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A protein-arginine methyltransferase binds to the intracytoplasmic domain of the IFNAR1 chain in the type I interferon receptor

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The intracytoplasmic domain (IC) of cytokine receptors provides docking sites for proteins which mediate signal transduction. Thus, in interferon-α,β receptors (IFNAR1 and 2), the IC region binds protein-tyrosine and -serine/threonine kinases which phosphorylate the receptor and the associated Stat transcription factors. A two-hybrid screening was carried out to identify additional proteins which could interact with the IC domain of the IFNAR1 chain of the IFN-α,β receptor. Several positive clones representing a protein sequence designated IR1B4 were recovered from a human cDNA library. IR1B4 was identified as the human homolog of PRMT1, a protein-arginine methyltransferase from rat cells. Flag-IR1B4 fusion proteins bind to the isolated IFNAR1 intracytoplasmic domain produced in *Escherichia coli*, as well as to the intact IFNAR1 chain extracted by detergent from human U266 cell membranes. *S*-Adenosylmethionine-dependent methyltransferase activity was precipitated by anti-IFNAR1 antibodies from untreated human cells. IR1B4/PRMT1 is involved in IFN action since U266 cells rendered deficient in this methyltransferase by antisense oligonucleotides become more resistant to growth inhibition by IFN. Methylation of proteins by enzymes which can attach to the IC domains of receptors may be a signaling mechanism complementing protein phosphorylation. Among substrates methylated by PRMT1 are RNA-binding heterogeneous nuclear ribonucleoproteins (hnRNPs) which are involved in mRNA processing, splicing and transport into the cytoplasm.

Keywords: interferon/methyltransferase/protein methylation/receptor/signaling

Introduction

Type I interferons (IFN-α and -β subtypes) produce pleiotropic effects on cells, such as inhibition of virus replication (antiviral effects), inhibition of cell proliferation (antitumoral effects) and modulation of immune cell activities (immunoregulatory effects). These multiple effects of IFNs are correlated with morphological and biochemical modifications of cells (for reviews, see Revel, 1984; Tamm *et al.*, 1987). Several cellular genes are induced or down-regulated by IFNs, their mRNA levels being determined through both transcriptional and post-transcriptional controls (Friedman *et al.*, 1984; Revel and Chebath, 1986).

Interferons exert their activities through species-specific receptors. For type I IFNs, two transmembrane receptor chains have been identified: IFNAR1 (Uze *et al.*, 1990) and IFNAR2-2 (or IFNAR2-c, Domanski *et al.*, 1995; Lutfalla *et al.*, 1995), which is a long form of IFN-α/βR (Novick *et al.*, 1994). Transduction of the signal generated by IFN-α,β,ω involves protein tyrosine kinases of the Janus kinases (Jak) family and transcription factors of the Stat family (Velazquez *et al.*, 1992; Darnell *et al.*, 1994). Proteins of the Jak–Stat pathways have been shown to bind to the intracytoplasmic (IC) domains of the IFNAR1 and 2 receptor chains. Jak1 is constitutively associated with IFNAR2 (Novick *et al.*, 1994), whereas tyk2 is bound to the IC domain of IFNAR1 (Abramovich *et al.*, 1994b; Colaminici *et al.*, 1994). IFNAR1 can also bind Stat2 (Abramovich *et al.*, 1994b), and the docking site for latent Stat2 was identified as a peptide containing phosphotyrosine Y466, adjacent to the tyk2 binding site (amino acids 479–511) in the 100 amino acid long IFNAR1-IC region (Yan *et al.*, 1996). Stat2 would then recruit Stat1 to form the IFN-induced ISGF3 transcription complex (Leung *et al.*, 1995). Activation of Stat3-containing transcription complexes is induced by IFN-β (Harroch *et al.*, 1994a,b), and an IFN-dependent binding of Stat3 to IFNAR1-IC was observed (Yang *et al.*, 1996). Protein-tyrosine phosphatases PTP1C and D reversibly associate with IFNAR1 upon IFN addition (David *et al.*, 1995a). In addition, two serine/threonine protein kinases, the 48 kDa ERK2 MAP kinase (David *et al.*, 1995b) and the cAMP-activated protein kinase A (PKA; David *et al.*, 1996) bind to the isolated membrane-proximal 50 residues of IFNAR1-IC. Therefore, the IC domains of type I IFN receptors serve as docking sites for multiple proteins involved in phosphorylation and dephosphorylation. Both IFNAR1 and IFNAR2 become tyrosine phosphorylated in response to IFN treatment, and binding of a tyrosine-phosphorylated protein (βPTyr) to IFNAR1 is seen specifically upon IFN-β (but not IFN-α) treatment (Abramovich *et al.*, 1994b; Platanias *et al.*, 1994; Constantinescu *et al.*, 1995). Many of the docking proteins (Jak1, tyk2, Stat2, Stat3 and PTP1C) bind to the phosphotyrosines through their SH2 domains, but the use of a recombinant glutathione-*S* transferase (GST)-fused IFNAR1-IC segment showed that some of the proteins (tyk2, ERK2 and PKA) have intrinsic affinities for polypeptide sequences of the receptor (David *et al.*, 1995b, 1996).

The two-hybrid screening system is a potent method for identifying proteins which bind to defined polypeptide sequences (Fields and Song, 1989). By a two-hybrid screening with the entire IC domain of the IFNAR1 receptor, we have identified two new human proteins

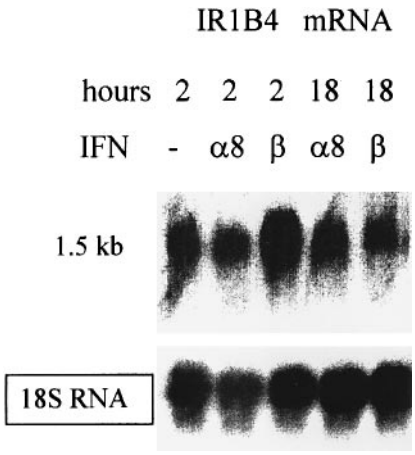

Fig. 1. Human mRNA hybridizing to IR1B4 plasmid. Human U266S cells were treated for the indicated time with IFN or left untreated. RNA was analyzed in formaldehyde–agarose gels blotted on GeneScreen (DuPont-New England Nuclear). The 28S and 18S RNA were used as size markers (not shown). The lower lanes represent subsequent hybridization with 18S cDNA. On the same blot, IR1B4 RNA runs just below the 18S RNA.

Number	pACT	pAS or pGBT10	Number	pACT	pAS or pGBT10
1	IR1B4	vector	5	IR1B4	tat
2	IR1B4	p53	6	IR1B4	rev
3	IR1B4	IFNAR1-IC	7	-	lacZ control -
4	IR1B4	cdk	8	IR1B4	lamin

Fig. 2. Two-hybrid interaction analysis. The yeast SFY526 Gal1–lacZ reporter strain was co-transformed with one of the indicated plasmids with Gal4 activation domain fusion protein (pACT) and one of the indicated plasmids with Gal4 DNA binding domain fusion protein (pAS or PGBT10). Colonies were selected in SC medium minus trp and leu, and filters were stained by X-gal reagent for β-galactosidase activity.

showing specific binding. A search through computer databases revealed that one of the proteins, designated IR1B4 (interferon receptor-1-bound protein 4) has a conserved homology domain found in methyltransferases and is highly homologous to the recently cloned rat protein-arginine methyltransferase PRMT1 (Lin *et al.*, 1996). This enzyme methylates proteins, in particular heterogeneous nuclear ribonucleoprotein (hnRNP) involved in RNA processing. Protein methylation, like phosphorylation, may be an important signaling mechanism for certain cytokine receptors.

Results

Two-hybrid screen for protein binding to the IFNAR1 intracytoplasmatic domain

A cDNA fragment encoding the entire IFNAR1-IC domain (Lys458–Val557 of Uze *et al.*, 1990) was amplified by PCR and introduced in the pGBT10 vector for the two-hybrid screen (CloneTech) so that it is fused in-frame to the Gal4 DNA binding domain. This plasmid was used as bait for a library of human cDNAs fused to the Gal4 activation domain of the pACT plasmid for dual-selection two-hybrid screening (Durfee *et al.*, 1993). Nine yeast clones which expressed Gal1UAS-dependent β-galactosidase and His3 activities in a specific manner (i.e. not alone and not when a Gal4 DNA binding domain fused to lamin was used as bait) were studied further. From each yeast, pACT plasmids were recovered in two independent *Escherichia coli* transformants and were found by partial sequencing to fall into two groups. One of the groups, designated IR1B4, is described here. IR1B4 cDNA hybridized to a 1.5 kb poly(A)$^+$ RNA constitutively expressed in various human cells including U266 myeloma cells (Figure 1). The pACT-IR1B4 plasmids were verified to be negative when subjected to a two-hybrid test with various other baits including lamin, p53, cdk2, tat and rev, as well as in other control combinations (Figure 2). A positive signal was obtained only when the IFNAR1-IC domain was used as bait.

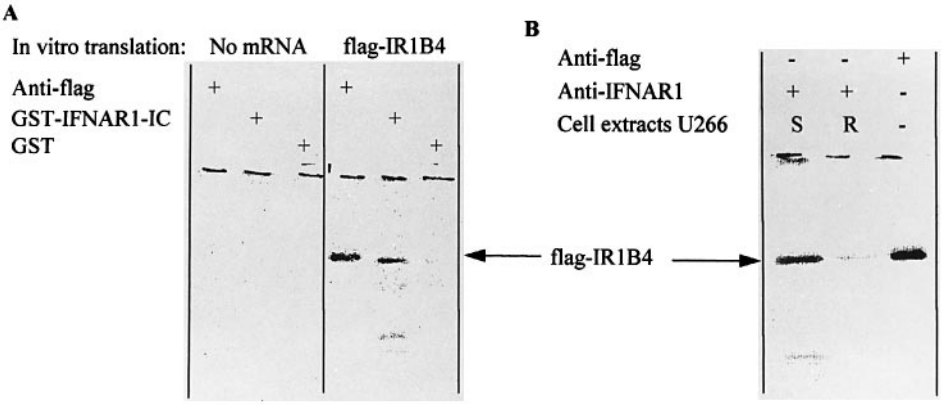

Fig. 3. *In vitro* translated IR1B4 protein binds to the IFN receptor IFNAR1 chain. (**A**) The [^{35}S]methionine-labeled translation products with or without flag-IR1B4 *in vitro* transcripts were either immunoprecipitated (10 μl) with anti-flag M2 beads (lanes 1 and 4), or reacted (50 μl) with glutathione beads coupled to GST fused to the 100 amino acid long IFNAR1-IC domain (lanes 2 and 5) or coupled to GST alone (lanes 3 and 6). After overnight incubation at 4°C (final volume 100 μl), the beads were washed and SDS-eluted proteins boiled in reducing conditions before SDS–PAGE. (**B**) U266S (lane 1) or U266R cells (lane 2) were extracted with Brij buffer and antiproteases (Abramovich *et al.*, 1994b) and 0.35 ml (10^7 cells) was incubated with 75 μl of [^{35}S]methionine-labeled translation products of flag-IR1B4 transcripts overnight at 4°C. Anti-IFNAR1 McAb R3 immobilized on protein G beads (25 μl) was added for 2.5 h, washed in Brij buffer and SDS-eluted, boiled and reduced proteins analyzed by SDS–PAGE. A control with anti-flag M2 beads as above was run (lane 3). The dried gels were visualized in a Phosphor-Imager.

IR1B4 cDNA

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      GCC GCG AAC TGC ATC ATG GAG AAT TTT GTA GCC ACC TTG GCT   42
  1                   M   E   N   F   V   A   T   L   A
      AAT GGG ATG AGC CTC CAG CCG CCT CTT GAA GAA GTG TCC TGT   84
 10   N   G   M   S   L   Q   P   P   L   E   E   V   S   C
      GGC CAG GCG GAA AGC AGT GAG AAG CCC AAC GCT GAG GAC ATG  126
 24   G   Q   A   E   S   S   E   K   P   N   A   E   D   M
      ACA TCC AAA GAT TAC TAC TTT GAC TCC TAC GCA CAC TTT GGC  168
 38   T   S   K   D   Y   Y   F   D   S   Y   A   H   F   G
      ATC CAC GAG GAG ATG CTG AAG GAC GAG GTG CGC ACC CTC ACT  210
 52   I   H   E   E   M   L   K   D   E   V   R   T   L   T
      TAC CGC AAC TCC ATG TTT CAT AAC CGG CAC CTC TTC AAG GAC  252
 66   Y   R   N   S   M   F   H   N   R   H   L   F   K   D
      AAG GTG GTG CTG GAC GTC GGC TCG GGC ACC GGC ATC CTC TGC  294
 80   K   V   V   L   D   V   G   S   G   T   G   I   L   C
      ATG TTT GCT GCC AAG GCC GGG GCC CGC AAG GTC ATC GGG ATC  336
 94   M   F   A   A   K   A   G   A   R   K   V   I   G   I
      GAG TGT TCC AGT ATC TCT GAT TAT GCG GTG AAG ATC GTC AAA  378
108   E   C   S   S   I   S   D   Y   A   V   K   I   V   K
      GCC AAC AAG TTA GAC CAC GTG GTG ACC ATC ATC AAG GGG AAG  420
122   A   N   K   L   D   H   V   V   T   I   I   K   G   K
      GTG GAG GAG GTG GAG CTC CCA GTG GAG AAG GTG GAC ATC ATC  462
136   V   E   E   V   E   L   P   V   E   K   V   D   I   I
      ATC AGC GAG TGG ATG GGC TAC TGC CTC TTC TAC GAG TCC ATG  504
150   I   S   E   W   M   G   Y   C   L   F   Y   E   S   M
      CTC AAC ACC GTG CTC TAT GCC CGG GAC AAG TGG CTG GCG CCC  546
164   L   N   T   V   L   Y   A   R   D   K   W   L   A   P
      GAT GGC CTC ATC TTC CCA GAC CGG GCC ACG CTG TAT GTG ACG  588
178   D   G   L   I   F   P   D   R   A   T   L   Y   V   T
      GCC ATC GAG GAC CGG CAG TAC AAA GAC TAC AAG ATC CAC TGG  630
192   A   I   E   D   R   Q   Y   K   D   Y   K   I   H   W
      TGG GAG AAC GTG TAT GGC TTC GAC ATG TCT TGC ATC AAA GAT  672
206   W   E   N   V   Y   G   F   D   M   S   C   I   K   D
      GTG GCC ATT AAG GAG CCC CTA GTG GAT GTC GTG GAC CCC AAA  714
220   V   A   I   K   E   P   L   V   D   V   V   D   P   K
      CAG CTG GTC ACC AAC GCC TGC CTC ATA AAG GAG GTG GAC ATC  756
234   Q   L   V   T   N   A   C   L   I   K   E   V   D   I
      TAT ACC GTC AAG GTG GAA GAC CTG ACC TTC ACC TCC CCG TTC  798
248   Y   T   V   K   V   E   D   L   T   F   T   S   P   F
      TGC CTG CAA GTG AAG CGG AAT GAC TAC GTG CAC GCC CTG GTG  840
262   C   L   Q   V   K   R   N   D   Y   V   H   A   L   V
      GCC TAC TTC AAC ATC GAG TTC ACA CGC TGC CAC AAG AGG ACC  882
276   A   Y   F   N   I   E   F   T   R   C   H   K   R   T
      GGC TTC TCC ACC AGC CCC GAG TCC CCG TAC ACG CAC TGG AAG  924
290   G   F   S   T   S   P   E   S   P   Y   T   H   W   K
      CAG ACG GTG TTC TAC ATG GAG GAC TAC CTG ACC GTG AAG ACG  966
304   Q   T   V   F   Y   M   E   D   Y   L   T   V   K   T
      GGC GAG GAG ATC TTC GGC ACC ATC GGC ATG CGG CCC AAC GCC 1008
318   G   E   E   I   F   G   T   I   G   M   R   P   N   A
      AAG AAC AAC CGG GAC CTG GAC TTC ACC ATC GAC CTG GAC TTC 1050
332   K   N   N   R   D   L   D   F   T   I   D   L   D   F
      AAG GGC CAG CTG TGC GAG CTG TCC TGC TCC ACC GAC TAC CGG 1092
346   K   G   Q   L   C   E   L   S   C   S   T   D   Y   R
      ATG CGC TGA GGC CCG GCT CTC CCG CCC TGC ACG AGC CCA GGG 1134
360   M   R
      GCT GAG CGT TCC TAG GCG GTT TCG GGG CTC CCC CTT CCT CTC 1176
      CCT CCC TCC CGC AGA AGG GGG TTT TAG GGG CCT GGG CTG GGG 1218
      GGA TGG GGA GGG CAC ATT GGG ACT GTG TTT TTC ATA AAT TAT 1260
      GTT TTT ATA TGG TTG CAT TTA ATG CCA ATA AAT CCT CAG CTG 1302
      GGG AAA                                               1308
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Fig. 4. Nucleotide sequence of IR1B4 cDNA, a human homolog of the rat protein-arginine methyltransferase PRMT1 (see text). Nucleotides are numbered on the right and amino acids on the left. High homology (88%) to the nucleotide sequence of rat PRMT1 (Genbank sequence I.D. 1390024; accession No. U60882) starts from nucleotide 73 and ends shortly after the TGA codon. The protein homology domain to other *S*-adenosylmethinionine-dependent methyltransferases, such as ribosomal protein L11 methyltransferase (accession No. 16811), is underlined.

In vitro binding of IR1B4 to the IC domain of IFNAR1

The insert of $pGBT_{10}$-IR1B4 was introduced into the pECE-flag plasmid (Ellis *et al.*, 1986) and the flag-IR1B4 was recloned in BlueScript (BS) following the T3 promoter. This plasmid DNA was used for *in vitro* transcription using T3 polymerase. The mRNA transcripts were translated in reticulocyte lysates with [^{35}S]methionine. A protein product of ~32 kDa was observed when the translation products were immunoprecipitated by anti-flag antibodies (Figure 3A and B). The protein was not observed in reticulocyte lysate products without added flag-IR1B4 mRNA.

The IFNAR1-IC domain was prepared as a fusion product with GST and expressed in *E.coli*. The bacterial extract was bound to glutathione–Sepharose beads, and the beads were added to the labeled translation products. Analysis on SDS–PAGE showed that the immobilized GST–IFNAR1-IC was able to bind the 32 kDa protein produced in reticulocyte lysates by flag-IR1B4 mRNA (Figure 3A). No such band was observed when GST alone was used or when the mRNA had been omitted from the translation reaction.

To verify that IR1B4 interacts with the IFNAR1 protein from human cells, we used detergent extracts from human myeloma U266 cells. The U266S line has abundant membranal IFNAR1 which can be immunoprecipitated from Brij extracts by an antibody to a C-terminal peptide of IFNAR1 (Ab 631), whereas the U266R variant lacks the membranal IFNAR1 (Abramovich *et al.*, 1994a,b). The translation product of flag-IR1B4 mRNA in reticulocyte lysates was added to Brij extract of U266S cells. The proteins were immunoprecipitated by monoclonal antibody IFNaR3, specific for the ectodomain of IFNAR1 (Colamonici *et al.*, 1990). Analysis by SDS–PAGE showed that the 32 kDa flag-IR1B4 was immunoprecipitated by these anti-IFNAR1 antibodies but, when the same reaction was done with U266R extracts, the 32 kDa band was absent (Figure 3B). The 32 kDa band similarly was seen when U266S extracts were reacted with Ab 631 against the C-terminal peptide of IFNAR1, and IFNAR1 was precipitated by anti-flag when Cos-7 cells were transfected by flag-IR1B4 and human IFNAR1 cDNAs (not shown). These results demonstrate that the flag-IR1B4 interacts specifically with IFNAR1 from human cells and with the isolated IC domain of IFNAR1 prepared in *E.coli*.

IR1B4 is a protein-arginine methyltransferase

The sequence of the longest IR1B4 cDNA showed an open reading frame encoding a 361 amino acid long protein (Figure 4). The IR1B4 sequence had 51% homology to a yeast protein HMT1 (ODP-1, accession Sw: P38074). HMT1 has a domain characteristic of several methyltransferases which methylate RNA binding proteins such as the ribosomal protein L11 methyltransferase (P28637). This domain, involved in *S*-adenosylmethionine binding (Kagan and Clarke, 1994), was clearly conserved in IR1B4 (amino acids 80–108) as revealed by a search with the Blast ProDom algorithm (Altschul *et al.*, 1990). More recently, HMT1 was shown to be the predominant protein-arginine methyltransferase (RMT1) from *Saccharomyces cerevisiae* (Gary *et al.*, 1996; Henry and Silver, 1996). The rat protein-arginine *N*-methyltransferase (PRMT1) was then reported (Lin *et al.*, 1996) and IR1B4 showed almost complete identity except for the N-terminal 11 amino acids and for Tyr –169 instead of His in PRMT1. Binding of IR1B4 to the IFNAR1-IC domain *in vitro* (such as in Figure 3) was observed with flag fusion constructs starting from Lys –78 and, therefore, containing (Figure 4) all the predicted methyltransferase regions I (amino acids 82–90), II (144–151) and III (174–183) as in rat PRMT1 (Lin *et al.*, 1996).

Methyltransferase activity could be co-immunoprecipitated from human cell extracts with the IFNAR1 receptor. Brij detergent extracts of U266S cells were reacted with anti-IFNAR1 antipeptide Ab 631 and protein A beads, or with protein A beads alone. The beads were incubated with [^{14}C](methyl)-*S*-adenosylmethionine and histones were used as substrate. The radioactivity in the histone band was analyzed after SDS–PAGE and exposure in the Phosphor-imager. A [^{14}C]methyl labeling of the histones was seen with the beads coated with anti-IFNAR1 but not

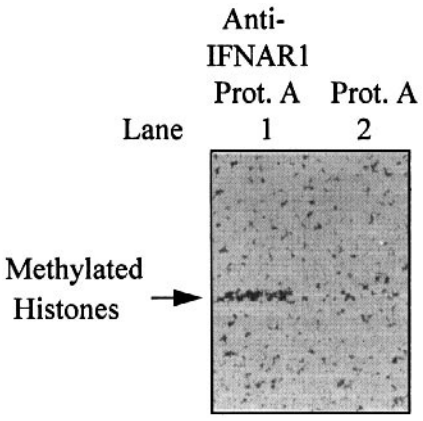

Fig. 5. Methyltransferase activity bound to IFNAR1 from human cells. U266S cells (2.25×10^7 logarithmically growing cells) were extracted in 1 ml of Brij buffer with antiproteases (Abramovich *et al.*, 1994b) and left overnight at 4°C with anti-IFNAR1 C-terminal peptide Ab 631 (10 μl) or without antibodies. Protein A beads (40 μl of a 50% suspension of IPA-400 fast flow, Repligen) were added for 1 h. The beads were washed and incubated in 0.1 ml of 25 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA with 100 μg of histones (type IIA from calf thymus; Sigma) and [^{14}C](methyl)-*S*-adenosylmethionine (0.25 μCi; 50 μM) for 30 min at 30°C. The supernatants were subjected to SDS–PAGE (15% acrylamide), the gels stained with Coomassie to visualize the histones bands, treated with Amplify (Amersham) for 30 min, dried and analyzed in the Phosphor-Imager. Lane 1, with anti-IFNAR1; lane 2, without anti-IFNAR1.

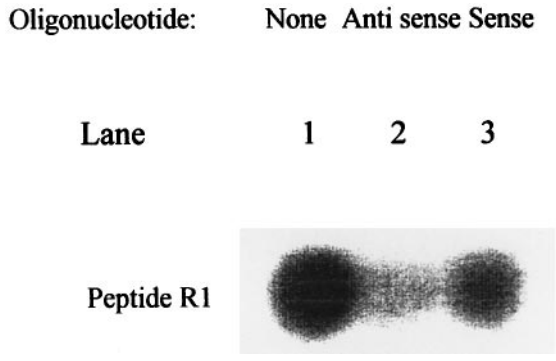

Fig. 6. Protein-arginine methyltransferase activity in U266S cells treated by antisense oligonucleotides. Cells were cultured for 3 days in wells of a 96-well microplate with 10 μM oligodeoxynucleotide phosphorothioates as described in Materials and methods. Extracts were incubated with [^{3}H](methyl)-*S*-adenosyl methionine and peptide GGFGGRGGFG, a specific protein-arginine methyltransferase substrate (Najbauer *et al.*, 1993). After SDS–PAGE, the peptide methylation was measured by autoradiography. Lane 1, cultures without oligonucleotides; lane 2, with antisense-1; lane 3, with sense-1 oligonucleotides.

in the control reaction (Figure 5). Therefore, protein methyltransferase activity appears to be associated constitutively with the IFN receptor chain of these human cells. A similar enzyme activity was recovered when IFNAR1 was immunoprecipitated 5 min after addition of IFN-β to the U266S cells.

Involvement of IR1B4/PRMT1 in IFN action

An antisense oligodeoxynucleotide phosphorothioate, which was able to reduce strongly the protein-arginine methyltransferase activity in U266S cells (Figure 6), was used to investigate the role that this enzyme may play in IFN action. The growth inhibitory activity of IFN was chosen because it can be quantified most directly on cells and because an interaction of PRMT1 with growth-related gene products has been observed (Lin *et al.*, 1996). As seen in Table I, addition of the antisense-1 oligonucleotide, complementary to the sequence around the initiation codon of IR1B4/PRMT1 cDNA, reduced the growth inhibitory effect of IFN-β on U266S cells in two independent experiments. In the presence of antisense-1, the IFN-treated cells exhibited a higher growth, excluding a toxic effect of phosphorothioates. The growth in the absence of IFN was not affected significantly. The sense-1 oligonucleotide corresponding to the same cDNA region had only a small effect as compared with antisense-1 (Table I) and also had only a slight inhibitory effect on the level of enzyme activity (Figure 6). Another antisense phosphorothioate oligonucleotide, directed to the middle of the cDNA, had almost no effect (Table I). The 2- to 5-fold reduction in the growth inhibitory effect of IFN-β on the myeloma cells rendered partially deficient in PRMT1 activity by antisense-1 oligonucleotide indicates that the association of this enzyme with the IC domain of the IFNAR1 receptor is of functional significance.

Discussion

The proteins found to date to be associated with the cytoplasmic domains of the IFN receptor were proteins involved in phosphorylation (tyrosine and serine/threonine protein kinases or phosphotyrosine binding proteins) or in dephosphorylation (see Introduction). The two-hybrid screening with the entire IC domain of IFNAR1 revealed that a methyltransferase is specifically bound to the receptor. The function of this human enzyme, highly homologous to the rat PRMT1, will be interesting to investigate. Lin *et al.* (1996) showed that among the rat PRMT1 substrates are histones, a 55 kDa cytosol protein and hnRNP A1, which binds to pre-mRNA in the nucleus and participates in processing, alternative splicing and mRNA transport to the cytoplasm (Dreyfuss *et al.*, 1993; Mayeda *et al.*, 1993; Burd and Dreyfuss, 1994; Liu and Dreyfuss, 1995). The hnRNPs contain 65% of the N^G, N^G-dimethylarginine residues found in cell nuclei, and 12% of the arginines in different hnRNPs are methylated, often in the RGG motif of RNA binding sites (Boffa *et al.*, 1977; Liu and Dreyfuss, 1995). Methylarginine residues are also abundant in proteins binding pre-rRNA and involved in its processing, such as nucleolin and fibrillarin (Najbauer *et al.*, 1993). These proteins are probably also PRMT1 substrates, whereas myelin basic protein is not a substrate (Lin *et al.*, 1996). There may well be other proteins methylatable by PRMT1 in cells.

Genetic information on the function of protein-arginine methyltransferase comes from the study of *S.cerevisiae* HMT1 (Henry and Silver, 1996), which is identical to ODP1 (YBR0320, Smits *et al.*, 1994) and RMT1 (Gary *et al.*, 1996). HMT1 was shown to complement a mutation in the Npl3p yeast hnRNP which, like the mammalian hnRNP A1, shuttles between nucleus and cytoplasm, binds poly(A)$^+$ RNA and may act as a carrier for mRNA export to the cytoplasm. HMT1 is not essential for viability in normal yeasts but HMT1 overexpression restores viability to the Npl3p mutant strain, suggesting that the methyltransferase enhances the function of the hnRNP in RNA maturation or in its transport from nucleus to cytoplasm

Table I. An antisense IR1B4/PRMT1 oligonucleotide reduces cell growth inhibition by IFN

Experiment	IFN added	Cell density, OD×1000 (growth inhibition, percentage) Oligodeoxynucleotide phosphorothioate added			
		None	Antisense-1	Sense-1	Antisense-2
1	none	67 (0%)	78 (0%)	83 (0%)	82 (0%)
	IFN-β, 64 U/ml	35 (48%)	71 (9%)	51 (38%)	51 (38%)
	IFN-β, 125 U/ml	19 (71%)	52 (33%)	39 (53%)	34 (58%)
2	none	75 (0%)	72 (0%)	73 (0%)	69 (0%)
	IFN-β, 125 U/ml	41 (45%)	55 (23%)	36 (50%)	34 (50%)

Human myeloma U266S cells cultured for 3 days with the indicated amounts of IFN and 10 μM oligonucleotide. Antisense-1 is complementary to the sequence around the ATG initiation codon of IR1B4/PRMT1 (Figure 4) and sense-1 is the same region. Antisense-2 is complementary to a sequence in the middle of the PRMT1 cDNA. Cell density was measured by alamar blue dye assay. Extracts of cells at day 3 (Experiment 1) assayed for methyltransferase activity by [^{3}H](methyl)-*S*-adenosylmethionine labeling of the R1 peptide as in Najbauer *et al.* (1993) indicated a >5-fold reduction with antisense-1 and a <2-fold reduction by sense-1 oligonucleotide (see Figure 6).

(Henry and Silver, 1996). Moreover, the yeast mutant *ire*15 was shown to be suppressed by human proteins, one of which is a transforming growth factor-β (TGF-β) receptor (Nikawa, 1994) and another a protein with homology to ODP1/HMT1 which was called HCP1 (Nikawa *et al.*, 1996, accession No. D66904). In the course of our work, we noticed that the nucleotide and amino acid sequences of HCP1 were identical to IR1B4 (except for two base deletions producing a frameshifted amino acid sequence between amino acids 147 and 175 as compared with IR1B4). Although the methyltransferase activity of HCP1 was not established, it is interesting that the *ire*15 mutation results in a low level of mRNA expression for inosine synthase (INO1) and inosine transferase (ITRF1) and that HCP1 restores the level of INO1 mRNA, indicating that HCP1 regulates the level of expression of several mRNAs (Nikawa *et al.*, 1996). Such regulation could be explained by an effect on hnRNP functions.

The presence of the IR1B4/PRMT1 protein-arginine methyltransferase associated with the cytoplasmic domain of the type I IFN receptor IFNAR1 chain appears to correlate with some function in IFN action. Cells made deficient in PRMT1 activity by an antisense oligonucleotide become less sensitive to the antiproliferative effect of IFN. Although this implicates IR1B4/PRMT1 in the cascade of events mediating IFN action on cells, the precise reaction(s) in which this protein is involved and how its function may be modulated by IFN remain intriguing. Lin *et al.* (1996) have observed that PRMT1 is present in a large macromolecular complex in the cytosol of rat cells but is in a latent form unable to methylate certain proteins. They found that PRMT1 binds to TIS21, an early response protein induced by cytokines and mitogens, and that binding to GST–TIS21 activates the methyltransferase and modulates its specificity possibly by displacing PRMT1 from some inhibitor. Interaction with the TIS21-related BTG1, an antiproliferative gene product disrupted in certain leukemias (Rouault *et al.*, 1992), also activates PRMT1 from rat cytosol (Lin *et al.*, 1996). The binding to the IFNAR1-IC domain could modulate PRMT1 activity similarly and mediate some signal related to IFN′s antiproliferative action or, more generally, to the way in which IFN modifies expression of genes, for example oncogenes and anti-oncogenes which regulate cell growth.

Based on our present knowledge, methylation by PRMT1 could be an important regulatory modification for proteins involved in post-transcriptional regulation, mRNA maturation, transport and stability. Expression of many IFN-α,β-induced genes is under both transcriptional and post-transcriptional controls (Friedman *et al.*, 1984), and the latter control may involve modifications of RNA-bound proteins. Some post-transcriptional control of rRNA expression by IFNs was observed (Radzioch *et al.*, 1987). Sequence-specific alternative splicing of mRNA is another possible function of PRMT1/IR1B4 through modification of hnRNPs (Burd and Dreyfuss, 1994). Interestingly, the IFN receptor IFNAR1 shows various forms produced by alternative splicing, and some splice variants selected by IFN lack signaling activity (Abramovich *et al.*, 1994a) while others have a differential response to IFN species (Cook *et al.*, 1996). IFNAR2 has complex alternative splicing (Lutfalla *et al.*, 1995) and different forms of IFN-induced 2′-5′ A synthetase are produced by differential splicing (Revel and Chebath, 1986). Such post-transcriptional controls through modification of hnRNPs could be involved in the specific inhibition of viral mRNA translation which can occur while host mRNA translation continues (Revel, 1984). However, other targets for protein-arginine methylation may exist, producing transcriptional and post-transcriptional regulation of genes.

PRMT1/IR1B4 is bound to IFNAR1 without IFN, but its activity or localization could be affected by the subsequent assembly of signaling proteins (e.g. Stat factors) on the tyrosine-phosphorylated IC domains of IFNAR1 or IFNAR2 after addition of IFN. PRMT1 may then methylate targets, such as RNA or DNA binding proteins, in the cytoplasm and nucleus. A shuttling of protein-modifying enzymes which associate with transcription factors and are thereby targeted to specific domains in the chromatin has been discovered recently (Wollfe, 1996). Thus, PRMT1 could well be a part of ligand-induced signal transduction pathways (Lin *et al.*, 1996) affecting expression of genes. This first example of a protein methyltransferase bound to a cytokine receptor, and involved in some functions mediated by this receptor, opens new perspectives on the role of protein methylation in cell regulatory events.

Materials and methods

Two-hybrid screen

A cDNA fragment encoding the entire IFNAR1-IC domain, amplified by PCR using a *Bam*HI sense primer (5′ ctga**ggatcc**AAAGTCTTCTTG-AGATGCATC) and an *Eco*RI antisense primer (5′ tgac**gaattc**ctaTCATA-CAAAGTC), was cloned in a BlueScript vector (BS-SK$^+$, Stratagen). The *Bam*HI–*Sal*I fragment from BS-IFNAR1-IC was introduced in the pGBT$_{10}$ vector for two-hybrid screen (CloneTech) fused in-phase after the Gal4 DNA binding domain (pGBT$_{10}$-IFNAR1-IC). The two-hybrid screening (Fields and Song, 1989) was carried out with the modified procedure of Durfee *et al.* (1993) using the pACT plasmid cDNA library from human Epstein–Barr virus-transformed B-lymphocytes to co-transform yeast reporter strain Y153 with pGBT$_{10}$-IFNAR1-IC. Colonies selected in medium SC –trp, –leu, –his with 25 mM 3-aminotriazole (i.e. for histidine prototrophy) were tested for β-galactosidase activity by the X-gal filter assay (Breeden and Naysmith, 1985). Positive yeasts were used to prepare the pACT plasmid DNA and, from each, two *E.coli* HB101 leu$^+$ transformants were isolated. Each library DNA pACT plasmid was subjected to specificity tests by co-transformation of the SFY526 yeast reporter strain (Bartel *et al.*, 1993) with pAS plasmids harboring lamin, cdk2 and p53 or other controls inserts (CloneTech). Colonies which grew in SC –trp, –leu were tested for β-galactosidase expression. From the specifically positive pACT plasmids, inserts were excised with *Xho*I, cloned into BS-KS and subjected to sequencing from T7 and T3 promoters using the DyeDeoxy Terminator Cycle Sequencing kit in a 373A DNA sequencer (Applied Biosystems).

In vitro and in vivo binding to IFNAR1

The pACT-IR1B4 DNA cut with *Xho*I and filled-in by Klenow enzyme, was cloned in the PECE-flag expression vector (Ellis *et al.*, 1986) cut with *Eco*RI and filled-in. The *Not*I–*Bam*HI fragment containing the in-frame flag-IR1B4 fusion was recloned in BS-SK cut with *Not*I–*Bam*HI. The sequence of the flag fusion was verified by sequencing from the T3 promoter. *In vitro* transcription (Promega kit) was done with T3 polymerase and 1 μg of *Bam*HI-linearized BS-flag-IR1B4 DNA. *In vitro* translation was carried out in rabbit reticulocyte lysates (Promega kit) with [^{35}S]methionine (Amersham) and 5 μg of RNA transcripts for 1 h at 30°C. The products were RNase treated before use. The GST–IFNAR1-IC fusion protein was prepared by cloning the *Bam*HI–*Eco*RI insert of BS-IFNAR1-IC (see above) into the same sites of pGEX2 (Pharmacia Biotech). GST and GST–IFNAR1-IC were expressed in *E.coli* and recovered bound to glutathione–agarose beads (Sigma).

Anti-flag M2 agarose beads were from Kodak Scientific Imaging Systems. Monoclonal antibodies IFNaR3 to the α-component of the IFN receptor (IFNAR1) were a kind gift of Dr O.Colamonici (Colamonici *et al.*, 1990) and were used at 1:100 dilution. Rabbit antibodies to the C-terminal peptide of IFNAR1-IC (Ab 631) were prepared and used for immunoprecipitation of IFNAR1 from Brij extracts (0.75 ml) of 2×10^7 human myeloma U266S and U266R cells with antiproteases as previously detailed (Abramovich *et al.*, 1994b) except that protein G beads (Pharmacia) were used with McAb IFNaR3. SDS–PAGE and analysis in a Fujix BAS1000 Phosphor-Imager were as before (Harroch *et al.*, 1994b). *In vitro* methylation of histones was carried out under the conditions described by Lin *et al.* (1996) with [^{14}C](methyl)-*S*-adenosyl-methionine (Amersham).

Cell cultures and RNA analysis

Human myeloma U266S cells were cultured in RPMI 1640 (Bio-Lab, Israel) with 10% heat-inactivated fetal calf serum (FCS) at 37°C in 5% CO_2. The U266R cells are an IFN-α,β-resistant derivative cell line from U266, lacking the transmembranal IFNAR1 mRNA (Abramovich *et al.*, 1994a). Human recombinant IFN-α8 (2×10^8 IU/mg) produced in *E.coli* was a gift from Dr M.Grutter (Ciba-Geigy) and IFN-β (Rebif, 3×10^8 IU/mg) produced in CHO cells was from InterPharm (Ares-Serono group). RNA was extracted with Tri-reagent (Molecular Research Center) and Northern blots were carried out with 10 μg of RNA/slot with 10^6 c.p.m./ml of IR1B4 cDNA labeled with Rediprime kit (Amersham) as described (Harroch *et al.*, 1994a).

Antisense oligonucleotide inhibition of protein-arginine methyltransferase.

Oligonucleotide 3′,5′ phosphorothioates (Stein *et al.*, 1989) corresponding to sequences of the human IR1B4 cDNA (see Figure 4) were synthesized: antisense-1, complementary to bases 12–33, 5′-GGCTACA-AAATTCTCCATGATG; antisense-2, complementary to bases 572–592, 5′-TGGCCGTCACATACAGCGTGG; and sense-1, 5′-CATCATGGAG-AATTTTGTAG, complementary to bases 12–31. The oligonucleotides were added to U266S cells seeded in 96-well microplates (8000 cells/well/0.2 ml RPMI, 10% FCS) at a final concentration of 10 μM on day 0 and re-added at 5 μM on day 2. IFN-β was added at 64 or 125 IU/ml on day 0. After 3 days of culture, 20 μl of alamar Blue, a colorimetric cell density indicator based on oxido-reduction (BioSource, Camarillo, CA), was added to each well and incubation continued for 6–7 h. Color was measured in a microplate ELISA reader (test filter 530 nm, reference filter 630 nm) with multiple reading of duplicate wells. Correlation of the growth curves by live cell number and by OD was verified. To measure methyltransferase, cells from pooled wells were lysed by freeze–thawing in 25 μl/well of 25 mM Tris–HCl pH 7.4, 1 mM EDTA, 1 mM EGTA, 40 μg/ml leupeptin and aprotinin, 20 μg/ml pepstatin, 1 μM phenylmethylsulfonyl fluoride (PMSF). Reactions were in 50 μl with 25 μl of cell extracts, 100 μM peptide GGFGGRGGFG (R1 of Najbauer *et al.*, 1993; obtained from Genosys, Cambridge, UK), 3 μCi of [^{3}H](methyl)-*S*-adenosylmethionine (Amersham, 73 Ci/mmol) for 30 min at 30°C. After electrophoresis in SDS–polyacrylamide (16%) gel, fixation in 50% methanol, 10% acetic acid and treatment by Amplify® (Amersham), autoradiography was carried out for 8 days.

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