C-terminal heparin-binding domain of fibronectin regulates integrin-mediated cell spreading but not the activation of mitogen-activated protein kinase

Jungyean KIM¹, Innoc HAN¹, Yeonhee KIM, Seungin KIM and Eok-Soo OH²

Department of Life Sciences, Division of Molecular Life Sciences and Center for Cell Signaling Research, Ewha Womans University, Daehyun-dong, Seodaemoon-Gu, Seoul 120-750, Korea

Fibronectin (FN) stimulates multiple signalling events including mitogen-activated protein kinase (MAPK) activation. During cell spreading, both the cell-binding domain and the C-terminal heparin-binding domain (HepII) of FN co-operatively regulate cytoskeleton organization. However, in comparison with the large number of studies on the functions of cell-binding domain, there is little information about the role of HepII. We therefore investigated the effect of HepII on integrin-mediated cell spreading and adhesion on FN and MAPK activation. In contrast with cells on FN substrates, rat embryo fibroblasts on FN120, which lacks HepII, were less spread, had weaker adhesion to FN and failed to form focal adhesions and actin stress fibres. Phosphotyrosine was present in the focal contacts of rat embryo fibroblasts on FN within 30 min but was absent from cells on FN120. Overall, tyrosine phosphorylation was much less in cell lysates from cells on FN120, with decreased phosphorylation of focal adhesion kinase (‘pp125FAK’) on tyrosine-397, implying additional regulation of tyrosine phosphorylation by HepII. Nevertheless, adhesion-mediated MAPK activity was similar in cells on FN and on FN120. Furthermore, cells spread on FN and on FN120 substrates showed similar MAPK activation in response to treatment with epidermal growth factor and with platelet-derived growth factor. Consistently, overexpression of syndecan-4, which binds to HepII, enhanced cell spreading and adhesion on FN but did not affect integrin-mediated MAPK activation. We therefore conclude that both HepII and syndecan-4 regulate integrin-mediated cell spreading but not MAPK activation.

Key words: fibronectin, focal adhesion kinase, syndecan-4, tyrosine phosphorylation.

INTRODUCTION

Integrin-mediated cell–FN interaction stimulates multiple signalling events, including the tyrosine phosphorylation of proteins involved in cytoskeleton organization [1–3]. At an early stage of cell spreading, tyrosine phosphorylation is a common and ubiquitous response to integrin engagement [4–6]. Clustering of cell surface integrins by either immobilized extracellular molecules or antibodies against integrin stimulates the tyrosine phosphorylation of several cytoskeleton proteins, including focal adhesion kinase (pp125FAK), paxillin, tensin and p130Cas [5,7,8]. Although the extent of tyrosine phosphorylation varies with cell type, the major phosphorylated protein is pp125FAK [4,9]. This tyrosine phosphorylation is crucial for cytoskeleton organization; the inhibition of tyrosine phosphorylation blocks cell spreading [10]. This integrin engagement is also important for stabilizing the interaction between the cell and the extracellular matrix through the formation of focal adhesions and intracellular cytoskeleton organization [1,2,5]. For the assembly of focal adhesion and stress fibres, at least, two adhesion-receptor-mediated signals are known to be required in primary fibroblasts on FN substrates [11–13]. One of the signals is mediated through the interactions of integrins with the RGD-motif-containing cell-binding domain of FN. This is critical for cell attachment to FN and for initial cytoskeleton organization [13]. The other signal is mediated through the interaction of cell-surface heparan sulphate proteoglycans, probably syndecan-4, with the C-terminal heparin-binding domain (HepII) of FN [13–15]. These two signals combine to activate the small G-proteins to regulate further signals for the formation of focal adhesions and stress fibres [16,17].

Integrin-mediated cell–FN interaction regulates not only cytoskeleton organization but also the integrin-mediated activation of mitogen-activated protein kinase (MAPK) [6,18]. On integrin engagement, pp125FAK is autophosphorylated on tyrosine-397, which becomes a binding site for the cytosolic tyrosine kinase c-Src. The association of c-Src leads to additional pp125FAK tyrosine phosphorylation and creates binding sites for SH2-containing proteins such as growth-factor-receptor-bound protein 2 (Grb2), which in turn activate the Ras cascade pathway [6,18]. The tyrosine phosphorylation cascade, including pp125FAK phosphorylation, is a crucial step for the adhesion-mediated activation of MAPK [6,18]. However, there is some controversy over the role of pp125FAK. Lin et al. [19] have reported that integrin-mediated MAPK activation is independent of pp125FAK activity.

It has been shown that (1) HepII, in addition to the cell-binding domain and integrin, is required for complete adhesion and cytoskeleton organization. (2) HepII interacts with the extracellular domain of syndecan-4, and (3) syndecan-4 functions as a co-receptor for integrin during the formation of focal adhesions and stress fibres. Taking these results together, HepII might be involved in the regulation of integrin-mediated signal transduction pathways. We therefore investigated the effect of HepII and syndecan-4 on both cell spreading and integrin-mediated MAPK activation. We found that HepII and syndecan-4 regulated cytoskeleton organization. However, they were not involved in integrin-mediated MAPK activation.

Abbreviations used: EGF, epidermal growth factor; ERK, extracellular signal-regulated protein kinase; FN, fibronectin; HepII, C-terminal heparin-binding domain of fibronectin; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; PDGF, platelet-derived growth factor; pp125FAK, focal adhesion kinase; REF, rat embryo fibroblast; TRITC, tetramethylrhodamine β-isothiocyanate.

¹ These authors contributed equally to this study.
² To whom correspondence should be addressed (e-mail ohes@mm.ewha.ac.kr).
EXPERIMENTAL

Materials
Monoclonal anti-phosphotyrosine antibody (anti-pTyr, 4G10) and pp125FAK were obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Monoclonal anti-paxillin antibodies were from Transduction Laboratories (Lexington, KY, U.S.A.). Monoclonal anti-phosphoERK (anti-pERK; ERK is extracellular signal-regulated protein kinase) and polyclonal anti-ERK2 antibody were purchased from Santa Cruz; phosphorylation-site-specific pp125FAK antibodies were purchased from BioSource International (Camarillo, CA, U.S.A.). Affinity-purified FITC-conjugated goat anti-mouse IgG was from Kirkegaard & Perry (Gaithersburg, MD, U.S.A.), and purified FN and FN120 (an N-terminal FN fragment lacking the heparin-binding domain) were from Gibco BRL. Tetramethylrhodamine β-isothiocyanate (TRITC)-conjugated phalloidin and other chemicals were purchased from Sigma.

Cell culture and plating experiments
Rat embryonic fibroblasts (REFs) were maintained in α-modified Eagle’s medium (αMEM; Gibco BRL) supplemented with 5% (v/v) FBS (fetal bovine serum), penicillin (100 i.u./ml) and streptomycin (100 µg/ml). HEK-293 cells were maintained in DMEM (Dulbecco’s modified Eagle’s medium; Gibco BRL) supplemented with 10% (v/v) FBS, penicillin and streptomycin. For cell adhesion assays, cells were starved in culture medium for 24 h. HEK-293 cells (2 × 10^6) were plated on culture dishes 6 cm in diameter, incubated at 37 °C for 24 h, then transfected with 4 µg of pcDNA3 or syndecan-4-pcDNA3 in the presence of 25 µg of chloroquine with the use of alkaline CaCl₂ reagents. After 7 h at 37 °C, the medium was replaced with 4 ml of fresh DMEM containing 10% (v/v) FBS.

Microscopic analysis
REFs were fixed for 10 min with 4% (w/v) paraformaldehyde in PBS at 25 °C, permeabilized with 0.5% (v/v) Triton X-100 in PBS and blocked for 45 min with 0.5% BSA and 0.05% gelatin in PBS. After being washed, cells were stained with TRITC-conjugated phalloidin for F-actin, phosphotyrosine or paxillin antibody, followed by affinity-purified FITC-conjugated goat anti-mouse IgG. After being rinsed three times with PBS and being mounted in 50% (v/v) glycerol in PBS, cells were photographed at ×40 magnification with a fluorescence microscope (Zeiss). For cell morphology, cells were observed with an inverted microscope (Zeiss) at ×40 magnification.

Transient transfections
HEK-293 cells (2 × 10⁶) were plated on culture dishes 6 cm in diameter, incubated at 37 °C for 24 h, then transfected with 4 µg of pcDNA3 or syndecan-4-pcDNA3 in the presence of 25 µg of chloroquine with the use of alkaline CaCl₂ reagents. After 7 h at 37 °C, the medium was replaced with 4 ml of fresh DMEM containing 10% (v/v) FBS.

Cell lysis and immunoblotting
After cultures had been washed twice with PBS (500 µl per 10 cm plate), the cells were lysed in RIPA buffer [50 mM Tris/HCl (pH 8.0)/150 mM NaCl/1% (v/v) Nonidet P40]/10 mM NaF/2 mM Na₂VO₃ containing a protease inhibitor cocktail (1 µg/ml aprotinin, 1 µg/ml antipain, 5 µg/ml leupeptin, 1 µg/ml pepstatin A and 20 µg/ml PMSF). Cell lysates were clarified by centrifugation at 10 000 g for 15 min at 4 °C, denatured with SDS/PAGE sample buffer, boiled and analysed by SDS/PAGE. Proteins were transferred on PVDF membranes (Amersham Pharmacia Biotech) and probed with an appropriate primary antibody followed by species-specific horseradish peroxidase-conjugated secondary antibodies (Amersham Life Science). Signals were detected by enhanced chemiluminescence (ECL⁺; Amersham Life Science).

ERK activity assay
ERK activity in cell lysates was measured by phosphorylation of myelin basic protein (MBP) as a substrate. ERK was immunoprecipitated by incubating 500 µg of cell lysate with 2 µg of anti-ERK2 antibody for 2 h and then adding 20 µl of Protein G-agarose (Santa Cruz Biotechnology, Los Angeles, CA, U.S.A.). After incubation for 2 h at 4 °C, the immune complex was recovered by centrifugation and washed three times with lysis buffer and once with washing buffer [20 mM Heps (pH 8.0)/10 mM MgCl₂/200 mM Na₂VO₃/1 mM dithiothreitol/1 mM EGTA]. The ERK pellets were resuspended in reaction buffer [20 mM Heps (pH 8.0)/10 mM MgCl₂/1 mM dithiothreitol/1 mM EGTA/200 µM ATP] containing 5 µg of MBP and 2 µCi of [γ-³²P]ATP in a final volume of 20 µl. Kinase reactions took place for 15 min at room temperature and were stopped by the addition of 5 × SDS sample buffer. After resolution by SDS/PAGE [15% (w/v) gel], samples were dried and autoradiographed.
RESULTS

HepII regulates integrin-mediated cell spreading

Because it is known that cytoskeleton organization is co-operatively regulated by two functional domains of FN, the cell-binding domain and HepII [13,16], we investigated the effect of HepII on integrin-mediated cell spreading, adhesiveness on FN and the formation of focal adhesions and stress fibres during cytoskeleton organization. When REFs were plated on FN, cells attached, spread and started to form focal contacts within 15 min. By 30 min most cells had completed spreading and had developed actin stress fibres as seen by staining with TRITC-conjugated phalloidin (Figure 1A). In contrast, cells on FN120 exhibited membrane ruffling at 15 min; by 30 min cells had completed spreading but had failed to form focal adhesions or actin stress fibres. Tyrosine-phosphorylated proteins were found

Figure 1 Decreased stress fibre formation is correlated with a lack of HepII

(A) REFs were plated on either FN-coated or FN120-coated plates for the periods (in min) indicated at the top, then stained with TRITC-conjugated phalloidin for the observation of actin filaments or with anti-phosphotyrosine antibody (P-Tyr). (B) HepII fragments (31K) were added to cells on FN120-coated plates. To observe the focal contacts, REFs were stained with anti-paxillin antibody. Fluorescence micrographs are shown at 30 min. Scale bars, 10 μm.
Figure 2 HepII enhances the adhesion of REFs on FN

REFs were plated on FN-coated or FN120-coated tissue culture plates for the periods indicated in parentheses (in min) and centrifuged the inverted plates for 30 min at 2000 g. The number of detached cells was counted with a haemocytometer and percentage of cells detached is shown. Results are means ± S.E.M. for three independent experiments. Open bars, detached cells from FN; hatched bars, detached cells from FN120.

Figure 3 Integrin-mediated FAK phosphorylation is dependent on HepII

REFs were plated for the periods indicated at the top (in min) on either FN-coated or FN120-coated tissue culture plates in SFM. After being plated, cells were lysed with RIPA buffer and each tyrosine phosphorylation of pp125FAK was analysed with phosphorylation-specific antibodies (α) against pp125FAK (anti-p397, anti-p407, anti-p577 and anti-p861) followed by stripping and reprobing with anti-pp125FAK antibody. Extensively in focal contacts of REFs on FN by 30 min but were not detected in focal structures in cells on FN120 even after 60 min. The addition of the soluble HepII-domain fragment, 31K, enhanced the formation of focal adhesions and stress fibres (Figure 1B). This confirmed that, during FN-mediated cell spreading, the C-terminal HepII binding domain was required for integrin-mediated tyrosine phosphorylation [20], leading to the formation of actin stress fibres and focal adhesions [13,14] and the insertion of tyrosine-phosphorylated components into focal adhesions.

Figure 4 HepII is not involved in integrin-induced MAPK activation

REFs were plated for the periods indicated at the top (in min) on either FN-coated or FN120-coated tissue culture plates in SFM. (A) Integrin-mediated MAPK activation was assessed either by using phospho-specific antibodies (anti-phospho-ERK2) followed by stripping and reprobing with anti-ERK2 antibody or with an ERK2 immune complex kinase assay. Abbreviations: α-pERK, anti-(phospho-ERK); α-Erk1/2, anti-ERK1/2. (B) Autoradiography of phosphorylated MBP. HepII fragments (31K) and the most active peptide from this domain (peptide V) were added separately to two of the samples. Both ERK1 (p44) and ERK2 (p42) are indicated at the right. Abbreviation: α-Erk2, anti-ERK2.

We next investigated cell-substrate adhesiveness with an inverted centrifugal detachment assay (Figure 2). After REFs had been spread on either FN or FN120, each culture dish was centrifuged and the number of detached REFs was quantified. At a force that would result in the detachment of approx. 25% of the cells on FN120, only 2% of REFs on FN were detached at 60 min after plating. This was consistent with the concept that, in addition to the cell-binding domain, HepII is required for REFs to adhere tightly on FN.

Overall tyrosine phosphorylation in total cell lysates was much less in cells on FN120 substrates than in those on FN substrates at all observed times (results not shown) [20]. During the early stage of spreading, a major decrease in tyrosine phosphorylation was found in paxillin and pp125FAK [20]. To define the relationship between the tyrosine phosphorylation of pp125FAK and MAPK activation, we investigated different tyrosine phosphorylations of pp125FAK with phosphorylation-site-specific antibodies after replating on either FN or FN120 (Figure 3). After plating of the cells, increased phosphorylation of tyrosine-397 was observed on FN but to a smaller extent on FN120, implying that HepII was involved in the phosphorylation of tyrosine-397. Phosphorylation of tyrosine-577 and tyrosine-861 showed a similar pattern to that of tyrosine-397, with a relatively low level of tyrosine phosphorylation overall. With tyrosine-407 there was little difference between tyrosine phosphorylations. These results support previous reports that the HepII, together with the cell-binding domain, co-operatively regulates the tyrosine phosphorylation of pp125FAK [8,20]. It is therefore likely that HepII is involved in several integrin-mediated cytoskeleton...
Heparin-binding domain, syndecan-4 and mitogen-activated protein kinase activation

Heparin-binding domain, syndecan-4 and mitogen-activated protein kinase activation

Figure 5 Integrin-mediated cell spreading is sufficient for growth-factor-induced MAPK activation

REFs were plated for 2 h on either FN-coated or FN120-coated tissue culture plates in SFM and treated with 50 ng/ml PDGF (A) or EGF (B) for the periods indicated at the top (in min). MAPK activation was assessed by using phospho-specific antibodies (phospho-ERK2) followed by stripping and reprobing with anti-ERK2 antibody (ERK2). Both ERK1 (p44) and ERK2 (p42) are indicated at the right. The positions of molecular mass markers are indicated at the left. Abbreviations: α-pTyr, anti-phosphotyrosine; α-pErk, anti-(phospho-ERK); α-Erk1/2, anti-ERK1/2.

HepII-mediated cytoskeleton organization did not affect integrin-mediated MAPK activation

Tyrosine phosphorylation of pp125FAK has been demonstrated in cells on FN substrates and pp125FAK is a major phosphotyrosine protein with a crucial role in integrin-mediated tyrosine phosphorylation of cytoskeleton proteins and in MAPK activation [1,2,6,17]. We therefore investigated the effect of HepII on integrin-mediated MAPK activation.

REFs were plated on FN and FN120; the activation of both ERK1 and ERK2 was monitored by immunoblotting with phospho-specific antibodies (Figure 4A) or by an ERK immune complex kinase assay with MBP as a substrate (Figure 4B). Because of weak attachment of cells, we could not analyse MAPK activation in cells on 31K-coated plates (results not shown). Although lysates from cells on FN120 substrates showed marked decreases in integrin-mediated tyrosine phosphorylation, the activation of both ERK1 and ERK2 was very similar to that of lysates from cells on FN substrates. The addition of either peptide V or 31K induced the formation of focal adhesions and stress fibres (Figure 1) [13,14] but did not affect integrin-mediated MAPK activation. This implies that, although HepII regulates cytoskeleton organization, it is not involved in integrin-mediated MAPK activation. In other words, the interaction of the cell-binding domain with integrin is sufficient to regulate MAPK activation.

It is known that cytoskeleton organization is essential for growth-factor-mediated MAPK activation; pp125FAK is an important linker between the growth-factor-receptor and integrin signalling pathways [21–23]. Our results above indicate that HepII transduces one or more signals for the regulation of cytoskeleton organization, together with pp125FAK. To investigate the effect of HepII on growth-factor-mediated MAPK activation, REFs were incubated for 2 h on FN and on FN120 substrates to allow cell spreading and cytoskeleton organization [13]. Then 50 ng/ml epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) was added. Although there was a clear decrease in tyrosine phosphorylation in lysates from cells on FN120, both EGF and PDGF activated MAPK to similar extents on each substrate (Figure 5). Thus the interaction of integrin with the cell-binding domain is sufficient to form a

© 2001 Biochemical Society
Syndecan-4 regulates adhesion and spreading on FN but not integrin-mediated MAPK activation

Figure 7  Syndecan-4 regulates adhesion and spreading on FN but not integrin-mediated MAPK activation

(A) REFs and REF-Syn4 cells were plated on FN-coated (a, c, e, g) and on FN120-coated (b, d, f, h) tissue culture plates in SFM for the periods indicated in parentheses (in min). Before (a–h) and after (a′–h′) centrifugation for 30 min at 2000 g, cells on the plates were photographed by phase-contrast microscopy with a digital camera. (B) Detached cells were collected by centrifugation and counted with a haemocytometer. The percentages of cells detached are shown: open columns, detached cells from FN; hatched columns, detached cells from FN120. Results are means ± S.E.M. for three independent experiments. (C) REFs or REF-Syn4s were plated for the periods indicated and integrin-mediated MAPK activation was assessed as described in the legend to Figure 5.

Syndecan-4 regulates integrin-mediated cell spreading but not integrin-mediated MAPK activation

HepII interacts with the cell-surface heparan sulphate proteoglycan syndecan-4, which is known to be involved in later stages of the formation of focal adhesions and stress fibres [13,14]. We reasoned that if HepII regulated integrin-mediated signals, this might be through syndecan-4.

We first investigated the effect of the heparan sulphate chain on integrin-mediated MAPK activation. After REFs had been pretreated with 75 mM NaClO₄ for 24 h to inhibit the maturation of the heparan sulphate chain on the cell-surface heparan sulphate proteoglycan, integrin-mediated MAPK activation was analysed. As shown in Figure 6, decreased sulphation of heparan sulphate did not affect integrin-mediated MAPK activation. We then investigated whether syndecan-4 could regulate integrin-mediated signals. REFs were compared with REFs overexpressing full-length syndecan-4 core protein (REF-syn4) in terms of spreading, adhesion on FN and integrin-mediated MAPK activation (Figure 7). REF-syn4 showed better spreading (Figure 7A) and stronger adhesion on FN as judged by the inverted centrifugal detachment assay (Figure 7B). However, integrin-mediated MAPK activation was not affected by the overexpression of syndecan-4 (Figure 7C). These results were similar to those in HEK-293 cells transiently transfected with full-length syndecan-4. As shown in Figure 8, overexpression of syndecan-
4 enhanced both cell spreading (results not shown) and adhesion (Figure 8A) on FN, confirming the role of HepII. However, syndecan-4 did not affect integrin-mediated MAPK activation (Figure 8B). Therefore both HepII and its receptor syndecan-4 regulated cytoskeleton organization but not integrin-mediated MAPK activation.

**DISCUSSION**

In the present study we have shown that HepII and its receptor syndecan-4 regulate integrin-mediated cell spreading and adhesion on FN but are not involved in the regulation of integrin-mediated MAPK activation. In addition, we have demonstrated that a lack of HepII does not prevent EGF-regulated or PDGF-regulated MAPK activation.

Integrin engagement itself induces tyrosine phosphorylation on cytoskeleton proteins including Paxillin and pp125FAK, and regulates cytoskeleton organization [1,2,5,17]. However, this tyrosine phosphorylation is not sufficient for the formation of focal adhesions and stress fibres [13] or for tight adhesion to the substrate. These require one or more additional signals from HepII but it is not known how this signalling is done.

One or more signals from HepII mediate additional tyrosine phosphorylation on pp125FAK, demonstrating that HepII can regulate integrin-mediated tyrosine phosphorylation signals [20]. In particular, decreased tyrosine phosphorylation of pp125FAK in cells on FN120 substrates in comparison with those on FN was clear. Because pp125FAK is crucial to the MAPK cascade [6,17,21–23], we expected that a decrease in tyrosine phosphorylation of pp125FAK on tyrosine-397 would result in a decrease in the formation of the pp125FAK/Grb2 complex. This in turn would decrease MAPK activation. However, integrin-mediated MAPK activation was not affected by a lack of interaction with HepII. Therefore, during cell spreading on FN, pp125FAK might have two separate roles: one involved in adhesion-mediated MAPK activation and the other in initial attachment and spreading. If this is so, MAPK activation requires a minimum level of pp125FAK phosphorylation, whereas the formation of focal adhesions and stress fibres require additional tyrosine phosphorylation on pp125FAK and its substrate Paxillin. This might be because integrin-mediated MAPK activation is a rapid response, whereas focal adhesion formation occurs only after spreading. Alternatively, these results could be explained by a pp125FAK-independent pathway for MAPK activation [19]. In both cases, however, HepII-mediated cytoskeleton organization is not needed for integrin-mediated MAPK activation.

HepII interacts with the extracellular domain of syndecan-4; this interaction induces the formation of focal adhesions and stress fibres [13,15]. It is therefore likely that syndecan-4 regulates HepII-mediated signal transduction. Syndecan-4 overexpression increased initial cell spreading and adhesion on FN but did not alter integrin-mediated MAPK activation. Thus the regulation of cytoskeleton organization by syndecan-4 is not correlated with MAPK activation. Therefore, although the two signalling mechanisms co-operate in adhesion, integrin signalling, independently of syndecan-4 signalling, is sufficient for MAPK activation. Thus all signals from FN are segregated by specific receptors and these signals are selectively transduced in different responses to the same matrix molecule.

We are grateful to Dr J. R. Couchman and Dr A. Woods for the gift of REF-syn4 cells, for advice and for reading the manuscript critically. This work was supported by Korea Science and Engineering Foundation (KOSEF) through the Center for Cell Signaling Research at Ewha Womans University, MOST through the Womans University Research Fund and in part by Korea Research Foundation Grant (KRF-99-042.D00096 to E.S.O). J.K. was supported by a fellowship from Brain Korea 21 project.

**REFERENCES**


Received 15 June 2001/4 September 2001; accepted 24 September 2001