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Alternatively Spliced EDA Segment Regulates Fibronectin-dependent Cell Cycle Progression and Mitogenic Signal Transduction*

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Fibronectin (FN) is comprised of multiple isoforms arising from alternative splicing of a single gene transcript. One of the alternatively spliced segments, EDA, is expressed prominently in embryonic development, malignant transformation, and wound healing. We showed previously that EDA$^+$ FN was more potent than EDA$^-$ FN in promoting cell spreading and cell migration because of its enhanced binding affinity to integrin $\alpha5\beta1$ (Manabe, R., Oh-e, N., Maeda, T., Fukuda, T., and Sekiguchi, K. (1997) *J. Cell Biol.* 139, 295–307). In this study, we compared the cell cycle progression and its associated signal transduction events induced by FN isoforms with or without the EDA segment to examine whether the EDA segment modulates the cell proliferative potential of FN. We found that EDA$^+$ FN was more potent than EDA$^-$ FN in inducing G$_1$-S phase transition. Inclusion of the EDA segment potentiated the ability of FN to induce expression of cyclin D1, hyperphosphorylation of pRb, and activation of mitogen-activated protein kinase extracellular signal regulated kinase 2 (ERK2). EDA$^+$ FN was also more potent than EDA$^-$ FN in promoting FN-mediated tyrosine phosphorylation of p130^{Cas}, but not focal adhesion kinase, which occurred in parallel with the activation of ERK2, suggesting that p130^{Cas} may be involved in activation of ERK2. These results indicated that alternative splicing at the EDA region is a novel mechanism that promotes FN-induced cell cycle progression through up-regulation of integrin-mediated mitogenic signal transduction.

Fibronectins (FNs)[1] are extracellular matrix glycoproteins that play a role in cell adhesion and migration during embryonic development, wound healing, and tumor progression (1). Proteolytic digestion of FN has revealed that FN has several cell binding domains: the central cell binding domain (CCBD), the COOH-terminal heparin binding domain (Hep2), and the CS1 region within the type III connecting segment (IIICS) (2). CCBD is the major cell-adhesive domain of FN and contains the Arg-Gly-Asp (RGD) motif that is recognized by members of the integrin family of cell adhesion receptors including $\alpha5\beta1$, $\alpha v\beta1$, $\alpha v\beta3$, $\alpha v\beta5$, $\alpha v\beta6$, αIIb$\beta3$, and $\alpha8\beta1$ (3–6). There is evidence that FN acts not only as a cell-adhesive substrate but also transduces biochemical signals across the plasma membrane via integrin receptors, thereby regulating cell proliferation, differentiation, and apoptosis (7–9). Interaction of FN with integrins stimulates tyrosine phosphorylation of several cellular proteins including FAK (10–12) and p130^{Cas} (13–15) and also stimulates activation of Src family protein tyrosine kinases (7) and the extracellular signal-related kinases ERK1 and ERK2 (16–19). Furthermore, FN induces expression of cyclin D1 and subsequent inactivation of pRb by hyperphosphorylation and thus mediates cell cycle progression through the G$_1$ phase (20).

FN contains three alternatively spliced segments: EDA, EDB, and IIICS (21–24). The EDA and EDB segments are each encoded by a single exon, and both comprise an intact type III repeat (22). FNs expressed in fetal and tumor tissues contain a greater percentage of EDA and EDB segments than those expressed in normal adult tissues (25–29). Increased expression of FNs containing the EDA and/or EDB segments has also been observed during wound healing (30). Because FNs containing the EDA and/or EDB segments are highly expressed in tissues that are populated with cells showing high proliferative and migratory potential, it seems likely that FNs containing these extra segments play an important role in promoting cell proliferation and migration. Despite accumulated evidence for regulated expression of EDA- and/or EDB-containing FNs *in vivo*, the biological functions of these isoforms are poorly understood (31).

Recently, we found that FN isoforms containing the EDA segment (EDA$^+$ FN) were more potent than those lacking the EDA segment (EDA$^-$ FN) in promoting cell adhesion and migration, irrespective of the presence or absence of the EDB segment. The increased cell adhesion and migration were caused by increased binding of EDA$^+$ FN to integrin $\alpha5\beta1$ (32). In the present study, we investigated whether alternatively spliced EDA and EDB segments regulate the potential of FNs to promote cell proliferation. We found that insertion of the EDA, but not EDB, segment potentiated the ability of FN to promote cell cycle progression. The enhanced cell cycle progression was associated with increased tyrosine phosphorylation of p130^{Cas}, activation of ERK2, increased expression of cyclin D1, and hyperphosphorylation of pRb. These results indicated that alternative splicing in the EDA region regulates FN-mediated extracellular signals and subsequent cell cycle progression via modulation of binding affinity to integrin $\alpha5\beta1$.

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[1] The abbreviations used are: FN, fibronectin; EDA$^+$ FN, FN isoform(s) containing the EDA segment; EDB$^+$ FN, FN isoform(s) containing the EDB segment; EDA$^-$ FN, FN isoform(s) lacking the EDA segment; CCBD, central cell binding domain of FN; FAK, focal adhesion kinase; Cas, Crk-associated substrate; ERK, extracellular signal-regulated kinase; CHO, Chinese hamster ovary; FBS, fetal bovine serum; mAb, monoclonal antibody; pRb, retinoblastoma protein; SH2, Src homology 2.

EXPERIMENTAL PROCEDURES

Purification of Recombinant FN Isoforms—Construction of the cDNA expression vectors for three different full-length FN isoforms, EDA$^-$/EDB$^-$ FN, EDA$^+$/EDB$^-$ FN, and EDA$^+$/EDB$^+$ FN, was described pre-

viously (32). To construct the expression vector for EDA$^-$/EDB$^+$ FN, the whole inserts of pHCF93B$^+$ encoding Val^{527}-Arg^{1449} including the EDB segment (32) were excised by double digestion with *Sal*I/*Bam*HI and ligated to *Sal*I/*Bam*HI-cleaved pAIFNC, which encodes a full-length FN isoform lacking both EDA and EDB segments. The resulting cDNA expression vector for EDA$^-$/EDB$^+$ FN was designated pAIFNBC. Recombinant FNs were expressed in CHO DG44 cells and purified from the culture supernatants of the stable transfectants by gelatin-affinity chromatography as described previously (32).

Cell Culture—CHO-K1 and CHO DG44 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and Dr. Lawrence Chasin (Columbia University), respectively. CHO-K1 cells were grown in Ham's F-12 medium supplemented with 10% FBS. CHO DG44 cells deficient in dihydrofolate reductase activity were maintained in α-minimal essential medium containing ribonucleosides/deoxyribonucleosides and 10% FBS. Stable transfectants of CHO DG44 cells overexpressing recombinant FNs were maintained in α-minimal essential medium without ribonucleosides/deoxyribonucleosides in the presence of 10% FBS (for routine passage) or 1% FN-depleted FBS (for large scale culture to purify recombinant proteins). All cells were maintained under a humidified 5% CO_2 atmosphere at 37 °C.

Antibodies—Polyclonal antibodies against cyclin D1 (R-124), p130^{Cas} (N-17), and ERK2 (C-14), and horseradish peroxidase-conjugated anti-phosphotyrosine mAb (PY20) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The mAb against pRb (G3-245) was obtained from Pharmingen (San Diego). Anti-FAK mAb 2A7 was obtained from Upstate Biotechnology (Lake Placid, NY). Horseradish peroxidase-conjugated secondary antibodies against mouse or rabbit IgG were from Cappel Worthington Biochemicals (Malvern, PA).

Cell Spreading Assay—Cell spreading assays were performed using 96-well microtiter plates coated with 5 μg/ml recombinant FNs and blocked with 1% bovine serum albumin. Amounts of recombinant FNs immobilized on plates were determined by enzyme-linked immunosorbent assay using anti-human FN antiserum or anti-FN mAbs as described previously (32). CHO-K1 cells grown in Ham's F-12 medium containing 10% FBS were trypsin treated and washed with serum-free medium. The cells were plated at a density of 3×10^4 cells/well in serum-free medium and incubated at 37 °C for 40 min. Nonadherent cells were removed by washing with serum-free medium, and attached cells were fixed with 3.7% formaldehyde and then stained with Giemsa. Cells adopting a well spread morphology (*i.e.* cells that had become flattened with the long axis more than twice the diameter of the nucleus) were counted/mm^2.

Integrin-liposome Binding Assay—Integrins α5β1 and αvβ3 were purified and reconstituted into liposomes as described previously (32). Integrin-liposomes in TBS(+) (25 mM Tris-HCl, pH 7.5, 0.13 M NaCl, 1 mM CaCl$_2$, and 1 mM MgCl$_2$) containing 0.2% bovine serum albumin were added to the wells of microtiter plates precoated with recombinant FNs (20 μg/ml) and incubated for 6 h at room temperature. The wells were washed with TBS(+), and bound liposomes were recovered in 1 N NaOH. The radioactivity of bound liposomes was quantified using an Aloka LSC-3500 scintillation counter (Aloka Co., Ltd., Tokyo).

Induction of G_1-S Phase Transition in Synchronized Cells—CHO-K1 cells were grown to confluence and synchronized at G_0 by serum starvation for 48 h in Ham's F-12 supplemented with 0.2% FN-depleted FBS, 50 μg/ml streptomycin, and 50 units/ml penicillin (starvation medium) according to Orren *et al.* (33). The cells were dissociated with phosphate-buffered saline containing 0.2% trypsin and 2 mM EDTA, and trypsin treatment was stopped by adding soybean trypsin inhibitor (1 mg/ml). The cells were washed with serum-free medium, incubated in suspension in starvation medium for 90 min at 37 °C, and seeded on plates that had been precoated with 5 μg/ml recombinant FNs or 20 μg/ml poly-L-lysine and then blocked with 1% bovine serum albumin. The amounts of immobilized recombinant FNs on the plates were determined by enzyme-linked immunosorbent assay using anti-human FN antiserum to confirm the equality of adsorbed FNs.

Determination of S Phase Entry—Quiescent cells were kept in suspension for 90 min and then plated at a density of 2×10^4 cells/well in starvation medium. [^{3}H]Thymidine (5 μCi/ml) was added 12–24 h after cell plating. The cells were washed three times with serum-free medium at the indicated time points, fixed with ice-cold 10% trichloroacetic acid for 1 h, and washed once with 10% trichloroacetic acid. The acid-insoluble precipitates were dissolved with 0.1% SDS and 0.2 N NaOH, neutralized with 0.2 N HCl, and transferred to vials containing scintillation fluid. Radioactivities in the precipitates were determined with an Aloka LSC-3500 scintillation counter. To determine the percentage of cells entering S phase, bromodeoxyuridine was added to the cultures 12–24 h after plating, and the cells were incubated with anti-bromodeoxyuridine mAb followed by visualization of bound mAb with 3,3′-diaminobenzidine tetrahydrochloride according to the manufacturer's instructions (Amersham Pharmacia Biotech).

Immunoprecipitation and Immunoblotting—Quiescent cells cultured on the substratum were extracted in lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 40 mM β-glycerophosphate, 2 mM orthovanadate, 0.5 mM sodium fluoride, 20 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). The cell lysates were clarified by centrifugation at $14,000 \times g$ for 10 min at 4 °C, and protein concentration was determined using a DC protein assay kit (Bio-Rad). The whole lysates were boiled in SDS-sample buffer (8% SDS, 100 mM dithiothreitol, 266 mM Tris, pH 6.8, 40 mM EDTA), and equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis with 8, 12, or 12.5% gels for detection of tyrosine-phosphorylated proteins and pRb (8%), cyclin D1 (12%), and ERK2 (12.5%). Proteins were detected immunologically after electrotransfer onto nitrocellulose membranes as described (32).

For immunoprecipitation of FAK, cell lysates were incubated with protein A-conjugated anti-FAK mAb 2A7 for 5 h. For immunoprecipitation of p130^{Cas}, cell lysates were incubated with anti-p130^{Cas} antibody for 1 h followed by incubation with protein G-Sepharose (Amersham Pharmacia Biotech) for 4 h. The immune complexes were precipitated by brief centrifugation and washed four times with lysis buffer. The immunoprecipitates were separated on 8% polyacrylamide gels to determine the levels of tyrosine phosphorylation and the amounts of precipitated proteins as described above.

RESULTS

EDA but Not EDB Regulates Cell Adhesive Functions of FN—We showed previously that the presence or absence of the EDB segment did not affect the ability of EDA$^+$ FN isoforms to promote cell adhesion and spreading (32), although we could not exclude the possibility that inclusion of the EDB segment alone might potentiate the cell-adhesive activity of FN. To explore this possibility, we produced a recombinant FN isoform containing the EDB but not the EDA segment (designated rFN(BC); see Fig. 1) and compared its cell attachment and spreading activities with those of rFN(C), rFN(AC) and rFN-(BAC). CHO-K1 cells were used in these assays because they have long been used as a standard model in cell cycle analysis and are more easily synchronized at the G_0 state than HT1080 cells, which were used in the previous study (32). Significant differences were observed in both cell attachment and spreading among the four different FN isoforms, EDA$^+$ FNs (*i.e.* rFN(AC) and rFN(BAC)) being more potent in promoting cell attachment than EDA$^-$ FNs (*i.e.* rFN(C) and rFN(BC)) (Fig. 2A). EDA$^+$ FNs were also more than twice as effective as EDA$^-$ FNs in inducing cell spreading (Fig. 2B). No significant differences were observed in cell spreading activity between rFN(AC) and rFN(BAC) or between rFN(C) and rFN(BC) (Fig. 2B), indicating that the EDB segment is not involved in regulation of the cell-adhesive activity of FN. Because CHO-K1 cells adhere to FN-coated substrates predominantly via integrin α5β1 (34) and the increased cell-adhesive activity of EDA$^+$ FNs is the result of enhanced binding of CCBD to integrin α5β1 (32), the integrin binding activity of EDB$^+$ FN was compared with that of EDB$^-$ FN. There were no significant differences in integrin α5β1 binding between rFN(C) and rFN(BC), although rFN(AC) was more than twice as active as rFN(C) and rFN(BC) in integrin α5β1 binding (Fig. 3). These results support the previous conclusion that the cell-adhesive functions of FN are specifically regulated by insertion of the EDA but not the EDB segment.

EDA$^+$ FN Is More Potent than EDA$^-$ FN in Promoting Cell Cycle Progression—The *in vivo* expression patterns of EDA$^+$ FN suggested a potential role of EDA$^+$ FN in cell proliferation (31). To investigate whether insertion of the EDA and/or EDB segment affects the ability of FN to promote cell proliferation, CHO-K1 cells were synchronized at the G_0 phase by serum starvation, incubated in suspension for 90 min, and replated on substrates precoated with different FN isoforms to stimulate

FIG. 1. **Structure of recombinant FNs.** Modular structures of recombinant FNs are shown schematically on the basis of internally homologous modules termed types I, II, and III. The EDA and EDB segments are shown by *filled rectangles*, and the IIICS segment is shown by a *hatched oval*. All recombinant FNs contain the complete IIICS sequence of 120 amino acids. Functional domains that interact with heparin (Hep1, Hep2), fibrin (Fib1, Fib2), bacteria, collagen, and integrin $\alpha 5\beta 1$ are indicated above the figures.
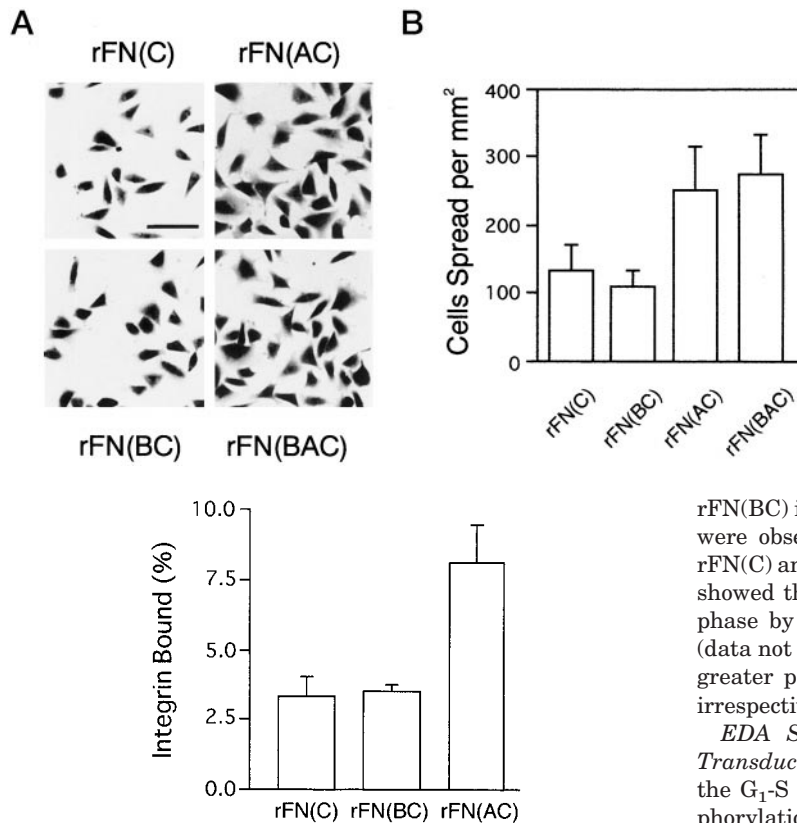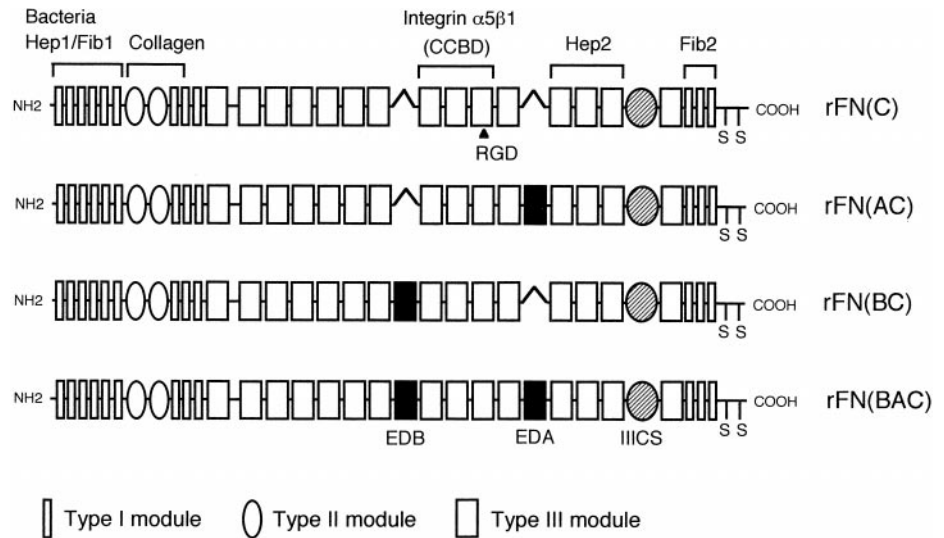

FIG. 2. **Attachment and spreading of CHO cells on recombinant FN-coated substrata.** *Panel A*, CHO cells (3×10^4) were seeded on 96-well microtiter plates coated with 5 μg/ml of different FN isoforms and incubated for 40 min at 37 °C. The cells were rinsed, fixed, and stained with Giemsa. *Bar*, 100 μm. *Panel B*, spreading of CHO cells was quantified as described under "Experimental Procedures." The S.D. of multiple determinations ($n = 3$) are indicated at the *top* of the *bars*.

FIG. 3. **Binding of integrin $\alpha 5\beta 1$ to recombinant FNs.** Integrin $\alpha 5\beta 1$ was purified from human placenta and reconstituted in phosphatidylcholine liposomes as described under "Experimental Procedures." The integrin $\alpha 5\beta 1$-liposomes were added to microtiter plates precoated with 20 μg/ml of different FN isoforms and incubated for 6 h at room temperature. Quantities of bound integrin $\alpha 5\beta 1$-liposomes are expressed as percentages of the total input radioactivity after subtraction of the radioactivity bound to plates coated only with bovine serum albumin. Each *bar* represents the mean S.D. ($n = 6$).

the cells to reenter the cell cycle. To confirm that the effects of FN isoforms on cell proliferation were primarily mediated via integrins, the cells were also plated on the substrate coated with poly-L-lysine, which mediates integrin-independent cell adhesion. All recombinant FNs significantly promoted entry into S phase when compared with poly-L-lysine (Fig. 4). The levels of DNA synthesis were different among the FN isoforms; rFN(AC) and rFN(BAC) were more potent than rFN(C) and

rFN(BC) in inducing DNA synthesis. No significant differences were observed between rFN(AC) and rFN(BAC) or between rFN(C) and rFN(BC). Labeling of cells with bromodeoxyuridine showed that more than 70% of the plated cells entered the S phase by 24 h after replating onto the FN-coated substrata (data not shown). These results indicated that EDA$^+$ FN has a greater potential than EDA$^-$ FN to induce cell proliferation irrespective of the presence or absence of the EDB segment.

EDA Segment Up-regulates Cell Cycle-associated Signal Transduction—It has been shown that pRb plays a key role in the G$_1$-S phase transition. Inactivation of pRb by hyperphosphorylation occurs at the restriction point in G$_1$ as the cell enters the S phase (35). We investigated whether the presence or absence of the EDA segment could modulate FN-induced phosphorylation of pRb. Quiescent cells were trypsin treated, held in suspension to reset anchorage-dependent signals, and replated onto dishes coated with rFN(C), rFN(AC) or poly-L-lysine. Hypo- and hyperphosphorylated forms of pRb were distinguished by the difference in their mobility on SDS-polyacrylamide gel electrophoresis. Hyperphosphorylation of pRb was induced on the substrates coated with rFN(C) and rFN(AC) compared with those with poly-L-lysine (Fig. 5, *upper panel*). The level of pRb hyperphosphorylation was higher with cells plated on rFN(AC) than on rFN(C) through 16–24 h after plating, indicating that the increased cell cycle progression driven by EDA$^+$ FN was associated with the increased hyperphosphorylation of pRb. Although pRb hyperphosphorylation was detectable even on poly-L-lysine upon prolonged incubation, this could be the result of deposition of endogenous FN by CHO cells.
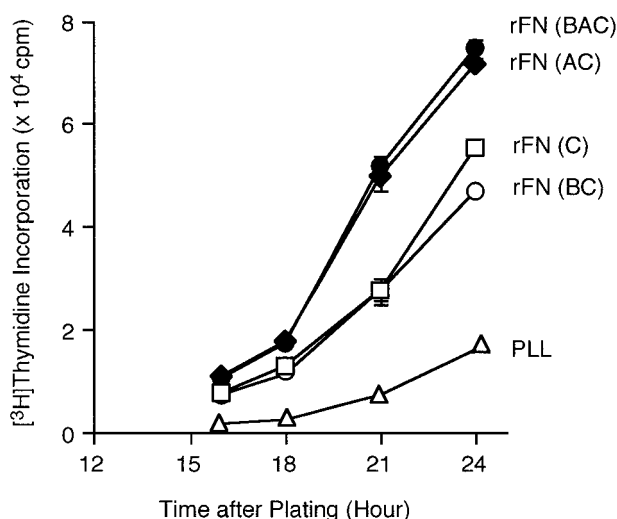
FIG. 4. **Induction of DNA synthesis upon adhesion of cells onto recombinant FN-coated substrata.** Quiescent CHO cells were replated onto wells precoated with 5 μg/ml rFN(C), rFN(BC), rFN(AC), rFN(BAC), or 20 μg/ml poly-L-lysine (*PLL*) in starvation medium. [3H]Thymidine was added to the wells 12 h after replating, and the cells were cultured for the indicated periods. S phase entry was quantified by measuring [3H]thymidine incorporation into DNA as described under "Experimental Procedures." Each *bar* represents the mean S.D. (*n* = 6).
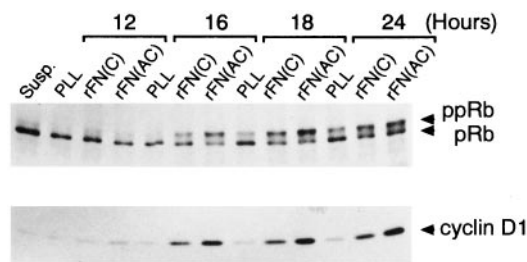

FIG. 5. **pRb phosphorylation and cyclin D1 accumulation in response to cell adhesion onto recombinant FN-coated substrata.** Quiescent CHO cells were seeded onto substrates coated with 5 μg/ml rFN(C) or rFN(AC) or 20 μg/ml poly-L-lysine (*PLL*), or they were kept in suspension in starvation medium for varying periods as indicated. Cells were then solubilized in Nonidet P-40 lysis buffer, and equal amounts of whole cell lysates were subjected to SDS-polyacrylamide gel electrophoresis. Protein levels of pRb (*upper panel*) and cyclin D1 (*lower panel*) were detected by Western blotting with antibodies specific for pRb or cyclin D1 after electrotransfer onto nitrocellulose membranes. The positions of hyper- and hypophosphorylated pRbs are indicated as *ppRb* and *pRb*, respectively. The reproducibility of the results was confirmed in three separate experiments.

pRb has been shown to be phosphorylated by cyclin D1·cdk4/6 complexes, with concomitant accumulation of cyclin D1 during the G_1 phase (35). Therefore, we examined whether the level of expression of cyclin D1 was also modulated by the EDA segment upon cell adhesion onto FN-coated substrates. When cells were plated onto substrates coated with recombinant FNs with or without the EDA segment, the expression of cyclin D1 was significantly induced by 24 h after replating, although no obvious induction of cyclin D1 expression was observed on the poly-L-lysine-coated substrate (Fig. 5, *lower panel*). The expression of cyclin D1 was more pronounced with cells plated onto rFN(AC) than onto rFN(C) with a time course profile similar to that of pRb hyperphosphorylation. These results indicated that expression of cyclin D1 and phosphorylation of pRb were coordinately regulated by the EDA segment upon cell adhesion to FN-coated substrates.

EDA Segment Enhances FN-dependent MAP Kinase Activation—Because activation of MAP kinase has been shown to induce expression of cyclin D1 and subsequent hyperphospho-
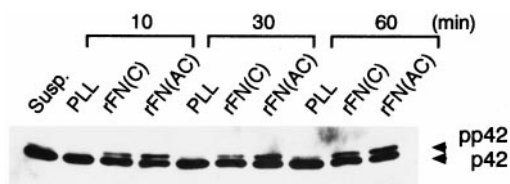

FIG. 6. **MAP kinase activation induced by recombinant FNs.** Quiescent cells were either held in suspension or plated on substrates coated with 5 μg/ml rFN(C) or rFN(AC) or 20 μg/ml poly-L-lysine (*PLL*) in starvation medium for various times. Cells were lysed, and MAP kinase activity was analyzed by a band shift assay. The phosphorylated, activated forms of ERK2 (*pp42*) display more reduced electrophoretic mobility than the unphosphorylated, nonactivated forms of ERK2 (*p42*). The reproducibility of the results was confirmed in three separate experiments.

rylation of pRb (36), we investigated whether ERK2 MAP kinase was stimulated differently by EDA+ and EDA− FNs. When quiescent cells were detached and either held in suspension or replated on dishes coated with rFN(C), rFN(AC), or poly-L-lysine, the activated form of ERK2 was detectable as early as 10 min after replating and persisted for over 60 min (Fig. 6). In contrast, no significant activation was observed in cells kept in suspension or replated on the substratum coated with poly-L-lysine. Activation of ERK2 was more prominent on surfaces coated with rFN(AC) than on those coated with rFN(C), indicating that the EDA segment potentiates the ability of FN to activate MAP kinases via integrin-dependent signaling and thereby promotes cell cycle progression.

EDA Increases FN-induced Tyrosine Phosphorylation of $p130^{Cas}$—Protein tyrosine phosphorylation triggered by integrin-mediated cell-matrix interaction has been shown to be caused by activation of protein tyrosine kinases including the Src family kinases, FAK and FAK-related kinases (7). These kinases phosphorylate tyrosine residues of their specific substrate proteins, providing binding sites for SH2-containing molecules that are involved in cell proliferation (7). We examined whether the EDA segment modulates the ability of FNs to stimulate tyrosine phosphorylation of cellular proteins. In parallel with the analysis of FN-dependent activation of ERK2, quiescent cells were kept in suspension or replated on dishes precoated with rFN(C), rFN(AC), or poly-L-lysine and incubated for different periods. As shown in Fig. 7A, proteins migrating at positions corresponding to 150, 130, and 80–60 kDa were tyrosine phosphorylated irrespective of the type of substrate. Among these, tyrosine phosphorylation of the 130-kDa protein was significantly induced in cells plated on substrates coated with recombinant FNs but not in cells either plated on poly-L-lysine-coated substrates or kept in suspension. The kinetics of tyrosine phosphorylation of the 130-kDa protein were similar to those of ERK2 activation (Fig. 6). The 130-kDa protein was tyrosine-phosphorylated at 10 min after replating on FN-coated surfaces, and the levels of tyrosine phosphorylation persisted for more than 60 min. The 130-kDa protein was more prominently tyrosine-phosphorylated in cells on rFN(AC) than those plated on rFN(C).

Cell adhesion to FN-coated substrates has been shown to induce tyrosine phosphorylation of $p125^{FAK}$ (10–12) and $p130^{Cas}$ (13–15). Immunoprecipitation with anti-FAK and anti-$p130^{Cas}$ antibodies showed that $p130^{Cas}$ was phosphorylated in CHO-K1 cells plated onto FN-coated surfaces, although no significant tyrosine phosphorylation was observed with FAK in the same cells (Fig. 7B, *upper panel*). These results indicated that the tyrosine-phosphorylated 130-kDa protein was $p130^{Cas}$ but not FAK. Tyrosine phosphorylation of $p130^{Cas}$ was more pronounced on the substrates coated with rFN(AC) than on those coated with rFN(C). The enhanced phosphorylation of the
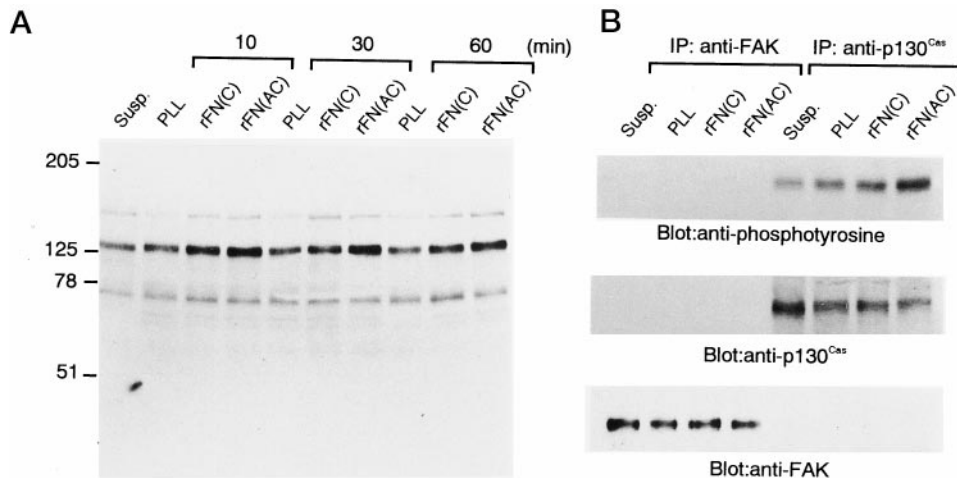
FIG. 7. **Tyrosine phosphorylation of p130^{Cas} and FAK upon cell adhesion onto recombinant FNs.** *Panel A*, quiescent cells were either held in suspension or plated on substrates coated with 5 μg/ml rFN(C) or rFN(AC) or 20 μg/ml poly-L-lysine (*PLL*) in starvation medium for various times. Whole cell lysates were prepared, and tyrosine phosphorylation of cellular proteins was analyzed by immunoblotting with anti-phospho-tyrosine antibody. The positions of molecular weight marker proteins are indicated in the *left* margin. *Panel B*, whole cell lysates prepared 30 min after replating were immunoprecipitated with anti-p130^{Cas} or anti-FAK antibody. The immunoprecipitates were subjected to immunoblotting with antibodies against phosphotyrosine (*upper*), FAK (*middle*), and p130^{Cas} (*bottom*). The reproducibility of the results was confirmed in two separate experiments.

130-kDa protein was not the result of an increase in p130^{Cas} expression because the amount of immunoprecipitated p130^{Cas} was decreased rather slightly on rFN(AC) than that on rFN(C) (Fig. 7*B*, *bottom panel*). Although we could not exclude the possibility that FAK was tyrosine-phosphorylated at levels below the limits of detection, it is likely that p130^{Cas} is the main target of protein tyrosine kinases activated by integrin-mediated adhesion onto FN-coated substrates and therefore is involved in activation of ERK2 and subsequent cell cycle progression.

<center>DISCUSSION</center>

Regulation of FN-mediated Signal Transduction by the EDA Segment—It has been shown that the EDA segment is included in FN species expressed in embryonic tissues, but is spliced out of the molecule as embryonic development proceeds (28, 31). In adults, EDA$^+$ FN reappears at sites of tissue injury and inflammation (30, 38). Despite the close association of the expression of EDA$^+$ FN with tissues populated with cells showing high proliferative potential, the physiological significance of this phenomenon is not known.

In this study, we found that EDA$^+$ FN was more potent than EDA$^-$ FN in inducing G$_1$-S phase transition. The increased ability of EDA$^+$ FN to induce G$_1$-S phase transition was closely associated with enhanced ERK2 activation, cyclin D1 expression, and pRb phosphorylation. Given that EDA$^+$ FN is more potent than EDA$^-$ FN in promoting cell adhesion through enhanced binding to integrin α5β1 (32) and that ligand ligation of integrin α5β1 promotes DNA synthesis through activation of intercellular signal transduction pathways involving ERK2, cyclin D1, and pRb (1, 20), it is conceivable that inclusion of the EDA segment up-regulates FN-dependent mitogenic signal transduction through increased binding of FN to integrin α5β1. Because EDA$^+$ FN is expressed preferentially in embryonic and tumor tissues, alternative splicing at the EDA region of FN may be instrumental in supporting the high proliferative potential of cells in these tissues.

FN contains two major integrin binding regions: CCBD and CS-1. CCBD is recognized by integrin α5β1 and αv-containing integrins, whereas CS-1 is recognized by integrin α4β1. Besides the distinct binding specificities, the integrin binding activities of these two regions are regulated differently at the level of RNA splicing. Thus, the CS-1 region itself is excluded

from the FN molecule by alternative splicing (39–41), regulating its cell adhesive activity in an all-or-none manner. In contrast, CCBD is encoded by constitutive exons, and its cell adhesive activity is regulated by the alternatively spliced EDA segment in a range of 2~3-fold increments. The difference in the mode of regulation of their activities by alternative splicing appears to reflect the physiological roles of these cell adhesive regions. Integrin α5β1, the major receptor for CCBD, is expressed on a wide variety of cell types and plays a central role in adhesion-dependent signaling events involved in proliferation, migration, and survival of cells (1, 9). The interaction of integrin α5β1 with CCBD may provide housekeeping signals that need to be maintained strictly at the minimal level required for cell survival. In contrast, integrin α4β1, the specific receptor for CS-1, is expressed on restricted cell types such as lymphocytes (39, 42) and elicits a cell type-specific proliferation signal (43). All-or-none regulation of CS-1 activity by alternative splicing may be suitable for such an auxiliary signal, leaving the basal survival signal from CCBD uncompromised.

The interaction of cells with FN is regulated not only by the alternative splicing at the EDA region but also by the affinity states of integrin α5β1. Integrins exist at different affinity states ranging from inactive to fully activated forms (44). The affinity states of integrins are modulated by cellular stimulation by chemoattractants (44) and interaction with membrane-associated proteins such as IAP (integrin-associated protein) (45). Modulation of the ligand binding affinity of integrin α5β1 has been shown to affect FN-dependent cell proliferation (46) and differentiation (47) by modulation of integrin α5β1-mediated signal transduction. It is conceivable, therefore, that alternative splicing of FN and affinity modulation of integrin α5β1 coordinately regulate the integrin-mediated, extracellular signals required for proliferation, differentiation, and survival of cells.

Role of p130^{Cas} in FN-mediated Signal Transduction—We found that cell adhesion to FN-coated substrata activates ERK2 without significant tyrosine phosphorylation of FAK. Although there is some evidence suggesting that FAK can activate the ERK signaling pathway in response to cell adhesion (16, 17, 19), it is unclear whether FAK plays a physiological role in the ERK signaling pathway. In fact, recent studies

have shown that the activation of ERK in response to integrin ligation is mediated by the adaptor protein Shc independently of FAK (48). In support of this observation, introduction of a dominant-negative version of FAK did not impair the ERK activation elicited by cell adhesion to FN (49). Our results also showed that p130^{Cas}, but not FAK, was prominently tyrosine-phosphorylated in response to cell adhesion onto FN-coated substrata with kinetics similar to those of ERK2 activation. Although the precise molecular mechanisms of p130^{Cas}-dependent activation of ERK remain to be defined, tyrosine phosphorylation of p130^{Cas} was suggested to be involved in ERK activation by the following observations. First, overexpression of p130^{Cas} or FN-stimulated tyrosine phosphorylation of p130^{Cas} promotes the SH2-mediated binding of the adaptor proteins Crk and/or Nck to p130^{Cas} and subsequent association of Crk and/or Nck with the GDP-GTP exchange protein Sos, which could result in Ras activation (50, 51). Second, overexpression of protein tyrosine phosphatase 1B, which can also associate with p130^{Cas}, resulted in dephosphorylation p130^{Cas} and inhibition of ERK activation, whereas a protein tyrosine phosphatase 1B mutant unable to bind p130^{Cas} failed to dephosphorylate p130^{Cas} and therefore failed to inhibit ERK activation (52). Although these observations seem to support the involvement of p130^{Cas} in ERK activation, further studies are required to define the role of tyrosine phosphorylation of p130^{Cas} in EDA-dependent enhancement of cell cycle progression.

Adhesive Function of FN Is Independent of the EDB Segment—Our results showed that the abilities of rFN(BC) to promote cell adhesion, integrin $\alpha5\beta1$ binding, and cell cycle progression were the same as those of rFN(C) and were significantly lower than those of rFN(AC), excluding the possibility that inclusion of the EDB segment alone might potentiate the adhesive activity of FN. Adhesive functions of FN are therefore regulated exclusively by alternative splicing at EDA but not EDB. Based on the three-dimensional structure of a recombinant FN fragment consisting of III$_7$–III$_{10}$ modules (53), we proposed that insertion of the EDA segment alters the global conformation of the FN molecule through rotating the NH$_2$-terminal portion of the FN polypeptide relative to the COOH terminus, thereby resulting in the enhanced binding of EDA$^+$ FN to integrin $\alpha5\beta1$ because of an increased exposure of the RGD motif in III$_{10}$ module and/or local unfolding of the module (32). Failure of the EDB segment to substitute for EDA in altering the global conformation of the FN so as to increase the binding affinity to integrin $\alpha5\beta1$ indicates that the conformational activation of FN by insertion of an extra type III module is dependent on the position and/or sequence of the inserted module, making the EDA segment most suited for modulation of the binding affinity to integrin $\alpha5\beta1$.

Although the presence or absence of the EDB segment did not affect the cell adhesive activity of FNs, the EDB segment may modulate other biological functions of FN. Expression of EDB$^+$ FN *in vivo* is more restricted than that of EDA$^+$ FN (28), suggesting that the EDB segment may have an important function only in highly specific situations such as angiogenesis (54) and lung development (55). Regulated *in vivo* expression of recombinant FN isoforms differing with respect to the presence or absence of the EDA and EDB segments should provide insight into the physiological and pathological roles of the alternatively spliced segments during development, tumorigenesis, and inflammatory processes.

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Alternatively Spliced EDA Segment Regulates Fibronectin-dependent Cell Cycle Progression and Mitogenic Signal Transduction

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