Biochemical Analysis of the Arginine Methylation of High Molecular Weight Fibroblast Growth Factor-2*

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Sharon Klein‡, James A. Carroll¶, Yan Chen‡, Michael F. Henry**, Pamela A. Henry**, Izabela E. Ortonowskï, Giuseppe Pintucci§§, Ronald C. Beavis¶¶, Wilson H. Burgess¶¶, and Daniel B. Rifkin‡

From the ‡Department of Cell Biology and the Kaplan Cancer Center, New York University Medical School, and the ¶Department of Pharmacology and Skirball Institute of Biomedical Research, New York University Medical School, New York, New York 10016 and 5402, the Department of Chemistry, New York University, New York, New York 10013, the §§Department of Molecular Biology, University of Medicine and Dentistry of New Jersey, Stratford, New Jersey 08084, and the ¶¶Department of Tissue Biology, Holland Laboratory, American Red Cross, Rockville, Maryland 20855

The post-translational methylation of the N-terminally extended or high molecular weight (HMW) forms of fibroblast growth factor-2 (FGF-2) has been shown to affect the nuclear accumulation of the growth factor. In this study, we determined the extent and position of methyl groups in HMW FGF-2. Using mass spectrometry and amino acid sequence analysis, we have shown that the 22- and 22.5-kDa forms of HMW FGF-2 contain five dimethylated arginines located at positions -22, -24, -26, -38, and -38 using the methionine residue normally used to initiate the 18-kDa form as position 0. The 24-kDa form of HMW FGF-2 contains seven to eight dimethylated arginines located at positions -48, -50, and -52, in addition to positions -22, -24, -26, -36, and -38. In vitro methylation reactions demonstrate that the N-terminal extension of HMW FGF-2 acts as a specific substrate for yeast Hmt1p and human HRMT1L2 arginine methyltransferases. These findings indicate that HMW FGF-2, with the presence of five or more dimethylated Gly-Arg-Gly repeats, contains an RGG box-like domain, which may be important for protein-protein and/or protein-RNA interactions.

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‡ To whom correspondence should be addressed. Present address: Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, 1275 York Ave., New York, NY 10021. Tel.: 212-639-7001; Fax: 212-794-6236; E-mail: s-klein@ski.mskcc.org.

¶ Present address: Monsanto Co., 700 Chesterfield Pkwy. N., St. Louis, MO 63198.

§§ Recipient of support from the Commission of the European Communities (Abruzzo) and the Ernst Schering Research Foundation. Present address: Dept. of Cardiothoracic Surgery, New York University Medical Center, 550 First Ave., New York, NY 10016.

** Present address: ProteoMetrics, LLC, 38 West 38th Street, New York, NY 10018.

The abbreviations used are: FGF-2, fibroblast growth factor-2; HMW, high molecular weight; LMW, low molecular weight; hNRNP, heterogeneous nuclear ribonucleoprotein; MALDI, matrix-assisted laser desorption/ionization; MES, 4-morpholineethanesulfonic acid; HPLC, high pressure liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid; SAM, S-adenosyl-l-methionine; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase.
FGF-2. The coexpression of a dominant negative FGF receptor reverts the phenotype of cells transformed by LMY FGF-2 but has no effect on the phenotype of cells transfomed by HMW FGF-2. Thus, different forms of FGF-2 affect the cell phenotype through autocrine FGF receptor-dependent or intracellular FGF receptor-independent pathways. These observations have focused our interest on the mechanisms that determine the nuclear localization of the HMW FGF-2 forms.

By pulse-chase experiments, newly synthesized HMW FGF-2 forms were shown to accumulate rapidly in the nuclei of transfected NIH 3T3 cells and displayed a gradual increase in their apparent molecular weight (21). The molecular weight increase was dependent on the presence of methionine, the precursor of the methyl donor S-adenosyl-l-methionine (SAM). In addition, the transmethylation inhibitor 5'-deoxy-5'-methylthioadenosine abolished the modification of HMW FGF-2, indicating that the post-translational increase in molecular weight was due to methylation. Moreover, inhibition of protein methylation by 5'-deoxy-5'-methylthioadenosine treatment decreased the nuclear accumulation of HMW FGF-2 (21). These findings indicated that post-translational methylation is critical for the proper nuclear transport or retention of HMW FGF-2.

Sommer et al. (22) showed by limited amino acid sequence analysis that guinea pig brain-derived HMW FGF-2 contained nonstandard phenylthiohydantoin-derivatives at three positions where the human cDNA sequence predicts arginine residues. Burgess et al. (23) later demonstrated that these amino acids, at positions −22, −24, and −26, numbering from the initiator methionine for the LMW FGF-2, were methylated arginines.

Arginine methylation is a post-translational modification found in nuclear proteins, including heterogeneous nuclear ribonucleoproteins (hnRNPs), nucleolin, and fibrillarin (24–26). The hnRNPs are proposed to function in all known steps of mRNA maturation including pre-mRNA packaging, splicing, and mRNA nuclear export. The nucleolar proteins nucleolin and fibrillarin are involved in pre-rRNA transcription and in pre-rRNA maturation, respectively. Furthermore, both of these proteins are implicated in ribosome biogenesis.

A distinctive feature of the N-terminal extension of HMW FGF-2 is the high percentage of arginine and glycine residues (57% in the 54-amino acid extension of the longest form) including six arginines flanked by glycyl residues on each side. The Gly-Arg-Gly motif is a general sequence recognized by arginine methyltransferases in vitro and in vivo (27). This suggests that the HMW FGF-2 N-terminal region may contain more than the three methylation sites originally described (23).

To elucidate the role of methylation in nuclear accumulation and HMW FGF-2 function, the degree and position of methylation must first be determined. We purified HMW FGF-2 from transfected NIH 3T3 cells, and, using mass spectrometry and protein sequencing approaches, characterized the post-translational modifications present in the N-terminal region.

MATERIALS AND METHODS

Reagents—Recombinant human FGF-2 (18 kDa) was a gift from Amgen, Inc. (Boulder, CO) and Scios Nova (Mountain View, CA). Aprotinin was a gift from Bayer AG (Wuppertal, Germany).

Cells—NIH 3T3 cells were transfected with the Zip-neo vector containing the human 1.1-kilobase FGF-2 cDNA encoding only for the 24–22-kDa FGF-2 and the pCEP4 vector (clone 365/365c14 and cultured as described previously (11, 12).

Purification of HMW FGF-2 Proteins—NIH 3T3 cells (clone 365/365c14) were washed and scraped on ice with phosphate-buffered saline containing aprotinin (50 μg/ml). The cells were resuspended in 20 ml of 0.5 M NaCl, 20 mM MES, pH 6.0, containing leupeptin (10 μg/ml) (Sigma), aprotinin (50 μg/ml), and 1 mM 4-(2-aminoethyl)-benzenesulfonyl-fluoride, hydrochloride (Sigma), sonicated, and centrifuged at 7,000 rpm in an SS34 rotor for 2 h at 4 °C. The supernatant was centrifuged at 24,000 rpm in a Ti60 rotor for 45 min at 4 °C. This supernatant was loaded on a 1 ml fast protein liquid chromatography heparin-Sepharose column (Amersham Pharmacia Biotech), and the column was washed with 0.5 and 0.8 M NaCl, 20 mM MES, pH 6.0. The 0.5 M eluate was diluted in high pressure liquid chromatography (HPLC) C8 column (RP300, 2.1 × 30 mm) (Microm Biosources USA System, Auburn, CA). HMW FGF-2 was eluted using 0.1% trifluoroacetic acid (buffer A) and 0.08% trifluoroacetic acid in 70% acetonitrile (buffer B) with a linear gradient of 0–95% buffer B in 60 min.

Protease Treatement of HMW FGF-2 Proteins—HMW FGF-2 samples from reverse-phase HPLC were dried, resuspended in 100 mM NH₄HCO₃, and digested either with Lys-C protease (9 μg/ml) (Sigma) for 4 h at room temperature or V8 protease (45 μg/ml) (Sigma) for 16 h at room temperature. For some experiments, the Lys-C digest was subjected to a second digestion with Asp-N protease (24 μg/ml) (Sigma) for 16 h at room temperature.

Matrix-assisted Laser Desorption Mass Spectrometry—Protein and peptide samples were applied on-matrix-assisted laser desorption/ionization (MALDI) mass spectrometry using the dried droplet method (28). The matrix used was recrystallized α-cyano-4-hydroxycinnamic acid (Sigma). The sample was added to a saturated solution of matrix prepared 2:1 aqueous 0.1% trifluoroacetic acid/acetonitrile at room temperature so that the final sample concentration was 1~10 μM. 0.5 μl of the solution was placed on the probe of the mass spectrometer and allowed to dry. The sample was analyzed using a linear time-of-flight mass spectrometer with a 1-m flight path and a single-field acceleration region (+40 kV). Ions were formed using a nitrogen laser (LSI 337ND, Newton, MA) as the light source. For the spectra shown, 100 scans were averaged.

Protein Sequencing—Automated Edman degradation of HMW FGF-2 proteins was performed on a 492 protein sequencer, and the resulting phenylthiodyantoin derivatives identified using an on-line phenylthiohydantoin derivative analyzer (Applied Biosystems).

Peptides—The peptides GGRGRGARPPVVGRG (F1), DGRGRG-RALPPG (F2), and GGRGGGFGRGGFGGRGG (R3) were synthesized using an Applied Biosystems model 431A peptide synthesizer and smaller scale Fmoc (N-(9-fluorenyl)methoxycarbonyl) cycles supplied by the manufacturer. Peptides were purified by reverse-phase HPLC and characterized by amino acid analysis and mass spectroscopy. The peptide ALAQQPVPYLVGL (C)-was kindly provided by John Albert (Dana Farber Cancer Institute).

Purification of HMW FGF-2 and Npl3 from Escherichia coli—The E. coli pGST-HMWFGF-2 expression vector was constructed by subcloning the Xhol/EcoRI fragment from vector CU024kDFGF-2 (29) into the expression vector pGEX-F1 (gift from Mindong Ren, New York University Medical School). The bacterial strain HB101 was transformed with the plasmid pGST-HMWFGF-2, and fusion protein expression was induced with isoprropyl-1-thio-β-D-galactopyranoside (1 mM) for 6 h at 30 °C. The recombinant protein was purified using Bulk GST Purification Module (Amersham Pharmacia Biotech), and the eluted fraction was diluted 5-fold with 0.5 M NaCl, 20 mM MES, pH 6.0, dialyzed against the same buffer, and loaded onto an fast protein liquid chromatography heparin Sepharose column. The column was washed with 0.8 M NaCl, 20 mM MES, pH 6.0, and protein was eluted with 2 M NaCl, 20 mM MES, pH 6.0. The eluted protein was dialyzed against 50 mM Tris-HCl, 100 mM NaCl, 0.5% Tween 20, 5% glycerol, 1 mM EDTA, pH 8.0, and precipitated with 2 M NaCl. The precipitated protein was redissolved in dialysis buffer containing 7 mM urea and renatured by dialysis using decreasing concentrations of urea. Recombinant Npl3, produced in E. coli and purified to homogeneity, was kindly provided by Ethan Ford and Adrian Krainer (Cold Spring Harbor Laboratory).

In Vitro Methylation Reactions—Methylation reactions were performed as described previously (30). 40 ml reactions contained 50 mM MOHA, 30 μg SAM, 30 μg HRMT1L2 or yeast Hmt1p (hnRNP methyltransferase 1 protein) and sub- strate protein. Soluble E. coli extracts were added at 10 μg/reaction, whereas purified substrate proteins were supplemented at 1 μg/reac- tion. Reactions were initiated by the addition of either human HMRTIL2 or yeast Hmt1p (hnRNP methyltransferase 1 protein) and incubated at 30 °C for 40 min. Competition reactions were performed in the same manner except for the presence of the R1, P1, P2, or C-
peptides (500 μM). Reactions were terminated by the addition of SDS-PAGE reducing sample buffer and boiling for 5 min. Proteins were resolved by SDS-PAGE followed by Coomassie staining and fluorography.

RESULTS

Purification of HMW FGF-2 from NIH 3T3 Cells Stably Transfected with HMW FGF-2 cDNA—To characterize the extent and position of arginine methylation in HMW FGF-2, we purified HMW FGF-2 proteins from NIH 3T3 cells (clone 365/365FGFc14) stably transfected with cDNA encoding exclusively HMW FGF-2 forms. HMW FGF-2 proteins were isolated from NIH 3T3 cell extracts by chromatography on two consecutive fast protein liquid chromatography heparin-Sepharose columns followed by HPLC as described under “Materials and Methods.” Fig. 1A (inset) shows a Coomassie Blue-stained SDS-PAGE gel of an aliquot of the purified HMW FGF-2 proteins. Fig. 1B illustrates the amino acid sequences of these forms based upon the cloned cDNA (31, 32).

Mass Spectrometric Analysis and Edman Degradation of HMW FGF-2 Proteins—MALDI mass spectrometry, in conjunction with enzymatic degradation, was used to determine the accurate molecular mass, amino acid sequence, and the extent of post-translational modification of the HMW FGF-2 proteins. Fig. 1A shows the MALDI mass spectrum of the purified forms of HMW FGF-2. The molecular mass of the short form of HMW FGF-2 was 21,170 Da (Fig. 1C). The difference in mass found is due to modifications.

To determine the cause of the mass increase, enzymatic digestion and analysis of the peptides generated by MALDI mass spectrometry was performed. The mixture of HMW FGF-2 forms was first digested with Lys-C, which cleaves specifically at the C-terminal side of lysine (Fig. 1B), and the Lys-C digest mixture was subjected to mass spectrometry. Following Lys-C digestion, the N-terminal fragments, the largest peptides in the digestion mixture (illustrated in the peptide map in Fig. 5A), are clearly separated from the remainder of the HMW FGF-2 peptides on the mass spectrum. Fig. 2 (A and B) shows the predicted sequences of the N-terminal peptides from the digest and the resulting MALDI mass spectrum of the peptides. The peaks labeled P1 and P2 at m/z 6527 and 6570 correspond to modified forms of the N-terminal peptide of the short form of HMW FGF-2. The smallest peptide P1 differs from the expected mass by 142 Da (Fig. 2C), indicating a post-translational modification present in the N-terminal region of the protein. In addition, the difference in mass between P1 and P2 (43 Da) approximates the mass of a single acetyl group (42 Da), suggesting that an additional fraction of the short form of HMW FGF-2 is acetylated.

To localize the sites of modification, the mixture of the three forms of HMW FGF-2 was subjected to Glu-C digestion with V8 protease, which cleaves specifically at the C-terminal side of glutamic acids (Fig. 1B). There are nine sites of cleavage in the protein, one of which is present in the N-terminal extension containing the nuclear localization sequence (Fig. 1B). Fig. 2 (D and E) illustrates the predicted sequences of the N-terminal peptides from...
a Glu-C digest of HMW FGF-2 and the mass spectrum showing the N-terminal peptides of all three forms of HMW FGF-2. Consistent with the results from the Lys-C digest, the two forms of the smallest peptide (P5 and P6), differing by 42 Da, indicate that a portion of the short form of HMW FGF-2 is acetylated at the N terminus. Moreover, the 56-Da difference between the measured and theoretical mass of the P5 peptide (at m/z 1068; Fig. 2F) predicts that four methyl groups (each 14 Da) are located in the peptide.

The mass of larger peptides was determined by examining a different region of the mass spectrum. The peptide illustrated in Fig. 3A representing amino acids 2 to 56 derived from a Glu-C digest is common to all three forms of HMW FGF-2 and contains seven arginines, three of which are flanked by glycines. The mass spectrum (Fig. 3B) reveals that the peptide (P11 at m/z 4219; Fig. 2F) predicts that four methyl groups (each 14 Da) are located in the peptide.

To identify unambiguously the position of the 10 methyl groups, the mixture of intact HMW FGF-2 proteins was transferred to polyvinylidene difluoride membrane and subjected to Edman degradation. The short and middle forms do not have coincidental Arg residues that would be generated in the same cycle. In addition, as the long form is blocked at the N terminus (see below), methylated Arg residues can be subscribed to their respective HMW FGF-2 form during sequencing. This analysis confirmed the amino acid sequence of the N-terminal extension (Gly240 through Thr1) of the short form and identified 5 of the 10 arginines having methyl groups.

The mass spectrometry analysis of the mixture of intact HMW FGF-2 also revealed that the middle form of HMW FGF-2 differs from the theoretical mass by 131 Da (minus the first amino acid; Fig. 1A). Furthermore, proteolytic fragments obtained by Lys-C digestion demonstrated that the 143-Da mass increase was due to post-translational modification located in the N-terminal region of the protein (peak P3 at m/z 7009; Fig. 2B). Consistent with the pattern of methylation in the short form of HMW FGF-2, the N-terminal peptide derived from Glu-C digestion (peak P7 at m/z 1549; Fig. 2E) showed a 55-Da increase in mass corresponding to four methyl groups (Fig. 2F).

For the long form of HMW FGF-2, mass spectrometry of the mixture of intact proteins showed a 252-Da difference with
respect to the theoretical mass of the protein lacking the first amino acid (Fig. 1, A and C). Lys-C digestion indicated that the post-translational modifications in the long form were present in the N-terminal peptide (P4 at m/z 8063; Fig. 2B). Interestingly, mass spectrometry of the Glu-C digest showed that the N-terminal peptide of 24 amino acids exists as three species (P8, P9, and P10), differing in mass of a single methyl group (Fig. 2E and F). This result indicated that the methylation of the peptide is heterogeneous. N-terminal sequencing of the intact protein was not possible, indicating that the first residue contains a blocking group. To determine the nature of the blocking group, the mixture of HMW FGF-2 forms were subjected to Glu-C digestion followed by digestion with Asp-N protease, which cleaves at the N terminus of aspartic acids (Fig. 1B). The mass region corresponding to the long form before and after digestion with Asp-N protease is shown in Fig. 4(B and C). The shift in mass corresponds to an acetylated glycine equaling 99 Da. Consequently, the masses of peptides P12, P13, and P14 (Fig. 4D) differ from the masses of the related peptides P8, P9, and P10 (Fig. 2F) by the absence of 42 mass units corresponding to an acetyl group. Therefore, by subtraction of mass, the N-terminal Glu-C peptide must contain 8–10 methyl groups as well as an acetyl group. To determine the position(s) of the heterogeneity in methylation, the N-terminal peptide of the long form was purified by reverse-phase HPLC after Asp-N digestion of the mixture of HMW FGF-2 forms and sequenced. The sequence analysis revealed that the only arginine that is heterogeneous in its methylation is Arg<sup>248</sup>, which contains 0–2 methyl groups (data not shown). Thus, a total of 14–16 methyl groups (8–10 methyl groups in peptides P8, P9, and P10 (Gly<sup>254</sup> through Glu<sup>31</sup>; Fig. 4B) and 6 methyl groups in peptide P11 (Arg<sup>30</sup> through Glu<sup>34</sup>; Fig. 3)) are post-translationally added to the long form of HMW FGF-2.

As another means to verify the primary structure of the intact HMW FGF-2 proteins, a peptide mass map was generated for the three HMW FGF-2 forms digested with Lys-C protease (Fig. 5A). The measured mass of peptide 1, which corresponds to the N terminus of the long form, matches the theoretical mass (8056.9 Da) adjusted by the addition of one acetyl group and fifteen methyl groups. Peptides 2 and 3, which correspond to the N termini of the middle and short forms, respectively, match their theoretical masses (7005.8 and 6525.2 Da) when 10 methyl groups are added to each peptide mass. The measured masses of peptides 4–10, which correspond to the remaining 70% of the protein, confirm the predicted sequence without modifications.

A second mass map was generated from a Glu-C digest of the mixture of HMW FGF-2 forms (Fig. 5B). The mass of peptide 1, which corresponds to the N terminus of the long form, matches the theoretical mass (2599.9 Da) when one acetyl and nine methyl groups are added. The masses of peptides 2 and 3, which correspond to the N termini of the middle and short forms, respectively, match the theoretical masses (7005.8 and 6525.2 Da) when 10 methyl groups are added to each peptide mass. The measured masses of peptides 4–10, which correspond to the remaining 70% of the protein, confirm the predicted sequence without modifications.
The mass of peptide 4, present in all HMW FGF-2 forms, matches the theoretical mass (4219.7 Da) when six methyl groups are added. The masses of peptides 5–11, common to all three forms, confirm the predicted sequence of the remaining protein without modifications. The Lys-C and Glu-C peptide mass maps illustrate that all of the modifications of the primary structure are localized to the N termini of the HMW FGF-2 proteins.

Hmt1 and HRMT1L2 Proteins Methylate HMW FGF-2 in Vitro—The yeast Hmt1p and human HRMT1L2 arginine methyltransferases have been shown to methylate several proteins containing RGG box motifs, including the yeast Npl3p and mammalian hnRNPA1 proteins (30, 33, 34). To determine whether HMW FGF-2 was also a substrate, in vitro methylation reactions were performed utilizing both methyltransferases. As a stringent test for the specificity of each methyltransferase, recombinant GST-HMW FGF-2 fusion protein (24-kDa form) was assayed directly in E. coli expression extracts. Under these conditions both methyltransferases methylated GST-HMW FGF-2 (Fig. 6A). Furthermore, only the FGF derivatives were methylated in this complex protein mixture (Fig. 6A, FGF extract lanes). When extracts prepared from bacterial cells expressing the GST moiety alone were used in the methylation reaction, no labeling was detected (Fig. 6A, GST extract lanes). The presence of thrombin in the methyltransferase preparations (see "Materials and Methods") results in cleavage at the GST-HMW FGF-2 junction sequence to regenerate intact GST and FGF-2 proteins (Fig. 6A, FGF extract lanes). All radiolabeled proteins were confirmed to be FGF-2 derivatives by Western blot analysis using polyclonal FGF-2 antibody (data not shown). Thus, HMW FGF-2 is a substrate for yeast and human arginine methyltransferases.

To extend our analysis, LMW FGF-2 and HMW FGF-2 proteins were tested for their ability to act as substrates for the human methyltransferase HRMT1L2. Recombinant LMW FGF-2, purified in its native form, and HMW FGF-2, purified from E. coli as a GST fusion protein, were utilized for these reactions. HRMT1L2 methylated GST-HMW FGF-2 (Fig. 6B, lane 2). Interestingly, the human enzyme more efficiently radiolabeled FGF-2 released from the fusion protein than the uncleaved fusion protein, indicating that the N-terminal GST moiety may partially block access of the methyltransferase to the methylation sites (located at the N terminus of HMW FGF-2). In contrast, recombinant human LMW FGF-2 was not methylated by HRMT1L2 (Fig. 6B, lane 1). These results indicate that HRMT1L1 methylation occurs within the N-terminal extension of HMW FGF-2.

To provide further evidence that the HMW FGF-2 N-termini...
Arginine Methylation of HMW FGF-2

During extension was the site of HRMT1L2 arginine methylation, we performed competition experiments using synthetic peptides derived from this region. Methylation reactions were performed using the yeast Npl3 substrate in the presence of a variety of synthetic peptides. Peptides F1 and F2 consisted of the N-terminal 16 residues of the short form of HMW FGF-2 and the N-terminal 12 residues of the long form, respectively. The R3 control peptide (18 residues) has previously been shown to inhibit the arginine methylation of several protein substrates and is based on arginine methylated sequences present to inhibit the arginine methylation of HRMT1 and Hmt1 demonstrate that these methyltransferases, which were previously thought to be specific for HRMT1L2 and Hmt1 enzymes. The most striking finding from our analysis is that the N-terminal extension of HMW FGF-2, with the presence of 5–6 methylated Gly-Arg-Gly repeats, closely resembles an RGG box domain, a domain found in many hnRNPs. The amino acid sequence of many hnRNPs contains both an nuclear localization signal and an RGG box. The RGG box is conserved RNA-binding domain found among these proteins that is methylated in vivo (36). The domain consists of 20–25 amino acids usually interrupted by aromatic amino acids (37). The RGG box is implicated in diverse aspects of protein-RNA metabolism (38). For certain hnRNPs, such as the U protein, RNA binding is exclusively due to the RGG box. For others, such as the A1 protein, the RGG box cooperates with two additional RNA-binding domains in binding RNA. The RGG box also mediates protein-protein interactions and may contribute to nuclear entry and/or export (39).

Liu and Dreyfuss (36) demonstrated that most hnRNPs are asymmetrically dimethylated in the RGG box region both in vivo and in vitro. The presence of this modification suggests that methylation modulates the activity of the RGG box and thus affects protein-protein interactions and binding to mRNA. Our results indicating that HMW FGF-2 can serve as a substrate for HRMT1 and Hmt1 demonstrate that these methyltransferases, which were previously thought to be specific for RGG motif RNA-binding proteins, can methylate a nuclear growth factor. Preliminary analysis of the modified arginines ranges from 4 to 6 methyl groups with the heterogeneity residing at Arg \(^{45}\). As shown in Fig. 1B, two methylated arginine residues, Arg \(^{52}\) and Arg \(^{48}\), are not in a Gly-Arg-Gly context but are instead in an Asp-Arg-Gly or Gly-Arg-Ala context, respectively. The presence of a glycine on the carboxyl side of the arginine residue seems to be a stricter requirement for recognition by the arginine methyltransferase than the presence of a glycine on the amino side (35). This requirement may explain why Arg \(^{48}\) but not Arg \(^{52}\) is heterogeneously methylated.

Previous observations indicated that the modification of specific arginines located in the N-terminal extension of HMW FGF-2 may help direct or retain these isoforms in the nucleus, perhaps by stabilizing protein-protein interactions (21). Within the nucleus and/or perinuclear region, HMW FGF-2 may stimulate a set of intracellular signaling pathways distinct from those elicited by interaction with the plasma membrane receptor. Therefore, post-translational methylation may provide a mechanism for regulating the action of different forms of FGF-2.

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Liu and Dreyfuss (36) demonstrated that most hnRNPs are asymmetrically dimethylated in the RGG box region both in vivo and in vitro. The presence of this modification suggests that methylation modulates the activity of the RGG box and thus affects protein-protein interactions and binding to mRNA. Our results indicating that HMW FGF-2 can serve as a substrate for HRMT1 and Hmt1 demonstrate that these methyltransferases, which were previously thought to be specific for RGG motif RNA-binding proteins, can methylate a nuclear growth factor. Preliminary analysis of the modified arginines ranges from 4 to 6 methyl groups with the heterogeneity residing at Arg \(^{45}\). As shown in Fig. 1B, two methylated arginine residues, Arg \(^{52}\) and Arg \(^{48}\), are not in a Gly-Arg-Gly context but are instead in an Asp-Arg-Gly or Gly-Arg-Ala context, respectively. The presence of a glycine on the carboxyl side of the arginine residue seems to be a stricter requirement for recognition by the arginine methyltransferase than the presence of a glycine on the amino side (35). This requirement may explain why Arg \(^{48}\) but not Arg \(^{52}\) is heterogeneously methylated.

Previous observations indicated that the modification of specific arginines located in the N-terminal extension of HMW FGF-2 may help direct or retain these isoforms in the nucleus, perhaps by stabilizing protein-protein interactions (21). Within the nucleus and/or perinuclear region, HMW FGF-2 may stimulate a set of intracellular signaling pathways distinct from those elicited by interaction with the plasma membrane receptor. Therefore, post-translational methylation may provide a mechanism for regulating the action of different forms of FGF-2.
Arginine Methylation of HMW FGF-2

(don't show) indicates that asymmetric dimethylation is the major form of methylation. This finding is consistent with the ability of HRMT1L2 and Hmt1 to methylate HMW FGF-2 in vitro because it is known that these enzymes methylate asymmetrically.

Methylation may also regulate the localization of proteins in the nucleus. The major nucleolar protein, nucleolin, shuttles between the nucleolus and cytoplasm and functions in rRNA transcription, rRNA packaging, and ribosome assembly. Nucleolin uses a bipartite nuclear localization signal to enter the nucleus and uses its glycine/arginine-rich domain (similar to the RGG box but restricted to nucleolar proteins) in cooperation with other RNA-binding domains to accumulate within the nucleolus (40). Our recent studies indicate that HMW FGF-2 also localizes within the nucleolus (29). However, these studies using chimeras of LMW FGF-2 fused with the nucleolar localization signal of SV40 T Ag suggest that the nucleolar localization of HMW FGF-2 is driven by the LMW FGF-2 sequence (29). Therefore, methylation of the N-terminal extension must not be directly involved in the accumulation of the growth factor within the nucleolus but rather may serve to stabilize its interaction with other molecules or to increase its concentration within the nucleus.

By virtue of this RGG box-like domain and by analogy with RGG box-containing proteins, we can speculate on several functions of HMW FGF-2 associated with methylation. Within the nucleolus, HMW FGF-2 may bind RNA and affect mRNA processing and mRNA export mediated by hnRNPs. Another possibility for HMW FGF-2 action is in ribosome assembly and/or transport (41). This intriguing scenario is suggested by the fact that HMW FGF-2 localizes to the nucleolus, nucleoplasm, and within the cytoplasm, HMW FGF-2 binds to ribosomes (42, 43). Although our results indicate that HMW FGF-2 purified from brain (22, 23) or expressed in fibroblasts contains a methylated domain, the potential variation in other cell types is possible and may be important for the function of HMW FGF-2. Further experiments are required to determine whether HMW FGF-2 does have specific nucleolar and/or nuclear activities, and if so what they are.

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