Rat embryo fibroblasts require both the cell-binding and the heparin-binding domains of fibronectin for survival

Jinsook JEONG*, Innoc HAN*, Yangmi LIM*, Jungyean KIM*, Ilseon PARK*, Anne WOODS†, John R. COUCHMAN† and Eok-Soo OH*[1]

*Division of Molecular Life Sciences and Center for Cell Signaling Research, Ewha Womans University, Daehyun-dong, Seodaemoon-Gu, Seoul 120-750, South Korea, and †Department of Cell Biology and Cell Adhesion and Matrix Research Center, University of Alabama at Birmingham, Birmingham, AL 35294, U.S.A.

Fibronectin (FN) is known to transduce signal(s) to rescue cells from detachment-induced apoptosis (anoikis) through an integrin-mediated survival pathway. However, the functions of individual FN domains have not been studied in detail. In the present study we investigated whether the interaction of the cell-binding domain of FN with integrin is sufficient to rescue rat embryo fibroblasts (REFs) from detachment-induced apoptosis. REFs attached and spread normally after plating on substrates coated with either intact FN or a FN fragment, FN120, that contains the cell-binding domain but lacks the C-terminal heparin-binding domain, HepII. REFs on FN maintained a well-spread fibroblastic shape and even proliferated in serum-free medium at 20 h after plating. In contrast, previously well-spread REFs on FN120 started losing fibroblastic shape with time and detached from FN120-coated plates after approx. 8 h. Nuclear condensation indicated apototic cell death. This was due to the decreased activity/stability of focal adhesion kinase (pp125FAK) in the absence of HepII domain. A peptide in the HepII domain [peptide V, WQPPRARI (single-letter amino acid codes)], which has previously been implicated in cytoskeletal organization, rescued apoptotic changes. Consistently, pp125FAK phosphorylation was increased, and both cleavage of pp125FAK and activation of caspase 3 on FN120 were partly blocked by peptide V. Thus the interaction of the cell-binding domain with integrin has a major role in cell survival but is itself not sufficient for cell survival. One or more additional survival signals come from the HepII domain to regulate pp125FAK activity/stability.

Key words: anoikis, caspase, focal adhesion kinase, integrin.

INTRODUCTION

Fibronectin (FN), a well-studied extracellular matrix (ECM) molecule, regulates multiple cellular functions such as cytoskeleton organization and gene expression through interaction with cell-surface receptors [1,2]. It has been shown that FN transduces a signal to rescue cells from detachment-induced apoptosis (anoikis) and that integrin-mediated signalling pathway(s) are involved in this protective effect [2–6]. It is thought that the cell-binding domain of FN interacting with integrin $\alpha5\beta1$ regulates cell survival by the activation of focal adhesion kinase (pp125FAK) [5,6]. This integrin–pp125FAK signalling might regulate cell survival by one of the following mechanisms: (1) suppression of the p53-dependent apoptosis in fibroblasts [7], (2) the activation of Akt through phosphoinositide 3-kinase to regulate the Bcl-2 cell survival pathway [8,9], or (3) other pathways [10–13]. Thus pp125FAK activity has a crucial role in protecting cells from anoikis [6]. Because FN is composed of several functional domains, cells interact with FN at multiple sites through specific functional receptors. However, the question of which domain of FN specifically regulates cell survival has not been well studied. In addition, it is not clear that the interaction of the cell-binding domain of FN with integrin is sufficient for this signal.

To study the function of the cell-binding domain, synthetic peptides containing the Arg-Gly-Asp (RGD) motif have been used extensively as inhibitors of integrin–ligand interactions because the integrin-recognition RGD motif is found in many ligands. Many cell lines, including fibroblasts and endothelial, mesangial, thyroid and Jurkat cells treated with RGD, have been reported to undergo apoptosis [14–18]. However, in all these experiments, to interrupt the interaction of FN with integrin, large amounts of peptide (0.5–1.0 mg/ml) were used, indicating that the RGD peptide has a limited ability to inhibit FN–integrin interactions. Apoptotic human endothelial cells plated on the polymer poly(hydroxyethylmethacrylate) were not rescued from apoptosis by RGD-coated microbeads but only by intact FN [19]. This indicates that the attachment of cells to the matrix or the binding of integrin might not itself be sufficient for maintaining cell survival and that cells might need some minimal degree of cytoskeleton organization or one or more additional signals for cell survival. It has recently been reported that exogenous RGD-containing peptides can induce apoptosis directly without any requirement for integrin-mediated cell clustering or signals through the direct activation of caspase 3 [20]. Although it might not explain all apoptosis induced by RGD peptides, it could provide an alternative molecular explanation for exogenous apoptosis induced by RGD-containing peptides.

It has been reported from two laboratories that there are one or more possible additional signals for cell survival. A peptide [WQPPRARI (single-letter amino acid codes); peptide V] derived from the C-terminal heparin-binding domain of FN (HepII) disrupted the ligation of integrin $\alpha5\beta1$ on FN, and ultimately induced the apoptosis of human umbilical-vein endothelial cells (HUVEC). The HepII domain might be needed to stabilize the interaction of integrin $\alpha5\beta1$ with the cell-binding domain, because

Abbreviations used: Ac-DEVD-AMC, *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; DAPI, 4,6-diamidino-2-phenylindole; ECM, extracellular matrix; ERK, extracellular signal-regulated protein kinase; FN, fibronectin; FN120, fibronectin fragment lacking C-terminal heparin-binding domain; HepII, C-terminal heparin-binding domain; HUVEC, human umbilical-vein endothelial cells; MAP, mitogen-activated protein; PKC, protein kinase C; pp125FAK, focal adhesion kinase; RGD, Arg-Gly-Asp; REF, rat embryo fibroblast; SFM, serum-free medium.

[1] To whom correspondence should be addressed (e-mail ohes@mm.ewha.ac.kr).

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both the tyrosine phosphorylation of pp125FAK and the expression of anti-apoptotic Bcl-2 protein, which are normally induced by the adhesion of HUVEC to FN, were reversed by peptide V [18]. Alternatively, the HepII domain itself might transduce an additional cell survival signal for HUVEC. When cultured on substrates coated with a recombinant mutant of FN lacking the HepII domain, human periodontal ligament fibroblasts showed decreased pp125FAK levels and increased apoptosis, which were again rescued by intact FN [21]. Interestingly, both reports imply the involvement of the HepII domain in cell survival.

The HepII domain can regulate cytosleketon organization. Previous studies with FN fragments have shown that this domain can regulate the late phase of focal adhesion and stress fibre formation [22,23], possibly via activation of the small G-protein Rho [24]. The cell-surface heparan sulphate proteoglycan syndecan-4 has been implicated as being the receptor for the HepII domain (reviewed in [25,26]). Thus cell survival might be regulated not only by integrins but also by collaborative actions from other receptors that interact with the HepII domain. We therefore investigated the role of the HepII domain in the regulation of integrin-mediated cell survival signals.

EXPERIMENTAL

Materials and antibodies

Synthetic peptides corresponding to the most active peptide sequence, peptide V, from the C-terminal heparin domain of FN were synthesized by using an improved version of the solid phase method (Peptron, Taejon, South Korea).

Monoclonal anti-phosphotyrosine (anti-pTyr) antibodies (4G10), anti-Src kinase antibody (clone GD11) and pp125FAK were obtained from Upstate Biotechnology (Lake Placid, New York, NY, U.S.A.). Monoclonal anti-paxillin antibodies were from Transduction Laboratories (Lexington, KY, U.S.A.) and polyclonal anti-(caspase 3) antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). [γ-^{32}P]ATP was obtained from Amersham Pharmacia Biotech. Affinity purified FITC-conjugated goat anti-mouse IgG was from Kirkegaard & Perry (Gaithersburg, MD, U.S.A.). Tetramethylrhodamine-isothiocyanate-conjugated phalloidin, enolase, 4,6-diamidino-2-phenylindole (DAPI) and other chemicals were purchased from Sigma.

Cell culture and plating experiments

Rat embryo fibroblasts (REFs) were maintained in α-modified Eagle's medium (Gibco BRL) supplemented with 5 % (v/v) fetal bovine serum together with penicillin (100 units/ml) and streptomycin (10 μg/ml, Gibco BRL). For cell adhesion assays, cells were starved in culture medium containing 0.2 % (v/v) fetal bovine serum for 24 h, then plated on tissue culture plates or coverslips coated with either purified FN or the N-terminal FN fragment lacking the HepII domain (FN120; Gibco BRL) as described previously [22]. In brief, both FN and FN120 were diluted in serum-free medium (SFM), added to tissue culture plates (final coating concentration 2 μg/cm^2 for FN or 1.3 μg/cm^2 for FN120) and incubated at room temperature for at least 1 h to allow adsorption on plates. After being washed with PBS, plates were blocked with 0.2 % heat-inactivated BSA for 1 h and then washed three times with SFM (5 min each wash). During equilibration of SFM with FN- or FN120-coated tissue culture plates at 37 °C under air/CO_2 (9:1), REFs (approx. 80–90 % confluent) were detached with 0.05 % trypsin/0.53 mM EDTA (Gibco BRL), suspended in SFM containing 0.25 mg/ml soybean trypsin inhibitor and then centrifuged. Cells were resuspended in SFM, maintained at 37 °C for 30–60 min, plated on either FN-coated or FN120-coated plates, then incubated at 37 °C for various periods. To study the effect of the HepII domain, peptide V (0.01 μg/ml) was added 2 h after plating. For suspension culture, cells were plated on heat-inactivated BSA-coated Petri dishes. In every experiment, 15 min after plating, REFs were washed with fresh SFM to remove unattached cells.

Microscopic analysis

REFs, plated on the coated coverslips, were fixed with 4 % (w/v) paraformaldehyde in PBS for 10 min at room temperature, permeabilized with 0.5 % (v/v) Triton X-100 in PBS and blocked with 0.5 % BSA/0.05 % gelatin in PBS for 45 min. Cells were then stained with DAPI for 10 min. After being rinsed three times with PBS and then mounted in 50 % (v/v) glycerol in PBS, cells were photographed at $\times$40 magnification with a fluorescence microscope (Zeiss, Oberkochen, Germany). For cell morphology, cells were observed under an inverted microscope (Zeiss) at $\times$40 magnification.

Caspase assay

After being plated on dishes as described above, REFs were washed three times with PBS at the indicated time (floating cells were combined with attached cells) and a hypotonic solution (20 mM Tris/HCl, pH 7.5) was added to release cytosol. Caspase activity in the cytosol was assayed in assay buffer [20 mM Pipes (pH 7.4)/100 mM NaCl/10 mM dithiothreitol/0.1 mM EDTA/0.1 % CHAPS/10 % (w/v) sucrose] with 10 μM *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) (Alexis Corporation) as substrate. Released AMC from the substrate was analysed at excitation and emission wavelengths of 360 and 460 nm respectively for 30 min at 30 °C with a microplate fluorescence reader (Bio-Tec Instruments, Winooski, VT, U.S.A.).

Immunoprecipitation and immunoblotting

After cultures had been washed twice with PBS (500 μl per 10 cm diameter 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 Na_3VO_4] containing a protease inhibitor cocktail (1 μg/ml aprotinin/1 μg/ml antipain/5 μg/ml leupeptin/1 μg/ml pepstatin A/20 μg/ml PMSF). Cell lysates were clarified by centrifugation at 10000 ***g*** for 15 min at 4 °C, denatured with SDS/PAGE sample buffer, boiled and then analysed by SDS/PAGE. For immunoprecipitations, each sample (containing 200–1000 μg of total protein) was incubated for 2 h with the relevant antibody at 4 °C; this was followed by the addition of Protein G–Sepharose beads (Sigma) for 1 h. Immune complexes were collected by centrifugation, washed three times with Nonidet P40 buffer, resuspended in SDS sample buffer, boiled and then analysed by SDS/PAGE. Proteins were transferred to PVDF membranes (Amersham Pharmacia Biotech) and probed with appropriate primary antibodies, followed by species-specific horseradish peroxidase-conjugated secondary antibodies (Amersham Life Science). Signals were detected by enhanced chemiluminescence (ECL®; Amersham Life Science).

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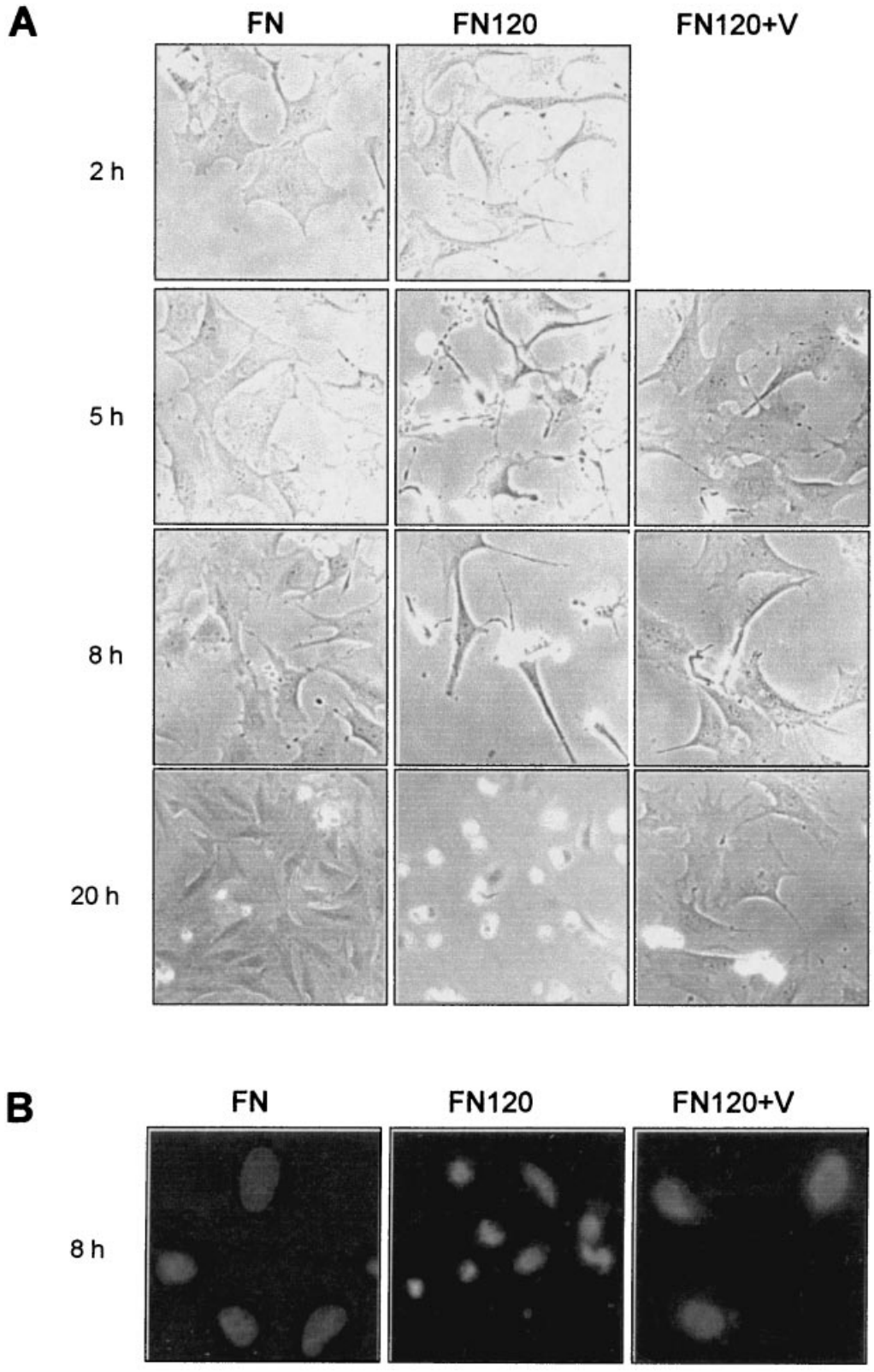

Figure 1 HepII domain is required for cell survival

(**A**) REFs were plated on to FN or FN120 substrates in SFM and incubated at 37 °C for the indicated durations. Peptide V (0.01 μg/ml) was added 2 h after plating on FN120. Phase-contrast micrographs are shown (magn. $\times$36). (**B**) Cells incubated as above were stained with DAPI at 8 h after plating on either FN or FN120 substrates. Fluorescence micrographs are shown (magnification $\times$36).

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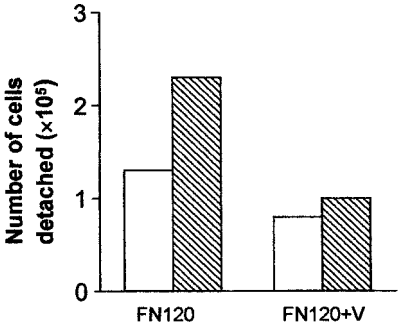

Figure 2 Peptide V significantly decreases the detachment of cells on FN120

REFs (4×10^6) were plated on FN120 substrates in SFM and incubated at 37 °C. Detached REFs were collected at 8 h (open bars) or 10 h (hatched bars) and counted with a haemocytometer. Peptide V (0.01 μg/ml) was added 2 h after plating on FN120.

RESULTS

HepII domain is needed for the survival of REFs

To study the role of the cell-binding domain of FN in cell survival, we used either whole FN or FN120 as the substrate for REF cells. FN120 contains the intact cell-binding domain but lacks Hep II [22]. We investigated cell attachment, spreading, morphology and cell survival (Figure 1). REFs on FN showed normal spreading and cytoskeleton organization in SFM, maintained a well-spread fibroblastic shape (Figure 1A) and even proliferated in SFM by 20 h after plating. Thus the interaction of REFs with FN was sufficient for transducing one or more cell survival signals under our experiment conditions. REFs on FN120 showed normal attachment and spreading at early time points after plating. However, well-spread REFs on FN120 suddenly started losing fibroblastic shape at approx. 5–6 h, becoming thin with retraction fibres. By 8 h, 30 % of cells on FN120 had become rounded and had detached (Figure 2) and showed nuclear condensation and fragmentation, indicating apotosis (Figure 1B). At 10 h, approx. 60 % of cells on FN120 were detached (Figure 2); at 20 h, most cells were detached from the plates, with those remaining being round (Figure 1A).

We next tested whether the HepII domain by itself could rescue REF cell survival on FN120. REFs were allowed to attach and spread on FN120 before the addition of peptide V, previously implicated in cytoskeletal organization [23]. Peptide V blocked the retraction of cells on FN120 (Figure 1A) and also prevented nuclear condensation at 8 h (Figure 1B). Consistently, detachment of cells from FN120 was significantly decreased by the addition of peptide V (Figure 2). Thus, although interaction of the cell-binding domain with integrin is sufficient for initial attachment and spreading, prolonged adhesion and survival require an additional signal that can be provided by a constituent peptide from the HepII domain.

Because the first step in apoptosis is to round up and detach from the substrate, it is possible that REFs on FN120 detached owing to weaker interactions and that this detachment itself, rather than a lack of more direct signalling, resulted in apoptosis. We therefore investigated the possibility that major cell adhesion receptor integrin was functional under our experimental conditions (Figure 3). In cells on either FN or FN120, integrin β1 levels were similar until 6 h, when REFs on FN120 started to lose fibroblastic shape (Figure 3, bottom panel). Although lysates from REFs on FN120 showed decreases in tyrosine phosphorylation (Figure 3, top panel), the activation of both extracellular signal-regulated protein kinase (ERK) 1 and ERK2 was very similar to that of lysates from REFs on FN (middle panels). Therefore integrin at the surface of REFs on either FN or FN120 normally transduced an integrin-mediated mitogen-activated protein (MAP) kinase activation signal. We also tested whether detached cells from FN120-coated plates could be rescued by being replated on FN-coated plates; however, most of these cells failed to attach to FN (results not shown). These results all strongly supported the idea that cells on FN120 were committed to apoptosis not because of detachment but because of the lack of a supportive survival signal from the HepII domain.

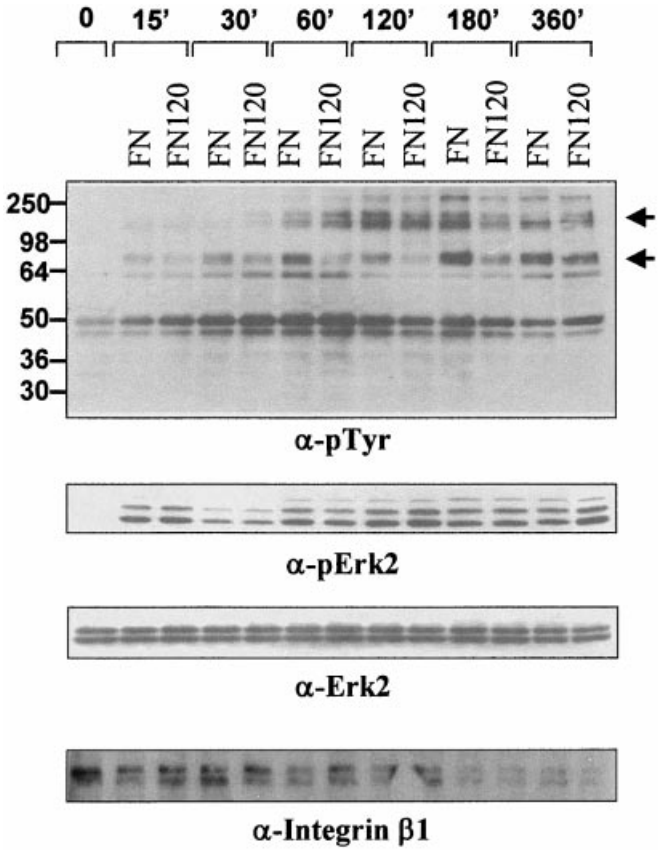

Figure 3 Integrin-mediated MAP kinase activation is normal on FN120 substrate

REFs were plated for the indicated durations (min, shown as ') on tissue culture plates coated with either FN or FN120 in SFM. Decreased tyrosine phosphorylation of total cell lysate on FN120 was shown by immunoblotting with anti-('α-')pTyr (α-PTyr) (top panel). The level of integrin β1 was also shown by immunoblotting with anti-(integrin β1) (α-integrin β1) (bottom panel). Integrin-mediated MAP kinase activation was assessed by using phospho-specific antibodies against phospho-ERK2 (α-pErk2) followed by stripping and reprobing with anti-ERK2 antibody (α-Erk2).

HepII domain regulates integrin-mediated pp125FAK activity/stability

During early spreading on FN120, a major decrease in tyrosine phosphorylation was found in proteins of molecular mass approx. 68 and 125 kDa (see Figure 3). We therefore investigated further differences in paxillin and pp125FAK phosphorylation in cells on FN or FN120 (Figure 4). When paxillin and pp125FAK were immunoprecipitated from cells on either FN or FN120 substrate,

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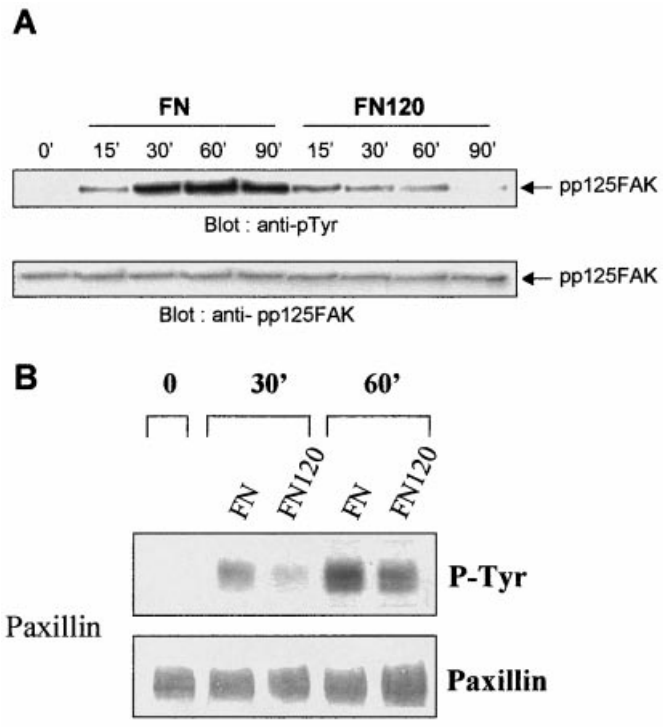

Figure 4 Decreased integrin-mediated tyrosine phosphorylation of pp125FAK in the absence of the HepII domain

REFs were plated for the indicated durations (min, shown as ') on tissue culture plates coated with either FN or FN120 in SFM. Tyrosine phosphorylation of pp125FAK (**A**) and paxillin (**B**) was analysed by immunoprecipitation with each antibody followed by immunoblotting with anti-pTyr (upper panels). The amounts of proteins in the immunocomplexes were monitored by stripping and reblotting with anti-pp125FAK or anti-paxillin (lower panels).

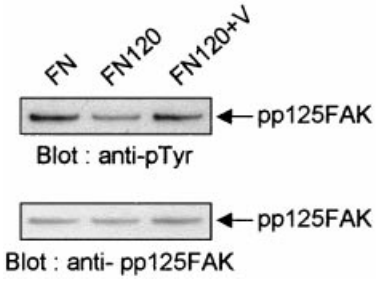

Figure 5 Peptide V restores the tyrosine phosphorylation of pp125FAK in cells on FN120 substrates

REFs were plated on FN or FN120 substrates in SFM and incubated at 37 °C to allow cell spreading. Peptide V (final concentration 0.01 μg/ml) was added 2 h after the plating of cells on FN120 substrates; the incubations continued for a further 1 h. Tyrosyl phosphorylation of pp125FAK was analysed by immunoprecipitation with anti-pp125FAK followed by immunoblotting with anti-pTyr (upper panel). The amounts of proteins in the immunocomplexes were monitored by stripping and reblotting with anti-pp125FAK (lower panel).

immunoblotting with anti-phosphotyrosine showed a major decrease in their phosphorylation. This decreased tyrosine phosphorylation of pp125FAK was evident at 3 h after plating; peptide V rescued the defective tyrosine phosphorylation of pp125FAK (Figure 5, lane 3). This implies that the HepII domain, co-operatively with the cell-binding domain, regulates

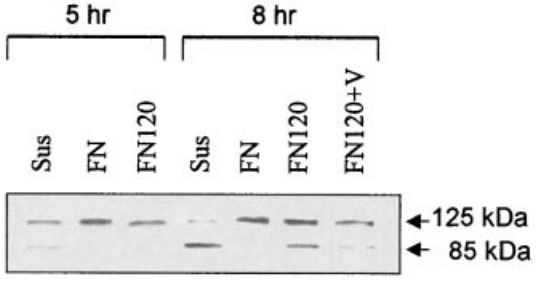

Figure 6 Cleavage of pp125FAK in cells on FN or FN120 substrates

Cells were incubated as described in the legend to Figure 4. Total cell lysates were analysed at 8 h by using antibody against pp125FAK. Arrows indicate intact pp125FAK and the cleaved 85 kDa fragment. Peptide V was added 2 h after plating on FN120. Sus, suspension culture.

the tyrosine phosphorylation of pp125FAK and its substrate, paxillin.

The importance of pp125FAK in transducing an anti-apoptotic signal is well known [6,27] and cleavage of pp125FAK by caspase has been demonstrated during apoptosis [28–30]. We therefore investigated the stability of pp125FAK (Figure 6). Cleavage of pp125FAK did not occur in cells on plates coated with either FN or FN120 until 5 h. However, although no cleavage was evident in cells on FN (Figure 6, lane 5), cleavage of pp125FAK was observed in lysate from cells on FN120-coated plates at 8 h (lane 6). This was less than that in suspended cultures (Figure 6, lane 4), so major inhibition of cleavage might be through interaction with the cell-binding domain, with the HepII domain augmenting the stability of pp125FAK. Consistent with its restoration of pp125FAK tyrosine phosphorylation, peptide V further blocked pp125FAK cleavage (Figure 6, lane 8). Thus the HepII domain additionally regulates both the integrin-mediated activity and stability of pp125FAK.

HepII domain is required for the inhibition of caspase 3 activation

It has been reported that several caspases, including caspase 3, are activated during detachment-induced apoptosis [12,13,31]. We therefore investigated the cytosolic caspase activity in lysates from cells on plates coated with either FN or FN120, or from suspended cells as control (Figure 7). Consistent with morphology was the observation that caspase 3-like activity was minimal in all cell lysates at early time points (results not shown) and by 8 h was evident in suspended cells. Caspase 3-like activity was significantly decreased in comparison with suspended cells when cells had been plated on FN or FN120 substrate on the basis of an assay *in vitro* with Ac-DEVD-AMC as substrate (Figure 7A, left panel). However, apoptosis occurred in cells on FN120 substrates but not those on FN substrates, indicating that the level of inhibition of caspase activity is important. Consistent with its anti-apoptotic effect, peptide V further inhibited caspase 3-like activity in cells on FN120 substrates (Figure 7A, right panel). Similar results were found by Western blotting with antibody against caspase 3. The production of the cleaved, active form of caspase 3 was significantly decreased by FN substrates (Figure 7B, compare lanes 1 and 2) but much less by FN120 substrates (lane 3). Consistent with morphological recovery was the observation that the activation of cytosolic caspase 3 was decreased in cells on FN120 substrates by the addition of peptide V (Figure 7B, compare lanes 3 and 4). Thus, during cell–FN interaction, integrin engagement has a major role in blocking the

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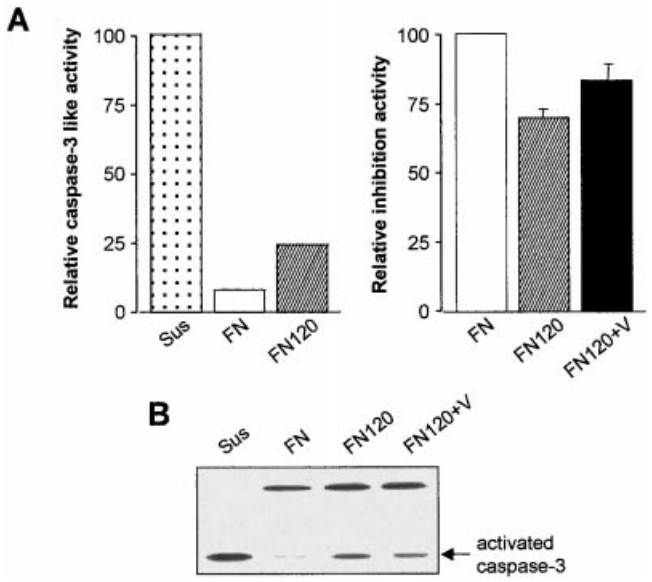

Figure 7 HepII domain is required for the inhibition of caspase 3 activation

Cells were incubated as described in the text. (**A**) Cytosol was isolated at 8 h and caspase activity was analysed with Ac-DEVD-AMC as substrate. Results are representative of three separate experiments. (**B**) Cytosolic fractions were prepared at 8 h and caspase 3 activity was analysed by SDS/PAGE [12.5% (w/v) gel] followed by immunoblotting with anti-(caspase 3). The arrow indicates activated caspase 3. Results are representative of three separate experiments.

activation of one or more apoptotic signals, with the augmentation of survival signals by the HepII domain.

DISCUSSION

It has previously been shown that the cell-binding domain of FN interacting with integrin $\alpha5\beta1$ regulates cell survival by the activation of pp125FAK [5,6]. Thus pp125FAK activity has a crucial role in protecting cells from anoikis [6]. However, we show here that this interaction is not sufficient for cell survival. In contrast with cells on FN substrates, those on substrates containing FN120, which lacks the HepII domain, retract and show nuclear condensation/fragmentation. Furthermore, these cells on FN120 substrates showed decreased phosphorylation and increased cleavage of pp125FAK. Interestingly, treatment with peptide V, which is a constituent peptide from the HepII domain that can substitute for the HepII domain in cytoskeletal organization [23], reversed or decreased these differences in cells on FN120. Thus, for sustained cell survival, it seems that REFs require at least two signals: one, which is the major survival signal, is via interaction with the cell-binding domain of FN, presumably through interaction with integrin $\alpha5\beta1$, which activates pp125FAK; the other is via interactions with the HepII domain, which augments pp125FAK activation. Thus these two signals co-operatively regulate FN-mediated cell survival.

To test the involvement of the heparan sulphate chain at the cell surface, we treated REFs with heparitinase to remove heparan sulphate, then plated them on FN-coated plates. However, we observed no significant increase in cell death, as seen in REFs on FN120 (results not shown). It therefore seemed that, for sustained survival, both the heparan sulphate chain and the core protein are important. At present, two receptors have been suggested to interact with the HepII domain. Sharma et al. [32] have shown that $\alpha4\beta1$ integrin can interact with the HepII domain and that this can be involved in the regulation of early cytoskeleton organization. We have recently reported that the HepII domain interacts directly with the extracellular domain of syndecan-4 [33], a transmembrane heparan sulphate proteoglycan [34]. Syndecan-4 and integrin $\alpha5\beta1$ co-operatively regulate cytoskeleton organization during cell–ECM interaction [23,24].

We and others have reported that syndecan-4 can both bind protein kinase Cα (PKCα) and regulate its activity [35,36]. Several papers have suggested a role for PKC in pp125FAK activation. pp125FAK is known to be phosphorylated on plating on FN [37]; PKC activity enhances the tyrosine phosphorylation of pp125FAK [38,39]. During focal adhesion and stress fibre formation, PKCα activity is required, and multimerized syndecan-4 cytoplasmic domain can bind PKCα and potentiate its activation by PtdIns(4,5)P_2-activated PKCα [35]. The interaction of the HepII domain or peptide V with syndecan-4 might drive oligomerization, effectively localizing PKCα to form focal adhesions, where it is highly activated. Thus the interaction of syndecan-4 with the HepII domain might cause the relocation and activation of PKCα, which then regulates the phosphorylation, and therefore the activity/stability, of pp125FAK. In addition to binding the heparin-binding domains of ECM components, cell-surface heparan sulphate proteoglycans can bind growth factors [34]. Indeed, the binding of basic fibroblast growth factor to quiescent cells induces a dephosphorylation of syndecan-4 that permits the multimerization and activation of PKCα. Thus the interaction of syndecan-4 with the HepII domain might transduce the signal(s) for cell survival.

In summary, REFs required at least two signals from FN for cell survival. Signalling via integrin interaction with the cell-binding domain is clearly not sufficient because cells become committed to apoptosis and detach from FN120 substrate by 5–8 h, whereas cells on FN substrates undergo cell proliferation. This implies the need for additional signalling via the HepII domain. Abnormal survival signals via differences with pp125FAK phosphorylation at early time points seem to underlie the subsequent pp125FAK cleavage; caspase 3 activation was found at later time points in cells on FN120 substrates. These differences could be reversed or decreased by the addition of peptide V, even after 2 h of cell spreading (i.e. after initial differences would have occurred), indicating that initial downstream signalling via pp125FAK in cells on FN can be regulated by the ligation of cell-surface heparan sulphate proteoglycan. However, the lack of signal at early time points cannot be reversed once detachment has occurred, because cells detached from FN120 substrates still undergo apoptosis when replated on FN.

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