An Amino-Terminal Fibronectin Fragment Stimulates the Differentiation of ST-13 Preadipocytes

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ABSTRACT: Differentiation of ST-13 preadipocytes into adipocytes was inhibited almost completely by addition of rat plasma fibronectin (FN) (~100 μg/mL), but was reversed by GRGDSP cell recognition peptide (1.5 mM) and anti-$\alpha_5\beta_1$. On the contrary, the thermolysin digest of FN stimulated adipocyte differentiation in a dose-dependent manner, in which remarkable increases in the values of the differentiation indexes, the number of adipocytes (8-fold above the control), glycerophosphate dehydrogenase (GPD) activity (12-fold), and triacylglycerol content (5-fold), were observed by inclusion of the thermolysin digest (100 μg/mL). The increase in GPD activity by the thermolysin digest was inhibited remarkably (about 70% inhibition) by an antibody directed to the amino-terminal fibrin-binding (Fib 1) domain of FN and slightly (about 15%) by an antibody directed to the central cell-binding (Cell) domain, but not by anti-gelatin-binding domain and anti-carboxy-terminal fibrin-binding domain. Treatment of ST-13 cells by a purified 24K fragment (100 μg/mL) derived from the Fib 1 domain caused an over 20-fold augmentation of the GPD activity, accounting for a major part of the differentiation stimulatory activity of the thermolysin digest. The differentiation stimulatory effect of the 24K Fib 1 fragment was not affected by either GRGDSP peptide or anti-$\alpha_5\beta_1$. Thus, FN can regulate adipose development of ST-13 cells by its two antipodal, inhibitory and stimulatory, activities, the latter of which is expressed only upon fragmentation. Proteolytic cleavage of FN may play an important role in controlling the action of FN on adipocyte differentiation.

Adipose cell differentiation has been primarily investigated in adipose precursor cells from established clonal lines, such as 3T3-L1 (Green & Meuth, 1974), 3T3-F442A (Green & Kehinde, 1976), and ST-13 (Hiragun et al., 1980) cells. Differentiation of these preadipocytes is characterized by major changes in cell morphology from fibroblastic to a polygonal shape and by induction of gene expression related to lipid metabolism (Spiegelman & Farmer, 1982; Spiegelman & Ginty, 1983; Bernlohr et al., 1985; Fernandez & Ben-Ze'ev, 1989). Such morphological change can be considered to result from lowered interaction between extracellular matrix (ECM)[1] components and cells, followed by a decrease in the expression of ECM components, integrins, and cytoskeletal proteins. Study of adipocyte differentiation using these cell lines would provide an important basis to understand the involvement of ECMs in differentiation.

Fibronectin (FN) is one of the key elements of the ECM, and it has been known that the protein has an ability to regulate cell differentiation including adipose conversion (Spiegelman & Farmer, 1982; Sieber-Blumn et al., 1981; Loring et al., 1982; Macig et al., 1982; Menko & Boettiger, 1987; Adams & Watt, 1989). On the other hand, a large number of studies have indicated that proteolytic degradation of FN often yields biological activities not present in intact FN. For example, FN degradation products containing the Arg-Gly-Asp-Ser (RGDS) sequence stimulate human neutrophile degranulation, whereas intact FN does not (Wachtfogel et al., 1988). Fragments derived from the central cell-binding (Cell) domain and the amino-terminal fibrin/heparin-binding (Fib 1) domain are much more active in promoting chemotactic migration of human peripheral blood monocytes than intact FN (Clark et al., 1988; Lohr et al., 1990). Degradation products of FN, not intact FN, induce collagenase and stromelysin gene expression (Werb et al., 1989). Furthermore, growth of bovine aortic endothelial cells is inhibited by FN fragments but not by intact FN (Homandberg et al., 1986). The same authors recently reported that FN fragments cause chondrolysis of bovine articular cartilage slices in culture whereas intact FN does not (Homandberg et al., 1992). We also demonstrated that fragmentation of intact FN yields a unique chemotactic fragment not detectable in intact FN (Fukai et al., 1991). Some other functions to regulate cell differentiation may be concealed in the FN molecule. From this point of view, we have studied the effects of FN on adipocyte differentiation using ST-13 preadipocytes. Here we present the results which indicate that FN has not only the differentiation inhibiting activity but also a cryptic activity to stimulate adipose conversion. Moreover, FN fragments having the differentiation stimulatory activity were isolated.

MATERIALS AND METHODS

Materials. Rat plasma FN was purified as described earlier (Fukai et al., 1991). The thermolysin digest of FN was prepared as described previously (Fuka et al., 1991) with some modifications as follows. Purified FN (40 mg) in 24 mL of

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[1] Abbreviations: FN, fibronectin; Fib 1 domain, amino-terminal fibrin-binding domain; Gel domain, amino-terminal gelatin-binding domain; Cell domain, central cell-binding domain; Hep 2 domain, carboxy-terminal heparin-binding domain; Fib 2 domain, carboxy-terminal fibrin-binding domain; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; DMEM, Dulbecco's modified Eagle's medium; HPF, high-powered fields; GPD, glycerophosphate dehydrogenase (EC 1.1.1.8); TG, triacylglycerol; NADH, β-nicotinamide adenine dinucleotide; PBS(−), Dulbecco's phosphate-buffered saline (pH 7.4); Tris-HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride; BSA, bovine serum albumin; GRGDSP, Gly-Arg-Gly-Asp-Ser-Pro.

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20 mM Tris-HCl buffer containing 2 mM $CaCl_2$ and 50 mM NaCl, pH 7.4, was incubated (25 °C, 3 h) with thermolysin (62 μg)(Sigma, protease X). After the proteolysis, thermolysin was inactivated by incubating (25 °C, 30 min) the reaction mixture with 10 mM $HgCl_2$, and the thermolysin digest was exhaustively dialyzed against PBS(–). There was no detectable proteolytic activity in the thermolysin digest as evaluated by the sensitive assay method using a fluorescein isothiocyanate-conjugated casein (Sigma Chemical)(Twining, 1984) that allowed detection of 0.5 ng/mL protease. A monospecific rabbit antiserum raised against rat plasma FN was obtained as reported elsewhere (Nagai et al., 1988). Rabbit polyclonal antibodies recognized specifically the functional domains of human plasma FN [the amino-terminal fibrin-binding (Fib 1), the central cell-binding (Cell), the gelatin-binding (Gel), and the carboxy-terminal fibrin-binding (Fib 2) domains] were prepared as previously described (Sekiguchi et al., 1985). Each of the domain-specific antibodies recognized monospecifically the corresponding functional domain of rat plasma FN (data not shown). A polyclonal antibody generated to human fibronectin receptor ($\alpha_5\beta_1$) was purchased from Telios Pharmaceuticals, Inc. These antisera were subjected to isolation of the IgG fraction by using a protein A–Sepharose (Pharmacia Biotechnology) column. An immunoaffinity matrix was prepared by coupling of anti-Fib 1 IgG or anti-Cell IgG to CNBr–Sepharose (Pharmacia Biotechnology) according to the method reported by Cuatrecasas et al. (1968).

Cell Culture. A clonal preadipose cell line, ST-13 (Hiragun et al., 1980), was a gift of Dr. Mayumi Sato, The Tokyo Metropolitan Institute of Medical Science. The cells were maintained in DMEM/Ham F-12 (1:1)(D/H) medium supplemented with 10% calf serum (growth medium). For induction of adipose conversion, the cells were plated at a density of 4×10^3 cells/cm^2 with the growth medium (day 0), and were refed with the D/H medium supplemented with 10% fetal bovine serum and 10 μg/mL insulin (induction medium) on the following day (day 1)(Sato et al., 1985). Treatment with intact FN or its thermolysin digest was started on day 1. Each medium was changed every 3 days. FN contents in both serum-containing media were within 2–5 μg/mL as determined by ELISA using anti-rat plasma FN antibody and bovine plasma FN (IWAKI GLASS, Tokyo) as standards. Immunoblot analysis indicated that at least 90% of the FN was of the intact form (data not shown).

Evaluation of Adipocyte Differentiation. The extent of ST-13 cell differentiation was evaluated by the following three indexes. *The number of adipocytes*: The number of cells containing lipid droplets (adipocytes)(see Figure 1) was counted daily in 10 high-powered fields (10 HPF) under a microscope at 200×. Total numbers of cells were kept constant [$(4.5 \pm 0.76) \times 10^4$/cm^2] after confluency was reached on day 4 under the culture conditions used in this study. Therefore, the number of adipocytes was regarded as one of the indexes for ST-13 cell differentiation. For counting of the adipocytes, lipid droplets accumulated in the cells were stained with Oil Red O. *Glycerophosphate dehydrogenase (GPD) activity*: GPD (EC 1.1.1.8) activity of the cytosolic fraction of ST-13 cells was measured from the oxidation of NADH at 340 nm as described earlier (Kozak & Jensen, 1974). *Triacylglycerol (TG) content*: TG accumulated in the cells was extracted with 2-propanol and quantitated colorimetrically according to the method of Fletcher (1968).

Purification of FN Fragments Showing Differentiation Stimulating Activity. To purify the active fragment relating to the Fib 1 domain of FN, a part of the thermolysin digest of intact FN was mixed with anti-Fib 1 IgG-immobilized Sepharose and shaken at 37 °C for 1 h. After the gel ws washed with PBS(–), fragments bound to the gel were eluted with 0.1 M glycine hydrochloride buffer containing 20% ethylene glycol, pH 2.5. The sample was immediately neutralized with 1 M Tris solution, dialyzed against 20 mM Tris-HCl buffer containing 0.5 mM EDTA and 50 mM NaCl, pH 7.4, and then applied to a heparin–Sepharose column equilibrated with the dialyzing buffer. After the column was washed extensively with the same buffer, the fragment bound to the column was eluted with 20 mM Tris-HCl buffer containing 1 M NaCl, pH 7.4. The protein peak was pooled and dialyzed against PBS(–). For purification of the Cell domain-relating active fragment, another part of the thermolysin digest was subjected to immunoaffinity chromatography using anti-Cell domain IgG-immobilized Sepharose as described above. The sample eluted from the immunoaffinity matrix was dialyzed against PBS(–). The tryptic Fib 1 fragment was purified from the short-term tryptic digest of FN [trypsin: FN ratio = 1:1000 (w/w), at 25 °C for 12 min] by column chromatography using gelatin–Sepharose (passed through), heparin–Sepharose (bound and eluted with 1 M NaCl), and then Sephadex G-75 (Sekiguchi et al., 1985). The purified Fib 1 fragment showed a single protein band of M_r 28K on SDS–polyacrylamide gel electrophoresis followed by protein staining with Coomassie blue.

Amino Acid Sequencing. The amino-terminal end of FN fragments was sequenced as reported previously (Yano et al., 1989), using an Applied Biosystems gas-phase sequenator 470A.

RESULTS

Inhibitory Effect of Intact FN on Adipocyte Differentiation of ST-13 Preadipocytes. Differentiation of ST-13 preadipocytes cultured in the induction medium was followed by the change in cell morphology from a fibroblastic to a polygonal shape, and included massive accumulation of intracellular lipid droplets, as visualized in Figure 1A,B. The differentiation of ST-13 cells was quantitated by increases in the number of the adipocytes, TG content, and GPD activity (see Table I and Figure 2). To examine the effects of FN on adipose conversion, ST-13 cells were treated continuously with intact FN. Inclusion of intact FN (100 μg/mL) neither induced any significant morphological changes in ST-13 preadipocytes nor resulted in adipose conversion during 10-days culture with the induction medium (Figures 1C and 2A). The increase in GPD activity observed during differentiation was suppressed dose-dependently by intact FN added exogenously, and the activity reached a plateau at the lowered level corresponding to that of preadipocytes by inclusion of 100 μg/mL FN (Figure 2B). No significant TG accumulation in the cells was observed by addition of intact FN (100 μg/mL)(Figure 2 and Table I). Intact FN showed the inhibitory effect also when coated on the culture dish, although somewhat less significant than FN added as a soluble factor (Table I). Thus, intact FN acted inhibitory on the adipocyte differentiation of ST-13 cells, being essentially in agreement with the previous reports using 3T3-F442A and 3T3-L1 cells. The block to adipose conversion by intact FN was reversed by GRGDSP peptide (1.5 mM), but not by GRGESP control peptide (Table I). The polyclonal antibody directed to $\alpha_5\beta_1$-integrin reversed the inhibitory activity of intact FN (Table I). The inhibitory activity of intact FN would be associated with ST-13 cell adhesion dependent on the RGDS–$\alpha_5\beta_1$-integrin interaction.

Stimulation of Adipocyte Differentiation by the Thermolysin Digest of FN. We next examined the effects of FN

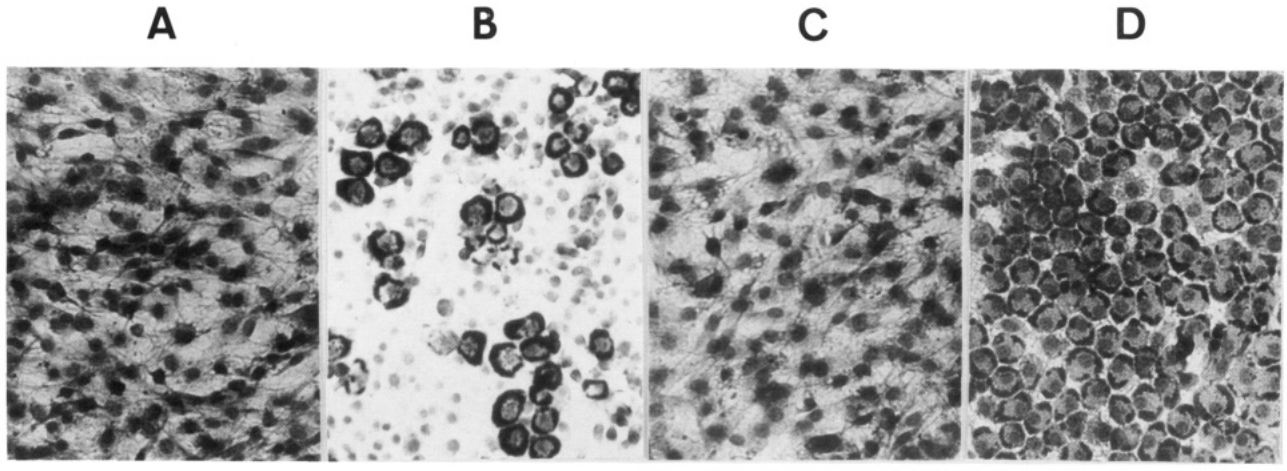

FIGURE 1: Photomicrographs of ST-13 cells. ST-13 cells were cultured for 10 days either with the growth medium (A), with the induction medium (B), with the induction medium including intact FN (100 μg/mL)(C), or with the induction medium including the thermolysin digest of FN (100 μg/mL)(D). The cells were stained with Oil Red O and then with hematoxilin–eosin. Photomicrographs taken at 70× magnification.

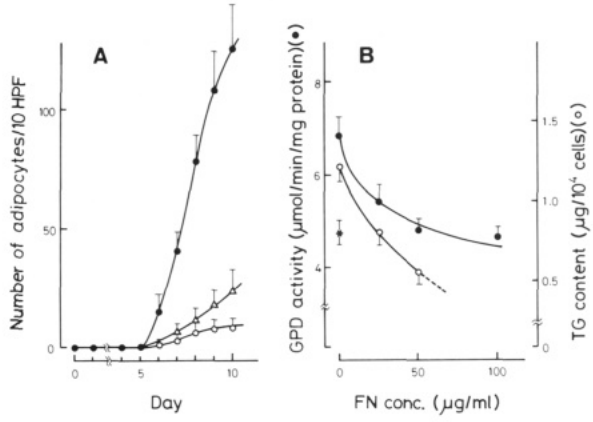

FIGURE 2: Suppression of adipose conversion of ST-13 cells by intact FN. (A) The number of the adipocytes was counted daily on cells cultured either with the induction medium (●) or with the induction medium mixed with 50 μg/mL (△) or 100 μg/mL (○) intact FN. (B) Cytosolic GPD activity (●) and cellular TG content (○) of ST-13 cells cultured for 10 days with the induction medium with the indicated concentrations of intact FN. GPD activity determined on cells cultured with the growth medium for 10 days is represented with an asterisk. The TG content of cells cultured with 100 μg/mL intact FN was not detected. Data points represent the means ± SE of four determinants.

Table I: Effects of FN on Adipocyte Differentiation of ST-13 Cells

culture conditions	no. of adiopocytes	GPD act. [μmol min^{-1} (mg of protein)$^{-1}$]	TG content (μg/10^4 cells)
growth medium	0*	3.6 ± 0.4*	ND[a]*
induction medium	112 ± 25†	8.3 ± 0.2†	1.25 ± 0.26†
+FN, 100 μg/mL	7 ± 3*	3.5 ± 0.2*	ND[a]*
+FN + GRGDSP[b]	86 ± 12†	6.5 ± 0.4†	1.10 ± 0.14†
+FN + GRGESP[c]	9 ± 3*	3.1 ± 0.4*	ND[a]*
+FN + anti-$\alpha_5\beta_1$[d]	79 ± 23†	6.0 ± 1.2†	0.90 ± 0.33†
FN-coated[e]	22 ± 9*	5.5 ± 0.7*	0.73 ± 0.14*†

[a] ND, not detected (below 0.1, μg/10^4 cells). [b] Mixture of 100 μg/mL FN and 1.5 mM GRGDSP peptide. [c] Mixture of 100 μg/mL FN and 1.5 mM GRGESP peptide. [d] Mixture of 100 μg/mL FN and 60 μg/mL anti-$\alpha_5\beta_1$ IgG. [e] Cells were cultured with the induction medium on the dish coated with 100 μg/mL FN. Values are shown as mean ± SE of three determinants on day 10. Analysis of variance as assessed by Student's *t*-test: induction medium vs others (asterisk), $P < 0.05$; +FN vs +FN+GRGDSP or +FN+anti-$\alpha_5\beta_1$ (dagger), $P < 0.05$.

fragments on adipocyte differentiation. Intact FN was proteolyzed by thermolysin, and the resulting FN digest which consists of the fragments of M_r 21–160K (Figure 3A) was then added to the culture system using the induction

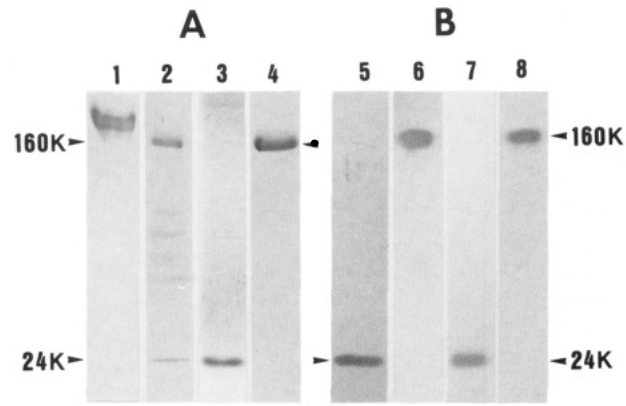

FIGURE 3: Fragment patterns and their immunoblot analysis. FN (lane 1), the thermolysin digest of FN (lanes 2, 5, and 6), the fragment purified by anti-Fib 1 IgG–Sepharose and heparin–Sepharose (lanes 3 and 7), and the fragment purified by anti-Cell IgG–Sepharose (lanes 4 and 8) were applied to SDS–gel electrophoresis using a 10.7–16% polyacrylamide gel. (A) Protein staining with Coomassie blue. (B) Immunostaining of the nitrocellulose blots with anti-Fib 1 (lanes 5 and 7) and anti-Cell (lanes 6 and 8).

medium. As can be seen from Figure 1D, the thermolysin digest of FN remarkably accelerated, instead of suppressed, the appearance of adipocytes. The digest at 100 μg/mL caused about an 8-fold rise above the control in the number of adipocytes on day 10 (Figure 4A). Moreover, the digest (100 μg/mL) induced remarkable increases in GPD activity (about 12-fold above the control) and cellular TG content (5-fold)-(Figure 4B). Table II summarizes the effects of the thermolysin digest on adipocyte differentiation under the various culture conditions. The stimulatory effect of the thermolysin digest was also demonstrated when the examination was carried out with the growth medium. GRGDSP peptide (up to 1.5 mM) could not prevent the stimulatory effect of the thermolysin digest, suggesting that the stimulatory effect may be due to a fragment(s) released from outside of the RGDS-relating molecular region of FN. On the other hand, no residual proteolytic activity was detected in the thermolysin digest (see Materials and Methods), and, additionally, the thermolysin inactivated with $HgCl_2$ has only basal activity (data not shown). Moreover, the differentiation stimulatory activity of the thermolysin digest was neutralized by the polyclonal antibody generated to intact FN (Figure 5). These results seem to exclude the possibility that the stimulatory activity may be due to some contaminants other than FN fragments.

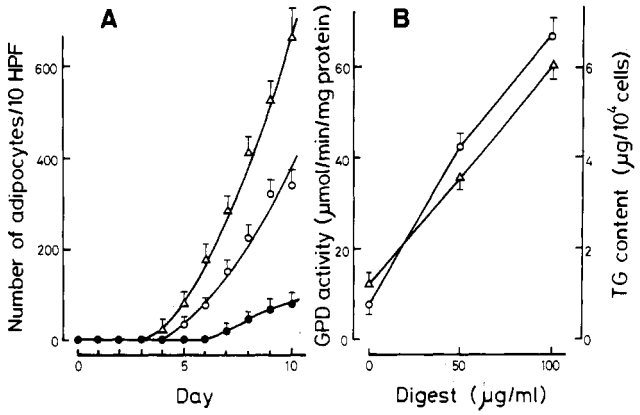

FIGURE 4: Stimulation of adipocyte differentiation of ST-13 cells by the thermolysin digest of FN. (A) The number of adipocytes counted daily on ST-13 cells cultured either with the induction medium (●) or with the induction medium mixed with 50 μg/mL (○) or 100 μg/mL (△) thermolysin digest. (B) GPD activity (○) and TG content (△) in ST-13 cells cultured for 10 days with the induction medium mixed with the indicated concentrations of thermolysin digest. Data represent the means ± SE of four determinants.

Table II: Effects of the Thermolysin Digest of FN on Differentiation of ST-13 Cells

culture conditions	no. of adipocytes	GPD act. [μmol min⁻¹ (mg of protein)⁻¹]	TG content (μg/10⁴ cells)
growth medium	0	4.2 ± 0.5	ND[a]
+digest, 50 μg/mL	61 ± 12*	5.5 ± 0.6	0.65 ± 0.10*
+digest, 100 μg/mL	129 ± 20*	7.6 ± 0.4*	1.30 ± 0.12*
induction medium	138 ± 45	7.3 ± 0.8	1.25 ± 0.61
+digest, 100 μg/mL	797 ± 86†	62.8 ± 6.0†	6.12 ± 0.54†
+digest + GRGDSP[b]	836 ± 104†	72.6 ± 11.3†	7.10 ± 1.05†

[a] ND, not detected (below 0.1 μg/10⁴ cells). [b] Mixture of 100 μg/mL digest and 1.5 mM GRGDSP peptide. Values are shown as mean ± SE ($N = 3$). Analysis variance: growth medium vs +digest (asterisk), $P < 0.05$; induction medium vs treated (dagger), $P < 0.05$.

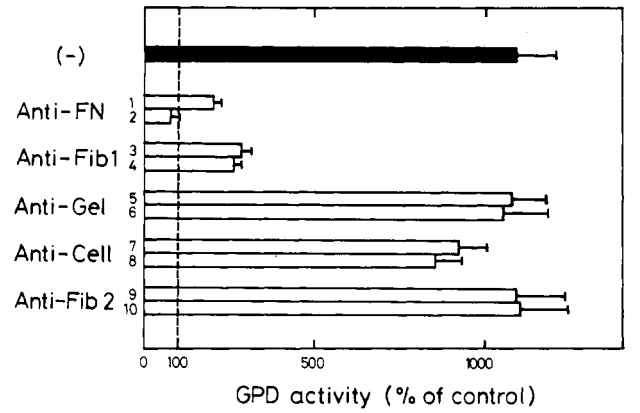

FIGURE 5: Neutralization of the differentiation stimulatory activity of the thermolysin digest by domain-specific antibodies. ST-13 cells were cultured for 10 days with the induction medium containing 100 μg/mL thermolysin digest in the absence (closed bar) or presence of the following antibodies: anti-FN IgG, 30 and 75 μg/mL (bars 1 and 2); anti-Fib 1 IgG, 50 and 100 μg/mL (bars 3 and 4); anti-Gel IgG, 50 and 100 μg/mL (bars 5 and 6); anti-Cell IgG, 50 and 100 μg/mL (bars 7 and 8); anti-Fib 2 IgG, 75 and 150 μg/mL (bars 9 and 10). GPD activity of cells cultured with the induction medium without the thermolysin digest is defined as 100%. Data represent means ± SE of three determinants.

Identification of the Active FN Fragment. To identify an active FN fragment(s) in the thermolysin digest, polyclonal antibodies directed to four functionally distinct FN domains (Fib 1, Gel, Cell, and Fib 2 domains) were used as specific probes. The effects of these domain-specific antibodies on adipocyte differentiation stimulated by the thermolysin digest were examined. There was no change in the appearance of the adipocytes by treatment with anti-Gel and anti-Fib 2, whereas anti-Fib 1 markedly suppressed adipose conversion (data not shown). Anti-Cell delayed the appearance of adipocytes. Then the effects of these antibodies were further assessed by quantifying the GPD activity in ST-13 cells on day 10. As shown in Figure 5, anti-Fib 1, but not anti-Gel and anti-Fib 2, strongly prevented augmentation of the GPD activity induced by the thermolysin digest. About 70% inhibition was recorded by 100 μg/mL anti-Fib 1 IgG, but the antibody at higher doses did not produce complete inhibition. Slight inhibition (about 15%) was also observed with anti-Cell (Figure 5). However, mixing of anti-Fib 1 and anti-Cell did not elicit complete inhibition (data not shown), suggesting that one or more fragments may be involved. Any of these domain-specific antibodies by themselves did not affect adipocyte differentiation (data not shown). Preimmune normal rabbit IgG was neither inhibitory nor stimulatory on the the differentiation (data not shown).

Table III: Effects of Purified 24K Fib 1 Fragment on Differentiation of ST-13 Cells

culture conditions	no. of adipocytes	GPD act. [μmol min⁻¹ (mg of protein)⁻¹]	TG content (μg/10⁴ cells)
induction medium	86 ± 25†	8.3 ± 0.3†	1.08 ± 0.41†
+24K (100 μg/mL)	1657 ± 203*	181.3 ± 27.5*	22.69 ± 2.36*
+24K + GRGDSP[a]	1732 ± 219*	186.7 ± 19.75*	
+24K + anti-$\alpha_5\beta_1$[b]	1800 ± 222*	197.4 ± 29.92*	
24K-coated[c]	91 ± 34†	8.7 ± 0.4†	1.26 ± 0.51†

[a] Mixture of 100 μg/mL 24K fragment and 1.5 mM GRGDSP peptide. [b] Mixture of 100 μg/mL 24K fragment and 60 μg/mL anti-$\alpha_5\beta_1$ IgG. [c] Cells were cultured for 10 days with the induction medium on the dish coated with 100 μg/mL 24K fragment. Values on day 10 are shown as mean ± SE ($N = 3$). Analysis of variance: induction medium vs treated (asterisk), $P < 0.05$; +24K vs +24K+GRGDSP or +24K+anti-$\alpha_5\beta_1$ (dagger), $P < 0.05$.

Immunoblot analyses of the thermolysin digest using anti-Fib 1 and anti-Cell were carried out. As shown in Figure 3B, anti-Fib 1 and anti-Cell reacted with fragments of M_r 24K and 160K, respectively, among the thermolysin fragments. Since the Fib 1 domain is known to have a specific affinity also toward heparin, the 24K fragment was purified to homogeneity by a combination of affinity chromatographies utilizing an anti-Fib 1 IgG-immobilized Sepharose and heparin–Sepharose (Figure 3). The amino-terminal 10 amino acids of the purified fragment were determined to be Val-Ser-Gln-Ser-Lys-Pro-Gly-X-Phe-Asp (X stands for an unidentified amino acid, and it was presumed to be a cysteine residue). The sequence corresponds to residues 14–23 of rat plasma FN (Patel et al., 1987; Schwarzbauer et al., 1987). The results directly indicated that the 24K fragment is released from the Fib 1 domain of FN. Another putative active fragment, the 160K fragment, was also purified from the thermolysin digest (Figure 3A). The amino-terminal 10 amino acids determined, Val-Val-Ala-Thr-Ser-Glu-Ser-Val-Thr-Glu, which corresponds to residues 688–697 of rat plasma FN, indicated that this fragment contained the Cell and Hep 2 domains. When ST-13 preadipocytes were treated with 100 μg/mL purified 24K Fib 1 fragment, the number of adipocytes on day 10 reached nearly 20-fold of the control (Table III). The GPD activity of the cells also exceeded 20-fold over the control (Table III and Figure 6). The differentiation stimulatory effect of the purified 24K Fib 1 fragment nearly reached a plateau at 150 μg/mL. Anti-Fib 1 neutralized the differentiation stimulatory activity of the 24K Fib 1 fragment

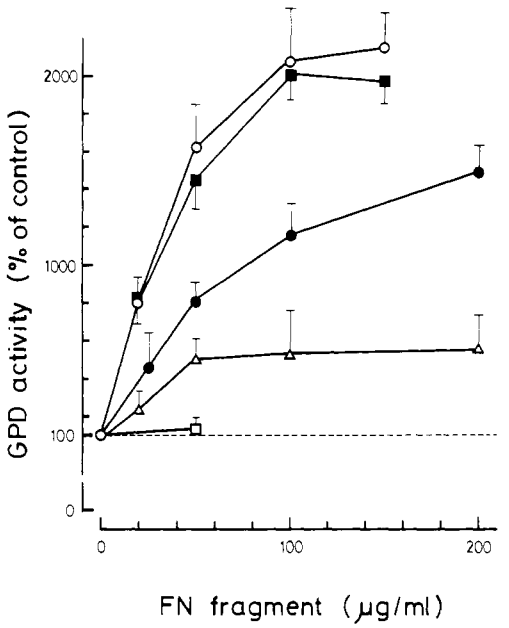

FIGURE 6: Dose-dependent increase in the GPD activity of ST-13 cells by FN fragments. ST-13 cells were cultured with the induction medium mixed with the indicated concentrations of either the purified 24K Fib 1 fragment (○), the purified 24K fragment (50 μg/mL) treated with anti-Fib 1 IgG (170 μg/mL) (□), the purified 160K Cell Hep 2 fragment (△), the purified 28K tryptic Fib 1 fragment (■), or the thermolysin digest of FN (●). Data represent the means ± SE of three determinants.

(Figure 6). The purified 160K fragment also stimulated significantly adipose development, whereas the increase in GPD activity reached a plateau at a much lower level than that observed with the Fib 1 fragment (Figure 6). The results indicate that most of the differentiation stimulatory activity of the thermolysin digest may be accounted for by the 24K Fib 1 fragment.

In contrast to the inhibitory effect of intact FN, the stimulatory activity of the 24K fragment was not affected by the GRGDSP peptide (up to 1.5 mM) (Table III). Anti-$\alpha_5\beta_1$-integrin could not prevent stimulation of differentiation by the 24K fragment (Table III). When the 24K fragment was coated on the culture dish, no stimulatory effect was observed (Table III). Another Fib 1 fragment, the 28K Fib 1 fragment obtained from the tryptic digest of FN, also showed differentiation stimulatory activity at doses similar to those of the 24K Fib 1 fragment (Figure 6).

DISCUSSION

Inhibitory effects of FN on adipose cell differentiation were first described by Spiegelman and Ginty using 3T3-F442A preadipose cells (Spiegelman & Ginty, 1983). They demonstrated that FN coated on the culture dish prevents cytoskeletal and morphological changes necessary for adipocyte differentiation. Our results also showed that FN in solution as well as FN matrices inhibits differentiation of ST-13 preadipocytes, in which the inhibitory effect is more evident on FN added continuously as a soluble factor. FN added as a soluble factor might be able to adsorb to the culture dish surface, in compliance with the consumption of FN matrices, and thereby might strengthen cell adhesion. Since inhibition of differentiation was reversed by either the GRGDSP cell recognition peptide or anti-$\alpha_5\beta_1$, the effect would be associated with the RGDS–$\alpha_5\beta_1$-integrin interaction. Similar to 3T3-F442A cells, the morphological change from flattened to spherical seems to be a key event in the adipocyte differentiation of ST-13 cells. Intact FN would prevent such a morphological change by acting as an element of ECM proteins and consequently inhibit the differentiation.

Interestingly, fragmentation of intact FN revealed a potent differentiation stimulating activity instead of the inhibitory activity. We first suspected that this differentiation stimulating activity may be derived from the thermolysin added for cleavage of FN. However, we could exclude the possibility as assessed by the following: (1) no detectable proteolytic activity remained in the thermolysin digest of FN after inactivation with $HgCl_2$ (see Materials and Methods); (2) the thermolysin–Hg complex was neither stimulatory nor inhibitory on adipocyte differentiation; (3) the stimulatory effect of the thermolysin digest was neutralized almost completely by anti-FN polyclonal antibody. An immunotitration experiment using the domain-specific antibodies showed that the FN fragment relating to the Fib 1 domain is mainly involved in the stimulation of adipocyte differentiation. Indeed, the purified 24K Fib 1 fragment exhibited a potent stimulatory activity to induce adipocyte differentiation. Additionally, another Fib 1-derived fragment, the 28K tryptic Fib 1 fragment, similarly stimulated adipose conversion at doses equivalent to those of the thermolysin Fib 1 fragment. The data suggest that the differentiation stimulatory activity is one of the functional characteristics of Fib 1-derived fragments. Although we could not exclude completely the possibility that some other FN fragments and some trace amounts of contaminants including growth factors such as transforming growth factor-β and insulin-like growth factors might be involved, a major part of the differentiation stimulatory activity of the thermolysin digest should be attributed to the 24K Fib 1 fragment.

Cellular receptors or binding activities directed to FN and its fragments have been described by many investigators. Many of the FN–cell interactions can be attributed to the binding of FN with the β_1-class integrins (Gehlsen et al., 1988; Pytela et al., 1987; Wayner et al., 1987, 1989). The ST-13 preadipocytes used here express detectable amounts of the β_1 class integrins, $\alpha_3\beta_1$, $\alpha_4\beta_1$, and $\alpha_5\beta_1$ (unpublished data). As mentioned above, the inhibitory effect of intact FN seems to depend on the RGDS–$\alpha_5\beta_1$-integrin interaction. On the contrary, the differentiation stimulatory activity of the 24K Fib 1 fragment was not affected by either the RGDS peptide or the anti-$\alpha_5\beta_1$-integrin. The effect of the Fib 1 fragment would be transduced via a putative receptor distinct from at least $\alpha_5\beta_1$-integrin. On the other hand, the amino-terminal region of the FN molecule is considered to play an important role in FN matrix assembly (McKeown-Longo & Mosher, 1985; Quade & McDonald, 1988; Ichhara-Tanaka et al., 1992). This process requires initial cellular binding of FN via the Fib 1 domain. McKeown-Longo and Mosher (1985) and Quade and McDonald (1988) have found that amino-terminal FN fragments inhibit incorporation of intact FN during the matrix assembly process. Recently, Limper et al. (1991) identified a cell-surface molecule that specifically binds to a 29K Fib 1 fragment in an interaction dependent on divalent cations. This putative receptor for the 29K Fib 1 fragment cannot be immunoprecipitated by polyclonal antibody generated to $\alpha_5\beta_1$-integrin. This knowledge may offer an important direction to understand how our 24K Fib 1 fragment stimulated adipocyte differentiation. It might be likely that the adipose development of ST-13 cells is accompanied by alteration of FN matrix assembly. Further study is needed to examine the alteration of matrix assembly during the adipocyte differentiation induced by our 24K Fib 1 fragment.

It is also important to define whether the same receptor for the Fib 1 domain is involved in the matrix assembly and the stimulation of ST-13 cell differentiation.

Why could the intact FN not show the differentiation stimulatory activity despite bearing the Fib 1 domain intact? Previous studies for the secondary and tertiary structures of FN have suggested that the two halves of the FN molecule are each folded back on themselves under physiological conditions (Williams et al., 1992; Erickson & Carrel, 1983; Tooney et al., 1983; Rocco et al., 1983). The Fib 1 domain may be sterically concealed in the intact FN molecule. Of close relevance to the study presented here, Homandberg and Erickson (1986) have shown that the amino-terminal region of the FN molecule interacts with the Hep 2 domain with high affinity ($K_d = \sim 10^{-9}$ M). They proposed that the Fib 1 domain participates in folding of the molecule in the native structure. Such interdomain interaction and the resulting foldover structure may provide a barrier to conceal the differentiation stimulatory activity. There have been many reports indicating that intact FN possesses the cryptic biological activity within the molecule. Proteolytic cleavage may be an efficient trigger to liberate such cryptic activity. In this sense, the mode of proteolysis of intact FN would be an important determinant to release selectively the active FN fragments without loss of their functions. On the other hand, the Fib 1 domain consists of five loops formed by a disulfide bridge. It is also of interest to know whether or not such a structural feature characteristic of the Fib 1 fragment is required for the differentiation stimulatory activity. Furthermore, since the Fib 1 domain is known to bind with heparin as well as with fibrin, the effect of these biopolymers on the Fib 1 activity should be tested. In any case, it would be essentially needed to define how the active FN fragments are actually generated in vivo. Although FN fragments are often found in inflammatory fluids (Carsons et al., 1989; Clemmensen & Andersen, 1982; Dal Nogare et al., 1987; Gervaix et al., 1992), there has been little information about the presence of FN fragments in the fluids surrounding developmental and differentiating cells. Further studies including this essential subject would be needed to establish the physiological significance of our observations.

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