Integrin Regulation by Endogenous Expression of 18-kDa Fibroblast Growth Factor-2*

(Received for publication, February 15, 1996, and in revised form, May 30, 1996)

Sharon Klein†‡, Andreas Bikfalvi‡, Thomas M. Birkenmeier, Filippo G. Giancotti**††‡‡, and Daniel B. Rifkin†††‡‡

From the Departments of †††Cell Biology and **Pathology, the ‡‡‡Raymond and Beverly Sackler Foundation Laboratory, and the Kaplan Cancer Center, New York University Medical Center, New York, New York 10016, the ¶Laboratory of Growth Factors and Cell Differentiation, University of Bordeaux I, Avenue des Facultés, 33405 Talence, France, and the **Department of Internal Medicine, Washington School of Medicine, St. Louis, Missouri 63110

The three high molecular weight (HMW) forms of fibroblast growth factor-2 (FGF-2) have a distinct intracellular localization and differentially affect cell mobility and growth compared with the fourth 18-kDa form. To characterize further the effects of the 18-kDa and HMW forms of FGF-2, we have examined their ability to modulate integrin expression. Transfected NIH 3T3 cells expressing only 18-kDa FGF-2 exhibited increased cell surface levels of a5b1, whereas cells expressing only HMW FGF-2 exhibited cell surface a5b1 levels similar to parental cells. When cells synthesizing 18-kDa FGF-2 were transfected with a cDNA encoding a dominant negative FGF receptor, a5b1 cell surface levels decreased. Immunoprecipitation of biosynthetically labeled cells indicated that expression of 18-kDa FGF-2 increased the biosynthesis and rate of maturation of a5. Northern blot analysis showed that 18-kDa FGF-2 increases the level of the a5 subunit mRNA but does not affect b1 subunit transcript levels. Experiments utilizing luciferase reporter gene activity revealed increased a5 promoter activity in cells expressing 18-kDa FGF-2 indicating that the enhanced a5 transcript level is due to modulation of the transcription rate. Therefore, interaction of 18-kDa FGF-2 with FGF receptors results in changes in a5b1 biosynthesis and processing. In contrast, endogenous expression of HMW FGF-2 does not mediate this effect.

Fibroblast growth factor-2 (FGF-2)1 belongs to the fibroblast growth factor family that consists of nine members that are able to promote the proliferation of cells of mesodermal, epithelial, and neuroectodermal origin (1, 2). FGF-2 is the prototypic angiogenic factor involved in wound-healing processes and tumor neovascularization (3, 4). The responses of cells to FGF-2 are mediated through a dual receptor system consisting of high affinity binding transmembrane receptors and lower affinity cell surface and extracellular matrix heparan sulfate proteoglycan binding sites (5–10). Four distinct high affinity tyrosine kinase plasma membrane receptors encoded by four different genes have been described for FGF (1, 11). Each of these genes encodes multiple variants derived from alternative mRNA splicing (12–15). Although FGF-2 does not contain a signal sequence, it is released from cells and can act as an autocrine and/or paracrine regulator (16–17). The mechanism for FGF-2 release remains unknown. However, it has been shown to be released independent of the endoplasmic reticulum/Golgi pathway (18).

FGF-2 is synthesized by a wide variety of cells including primary endothelial cells (5, 19). FGF-2 induces angiogenesis as it increases endothelial cell proliferation, migration, and proteolytic activity (19–22). We previously reported that exogenous FGF-2 modulates integrin expression in microvascular endothelial cells (23). Integrins are heterodimeric receptors composed of a and b subunits. At present, there are eight different b and 15 different a subunits that can combine to form 21 receptors with distinct ligand specificities (24). Integrins are involved in the processes of cell proliferation, motility, survival, and mesoderm induction (24–29). Endothelial cell integrins also function with other families of adhesion molecules during vasculogenesis, angiogenesis, inflammation, and wound healing (30, 31). Treatment of endothelial cells with FGF-2 caused significant changes in the surface expression of nine different integrins (23). Thus, modulation of integrins may be one of the FGF-2-induced effects on endothelial cells during angiogenesis.

Several forms of FGF-2 are produced in vivo resulting from alternative initiation of translation either at an AUG codon or at three in-frame CUG codons 5’ to the AUG (32, 33). This results, respectively, in the synthesis of a form of 18 kDa and three high molecular weight (HMW) forms of 22,000, 22,500, and 24,000 (32–35). The complete sequences of the smaller forms are contained in the larger forms. The relative amounts of the individual molecular weight forms have been reported to differ substantially among various cell lines and tissues during development, implying that the alternative codon usage is highly regulated (36–39). It has been suggested that cis-acting elements in the FGF-2 mRNA are involved in regulating the translation of the different forms of FGF-2 at the four initiation sites (40).

The process of alternative initiation of translation has varying consequences for the ultimate fate of the different FGF-2 forms (41–44). The three HMW forms of FGF-2 contain a
nuclear localization sequence that concentrates the growth factor in that organelle. In contrast, 18-kDa FGF-2, which lacks a nuclear localization sequence, is primarily cytosolic.

The existence of multiple forms of FGF-2 with different subcellular localizations raises the question of whether these different species of FGF-2 have specialized functions. We approached this question by creating stably transfected NIH 3T3 cell lines that express exclusively 18-kDa FGF-2, HMW FGF-2, or all forms of FGF-2 (45, 46). Both 18-kDa FGF-2 and HMW FGF-2 alone expressed at high levels transformed NIH 3T3 cells. Cells expressing only 18-kDa FGF-2 had high motility and surface-associated 18-kDa FGF-2, whereas cells expressing exclusively HMW FGF-2 had low motility and virtually no surface-associated FGF-2. FGF receptors were down-regulated in cells expressing 18-kDa FGF-2 but not in cells expressing HMW FGF-2. Cells expressing HMW FGF-2 had a reduced serum requirement for growth, but cells expressing 18-kDa FGF-2 proliferated poorly in low serum. These results showed that 18-kDa and HMW FGF-2 have both unique and shared biological activities. Expression of a dominant negative FGF receptor in cells expressing 18-kDa FGF-2 inhibited their migration and suppressed their growth in soft agar as well as their saturation density. In contrast, expression of the dominant negative receptor in cells expressing HMW FGF-2 had no effect on their growth. Thus, 18-kDa and HMW FGF-2 may mediate certain functions through distinct mechanisms. 18-kDa FGF-2 modulates cell motility and proliferation through the interaction with its cell surface receptors, whereas HMW FGF-2 appears to act as a mitogen and an inducer of anchorage-independent growth through an intracellular mechanism.

Our aim in the present study was to characterize additional functional differences between 18-kDa and HMW FGF-2. To this end, we chose to analyze the regulation of integrin expression by FGF-2 in NIH 3T3 cells expressing the various FGF-2 forms. We have determined which form of FGF-2 modulates integrin expression in these cells as well as the mechanism mediating this effect. We found that endogenous expression of 18-kDa FGF-2 modulates β1 integrin expression in NIH 3T3 cells, although endogenous expression of HMW FGF-2 does not. The induced expression of α5β1 on the cell surface mediated by endogenous expression of 18-kDa FGF-2 is a result of the combined effects of an increased level of transcript coding for the α5 subunit and an increased rate of processing of the α5 and β1 subunits.

MATERIALS AND METHODS

Reagents

Recombinant human FGF-2 (18 kDa) was a gift from Synergen, Inc. (Boulder, CO) and Scios Nova (Mountain View, CA). Na[125I] and 35S-Trans-label were purchased from DuPont NEN, Pont Radiochemicals (Boston, MA), and 125I-protein A from ICN Biobodies (Boulder, CO) and Scios Nova (Mountain View, CA). Na125I and 35S-labeled cell lysates were immunoprecipitated as described (45, 46). The cells were grown in DME (Bio-Whittaker) containing 10% FCS plus 500 µg/ml Geneticin.

Secondary Transfection of Cells with 18-kDa FGF-2 or HMW FGF-2 cDNA—NIH 3T3 cells transfected with the Zip-neo vector containing HMW-RF-2 cDNA were subsequently retransfected either with 18-kDa FGF-2 or with HMW FGF-2 cDNA inserts in the Zip-neo vector plus the pCEP4 vector containing a hygromycin resistance gene (kindly provided by Dr. C. Basilico, New York University Medical Center, New York) at a molar ratio of 8:1. Hygromycin-resistant clones were selected in DME containing 10% FCS, 200 µg/ml hygromycin, and 250 µg/ml Geneticin. Secondary transfectants were characterized by Western blotting of cell extracts with anti-FGF-2 antibodies. The cells used were HMW clone transfected with 18-kDa FGF-2 cDNA, 365/43NC33; HMW clones transfected with HMW FGF-2 cDNA, 365/365FGFc14, 365/365FGFc8; control clone transfected with hygromycin-resistant gene alone, 365FGFc5h3.

Transfection with a Dominant Negative FGF Receptor cDNA—NIH 3T3 cells transfected with the Zip-neo vector containing either HMW, 18 kDa, or WT FGF-2 cDNAs were cotransfected with pK5 containing a 1.3-kilobase insert of a human bek (FGF receptor 2) cDNA that lacks the C-terminal tyrosine kinase domain (dominant negative FGF receptor; kindly provided by Dr. J. Schlessinger, New York University Medical Center) and the pCEP4 vector. Hygromycin-resistant clones were selected in DME containing 10% FCS, 200 µg/ml hygromycin, and 250 µg/ml Geneticin. Resistant clones were tested for high affinity FGF-2 receptors according to Moscatelli (5) and by cross-linking to cell surface receptors with 125I-FGF-2. The cells used in this study were HMW FGF-2 cDNAs transfected with dominant negative FGF receptor cDNA, 365DNc5, 365DNc7; 18-kDa FGF-2 clone transfected with dominant negative FGF receptor cDNA, 43DNc11; WT FGF-2 clone, WTDFc2.

Cell Surface Labeling

NIH 3T3 cells were plated at subconfluence in 15-cm dishes (Falcon, Becton Dickinson, Lincoln Park, NJ). Cells were incubated for 48 h in fresh DME containing 5% FCS in the presence or absence of 15 ng/ml FGF-2. Cells were washed with phosphate-buffered saline (PBS) and detached with 5 mM EDTA. The suspended cells were washed three times with PBS, and surface proteins were labeled with 158 Ci/ml 35S-Trans-label. The 125I- and 35S-labeled cell lysates were immunoprecipitated as described (45, 46).

Northern Blot Analysis

Northern blot analysis was performed with total cellular RNA isolated from NIH 3T3 cells using single-step guanidinium thiocyanate/phenol/chloroform extraction using Trizol reagent. Mouse α5 and mouse α5 cDNA probes were kindly provided by Dr. H. S. Baldwin (Wistar Institute, Philadelphia), and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe was kindly provided by Dr. A. M. Curatola (New York University Medical Center). Radioactive probes were made by using an oligonucleotide random priming procedure (Boehringer Mannheim) and

Antibodies

The anti-integrin antibodies used in this study were raised by immunization of rabbits with synthetic peptides reproducing C-terminal portions of individual integrin subunits. The cytosplasmic peptide antibodies to α5 and β1 (47), α6 (48), αv (49), and α3 (23) were previously described. Polyclonal rabbit antisera against human recombinant FGF-2 was used in Western blot analysis to detect levels of FGF-2 in the cell clones.
Luciferase Plasmid Construct and Luciferase Assay

To create the plasmid p5-926LUC, the region of the human α5 gene from −926 to +23 bp was amplified using PCR with the template PstI-α5 as described previously (50). The resulting PCR product was digested with SalI and PstI and cloned into the plasmid SK BlueScript (Stratagene, La Jolla, CA). The insert was sequenced to ensure that no mutations occurred during PCR. To create the α5-luciferase expression vector, this plasmid was digested with the enzymes SmaI (contained immediately 5′ of the PstI site in the SK plasmid) and SalI, and the fragment was gel-purified and cloned into the SmaI and XhoI sites in the vector pβL2-Basic (Promega, Madison, WI).

NIH 3T3 cells were plated at subconfluence on 10-cm tissue culture dishes in DMEM containing FCS (10%). 18 h later, cells were transiently transfected using lipofectamine reagent with expression construct p5-926LUC or with a control construct, pLuc, containing a promoterless luciferase gene and a β-galactosidase (β-gal) cDNA. 48 h after transfection, cells were washed once with PBS, scraped, and pelleted by centrifugation. Cells were resuspended in 100 μl of 0.25 % Tris–Cl, pH 7.8, and lysed by three freeze-thaw cycles. β-Gal activity was measured as described previously (51). Results of β-gal activity were normalized according to protein concentration in each cell extract determined by the bicinchoninic acid method (Pierce). 35S-labeled integrin levels were iodinated, and cell extracts were immunoprecipitated with integrin subunit cytoplasmic domain antibodies indicated that the two most abundant integrins, αβ integrins, were iodinated, and cell extracts were immunoprecipitated with integrin subunit cytoplasmic domain antibodies. Samples were boiled in nonradioactive sample buffer and analyzed by SDS-PAGE. Scanning analysis of protein bands was performed by PhosphorImage software. A. Immunoprecipitation with antibodies to α5, α6, and β1. B. Immunoprecipitation with antibodies to α3 and αv. This pattern is representative of three experiments. Two control clones tested yielded similar results.

Table I

| Cell Type | αβ Integrin | α6β1 | Fold Increase |
|-----------|-------------|------|--------------|
| Control   | 3.2         | 3.5  | 1.1          |
| HMW FGF-2 | 4.1         | 2.1  | 1.0          |
| 18-kDa FGF-2 | 1.7     | 1.8  | 1.0          |
| WT FGF-2  | 1.0         | 1.0  | 1.0          |

Endogenous 18-kDa FGF-2 but Not HMW FGF-2 Increases Surface β1 Integrin Levels—Previously we demonstrated that integrin expression in cultured endothelial cells could be modulated by exogenous FGF-2 (231). In order to explore the effects of endogenous FGF-2 expression on integrins, we chose to examine integrin levels in NIH 3T3 cells as these cells expressing different forms of FGF-2 are a useful model to study FGF-2 activity (45, 46). To establish that FGF-2 was capable of modulating integrin levels in NIH 3T3 cells, control cells were treated for 48 h with and without FGF-2 (15 ng/ml), cell surface proteins were iodinated, and cell extracts were immunoprecipitated with anti-integrin antibodies. In agreement with previous observations demonstrating the induction of β1 integrins in capillary endothelial cells, addition of FGF-2 increased β1 integrin levels. Treatment of NIH 3T3 fibroblasts as well as in capillary endothelial cells.

To test whether endogenous FGF-2 synthesis also affects integrin expression, we examined the cell surface integrins in NIH 3T3 cells stably transfected with the FGF-2 cDNA encoding all forms of FGF-2 (wild type (WT) FGF-2). Immunoprecipitation of surface-labeled cells with anti-β1 antibody indicated that overall cell surface β1 integrins were increased by 3.1-fold compared with control cells (Fig. 1A). Specifically, immunoprecipitation with anti-α5 and anti-α6 antibodies revealed that the αβ1 and α6β1 integrins were enhanced by 3.1- and 2.3-fold, respectively. In addition, we checked whether the levels of other β1 integrins were also modulated by endogenous FGF-2. The results obtained by immunoprecipitation of cells synthesizing WT FGF-2 using anti-α3 and anti-αv antibodies indicated that α3β1 levels were decreased 2-fold, and αvβ1 levels were not changed compared with control cells (Fig. 1B). Other β1 integrins were not detected in these cells by our analysis. Our results thus show that endogenous FGF-2 differentially regulates cell surface levels of specific β1 integrins in NIH 3T3
cells, with antibodies against the α5 and β1 subunits revealed that the amount of α5β1 integrin was increased compared with control levels. In contrast to the results obtained with cells synthesizing 18-kDa FGF-2, immunoprecipitation of cell extracts from NIH 3T3 cells expressing only HMW FGF-2 with antibodies to β1 and α5 subunits indicated that no change occurs in the level of β1 integrins. Thus, endogenous 18-kDa FGF-2 increases the β1 surface integrins on NIH 3T3 cells, but HMW FGF-2 does not. Similar results were obtained with three additional clones of each cell type synthesizing comparable levels of FGF-2 as determined by Western blot analysis (data not shown).

We investigated whether exogenously added FGF-2 increases integrin levels in NIH 3T3 cells transfected with HMW FGF-2 cDNA. As in control cells, addition of FGF-2 increased cell surface levels of β1 integrins including the α5β1 and α6β1 receptors in cells expressing only HMW FGF-2 (Table I). Thus, the low level of integrins in NIH 3T3 cells synthesizing only HMW FGF-2 does not result from an inability to respond to extracellular FGF-2. In contrast to control cells and cells expressing HMW FGF-2, FGF-2 addition to cells synthesizing WT FGF-2 did not further increase integrin levels. In cells synthesizing 18-kDa FGF-2, integrin levels were further induced only slightly by exogenous addition of FGF-2 (Table I).

The previous results support the hypothesis that endogenous HMW FGF-2 does not increase β1 integrins above levels observed in control cells, although exogenous FGF-2 can mediate this effect on these cells. To test further whether the synthesis of 18-kDa FGF-2 is responsible for changes in integrin levels, cells synthesizing only HMW FGF-2 were transfected with cDNAs encoding 18-kDa FGF-2 and a hygromycin-resistant gene. As shown in Fig. 2B, cells expressing both HMW FGF-2 and 18-kDa FGF-2 displayed a 3-fold increase in β1 integrins compared with cells expressing HMW FGF-2 alone. This result confirms the hypothesis that 18-kDa FGF-2 modulates integrin cell surface levels in NIH 3T3 cells.

**Induction of Surface β1 Integrins by Endogenous 18-kDa FGF-2 Is FGF Receptor-mediated**—Although the FGF-2 translation product lacks a signal peptide sequence normally required for secretion, the protein is released from cells. Bikfalvi et al. (46) demonstrated that in NIH 3T3 cells, extracellular interaction of 18-kDa FGF-2 with its cell surface receptor is required for the growth factor's biological activities including increases in cell migration, cell proliferation, and FGF receptor down-regulation. In contrast, interaction with cell surface high affinity receptors is not necessary for the biological activities stimulated by HMW FGF-2 such as increases in cell proliferation and growth in low serum. Therefore, we next questioned whether the regulation of integrin expression by 18-kDa FGF-2 was dependent on the presence of high affinity FGF receptors. In order to test this hypothesis, cells were transfected with a cDNA encoding a dominant negative mutant type-2 FGF receptor lacking the tyrosine kinase domain. Previously, it was demonstrated that the effect of this mutant receptor is trans-dominant (i.e. it inhibits signaling by all FGF receptor types) (52).

Fig. 3 illustrates that cells expressing 18-kDa FGF-2 or WT FGF-2 plus the dominant negative FGF receptor showed a decrease in surface β1 integrins compared with cells expressing 18-kDa or WT FGF-2 forms alone. This decrease was 4.5-fold as determined by PhosphorImager scanning. Thus, the presence of the dominant negative receptor prevented the signaling necessary for the increase in integrin levels by 18-kDa FGF-2. Therefore, regulation of integrin levels by 18-kDa FGF-2 occurs through an FGF receptor-mediated pathway.

**Effect of Endogenous 18-kDa FGF-2 on the Biosynthesis of the**
Integrin Regulation by FGF-2

β1 Integrin Subunit—Of the integrins regulated by 18-kDa FGF-2, we have focused our studies on α5β1, which has been shown to control migration of neural crest-like cells and Chinese hamster ovary cells (53, 54). To characterize the mechanism by which 18-kDa FGF-2 increases cell surface α5β1 levels, we first examined the biosynthesis of the β1 subunit. Cells were metabolically labeled under steady-state conditions, and cell extracts were immunoprecipitated with β1-specific antibody. Fig. 4A illustrates that the combined amount of precursor and mature β1 forms was not affected by expression of 18-kDa FGF-2 compared with control cells. However, endogenous expression of 18-kDa FGF-2 increased the amount of mature β1 subunit by 4-fold that was paralleled by a decrease of 4-fold in the precursor form. In control cells, 20% of the total of precursor and mature β1 forms was present as mature β1, whereas, in the two clones synthesizing 18-kDa FGF-2, 75% and 40% was present as mature β1. The effect appeared to be dependent on the expression level of 18-kDa FGF-2 because of the two clones examined (lanes 2 and 3); the clone synthesizing a higher level of 18-kDa FGF-2 (lane 3) had the higher percent mature β1. Both clones of cells that expressed only HMW FGF-2 did not significantly differ in the level of either premature or mature β1 with respect to control cells even though the cells analyzed produced levels of HMW FGF-2 comparable with the levels of FGF-2 in the two clones transfected with 18-kDa FGF-2 cDNA. This experiment demonstrates that 18-kDa FGF-2 expression increases the amount of precursor β1 converted to the mature form. To confirm this result, Western blot analysis was performed because it is another method to detect steady-state β1 integrin levels. The results were in agreement with the previous findings as more mature β1 was present in cell extracts from cells expressing 18-kDa FGF-2 compared with control cells. In addition, there was a parallel decrease in precursor β1 (data not shown).

Endogenous 18-kDa FGF-2 Increases the Rate of Processing of the β1 Subunit—A pulse-chase experiment was performed to test whether 18-kDa FGF-2 affects the rate of conversion of premature β1 to mature β1 subunit. Control cells and cells expressing 18-kDa FGF-2 were metabolically labeled for 1 h and chased for various lengths of time. The cell extracts were immunoprecipitated with β1 antibody and the antigens separated by SDS-PAGE. Fig. 5A shows that the β1 subunit was synthesized as an immature form that, after 3 h of chase, was partially converted to the mature form. Between 12 and 24 h of chase, the protein was completely converted to the mature form. The kinetics of processing were faster in cells expressing 18-kDa FGF-2. After 3 h of chase the amount of mature β1 was twice the level found in control cells. After 6 h of chase, the premature form was almost completely converted to the mature form. As observed above, the total amount of β1 subunit synthesized was not affected. In addition, control cells and cells expressing HMW FGF-2 were pulse-labeled and immunoprecipitated with anti-β1 antibody. The kinetics of processing of β1 was unaffected by HMW FGF-2 expression (data not shown).

Biosynthesis of the α5 Subunit Is Increased by 18-kDa FGF-2 Expression—To investigate the synthesis of the α5 subunit, five clones of cells (one control clone, two clones expressing only 18-kDa FGF-2, and two clones expressing only HMW FGF-2) were metabolically labeled, and cell extracts were immunoprecipitated with α5-specific antibody. Fig. 4B shows that the synthesis of the α5 subunit was increased 5-fold. The effect on α5 subunit synthesis was dependent on the expression level of 18-kDa FGF-2 because the clone expressing higher 18-kDa FGF-2 (lane 2) synthesized more α5 subunit. In one clone expressing only HMW FGF-2 (lane 4), the synthesis of the α5 subunit was not affected. In the second clone (lane 5), the α5 level was slightly higher than control levels. However, additional experiments indicate that this increase was not reproducible (data not shown). Western blot analysis using anti-α5 antibody confirmed the previous result demonstrating that 18-kDa FGF-2 but not HMW FGF-2 increases the biosynthesis of the α5 subunit (data not shown). To study whether 18-kDa FGF-2 affects the rate of processing of the α5 subunit, a pulse-chase experiment using cell extracts from control cells and cells synthesizing 18-kDa FGF-2 was performed. The results obtained using control cells showed that α5 was synthesized as a precursor form, which after 30 min of chase was partially converted to the mature form (Fig. 5B). After 3 h of chase the protein was completely converted to the mature form and was associated with the β1 subunit. In contrast, 18-kDa FGF-2 synthesis increased the rate of processing of the α5 subunit as mature α5 was already visible at the start of chase and the α5β1 complex was observed by 1 h of chase. Thus, 18-kDa FGF-2 increased the rate of conversion of immature α5 to mature α5, which in turn increased the rate of processing of the α5β1 complex.

**Fig. 3. Effect of a dominant negative FGF receptor cDNA on cell surface integrin levels.** Surface labeling of cells, immunoprecipitation, SDS-PAGE, and PhosphorImager analysis were performed as described under “Materials and Methods.” Cells expressing all FGF-2 forms (clone WTFGFc3), all FGF-2 forms with the dominant negative FGF receptor (WT FGF-2/DN, clone WTDNc2), 18-kDa FGF-2 (clone 43FGFc31), or 18-kDa FGF-2 with the dominant negative FGF receptor (18-kDa FGF-2/DN, clone 43DNC11) were analyzed with anti-α5 and anti-β1 antibodies. This pattern is representative of two experiments performed that yielded similar results.

**Fig. 4. Effect of 18-kDa or HMW FGF-2 forms on the biosynthesis of the β1 and α5 integrin subunits in NIH 3T3 cells.** Cultures of cells were incubated in the presence of 100 μCi/ml [35S]-Trans-label for 16 h in Met/Cys-free medium. Cells were harvested, and aliquots of detergent-soluble cell extracts were immunoprecipitated with antibodies to β1 and α5 subunits. Immunoprecipitated proteins were analyzed by gel electrophoresis, and scanning analysis of protein bands was performed by PhosphorImager. A, immunoprecipitation with anti-β1; control cells, clone Zipneo2c (lane 1); cells expressing 18-kDa FGF-2, clones 43FGFc21 (lane 2), 43FGFc21 (lane 3); cells expressing HMW FGF-2, clones 365/365FGFc14 (lane 4), 365/365FGFc38 (lane 5). B, immunoprecipitation with anti-α5; control cells, clone Zipneo2c (lane 1); cells expressing 18-kDa FGF-2, clones 43FGFc21 (lane 2), 43FGFc21 (lane 3); cells expressing HMW FGF-2, clones 365/365FGFc9 (lane 4), 365FGFc2 (lane 5). This pattern is representative of three experiments that yielded similar results with the exception of lane 5 (B), which showed a lower level of α5 in the additional two experiments. Comparable levels of β1 and α5 levels were obtained with five clones of control cells.
FGF-2 increases the biosynthesis as well as the rate of maturation of the \( \alpha_5 \) subunit. Whereas the rate of maturation of the \( \beta_1 \) subunit is similarly increased by 18-kDa FGF-2, the biosynthesis of \( \beta_1 \) is unaffected.

Pre-translational Regulation of the \( \alpha_5 \) and \( \beta_1 \) Subunits by FGF-2 Expression—To analyze further the mechanism of increased \( \alpha_5 \) synthesis, Northern blot analysis was performed. Total RNA extracted from control cells and cells synthesizing 18-kDa FGF-2 was hybridized with \( \alpha_5 \)-specific, \( \beta_1 \)-specific, and GAPDH-specific cDNA probes. The results showed that, after normalization to GAPDH mRNA levels by PhosphorImager scanning analysis, the level of mRNA coding for the \( \alpha_5 \) subunit was increased by 3-fold by 18-kDa FGF-2 synthesis (Fig. 6A). However, the level of \( \beta_1 \) mRNA was unchanged by 18-kDa FGF-2 (Fig. 6B). The level of \( \beta_1 \) or \( \alpha_5 \) mRNAs did not change with synthesis of HMW FGF-2 (data not shown). Thus, endogenous 18-kDa FGF-2 specifically increases the message level of the \( \alpha_5 \) subunit.

Increase in \( \alpha_5 \) mRNA Levels by FGF-2 Expression Is Due to Enhanced Transcriptional Activity and Not mRNA Stabilization—The increase in \( \alpha_5 \) mRNA levels could reflect increased transcription of the gene or an increase in message stability. The stability of \( \alpha_5 \) mRNA was determined in NIH 3T3 cells by treatment with actinomycin D (5 \( \mug/ml \)) to inhibit transcription (Fig. 7). By this method, the half-life of \( \alpha_5 \) mRNA was determined to be approximately 1 h in control cells. In cells synthesizing 18-kDa FGF-2, the half-life was also approximately 1 h. These data suggest that 18-kDa FGF-2 expression does not result in an increase in \( \alpha_5 \) mRNA stability.

To determine whether transcription of the \( \alpha_5 \) gene was increased by 18-kDa FGF-2, the \( \alpha_5 \) promoter activity was measured in control cells and cells synthesizing 18-kDa FGF-2. Cells were transiently transfected with either \( \alpha_5 \)-926LUC or pLUC and a transfection control \( \beta \)-gal plasmid. Luciferase activity in extracts prepared from cells transfected with either luciferase construct were normalized to \( \beta \)-gal activity. In cells synthesizing 18-kDa FGF-2, there was a 6-fold increase in luciferase activity with expression of \( \alpha_5 \)-926LUC compared with control cells (Fig. 8). Thus, 18-kDa FGF-2 acts on the \( \alpha_5 \) promoter to drive expression of the luciferase reporter gene. As a further control, cells synthesizing HMW FGF were transfected with the luciferase constructs. HMW FGF-2 expression did not increase \( \alpha_5 \) promoter activity significantly above that in control cells (data not shown). Thus, 18-kDa FGF-2 enhances \( \alpha_5 \) mRNA levels by increasing the transcription of the \( \alpha_5 \) gene.

**DISCUSSION**

The data reported in this paper show that the pattern of expression of \( \beta \) integrins at the cell surface of NIH 3T3 cells is influenced by endogenous 18-kDa FGF-2 but not by HMW FGF-2. This conclusion is based on the following observations.

1. **(a)** Cells expressing 18-kDa FGF-2 have increased cell surface \( \alpha_5 \beta_1 \) and \( \alpha_6 \beta_1 \) and decreased \( \alpha_3 \beta_1 \) integrins, whereas cells expressing HMW FGF-2 have levels of \( \beta_1 \) integrins comparable with control NIH 3T3 cells. **(b)** Transfection of cells expressing HMW FGF-2 with 18-kDa FGF-2 cDNA results in increased cell surface \( \alpha_5 \beta_1 \). **(c)** Coexpression of a dominant negative FGF receptor inhibits the changes in integrin levels at the cell
Integrin Regulation by FGF-2

surface mediated by 18-kDa FGF-2. These results add support to the model that 18-kDa FGF-2 is released from cells and interacts with cell surface FGF receptors, which induces receptor phosphorylation and signal propagation, and ultimately triggers various biological responses. These include down-regulation of FGF receptors, increases in motility, stimulation of growth, and modulation of integrin expression. The responses we have observed are dependent upon the absolute amount of 18-kDa FGF-2 because clones of cells synthesizing low levels of 18-kDa FGF-2 mediate these effects to a lesser extent than cells synthesizing high levels, probably because less growth factor is released and available to interact with receptors. Clones of NIH 3T3 cells expressing high levels of HMW FGF-2 do not regulate integrin levels probably because the growth factor is not released from cells in sufficient quantity (46). This may be due to the nuclear localization sequence that efficiently targets HMW FGF-2 into the nucleus. Whereas certain biological activities such as growth in low serum may be mediated by HMW forms of FGF-2, integrin modulation is not.

The most abundant integrin in the parental NIH 3T3 cells was α5β1, which displayed a striking up-regulation at the cell surface in cells synthesizing 18-kDa FGF-2. Metabolic labeling of NIH 3T3 cells expressing FGF-2 followed by immunoprecipitation with anti-α5 antibody showed that the modulation of α5β1 appearance at the cell surface reflects a concomitant modification of the biosynthesis of the α5 subunit. Northern blot analysis demonstrated that the change in the rate of biosynthesis is a result of an increase in the transcript level of α5, and luciferase assays indicated that this increase is a consequence of modulation of the rate of transcription of the α5 gene. In contrast to α5, the biosynthesis of β1 is not enhanced by endogenous 18-kDa FGF-2, probably because an excess pool of precursor β1 already exists in control cells. However, 18-kDa FGF-2 dramatically increases the rate of processing of β1 as measured by pulse-chase experiments, and this enhanced rate of processing increases the level of mature β1. Similarly, the rate of processing of α5 is stimulated by 18-kDa FGF-2.

We previously observed that NIH 3T3 cells expressing 18-kDa FGF-2 are more migratory than control cells, whereas cells expressing HMW FGF-2 migrated to the same degree as control cells. These differences in migration between the cells expressing 18-kDa and HMW FGF-2 may be due to differences in the levels of α5β1. In neural crest-like cells, the repertoire of integrins and the extent of integrin expression determined the rate of cell migration and the particular pathway of cell migration (53). Expression of α5β1 or α4β1 in mouse sarcoma S180 cells, which behave similarly to neural crest cells and normally synthesize low levels of these integrins, promoted an increase in cell motility in vitro. When these cells were plated onto an endothelial monolayer, they migrated in distinct pathways compared with parental cells. The cells expressing α5 migrated simultaneously in both ventral and dorsolateral pathways in contrast to the parental cells that migrated only in the ventral path. Similarly, cells expressing low levels of α4 migrated in both ventral and dorsolateral pathways. However, the cells expressing high levels of α4 remained nonmigratory. Thus, the repertoire and levels of integrins enabled the cells to utilize different pathways of migration and regulate their speed of migration in vivo. Based on these observations, it is likely that increased α5β1 levels in NIH 3T3 cells synthesizing 18-kDa FGF-2 play a role in the enhanced migration compared with control cells, but this hypothesis still remains to be proven.

Other studies support the above hypothesis. Variants of Chinese hamster ovary cells were selected that expressed reduced levels of α5β1 (54). These cells exhibited slower migration than the parental cell line. This result taken together with the studies described above, strongly suggests a direct correlation between the concentration of α5β1 and speed of migration. However, if the level of α5β1 is increased significantly, cell migration is decreased (47). One explanation for this effect is that very high levels of α5 may produce an affinity to the substratum that reduces rather than increases motility. As increased expression of the α5 subunit enhances fibronectin assembly at the cell surface, this could immobilize the cells (47, 53).

In addition to regulating migration of the NIH 3T3 cells expressing 18-kDa FGF-2, α5β1 may contribute to the proliferation of these cells. α5β1 expression by HT29 colon carcinoma cells decreases cell proliferation by inducing the transcription of growth arrest gene 1, a gene product that induces growth arrest and blocks transcription of several immediate early genes (55). These changes occur in the absence of cell attachment to fibronectin. However, ligation of α5β1 to fibronectin down-regulates growth arrest-specific gene 1 expression, activates immediate early gene transcription, and induces cell proliferation. Thus, α5β1 can generate both positive and negative signals depending on whether it is bound to its substrate fibronectin. Therefore, α5β1 expression in NIH 3T3 cells transformed by 18-kDa and HMW FGF-2 may contribute to enhanced cell proliferation.

Integrins have been shown to be required during angiogenesis. Several studies have demonstrated that blocking the activity of integrins affects angiogenesis. In particular, antibodies against αvβ3 or αvβ5 severely perturbed angiogenesis induced in the choroidafticochoroid membrane by FGF-2 or VEGF, respectively (56, 57). In fact, antibodies against αvβ3 induced apoptosis in proliferative angiogenic vascular cells suggesting that ligation of αvβ3 may be required for the survival and maturation of newly forming blood vessels (56). Antibodies against β1 or αvβ3 integrins injected into quail embryos arrest or severely disrupt vasculogenesis indicating an important role for both β1 and αvβ3 integrins during vasculogenesis (58, 59). However, it is more likely that the specific integrins playing a role during angiogenesis depend on the tissue type. Although it is clear that many cytokines and growth factors can induce angiogenesis, little is known about the molecular mechanisms underlying this activity. Changes in the level of expression or function of integrins may be necessary during angiogenesis. We have previously demonstrated that FGF-2 can regulate inte-
Integrin Regulation by FGF-2

Grin levels in microvascular endothelial cells. We show here that the 18-kDa form of FGF-2 is the only endogenous form that mediates this effect. HMW FGF-2 does not modulate integrin production and, therefore, blocking the extracellular activity of the 18-kDa form may be sufficient to block integrin modulation by FGF-2 in vivo. It is possible that blocking the FGF-2-induced modulation of integrin levels is sufficient to inhibit angiogenesis.

In summary, we have shown that endogenous expression of 18-kDa FGF-2, but not HMW FGF-2, modifies surface integrin levels. This involves 18-kDa FGF-2 interaction with FGF receptors and signaling of changes in integrin biosynthesis and processing. Enhanced α5β1 levels caused by endogenous 18-kDa FGF-2 may play a role in the increased migration and proliferation of the cells. Furthermore, modification of integrin expression in vitro by 18-kDa FGF-2 may be important during several FGF-2-mediated processes including mesoderm formation, wound healing, and angiogenesis.

Acknowledgments—We are especially grateful to Dr. Giuseppe Pintucci for his advice and support throughout this study. We thank Dr. Natalina Quarto for helpful suggestions and Drs. J. Schlessinger, C. Basilico, and A. M. Curatola for providing constructs.

REFERENCES

1. Basilico, C., and Moscatelli, D. (1992) Adv. Cancer Res. 59, 115–165
2. Miyamoto, M., Naruo, K., Seko, C., Matsumoto, S., Kondo, T., and Kurokawa, T. (1993) Mol. Cell. Biol. 13, 4251–4259
3. Gospodarowicz, D., Neufeld, G., and Schweigerer, L. (1986) Mol. Cell. Endocrinol. 46, 187–204
4. Kandel, J., Bissantz, E., Radonjić, V., Klagsbrun, M., Folkman, J., and Hanahan, D. (1991) Cell 66, 1095–1104
5. Moscatelli, D. (1987) J. Cell. Physiol. 131, 123–130
6. Bashkin, P., Docutrow, S., Klagsbrun, M., Svahn, C. M., Folkman, J., and Vlodavsky, I. (1989) Biochemistry 28, 1737–1743
7. Yayon, A., Klagsbrun, M., Eskin, J. D., Leder, P., and Ornitz, D. M. (1991) Cell 64, 641–648
8. Ornitz, D. M., Yayon, A., Flanagan, J. G., Svahn, C. M., Levi, E., Leder, P. (1992) Mol. Cell. Biol. 12, 240–247
9. Roghani, M., Mansukhani, A., Dell’Era, P., Bellosta, P., Basilico, C., Rifkin, D. B., and Moscatelli, D. (1994) J. Biol. Chem. 269, 3976–3984
10. Aviezer, D., Hecht, D., Safran, M., Eisinger, M., David, G., and Yayon, A. (1994) Cell 79, 1005–1013
11. Jaye, M., Schlessinger, J., Dionne, C. (1992) Biochem. Biophys. Acta 1135, 185–199
12. Houssaint, E., Blanquet, P., Champion-Arnaud, P., Gesnel, M. C., Torrigia, A., Courtois, Y., and Breathnach, R. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 8180–8184
13. Johnson, D. E., Lu, J., Chen, H., Werner, S., and Williams, L. T. (1991) J. Biol. Chem. 266, 4627–4634
14. Miki, T., Botta, D. P., Fleming, T. P., Smith, C. L., Burgess, W. H., Chan, A. M., and Aaronson, S. A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 246–250
15. Werner, S., Daun, D. S., de Vries, C., Peters, K. G., Johnson, D. E., and Williams, L. T. (1992) Mol. Cell. Biol. 12, 82–88
16. Abraham, J. A., Mergia, A., Whang, J. L., Tumolo, A., Friedman, J., Heijrild, K. A., Gospodarowicz, D., and Fiddes, J. C. (1986) Science 233, 545–548
17. Mignatti, P., Morimoto, T., and Rifkin, D. B. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 11007–11011
18. Mignatti, P., Morimoto, T., and Rifkin, D. B. (1992) J. Cell. Physiol. 151, 81–93
19. Schweigerer, L., Neufeld, G., Friedman, J., Abraham, J. A., Fiddes, J. C., and Gospodarowicz, D. (1987) Nature 325, 257–259
20. Montesano, R., Vassalli, J. D., Baird, A., Guillemin, R., and Orci, L. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 7297–7301
21. Sato, Y., and Rifkin, D. B. (1988) J. Cell Biol. 107, 1199–1205
22. Tsai, H., Sato, Y., and Rifkin, D. B. (1990) J. Cell. Biol. 110, 511–517
23. Klein, S., Giancotti, F. G., Presta, M., Albelda, S. M., Back, C. A., and Rifkin, D. B. (1993) Mol. Biol. Cell. 4, 973–982
24. Hytkowycz, R. O. (1992) Cell 68, 11–25
25. Smith, J. C., Symes, K., Hynes, R. O., and DelSimone, D. (1990) Development 108, 229–238
26. Bursac, G. S., Damsky, C., and Pedersen, R. A. (1993) Development 118, 1836–1840
27. Giancotti, F. G., and Mainiero, F. (1994) Biochem. Biophys. Acta 1198, 47–64
28. Rusnati, E., and Reed, J. C. (1994) Cell 77, 477–478
29. Clark, E. A., and Brugge, J. S. (1993) Science 260, 233–239
30. Luscinskas, F. W., and Lawler, J. (1994) PASEB J. 8, 929–938
31. Bischof, J. (1995) Trends Cell Biol. 5, 69–74
32. Prats, A. C., Vagner, S., Prats, H., and Amaral, F. (1992) Mol. Cell. Biol. 12, 4796–4805
33. Bugler, B., Amaral, F., and Prats, H. (1991) Mol. Cell. Biol. 11, 573–577
34. Giancotti, F. G., Baird, A., and Gonzalez, A.-M. (1991) Growth Factors 4, 265–275
35. Quarto, N., Finger, F. P., and Rifkin, D. B. (1991) J. Cell Physiol. 147, 311–318
36. Renko, M., Quarto, N., Morimoto, T., and Rifkin, D. B. (1990) J. Cell. Physiol. 144, 108–114
37. Depalma, C. L., Ruoslahti, E., and Dean, D. C. (1991) Integrin Regulation by FGF-2
