Fibronectin Peptides Derived from Two Distinct Regions Stimulate Adipocyte Differentiation by Preventing Fibronectin Matrix Assembly†

Sadahiro Kamiya,[‡,§] Rina Kato,[‡,§] Masayoshi Wakabayashi,[‡] Takehiro Tohyama,[‡,§] Isao Enami,[∥] Masaaki Ueki,[⊥] Hirofumi Yajima,[⊥] Tadahiro Ishii,[⊥] Hiroshi Nakamura,[§] Takashi Katayama,[‡] Junichi Takagi,[#] and Fumio Fukai*[,‡]

Department of Patho-Physiology and Analytical Chemistry, Faculty of Pharmaceutical Sciences, and Department of Biology and Applied Chemistry, Faculty of Science, Science University of Tokyo, 12 Ichigaya Funagawara-Machi, Shinjuku-Ku, Tokyo 162-0826, Japan, and Department of Biological Sciences, Tokyo Institute of Technology, 4259 Nagatsuta-Cho, Midori-Ku, Yokohama 226-0026, Japan

Received August 8, 2001; Revised Manuscript Received November 26, 2001

ABSTRACT: Here, we show that fibronectin (FN) peptides derived from two distinct regions promote the insulin-induced adipocyte differentiation of ST-13 cells by preventing FN fibrillogenesis. ST-13 cells formed numerous FN fibrils under nonadipogenic conditions, whereas this FN fibrillogenesis was suppressed by adipose induction with insulin. The insulin-induced adipocyte differentiation was promoted by an amino-terminal 24-kDa fragment of FN, accompanied by further suppression of FN fibrillogenesis. The 24 K fragment prevented FN matrix assembly by direct incorporation into the FN matrix. Like the 24 K fragment, a peptide from the 14th type III repeat, termed FNIII14, which suppressed the integrin $\alpha5\beta1$-mediated adhesion of ST-13 cells to FN, accelerated the adipocyte differentiation by preventing FN fibrillogenesis without direct incorporation into the FN matrix. FNIII14 induced the conformation change of $\beta1$ integrins of K562 cells from active to resting, as judged by FACS analysis using a monoclonal antibody AG89 directed to an active $\beta1$ integrin-dependent epitope. Binding of a ^{125}I-labeled FN fragment containing the RGD cell adhesive site to ST-13 cell surface was dissociated by FNIII14, with a concomitant binding of FNIII14 itself to the cell surface. The affinity labeling of ST-13 cells using biotinylated FNIII14 showed that FNIII14 specifically bound to a nonintegrin membrane protein with M_r of around 50 kDa. Thus, the results indicated that prevention of FN fibrillogenesis by the 24 K Fib 1 fragment and FNIII14 caused the promotion of adipocyte differentiation of ST-13 cells and that the former was due to the direct incorporation into the FN matrix and that the latter might be interpreted by negative regulation of FN receptor $\alpha5\beta1$ activity.

Cell adhesion to the extracellular matrix (ECM)[1] is a crucial event in multicellular organisms for the regulation of fundamental cellular processes such as growth, differentiation, survival/apoptosis, and gene expression (*1*−*3*). ECM proteins, such as fibronectin (FN), collagen, and laminin, form characteristic protein networks that show tissue specific variation in composition and architecture. Therefore, ECM provides positional and environmental information that is essential for tissue function. FN, one of the key elements of ECM proteins, is a large, dimeric glycoprotein that is found in plasma and most body fluids and is an insoluble constituent of loose connective tissue and basement membranes. A wide variety of cell types can synthesize FN and assemble it into detergent-insoluble disulfide cross-linked fibrils in vitro, which closely resemble the FN matrix fibrils found in vivo (*4*−*8*). It is increasingly apparent that the FN matrix has profound effects on cell functions. Indeed, a number of studies have demonstrated the involvement of FN fibrillogenesis in cell growth (*9*−*12*), migration (*13*, *14*), and even differentiation (*15*).

Adipocyte differentiation in adipose precursor cell lines, such as 3T3-F442A (*16*), 3T3-L1 (*17*), and ST-13 (*18*), is characterized by major changes in cell morphology from a flattened/fibroblastic to a rounded/spherical shape. This morphological change, which is due to a lowered interaction of cells to ECM, is considered to be a key event in the adipocyte differentiation of these cell lines (*19*−*24*). Spiegelman and Ginty first described the importance of integrin-mediated adhesive interactions in adipose cell differentiation (*20*). They demonstrated that FN coated on the culture dish inhibited differentiation, being accompanied by the prevention of cytoskeletal and morphological changes. Using ST-13 preadipocytes, we also showed, previously, the inhibition of adipocyte differentiation by FN (*25*, *26*). In our study,

† This work was supported by a Grant-in-Aid for Scientific Research (11680615) provided by the Ministry of Education, Science and Culture of Japan.

* To whom correspondence should be addressed. Fumio Fukai, Ph.D., Department of Patho-Physiology, Faculty of Pharmaceutical Sciences, Science University of Tokyo, 12 Ichigaya Funagawara-Machi, Shinjuku-Ku, Tokyo 162-0826, Japan. Phone: +81(3)3260-6725. Fax: +81(3)3268-3045. E-mail: fukai@ps.kagu.sut.ac.jp.

‡ Department of Patho-Physiology, Science University of Tokyo.

§ Department of Analytical Chemistry, Science University of Tokyo.

∥ Department of Biology, Science University of Tokyo.

⊥ Department of Applied Chemistry, Science University of Tokyo.

Department of Biological Sciences, Tokyo Institute of Technology.

[1] Abbreviations: FN, fibronectin; ECM, extracellular matrix; 24 K Fib 1 fragment, a 24-kDa fragment derived from the amino-terminal fibrin-binding domain of fibronectin; 110 K FN fragment, a 110-kDa fragment derived from the central cell-binding domain of fibronectin; GRGDSP, Gly-Arg-Gly-Asp-Ser-Pro; GRGESP, Gly-Arg-Gly-Glu-Ser-Pro; EIA, enzyme immunoassay; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; ECL, enhanced chemiluminescence.

10.1021/bi015660a CCC: $22.00
Published on Web 02/01/2002

the inhibitory effect of FN was more evident on FN added as a soluble supplement than on FN coated as an immobilized substrate. It has been believed that the active form of FN is a fibrillar matrix form. It might be possible that exogenously added FN was incorporated into FN matrix fibrils surrounding ST-13 cells and then inhibited adipocyte differentiation by preventing the cytoskeletal and morphological changes necessary for adipocyte differentiation.

In contrast to the inhibitory effect of FN on adipocyte differentiation, our previous study also showed that a 24-kDa FN fragment derived from the amino-terminal fibrin-binding (Fib 1) domain promoted insulin-induced differentiation (*25*, *26*). On the analogy of this possible action mechanism of FN, the differentiation stimulatory effect of the 24 K Fib 1 fragment may also be interpreted by FN matrix assembly, because it is now established that FN fragments containing the Fib 1 domain inhibit FN matrix assembly (*27*−*31*). It might be possible that our 24 K Fib 1 fragment prevented FN matrix assembly of ST-13 cells and, consequently, facilitated the morphological change of ST-13 preadipocytes to spherical. In the present study, we show that the opposing effects of FN and the 24 K Fib 1 fragment on the adipocyte differentiation of ST-13 cells can be attributed to the antipodal activities of these factors on the FN fibrillogensis of ST-13 cells. On the other hand, we previously found that FN has a functional site opposing to cell adhesion to FN (*32*, *33*); a peptide derived from the 14th type III repeat (III_{14}) (1835−1855 of FN, termed FNIII14) acts inhibitory on the integrin-mediated signaling in a reversible manner (*33*−*36*). In addition to the Fib 1 domain, we also show that FNIII14 is involved in the positive regulation of adipocyte differentiation of ST-13 cells by preventing FN fibrillogenesis. Both the 24 K Fib 1 fragment and peptide FNIII14 can prevent FN fibrillogenesis, whereas their action mechanisms on FN matrix assembly are shown to be entirely different.

EXPERIMENTAL PROCEDURES

Materials. Human plasma FN, a 24-kDa fragment derived from the Fib 1 domain, and a 110-kDa fragment containing the central cell-binding domain were isolated, as described earlier (*25*, *37*). FNIII14 (TEATITGLEPGTEYTIYVIALC), its control inactive peptide FNIII14scr (TEATITGLEPGTELIVYATYIC) (*34*, *35*), and CS-1 peptide (CLHPGEILDVPST) were obtained from Sawady Technology (Tokyo, Japan) (*34*, *35*). Anti-murine β1 integrin (9EG7), which induces the activation of cell adhesion to FN (*38*−*40*), was purchased from BD-Pharmingen (San Diego, CA). An mAb directed to an active conformation-dependent epitope of human β1 integrin (AG89) was prepared as described earlier (*41*, *42*). Anti-integrin $\alpha5\beta1$ polyclonal antibody (pAb) and anti-α4 mAb were purchased from Chemicon Intenational Inc. (Temecula, CA) and Seikagaku Corporation (Tokyo, Japan), respectively.

Cell Culture. ST-13 cells were grown in DMEM/Ham F-12 (D/H) medium containing 10% calf serum (growth medium). For the induction of adipose conversion, cells were plated in 24- or 96-well culture plates with the growth medium (day 0) and were cultured with the D/H medium containing 10% FBS and insulin (10 μg/mL) (induction medium) on day 1 (*25*). FN in the seru-containing medium was depleted by passing through a gelatin affinity column.

Evaluation of Adipocyte Differentiation. ST-13 cell differentiation was evaluated either by counting the number of adipocytes or by quantifying glycerophosphate dehydrogenase (GPD) activity of ST-13 cells, as described previously (*25*, *26*).

Quantitation of FN Fibrils Formed by ST-13 Preadipose and Adipose Cells. On days 1, 3, 6, and 9, ST-13 cells cultured as described previously were washed 5 times with the D/H medium and then lysed to extract the deoxycholate-soluble (pool I) and insoluble (pool II) fractions, as established previously (*27*). FN in pool I and pool II were quantified by EIA (*29*).

FN Matrix Assembly Assay. ST-13 cells in the growth medium were plated in 24-well plates at confluent and cultured with biotinylated FN (50 μg/mL) in the presence or absence of the sample to be tested. After a 2-day culture, cells were washed 5 times with the D/H medium and lyzed to obtain pool I and pool II, as described previously. FN in pools I and II were quantified by EIA using either avidine conjugated peroxidase (POD) or anti-FN pAb, as described previously.

Immunofluorescence Microscopy. ST-13 cell suspension with the growth medium was seeded onto glass coverslips and cultured under adipogenic and nonadipogenic conditions, as described previously. FN fibrils were detected with a FITC-labeled anti-FN pAb and visualized using an Olympus fluorescence microscope.

Cell Adhesion Assay. Cell adhesion assays of ST-13 cells and K562 cells were performed as described previously (*25*, *34*). For ST-13 cells, adhesion was evaluated by the number of cells spread and the total number of cells adhered.

Effect of FNIII14 on the Binding of the 110 K FN Fragment to ST-13 Cells. ST-13 cell suspension (7.5×10^5 cells/100 μL) in the D/H medium was incubated with ^{125}I-labeled 110 K FN fragment (40 μg/mL) in the presence or absence of increased concentrations of FNIII14 at room temperature for 30 min. Each cell suspension was layered on 10 mL of 20% Percoll containing a 0.1 M sucrose solution and then centrifuged at 1500*g* for 30 min. Each supernatant (9.8 mL) was gently aspirated, and the radioactivity of the remain was counted. Another series of the experiments was carried out using the 110 K FN fragment and ^{125}I-labeled FNIII14 under the same conditions.

Flow Cytometric Analysis. Human erythroleukemia K562 cells were incubated with or without FNIII14 (100 μg/mL) in the presence or absence of 5 mM Mn^{2+} for 30 min at 37 °C and then incubated with 10 μg/mL FITC-conjugated AG89 or FITC-conjugated normal mouse IgG for 30 min on ice. Fluorescence was measured using a flow cytometer (Cytoron, Ortho).

Affinity Label. ST-13 cells were incubated with biotinylated FNIII14 in the presence or absence of 20-fold molar excess of unlabeled FNIII14 or FNIII14scr at 37 °C for 1 h. After unbound peptides were washed, peptides bound to cell surface proteins were cross-linked with EDC (5 mM) (Pierce, Rockford, IL). Cell lysates with equal amount of proteins were separated on SDS−PAGE, transblotted to PVDF membrane, and incubated with POD-conjugated streptavidine followed by visualization with ECL (Amersham Biosciences Inc., Piscataway, NJ).

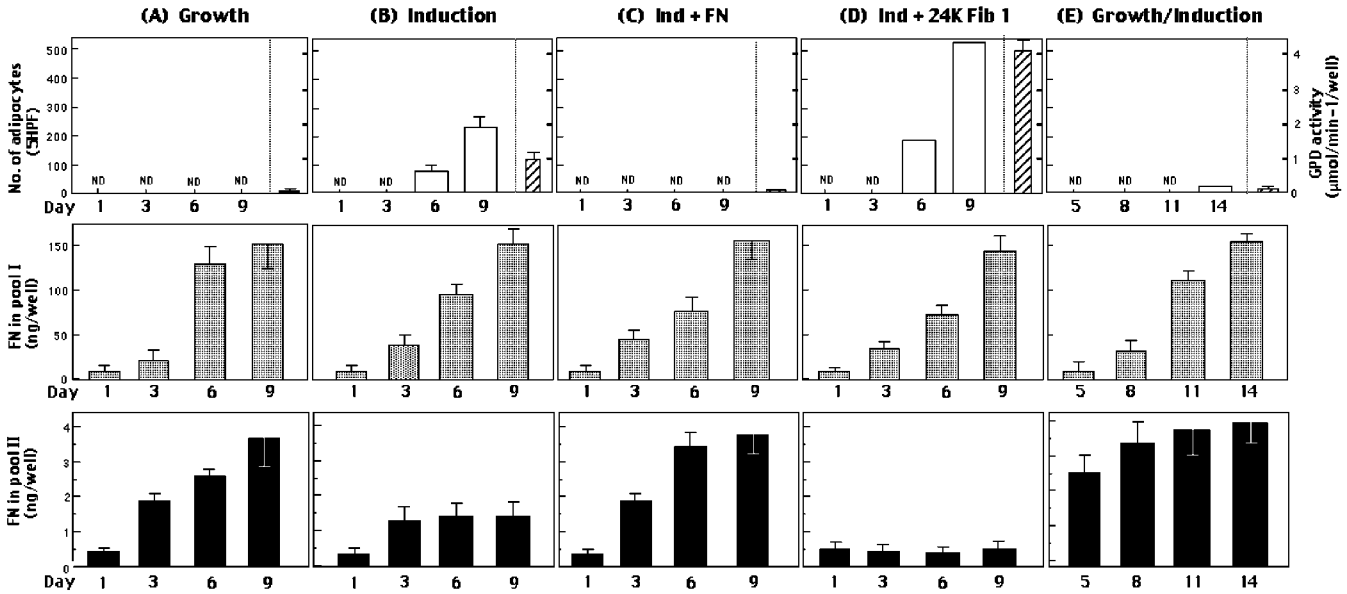

FIGURE 1: FN incorporation into cell matrix during the adipocyte differentiation of ST-13 cells. ST-13 cells seeded on 96-well plates were cultured with the growth medium (A), the induction medium (B), or the induction medium supplemented with FN (100 μg/mL) (C) or the 24 K Fib 1 fragment (100 μg/mL) (D). Alternatively, ST-13 cells, which were cultured with the growth medium for 5 days, were then stimulated with the induction medium (E). On the indicated day, the number of adipocytes (open bars) and FN incorporation into pool I (shaded bars) and II (closed bars) were determined. GPD activity (hatched bars) as a differentiation marker was assayed on days 10 (A−D) or 14 (E). Data represent the means of three determinations ± SD.

RESULTS

FN Fibrillogenesis Regulates the Insulin-Induced Adipocyte Differentiation of ST-13 Cells. FN fibrils were quantified in ST-13 cells cultured under adipogenic and nonadipogenic conditions. When ST-13 cells were cultured without insulin, the accumulation of FN in both pools I and II increased daily (Figure 1A). On day 10, most of the ST-13 cells remained fibroblastic and undifferentiated, whereas well-developed FN fibrils were observed by immunofluorescence microscopy (Figure 2A). In contrast, when ST-13 cell differentiation was induced by insulin, FN incorporation into pool II was suppressed, although the pool I FN increased up to a level similar to that under nonadipogenic conditions (Figure 1B). In agreement with these data, ST-13 cells formed thin and sparse FN fibrils without insulin (Figure 2B). On the other hand, exogenously added FN inhibited the adipocyte differentiation, and simultaneously, FN incorporation into pool II was restored (Figure 1C), where ST-13 cells formed numerous FN fibrils (Figure 2C). Conversely, FN incorporation into pool II was a minimum in ST-13 cells cultured with insulin in the presence of the 24 K Fib 1 fragment (Figure 1D), where many ST-13 cells differentiated and formed only frail FN fibrils (Figure 2D). Also in this case, the pool I FN increased regardless of differentiation. When ST-13 cells were cultured once under nonadipogenic conditions for 5 days, insulin could no longer induce adipocyte differentiation, where FN was accumulated in pool II (Figure 1E).

This reversed correlation between the adipocyte differentiation and FN fibrillogenesis may be further correlated to FN secretion by ST-13 cells. A high level of FN was detected in the medium of ST-13 cells cultured under nonadipogenic conditions (Table 1, data A). The FN content was dramatically reduced when ST-13 cell differentiation was induced by insulin (data B), in parallel with the suppressed FN fibrillogenesis in ST-13 cells cultured with insulin. When ST-13 cells were cultured with insulin in the presence of exogenously added FN, the FN content remained high; FN detected in the medium (data C) was much higher than the value of data B plus exogenously added FN (10 μg/well). Taken together with the data that ST-13 cells cultured under nonadipogenic conditions, which already accumulated FN in pool II, could no longer differentiate, it was conceivable that exogenously added FN was incorporated into the FN matrix and caused the inhibition of adipocyte differentiation. On the other hand, the reduced FN content in the medium, which occurred on the culture with insulin, was not further reduced by addition of the 24 K Fib 1 fragment (data D), although adipocyte differentiation was potently accelerated by this fragment (Figure 1D), indicating that the 24 K Fib 1 fragment prevented FN matrix assembly without affecting the FN content in the medium.

We then examined if ST-13 cells actually incorporate exogenously added FN into their matrix and if the 24 K Fib 1 fragment can prevent it. ST-13 cells were incubated with biotinylated FN in the presence or absence of the 24 K Fib 1 fragment, and FN accumulation in pools I and II was quantified by EIA using either avidine-POD (for the determination of exogenously added FN) or anti-FN pAb (for the determination of exogenous FN plus endogenous FN) (Figure 3). Using either EIA system, the accumulation of FN into pool II, not pool I, was observed and was inhibited by the 24 K Fib 1 fragment in a dose-dependent manner. In parallel with the prevention of FN incorporation into pool II, the 24 K Fib 1 fragment was incorporated into pool II (Figure 3B).

Taken altogether, it was suggested that FN fibrillogenesis was involved in the negative regulation of adipocyte differentiation of ST-13 cells and that the 24 K Fib 1 fragment promoted differentiation by preventing FN fibrillogenesis.

Peptide FNIII14 Derived from the 14th Type III Repeat Accelerates the Adipocyte Differentiation by Preventing FN

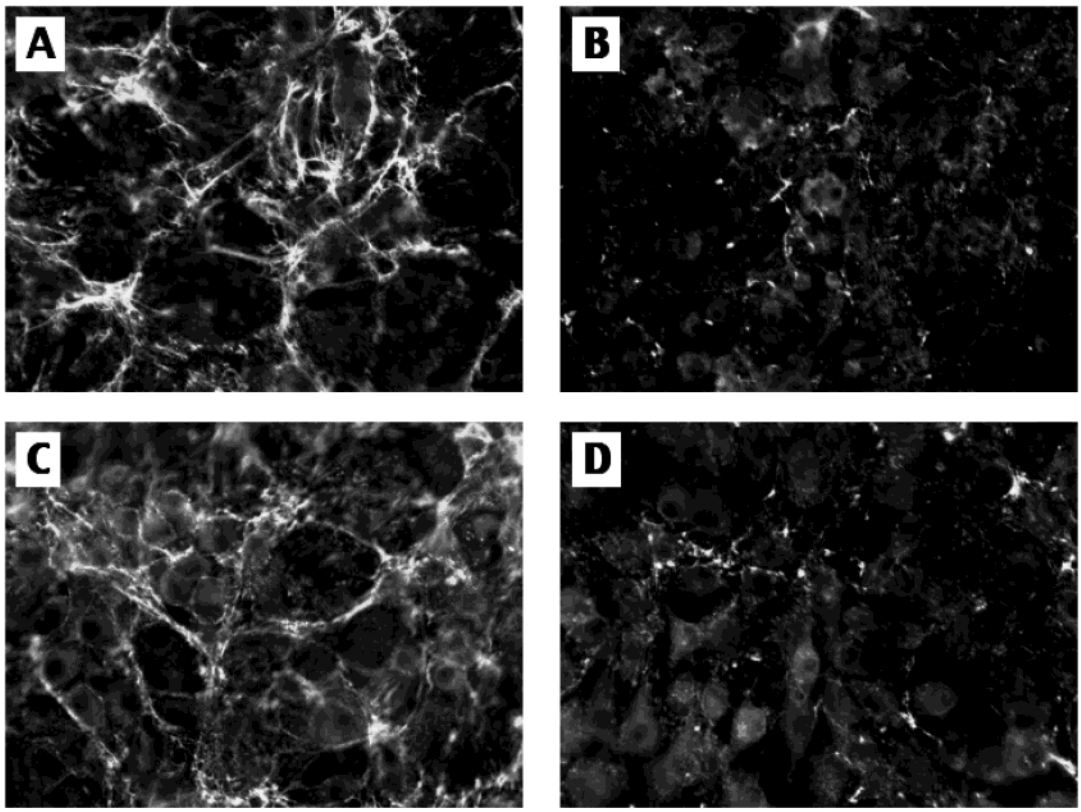

FIGURE 2: Indirect immunofluorescence microscopy of FN fibrils formed surrounding ST-13 cells cultured under adipogenic and nonadipogenic conditions. ST-13 cells were cultured with the growth medium (A), the induction medium (B), and the induction medium containing FN (C) or the 24 K Fib 1 fragment (D).

Table 1: FN Content in the Culture Medium of ST-13 Cells under Adipogenic and Nonadipogenic Conditions[a]

	FN content (μg/well)			
culture conditions	day 1 (0−1)	2 (2−4)	7 (5−7)	10 (8−10)
(A) growth medium	54.1 ± 3.5	182.2 ± 22.9	203.1 ± 26.5	nt[b]
(B) induction medium	8.2 ± 3.4	26.7 ± 4.6	29.7 ± 9.1	nt[b]
(C) ind + FN (10 μg/well)	33.7 ± 5.1	109.9 ± 20.3	nt[b]	178.4 ± 19.9
(D) ind + 24 K Fib 1	7.0 ± 3.7	27.6 ± 5.4	30.1 ± 8.6	nt[b]

[a] ST-13 cells were cultured in 96-well plates with the growth medium (A), the induction medium (B), and the induction medium containing FN (100 μg/mL) (C) or the 24 K Fib 1 fragment (100 μg/mL) (D), respectively. While changing of each medium every 3 days, cells were cultured for 10 days. Each medium collected was examined for EIA using anti-FN pAb, as described in Experimental Procedures. Data represent the means of three measurements ± SD. [b] Not tested.

Matrix Assembly. The aforesaid data indicated the importance of the cell-to-FN interaction in the adipocyte differentiation of ST-13 cells. We have previously found that an FN-derived peptide FNIII14 has the ability to negatively regulate integrin-mediated adhesion and signaling (*33*−*35*). FNIII14 may affect the adipocyte differentiation of ST-13 cells. We first characterized ST-13 cell adhesion to an FN substrate. As shown in Figure 4A, ST-13 cell adhesion to FN was suppressed by incubation with an anti-integrin $\alpha5\beta1$ pAb and GRGDSP peptide, but not with anti-$\alpha4$ mAb and CS-1 and GRGESP peptides, indicating that ST-13 cells adhered to FN mainly via $\alpha5\beta1$. In support of this, FACS analysis showed that ST-13 cells expressed a detectable level of $\alpha5$ but not $\alpha4$ (data not shown). This $\alpha5\beta1$-mediated adhesion of ST-13 cells to FN was suppressed by FNIII14 (Figure 4A), where ST-13 cells represented a rounded/spherical morphology. The ST-13 cell, which became rounded, spread again following the removal of FNIII14 from the medium. An inactive control peptide, FNIII14scr, had no effect on ST-13 cell adhesion to FN.

We next examined the effect of FNIII14 on adipocyte differentiation. When ST-13 cell differentiation was induced by insulin in the presence of FNIII14, a marked dose-dependent acceleration of adipocyte differentiation was observed (Table 2). Like the 24 K Fib 1 fragment, this acceleration of adipocyte differentiation by FNIII14 was accompanied by suppression of FN incorporation into pool II, not pool I (Table 2). FNIII14scr showed no significant effects on both the adipocyte differentiation and FN incorporation into pool II (Table 2). Furthermore, FNIII14 but not FNIII14scr inhibited matrix assembly of FN either secreted by ST-13 cells or added exogenously (Figure 5). However, unlike the 24 K Fib 1 fragment, we failed to detect a clear incorporation of FNIII14 into pool II; FNIII14 binding to ST-13 cells was recovered in pool I (data not shown). FNIII14 might promote adipocyte differentiation by prevent-

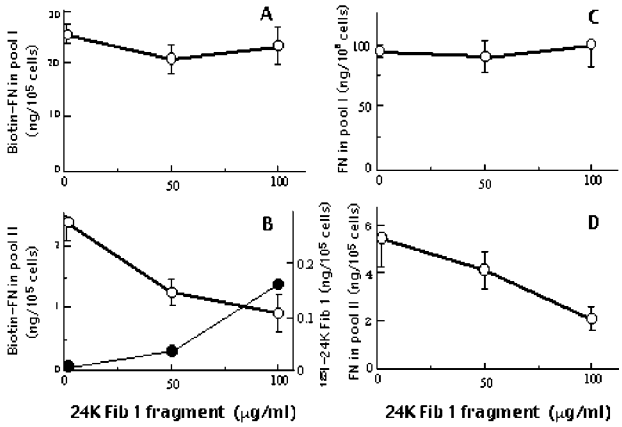

FIGURE 3: Effects of the 24 K Fib 1 fragment on FN matrix assembly of ST-13 cells. ST-13 cell monolayers were cultured in 24-well plates with the growth medium containing biotinylated FN (50 μg/mL) in the presence of the indicated concentrations of the 24 K Fib 1 fragment for 2 days. FN in pools I (A,C) and II (B,D) were quantified using avidin-POD (A,B) or anti-FN pAb (C,D), as described in Experimental Procedures. Incorporation of the 24 K Fib 1 fragment into ST-13 cell matrix was examined using a ^{125}I-labeled 24 K Fib 1 fragment under the same conditions as in panel B. Data represent the means of three determinations ± SD.

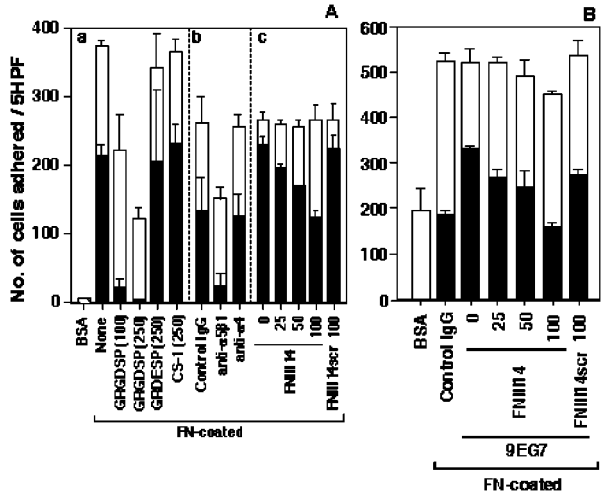

FIGURE 4: Effect of FNIII14 on ST-13 cell adhesion to FN substrate. (A) ST-13 cells suspended with the D/H medium (panels a and b) or the growth medium (panel c) were seeded on 96-well plates coated with FN in the presence of the indicated concentrations of peptides (μg/mL) or antibodies (20 μg/mL). After a 60−80 min incubation at 37 °C, the number of ST-13 cells adhered (attached plus spread, open bars; spread, closed bars) was counted, as described previously (*25*, *34*). (B) The adhesion assay was performed in the D/H medium containing the β1 integrin-activating mAb 9EG7 (30 μg/mL) or the control IgG, as described above. Data represent the means of three determinations ± SD.

ing FN matrix assembly via a way distinct from that of the 24 K Fib 1 fragment.

FN matrix assembly is believed to be initiated by the binding of FN to its receptor integrin. To examine the action of FNIII14 to the FN receptor $\alpha5\beta1$, ST-13 cells were incubated with anti-β1 integrin mAb (9EG7) that induces the β1 integrin-mediated adhesion of cells to FN (*39*, *40*) (Figure 4B). The 9EG7 at 30 μg/mL caused the increase in the number of ST-13 cells spread on FN. FNIII14, but not FNIII14scr, dose-dependently reversed the enhanced adhesion of ST-13 cells in response to 9EG7. To define the FNIII14 action to the FN receptor $\alpha5\beta1$ more clearly, we then performed FACS analysis using an mAb AG89 that recognizes an active conformation-dependent epitope of β1 integrin and a nonadherent cell type K562 that expresses the FN receptor $\alpha5\beta1$ as its only β1 integrin (Figure 6A). Exposure of K562 cells to an integrin activator Mn^{2+} increased the cell populations expressing the AG89 epitope, accompanied by K562 cell adhesion to FN (Figure 6B). Both the expression of the AG89 epitope and the K562 cell adhesion to FN were blocked by FNIII14 but not by FNIII14scr. FNIII14 also suppressed K562 cell adhesion induced by another integrin activator, phorbor myristate acetate (data not shown), excluding the possibility that FNIII14 served as a scavenger of Mn^{2+}. To further clarify the effect of FNIII14 on the FN-$\alpha5\beta1$ interaction actually occurring on ST-13 cells, the binding of the 110 K FN fragment containing the central cell-binding domain to ST-13 cells, and the effect of FNIII14 on this binding, were observed (Figure 7). The ^{125}I-labeled 110 K FN fragment bound to ST-13 cells, and this binding was abrogated with GRGDSP. The binding of the ^{125}I-labeled 110 K fragment on ST-13 cells was dissociated by the addition of increased concentrations of FNIII14 but not FNIII14scr. When this experiment was performed using ^{125}I-labeled FNIII14, the binding of ^{125}I-labeled FNIII14 was detected. The ^{125}I-labeled FNIII14 bound to ST-13 cells was not released by GRGDSP peptide but was recovered with 1% deoxycholate (i.e., pool I).

FNIII14 might change the β1 integrin conformation by direct binding. To confirm this, we affinity-labeled ST-13 cells with biotinylated FNIII14. However, no bands of $\alpha5$ and β1 integrin (110−150 kDa), but a major 55 kDa band and a weak 38 kDa band, were detected (Figure 8, lane 1). These bands disappeared when the affinity labeling was carried out in the presence of an excess amount of unlabeled FNIII14 (lane 2) but not of FNIII14scr (lane 4). The 55 and 38 K bands could not be detected with biotinylated FNIII14scr (lane 3). Anti-β1 integrin mAb 9EG7 did not react with the 55 and 38 K bands but detected a clear band at around 130 kDa (lane 5). Of these two specific bands, the 38 K minor band was not necessarily detected at the same position but sometimes at lower sizes in other cell types responsible to FNIII14, suggesting that the 38 K band may result from proteolytic modification of the 55 K band. These results indicated that FNIII14 negatively regulated the β1 integrin function without direct interaction but possibly by binding to a membrane protein with M_r of around 50 kDa.

DISCUSSION

In the adipose precursor cell lines, such as 3T3-F442A, 3T3-L1, and ST-13, the lowered adhesion to ECM including FN appears to be a prerequisite for their differentiation (*19*−*23*). It was demonstrated that TGF-β inhibited 3T3-L1 cell differentiation by stimulating the expression of FN and collagen (*43*). Selvarajan et al. recently showed that the plasminogen activation by kallikrein is necessary for the induction of adipose development both in vitro and in vivo (*44*). They proposed that the plasminogen cascade fosters adipocyte differentiation by degradation of the FN-rich preadipocyte stromal matrix. Thus, previous reports have indicated the close correlation of FN, as a scaffolding for cell anchoring, to adipogenesis. The active form of FN is thought to be its polymerized fibrillar form. Adipogenesis

Table 2: Acceleration of the Adipocyte Differentiation of ST-13 Cells by FNIII14 Accompanied by the Suppression of FN Incorporation into Pool II[a]

culture conditions	no. of adipocytes (5HPF)	GPD activity (μmol/min^{-1}/10^3 cells)	FN in pool I (ng/10^3 cells)	FN in pool II (ng/10^3 cells)
induction medium	102 ± 34	9.2 ± 0.31	420 ± 27	15 ± 3.3
+FNIII14 (50 μg/mL)	504 ± 97	27.9 ± 0.86	386 ± 30	3.0 ± 0.7
+FNIII14 (100 μg/mL)	892 ± 116	58.4 ± 1.03	402 ± 38	1.7 ± 0.5
induction medium	86 ± 24	7.3 ± 0.22	320 ± 48	11 ± 2.5
+FNIII14 (100 μg/mL)	709 ± 93	60.3 ± 2.16	299 ± 30	0.9 ± 0.6
+FNIII14scr (100 μg/mL)	80 ± 21	8.4 ± 0.30	317 ± 38	15 ± 4.1

[a] ST-13 cells were cultured, in 24-well culture plates, with the induction medium in the presence or absence of FNIII14 at the indicated concentrations. Following a 10-day culture, adipocyte differentiation and FN fibril formation were assessed. Data represent the means of three measurements ± SD.

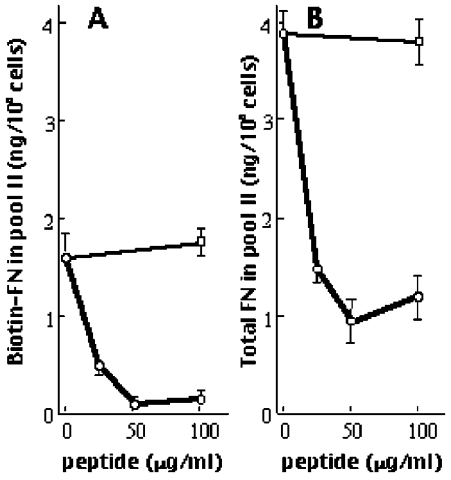

FIGURE 5: Prevention of FN incorporation into pool II by FNIII14. ST-13 cell monolayers were cultured in 24-well plates with the growth medium containing biotinylated FN (50 μg/mL) in the presence or absence of the indicated concentrations of FNIII14 (circles) or FNIII14scr (squares) for 2 days. Biotinylated FN and total FN in pool II were quantitated as in Figure 3. Data represent the means of three measurements ± SD.

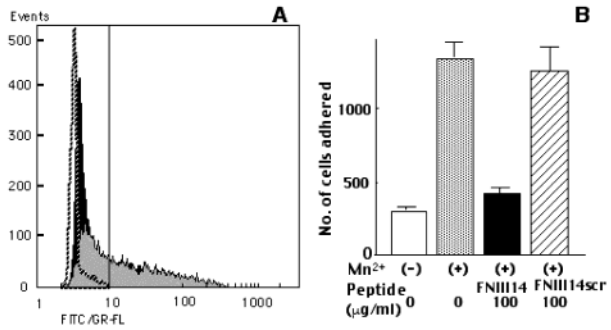

FIGURE 6: Conformation change of β1 integrin of K562 cells induced by FNIII14. (A) K562 cells (10^5) suspended with RPMI1640 medium were incubated with vehicle (open peak with dashed line), Mn^{2+} (5 mM) (gray peak), or Mn^{2+} plus FNIII14 (100 μg/mL) (blackened peak). FACScan analysis of the cells was performed using FITC-labeled anti-β1 integrin mAb AG89, as described under Experimental Procedures. (B) K562 cells were seeded in a 96-well plate coated with FN (5 μg/mL) in the presence or absence of FNIII14 (closed bar) or FNIIIscr (shaded bar) with or without Mn^{2+} (0.1 mM) (open bar). The number of K562 cells adhered was counted, as described previously (*25*, *34*). Data of K562 cell adhesion represent the means of three measurements ± SD.

should be depend on FN expression and its incorporation into fibrillar marix surrounding preadipose cells. The present study showed that the insulin-induced adipocyte differentiation of ST-13 cells was controlled depending on FN fibrillogenesis. Interestingly, ST-13 cells, which were cultured for 5 days under nonadipogenic conditions, could no longer differentiate, even after exposing to insulin for further 10 days. During the 5-day culture, ST-13 cells formed a considerable amount of FN fibrils. It is likely that this FN fibrils blocked adipocyte differentiation. In this regard, FN fibrillogenesis would be one of the earliest and crucial determinants for adipocyte differentiation.

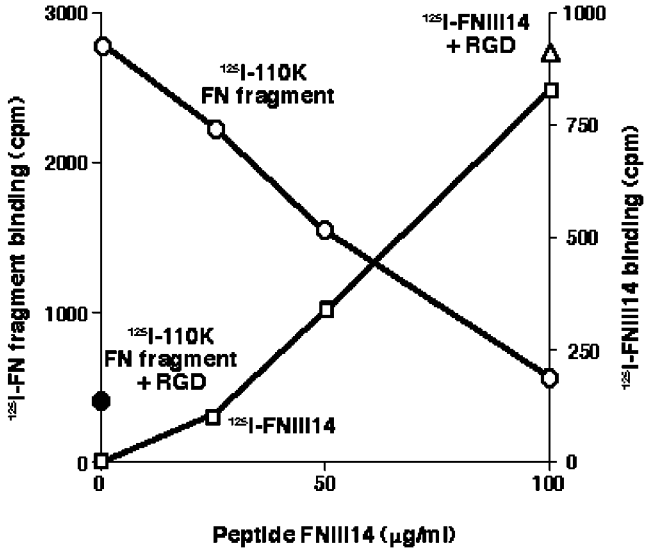

FIGURE 7: FNIII14 induces to dissociate the binding of the 110 K FN fragment to ST-13 cells. ST-13 cell suspension (7.5×10^5 cells/100 μL) in the D/H medium was mixed with a ^{125}I-labeled 110 kDa FN fragment (40 μg/mL) in the presence of the indicated concentrations of FNIII14 (open circles) or 300 μg/mL GRGDSP peptide (closed circle). After the incubation at room temperature for 30 min, cell suspension was centrifuged in 10 mL of 20% Percoll/0.1 M sucrose solution (1500*g*, for 30 min). Radioactivity of the remaining precipitates was counted. Another series of the experiments were carried out using ^{125}I-labeled FNIII14 in the absence (square) or presence (triangle) of GRGDSP peptide under the same conditions.

It has been well-established that FN fragments containing I_{1-5} inhibit FN fibrillogenesis (*27*−*31*). In the present study, the 24 K Fib 1 fragment promoted the adipocyte differentiation of ST-13 cells, with a concomitant suppression of FN incorporation into pool II. Prevention of FN fibrillogenesis by the 24 K Fib 1 fragment would liberate ST-13 preadipose cells from the suppression of adipocyte differentiation by the FN matrix. In addition to the Fib 1 domain, several FN sites recognized by integrins $\alpha5\beta1$ and $\alpha4\beta1$ are involved in FN matrix assembly (*28*, *45*−*50*). The FN−$\alpha5\beta1$/$\alpha4\beta1$

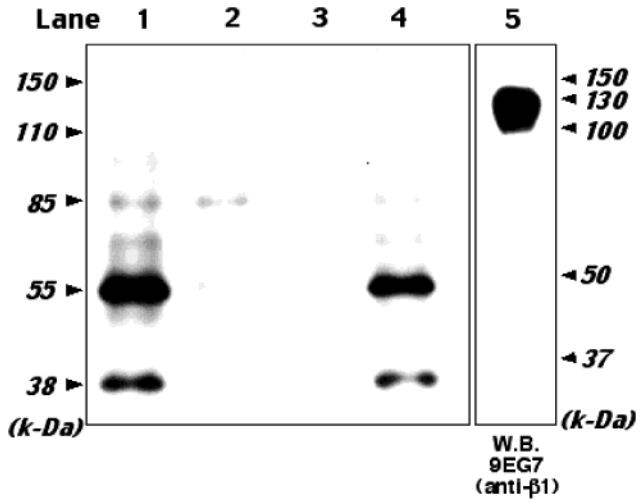

FIGURE 8: Affinity label of ST-13 cells using biotinylated FNIII14. ST-13 cells were incubated with biotinylated FNIII14 in the absence (lane 1) or presence of a 20-fold molar excess of unlabeled FNIII14 (lane 2) or unlabeled FNIII14scr (lane 4), as described in Experimental Procedures. Affinity label of ST-13 cells was also carried out using biotinylated FNIII14scr (lane 3). Peptides bound to cell surface proteins were cross-linked with EDC (5 mM). Cell lysates with equal amount of proteins were separated on SDS−PAGE, transblotted to PVDF membrane, and incubated with POD-avidine, followed by visualization with ECL. (Lane 5) ST-13 cell lysates separated onto PVDF membrane were probed with anti-β1 integrin mAb 9EG7, followed by POD-conjugated anti-mouse IgG (cross-reactive to rat IgG) and visualized with ECL.

interaction is necessary for triggering a series of sequential FN−FN interactions and its polymerization. With regard to $\alpha5\beta1$ that was expressed as a major FN receptor on ST-13 cells, factors inhibiting the FN-integrin interaction, such as the RGD-containing peptides and anti-$\alpha5\beta1$ mAbs, have been reported to block FN fibrillogenesis (*51*−*56*). We showed here that FNIII14 inhibited FN fibrillogenesis without its incorporation into FN matrix. Because FNIII14 is capable of suppressing the cell-to-FN interactions mediated by the FN receptor $\alpha5\beta1$ (*34*−*36*), it is conceivable that FNIII14 inhibited FN fibrillogenesis by negatively regulating the FN-$\alpha5\beta1$ interaction. The assumption was supported by three kinds of data, as follows. First, FNIII14 inhibited the conformation change of $\beta1$ integrin from resting to active. However, evaluation of the conformation-dependent epitope of $\beta1$ integrin was only observed in K562 cells but not in ST-13 cells. To evaluate using ST-13 cells, an mAb which can discriminate between the resting and active conformations of murine $\beta1$ integrin, is essentially needed. Second, the enhanced adhesion of ST-13 cells to FN, which was induced by the 9EG7 mAb in a murine $\beta1$ integrin-specific manner, was reversed by FNIII14. Third, FNIII14 induced the dissociation of the binding of a ^{125}I-labeled 110 K FN fragment to ST-13 cells. This binding of a ^{125}I-labeled 110 K FN fragment to ST-13 cells was abrogated with GRGDSP. With the data of ST-13 cell adhesion to FN taken together (Figure 4), it was presumed that FNIII14 has the potential to suppress FN fibrillogenesis of ST-13 cells by negatively regulating the FN-to-$\alpha5\beta1$ interaction.

On the other hand, Bultmann et al. reported that the Hep 2 domain was involved in FN fibrillogenesis (*57*). In their report, FN fragments containing III_{12-14} inhibit FN fibril formation and binding of ^{125}I-labeled FN or an amino-terminal 70-kDa fragment to the cell surface. Interactions between the I_{1-5} and 160-kDa fragment containing III_1 and III_{12-14} could be inhibited by either an anti-III_1 or anti-III_{13-14} monoclonal antibody. They proposed that fibronectin fibrillogenesis may involve direct interactions between I_{1-5} and III_{12-14}, although the aforesaid monoclonal antibodies failed to inhibit the binding of ^{125}I-labeled 70-kDa fragments to cells plated on the 160-kDa substrate. It might be possible that the inhibitory effects of their FN fragments containing III_{12-14} on FN matrix assembly included, in part, the effect of the FNIII14 region in III_{14}.

We recently showed that the YTIYVIAL active site of FNIII14 was usually buried, at least in plasma FN, within the Hep 2 domain structure but exposed either by the interaction of FN with heparin or by processing of FN with matrix metalloproteinase (MMP)-2 (*33*). Our previous data also showed that MMP-2 was much more active in releasing the Fib 1 fragments having the activity to promote the adipocyte differentiation of ST-13 cells, as compared to other MMPs such as MMPs-1, 3, and 9 (*26*). As proposed by Selvarajan et al. (*44*) (see previous discussion), MMP-2 may not only support adipocyte differentiation by degradation of FN fibrils surrounding preadipose cells but also aggresively promote adipose development by the release of FN fragments containing the Fib 1 domain and the YTIYVIAL active site.

REFERENCES

1. Yamada, K. M. (1989) in *Fibronectin* (Mosher, D. F., Ed.), pp 47−121, Academic Press, New York.
2. Hynes, R. O. (1992) *Cell 69*, 11−25.
3. Ruoslahti, E., and Engvall, E. (1997) *J. Clin. Invest. 100*, S53−S56.
4. Hay, E. D. (1991) *Cell biology of extracellular matrix*, Plenum Press, New York.
5. McDonald, J. A. (1986) *Annu. Rev. Cell Biol. 4*, 183−207.
6. Mosher, D. F., Sottile, J., Wu, C., and McDonald, J. A. (1992) *Curr. Opin. Cell Biol. 4*, 810−818.
7. Wu. C. (1997) *Histol. Histopathol. 12*, 233−240.
8. Schwarzbauer, J. E., and Sechler, J. L. (1999) *Curr. Opin. Cell Biol. 11*, 622−627.
9. Sechler, J. L., and Schwarzbauer, J. E. (1998) *J. Biol. Chem. 273*, 25535−25536.
10. Sottile, J., Hocking, D. C., and Swiatek, P. J. (1998) *J. Cell Sci. 11*, 2933−2943.
11. Mercurius, K. O., and Moria, A. O. (1998) *Circ. Res. 82*, 548−556.
12. Bourdoulous, S., Orend, G., MacKenna, D. A., Pasqualini, R., and Ruoslahti, E. (1998) *J. Cell Biol. 143*, 267−276.
13. Zhang, O., Magnusson, M. K., and Mosher, D. F. (1997) *Mol. Biol. Cell 8*, 1415−1425.
14. Nagel, M., and Winklbauer, R. (1999) *Development 126*, 1975−1984.
15. Serini, G., Bochaton-Piallat, M.-L., Ropraz, P., Geinoz, A., Zardi, L., and Gabbiani, G. (1998) *J. Cell Biol. 142*, 873−881.
16. Green, H., and Meuth, M. (1974) *Cell 3*, 105−113.
17. Green, H., and Khinde, O. (1976) *Cell 3*, 127−133.
18. Hiragun, A., Sato, M., and Mitsui, H. (1980) *In Vitro 16*, 685−693.
19. Spiegelman, B. M., and Faumer, S. R. (1982) *Cell 29*, 53−60.
20. Spiegelman, B. M., and Ginty, C. A. (1983) *Cell 35*, 157−166.
21. Castro-Munozledo, F., Marsch-Moreno, M., Beltran-Langarica, A., and Kuri-Harcuch, W. (1987) *Differentiation 36*, 211−219.

22. Richardson, R. L., Campion, D. R., and Hausman, G. J. (1988) *Cell Tisue Res. 251*, 123−128.
23. Fernandez, J. L. R., and Ben-Ze'ev, A. (1989) *Differentiation 42*, 65−74.
24. Hausman, G. J., Wright, J. T., and Richardson, R. L. (1996) *J. Anim. Sci. 74*, 2117−2128.
25. Fukai, F., Iso, T., Sekiguchi, K., Miyatake, N., Tsugita, A., and Katayama, T. (1993) *Biochemistry 32*, 5746−5751.
26. Fukai, F., Ohtaki, M., Fujii, N., Yajima, H., Ishii, T., Nishizawa, Y., Miyazaki, K., and Katayama, T. (1995) *Biochemistry 34*, 11453−11459.
27. McKeown-Longo, P. J., and Mosher, D. F. (1985) *J. Cell Biol. 100*, 364−374.
28. McDonald, J. A., Quade, B. J., Broekelmann, T. J., LaChance, R., Forsman, K., Hasegawa, E., and Akiyama, S. (1987) *J. Biol. Chem. 262*, 2957−2967.
29. Quade, B. J., and McDonald, J. A. (1988) *J. Biol. Chem. 263*, 19602−19609.
30. Chernousov, M. A., Fogerty, F. J., Koteliansky, V. E., and Mosher, D. F. (1991) *J. Biol. Chem. 266*, 10851−10858.
31. Sottile, J., and Wiley, S. (1994) *J. Biol. Chem. 269*, 17192−17198.
32. Fukai, F., Takahasi, H., Habu, Y., Kubushiro-Kamiya, N., and Katayama, T. (1996) *Biochem. Biophys. Res. Commun. 220*, 394−398.
33. Watanabe, K., Takahashi, H., Habu, Y., Kamiya-Kubushiro, N., Kamiya, S., Nakamura, H., Yajima, H., Ishii, T., Katayama, T., Miyazaki, K., and Fukai, F. (2000) *Biochemistry 39*, 7138−7144.
34. Fukai, F., Hasebe, S., Ueki, M., Mutoh, M., Ohgi, C., and Katayama, T. (1997) *J. Biochem. 121*, 189−192.
35. Fukai, F., Kamiya, S., Ohwaki, T., Goto, S., Akiyama, S. K., Goto, T., and Katayama, T. (2000) *Cell. Mol. Biol. 46*, 145−152.
36. Kato, R., Kamiya, S., Ueki, M., Yajima, H., Ishii, T., Nakamura, H., Katayama, T., and Fukai, F. (2001) *Exp. Cell Res. 265*, 54−63.
37. Fukai, F., Suzuki, H., Suzuki, K., Tsugita, A., and Katayama, T. (1991) *J. Biol. Chem. 266*, 8807−8813.
38. Lenter, M., Uhlig, H., Hamann, A., Jeno, P., Imhof, B., and Vestweber, D. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 9051−9055.
39. Bazzoni, G., Shih, D., T., Buck, C. A., and Hemler, M. E. (1995) *J. Biol. Chem. 270*, 25570−25577.
40. Shibayama, H., Anzai, N., Braun, S. E., Fukuda, S., Mantel, C., and Broxmeyer, H. E. (1999) *Blood 93*, 1540−1548.
41. Takagi, J., Isobe, T., Takada, Y., and Saito, Y. (1997) *J. Biochem. 121*, 914−921.
42. Tsuchida, J., Ueki, S., Saito, Y., Takagi, J. (1997) *FEBS Lett. 416*, 212−216.
43. Ignotz, R. A., and Massague, J. (1986) *J. Biol. Chem. 261*, 4337−4345.
44. Selvarajan, S., Lund, L. R., Takeuchi, T., Craik, C. S., and Werb, Z. (2001) *Nat. Cell Biol. 3*, 267−275.
45. Hocking, D. C., Sottile, J., McKeown-Longo, P. J. (1994) *J. Biol Chem. 269*, 19183−19187.
46. Morla, A., and Ruoslahti, E. (1992) *J. Cell Biol. 118*, 421−429.
47. Morla, A., and Ruoslahti, E. (1994) *Nature 367*, 193−196.
48. Sechler, J. L., Takada, Y., and Schwarzbauer, J. E. (1996) *J. Cell Biol. 134*, 573−583.
49. Schwarzbauer, J. E. (1991) *J. Cell Biol. 113*, 1463−1473.
50. Hocking, D. C., Smith, R. K., and McKeown-Longo, P. J. A. (1996) *J. Cell Biol. 133*, 431−444.
51. Akiyama, S. K., Yamada, S. S., Chen, W. T., and Yamada, K. M. (1989) *J. Cell Biol. 109*, 863−875.
52. Fogerty, F. J., Akiyama, S. K., Yamada, K. M., and Mosher, D. F. (1990) *J. Cell Biol. 111*, 699−708.
53. Darribere, T., Guida, K., Larjava, H., Johnson, K. E., Yamada, K. M., Thiery, J. P., and Boucaut, J. (1990) *J. Cell Biol. 110*, 1813−1823.
54. Nagai, T., Yanakawa, N., Aota, S., Yamada, S. S., Akiyama, S. K., Olden, K., and Yamada K. M. (1991) *J. Cell Biol. 114*, 1295−1305.
55. Wu, C., Bauer, J. S., Juliano, R. L., and McDonald, J. A. (1993) *J. Biol. Chem. 268*, 21883-21888.
56. Hocking, D. C., Sottile, J., and McKeown-Longo, P. J. (1998) *J. Cell Biol. 141*, 241−253.
57. Bultmann, H., Santas, A. J., and Peters, D. M. P. (1998) *J. Biol. Chem. 273*, 2601−2609.

BI015660A