPKs-dependent production of
NO in chondrocytes stimulated with an N-terminal fragment of
fibronectin (41). In another study, FAK contributes to the sub-
sequent inflammatory response in response to adenovirus type
19 infection of human corneal fibroblasts (42). Finally, in ovari-
narian carcinoma cells, FAK and ERK 1/2 are involved in integrin-
stimulated matrix metalloproteinase-9 secretion (26).

Consistent with experiments using FAK−/− cells, we also
found that overexpression of FRNK, the C-terminal non-cata-
lytic domain of FAK that includes the FAT domain as well as
the p130Cas, CAP, and Graf proteins-SH3 binding domains,
inhibited MAPKs phosphorylation and subsequent cytokine
synthesis in protein I/II-stimulated FAK+/+ cells. This was also
demonstrated in FLSs transiently transfected with FRNK.

### Table 1

| Time (min) | C | 15 | 30 | 0.5 | 1 | 2 | 30 |
|------------|---|----|----|-----|---|---|----|
| Protein I/II (125 pM) |   | +  | +  | +  | + | + |   |
| Cytochalasin D (1 μM) | - | -  | +  | +  | + | + | +  |

**Fig. 6.** Cytochalasin D has no effect on protein I/II-induced FAK, ERK 1/2, and JNKs phosphorylation and IL-6 and IL-8 release. FLSs pretreated (40 min at 37 °C) with cytochalasin D (1 μM) were incubated in medium alone (C) or stimulated with protein I/II (125 pM) for 15 and 30 min. Cell lysates were then analyzed by Western-blotting with phosphospecific antibodies to A, FAK (pFAKY397); B, ERK 1/2 (pERK 1/2); or C, JNKs (pJNKs). The same blot was stripped and reprobed with anti-FAK, -ERK 1/2, or -JNKs antibodies to confirm the presence of equal levels of FAK, ERK 1/2, or JNKs proteins between samples. Results shown are representative of three identical experiments. D, FLSs preincubated (40 min at 37 °C) with 0.5, 1, and 2 μM of cytochalasin D, were treated with protein I/II (125 pM) for 20 h at 37 °C. IL-6 [□], and IL-8 [■] levels in the cell supernatants were determined by a heterologous two-site sandwich ELISA. Results are expressed as concentrations of IL-6 and IL-8. Data are expressed as mean values ± S.D. for triplicate determinations from three identical experiments.

**PI 3-Kinase Is Not Involved in Protein I/II-induced IL-6 and IL-8 Release**—Interestingly, we also found that PI 3-kinase,
FRNK is known to function as a negative regulator of FAK activity. In most cells, overexpression of FRNK inhibits FAK-dependent cell spreading, cell migration, as well as growth factor-mediated signals to MAPKs (43). Previous observations indicated that the FAT domain is the principal region by which FRNK inhibits FAK but the proposal that FRNK displaces FAK from focal adhesion sites or diverts some critical partners from FAK remains to be proven (44). In the experiments reported here, FRNK is unlikely to function by displacing FAK from focal adhesion sites, because actin cytoskeleton disruption induced by cytochalasin D did not suppress signaling to MAPKs and subsequent cytokine synthesis. One hypothesis could be that FRNK might interfere as a competitive inhibitor for a yet unidentified binding partner that regulates FAK activation.

The fact that FAK is critical for cytokine synthesis in this model, is not fully consistent with studies from Barberis et al. (22) who proposed that, in primary fibroblasts, signaling to ERK 1/2 can proceed in the absence of FAK activation. There is some controversy in the literature concerning the connections linking integrins to the activation of MAPKs, and it is by no means obvious that the same mechanisms might take place in all cell types in response to various stimuli. In some primary cells, integrin-mediated activation of ERK 1/2 has been proposed to be dependent on the phosphorylation of the adaptor protein Shc at Tyr-239 or Tyr-317. Shc is an important intermediate of MAPKs activation by integrins. Shc−/− fibroblasts exhibit a decrease of ERK 1/2 activation in response to extracellular matrix proteins (45). However, in our model, even though Shc appears to be slightly phosphorylated in unstimulated FLSs, protein I/II did not increase tyrosine phosphorylation of Shc. Moreover, IL-6 and IL-8 synthesis was not blocked by overexpression of a dominant negative form of Shc, indicating that Shc is not required for protein I/II-dependent cytokine synthesis.

Our initial expectation was that phosphorylation of FAK at Tyr-397 would be implicated in MAPKs activation in response to protein I/II. This residue is essential for the biological and biochemical functions of FAK as a number of FAK-dependent signaling events, involving Src family kinases, the p85 subunit of PI 3-kinase, Shc, Grb7, and phospholipase C, are dependent on FAK phosphorylation at Tyr-397 (18). Here, we demonstrated that cytochalasin D, at a concentration that profoundly suppresses FAK phosphorylation at Tyr-397, does not impair ERK 1/2 and JNKs tyrosine phosphorylation induced by protein I/II. Moreover, preincubation of cells with concentrations of cytochalasin D that inhibit FAK phosphorylation had no effect on IL-6 and IL-8 synthesis, suggesting that FAK phosphorylation at Tyr-397 is not essential for MAPKs-dependent cytokine synthesis in response to protein I/II. In accordance with this hypothesis, it is interesting to note that PI 3-kinase signaling activity, which is regulated by FAK phosphorylation at Tyr-397, is not involved in protein I/II-induced cytokine synthesis.

Cytochalasin D is probably not the most suitable agent to address the role of FAK, because it has been reported previously in NIH 3T3 cells that cytochalasin D, which has no effect on the level of Src, may increase its intrinsic kinase activity and thus could mask the potential contribution of FAK to the activation of MAPKs and subsequent cytokine synthesis (46).
However, this was not found to be the case here, because in control experiments increasing amounts of cytochalasin D failed to induce IL-6 and IL-8 synthesis from unstimulated FLs (data not shown).

Our finding, that FAK phosphorylation at Tyr-397 may not be essential for the signaling pathway induced in response to protein I/II, is consistent with observations from several authors suggesting that the phosphorylation state of Tyr-397 is not always correlated with FAK activity. First, Izaguirre et al. (47) restored α-actinin phosphorylation normally induced by FAK by transfecting a Phe-397 mutant of FAK in cells lacking FAK. Second, Hamawy et al. (48) demonstrated that transfection of a variant (3B6) of the RBL-2H3 mast cell line with the FAK-Y397F mutant restored mast cell secretion. Third, Tachibana et al. (49) showed that a FAK-Y397F mutant was still able to phosphorylate Cas proteins. Moreover, by transfecting cells with a kinase-negative mutant of FAK or a FAK C-terminal domain that does not contain the kinase domain, Tachibana et al. (49) demonstrated that the kinase domain of FAK was required for Cas phosphorylation. Because cytochalasin D, which prevents Tyr-397 phosphorylation, does not inhibit either protein I/II-induced FAK kinase activity or cytokine synthesis, it is tempting to speculate that FAK may function in response to protein I/II, by initiating by itself, integrin-mediated tyrosine phosphorylation of a binding partner yet unidentified. However we cannot exclude that FAK may also function as an adaptor or linker protein in the protein I/II-induced signaling pathway. Given that integrins collaborate with growth factor receptors to signal to MAPKs and that this pathway is cytochalasin D-insensitive, it is also possible that growth factor receptors may function as binding partners in protein I/II-induced activation of FAK and subsequent cytokine synthesis (43). This remains to be proven, and further studies will be now necessary to define more precisely how FAK is activated and which FAK substrates are involved in cytokine synthesis in response to protein I/II→αβ integrin interaction.

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REFERENCES
1. Van der Flier, A., and Sonnenberg, A. (2001) Cell. Microbiol. 3, 157–168
2. Schlerf, P., Al-Okla, S., Chatenay-Rivauday, C., Klein, J. P., and Wachsmann, D. (1999) J. Immunol. 163, 5861–5868
3. Urai, K. K., Mariotti, A., Zurzolo, C., and Giancotti, F. G. (1998) Cell 94, 625–626
4. Sinha, B., Francois, P., Que, Y. A., Hussain, M., Heilmann, C., Moreillon, P., Lew, D., Krause, K. H., Peters, G., and Ferrmann, M. (2000) Infect. Immun. 68, 8671–8678
5. Rawadi, G., Ramez, V., Lemercier, B., and Roman-Roman, S. (1998) J. Immunol. 160, 1330–1339
6. Scherle, P. A., Jones, E. A., Favata, M. F., Daulerio, A. J., Covington, M. B., Schlaepfer, D. D., Hanks, S. K., and Hunter, T. (1998) J. Biol. Chem. 273, 12802–12805
7. Polte, T. R., and Hanks, S. K. (1997) J. Biol. Chem. 272, 5501–5509
8. Ilic, D., Dixas, C., and Yamamoto, T. (1997) J. Cell Sci. 110, 401–407
9. Alhourani, K., Mariotti, A., Zurzolo, C., and Giancotti, F. G. (1998) Cell 94, 625–626
10. Sinha, B., Francois, P., Que, Y. A., Hussain, M., Heilmann, C., Moreillon, P., Lew, D., Krause, K. H., Peters, G., and Ferrmann, M. (2000) Infect. Immun. 68, 8671–8678
11. Rawadi, G., Ramez, V., Lemercier, B., and Roman-Roman, S. (1998) J. Immunol. 160, 1330–1339
12. Scherle, P. A., Jones, E. A., Favata, M. F., Daulerio, A. J., Covington, M. B., Schlaepfer, D. D., Hanks, S. K., and Hunter, T. (1998) J. Biol. Chem. 273, 12802–12805
13. Urai, K. K., Mariotti, A., Zurzolo, C., and Giancotti, F. G. (1998) Cell 94, 625–626
14. Sinha, B., Francois, P., Que, Y. A., Hussain, M., Heilmann, C., Moreillon, P., Lew, D., Krause, K. H., Peters, G., and Ferrmann, M. (2000) Infect. Immun. 68, 8671–8678
15. Rawadi, G., Ramez, V., Lemercier, B., and Roman-Roman, S. (1998) J. Immunol. 160, 1330–1339
16. Schlerf, P., Al-Okla, S., Chatenay-Rivauday, C., Klein, J. P., and Wachsmann, D. (2000) PEMS Immunol. Med. Microbiol. 1290, 1–7
17. Izaguirre, G., Aguirre, L., Hu, Y. P., Lee, H. Y., Schlaepfer, D. D., Aneskievich, P., Lai, K. M., and Pawson, T. (2000) J. Biol. Chem. 275, 36769–36774
18. Schlerf, P. A., Jones, E. A., Favata, M. F., Daulerio, A. J., Covington, M. B., Schlaepfer, D. D., Hanks, S. K., and Hunter, T. (1998) J. Biol. Chem. 273, 12802–12805
19. Al-Okla, S., Chatenay-Rivauday, C., Klein, J. P., and Wachsmann, D. (1999) J. Immunol. 163, 5861–5868
20. Leonardi, U., Gieras, L. A., and Schofield, L. (1996) J. Biol. Chem. 271, 435–478
21. Giancotti, F. G., and Ruoslahti, E. (1999) J. Biol. Chem. 274, 26602–26605
22. Mortier, E., Cornelissen, F., van Hove, C., Dillen, L., and Richardson, A. (2001) Biochim. Biophys. Acta 1540, 1–2
23. Ilic, D., Damsky, C. H., Hunter, D., and Schlaepfer, D. D. (2000) J. Cell Sci. 114, 1601–1609
24. Lock, P., Abram, C., Gibson, T., and Courtneidge, S. (1998) Genes Dev. 12, 1132–1145
25. Calalb, M., Polte, T., and Hanks, S. K. (1995) Mol. Cell. Biol. 15, 945–963
26. Hauck, C. R., Sieg, D. J., Hsia, D. J., Loftus, J. C., Ilic, D., Dixas, C., and Yamamoto, T. (1997) J. Cell Sci. 110, 401–407
27. Giannone, G., Ronde, P., Guire, M., Haiech, J., and Takeda, K. (2002) J. Biol. Chem. 277, 26364–26371
28. Dechanet, J., Taupin, J. L., Chomarat, M. C., Moreau, F. J., Banchereau, J., and Moussac, P. (1994) Eur. J. Immunol. 24, 3222–3228
29. Arnett, F., Edworthy, S. M., Bloch, D. A., McShane, D. J., Fires, J. P., and Cooper, N. S., et al. (1988) Arthritis Rheum. 3, 315–324
30. Mosmann, T. (1983) J. Immunol. Methods 65, 55–61
31. Chatenay-Rivauday, C., Yamodo, I., Sciotto, M., Ogier, J. A., and Klein, J. P. (1998) Mol. Microbiol. 29, 39–48
32. Vernier, A., Diab, M., Soell, M., Haan-Archipoff, G., Beresz, A., Wachsmann, D., and Altruda, F., Tarone, G., and Giancotti, F. G. (1998) J. Biol. Chem. 273, 36532–36540
33. Polte, T. R., and Hanks, S. K. (1997) J. Biol. Chem. 272, 5051–5059
34. Parsons, J. T. (2003) J. Biol. Chem. 278, 435–478
35. Tachibana, K., Urano, T., Fujita, H., Ohashi, Y., Kamiguchi, K., Iwata, S., and Schlaepfer, D. D. (2000) J. Cell Biol. 150, 661–672
36. Morino, M., Nimjura, T., Hamasaki, K., Tobe, K., Ueki, K., Ikkichi, K., Takehara, K., Kadowaki, T., Yasaki, Y., and Nojima, Y. (1995) J. Biol. Chem. 270, 269–272
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