Mechanism of Interferon Action: Alpha and Gamma Interferons Differentially Affect mRNA Levels of the Catalytic Subunit of Protein Kinase A and Protein Mx in Human Cells

DANIEL C. THOMIS AND CHARLES E. SAMUEL*

Department of Biological Sciences, Division of Molecular and Cellular Biology, Graduate Program of Biochemistry and Molecular Biology, University of California, Santa Barbara, California 93106-9610

Received 23 October 1991/Accepted 27 December 1991

Treatment of human HeLa and amnion U cells with gamma interferon (IFN- γ), either alone or in combination with alpha interferon (IFN- α), reduced the steady-state level of mRNA encoding the catalytic (C) subunit of protein kinase A (PKA) as measured by Northern gel-blot (RNA) analysis. In addition, IFN- γ treatment increased the ratio of C α to C α 2 (the two splice-site variants of PKA C α subunit mRNA produced in HeLa cells) as measured by a polymerase chain reaction assay. IFN- γ greatly reduced the amount of a novel splice-site variant of PKA, C α 2, which retains introns G and H, relative to the amount of C α , which lacks introns G and H. IFN- α treatment in combination with IFN- γ did not further reduce the level of PKA C α transcripts beyond that of IFN- γ alone, as measured by Northern blots; however, IFN- α in combination with IFN- γ did cause a synergistic increase in the level of human Mx transcripts.

Protein kinase A (PKA; cyclic AMP [cAMP]-dependent protein kinase) occupies an important regulatory role in mammalian cells (10). cAMP-dependent protein phosphorylation catalyzed by PKA catalytic subunits modulates a variety of biochemical activities, including signal transduction, transcription, and intermediary metabolism (10, 17). PKA exists as an inactive holoenzyme, R₂C₂. Binding of cAMP to the regulatory (R) subunits causes dissociation of the R₂C₂ tetramer, yielding active monomer catalytic (C) subunits (28).

Interferon (IFN) is a fundamentally important cellular regulatory protein that affects a variety of processes, ranging from cell growth and differentiation and the immune response to the multiplication of viruses (21, 23). IFN treatment of animal cells leads to an increased expression of nearly two dozen different cellular genes encoding products whose biochemical functions have been identified (23, 24), among them protein Mx, which plays a central role in the IFN-induced antiviral response against influenza virus (24, 27). In addition, IFN treatment causes decreased expression of a limited number of cellular genes, including those for *c-myc* (9, 15, 16), immunoglobulin M μ chain (4, 20), and collagen (12). As part of our efforts to obtain a molecular cDNA clone of the human P1/eIF-2 α protein kinase, a number of cDNA clones of candidate protein kinases were isolated by screening human cDNA libraries with degenerate oligonucleotide probes corresponding to conserved kinase catalytic domains (15) coupled with a secondary screen involving Northern gel-blot (RNA) hybridization. Among the cDNA clones obtained was KIN27 (C α 2), a novel splice-site variant of the catalytic subunit of human PKA (30). We report here that gamma IFN (IFN- γ) causes a significant reduction in the steady-state level of the mRNA encoding the C subunit α isoforms of PKA in human HeLa cells.

Effect of IFN on the expression of human PKA and Mx mRNAs. The effect of IFN on the expression of human PKA

C α mRNA was examined for two human cell lines, amnion U and HeLa, grown in monolayer culture in modified Eagle's medium supplemented with 10% fetal bovine serum (HyClone). Cells were left untreated or were treated for the indicated periods of time (see figure legends) with either 300 IU of Sendai virus-induced human leukocyte IFN- α per ml (generously provided by K. Cantell, Helsinki, Finland), 100 IU of highly purified recombinant IFN- γ per ml (generously provided by D. Goeddel, Genentech, South San Francisco, Calif.), or a combination of the two IFNs. RNAs were examined by Northern gel-blot analysis (1, 2), using as probes either human PKA C α 2 KIN27 cDNA (30), GenBank accession no. M80335, or human MxA cDNA (1), GenBank accession no. M30817.

As shown by Fig. 1 and 2A, a single major PKA C α transcript of about 2.4 kb was observed for RNA isolated from HeLa (Fig. 1) or U (Fig. 2A) cells. The 2.4-kb PKA C α transcript observed for HeLa and U cells is characteristic of the size of the PKA C α mRNA previously described for human (3) and murine (33) cells. HeLa cells treated with IFN- γ , either alone or in combination with IFN- α , possessed a reduced level of PKA C α mRNA (Fig. 1, lanes c and d). Likewise, treatment of human U cells with IFN- γ , either alone or with IFN- α , caused a reduced level of C α mRNA (Fig. 2A, lanes d and e). However, type I IFN- α alone, as opposed to type II IFN- γ , did not significantly affect the steady-state level of PKA C α mRNA in either HeLa cells (Fig. 1, lane b) or U cells (Fig. 2A, lanes b and c). Equivalent amounts of RNA from untreated and IFN-treated cells, as assessed by the level of 18S and 28S ribosomal RNA and the level of KIN30 transcript present in Northern blots (quantitated with a LKB Ultrascan XL laser densitometer), were loaded onto the Northern blot gels shown in Fig. 1 and 2A; KIN30 cDNA encodes an RNA not known to be regulated by IFN (29).

As further controls for the activity of the IFN preparations with the U and HeLa cells and the quality of the RNA samples, hybridizations were also carried out with cDNA probes for human MxA (1) and human protein P1 kinase (29), two genes known to be IFN regulated in human cells (24). As

* Corresponding author.

FIG. 1. Northern gel-blot analysis of human PKA α mRNA expression in HeLa cells. RNA was isolated by the guanidinium thiocyanate-cesium chloride centrifugation technique (5) from HeLa cells not treated with IFN (lane a) or treated with IFN- α for 6 h (lane b), IFN- γ for 24 h (lane c), or a combination of IFN- α and IFN- γ for 24 h (lane d). Total RNA (10 μ g) was separated by formaldehyde-agarose gel electrophoresis, blotted to a Hybond-N nylon membrane, and, after UV cross-linking with a Stratalinker 1800, probed with the 0.9-kb 32 P-labeled *EcoRI-EcoRI* insert of the KIN27 PKA cDNA clone (2, 30).

shown in Fig. 2B, Northern analysis of human *Mx* transcripts, IFN- α treatment greatly increased the steady-state level of a 2.8-kb *Mx* mRNA in U cells (lanes b and c), whereas IFN- γ was a very poor inducer of the *Mx* transcript (lane d). These results are in full agreement with those previously obtained for IFN-treated human fetal lung cells (1). Somewhat unexpectedly, treatment of U cells with a combination of IFN- α and IFN- γ resulted in an enhanced level of *Mx* mRNA over that observed with cells treated with the IFNs alone (Fig. 2B, lane e). Although to our knowledge a synergistic effect of type I and type II IFNs on the level of *Mx* expression has not been previously described (24, 27), it

FIG. 2. Northern gel-blot analysis of human PKA α (A) and human *Mx* (B) expression in amnion U cells. Total RNA (10 μ g) was isolated from untreated or IFN-treated human U cells, separated by formaldehyde-agarose gel electrophoresis, blotted to a Hybond-N nylon membrane, and UV cross-linked. The gel blots were then probed with the 0.9-kb 32 P-labeled *EcoRI-EcoRI* insert of the KIN27 PKA α cDNA clone (29) (A), as for Fig. 1, or with the 0.9-kb 32 P-labeled *BglIII-BglIII* insert of the human *MxA* cDNA clone (1) (B). RNA was isolated from U cells not treated with IFN (lanes a) or treated with IFN- α for 6 h (lanes b), IFN- α for 24 h (lanes c), IFN- γ for 24 h (lanes d), or a combination of IFN- α and IFN- γ for 24 h (lanes e).

FIG. 3. Outline of the PCR strategy for measurement of the ratio of KIN27 Ca_2 mRNA to PKA Ca_1 mRNA. The exon-intron organization predicted for human PKA cDNA clone KIN27 (Ca_2) is based on the mouse PKA Ca gene structure (6). The numbers refer to the nucleotide position, with the A of the AUG codon defined as +1. Introns G and H (INTG and INTH), which are present only in KIN27 PKA Ca_2 and not in mouse or human PKA Ca_1 , are shaded. Plus-strand sense and minus-strand antisense primers specific for either KIN27 Ca_2 or PKA Ca_1 were prepared and used for the amplification of first-strand cDNA by PCR (22). The combination of 5'COM (5'-GGAAGGTTTCAGTGAGCC-3', KIN27 plus-strand nt 409 to 425) and 3'INT (5'-GAGCAAGATGGGGGGGC-3', KIN27 minus-strand nt 734 to 718) primers were designed to yield a KIN27 Ca_2 -specific 326-bp fragment. The combination of 5'COM and 3'EX (5'-GTTGTAGCCTTTGCTCAG-3', KIN27 minus-strand nt 746 to 738/642 to 634) primers were designed to yield a PKA Ca -specific 243-bp fragment.

is well established that treatment of human cells, including U cells, in culture with a combination of type I and type II IFNs may result in a synergistic antiviral effect as measured by virus yield reduction (7, 25). Finally, when the same RNA preparations that were previously probed with the human PKA Ca cDNA in the Northern blot of Fig. 1 were subsequently analyzed with the human P1 kinase cDNA (GenBank accession no. M85294), increased amounts of the 2.5-kb P1 RNA transcript were observed with IFN- α treatment relative to those observed with the untreated control or with IFN- γ treatment, both of which showed comparable P1 transcript levels (29).

Expression of two distinct human PKA α mRNA species in HeLa cells is differentially affected by IFN. Two splice variants of the α catalytic subunit of PKA have been identified in human cells, Ca (3, 19, 30) and Ca_2 (30). Although Ca is much more abundant than Ca_2 , both mRNAs are present in relatively low amounts (3, 19, 30–32). The Ca_2 mRNA, a novel splice-site variant that includes sequence derived from introns G and H which are excised from the mouse (6) and human (19, 30) Ca transcripts (Fig. 3), possesses all of the sequence motifs and single residues shown to be essential for serine/threonine protein kinase activity (14). However, Ca_2 is truncated at the C terminus to yield a 224-amino-acid protein rather than the 351-amino-acid Ca isoform; it is not yet known whether Ca_2 codes for an enzymically functional C subunit (30). Polymerase chain reaction (PCR) (11, 22) was used to analyze RNA isolated from human HeLa cells for PKA C subunit messages that correspond to Ca and Ca_2 .

The PCR amplification involved the use of two different antisense (minus-strand) oligonucleotide primers, 3'INT (nucleotides [nt] 734 to 718) and 3'EX (nt 746 to 738/642 to 634), and one sense (plus-strand) oligonucleotide primer, 5'COM (nt 409 to 425). The relative positions of the PCR primers are shown in Fig. 3, which presents an outline of the PCR

FIG. 4. The level of KIN27 Ca_2 mRNA is reduced relative to that of PKA Ca mRNA by treatment of HeLa cells with IFN- γ . Human HeLa cells were not treated with IFN (none) or were treated with IFN- α for 6 h (α), IFN- γ for 24 h (γ), or a combination of IFN- α and IFN- γ for 24 h ($\alpha + \gamma$). RNA was isolated and first-strand cDNA was prepared by reverse transcription according to the method of Gubler and Hoffman (13). cDNA equivalent to about 20 ng of poly(A)⁺ RNA was boiled for 3 min and quenched on ice before amplification by PCR (11, 22, 30), using 20 U of *Thermus aquaticus* DNA polymerase per ml and primers as indicated at a concentration of 0.25 μM . Primers were synthesized by using an Applied Biosystems 380A DNA synthesizer. Reactions contained either 3'INT and 5'COM primers (lanes 3, 5, 7, and 9) or 3'EX and 5'COM primers (lanes 4, 6, 8, and 10). Lane 2 contained all three primers but no template cDNA. Lane 11 contained 3'EX and 5'COM primers and 10^6 molecules of pBlue-KIN27 plasmid DNA as a control template. PCR reaction products were fractionated on a 3% Nu-Sieve 3:1 agarose gel, transferred to Hybond-N, and probed with *EcoRI*-*EcoRI* fragment of KIN27 PKA cDNA (26, 30). Lane 1 is a DNA size standard. The numbers to the left of the gel refer to the sizes (in nucleotides) of the standards.

strategy. The 5'COM primer was common to both the Ca and Ca_2 isoforms of PKA. The 3'INT primer was specific for intron G of PKA Ca_2 (KIN27) and was designed to yield a 326-bp Ca_2 KIN27 product. The 3'EX primer was specific for the Ca exon 7-8 junction and was designed to yield a 243-bp Ca PKA product.

As shown by Fig. 4, the Ca -specific product (PKA) was observed when the 5'COM and 3'EX primers were used to amplify cDNA prepared from RNA isolated from either untreated (lane 4) or IFN-treated (lanes 6, 8, and 10) HeLa cells. The amount of PKA Ca -specific PCR product was slightly increased by IFN- α treatment and was reduced by IFN- γ treatment. The PKA Ca_2 -specific product (KIN27) was clearly detectable in the absence of IFN treatment with the 5'COM and 3'INT primers (Fig. 4, lane 3). However, very little or no Ca_2 -specific KIN27 product was observed with cDNA prepared from RNA isolated from HeLa cells treated with IFN- γ (lane 7), IFN- α plus IFN- γ (lane 9), or IFN- α (lane 5).

Although attempts were made to carry out the PCR analyses under quantitative conditions (excess primer and *Taq* polymerase, ≤ 40 cycles), the reduction in the absolute amount of PKA Ca -specific transcript following IFN- γ treatment as measured by PCR (Fig. 4) was not as great as that revealed by Northern analysis (Fig. 1), no doubt reflecting the difficulty of performing quantitative PCR. However, less Ca_2 PCR product was always observed with cDNA prepared from IFN-treated cells than with cDNA prepared from untreated cells (Fig. 4). Quantitation of autoradiograms

revealed a Ca/Ca_2 ratio of 5.9 for untreated HeLa cells, whereas the ratio ranged from 33 to >100 for HeLa cells treated with IFN- γ .

Conclusions. The results reported herein demonstrate three important points. First, treatment of cells with IFN decreases the steady-state amount of mRNA encoding the catalytic subunit of cAMP-dependent protein kinase as revealed by Northern gel-blot analysis of U and HeLa human cells. Second, IFN treatment reduces the amount of the Ca_2 splice-site variant of PKA (KIN27) more extensively than the amount of the Ca isoform. Third, IFN treatment with a combination of IFN- α and IFN- γ has a synergistic effect on the induction of *Mx*-specific transcripts in U cells but does not have a synergistic effect on the reduction of PKA Ca -specific transcripts.

IFN is known to induce the expression of more than 20 different genes (21, 23), the products of some of which have been characterized extensively in biochemical and genetic terms as they relate to their surprisingly selective antiviral activities (24, 27). Considerable progress has been made in the understanding of the *cis*-acting elements and the *trans*-acting factors involved in the IFN-induced transcriptional activation of cellular genes. It appears that the activation of IFN-stimulated factor 3 is of central importance for transcriptional induction of genes via the IFN-stimulated response element (8, 18). By contrast, the number of genes whose expression is reduced in response to IFN treatment is small relative to the number of genes induced (24). Either a decrease in the rate of transcription or an increase in the rate of turnover, or both, may be responsible for the reduction in the steady-state amount of transcripts following IFN treatment, as revealed from studies of c-myc (9, 16), immunoglobulin M μ chain (20), and collagen (12) in human cells. It is unclear which mechanism is responsible for the reduction of PKA Ca transcript level in HeLa and U cells.

IFN- γ treatment has been reported in two studies to adversely affect the splicing of heterogeneous nuclear RNA in murine cells (4, 15). It also appears that IFN- γ treatment affects the distribution of the splice-site variants Ca and Ca_2 of PKA in human cells. It is of fundamental importance to elucidate the functional roles of the two PKA C subunit variants and how alteration in the levels of the Ca isoforms may be related to the pleiotropic effects of IFN.

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