Methylation of High Molecular Weight Fibroblast Growth Factor-2 Determines Post-translational Increases in Molecular Weight and Affects Its Intracellular Distribution

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The high molecular weight (HMW) forms (24, 22.5, and 22 kDa) of basic fibroblast growth factor-2 (FGF-2) contain an N-terminal extension responsible for their predominantly nuclear localization. These forms of FGF-2 are post-translationally modified, resulting in a 1- to 2-kDa increase in apparent molecular mass. Here we show that this post-translational modification is inhibited by methionine starvation and by the methyltransferase inhibitors 5'-deoxy-5'-methylthioadenosine (MTA) and 3-deaza-adenosine. Inhibition of the methylation-dependent modification results in a significant decrease in HMW FGF-2 nuclear accumulation, suggesting that methylation is relevant to the intracellular distribution of these forms of FGF-2. Treatment with MTA does not affect either the synthesis or the intracellular fate of another nuclear protein, the SV40 large T antigen, demonstrating that this drug does not have a generalized effect on nuclear protein accumulation. These results link HMW FGF-2 post-translational modification to its intracellular distribution.

INTRODUCTION

The fibroblast growth factor (FGF) family consists of nine heparin-binding polypeptides. These proteins can modulate a number of biological parameters—ranging from cell proliferation to plasminogen activation, integrin expression, cell migration, embryonic development, and cell differentiation—and may be involved in angiogenesis, malignant transformation, and differentiation (Burgess and Maciag, 1989; Rifkin and Moscatelli, 1989; Basilico and Moscatelli, 1992; Enenstein *et al.*, 1992; Klein *et al.*, 1993; Maciag *et al.*, 1994; Mason, 1994; Bikfalvi *et al.*, 1995). Interestingly, the prototypic members of this family, FGF-1 and FGF-2, lack a secretory sequence required for extracellular release through the classical membrane pathway

(Abraham *et al.*, 1986; Mignatti *et al.*, 1992), are synthesized on free ribosomes, and have all of the features of cytosolic proteins (Basilico and Moscatelli, 1992). However, both FGF-1 and FGF-2 are released from cells that express them (Mignatti *et al.*, 1991; Maciag *et al.*, 1994; Bikfalvi *et al.*, 1995). The low concentrations of these growth factors in the conditioned medium of cells has made the question of the mechanism of release difficult to approach. However, FGF-1 is released after heat shock (Jackson *et al.*, 1992; Opalenik *et al.*, 1995), and FGF-2 may be released after cell death or sublethal damage (McNeil *et al.*, 1989; Ku and D'Amore, 1995).

Although FGF-1 is expressed as a single polypeptide (Maciag *et al.*, 1994), FGF-2 is produced naturally in four forms that originate from alternative translation initiation within a single mRNA species (Florkiewicz and Sommer, 1989; Prats *et al.*, 1989). The lowest molecular weight form, 18-kDa FGF-2, initiates at an internal AUG codon, whereas the three high molecular weight (HMW) forms (22, 22.5, and 24 kDa) initiate at

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three CUG codons located 5' to the AUG. As a result, the three HMW FGF-2 forms are colinear N-terminal extensions of the 18-kDa FGF-2 and contain the entire sequence of the 18-kDa form at their C termini.

The various forms of FGF-2 have different intracellular distributions. The 18-kDa form is predominantly cytoplasmic, whereas the HMW FGF-2 forms are localized primarily in the nucleus (Renko *et al.*, 1990; Florkiewicz *et al.*, 1991). The intracellular distribution of the HMW forms of FGF-2 is determined by the N-terminal extensions that function as nuclear localization signals. Chimeric proteins consisting of the N-terminal domain of HMW FGF-2 joined to proteins that normally localize in the cytoplasm are targeted to the nucleus (Bugler *et al.*, 1991; Quarto *et al.*, 1991a). Thus, the addition of these N-terminal sequences to 18-kDa FGF-2 provides a structural feature that determines an alternative intracellular localization of the growth factor.

The biological functions of the FGFs are mediated by their interaction with both high- and low-affinity receptors located on the surfaces of target cells (for review, see Baird, 1994). A family consisting of four high-affinity tyrosine kinase FGF-receptor (FGF-R) types has been identified. The interaction of FGF-2 with its surface receptor induces autophosphorylation of the receptor and the phosphorylation of cytosolic substrates on tyrosine residues (for review, see Jaye *et al.*, 1992; Fantl *et al.*, 1993).

The overexpression of different FGF-2 forms confers unique cellular phenotypes on transfected cells (Bikfalvi *et al.*, 1995). The expression of HMW FGF-2 induces NIH 3T3 cell transformation as measured by enhanced saturation density, growth in soft agar, and growth in low serum. The expression of the 18-kDa form also enhances cell proliferation, saturation density, and growth in soft agar; in addition, it confers a spindle-shaped morphology, increased cell migration, and changes in integrin pattern. These latter three properties are unaffected by HMW FGF-2 expression (Bikfalvi *et al.*, 1995; Klein *et al.*, 1996). The coexpression of a dominant-negative FGF receptor reverts the phenotype of cells transformed by 18-kDa FGF-2 but has no effect on the phenotype of cells transformed by HMW FGF-2 (Bikfalvi *et al.*, 1995). Thus, different forms of FGF-2 with different subcellular distributions appear to affect the cell phenotype through autocrine FGF-R-dependent, or intracrine FGF-R-independent pathways. These observations have focused our attention on the mechanisms that determine the subcellular sorting of the FGF-2 forms.

Post-translational modification can regulate protein distribution and/or activity (for review, see Danpure, 1995). Three types of post-translational modification have been described for FGF-2. The 18-kDa FGF-2 can undergo phosphorylation either by a kinase associated with the cell surface (Vilgrain and Baird, 1991) or by a

nuclear kinase detected in SK-Hep1 cells (Vilgrain *et al.*, 1993). Boulle *et al.* (1995) reported that FGF-2 may also undergo ADP ribosylation in cultured SK-HEP1 cells. However, the occurrence of this modification of the HMW forms of FGF-2 has not been shown. An additional post-translational modification occurs specifically on the HMW forms of FGF-2. Sommer *et al.* (1989) and Burgess *et al.* (1991) demonstrated that in HMW FGF-2 three specific arginine residues (residues -26, -24, and -22 numbering from the initiator methionine for the guinea pig 18-kDa FGF-2) are mono- or dimethylated. By pulse-chase experiments, Florkiewicz *et al.* (1991) observed that the 24-kDa, but not the 18-kDa, FGF-2 undergoes a 1- to 2-kDa increase in its apparent molecular mass as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

In this paper, we report that the synthesis of HMW FGF-2 forms in NIH 3T3 fibroblasts transfected with a human wild-type (wt) FGF-2 cDNA that codes for all FGF-2 forms is accompanied by a progressive increase in size. This increase, which is detected in both the nuclear and the cytoplasmic HMW FGF-2, is not observed for 18-kDa FGF-2. The post-translational modification of HMW FGF-2 is methionine-dependent and is abolished by the methyltransferase inhibitors 5'-deoxy-5'-methylthioadenosine (MTA) and 3-deazaadenosine (DAA). Treatment with MTA also causes a reduction of the nuclear accumulation of HMW FGF-2. These results suggest that methylation may be essential for the nuclear accumulation of the HMW forms of FGF-2 and that, in the absence of this post-translational modification, the differences observed in the intracellular distribution and, ultimately, the biological function of HMW FGF-2 might be altered.

MATERIALS AND METHODS

Reagents

MTA was purchased from Sigma Chemical Co. (St. Louis, MO), DAA from Southern Research Institute (Birmingham, AL), [³⁵S]-methionine/L-cysteine JEXPRES³⁵S, ³⁵S Protein labeling mix (1,175 Ci/mmol) from DuPont Radiochemicals (Boston, MA), and geneticin (G-418) from Life Technologies (Gaithersburg, MD). The protease inhibitors 4-(2-aminoethyl)-benzenesulfonyl-fluoride hydrochloride (AEBSF) and leupeptin were purchased from Boehringer Mannheim (Indianapolis, IN). All other reagents were research grade.

Cells

NIH 3T3 cells were transfected with the Zip-neo vector containing the human FGF-2 cDNA (wt FGF-2 cells, clone c3) and cultured as described previously (Quarto *et al.*, 1991b; Bikfalvi *et al.*, 1995). The properties of these cells have been described previously (Quarto *et al.*, 1989, 1991b; Bikfalvi *et al.*, 1995). The clone used in this study expressed ~200 ng FGF-2/mg total protein as measured by an enzyme-linked immunosorbent assay (Brunner *et al.*, 1993).

BALB/c 3T3 cells transformed by wt SV40 (SVA31E7) (Carroll and Gurney, 1982) were obtained from the laboratory of Dr. Robert

B. Carroll (New York University Medical Center, New York, NY) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (CS).

Metabolic Labeling

Cells were starved for 45 min in cysteine/methionine-free DMEM (Life Technologies) in the presence or absence of methyltransferase inhibitors and pulsed for 30 min with 125 μ Ci/ml [35 S]methionine/cysteine in the same medium used during starvation with or without inhibitors. After a chase for the indicated times with complete DMEM containing 10% CS with or without inhibitors, the cells were gently washed with cold phosphate-buffered saline and lysed in RIPA buffer (300 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.05% Tween-20, 10 mM ethylenediaminetetra-acetate, and 20 mM Tris-HCl, pH 7.4) containing 10 μ g/ml leupeptin and 1 mM AEBSF. For the experiment illustrated in Figure 2, examining the methionine requirement for methylation, the cells were chased with cysteine/methionine-free DMEM containing 10% CS with or without methionine or cysteine supplementation. Cells were then treated as above. Alternatively, the cells were fractionated to prepare nuclei and cytoplasm (see next section).

Preparation of Nuclei and Cytoplasm

Nuclei were separated from cytoplasm as described by Renko *et al.* (1990). Briefly, confluent cells were scraped in cold Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl (in Tris-buffered saline), pelleted, and resuspended on ice in TKM solution (10 mM KCl, 1 mM MgCl₂, 10 mM Tris-HCl, pH 7.5) containing 10 μ g/ml leupeptin and 1 mM AEBSF. The cells were processed using a Dounce homogenizer with a tight pestle at 4°C. The homogenate was adjusted to 0.25 M sucrose, layered on top of a 1 M sucrose/TKM cushion, and centrifuged at 3000 rpm in a Sorvall HB-4 swinging bucket rotor for 10 min at 4°C. The supernatant (cytoplasmic fraction) was kept on ice while the pellet was centrifuged again through a 1 M sucrose cushion after resuspension in 0.3 M sucrose/TKM. Nuclear pellets were resuspended in 0.3 M sucrose/TKM plus 0.5% Triton X-100, layered on top of a 1 M sucrose cushion, and centrifuged as above. This step is required to obtain nuclei free of contamination with endoplasmic reticulum even though the procedure results in the loss of the outer nuclear envelope (Busch, 1967). Nuclei were resuspended in RIPA buffer containing protease inhibitors (10 μ g/ml leupeptin and 1 mM AEBSF). The purity of the nuclear and cytoplasmic fractions was assessed by observing with a phase-contrast microscope (Axiovert 35, objective LD Achroplan, 32 $\times$, Carl Zeiss, Inc., Thornwood, NY) and by analyzing the distribution of HMW and 18-kDa FGF-2 (Renko *et al.*, 1990).

Immunoprecipitation and Gel Electrophoresis

Nuclear and cytoplasmic fractions, or cell lysates prepared as described above, were sonicated for 15 s with a Vibra Cell Sonicator (Sonics & Materials, Inc., Danbury, CT). Each sample was clarified by centrifugation in an Eppendorf centrifuge at 14,000 rpm for 10 min at 4°C and assayed for protein content by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). Comparable amounts of protein from the cell lysates, nuclear and cytoplasmic fractions or, alternatively, the entire cytoplasmic and nuclear fractions (100% volume) were precleared with 80 μ l of a 50% slurry of protein A/agarose (Boehringer Mannheim) and immunoprecipitated with anti-FGF-2 rabbit serum (1/200 v/v) for 2 h at 4°C. For control experiments with SVA31E7 cells, a monoclonal antibody to SV40 large T antigen (a gift of Dr. Robert B. Carroll) was used for the immunoprecipitation. The beads were washed five times with RIPA buffer, boiled in reducing Laemmli sample buffer, and loaded onto a 12% SDS-polyacrylamide gel. After electrophoresis, the gels were dried and analyzed either by PhosphorImager (Molecular Dynam-

ics, Sunnyvale, CA) or by autoradiography using XAR-5 film (Eastman Kodak, Rochester, NY).

RESULTS

Nuclear Accumulation of HMW FGF-2 Forms Is Accompanied by a Post-translational Modification

During studies of intracellular signaling activated by the various forms of FGF-2, we analyzed the cytoplasmic and nuclear distributions of HMW and 18-kDa FGF-2 by a pulse-chase experiment with wt FGF-2 cells (clone C3) (Figure 1). As expected, newly synthesized 18-kDa FGF-2 rapidly accumulated in the cytoplasm, and its level was only slightly reduced after 8 h of chase (Figure 1). A lower molecular weight species of ~16 kDa, which probably represents a degradation product of the 18-kDa form (Bikfalvi *et al.*, 1989; Sommer and Rifkin, 1989), was also observed. In the nuclear fractions, there was a rapid accumulation of HMW FGF-2 (Figure 1). In addition, some 18-kDa FGF-2, the level of which was reduced during the 8-h chase, was initially present in the nucleus. Interestingly, the HMW forms displayed a gradual increase in their apparent molecular mass over the 8-h chase period (Figure 1). The final increase was calculated to be ~1.5 kDa for the 24-kDa form, and 0.5 kDa for the 22.5/22-kDa forms. The post-translational modification of HMW FGF-2 mobility was observed as early as 15 min after the start of the chase and was completed between 3 and 6 h as determined by immunoprecipitating the cell lysates with anti-FGF-2 serum (G. Pintucci, unpublished observations). A similar post-translational increase in apparent molecular mass was observed when NIH 3T3 cells transfected with a cDNA that permits expression only of HMW FGF-2 (Bikfalvi *et al.*, 1995) were analyzed.

Inhibition of Methylation Abolishes the Post-translational Modification of HMW FGF-2

The HMW FGF-2 isolated from guinea pig brain contains at least three arginine residues that are mono-

Figure 1. Nuclear and cytoplasmic distribution of FGF-2 forms and post-translational modification of HMW FGF-2. wt FGF-2 NIH 3T3 cells were metabolically labeled in a pulse-chase experiment as described in MATERIALS AND METHODS. At the indicated times, 80 μ g of nuclear and cytoplasmic fraction proteins were immunoprecipitated with anti-FGF-2 serum. The immunoprecipitates were analyzed by SDS-PAGE followed by autoradiography.

and/or dimethylated and are located in the HMW N-terminal extension (Burgess *et al.*, 1991). Because the post-translational modification of HMW FGF-2 was observed only in pulse-chase experiments in which the cells were starved of both methionine and cysteine before the start of the chase, and because methionine is the methyl donor for methylation reactions, we tested the effect of cysteine or methionine supplementation during the chase on the modification of HMW FGF-2 (Figure 2). Whole-cell lysates were analyzed rather than nuclear fractions to simplify the protocol. Consistent with the modification resulting from methylation, the increase in molecular weight of newly synthesized HMW FGF-2 immunoprecipitated from NIH 3T3 cells was strictly dependent on the presence of methionine, but not cysteine, in the medium during the chase (Figure 2). This modification was not affected by starving the cells of phosphate (Figure 2).

Methionine is the precursor of the methyl donor S-adenosyl-methionine (Finkelstein, 1990), which is the cofactor for the arginine-specific methyltransferases (Borchardt, 1980; Paik and Kim, 1980). To test the hypothesis that methylation caused the change in protein migration, we examined the effects of the methyltransferase inhibitors MTA and DAA on the post-translational modification of HMW FGF-2. Treatment of the cells with MTA (3 mM) and DAA (30 μ M) abolished the modification of HMW FGF-2 (Figure 3). These concentrations of the inhibitors were not cytotoxic as determined by cell counts and trypan blue exclusion. MTA (3 mM) has been shown to completely abolish protein methylation in BALB/c 3T3 cells (Maher, 1993). Therefore, we used this concentration of MTA in all subsequent experiments.

MTA Treatment Translationally Regulates the Expression of the Different FGF-2 Isoforms

The synthesis of proteins by alternative translation of the same messenger can be modulated by changes in

methionine	-	-	+	-	+	+
cysteine	-	-	-	+	+	+
phosphate	+	+	+	+	+	-
chase (h)	0	3	3	3	3	3

for 3 h (lane 2), the same medium supplemented with methionine (lane 3), with cysteine (lane 4), or with cysteine and methionine (lane 5). Lane 6 shows the effects of phosphate starvation during the 3-h chase. At the end of the chase period, equivalent amounts of protein from cell lysates were immunoprecipitated with anti-FGF-2 serum. The immunoprecipitates were analyzed by SDS-PAGE followed by autoradiography.

Figure 2. Methionine is required for the post-translational modification of HMW FGF-2. wt FGF-2 NIH 3T3 cells were metabolically labeled as described in MATERIALS AND METHODS and chased for 3 h in different media. At the start of the chase (lane 1), the cells were given fresh methionine-cysteine-free DMEM

	C	C	MTA	DAA
chase (h)	0	6	6	6

anti-FGF-2 serum. The immunoprecipitates were analyzed by SDS-PAGE followed by autoradiography. (Lane 1) Control at the start of the chase; (lane 2) control after 6-h chase; (lane 3) 3 mM MTA (6-h chase); (lane 4) 30 μ M DAA (6-h chase). Drug treatments were initiated during starvation (45 min before the pulse) and continued during the pulse (30 min) and chase (6 h).

Figure 3. Effect of methyltransferase inhibitors on the post-translational modification of HMW FGF-2. wt FGF-2 NIH 3T3 cells were metabolically labeled in the presence or absence of the methyltransferase inhibitors MTA or DAA as described in MATERIALS AND METHODS. After a 6-h chase, comparable amounts of cell lysate protein were immunoprecipitated with

the culture conditions (Hann *et al.*, 1992; Hann, 1994). Therefore, we tested the effect of MTA on the synthesis of the different forms of FGF-2. When cells were treated with MTA throughout the pulse and chase periods and when the amounts of FGF-2 were analyzed in cell lysates (Figure 4, lane 3), increased levels of the CUG-initiated forms (HMW FGF-2) and decreased amounts of the 18-kDa FGF-2 were observed in the cell lysates compared with the control (Figure 4, lane 2) (see also Figure 3). The same effect was observed as early as the start of chase (Figure 5A). This effect was not observed if the drug was administered to the cultures at the start of the chase (Figure 4, lane 4) or under conditions of methionine starvation (Figure 2, lanes 2 and 4). Decreased synthesis of 18-kDa FGF-2 was also observed when NIH 3T3 cells expressing only this form of FGF-2 (Bikfalvi *et al.*, 1995) were treated with MTA.

MTA Treatment of Transfected NIH 3T3 Cells Increases the Stability of HMW FGF-2

Because methylation may play a crucial role in regulating protein turnover (Hershko *et al.*, 1991; Clarke, 1992), we compared the stability of the FGF-2 forms in the presence or absence of the methyltransferase inhibitor MTA. As shown in Figure 5A, the presence of the inhibitor resulted in the appearance of increased

MTA			+	+
chase (h)	0	6	6	6

autoradiography. (Lanes 1 and 2) Control cells; (lanes 3 and 4) cells with MTA. Inhibitor was present throughout the experiment, starvation plus pulse and chase (lane 3), or added at the start of the chase (lane 4).

Figure 4. Effect of MTA on FGF-2 expression. wt FGF-2 NIH 3T3 cells were metabolically labeled as described in MATERIALS AND METHODS and exposed to MTA at the indicated times. Equivalent amounts of cell lysate protein were immunoprecipitated with anti-FGF-2 serum. Immunoprecipitates were analyzed by SDS-PAGE followed by

amounts of HMW FGF-2 in the cell lysate compared with the control at time zero. In addition, MTA did not increase the turnover of either HMW or 18-kDa FGF-2 but, instead, decreased it. This effect, which was maximum after 6-h chase, is evident when the relative amounts of FGF-2 forms, as measured by PhosphorImaging, are plotted as a function of time (Figure 5, B and C).

Figure 5. Stability of FGF-2 in the presence of MTA. Cells were pulse-labeled either in the absence or in the presence (+) of MTA as described in MATERIALS AND METHODS. Equivalent amounts of cell lysate protein were immunoprecipitated with anti-FGF-2 serum after chasing for the times indicated. The immunoprecipitates were analyzed by SDS-PAGE followed by autoradiography. Phosphor Imager units (PhImU) (percent values of the units calculated at time 0 using either control or MTA-treated cells) $\pm$ SE are plotted for the HMW forms (B) or the 18 kDa form (C) of FGF-2 immunoprecipitated from three independent experiments.

Inhibition of Methylation Results in a Decreased Nuclear Accumulation of HMW FGF-2

As shown in a previous report, the nuclear localization of HMW forms of FGF-2 is mediated by their N-terminal extensions (Quarto *et al.*, 1991a; Patry *et al.*, 1994a). Because the methylation is specific for HMW FGF-2 (see Figure 1), we tested whether the prevention of methylation would affect the nuclear accumulation of HMW FGF-2. Because a lag period might be necessary for the MTA to become fully effective (Seeley *et al.*, 1984), in this experiment we used the methyltransferase inhibitor throughout the starvation, pulse, and chase periods. To visualize the actual partitioning of HMW FGF-2 in the control and MTA-treated cells, total nuclear and cytoplasmic fractions were immunoprecipitated with anti-FGF-2 serum and analyzed after SDS-PAGE (Figure 6A). The relative percent values in the two intracellular compartments were calculated by taking as 100% the sum of the PhosphorImager units obtained from the analysis of either HMW or 18-kDa FGF-2 bands in the nuclei and the cytoplasm from either control or MTA-treated cells. This means that only values within a pair—nucleus and cytoplasm—can be compared directly. The quantitative changes in the distribution of FGF-2 are illustrated in Figure 6, B and C, in which the average percent values obtained from the PhosphorImager scans of the different species from three independent experiments are plotted. Consistent with previous reports, after a 6-h chase, >75% of HMW FGF-2 was found in the nucleus and <25% in the cytoplasm of control cells. After MTA treatment, a 55:45% distribution of the HMW forms was found in the two compartments, respectively. The ratio of 18-kDa FGF-2 in the nucleus to that in the cytoplasm was affected minimally by MTA (Figure 6, A and C). The absolute amount of 18-kDa FGF-2 was decreased because MTA inhibits the synthesis of this form of FGF-2 (Figures 4 and 5). A significant reduction (40–60%) in the nuclear accumulation of HMW FGF-2 was also observed when equivalent amounts of nuclear protein were immunoprecipitated from control or MTA-treated cells.

The relative values for the distribution of the HMW forms might be skewed because nuclear breakage or leakage from the nucleus to the cytoplasm may have occurred. To test this possibility, we examined the distribution of the nuclear SV40Tag. This molecule has a nuclear localization signal that determines its intracellular fate (Garcia-Bustos *et al.*, 1991). The nuclear accumulation of SV40Tag was not affected by MTA in pulse-chase experiments with metabolically labeled SV40-transformed BALB/c 3T3 cells (Figure 7A). With the cell fractionation conditions used in two independent experiments, a 70:30% distribution of SV40Tag was found between the nucleus and the

Figure 6. Nuclear accumulation of HMW FGF-2 in the presence of MTA. wt FGF-2 NIH 3T3 cells were metabolically labeled with [35 S]cysteine/methionine and subjected to cell fractionation after 6-h chase as described in MATERIALS AND METHODS. The entire nuclear and cytoplasmic fractions from control or MTA-treated cells were immunoprecipitated with anti-FGF-2 serum. The immunoprecipitates were analyzed by SDS-PAGE followed by autoradiography (A) and PhosphorImaging. Values obtained from the PhosphorImager scanning (PhImU, PhosphorImager units) (percent values, see description in the text) $\pm$ SE represent the values from three independent experiments plotted for the HMW forms (B) and the 18-kDa form (C) of FGF-2.

cytoplasm. However, by immunofluorescence, the protein was observed exclusively in the nucleus. Therefore, the values we obtained for HMW FGF-2 may reflect a minimum level of nuclear FGF-2 after some redistribution into the cytoplasm during cell fractionation.

To exclude the possibility that MTA caused a generalized impairment in nuclear protein accumulation, we analyzed the total protein from nuclear fractions of metabolically labeled MTA-treated or untreated cells. Figure 7B shows no obvious decrease in protein accumulation in the nuclei of the treated cells. However, immunoprecipitation of the same samples with anti-FGF-2 serum revealed a significant (40%) decrease of HMW FGF-2 in the nucleus (Figure 7B, bottom). These results indicate that MTA treatment does not have a generalized effect on nuclear protein import and/or accumulation.

DISCUSSION

Moscatelli *et al.* (1987) first described the existence of an FGF-2 molecule larger than the initially characterized 18-kDa FGF-2. This form of FGF-2 was shown to originate by translation initiation at alternative codons of the FGF-2 mRNA that generates 22-, 22.5-, and 24-kDa species in addition to the 18-kDa molecule (Florkiewicz and Sommer, 1989; Prats *et al.*, 1989). The HMW forms of FGF-2 have predominantly a nuclear localization, contain at least three residues of methyl arginine (Renko *et al.*, 1990; Burgess *et al.*, 1991; Florkiewicz *et al.*, 1991), and by pulse-chase experiments display a post-translational increase in molecular mass of 0.5–1.5 kDa. In this paper, we show that this

post-translational modification is dependent on methylation because either methionine starvation or inhibitors of transmethylation block the increase in molecular weight. Blockage of methylation also decreases the nuclear accumulation of HMW FGF-2, suggesting that methylation may contribute to the subcellular localization of HMW FGF-2.

The N-terminal extensions of HMW FGF-2 contain multiple repeats of the sequence G-R-G, believed to be the motif recognized by the arginine methyltransferase (Najbauer *et al.*, 1993). The presence of a glycine on the carboxyl side of the arginine residue seems to be a more strict requirement than the presence of a glycine on the amino side (Najbauer *et al.*, 1993). Analysis of the sequence of the N-terminal extension of 24-kDa FGF-2 (Figure 8) reveals that seven or, perhaps, eight arginine residues are potential targets of the methyltransferase according to these restrictions (Najbauer *et al.*, 1993). Burgess *et al.* (1991) identified a peptide in the N-terminal extension of guinea pig brain-derived HMW FGF-2 that contains three mono- or dimethylated arginine residues located in the indicated consensus sequence. How many of the additional potential target residues are actually methylated is not known. Experiments are currently in progress to isolate and sequence additional N-terminal peptides from HMW FGF-2. It is also possible that methylation of a second molecule, which determines the intracellular fate of HMW FGF-2, may be important for the observed nuclear accumulation. Our experiments do not exclude this possibility.

Obviously, even dimethylation of eight arginine residues cannot account for a mass increase of 0.5–1.5

Figure 7. Effect of MTA on nuclear accumulation of SV40Tag or total protein. (A) BALB/c 3T3 cells overexpressing SV40Tag were metabolically labeled in presence (+) or absence (-) of 3 mM MTA as described in MATERIALS AND METHODS. Nuclear fraction protein (200 μ g) was immunoprecipitated with an anti-SV40Tag monoclonal antibody. The immunoprecipitates were analyzed by SDS-PAGE followed by autoradiography. The migration of SV40Tag and of coprecipitating p53 is indicated on the right. (B) wt FGF-2 NIH 3T3 cells were metabolically labeled in the presence (+) or absence (-) of MTA and, after 24-h chase, nuclear and cytoplasmic fractions were prepared as described in MATERIALS AND METHODS. (Top) Comparable amounts of protein were analyzed by SDS-PAGE followed by autoradiography. (Bottom) Comparable amounts of protein from the same samples were immunoprecipitated with anti-FGF-2 serum and analyzed by SDS-PAGE followed by autoradiography.

kDa. However, methylation of HMW FGF-2 may generate conformational changes that alter its electrophoretic behavior in SDS. Patry *et al.* (1994b) analyzed a recombinant form of the 24-kDa CUG-initiated FGF-2 by mass spectrometry. Consistent with the in-

Figure 8. Amino acid sequence of the human FGF-2 N-terminal extension. Eight potential sites for arginine methylation exist (bold characters) according to the consensus requirement reported by Najbauer *et al.* (1993). The asterisks indicate the three arginine residues that were found to be methylated in the guinea pig brain-derived HMW FGF-2 (Burgess *et al.*, 1991). The three CUG (L) (for the 24-, 22.5-, and 22-kDa forms) and the AUG (M) (for the 18-kDa form) initiation sites are indicated by arrows.

ability of bacteria to form methyl-arginine (Clarke, 1992), the molecular mass of the prokaryotic recombinant 24-kDa FGF-2 corresponded to that from the predicted amino acid sequence. However, when Patry *et al.* (1994b) compared the electrophoretic behavior of this prokaryotic form of HMW FGF-2 with the HMW FGF-2 form isolated from eukaryotic cells, they observed that the eukaryotic form had an apparent molecular mass ~ 1 kDa higher than the prokaryotic form. It is also possible that an additional modification occurs and that the change in apparent molecular mass represents the sum of these modifications. We are testing this possibility by mass spectrometry analysis. Whatever the cause of the increase in the observed molecular mass, the results presented here show that inhibition of methylation abolishes the post-translational modification of HMW FGF-2 forms as determined by their electrophoretic mobility. It is noteworthy that Western blot analysis of the HMW FGF-2 failed to reveal the precursor form of HMW FGF-2 under non-pulse/chase conditions when cells were treated with MTA, probably because of the low level of FGF-2 synthesis and the rapid conversion of the hypomethylated form of HMW FGF-2 to the mature form. In addition, incubations with the drug for periods longer than 24 h result in cell death. These two aspects of the system prevented a potential immunofluorescence approach for the analysis of the intracellular distribution of the HMW FGF-2.

Almost all known proteins can be modified post-translationally, and >100 different post-translational modifications have been described (Rattan *et al.*, 1992). Methylation of specific amino acid residues, a general phenomenon, can occur on the nucleophilic groups of many different amino acid residues of a polypeptide (Clarke, 1992, 1993). The biological role(s) of post-translational methylation of amino acid residues depends on the specific residue and its location in the protein structure (for examples, see Clarke, 1992, 1993). Protein methylation can be reversible (C-meth-

ylation) or irreversible (N-methylation). Major examples of the first category are the methylation of cysteine in the ras protein (Clarke *et al.*, 1988) and the methylation of an aspartic acid in calmodulin (Johnson *et al.*, 1987). N-methylation has been described in several proteins, although its biological relevance has not been established in many cases (Clarke, 1992). Studies on apocytochrome *c* have revealed that methylation of a specific lysine residue correlates with an increased transport into the mitochondria and a decreased degradation rate (Park *et al.*, 1987). Arginine methylation has also been suggested to be functionally important. Mutant mice with a reduced activity of the arginine-specific methyltransferase display an abnormal organization in their myelin sheaths of the myelin basic protein, a protein that undergoes arginine methylation (Kim *et al.*, 1984). Here we show that methylation of HMW FGF-2 correlates with its nuclear accumulation.

The ability to modulate HMW FGF-2 distribution with methyltransferase inhibitors supports the view that nuclear accumulation and post-translational modification of HMW FGF-2 are related. In the absence of methylation, HMW FGF-2 distributes equally between the cytoplasm and the nucleus, in contrast to the nuclear accumulation observed in control cells. The decrease in nuclear HMW FGF-2 does not result from decreased synthesis or increased turnover. In MTA-treated cells, HMW FGF-2 synthesis seems to be favored over that of the 18-kDa form (Figures 4 and 5). In addition, MTA treatment does not increase FGF-2 turnover as analyzed by immunoprecipitation of comparable amounts of protein from cell lysates of control or MTA-treated cells with an anti-FGF-2 antibody (Figure 5). However, in our cell fractionation analyses, the total amount (nuclear plus cytoplasmic PhosphorImager units) of HMW FGF-2 immunoprecipitated from MTA-treated cells appears slightly lower than in control cells (Figure 6). This seems to contradict the finding of increased synthesis/stability of the HMW FGF-2 forms in MTA-treated cells (Figures 4 and 5). In this respect, it should be kept in mind that the experimental conditions for cell lysate preparation are different than those used for the cell fractionation (see MATERIALS AND METHODS). Especially noteworthy is the fact that cell lysate preparation probably includes some FGF-2 associated with the extracellular matrix, whereas cell fractionation experiments do not. Therefore, in our experimental system we cannot rule out the possibility that nonmethylated forms of HMW FGF-2 might be released from the cell and accumulate in the extracellular matrix. These might be included in cell lysis assays but might be lost in the cell fractionation assays, thereby accounting for the difference in FGF-2 levels obtained by the two techniques.

Maier (1993) reported that MTA can inhibit signaling by the FGF receptor in cultured cells as determined by the analysis of FGF-2-induced protein tyrosine phosphorylation. This result suggests that MTA might inhibit phosphorylation as well as methylation. However, in the experiments described by Maier (1993), MTA inhibited tyrosine phosphorylation induced by FGF-2, but not phosphorylation elicited by either epidermal growth factor or platelet-derived growth factor (Maier, 1993). Therefore, MTA is not a general inhibitor of protein phosphorylation. Moreover, treatment of cells with a second inhibitor of transmethylation, DAA, which blocks methylation by a different mechanism than MTA (Borchardt, 1980), also resulted in the inhibition of the increase in molecular mass of HMW FGF-2 (Figure 3). Cotransfection of a dominant-negative FGF-R into FGF-2-transfected cells has been shown to suppress the biological activity of 18-kDa but not of HMW FGF-2 (Bikfalvi *et al.*, 1995). The overexpression of this defective FGF-R did not affect either the increase in molecular mass or the intracellular distribution of HMW FGF-2 (G. Pintucci, unpublished observations). Therefore, if the effect of MTA on receptor-mediated responses and phosphorylation occurs in our system, it is unlikely to be responsible either for the blockade of post-translational modification or for the reduction in nuclear accumulation of HMW FGF-2.

The observations described in this paper suggest an interesting mechanism for regulating the action of different forms of FGF-2. The isoforms of this growth factor originate from alternative translation of the same mRNA (Florkiewicz and Sommer, 1989; Danpure, 1995) and exhibit distinct biological effects in transfected cells (Bikfalvi *et al.*, 1995). The post-translational modification of specific amino acids may direct the HMW forms to the nucleus, where these molecules stimulate a set of intracellular signaling reactions different from those elicited by interaction with the plasma membrane receptor. These latter effects require FGF-2 release from the cell. The HMW forms initiated from CUG codons show analogies with the different forms of the int-2 oncogene product (FGF-3). The CUG-initiated forms of both FGF-2 and FGF-3 are found primarily in the nucleus, whereas the AUG-initiated forms are found either in the cytoplasm (FGF-2) or extracellularly (FGF-3) (Acland *et al.*, 1990; Danpure, 1995). The proto-oncogene *c-myc* also exists in two alternative forms: one originating from an AUG initiation codon, the other, less abundant under normal conditions, originating from a CUG codon. Certain stress conditions or nutrient deprivation have been shown to shift the translation initiation from the AUG to the CUG codon (Hann *et al.*, 1992; Hann, 1994). This result raises the question of whether similar controls exist for other CUG/AUG-initiated proteins. The conditions and mechanisms that determine

in vivo expression of the AUG- versus the CUG-initiated forms of FGF-2 are not known currently, although patterns of differential expression of the FGF-2 alternative forms in different organs have been reported recently (Coffin *et al.*, 1995). Changes in methionine pools or in cellular metabolism, such as methylation inhibition achieved by the combined effect of methionine starvation and the use of methyltransferase inhibitors shown in our study, may shift both the ratio of FGF-2 forms synthesized and their degree of methylation. This, in turn, may affect their relative intracellular distribution and the cellular phenotype. Whether shifts in the methylation pattern of HMW FGF-2 alter the properties of cells expressing this growth factor remains to be examined. Moreover, several other proteins that contain methylarginine and have a nuclear localization, including nucleolin, have been described (Lapeyre *et al.*, 1986). It will be interesting to establish whether inhibition of methylation affects their localization and/or their function.

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