Enzymic methylation of arginyl residues in -Gly-Arg-Gly- peptides

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N^G-Methylation of arginine residues in many nucleic-acid-binding proteins are formed post-translationally, catalysed by *S*-adenosylmethionine:protein-arginine *N*-methyltransferase in their glycine-rich and arginine-rich motifs. The amino acid sequences of the stimulator of HIV-1 TAR (Tat-responsive element) RNA-binding protein (SRB) and fibronectin also show the presence of the internal -Gly-Arg-Gly- (-GRG-) sequence, which is potentially methylatable by the methyltransferase. To investigate the sequence requirement for methylation of these proteins, several synthetic oligopeptides with different chain lengths and sequences similar to the -GRG- regions of SRB and fibronectin were synthesized. Whereas the heptapeptide AGGRGKG (residues 16–22 in SRB) served as the methyl acceptor for the methyltransferase with a K_m of 50 μM, the 19-mer peptide (residues 10–28 in SRB) was methylated with a K_m of 8.3 μM, indicating that a greater peptide chain length yields a better methyl acceptor. Product analysis of the methylated [*methyl*-^{14}C]SRB-peptide by HPLC indicated the formation of N^G-monomethylarginine and N^G,N^G-dimethyl(asymmetric)-arginine. Synthetic peptides containing the cell attachment sequence [Arg-Gly-Asp ('RGD')] in fibronectin, GRGDSPK, GGRGDSPK and GGGRGDSPK, were also studied; whereas GRGDSPK was a poor methyl acceptor, the longer peptides were better methyl acceptors. To provide an understanding of the effect of methylation on fibronectin peptide, arginine-unmethylated and methylated GG**R**GDSPK were compared for their effect on the mitogenesis induced by β-hexosaminidase A and an agonistic antibody (mAb_{15}) in bovine tracheal smooth-muscle cells; whereas the former inhibited 35–67 % of mitogenesis at a concentration of 5–10 μM, the latter did not block mitogenesis. This lack of inhibition by the insertion of a methyl group on the arginyl residue of the cell attachment sequence might be due to the hindrance of the binding of fibronectin peptide to integrins.

Key words: *S*-adenosylmethionine, fibronectin-peptide, N^G-methylarginine, protein methylase I, RNA-binding protein.

INTRODUCTION

The methylation of protein-bound amino acid side chains is one of several post-translational modifications catalysed by highly specific methyltransferases [1,2]. Among these, protein methylase I (*S*-adenosylmethionine:protein arginine N-methyltransferase, EC 2.1.1.23) [also referred to as protein methylase I (PMI), and protein-arginine methyltransferase (PRMT1, RMT1 and HMT1)] catalyses transfer of the methyl group from *S*-adenosyl-L-methionine (AdoMet) to the guanidino nitrogen of specific arginine residues in polypeptide substrates, yielding N^G-mono-methylarginine, N^G,N^G-dimethyl(asymmetric)arginine and N^G,N'^G-dimethyl(symmetric)arginine with the release of *S*-adenosyl-L-homocysteine, the demethylated AdoMet [1,3].

Two subclasses of protein methylase I have been identified and purified to near homogeneity from calf brain and rat liver: myelin-basic-protein-specific methylase I and nuclear protein/histone-specific protein methylase I, both exhibiting distinct substrate specificities towards the respective methyl acceptor protein [4,5]. Rajpurohit et al. [5] have demonstrated that the nuclear protein/histone-specific protein methylase I methylates most efficiently arginine residues within the Arg-Gly-Gly (RGG) motif of the recombinant heterogeneous ribonucleoprotein particle (hnRNP) A1 protein. Earlier, the presence of N^G,N^G-dimethyl(asymmetric)arginine at residue 193 of helix-unwinding protein 1 ('UP1', the N-terminal proteolytic fragment of hnRNP A1 protein) [6] was reported. More recently, three additional N^G,N^G-dimethyl(asymmetric)arginine residues in the RGG motif (residues 205, 217 and 224) of hnRNP protein A1 isolated from HeLa nuclei have also been identified [7]. Thus hnRNP A1 protein is one of the most highly arginine-dimethylated proteins in nature.

The RGG motif occurs in several nucleic-acid-binding proteins and is known to be a conserved domain consisting of clusters of glycine and N^G,N^G-dimethyl(asymmetric)arginine residues interspersed with phenylalanine, often spanning over 10–20 chain lengths [8–10]. Compilation of several nucleic-acid-binding proteins, such as nucleolin and fibrillarin from different species, indicated a common sequence alignment within the methylatable site of the RGG motif [7]. In addition, further examination of the sequences around the N^G-methylated arginines indicated -G**R**G- as the preferred sequence for methylation, although the $n-1$ position of the Arg residue could be replaced by a few selected amino acids other than Gly [7]. This feature is in good agreement with the previous studies performed with several synthetic oligopeptides as the methyl acceptor for protein methylase I [11,12]. In an effort to extend these studies, we examined the primary sequences of several proteins and found the stimulator of HIV-1 Tat-responsive element (TAR) RNA-binding protein (SRB) [13] and fibronectin [14] to contain the -GRG- sequence.

The SRB protein is one of the three transactivators to regulate HIV-1 gene expression, consisting of 539 residues (accession number U38846) and containing a -G**R**G- sequence at residue 18–20 in the N-terminus [13]. One-quarter of the N-terminal amino acid sequence showed a high degree of similarity to a member of the chaperonin family of proteins as well as the P-loop

Abbreviations used: AdoMet, *S*-adenosyl-L-methionine; Hex, hexosaminidase; hnRNP, heterogeneous nuclear ribonucleoprotein particle; SRB, stimulator of HIV-1 TAR RNA-binding protein; TAR, Tat-responsive element.

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motif, a glycine-rich region capable of binding to ATP or GTP [13]. Fibronectin is one of the integrin families mediating cell adhesion in communicating with a receptor at the cell surface [15]; the attachment sequence is known to be the common tripeptide Arg-Gly-Asp (RGD) [14,16]. Therefore the fibronectin peptides containing RGD have often been used in many experimental systems [14,17,18].

In the present study we synthesized several oligopeptides that were identical or similar to the G**R**G-containing region of SRB and fibronectin peptides, and used them as substrates *in vitro* for the purified rat liver protein methylase I. In addition, the chemically synthesized fibronectin peptide containing the N^G-methylated and unmethylated arginine residues were compared for their effect on cell-surface receptor-mediated DNA synthesis and cell proliferation in tracheal smooth-muscle cells.

EXPERIMENTAL

Materials

Ado[*methyl*-^{14}C]Met (specific radioactivity 46.8–53.0 mCi/mmol), Ado[*methyl*-^{3}H]Met (75.0 Ci/mmol) and [^{3}H]thymidine (5 Ci/mmol) were purchased from Amersham Life Science. N^G,N^G-Dimethylarginine, Sephadex G-10 and histone (type II-AS) were from Sigma (St Louis, MO, U.S.A.). Fetal bovine serum was purchased from Hyclone Lab (Logan, UT, U.S.A.). The synthetic oligopeptides containing the -GRG- sequence with variable chain lengths (Table 1) were synthesized at the W. M. Keck Foundation Biotechnology Resource Laboratory of Yale University (New Haven, CT, U.S.A.). All other chemicals were of the reagent grade available from various commercial sources.

Purification of protein methylase I

The purification of protein methylase I from rat liver was performed essentially by the same method as described previously [19]. In brief, rat liver was homogenized in 4 vol. of 5 mM sodium phosphate, pH 7.4, containing 5 mM EDTA and 0.25 M sucrose, and the homogenate was centrifuged at 105000 ***g*** for 60 min. The supernatant was mixed with an equal volume of 10 mM Mops (pH 7.4)/2 mM EDTA/0.5 mM PMSF/10 mM 2-mercaptoethanol and chromatographed on DE-52 followed by Sephadex G-200. The purified enzyme transferred 28.4 pmol of methyl groups/min per mg of protein, with histone as the methyl acceptor substrate under the assay conditions.

Assay for protein methylase I

The enzymic activity for protein methylase I was determined as described previously [4,19]. A total 0.125 ml reaction mixture containing 0.1 M potassium phosphate buffer, pH 7.6, 0.5 mg of histone (type II-AS) and 0.04 mM Ado[*methyl*-^{3}H]Met (500 d.p.m./pmol) was incubated in the presence of rat liver methylase preparation (approx. 100 μg) at 37 °C for 30 min, unless otherwise specified. The reaction was terminated by the addition of 15 % (w/v) trichloroacetic acid and the resulting precipitates were repeatedly washed as described [4].

Protein concentration was estimated by the Coomassie Blue method of Bradford [20], as modified by Pierce Chemical Co., with BSA as the standard.

Table 1 List of synthetic oligopeptides used for methylation reactions

Single-letter amino acid abbreviations are used. The underlined bold letter **R** is the potential methyl-acceptor residue. Abbreviation: FN, fibronectin.

Peptide	Sequence	Comments	Reference
SRB P-1	HN_2-AGG**R**GKG-CO_2H	Residues 16–22 in SRB	[13]
SRB P-2	Acetyl-AGG**R**GKG-amide	Residues 16–22 in SRB (N-terminus and C-terminus blocked)	
SRB P-3	HN_2-GATAGAAGG**R**GKGA YQDRD-CO_2H	Residues 10–28 in SRB	[13]
FN P-1	NH_2-G**R**GDSPK-CO_2H	Cell attachment sequence	[16]
FN P-2	NH_2-GG**R**GDSPK-CO_2H		
FN P-2	NH_2-GGG**R**GDSPK-CO_2H		

Enzymic methylation of synthetic oligopeptides

Because the synthetic oligopeptides employed in the present study were not precipitable by trichloroacetic acid, the gel-filtration method described previously was employed [11]. Different amounts of each peptide substrate were incubated in a total 0.125 ml reaction mixture as described above, but with 0.04 mM Ado[*methyl*-^{14}C]Met (103–117 d.p.m./pmol, as indicated in the respective tables) in the presence of various concentrations of the rat liver methylase I (47–174 μg) at 37 °C for 12–60 min as indicated. The reaction was terminated by adjusting the pH of the mixture to 4.0 with 0.2 M sodium citrate and heated at 100 °C for 30 min. This treatment cleaves unreacted Ado[*methyl*-^{14}C]Met, resulting in 5′-[*methyl*-^{14}C]methylthioadenosine, thus enabling the [*methyl*-^{14}C]-peptide to be separated easily from the unreacted Ado[*methyl*-^{14}C]Met by subsequent molecular sieve chromatography. The total reaction mixture was applied to a column of Sephadex G-10 (0.5 cm × 110 cm) that had been pre-equilibrated with 0.01 M HCl; it was then eluted with the same solvent, collecting fractions of 0.5 ml. The aliquots of each of the fractions were counted for the radioactivity incorporated, and other aliquots were used for ninhydrin reaction to assess the peptide elution profile. A typical chromatographic profile is shown in Figure 1 below. To obtain K_m values for SRB peptides, the methylation reactions were performed with variable concentrations of peptide at a fixed concentration of 0.04 mM Ado[*methyl*-^{14}C]Met.

HPLC analysis of methylated amino acids

The *methyl*-^{14}C-labelled oligopeptide, purified by Sephadex G-10 chromatography, was filtered through a Centricon-10 (Amicon Co.) to remove the enzyme protein from the *methyl*-^{14}C-labelled peptide. The filtrate was subjected to hydrolysis in 6 M HCl at 110 °C for 24 h. The hydrolysate was washed with distilled water repeatedly to remove HCl completely; 10 μl of redrying agent [triethylamine/ethanol/water (2:2:1, by vol.)] was added to the hydrolysate. After drying under vacuum, 50 μl of derivatizing agent [ethanol/triethylamine/water/phenylisothiocyanate (7:1:1:1, by vol.)] were added to the mixture and left to react for 20 min at room temperature. The mixture was again dried completely and dissolved in 100 μl of solvent A [2.5 % (v/v) acetonitrile in 70 mM sodium phosphate buffer, pH 6.5]. The derivatized sample was then injected onto a C_{18} reverse-phase column (Shodex C_{18}, 5 μm spherical particles, 4.6 mm internal diam. × 25 cm) equilibrated in solvent A at 43 °C, and eluted at a flow rate of 1.2 ml/min for 10 min and then with solvent B [15 % (v/v) methanol in 45 % (v/v) acetonitrile] with the use of the following gradient (v/v): 0–10 min, 0 % B; 10–40 min, 5 % B; 40–55 min, 5 % B; 55–65 min, 5–15 % B; 65–69 min, 15–30 % B. The eluate was monitored at 254 nm and 1 min fractions were collected.

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Airway smooth-muscle cell culture

Primary cultures of bovine tracheal smooth-muscle cells were prepared from bovine trachealis muscle, as described previously [21]. Direct immunofluorescence staining of the cells with an FITC-conjugated monoclonal antibody against smooth-muscle isoactin (Sigma) demonstrated that cultures consisted of a homogeneous population of smooth-muscle cells [22]. Cells at passage 1–7 were used for experiments.

Assesment of proliferation

DNA synthesis rates were assessed by measuring [^{3}H]thymidine incorporation as follows: cells were seeded in microtitre wells (25000 cells per well) and allowed to grow to confluence. The medium was replaced with medium M199 (Cellgro; Mediatech, Washington, DC, U.S.A.) containing 0.4% (v/v) fetal bovine serum. After a 48 h starvation, the inhibitors to be tested were added 30 min before stimulation of the cells. Without a change of medium, purified hexosaminidase A (Hex A) or vehicle (PBS) was added [21]. In some experiments, an agonistic antibody (mAb$_{15}$; [23]) directed to the extracellular domain of human macrophage mannose receptors was used to stimulate cells. [^{3}H]Thymidine (0.5 μCi per well) was added to the cells 20 h after the stimulation; cells were harvested 18 h later. Radioactivity was quantified in a liquid-scintillation counter. Experiments were performed in quadruplicate and were repeated three times.

Parallel experiments were set up to assess cell counts and cytotoxicity by tetrazolium salt reduction with the use of a 3-[4,5-dimethylthiozol-2-yl]-2,5-diphenyl tetrazolium bromide (5 mg/ml; Millipore-filtered) (MTT) colorimetric assay, as described previously [21]. MTT dissolved in PBS was added to the adherent cells in microtitre wells (20 μl per well) at the end of the experimental period. After incubation for 4 h at 37 °C, medium was removed from the microtitre wells and acidified with 0.04 M HCl; 2-propane/propan-2-ol was then added to wells (200 μl per well). The plates were left at room temperature for 30 min and then read on an ELISA reader (EL340 Microplate; Bio-Tek Instruments, Winooski, VT, U.S.A.) at 570 nm. Cell numbers were derived from a standard curve generated from known cell numbers.

RESULTS

Enzymic methylation of oligopeptides derived from SRB

Previous studies had indicated that the arginine residue in -G**R**G- in polypeptide sequences was the most methylatable by protein methylase I; SRB was found to contain this tripeptide sequence (residues 18–20). Therefore, to investigate the methylation of SRB peptides, SRB P-1 (7-mer), SRB P-2 (7-mer with blocked N-terminus and C-terminus) and SRB P-3 (19-mer) were synthesized chemically. All three peptides served as methyl acceptors for the rat liver cytosolic protein methylase I (Table 2). However, the 19-mer was the best methyl acceptor, even though only less than one-third the amount of the 7-mer peptides was used for methylation. To examine whether free amino and carboxy groups in the peptide exerted any effect on methyl acceptability, the N-terminus and C-terminus were blocked with an acetyl and an amide group respectively; the blocked 7-mer (SRB P-2) was 14% less effective as a methyl acceptor than the unblocked (SRB P-1).

The K_m values for 7-mer and 19-mer peptides were then compared (Table 3). SRB P-3 (the 19-mer) showed a much lower K_m value than the 7-mer peptides (8.3 μM compared with 50.0 and 52.6 μM), indicating clearly that the longer peptide had a

Table 2 Methyl-acceptability of peptides derived from the SRB protein by rat liver protein methylase I

The methylation reaction was performed in a total reaction mixture of 0.125 ml containing 0.125 M potassium phosphate, pH 7.6, 0.04 mM Ado[*methyl*-^{14}C]-Met (103 d.p.m./pmol), 174 μg of protein methylase I and the indicated amounts of substrate at 37 °C for 12 min. The mixture was then adjusted to pH 4.0 and boiled for 30 min to terminate the reaction; methyl incorporation into the peptides was estimated with a Sephadex G-10 column as described in the Experimental section. *The methyl incorporations into hnRNP protein A1 and histone II-AS were quantified by the trichloroacetic acid precipitation method.

Substrate	Molecular mass (Da)	Amount used (nmol)	Incorporation of *methyl*-^{14}C	
			(d.p.m.)	(mol%)
SRB P-1	602	229	5060	0.021
SRB P-2	643	298	4410	0.014
SRB P-3	1820	68.0	5800	0.083
hnRNP protein A1*	34000	1.5	5340	3.453
Histone II-AS*	12000–14000	23.8	2980	0.121

Table 3 Kinetic constants for SRB peptides by rat liver protein methylase I

The experimental conditions were the same as those described in Table 2, except that variable concentrations of substrate peptides were used in the presence of a fixed amount of 0.04 mM Ado[*methyl*-^{14}C]Met.

Substrate	K_m (μM)	V_{max} (pmol of [*methyl*-^{14}C]/min per mg)
SRB P-1	50.0	3.03
SRB P-2	52.6	2.63
SRB P-3	8.3	3.39
hnRNP protein A1	0.5*; 0.19†	151†
Histone II-AS	21.0†	25.5†

* From [19].
† From [5].

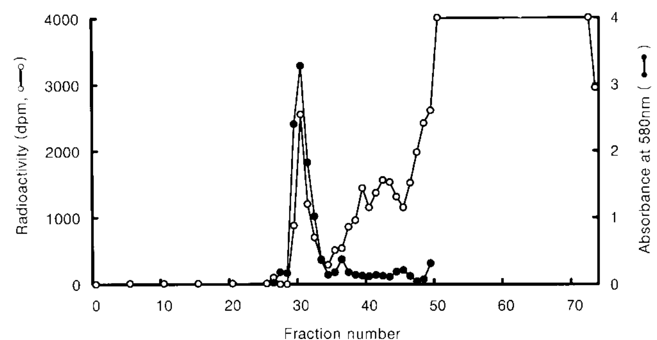

Figure 1 Sephadex G-10 chromatography of *methyl*-^{14}C-labelled SRB-P3

SRB-P3 (39.6 μg) was methylated in a standard reaction mixture with Ado[*methyl*-^{14}C]Met in the presence of protein methylase I for 60 min. After incubation, the mixture was adjusted to pH 4.0 and heated at 100 °C for 30 min to destroy unreacted AdoMet. The mixture was then chromatographed on a Sephadex G-10 column (0.5 cm × 110 cm) and 0.5 ml fractions were collected. Aliquots (0.05 ml) from each fraction were counted for radioactivity; other aliquots were analysed by ninhydrin reaction to obtain a peptide elution profile. The sum of the total radioactivities eluted in fractions 30–33 was taken as the total enzyme activity. Symbols: ●, ninhydrin at 580 nm; ○, radioactivity incorporated.

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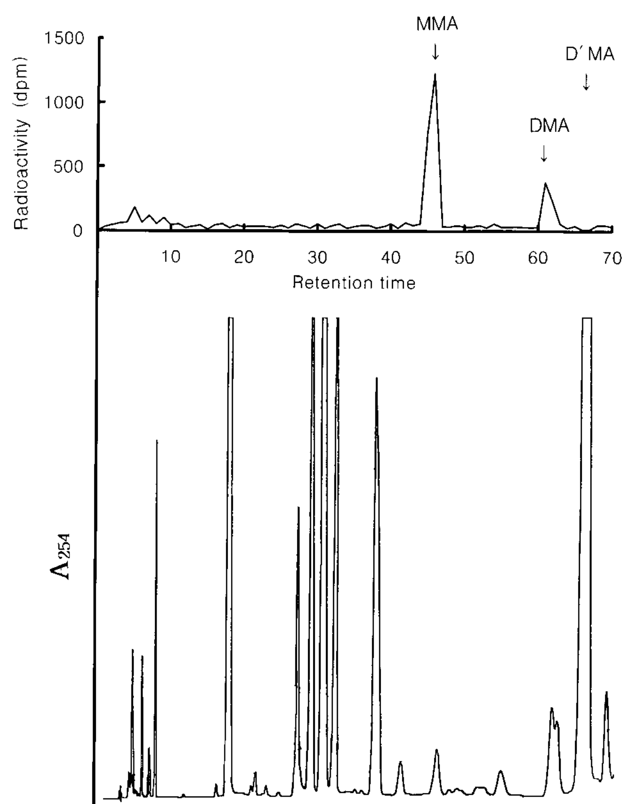

Figure 2 HPLC analysis of [*methyl*-^{14}C]SRB P-2

Details of the methylation and derivatization reactions are described in the Experimental section. The *methyl*-^{14}C-labelled SRB P-2 was hydrolysed in 6 M HCl at 110 °C for 24 h and the hydrolysate was prepared for HPLC analysis. The sample (37 nmol of [*methyl*-^{14}C]SRB P-2) was injected on a C_{18} reverse-phase column and analysed for methylated amino acids as described in the Experimental section. Abbreviations: MMA, N^G-monomethylarginine; DMA, N^G,N^G-dimethyl(asymmetric)arginine; D′MA, N^G,N'^G-dimethyl(symmetric)arginine.

higher affinity for the enzyme. It has also been previously reported that the K_m of hnRNP A1 protein was 0.5 μM; this high affinity was most probably due to the protein chain length as well as the presence of four methylatable arginine residues in A1 protein.

Identification of enzymically methylated [*methyl*-^{14}C]SRB P-2

To identify the enzymic product, the *methyl*-^{14}C-labelled SRB P-2 purified on a Sephadex G-10 column (Figure 1) was concentrated and subjected to hydrolysis with HCl. The hydrolysate was then analysed by HPLC for *methyl*-^{14}C-labelled amino acids. As shown in Figure 2, two radioactive peaks, N^G-monomethylarginine (78.6 %) and N^G,N^G-dimethylarginine (21.4 %), migrated together with respective authentic arginine derivatives, with retention times of 46 and 66 min, respectively, indicating that the enzymic products were indeed due to the protein arginine N-methyltransferase. No other methylated basic amino acids were seen.

Enzymic methylation of fibronectin peptide and its analogues

Several synthetic oligopeptides similar to the sequence of the cell attachment domain in fibronectin were examined as substrates for protein methylase I. As shown in Table 4, the synthetic heptapeptide Gly-Arg-Gly-Asp-Ser-Pro-Lys (FN P-1) showed negligible substrate capability. However, when the N-terminal glycine residue of FN P-1 was extended with extra glycine residues, the methyl acceptability increased in proportion to the number of residues added, indicating that the number of glycine residues at the N-terminus of the GRG motif was an important factor in increasing the methyl acceptability of the fibronectin peptide. However, because of very low methyl incorporation in these peptides, K_m values were not determined.

Table 4 Methyl-acceptability of fibronectin peptides by rat liver protein methylase I

The experimental conditions were the same as those described in Table 2, except that incubation was performed for 60 min at 37 °C, with 47 μg of protein methylase I. The specific radioactivity of the Ado[*methyl*-^{14}C]Met was 117 d.p.m./pmol. For histone II-AS the trichloroacetic acid precipitation method was used.

Substrate	Molecular mass (Da)	Amount used (nmol)	Incorporation of *methyl*-^{14}C (d.p.m.)
FN P-1	717	1390	174
FN P-2	774	1290	1750
FN P-3	831	1200	2970
Histone II-AS	12000–14000	76.9	9340

Table 5 Effect of arginine-methylated and unmethylated fibronectin peptides on mitogenesis induced by β-Hex A and mannose receptor agonist antibody in bovine tracheal smooth-muscle cells

Quiescent cells [starved in medium containing 0.4% (v/v) FBS] in microtitre wells were pretreated with one of the peptides [GGRGDSPK or GGR(CH_3)GDSPK, in which R(CH_3) indicates N^G,N^G-dimethyl(asymmetric)arginine] at a concentration of 5–10 μM dissolved in medium M199 for 30 min before the addition to the cells of Hex A (50 nM), vehicle for Hex A (PBS), mAb_{15} (25 nM) or non-immune mouse IgG_1. Parallel experiments were performed as described above. Viable cell numbers were obtained by MTT assay at the end of a 48 h experimental period as described in the Experimental section. Results are means ± S.E.M. ($n = 3$: three separate experiments of quadruplicate cultures from different cell populations each from a different animal). Single-letter amino acid abbreviations are used.

Agonist	Fibronectin peptide	Concentration (μM)	Inhibition (%)	
			DNA synthesis	Cell number
Hex A	None	0	0	0
	GGRGDSPK	5	39 ± 2	35 ± 5
	GGRGDSPK	10	62 ± 7	51 ± 5
	GGR(CH_3)GDSPK	5	0	0
	GGR(CH_3)GDSPK	10	0	0
mAb_{15}	None			0
	GGRGDSPK	5	36 ± 5	36 ± 6
	GGRGDSPK	10	67 ± 8	48 ± 3
	GGR(CH_3)GDSPK	5	0	0
	GGR(CH_3)GDSPK	10	0	0

Effect of arginine methylation of the fibronectin peptides on their β-Hex-induced DNA synthesis

It has been known that lysosomal hydrolases, such as human Hex A and the agonist antibody against the macrophage mannose receptor, induce DNA synthesis and cell proliferation in the mannose-receptor-mediated mitogenesis of bovine tracheal smooth-muscle cells [22,23]. To investigate the effect of methylation of the arginine residue in the cell attachment peptide, two analogues of fibronectin peptide (FN P-2; GRGDSPK) were synthesized chemically, one with arginine and the other with N^G,N^G-dimethyl(asymmetric)arginine. As shown in Table 5, the

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fibronectin peptide (FN P-2) containing an arginine residue inhibited the synthesis of DNA induced by both the ligand (Hex A) and an agonistic antibody (mAb_{15}), as well as cell division, in a concentration-dependent manner (35–67% inhibition, 5–10 μM). In contrast, FN P-2, containing dimethylarginine, inhibited neither of the agonist-induced mitogeneses.

DISCUSSION

N^G-Methylated arginine residues in protein are formed post-translationally in polypeptides only when the amino acid sequence and chain length are specifically recognized by protein arginine N-methyltransferase [1,2]. Previous studies indicated that the -GRG- motif within the methyl acceptor oligopeptides was the most favourable local sequence for methylation by the methyltransferase [7,11,12]. In extending the above observations in the present study, we chemically synthesized several oligopeptides with overlapping local sequences of SRB and fibronectin for methylation *in vitro*, because these proteins contain a -GRG- sequence and are biologically important.

SRB protein is an RNA-binding protein that acts as one of the cofactors in the regulation of HIV-1 gene expression. The regulation of the HIV-1 gene is dependent on a number of *cis*-acting regulatory elements in the long terminal repeat [24,25]; a double-stranded RNA structure transcribed from the HIV-1 long terminal repeat, known as TAR, is critical for increasing gene expression in response to the Tat transactivator protein [26]. Two cellular factors, RNA polymerase II and TRP-185, bind specifically to TAR RNA in the presence of cellular factors [13]. Three cellular proteins have been identified in the cofactor fraction: elongation factor 1-α, the polypyrimidine-tract-binding proteins and SRB. SRB has a high degree of similarity to a variety of cellular proteins known as chaperonins [13]. The primary structure of SRB contains a GRG motif at the N-terminus (residues 18–20). As shown in Table 2, comparing the methyl acceptability of the synthetic 7-mer (SRB P-1; residues 16–22) and 19-mer (SRB P-3; residues 10–28), it is clear that the longer peptide showed a much better substrate activity, with a lower K_m value, than the 7-mer peptide. These findings were predictable because our previous study had indicated that a hexapeptide was the minimum chain length for methylation; a tetrapeptide was completely inert as a substrate for myelin-basic-protein-specific protein methylase I (EC 2.1.1.126) [11]. When the N-terminus and the C-terminus of the 7-mer (SRB P-2) were blocked, an approx. 15% decrease in methyl acceptability was observed in comparison with the unblocked 7-mer (SRB P-1), indicating that the charge status of the N-terminus and the C-terminus of peptide did not greatly affect the methylation; rather, the chain length of the substrate was significant. It would constitute a great challenge to study whether the whole molecule of SRB protein has methyl-accepting activity and what effect methylation has on RNA-binding activity. Unfortunately, however, unmethylated SRB protein is not available at present. Nevertheless, when the arginine residue in the RGG motif was N^G-methylated (1.4 mol%), the binding activity of hnRNP A1 protein towards RNA was depressed [27]. Because the methylation would be expected to block hydrogen-bonding interactions between the phosphoryl group of RNA and the N^G-guanidino nitrogen that allows a single essential arginine residue in Tat protein to recognize the TAR RNA target [28], methylation might be expected to have a far-reaching effect on the regulation of HIV gene expression.

In general, enzymic post-synthetic modification of protein *in vitro* is not a stoichiometric reaction and often yields a fractional modification, thereby resulting in microheterogeneity on gel electrophoresis of the reaction product. Fractional modification was more obvious when peptide substrates were employed. To overcome this drawback, we chemically synthesized an analogue of the 8-mer cell-attachment peptide (GGRGDSPK; FN P-2) containing N^G,N^G-dimethyl-(asymmetric)-L-arginine in the place of L-arginine and studied its effect on the tracheal smooth-muscle cells in culture. As shown in Table 5, the arginine-unmethylated fibronectin peptide (FN P-2) inhibited an agonistic antibody of DNA synthesis induced by mannose receptor and Hex, as well as cell proliferation, in proportion to the concentration of peptide, whereas the methylated peptide did not block under the identical conditions. On activation of the receptors for Hex A in tracheal smooth-muscle cells, $\alpha_5\beta_1$ integrins rapidly associate directly with the receptor [29]. The present results further support the notion that the fibronectin peptide inhibits mannose-receptor-mediated signalling pathways and mitogenesis indirectly by binding the integrins. This in turn might inhibit the interaction between integrins and the mannose receptors in tracheal smooth-muscle cells.

Fibronectin interacts with extracellular matrix macromolecules and with receptors at the surface of most eukaryotic cells [15]; the interaction of fibronectin with cells results in the attachment and spreading of those cells on a surface covered with fibronectin. It has been known that the RGD sequence is required for the recognition of fibronectin in cells [14]. These sequences have also been identified in cell recognition sites of vitronectin, fibrinogen, von Willebrand factor, type I collagen and osteopontin, each of which can bind to one or more integrins [30,31]. The results presented here, that N^G-methylated arginine in fibronectin peptide abolished the inhibitory effect of fibronectin peptide on mannose-receptor-mediated reactions, suggest that the post-translational methylation of arginine in fibronectin-like proteins is involved in the regulation of the interactions of the RGD sequence with cells.

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