Regulació de la producció de gelatinases (MMP2 i MMP9) pels limfòcits.

Implicació en malalties inflamatòries i síndromes limfoproliferatives

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Marta Segarra Blasco

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Departament de Medicina

Facultat de Medicina

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RESULTATS

Mecanismes de Regulació de la Producció de Gelatinases mitjançant Integrines en Limfòcits T

PRIMER ESTUDI:

Anàlisi de les vies de senyalització mitjançades per integrines implicades en la regulació de l'expressió i l'alliberament de MMPs pels limfòcits. Paper de FAK (*Focal Adhesion Kinase*) en la coordinació d'aquest procés i la seva relació amb la invasió cel·lular.

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Dual function of focal adhesion kinase in regulating integrin-induced MMP-2 and MMP-9 release by human T lymphoid cells

Marta Segarra,* Carme Vilardell,* Kazue Matsumoto,[†] Jordi Esparza,* Ester Lozano,* Carles Serra-Pages,[‡] Alvaro Urbano-Márquez,* Kenneth M. Yamada,[†] and Maria C. Cid*

*Department of Internal Medicine and [‡]Immunology. Hospital Clínic, University of Barcelona, Institut d'Investigacions Biomèdiques August Pi i Sunyer, Barcelona, Spain; and [†]Craniofacial Developmental Biology and Regeneration Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda Maryland

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Correspondence: Maria C Cid, MD. Department of Internal Medicine. Hospital Clínic. Villarroel, 170 08036 Barcelona, Spain. E-mail: mccid@clinic.ub.es

ABSTRACT

Integrin engagement induces matrix metalloproteinase (MMP) production by lymphocytes, allowing their progression into tissues. Focal adhesion kinase (FAK) is a key component of integrin-mediated signaling pathways regulating cell migration. We explored the role of FAK in integrin-induced gelatinase production by Jurkat T cells. Elevation of FAK expression by transient transfection increased cell invasiveness and gelatinase production and release driven by fibronectin. FAK point mutants revealed that gelatinase release was not dependent on FAK kinase activity but did require Y397, a binding site for Src-type tyrosine kinases. Requirement of Src kinases was further demonstrated by transfection with Src kinase-deficient mutants and treatment with a Src inhibitor. Transfection of truncated forms demonstrated dual functional elements in the FAK molecule. The FRNK fragment decreased gelatinase release, whereas the FAT subfragment enhanced it. FRNK inhibitory signals were transduced through Src-dependent pCAS phosphorylation and subsequent ERK1/2 activation. In contrast, FAT stimulated gelatinase secretion, which was also dependent on Src-kinase activity, was associated with a decreased ERK1/2 phosphorylation. This dual function of FAK in gelatinase secretion is then associated with changes in ERK1/2 activation status, a pathway coordinating cycles of adhesion/release required for cell migration and defines a novel regulatory step in the complex control of MMP function.

Key words: matrix metalloproteinases • lymphocytes • fibronectin

Lymphocyte transmigration into tissues is essential for many physiological and pathological conditions, including immune surveillance, graft rejection, lymphoma dissemination, host defense against tumors, and the development of inflammatory infiltrates underlying chronic inflammatory diseases (1–4). Tissue infiltration by lymphocytes requires focal degradation of the basement membrane and interstitial matrix (5, 6). To accomplish this process, lymphocytes produce small amounts of proteases among which matrix metalloproteinases (MMP) have a significant role based on their ability to degrade virtually any component of the extracellular matrix.

Cells from the lymphoid lineage are able to produce several MMPs (7–10). Among them, gelatinases appear to have a major role in lymphocyte transmigration due to their ability to degrade type IV collagen, a major component of basement membranes. In addition to extracellular matrix breakdown, gelatinases and other MMPs have important additional functions by cleaving a wide array of proteins such as adhesion molecules, cytokines, chemokines and growth factors, by exposing functionally active cryptic sites of large extracellular matrix proteins and by allowing access of cells to growth factors sequestered in the surrounding matrix (4, 11–15). This newly expanded range of biological functions may have unexpected impact on the regulation of inflammatory disorders and in early stages of tumor development (16, 17).

The mechanisms regulating gelatinase production and secretion by lymphocytes are largely unknown. Several cytokines, chemokines, and growth factors have been demonstrated to induce pro-MMP9 and to a lesser extent pro-MMP2 in lymphocytes (18–20). However, the most effective mechanism not only in inducing but also in activating both gelatinases appears to be integrin-dependent lymphocyte adhesion (7, 21, 22). Integrin engagement by cell membrane counter receptors (VCAM-1) or by extracellular matrix proteins, particularly fibronectin (FN), can elicit not only gelatinase production but also MMP-2 activation by inducing coordinated expression of MMP-14 (7). Among integrins, $\alpha_4\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$ have been demonstrated to mediate adhesion-dependent gelatinase production and activation by T cells (7, 21, 22).

The signaling pathways involved in integrin-mediated gelatinase production by lymphocytes are unknown. On the basis of the results obtained from studies performed in other cell types, particularly fibroblasts, integrin engagement is followed by the recruitment of adaptor molecules connecting with cytoplasmic kinases and cytoskeletal proteins, leading to the formation of multimolecular complexes named focal adhesions (23, 24). These structures appear to be crucial for mediating the cycles of adhesion/release necessary for cell motility (25). Equivalent structures develop upon lymphocyte adhesion and migration (26).

A crucial molecule in focal contacts is focal adhesion kinase (FAK) (27). FAK and its paralog proline-rich tyrosine kinase-2 (Pyk-2) are expressed in cells of the lymphoid lineage (26). FAK is involved in the regulation of integrin-dependent signals regulating cell motility and survival (28–30). FAK has been shown to promote an aggressive phenotype in human tumors and is overexpressed in some lymphoproliferative disorders (31–35).

Upon integrin binding to the extracellular matrix, focal adhesion assembly occurs through incompletely characterized mechanisms (36, 37). FAK interacts with integrins at focal adhesions through direct binding of its N-terminal domain to the cytoplasmic tail of integrin β chains, or indirectly by binding of its C-terminal region to cytoskeletal proteins such as paxillin and talin

that bind to integrin chains (38, 39). Subsequently, FAK is activated by phosphorylation at residue Y397, providing a binding site for Src-type tyrosine kinases (40, 41), which, in turn, phosphorylate additional tyrosine residues, including Y925, located within the focal adhesion targeting (FAT) sequence of the FAK C-terminal domain. Phosphorylated Y925 has been suggested to recruit the adaptor protein Grb2, leading to the activation of the GTP binding protein Ras, which can trigger a signaling cascade, leading to ERK1/2 activation (42). Increasing evidence indicates that ERK1/2 can be also activated by alternative mechanisms and sites on FAK (43–45).

Interestingly, high activation of the Ras/Raf-1/MEK1/ERK1/2 pathway leads to a decrease in integrin avidity, facilitating focal adhesion turnover and promoting cell migration (46, 47). Moreover, FAK has two proline-rich domains that allow the assembly of additional signaling complexes, among which pCAS (Crk-associated substrate) and GRAF (GTPase-activating protein for Rho associated with FAK) are the best characterized (48, 49). The role of FAK in focal adhesion turnover is underscored by the increases in focal adhesion formation and cell adherence associated with reduced cell motility displayed by FAK–/– cells (50, 51).

Given the seminal role of FAK in integrin-mediated cell migration, the aim of our study was to assess the role of FAK in integrin-mediated gelatinase production by T lymphoid cells. By transfection of FAK wild-type and FAK-truncated forms and point mutants, we found that FAK transmits both stimulatory and inhibitory signals for gelatinase production and release. This dual role of FAK is achieved through its scaffolding function rather than through its intrinsic kinase activity. Stimulatory and inhibitory signals for gelatinase expression coordinated by FAK are transmitted through pathways known to control focal adhesion formation and disassembly required for cell migration.

MATERIALS AND METHODS

Cell culture

Human T lymphoblastoid cell lines (Jurkat and J.CaM1.6) were obtained from the European Collection of Cell Cultures (Salisbury, UK). Peripheral blood mononuclear cells were obtained from a healthy donor and depleted from monocytes and B cells by nylon wool adherence. After this procedure, primary T cell purity was 97% as assessed by flow cytometry. T lymphoblasts were generated by culturing primary T cells with phytohemagglutinin (Gibco Life Technologies, Grand Island, NY) at 2.5 μg/ml during the first 48 h and then stimulated with interleukin-2 at 25 U/ml until 2 days before use. Cells were cultured in RPMI 1640 (Gibco) supplemented with 10% fetal calf serum (Biological Industries, Kibbutz, Beit Haemec, Israel), 2 mM L-glutamine and 50 μg/ml gentamycin at 37°C in 5% CO_2, and adjusted to a concentration of 0.3×10^6 cells/ml the day before performing the experiments.

Inhibitors

PP2, GM6001, curcumin, and PD98059 were purchased from Calbiochem (La Jolla, CA). Actinomycin D, brefeldin A, monensin, and wortmannin were obtained from Sigma (St. Louis, MO). SP600125 was a generous gift from Dr. B. Bennett (Celgene, San Diego, CA). Inhibitors were added to the cells 30 min before addition of fibronectin (Calbiochem). Except when

otherwise indicated, cells were exposed to drugs during experiments for 4 h. Cell viability was confirmed by trypan blue exclusion.

Transient transfection

FAK wild-type cDNA and FAK point mutants K454R, Y397F and Y925F, were expressed using the pcDNA3 vector containing the hemagglutinin (HA) epitope generated, as described previously (45). The FAK truncation form termed FRNK and the point mutants FAK P712/715A and FRNK P712/715A were expressed using the pRK-VSV vector containing the vesicular stomatitis virus (VSV) epitope (43, 52). A shorter FAK construct containing FAT domain was constructed in pRK-VSV as follows: the fragment of FAK encoded by the DNA sequence 2630-3268 of GenBank accession number M95408 was PCR amplified from the HA-FAK plasmid using the following forward and reverse primers: 5′-GGATCCGGATCCCGAGGCAGCATCGACAGGGAAGA-3′ and 5′-TCTAGATCTAGATCAGTGTGGCCGTGTCTGCCCTA-3′. The PCR product was digested with BamHI and XbaI and cloned into pRK-VSV. The Src-type kinase-deficient mutants Fyn KD and Lck KD were inserted into pSRα-puro (a kind gift from Dr. O. Acuto, Molecular Immunology Unit, Institut Pasteur, France) and wild-type Lck was subcloned into pSRα (prepared by Dr. C. Serra, Immunology Department, Hospital Clinic, Barcelona, Spain).

Superfect Transfection Reagent (Qiagen, Valencia, CA) was used for transient transfection of Jurkat cells following the protocol advised by the manufacturer, using 2 μg of cDNA for 2.5 × 10^6 cells in a 6-well plate.

Primary T cells were transiently transfected with the Nucleofector kit (Amaxa, Cologne, Germany), following the protocol recommended for stimulated human primary T cells. Transfection efficiency was estimated by cotransfecting the cells with pEGFP-C3 or pSRα-HA-GFP. Cells were used for experimental procedures 48 h post-transfection. Equivalent levels of transfection were verified by Western blot analysis of cell lysates. Experiments were repeated at least 4 times with consistent results.

Gelatin zymography

Cultured Jurkat cells were washed twice with plain RPMI 1640 and resuspended at 0.5 × 10^6 cells/ml. FN was added at 10 μg/ml, and 5 × 10^6 cells per condition were incubated at 37°C in 5% CO_2. Unless otherwise specified, the supernatant fluid was collected 4 h later and concentrated 200-fold with Urifil-10 concentrator devices (Millipore, Molsheim, France). Concentrated samples were analyzed as described previously (7).

RT-PCR

RNA was extracted from 5 × 10^6 Jurkat cells using TRIzol Reagent (Gibco) following the manufacturer's instructions. One microgram of RNA was reverse-transcribed into cDNA using Superscript First-Strand Synthesis System (Invitrogen, Carlsbad, CA).

Primers for MMP-2 amplification were 5′-GGCACCCATTTACACCTACACCAA-3′ (position 1552-1575) (sense) and 5′-GCTTCCAAACTTCACGCTCTTCAG-3′ (position 2245-2222)

(antisense); for MMP-9, 5′-CTCCTGCTCCCCCTGCTCACG-3′ (position 376-395) (sense) and 5′-CGGGTGTAGAGTCTCTCGCT-3′ (position 855-836) (antisense) and for MMP-14, 5′-CAACATCACCTATTGGATCC-3′ (position 265-285) (sense) and 5′-CTCACCCCCATAAAGTTGCTG-3′ (position 1092-1072) (antisense). The expected sizes for the amplification products were 670 bp (MMP-2), 480 bp (MMP-9) and 828 bp (MMP-14). TIMP-1 and TIMP-2 were detected as described previously (7).

Thirty-five reaction cycles were run, each consisting of three steps of 45 s at 94°C, 57°C (for MMP-2 and MMP-9) or 60°C (MMP-14), respectively, followed by an elongation period of 10 min at 72°C. PCR products were analyzed in 1.2% agarose gels (Invitrogen). Multiplex amplification of β2-microglobulin was used as an internal control.

Adhesion assay

Ninety-six well plates were coated with FN (50 μg/well) overnight at 4°C, and the remaining fluid was aspirated. Cells were suspended in serum-free medium, plated on FN-coated wells at 0.15×10^6 cells/well and incubated at 37°C for 1 h. Nonadherent cells were aspirated and the remaining cells were fixed and stained with 0.2% crystal violet (Sigma) in 20% methanol. Wells were washed with distilled H_2O and air-dried. Dye was solubilized with 1% SDS, and optical density was read with a spectrophotometer at 600-nm wavelength. Conditions were tested in quadruplicate wells.

Invasion assay

Ten micrometer-pore polycarbonate filters (Nucleopore, Toronto, Canada) were coated with Matrigel (kindly provided by Dr. Hynda K. Kleinman, National Institutes of Health, Bethesda, MD) diluted in RPMI 1640 at 1:8, and placed between the lower and the upper compartment of 48-well Boyden chambers (Neuro Probe Inc., Gaithersburg, MD). The lower compartments were filled with 25 μl RPMI 1640 with 10% FCS and 0.1×10^6 cells in serum-free medium and were loaded onto the upper chambers. After 6 h-incubation at 37°C, the filter was removed, fixed with methanol, and stained with hematoxylin. Cells on the upper side were swept, and cell number/field in the lower side was counted in 6 randomly selected fields/well. Experiments were performed in quadruplicate wells.

Western blotting

5×10^6 cells per condition were incubated with FN at 10 μg/ml in serum-free RPMI 1640 medium and were lysed with 0.5 ml of modified RIPA buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton-X-100, 2 mM EDTA, 1% sodium deoxycholate, 0.1% SDS) supplemented with freshly added protease inhibitors (Complete, Boehringer Mannheim, Mannheim, Germany) and Na_3VO_4 at 200 μM. Protein content of lysates was measured with the BCA protein assay (Pierce, Rockford, IL).

Twenty micrograms of lysate per condition were subjected to SDS-PAGE and transferred onto nitrocellulose. Membranes were incubated overnight at 4°C with the appropriate primary antibody: FAK variants were detected with a rabbit polyclonal antibody against the carboxy-terminal domain (anti FAK C-20 from Santa Cruz Biotechnology, Santa Cruz, CA) at 1:400

dilution. A mouse monoclonal antibody anti-Lck (3A5) (Santa Cruz Biotechnology) was used to assess Lck content at 1:500 dilution. ERK activation state was evaluated using anti-phospho-p44/42 MAP kinase and then reprobed with p44/42 MAP kinase rabbit polyclonal IgG antibodies (Cell Signaling, Beverly, MA) at 1:1000 dilution. JNK MAP kinase required a preceding enrichment procedure. Cell lysates were incubated overnight at 4°C with SAPK/JNK rabbit polyclonal IgG antibody (Cell Signaling) at 1:100 dilution and immunoprecipitated with GammaBindPlus Sepharose beads (Amersham Biosciences, Uppsala, Sweden). Immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose. The phosphorylation state of JNK was detected using phospho-JNK/SAPK rabbit polyclonal IgG antibody (Cell Signaling) at 1:1000 dilution. In parallel experiments, a mouse monoclonal anti-phosphotyrosine (4G10) from Upstate Biotechnology at 1 μg/ml was used as a primary antibody and, after immunodetection, blots were stripped and reprobed with a rabbit polyclonal antibody against the N-terminal fragment of pCAS (N-17) (Santa Cruz Biotechnology) at 1:300 dilution.

Immunodetection was performed by incubating membranes with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit HRP-conjugated, Cell Signaling or anti-mouse HRP-conjugated, Transduction Laboratories, Lexington, KY) at 1:2000 dilution. Chemiluminescence signals were detected with LUMIGLO chemiluminescent reagent (Cell Signaling). Membranes were exposed to *X*-ray film Hyperfilm ECL (Amersham Life Science, Buckinghamshire, England) and developed with a Kodak RP-X-OMAT processor.

RESULTS

Focal adhesion kinase regulates integrin-dependent gelatinase (MMP-2 and MMP-9) expression and release by T lymphoid cells

Transient transfection of FAK wild-type cDNA into Jurkat T cells and into primary T cells increased MMP-2 and MMP-9 production in response to FN, as assessed by gelatin zymography of the conditioned medium, consistent with a role for FAK in the regulation of integrin-mediated gelatinase production (Fig. 1*A*). The increase in gelatinase production was followed by an increase in invasiveness, which was inhibited by the MMP inhibitor GM6001 (Fig. 1*B*). To assess whether FAK-stimulated MMP secretion was associated with higher rates of MMP expression, semiquantitative RT-PCR was performed with total RNA samples obtained from mock- and FAK- transfected cells. As shown in Fig. 1*C*, FAK transfected cells showed enhanced MMP-2, MMP-9, and TIMP-1 mRNA in response to FN with no significant changes in MMP-14 and TIMP-2 expression. FAK transfection alone was unable to increase gelatinase production by T cell lines without exposure to FN (data not shown), confirming that the increase in MMP production achieved by FAK transfection is dependent on additional signals driven by integrin engagement.

Given the important role of FAK in the focal adhesion turnover required for cell migration through the extracellular matrix, we developed the hypothesis that besides inducing MMP gene expression, FAK activation by integrin engagement might have an even more prominent role in regulating gelatinase release. As shown in Fig. 2*A* and *B*, upon FN exposure, both MMP-2 and MMP-9 appeared in the supernatant fluid long before an increase in gelatinase mRNA could be detected by RT-PCR. Furthermore, blocking transcription with actinomycin D, which was effective in abrogating gelatinase mRNA induction by FN (Fig. 2*C*), did not prevent gelatinase

detection in the cell culture supernatant medium and did not inhibit the increase in gelatinase secretion triggered by FAK transfection (Fig. 2*D*). This early release was markedly reduced by the protein secretion inhibitors monensin and brefeldin A, further supporting that FN stimulates secretion of prestored gelatinases (Fig. 2*E*). These findings indicate a prominent role for FAK in mediating post-transcriptional release of gelatinases by T lymphoid cells.

We next explored potential differences in integrin-driven signaling pathways leading to gelatinase expression and to gelatinase release. As shown in Fig. 2*F*, inhibition of Src-type tyrosine kinases with PP2, blocking ERK1/2 activation with PD98509, and inhibition of JNK activity by SP600125, all inhibited the FN-induced increase in gelatinase mRNA. However, although Src-tyrosine kinase and JNK inhibition also resulted in decreased gelatinases in the supernatant medium, inhibition of ERK activation and inhibition of PI 3-kinase with wortmannin produced a significantly increased release of gelatinases (Fig. 2*G*). In agreement with these findings, curcumin, which at low concentrations inhibits JNK and at higher concentrations also prevents ERK activation (53), displayed bimodal effects (Fig. 2*G*).

To better understand the mechanisms through which FAK regulates gelatinase production, point mutants of FAK were transiently transfected into Jurkat cells in order to determine which docking or signaling FAK domains are involved in MMP-2 and MMP-9 production and release induced by FN. Point mutation of tyrosine 397 (FAK Y397F) has been shown to prevent recruitment of tyrosine kinases of the Src family, while mutation of lysine 454 (FAK K454R) abolishes FAK intrinsic kinase activity and mutation of tyrosine 925 (FAK Y925F) has been suggested to prevent Grb2 binding and the subsequent activation of the Ras/Raf-1/ERK signaling pathway (42). As shown in Fig. 3*A*, mutation at the catalytic site did not decrease gelatinases in the conditioned medium, indicating that intrinsic FAK kinase activity is not required for gelatinase release. Mutation of Y925 also did not reduce gelatinase release compared with FAK wild-type transfected cells. In contrast, mutation at Y397 remarkably decreased MMP gelatinolytic signal, suggesting that recruitment of Src and possibly other kinases by FAK into focal adhesions is necessary for gelatinase production.

Src and Src-FAK interaction are crucial for integrin-mediated gelatinase release by T lymphoid cell lines

These results led us to focus on the role of Src-type protein kinases in gelatinase production induced by FN. As shown in Fig. 2*F* and 2*G*, PP2, a specific inhibitor of Src-type tyrosine kinases, strongly reduced gelatinase production induced by FN.

Given that Lck is a major Src-type tyrosine kinase in cells of lymphoid lineage, we explored gelatinase induction by FN in J.CaM1.6 cells, a Jurkat-derived Lck-deficient T cell line. JCaM1.6 cells displayed dramatically reduced production of gelatinases in response to FN compared with their Jurkat parental cell line (Fig. 3*C*). Gelatinase production by J.CaM1.6 cells in response to FN was restored by transfection with wild-type Lck. Although the levels were markedly decreased, J.CaM1.6 exhibited some gelatinase production, indicating that Lck function may be partially compensated by other Src-type tyrosine kinases with redundant functions (Fig. 3*C*). Subsequently, Jurkat cells were transiently transfected with Lck and Fyn kinase-deficient mutants. As shown in Fig. 3*D*, both mutants resulted in decreased gelatinase production elicited by FN, indicating that Src-family kinase activity contributes to gelatinase

induction by FN and providing further evidence for functional redundancy among Src-type tyrosine kinases.

The reduced gelatinase production in Y397F-transfected cells, combined with the role of Src tyrosine kinases in gelatinase induction, suggests that FAK/Src complex formation is crucial for integrin-mediated induction of gelatinases. In accord with this hypothesis, cotransfection of Lck and FAK wild-type into the Lck-deficient cell line J.CaM1.6 resulted in more increased MMP-2 and MMP-9 production than transfection with either Lck or FAK individually (Fig. 3*E*).

Integrin-mediated stimulatory and inhibitory signals for gelatinase release are orchestrated by distinct domains of FAK: functional divergence between FRNK and FAT fragments

We have previously shown that exposure to FN transduces stimulatory and inhibitory signals for MMP production by T lymphoid cells (7). We next explored whether these dual signals were coordinated by FAK by inducing overexpression of truncated forms. These expressed constructs consisted of the naturally occurring C-terminal domain FRNK, which lacks the kinase domain but retains binding sites for adaptor proteins (54) and FAT, a shorter C-terminal truncated form that retains binding sites for paxillin and talin but lacks the proline-rich domains present in FRNK (Fig. 4*A*).

As shown in Fig. 4*B*, FRNK and FAT fragments had intriguingly opposite effects on gelatinase release. FRNK overexpression elicited a much lower production of released gelatinases than FAK wild-type in response to FN. In contrast, FAT transfection produced a remarkable increase in both MMPs that was considerably higher than even FAK wild-type. Similar results were obtained when cells were exposed to soluble or to solid-phase FN (Fig. 4*B*).

The effects of FRNK and FAT were more prominent on gelatinase release than on gelatinase mRNA expression (Fig. 4*D*). Interestingly, FRNK and FAT also had an opposite effect on cell adhesion to FN: while FRNK decreased cell attachment, FAT increased it (Fig. 4*E*). These findings suggest that these mutants may decrease cell motility through different mechanisms: by decreasing cell attachment or by inducing cell arrest. Disturbed cell motility and altered gelatinase release resulted in a decrease in cell invasiveness in cells transfected with either truncated form (Fig. 4*E*).

We next tried to elucidate the molecular basis for the divergent effects of FRNK and FAT on gelatinase production. Both FRNK and FAT are able to displace native FAK from its physiological location at focal adhesions and compete with FAK for binding proteins resulting in inhibition of FAK-mediated effects on cell motility (50, 55, 56). Both lack the catalytic domain, indicating that, in accordance with the results obtained from FAK K454R-transfected cells (Fig. 3*A*), the absence of the catalytic domain in FRNK, and in FAT, is not a major determinant of their divergent effects. The key determinants for the opposing effects of FRNK and FAT fragments should instead reside in FRNK domains absent in FAT.

Compared with FRNK, FAT lacks two proline-rich domains with important functions (Fig. 4*A*). The proline-rich 1 domain is able to interact with SH3-containing pCAS (Crk-associated substrate) and the proline-rich 2 region is a binding site for GRAF (GTPase-activating protein for Rho associated with FAK) (57, 58). Activated GRAF, as well as phosphorylated pCAS, lead to

Rho inhibition, favoring focal adhesion disassembly and facilitating cell motility (59). In other cell types, FAK-recruited pCAS is phosphorylated by FAK itself and by locally recruited Src-type tyrosine kinases (60). pCAS interacts with Crk and activates Rac and, subsequently, JNK (61, 62). Both activated Rac and JNK regulate gene expression and have been shown to increase gelatinase production in other cell systems (63, 64). Crk is also able to activate PI 3-kinase and Ras/Raf-1/ERK (65, 66), which, according to our results, are inhibitory pathways for gelatinase secretion (7) (Fig. 2*G*).

pCAS-mediated signals could be, then, stimulatory (through JNK) or predominantly inhibitory (through Ras/ERK or PI 3-kinase activation) for gelatinase secretion. Since FRNK is able to bind pCAS but unable to phosphorylate it, we considered that FRNK might compete with pCAS binding to native FAK thereby preventing pCAS downstream signaling events leading to JNK activation. However, even though JNK is necessary for FN-induced MMP expression, as indicated by the abrogation of gelatinase expression and release obtained with JNK inhibitor SP600125 (Fig. 2*F* and *G*), the reduced integrin-mediated gelatinase production by FRNK does not appear to result from interference with JNK activation (Fig. 5).

FRNK is able to mediate pCAS downstream signaling events regulating gelatinase release through mechanisms requiring Src-kinase activity

The absence of FRNK inhibition on JNK phosphorylation may indicate that FRNK-recruited pCAS can be phosphorylated by other kinases and effectively transmit downstream-signaling events. In fact, it has been recently shown that pCAS is able to directly recruit Src-type tyrosine kinases (67). This hypothesis might explain, at least in part, the reduced JNK2 phosphorylation achieved by FAT, which lacks proline-rich regions and is unable to recruit pCAS (Fig. 5).

As a test of this hypothesis, Jurkat cells were transfected with FRNK mutated at prolines 712/715 (FRNK P712/715A), which is unable to recruit pCAS. As displayed in Fig. 6*A*, this mutation prevented the inhibitory effect of FRNK on gelatinase release. Supporting the role of Src-type tyrosine kinases in phosphorylating FRNK-bound pCAS, FRNK transfection into the Lck-deficient J.CaM1.6 cell line not only failed to reduce MMP production but, in fact, resulted in an enhancement of MMP release, even stronger than that achieved by FAT (Fig. 6*B*). Furthermore, the suppressing effect of FRNK was reversed by inhibiting Src-type tyrosine kinase activity with PP2 (Fig. 6*C*). The FRNK inhibitory effect is, consequently, dependent on Src-type tyrosine kinases, particularly Lck.

Further supporting the ability of FRNK to recruit pCAS and the ability of Src-type tyrosine kinases to phosphorylate it, FRNK overexpression did not interfere with phosphorylation of two pCAS family members present in lymphoid cells, CasL and Sin (Fig. 6*D*). However, both CasL and Sin phosphorylation were reduced by PP2 only in FRNK-transfected cells and not in the mock-transfected controls, supporting that FRNK cooperates with Src-type tyrosine kinases to transmit downstream CasL/Sin signaling (Fig. 6*D*). Consistent with this idea, FRNK-induced ERK phosphorylation was abrogated by the Src inhibitor PP2 (Fig. 6*E*). Importantly, as shown in Fig. 6*C*, the FRNK inhibitory effect on gelatinase release was reversed with the MEK1 inhibitor PD98059 indicating that FRNK transmits inhibitory signals for gelatinase release by a process requiring ERK activation.

FAT-increased gelatinase release requires Src activity and is associated with reduced ERK activation

FAT subfragment retains binding sites for important signaling and cytoskeleton proteins such as paxillin and talin and may compete with native FAK for focal adhesion location and for Y925 phosphorylation. As shown in Fig. 7*A*, FAT enhanced gelatinase release was inhibited by the Src-family kinase inhibitor PP2 and was virtually unmodified by curcumin and the more specific JNK inhibitor SP600125, indicating that Src kinase activity but not JNK activity are necessary for FAT-enhanced gelatinase release. As shown in Fig. 7*B*, FAT was, indeed, less efficient than FAK wild-type in inducing tyrosine phosphorylation of ERK1/2 in response to FN, suggesting that interference with ERK1/2 activation may be a mechanism through which FAT increases gelatinase release. In accord with this mechanism, further inhibition of ERK 1/2 activation by PD98509 elicited only a weak additional increase in gelatinase release induced by FAT (Fig. 7*A*). However, given the lack of increase in gelatinase release achieved by the point mutant Y925F, it appears likely that FAT decreases ERK activation and gelatinase release through additional mechanisms besides the Y925 site. Alternatively, FAT effects would not be mediated by competing with endogenous FAK and FAT itself with its bound molecules and devoid of inhibitory proline-rich regions would directly mediate gelatinase release through mechanisms requiring Src-kinase activity and ERK inhibition. Further experiments are needed to better identify the mechanisms involved in FAT-induced gelatinase release.

DISCUSSION

Gelatinase production by lymphocytes permits their migration through tissues where they fulfill crucial biological functions in health and disease. In this study, we show that FAK has an important role in regulating integrin-induced gelatinase production and, particularly, release by T lymphoid cells.

Transient transfection of Jurkat cells with full-length FAK increased MMP-2 and MMP-9 expression and release in response to FN, resulting in an enhanced invasiveness of transfected cells. It has been recently shown that, in melanoma cells, MMP-2 and MMP-9 are stored in vesicles, closely associated with microtubules (68). Reorganization of the cytoskeleton during FAK coordinated focal adhesion turnover, might then, promote gelatinase release.

Point mutation of FAK at Y397, which upon phosphorylation, becomes a binding site for SH2 domains of Src-type tyrosine kinases and other signaling molecules such as PLCγ, PI 3-kinase, and Grb7 (28, 29), decreased gelatinase production, suggesting that FAK interaction with Src-type tyrosine kinases and possibly other SH2-bearing signaling molecules is required for FAK-increased gelatinase production. In support of this mechanism, Lck-deficient Jurkat cells displayed reduced gelatinase expression in response to FN. Transfection with kinase-deficient mutants showed that, among Src-type tyrosine kinases, both Lck and Fyn participate in integrin-dependent gelatinase production by T lymphoid cell lines. This finding is consistent with previous studies demonstrating that constitutive Src activation or viral Src transformation is associated with cell invasiveness in malignant cells (69). Recently, Hsia et al. have demonstrated that, while viral Src infection rescues impaired motility in FAK–/– cells, interaction with FAK is necessary for viral Src-mediated cell invasiveness and MMP production (64). Our results indicate that interaction of FAK with endogenous Src-type tyrosine kinases is relevant not only

for invasiveness of v-Src-transformed cells but is also crucial for physiologic responses such as integrin-mediated induction of gelatinases in T lymphoid cells. Even though Src tyrosine kinase activity is essential for FN-induced MMP production and release, as indicated by the abrogation of gelatinase production achieved by the Src-type tyrosine kinase inhibitor PP2, intrinsic FAK kinase activity was not required for integrin-mediated gelatinase production, as demonstrated by the lack of any inhibitory effect of the FAK K454R kinase-dead point mutant.

Interestingly, as revealed by transient transfection of truncated forms, FAK is able to transduce both stimulatory and inhibitory signals for gelatinase production by T lymphoid cells through pathways known to regulate cell motility and survival in other cell types (70). Transient transfection of Jurkat cells with FRNK elicited a reduced release of gelatinases compared with full-length FAK, while transfection with FAT substantially increased gelatinase release. Although FAK truncated forms exhibited opposite effects on cell adhesion and gelatinase release, both resulted in decreased cell invasiveness, indicating that coordination of gelatinase release with focal adhesion turnover is essential for cell invasion.

FAT-increased gelatinase release required Src tyrosine kinase activity, as it was abrogated by the Src inhibitor PP2. The FAT-stimulated gelatinase release may occur, at least in part, by interfering with ERK activation. This is supported both by the fact that FAT elicited reduced ERK phosphorylation compared with FAK wild-type and that inhibition of ERK1/2 phosphorylation in nontransfected cells actually resulted in an increased gelatinase release in response to FN. However, transfection of FAK mutated at Y925 did not result in a substantial increase in gelatinase release, suggesting that FAT may inhibit ERK activation through pathways other than interfering with Y925-mediated Grb2 recruitment by endogenous FAK.

JNK activation has been implicated in MMP-9 production in FAK–/– cells infected with viral v-Src (64), as well as for MMP-3 and MMP-13 production in in vivo models of arthritis (63). JNK was, indeed, necessary for gelatinase production in response to FN, as demonstrated by the fact that SP600125 prevented the increase in both gelatinase mRNA and protein induced by FN. However, JNK was not involved in FAT-stimulated gelatinase release, as demonstrated by the decrease in JNK2 phosphorylation obtained with FAT overexpression and by the absence of any significant inhibitory effect of curcumin or SP600125 on FAT-mediated gelatinase release by T lymphoid cells. Moreover, interference with JNK, also does not appear to be a major mechanism underlying the reduced effect of FRNK on gelatinase production by T lymphoid cells: JNK2 was highly phosphorylated in Jurkat cells before exposure to FN (data not shown), and FRNK transfection did not result in a major decrease in JNK phosphorylation, contrary to the effects of FAT. Consequently, even though JNK2 activation is necessary for gelatinase production, other mechanisms may activate it more efficiently than integrin engagement. Serum factors, rather than integrin engagement, have been shown to induce JNK activation and localization, along with FAK, in lamellipodia and invadopodia in FAK restored and viral-Src infected FAK–/– cells (64).

We show that FRNK is able to transmit some stimulatory signals for gelatinase release through activation of JNK and perhaps other pathways. In our system, FRNK was inefficient in increasing FN-induced gelatinase production compared with the smaller FAT truncation, probably due to the parallel transduction of more powerful inhibitory signals from FRNK via ERK1/2 activation and possibly other pathways.

The ability of FRNK to transduce downstream predominantly inhibitory signals is dependent on pCAS binding to FRNK, as a FRNK mutant unable to recruit pCAS (FRNK P712/715A) reversed FRNK inhibitory effect. Moreover, FRNK inhibition is dependent on Src-type tyrosine kinases, which can be recruited by pCAS (71), given that Src inhibition substantially enhanced gelatinase release in FRNK-overexpressing cells, and FRNK expression in Lck-deficient J.CaM1.6 cells resulted in a strong gelatinase release. Src-dependent FRNK inhibitory signals were likely transduced through CasL/Sin phosphorylation and subsequent Crk-derived ERK activation, because in FRNK-overexpressing cells, Src inhibitors decreased CasL/Sin phosphorylation and inhibitors of ERK phosphorylation increased gelatinase release.

Interestingly, our findings indicate that FRNK is not a dominant-negative for all FAK functions. In support of our findings, it has been recently demonstrated that, as opposed to FAT, which induces apoptosis, FRNK can efficiently transduce survival signals for serum-deprived fibroblasts through Src-dependent pCAS phosphorylation and subsequent JNK activation (72). In our setting, FRNK dominant-negative function for gelatinase release also depended on Src-tyrosine kinase activity. FRNK has been recently demonstrated to inhibit invasiveness and MMP-2 secretion in viral v-Src-transformed NIH3T3 fibroblasts (55). In v-Src-transformed cells, where Src is constitutively active, Src-dependent FRNK inhibition of gelatinase release might be constantly activated, and FRNK could always function as a dominant-negative for gelatinase production. In contrast, during physiological migration on FN, Src-tyrosine kinases are only transiently activated and, through their interaction with FAK, may transduce stimulatory and inhibitory signals for gelatinase release.

Although T lymphocytes are not major agents in proteolytic tissue remodeling, they are able to produce tiny amounts of gelatinases that are crucial in allowing their progression into tissues in physiological and pathological conditions. Our findings indicate that, in T lymphoid cells, FAK regulates not only gelatinase production but also post-transcriptional gelatinase release in response to FN, and the scaffolding function of FAK—rather than its kinase activity—is the most relevant feature in this process. FAK emerges, then, as an important mediator in coordinating stimulatory and inhibitory signals for gelatinase release using pathways controlling cell migration. This fine control may adapt pulse release of gelatinases to focal adhesion turnover.

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Fig. 1

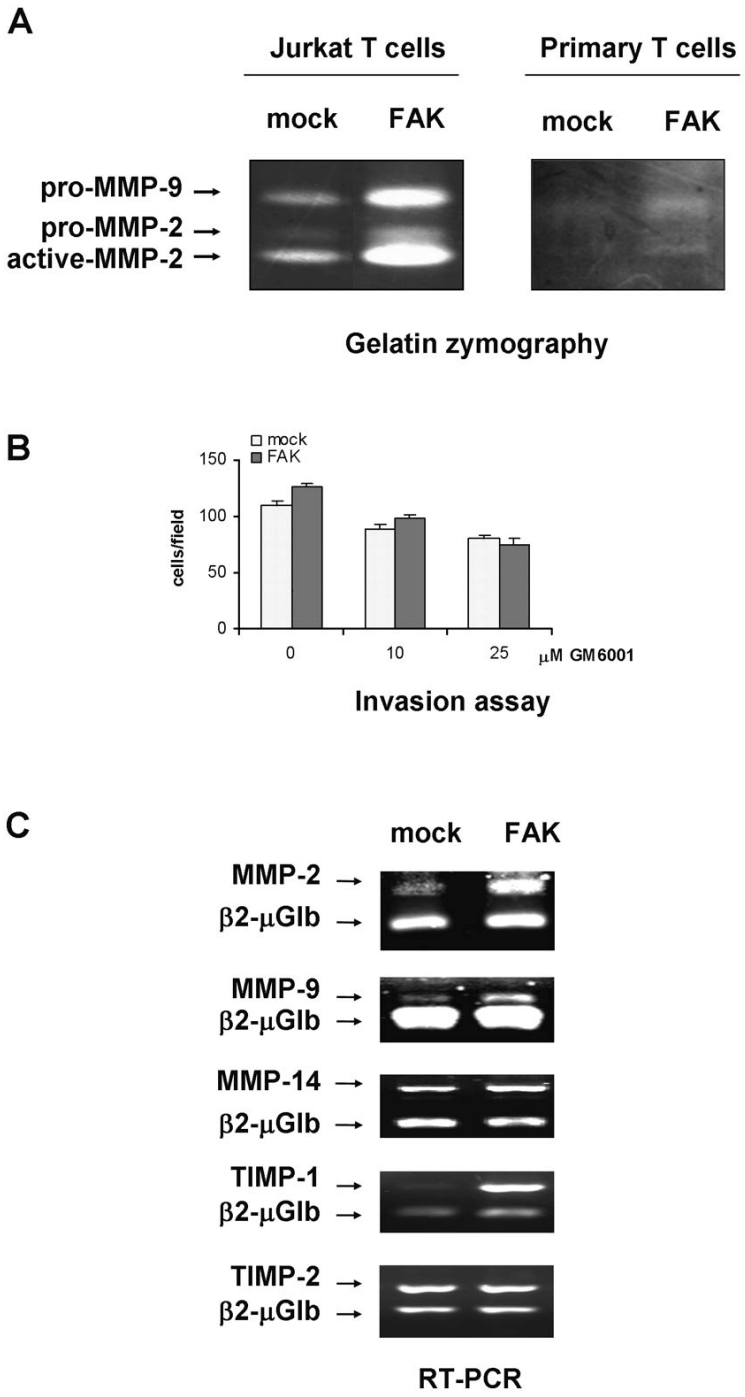

Figure 1. Transient transfection of FAK wild-type increases gelatinase production in response to fibronectin. ***A***) Gelatin zymography of concentrated conditioned medium obtained from mock and FAK-transfected Jurkat and primary T cells exposed to 10 μg/ml of fibronectin (FN) for 4 h. ***B***) Invasion of FAK and mock-transfected Jurkat cells through Matrigel-coated filters in the absence and in the presence of the MMP inhibitor GM6001. FAK vs. mock $P = 0.0134$; baseline mock vs. mock with GM6001 (10 μM), $P = 0.0039$; baseline mock vs. mock with GM6001 (25 μM) $P = 0.0018$; baseline FAK vs. FAK with GM6001 (10 μM and 25 μM) $P < 0.0001$ (Mann Whitney U test). ***C***) RT-PCR amplification of MMP-2, MMP-9, MMP-14, TIMP-1, TIMP-2, and β2-microglobulin (β2-μGlb), using RNA obtained from mock and FAK wild-type transfected Jurkat cells after exposure to FN for 4 h.

Fig. 2

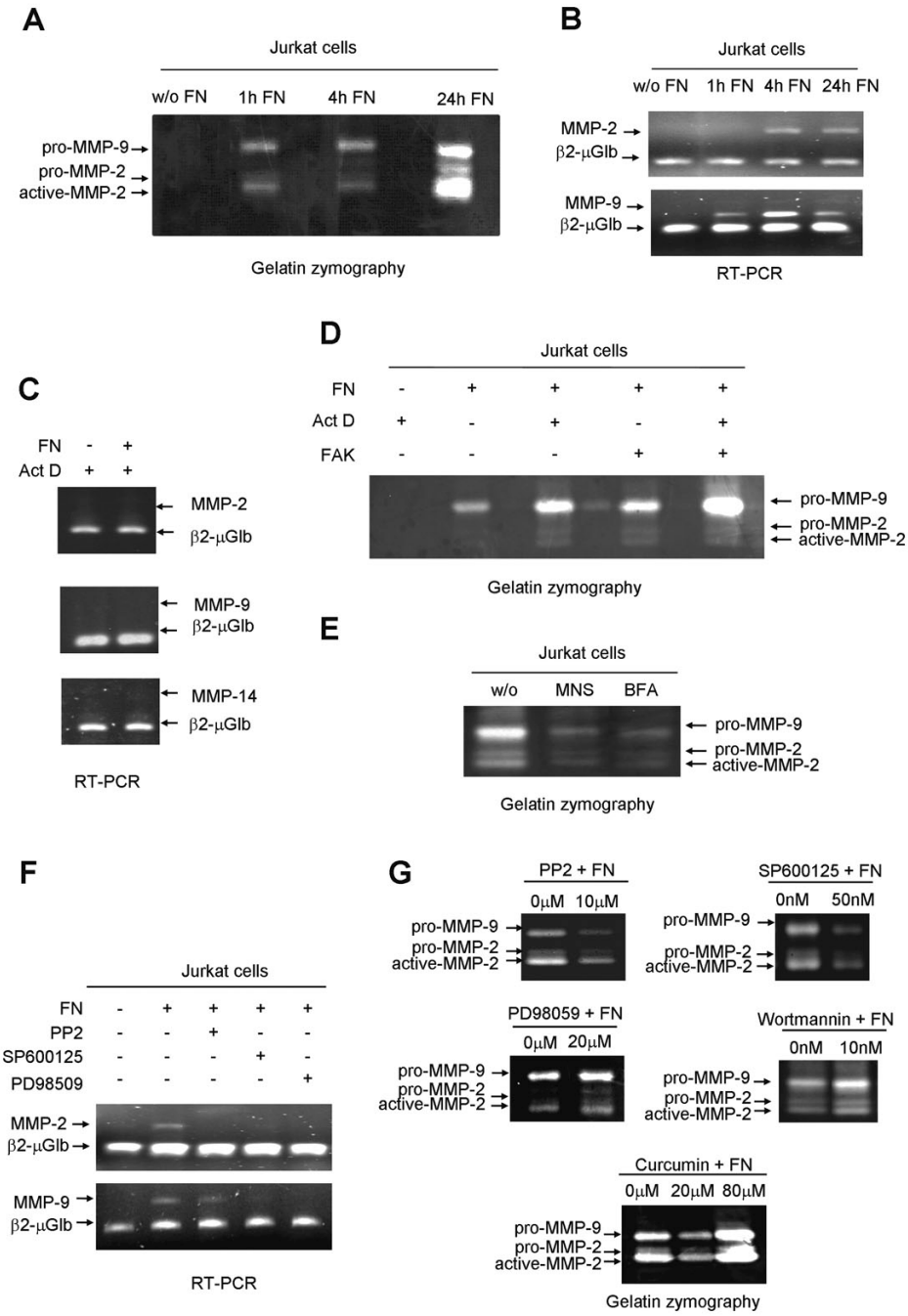

Figure 2. FAK mediates fibronectin-induced gelatinase release. ***A*)** Gelatin zymography of concentrated conditioned medium of Jurkat cells collected at the indicated time points after exposure to FN (10 μg/ml). ***B*)** RT-PCR detection of MMP-2, MMP-9, and β2-μGlb using RNA obtained from the same cells as in (***A***). ***C*)** RT-PCR amplification of MMP-2, MMP-9, MMP-14, and β2-μGlb of RNA obtained from Jurkat cells exposed to FN for 4 h with (+) or without (–) pretreatment with actinomycin D (Act D) at 2 μg/ml. ***D*)** Gelatin zymography of concentrated conditioned medium obtained from mock and FAK-transfected Jurkat cells exposed to FN for 4 h with or without pretreatment with actinomycin D. ***E*)** Gelatin zymography of Jurkat cells exposed to FN in the absence (w/o) and in the presence of monensin (MNS) or brefeldin A (BFA). ***F*)** RT-PCR amplification of MMP-2 and MMP-9 using RNA obtained from Jurkat cells under basal conditions compared with exposure to FN for 4 h, with or without pretreatment with the Src-tyrosine kinase inhibitor PP2 at 10 μM, JNK inhibitor SP600125 at 50 nM, or MEK1 inhibitor PD98509 at 20 μM. ***G*)** Gelatin zymography of the conditioned medium of Jurkat cells exposed to FN for 4 h and pretreated with PP2, SP600125, PD98059, wortmannin or curcumin at the indicated concentrations.

Fig. 3

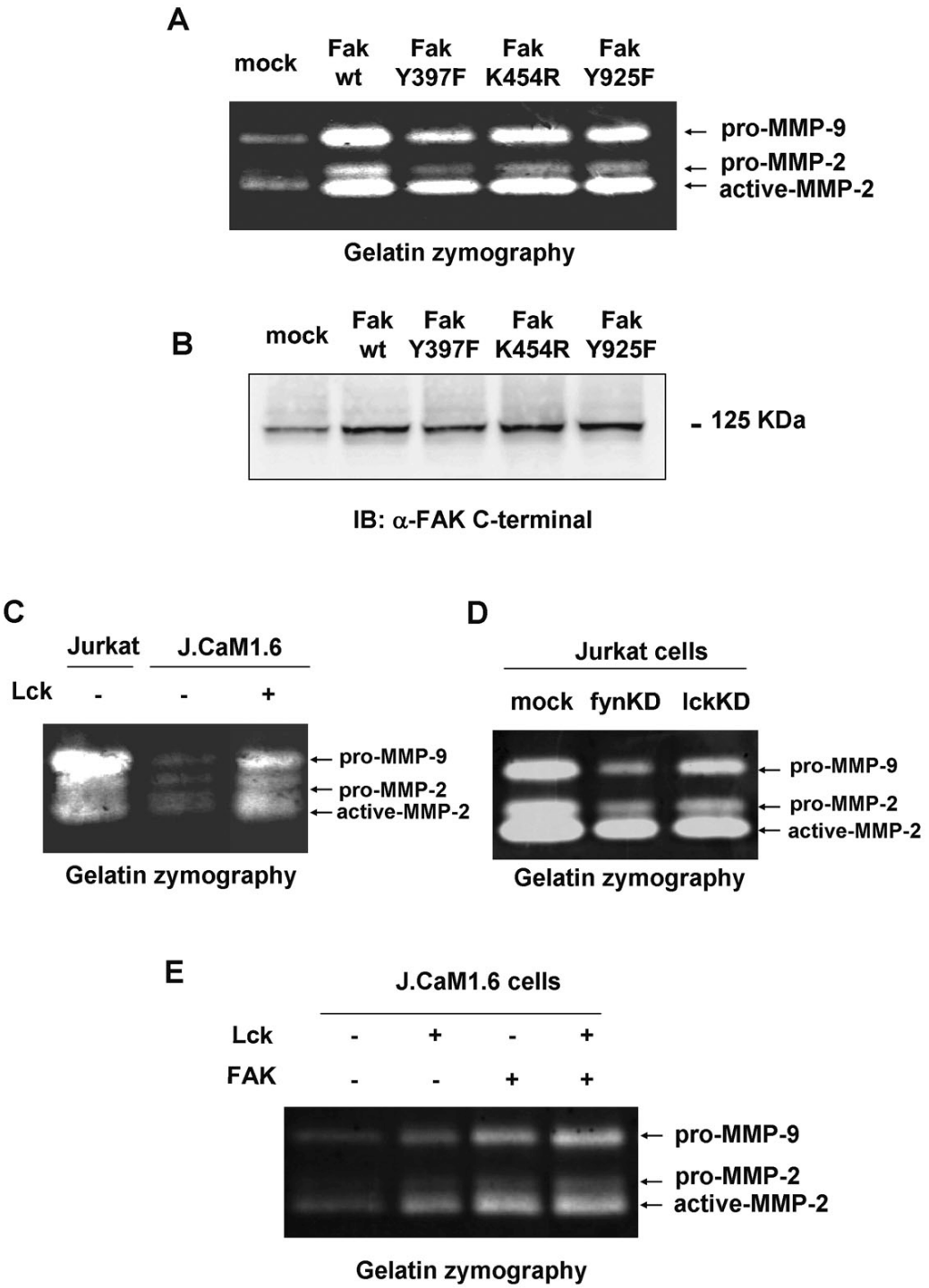

Figure 3. Functional participation of distinct FAK domains in gelatinase expression. Role of Src kinases Lck and Fyn. ***A***) FAK point mutants Y397F, K454R, and Y925F were transiently transfected into Jurkat cells and incubated with FN (10 μg/ml) for 4 h. Conditioned medium was concentrated and subjected to gelatin zymography. ***B***) Western blot assessing FAK protein content in lysates of transfected cells. ***C***) Gelatin zymography of conditioned medium from Jurkat cells, Lck-deficient JCaM1.6 cells, and J.CaM1.6 cells transiently transfected with wild-type Lck exposed to FN for 4 h. ***D***) Gelatin zymography of concentrated conditioned medium from mock-transfected Jurkat cells and transfected with kinase-deficient Fyn and Lck mutants exposed to FN for 4 h. ***E***) Gelatin zymography of conditioned medium of JCaM1.6 cells mock transfected (–) or transfected with wild-type Lck (+) or FAK (+), cultured with FN (10 μg/ml) for 4 h.

Fig. 4

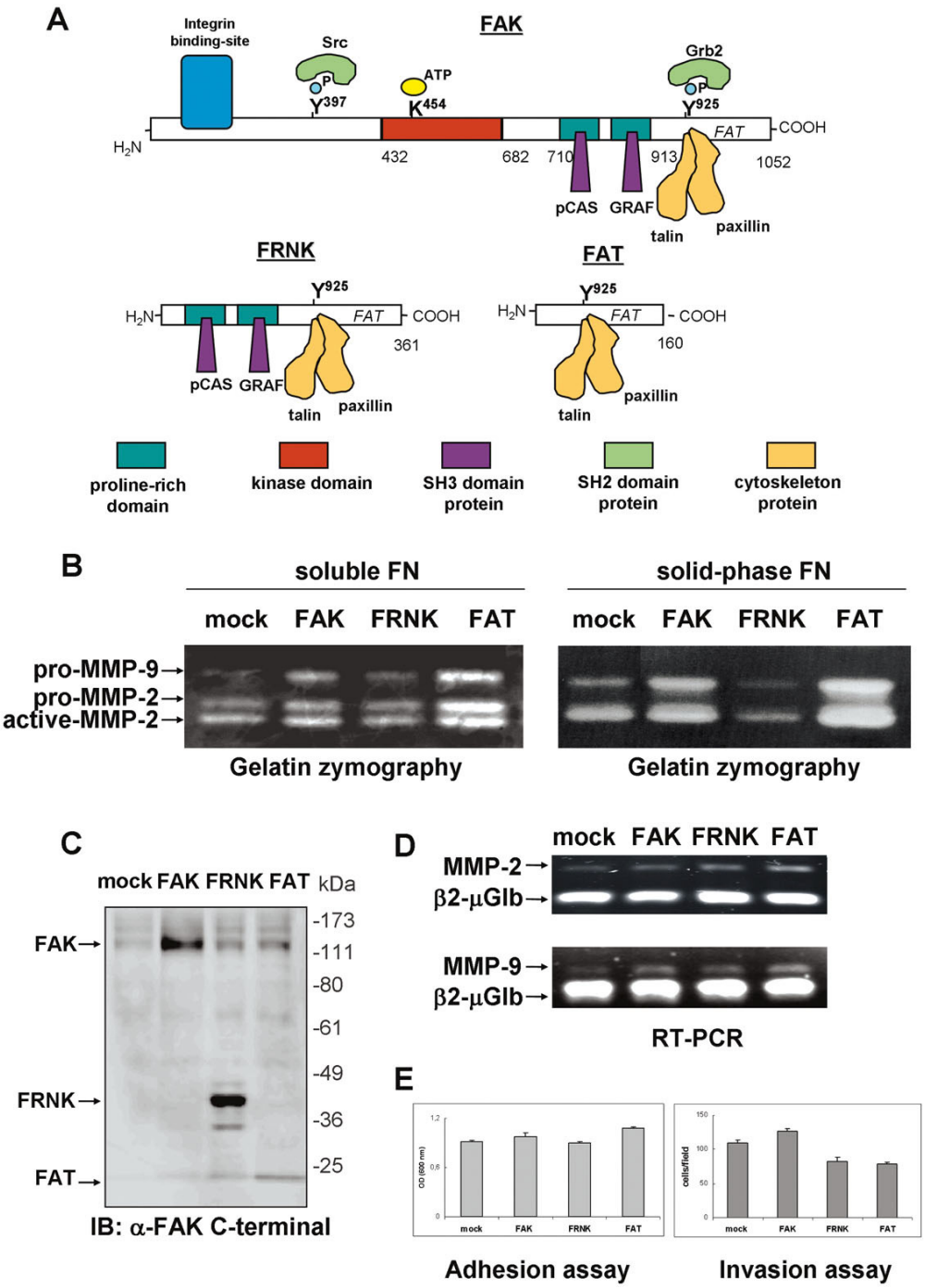

Figure 4. Different effects of FAK truncated forms on gelatinase expression and release. ***A*)** Schematic representation of FAK wild type and truncated forms FRNK and FAT used in this study. ***B*)** Gelatin zymography of concentrated conditioned medium from Jurkat cells mock-transfected or transfected with FAK wild-type or FAK truncated forms FRNK and FAT, after 4 h exposure to soluble FN (10 μg/ml) (***left***) or solid-phase FN (100 μg/ml) (***right***). ***C*)** Western blot performed with lysates of transfected cells indicating the relative expression of FAK wild-type, FRNK fragment, and FAT subfragment. ***D*)** RT PCR amplification of MMP-2, MMP-9 and β2-μGlb using RNA obtained from the same cells as in (***B***). ***E*)** Adhesion of transfected Jurkat cells to solid-phase FN and invasion through Matrigel-coated filters. For adhesion: FAK vs. mock, $P = 0.0571$; FAK vs. FRNK, FAK vs. FAT, and FRNK vs. FAT, $P = 0.0286$. For invasion: FAK vs. mock, $P = 0.0134$; FAK vs. FRNK and FAT, $P < 0.0001$ (Mann-Whitney *U* test).

Fig. 5

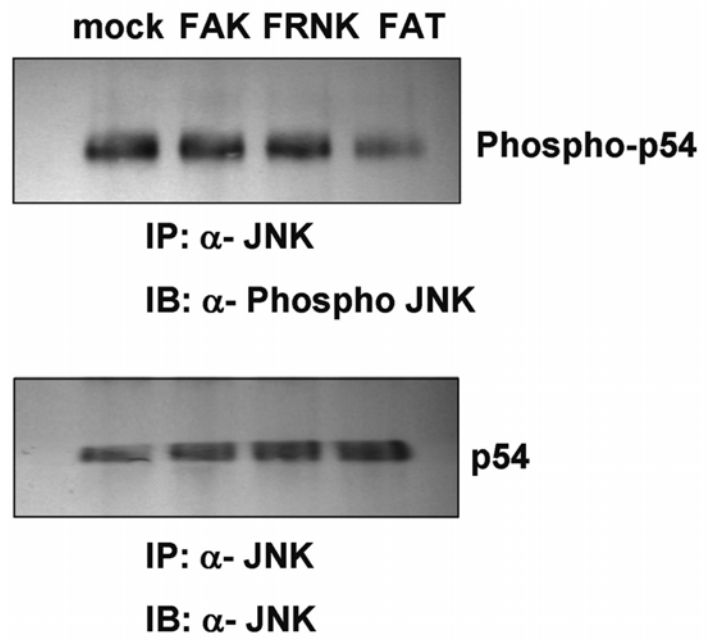

Figure 5. FRNK overexpression does not interfere with JNK activation. Cell lysates obtained from Jurkat cells mock transfected or transfected with FAK wild-type or FAK truncated forms FRNK or FAT and exposed to FN for 1 h were immunoprecipitated with anti-JNK antibody, subjected to SDS-PAGE and blotted onto nitrocellulose. Western blot analysis was performed with anti-phosphorylated JNK.

Fig. 6

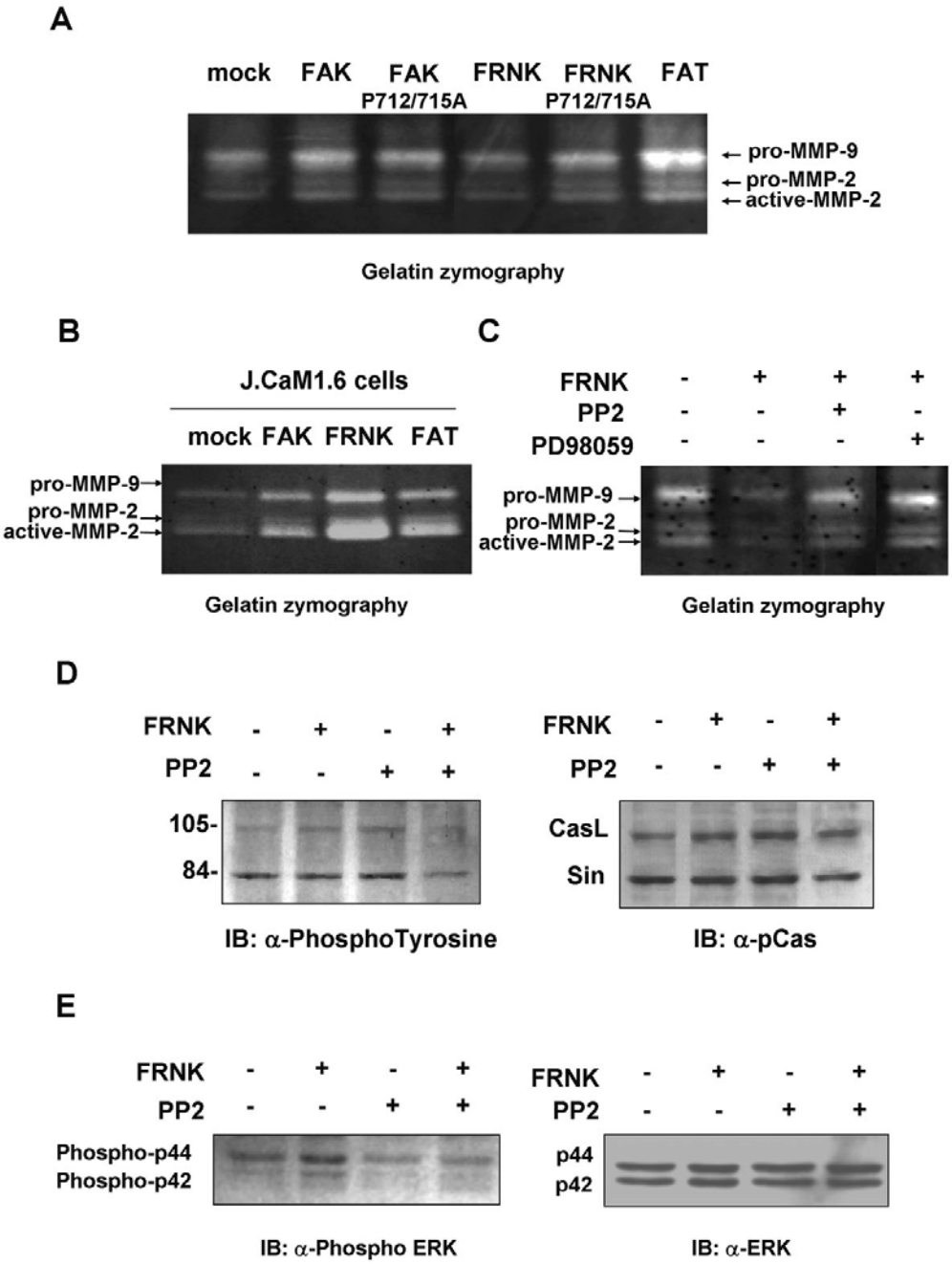

Figure 6. FRNK inhibitory effect on gelatinase release requires Src activity and is associated with CasL phosphorylation and ERK activation. ***A*)** Gelatin zymography of conditioned medium obtained from Jurkat cells transiently transfected with the indicated FAK mutants and truncated forms after 4 h exposure to FN (10 μg/ml). ***B*)** Gelatin zymography of concentrated conditioned medium obtained from baseline JCaM1.6 cells, and J.CaM1.6 cells transiently transfected with FAK wild-type or truncated forms FRNK and FAT. Gelatin zymography of concentrated conditioned medium obtained from Jurkat cells mock-transfected (–) and Jurkat cells transiently transfected with FRNK (+), untreated (–), or treated with Src-inhibitor PP2 (+) or MEK1 inhibitor PD98059 (+). All cells were exposed to FN for 4 h. ***C*)** Cell lysates obtained from Jurkat cells mock transfected (–) or transfected with FRNK (+), untreated (–), or treated with Src-inhibitor PP2 (+) and incubated with FN for 1 h were subjected to SDS-PAGE and blotted onto nitrocellulose. Western blot analysis was performed with a phosphotyrosine antibody; then the blot was stripped and reprobed with anti-pCAS antibody. ***D*)** Western blot analysis of cell lysates obtained from mock (–) or FRNK (+) transfected Jurkat cells, untreated (–), or treated with PP2, and exposed to FN for 1 h. ***E*)** Blots were incubated with anti-phosphorylated ERK antibody, stripped, and reprobed with anti-ERK antibody.

Fig. 7

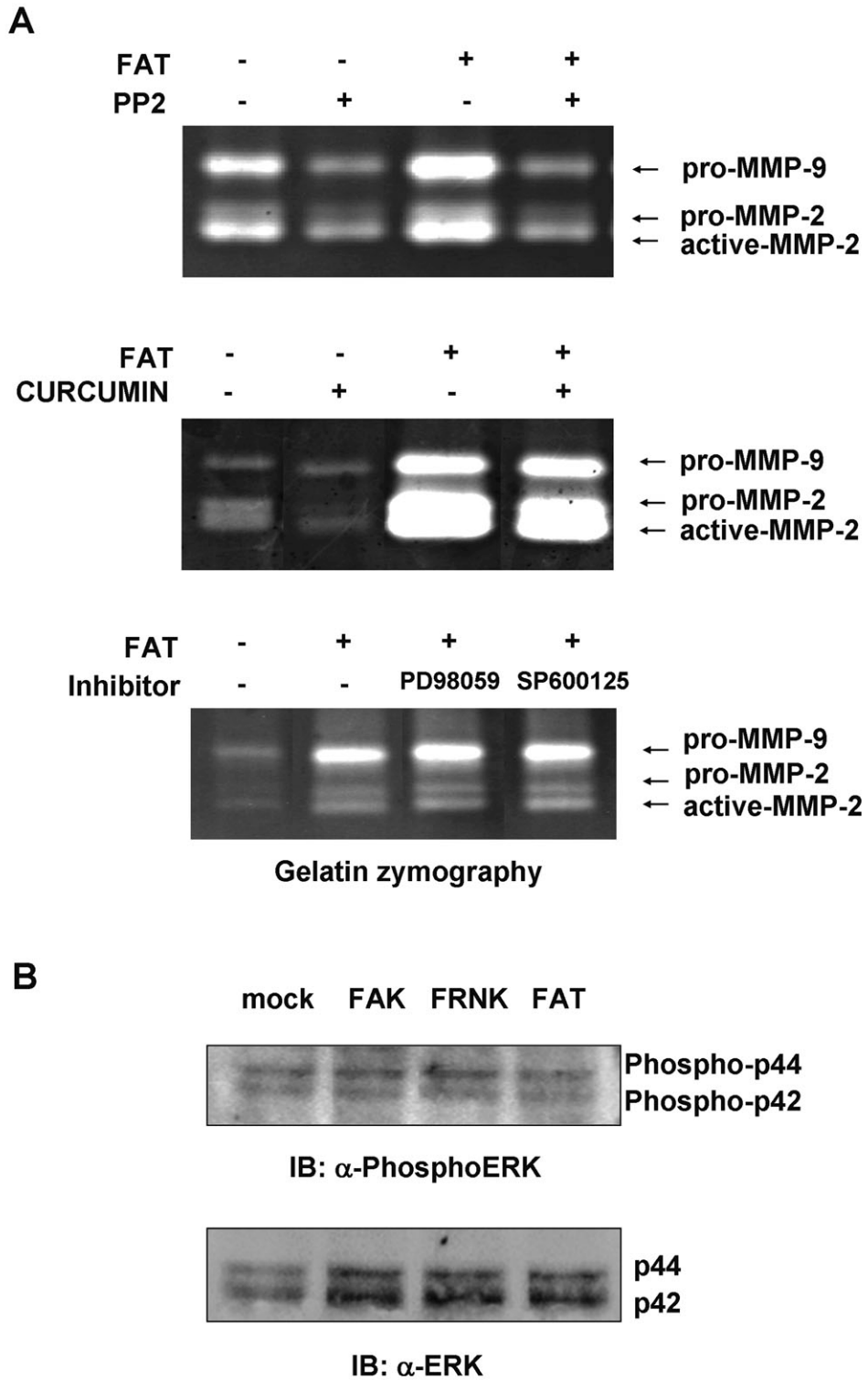

Figure 7. FAT enhanced gelatinase release requires Src activity and is associated with a reduction in ERK activation. ***A***) Gelatin zymography of concentrated conditioned medium obtained from Jurkat cells mock-transfected (–) or transfected with FAT (+), untreated (–), or treated with PP2 at 10 μM (+), curcumin at 20 μM (+), PD98509 at 20 μM (+), or SP600125 at 50 nM (+), as indicated in the figure. All cells were cultured with FN at 10 μg/ml for 4 h. ***B***) Western blot analysis of cell lysates from Jurkat cells mock-transfected or transfected with FAK wild-type or truncated forms FRNK and FAT, exposed to FN (10 μg/ml) for 1 h, and analyzed using anti-phosphorylated ERK antibody. The blot was subsequently stripped and reprobed using anti-ERK.

Dual function of focal adhesion kinase in regulating integrin-induced MMP-2 and MMP-9 release by human T lymphoid cells

Marta Segarra, Carme Vilardell, Kazue Matsumoto,[†] Jordi Esparza, Ester Lozano, Carles Serra-Pages,* Alvaro Urbano-Márquez, Kenneth M Yamada,[†] and Maria C Cid[1]

Department of Internal Medicine and *Immunology, Hospital Clínic, University of Barcelona, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain; and [†]Craniofacial Developmental Biology and Regeneration Branch. National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda Maryland, USA

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SPECIFIC AIMS

The aim of our study was to assess the role of focal adhesion kinase (FAK) in integrin-mediated gelatinase production by T lymphoid cells.

PRINCIPAL FINDINGS

1. FAK regulates integrin-dependent gelatinase (MMP-2 and MMP-9) expression and release by T lymphoid cells

Transient transfection of FAK wild-type cDNA into Jurkat T and primary T cells increased MMP-2 and MMP-9 production in response to fibronectin (FN), as assessed by gelatin zymography. FAK transfected cells showed enhanced MMP-2, MMP-9, and TIMP-1 mRNA in response to FN with no significant changes in MMP-14 and TIMP-2 expression.

However, both MMP-2 and MMP-9 appeared in the supernatant fluid long before an increase in gelatinase mRNA was detected by RT-PCR. Furthermore, blocking transcription with actinomycin D, which abrogated gelatinase mRNA induction by FN, did not inhibit the increase in gelatinase secretion triggered by FAK transfection. By contrast, blocking protein secretion with the inhibitors monensin and brefeldin A greatly reduced gelatinase production. Therefore, besides inducing MMP gene expression, FAK activation by integrin engagement has a prominent role in regulating gelatinase release.

To better understand the mechanisms through which FAK regulates gelatinase release, point mutants of FAK were transiently transfected into Jurkat cells. Point mutation of tyrosine 397 (FAK Y397F) has been shown to prevent recruitment of tyrosine kinases of the Src family, while mutation of lysine 454 (FAK K454R) abolishes FAK intrinsic kinase activity and mutation of tyrosine 925 (FAK Y925F) has been suggested to prevent Grb2 binding and the subsequent activation of the Ras/Raf-1/ERK signaling pathway. Mutation at the catalytic site did not decrease gelatinase production, indicating that intrinsic FAK kinase activity is not required for gelatinase production. Mutation of Y925 also did not reduce gelatinase release compared with FAK wild-type transfected cells.

In contrast, mutation at Y397 significantly decreased gelatinolytic signals, suggesting that recruitment of Src kinases by FAK into focal adhesions is necessary for gelatinase production.

2. Src and Src-FAK interaction are crucial for integrin-mediated gelatinase release by T lymphoid cell lines

Given that Lck is a major Src-type tyrosine kinase in cells of lymphoid lineage, we explored gelatinase induction by FN in J.CaM1.6 cells, a Jurkat-derived Lck-deficient T cell line. JCaM1.6 cells displayed dramatically reduced production of gelatinases in response to FN compared with Jurkat cells and it was restored by transfection with wild-type Lck.

The reduced gelatinase production in Y397F-transfected cells, combined with the requirement of Src tyrosine kinase activity in gelatinase induction, suggests that FAK/Src complex formation is crucial for integrin-mediated induction of gelatinases. In accord with this hypothesis, co-transfection of Lck and FAK wild-type into the Lck-deficient cell line J.CaM1.6 resulted in more strongly increased MMP-2 and MMP-9 production than transfection with either Lck or FAK individually.

[1] Correspondence: Department of Internal Medicine. Hospital Clínic. Villarroel, 170. 08036 Barcelona, Spain. E-mail: mccid@clinic.ub.es

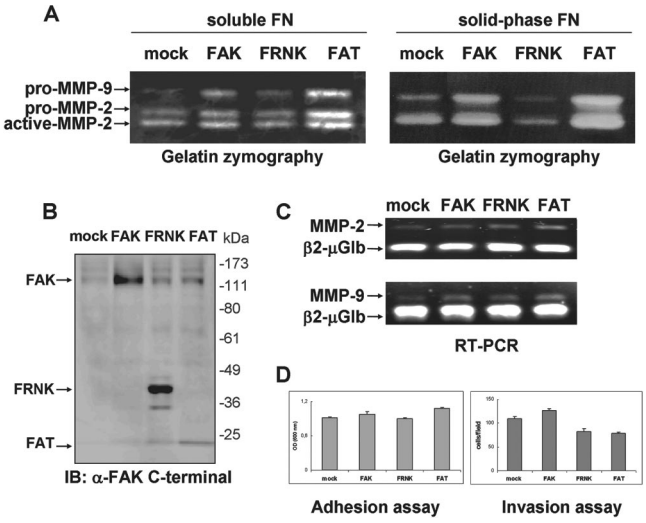

Figure 1. Opposite effects of FAK truncated forms on gelatinase expression and release. *A)* Gelatin zymography of concentrated conditioned medium from Jurkat cells mock-transfected or transfected with FAK wild-type or FAK truncated forms FRNK and FAT, after exposure to soluble FN (left) or solid-phase FN (right). *B)* Western blot performed with lysates of transfected cells indicating the relative expression of FAK wild-type, FRNK fragment, and FAT subfragment. *C)* RT-PCR amplification of MMP-2, MMP-9 and β2-μGlb using RNA obtained from the same cells as in panel *B. D)* Adhesion of transfected Jurkat cells to solid-phase FN and invasion through Matrigel-coated membranes.

3. Integrin-mediated stimulatory and inhibitory signals for gelatinase release are orchestrated by distinct domains of FAK: functional divergence between FRNK and FAT fragments

We previously showed that exposure to FN transduces stimulatory and inhibitory signals for MMP production by T lymphoid cells. We next explored whether these dual signals were coordinated by FAK by inducing overexpression of truncated forms. These consisted of the naturally occurring C-terminal domain FRNK, which lacks the kinase domain but retains binding sites for adaptor proteins and FAT, a shorter C-terminal truncated form that retains binding sites for paxillin and talin but lacks the proline-rich domains present in FRNK.

FRNK and FAT fragments had opposite effects on gelatinase release (**Fig. 1*A***). FRNK overexpression elicited a much lower release of gelatinases than FAK wild-type in response to FN. In contrast, FAT transfection produced an increase in both MMPs that was considerably higher even than FAK wild-type. The effects of FRNK and FAT were more prominent on gelatinase release than on gelatinase mRNA expression (Fig. 1*C*). FRNK and FAT also had opposite effect on cell adhesion to FN: while FRNK decreased cell attachment, FAT increased it (Fig. 1*D*). These findings suggest that these mutants may decrease cell motility through opposite mechanisms: by decreasing cell attachment or by inducing cell arrest.

We next tried to elucidate the molecular basis for the divergent effects of FRNK and FAT on gelatinase production. Compared with FRNK, FAT lacks two proline-rich domains with important functions. The proline-rich 1 domain is able to interact with SH3-containing pCAS (Crk-associated substrate) and the proline-rich 2 region is a binding site for GRAF (GTPase-activating protein for Rho associated with FAK). pCAS-mediated signals could be, then, stimulatory (through

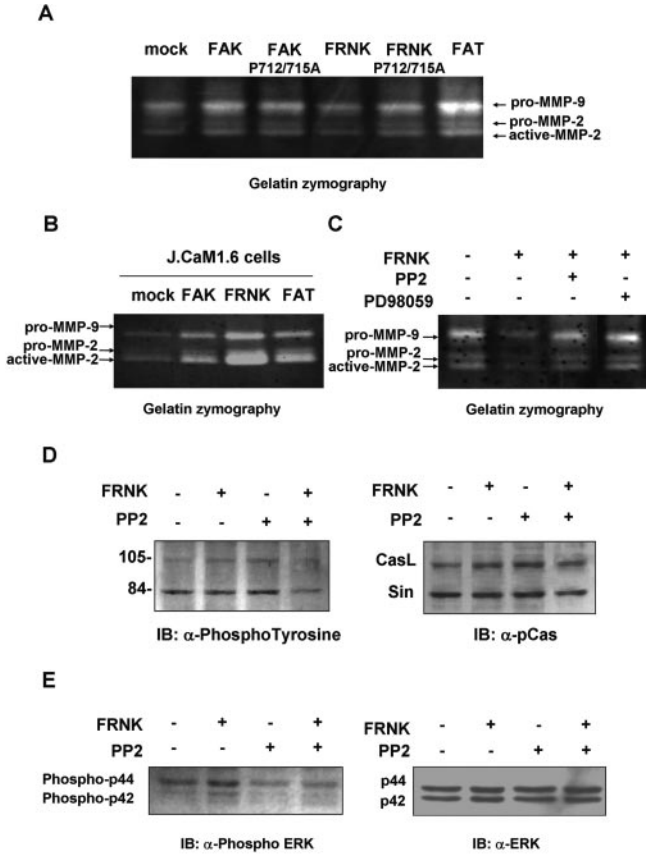

Figure 2. FRNK inhibitory effect on gelatinase release requires Src activity and is associated with CasL phosphorylation and ERK activation. *A)* Gelatin zymography of conditioned medium obtained from Jurkat cells transiently transfected with the indicated FAK mutants and truncated forms after exposure to FN. *B)* Gelatin zymography of concentrated conditioned medium obtained from baseline JCaM1.6 cells, and J.CaM1.6 cells transiently transfected with FAK wild-type or truncated forms FRNK and FAT. *C)* Gelatin zymography of concentrated conditioned medium obtained from Jurkat cells mock transfected (–) and Jurkat cells transiently transfected with FRNK (+), untreated (–) or treated with Src-inhibitor PP2 (+) or MEK1 inhibitor PD98059 (+). *D)* Cell lysates obtained from Jurkat cells mock transfected (–) or transfected with FRNK (+), untreated (–) or treated with Src-inhibitor PP2 (+) and incubated with FN were subjected to SDS-PAGE and blotted onto nitrocellulose. Western blot was performed with a phosphotyrosine antibody, then the blot was stripped and reprobed with anti-pCAS antibody. *E)* Western blot analysis of cell lysates obtained from mock (–) or FRNK (+) transfected Jurkat cells, untreated (–) or treated with PP2, and exposed to FN. Blots were incubated with anti-phosphorylated ERK antibody, stripped and reprobed with anti-ERK antibody.

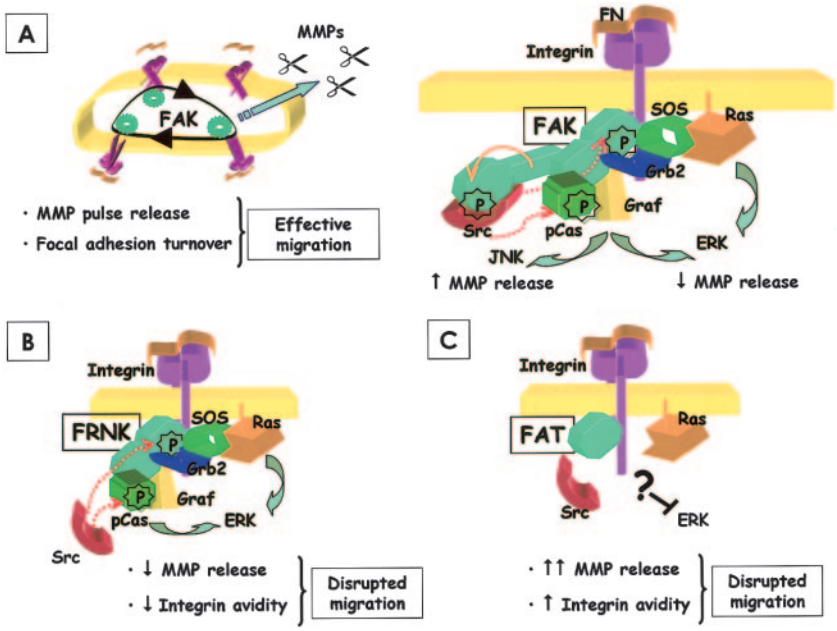

Figure 3. Schematic model of FAK-mediated pulse gelatinase release coordinated to focal adhesion turnover. Cyclic activation/inhibition of ERK1/2 mediated by distinct domains of FAK is proposed as one of the involved pathways. *A)* Effective migration through the extracellular matrix requires focal adhesion turnover and pulse gelatinase release coordinated by entire FAK. *B)* The truncated form FRNK is unable to mediate this process and decreases MMP release as well as interaction with FN. *C)* The shorter fragment FAT freezes the cell in an adherent status with unrepressed gelatinase release.

JNK) or predominantly inhibitory (through Ras/ERK or PI 3-kinase activation) for gelatinase secretion.

4. FRNK is able to mediate pCAS downstream signaling events regulating gelatinase release through mechanisms requiring Src-kinase activity

FRNK-recruited pCAS might be phosphorylated by other kinases and transmit downstream signaling events. It was recently shown that pCAS is able to directly recruit Src-type tyrosine kinases.

As a test of this hypothesis, Jurkat cells were transfected with FRNK mutated at prolines 712/715 (FRNK P712/715A), which is unable to recruit pCAS. As displayed in **Fig. 2*A***, this mutation prevented the inhibitory effect of FRNK on gelatinase release. Supporting a role for Src-type tyrosine kinases in phosphorylating FRNK-bound pCAS, FRNK transfection into the Lck-deficient J.CaM1.6 cell line not only failed to reduce MMP production but in fact resulted in an enhancement of MMP release even stronger than that achieved by FAT (Fig. 2*B*). The suppressing effect of FRNK was reversed by inhibiting Src-type tyrosine kinase activity with PP2 (Fig. 2*C*). The FRNK inhibitory effect, consequently, is dependent on Src-type tyrosine kinases.

Further supporting the ability of FRNK to recruit pCAS and the ability of Src-type tyrosine kinases to phosphorylate it, FRNK overexpression did not interfere with phosphorylation of two pCAS family members present in lymphoid cells CasL and Sin (Fig. 2*D*). However, both CasL and Sin phosphorylation were reduced by PP2 only in FRNK-transfected cells and not in the mock-transfected controls, supporting the idea that FRNK cooperates with Src-type tyrosine kinases to transmit downstream CasL/Sin signaling (Fig. 2*D*). Consistent with this, FRNK-induced ERK phosphorylation was abrogated by the Src inhibitor PP2 (Fig. 2*E*). As shown in Fig. 2C, the FRNK inhibitory effect on gelatinase release was reversed with the MEK1 inhibitor PD98059 indicating that FRNK transmits inhibitory signals for gelatinase release by a process requiring ERK activation.

5. FAT-increased gelatinase release requires Src-activity and is associated with reduced ERK activation

FAT subfragment lacks the predominantly inhibitory proline-rich regions but retains binding sites for important signaling and cytoskeleton proteins such as paxillin and talin. FAT enhanced gelatinase release was inhibited by the Src-family kinase inhibitor PP2 and was virtually unmodified by the JNK inhibitor SP600125, indicating that Src kinase activity but not JNK activity are necessary for FAT-enhanced gelatinase release.

CONCLUSIONS AND SIGNIFICANCE

Our findings indicate that, in T lymphoid cells, FAK regulates not only gelatinase production but also post-transcriptional gelatinase release in response to FN and that the scaffolding role of FAK rather than its kinase activity is the most relevant function in this process. FAK interaction with Src-type tyrosine kinases is essential in gelatinase release. As revealed by transient transfection of truncated forms, FAK is able to transduce both stimulatory and inhibitory signals for gelatinase release by T lymphoid cells through pathways leading to ERK1/2 inhibition and activation, respectively. Src-tyrosine kinase activity is required for both stimulatory and inhibitory functions. Inhibitory signals are mostly mediated by proline-rich regions, which also have an important role in regulating focal adhesion turnover. FAK emerges as an important mediator in coordinating stimulatory and inhibitory signals for gelatinase release. As depicted in **Fig. 3**, this fine control may adapt pulse release of gelatinases to focal adhesion turnover. FJ

RESUM DELS RESULTATS

1. La fibronectina indueix l'expressió i activació de les gelatinases (MMP9 i MMP2) en limfòcits T a través de la unió a integrines limfocitàries. Aquest efecte s'incrementa amb la sobreexpressió de FAK en aquestes cèl·lules i es tradueix en una major capacitat invasiva de les cèl·lules transfectades per FAK.

2. A més d'induir l'expressió dels transcrits de les gelatinases, la fibronectina promou la secreció de MMP2 i MMP9 en un ràpid procés post-transcripcional.

3. L'alliberament de gelatinases resultat de la unió de fibronectina a receptors integrina està estimulat per Src tirosina cinases i JNK MAPK, i reprimit per ERK MAPK i PI3K.

4. La transfecció de mutants puntuals de FAK en la línia cel·lular T, Jurkat, indica que l'augment en la producció de MMP2 i MMP9 induïda per fibronectina no depèn de la capacitat cinasa intrínseca de FAK. En canvi, és determinant la integritat de la tirosina 397 (Y397), definit com el lloc d'unió de Src tirosina cinases. La capacitat adaptadora de FAK és clau per a la modulació de la producció de MMPs.

5. Reforçant la rellevància de Src, la línia cel·lular deficient en Lck derivada de Jurkat, JCaM1.6, produeix menys gelatinases respecte Jurkat en resposta a fibronectina. No obstant, la reexpressió de Lck en JCAM1.6 recupera aquesta capacitat. La co-transfecció de Lck i FAK en cèl·lules JCaM1.6 genera una producció de MMP2 i MMP9 molt superior a la transfecció individual de FAK o Lck en aquestes cèl·lules.

6. La transfecció de formes truncades de FAK en cèl·lules Jurkat descobreix la capacitat de FAK de regular les senyals integrina de forma bimodal. Mentre el fragment FRNK, deficient en el domini catalític, redueix la producció de

gelatinases, FAT, el petit domini C-terminal de FAK, n'augmenta la secreció. Les senyals induïdes per la transfecció de FRNK i FAT també divergeixen en la regulació de la capacitat adhesiva a fibronectina: FRNK no estimula l'adhesivitat, en canvi FAT incrementa l'adhesió. Significativament, tant les cèl·lules transfectades amb FRNK o amb FAT presenten menors taxes d'invasió respecte les transfectades amb FAK.

7. Les senyals inhibidores transmeses per FRNK en la producció de gelatinases són dependents de la seva capacitat d'unió a pCAS i requereixen l'activitat tirosina cinasa de Src per fosforilar a pCAS. En concordança, l'activació de pCAS per Src en cèl·lules transfectades amb FRNK es redueix amb l'inhibidor específic de Src tirosina cinases, PP2. La fosforilació de pCAS resulta en l'activació de ERK MAPK que reprimeix l'alliberament de MMPs en cèl·lules transfectades amb FRNK.

8. La sobreproducció de gelatinases en cèl·lules transfectades amb el fragment FAT depèn de Src tirosina cinases. La sobreexpressió de FAT provoca una activació deficient de ERK MAPK respecte FAK. A més, el bloqueig de ERK per mitjà d'un inhibidor específic incrementa només lleugerament l'activació de gelatinases. La reducció de l'activació de ERK podria ser un possible mecanisme, però possiblement no l'únic, mitjançant el qual FAT promou l'alliberament de MMPs.

CONCLUSIONS

Els limfòcits, en condicions fisiològiques i patològiques, necessiten l'alliberament de petites quantitats de gelatinases per migrar a través dels teixits. Per a una invasió limfocitària efectiva, la producció de gelatinases hauria d'anar coordinada amb

mecanismes d'adhesió cíclics que permetin la seva motilitat. Dels resultats d'aquest estudi se'n dedueixen les següents conclusions:

1. FAK és un component clau en la senyalització integrina dels limfòcits T. Els mecanismes impulsats per la unió de fibronectina a receptors integrina de la superfície del limfòcits s'accentuen per la sobreexpressió de FAK i condueixen al ràpid increment de la degradació proteolítica que facilita la invasió a través de la membrana basal.

2. FAK amb la col·laboració de les Src tirosina cinases, especialment Lck, transmeten senyals per a la secreció de gelatinases utilitzant els mecanismes prèviament descrits en la regulació de la motilitat cel·lular induïda per integrines.

3. FAK conté elements duals en la seva estructura que coordinen la secreció de gelatinases i la capacitat migratòria. Tot i els efectes oposats dels fragments FRNK i FAT en la inducció de la producció de gelatinases i l'adhesió a fibronectina, tots dos resulten en la retenció del procés d'invasió dels limfòcits. Per tant FAK, a través dels diferents dominis adaptadors i senyalitzadors, podria ser l'eix coordinador central dels passos que vehiculitzen l'acoblament de la secreció/retenció de gelatinases amb els cicles d'adhesió/alliberament necessaris per a la progressió migratòria dels limfòcits.