The 100-kDa 2',5'-Oligoadenylate Synthetase Catalyzing Preferentially the Synthesis of Dimeric pppA2'p5'A Molecules Is Composed of Three Homologous Domains*

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The 2–5A synthetases represent a family of proteins implicated in the mechanism of the antiviral action of interferon. When activated by double-stranded RNA, these proteins polymerize ATP into 2'-5'-linked oligomers with the general formula pppA(2'5'A)n, n ≥ 1. Three forms of human 2–5A synthetases have been described corresponding to proteins of 40/46 (p40/p46), 69/71 (p69/p71), and 100 kDa (p100). Here we describe the molecular cloning and characterization of p100. By screening a cDNA expression library with a specific p100 polyclonal antibody, we first isolated a 550-nucleotide cDNA fragment which was subsequently used to isolate the full-length 6365-nucleotide cDNA. This cDNA recognizes a distinct interferon-induced messenger RNA of 7 kilobases. It has an open reading frame encoding a protein of 1087 amino acids including the sequence of seven peptides obtained by microsequencing of the natural p100 protein, which was purified from interferon-treated human cells. p100 is composed of three adjacent domains, each homologous to the previously defined catalytic unit of 350 amino acids, which is present as one unit in p40/p46 and as two units in p69/p71. The recombinant p100 synthesized preferentially dimeric 2',5'-oligoadenylate molecules and displayed parameters for maximum enzyme activity similar to the natural p100. These results confirm that the enzymatic activity of p100 is distinct compared with that of p40/p46 and p69/p71.

The 2–5A synthetases are interferon-induced proteins characterized by their capacity to catalyze the synthesis of 2',5'-linked oligomers of adenosine from ATP with the general formula pppA(2'5'A)n, where n ≥ 1 (1, 2). This mixture of oligonucleotides is referred to as 2–5A, and the enzymes that synthesize it are referred to under the generic term 2–5A synthetase (3) or as 2',5'-oligoadenylate synthetase (2',5'-OAS). Currently, the only known function of 2–5A is to bind and activate a latent endoribonuclease responsible for the degradation of viral and cellular RNAs (4, 5). This leads to inhibition of cellular protein synthesis, thus impairing viral replication (for reviews see Refs. 6–8). The action of 2–5A in cells is transient (9) because of a phosphodiesterase that cleaves preferentially 2',5'-linked oligonucleotides (10). In vitro, the synthesis of 2–5A requires activation of the 2',5'-OAS by double-stranded (ds) RNA or single-stranded RNA with a defined secondary structure, such as the 5'-untranslated region of all human immunodeficiency virus mRNAs that contains a stem-loop structure (11, 12). In intact cells or tissues, the 2',5'-OAS can become activated during virus infections due to the presence of genomic viral dsRNA molecules, or as a result of the production of viral dsRNA replicative intermediates during virus replication (13, 14). The 2',5'-OAS is present in most mammalian cells and tissues (15, 16). Natural occurrence of 2–5A has clearly been demonstrated in interferon-treated cells infected with EMCV (13), and results from several laboratories have suggested that the 2–5A system (the OAS, 2–5A, and the endoribonuclease) plays a role, at least in part, in the mechanism of the antiviral and antiproliferative action of interferon (6–8, 17–20).

Three major forms of 2',5'-OAS have been described in interferon-treated human cells corresponding to proteins of 40/46, 69/71, and 100 kDa (p40/p46, p69/p71, and p100, respectively; Refs. 21–24). The two isoforms p40/p46, are encoded by the same gene; they are identical for their first 346 amino acids but have different carboxy termini generated by differential splicing (25–27). Similarly, p69/p71 gathers two isoforms sharing a common amino terminus of 683 residues but with different carboxy termini, probably generated by differential splicing (28). Each form of human OAS is induced by interferon α, β, and γ, but in some cells there might be differential expression and induction by interferon (21–23, 29–31). The p40/p46 and p69/p71 are found to be associated with different subcellular fractions such as mitochondrial, nucleolar, and rough/smooth microsomal fractions, whereas p100 is mainly associated with the ribosomal fraction (21, 22, 24, 32). Ultrastructural localization studies on p69/p71 and p100 have confirmed their presence in the nucleus (33). Only p69/p71 is myristoylated (29), and, consistent with this, this form of OAS has been found to be associated with the nuclear and plasma membranes (29, 33). Interestingly, the three forms of OAS have distinct enzymatic parameters thus suggesting that they might have specific functions (21–23, 29–31, 34). In addition to the synthesis of 2–5A molecules, partially purified OAS preparations have been shown to catalyze in vitro the transfer of a nucleotide monophosphate moiety to the 2'-OH end of

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‡ The abbreviations used are: 2–5A synthetase, 2',5'-oligoadenylate synthetase; 2',5'-OAS, 2',5'-oligoadenylate synthetase; 2–5A, 2',5'-oligoadenylate-triphosphate molecules with the general formula pppA(2'5'A)n, where n > 1; OAS, oligoadenylate synthetase; ds, double-stranded; p40/p46, 40- and 46-kDa isoforms of the small 2',5'-oligoadenylate synthetase; p69/p71, 69- and 71-kDa isoforms of the medium-sized 2',5'-oligoadenylate synthetase; p100, the 100-kDa form of 2',5'-oligoadenylate synthetase; PAC, F1 artificial chromosome; Ap₅A, di(adenosine-5')tetraphosphate; tRNA, transfer RNA; kb, kilobase(s); aa, amino acid(s); RT, reverse transcriptase; PCR, polymerase chain reaction; PIPES, 1,4-piperazinediethanesulfonic acid.
a preformed 2–5A molecule or to a nucleotide with the structure RpA like NAD+, AtpA, and tRNA (35–38). However, biological relevance of these latter modified nucleotides is not yet clear. Moreover, GTP could be an alternative substrate for OAS p69 or P100 to catalyze the 2′–5′ transfer of a GMP moiety to a GTP molecule other than to a nucleotide with the structure RpA (34). This latter observation is in agreement with some reports suggesting a role for p100 in pre-messenger RNA splicing (39).

In this article, we purified p100 from interferon α-treated human Daudi cells, and after digestion with endo-lysine C the sequence of several peptides was obtained by microsequencing. In parallel, by using polyclonal antibodies specific to p100, we isolated a cDNA fragment corresponding to p100 by screening an expression cDNA library constructed with mRNAs from interferon-treated Daudi cells. The identity of this isolated cDNA as that of p100 was confirmed by the presence of the peptide sequences of the natural p100. Full-length cDNA encoding p100 was achieved by screening additional cDNA libraries and by RT-PCR approaches. The deduced amino acid sequence from this cDNA revealed its tripartite nature compared with the bipartite nature of p69/p71. Expression of the full-length cDNA generated a 100-kDa protein, which was recognized by polyclonal and monoclonal antibodies against p100, and manifested enzymatic activity similar to the natural p100 from interferon-treated human cells.

MATERIALS AND METHODS

Reagents—[α-32P]ATP (10 μCi/sample; 3000 Ci/mmol), [γ-32P]ATP (10 μCi/sample; >5000 Ci/mmol), poly[1(polyC),] and protein A-agarose were purchased from Amersham Pharmacia Biotech. TNT® coupled reticulocyte lysate system for coupled transcription translation of mRNAs from interferon-treated Daudi cells. The identity of this isolated cDNA as that of p100 was confirmed by the presence of the peptide sequences of the natural p100. Full-length cDNA encoding p100 was achieved by screening additional cDNA libraries and by RT-PCR approaches. The deduced amino acid sequence from this cDNA revealed its tripartite nature compared with the bipartite nature of p69/p71. Expression of the full-length cDNA generated a 100-kDa protein, which was recognized by polyclonal and monoclonal antibodies against p100, and manifested enzymatic activity similar to the natural p100 from interferon-treated human cells.

Isolation of cDNA Clones—The agt11 random-prime expression cDNA library (6.0 × 108 plaques) was screened using p100-specific murine polyclonal antibodies at 1:50 dilution and 32P-labeled goat anti-mouse antibodies. In order to improve the specificity of the antigen-antibody reaction, nitrocellulose filters after the plaque lift were washed extensively by a denaturating solution of a single-stranded DNA filter. Filters were incubated successively in guanidine-HCl buffer at 1, 0.75, 0.5, and 0.1. Screening of the keratinocyte cDNA library was performed using a cosmid clones containing the cluster of 2′,5′-OAS genes (41).

Construction of Library from a PAC Clone—1 μg of DNA of a PAC clone containing the gene encoding p100 (41) (PAC F336L20; from the human, genomic library developed by P. de Jong) was digested by XhoI following the manufacturer’s recommendations (New England Biolabs). Products of digestion were then subcloned in pBS/SK into the XhoI site. Positives clones containing the 5′ missing region of p100 were identified by screening using a labeled oligonucleotide located in the 5′ part of the cDNA REB2 (AGAGCAGCGGCGGACGACACGAC). RT-PCR Conditions—Total cytoplasmic RNA was prepared from interferon α-treated HeLa cells using the RNAzol reagent. Briefly, 5 μg of total RNA were heat-denatured at 95 °C, then a reverse transcription was carried out with the GSP3 primer located in the cDNA 3′ (AGG ATGTATGTGCGCGGCTTCGTTGAC) at 42 °C using 10 units of Moloney murine leukemia virus reverse transcriptase (Boehringer Mannheim) in a total reaction volume of 25 μl. Five μl of the resultant first-strand cDNA was amplified in 50–μl PCR reactions using combinations of the forward specific GSP1 primer located in cDNA REB2 (ATGTTGCTGACCTTCTCCGCTGTC) and the reverse GSP2 primer located in cDNA 3′ (AGG TGACGACCTTCTCCGCTGTC) amplification conditions were as follows: 30 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, followed by an extension step at 72°C for 10 min. Amplified products were analyzed by electrophoresis in 1.2% agarose gels. An addition of a single adenine at the 3′ of the fragment was performed using Taq polymerase (Cetus) as described previously (42). The fragment was gel-purified and ligated directly into a plasmid pTAG vector (R & D Systems). The resultant clones were characterized by sequencing.

Nucleotide Sequence Analysis—DNA sequences were determined by the Sanger dyeideoxy sequencing method using double-stranded DNA as a template and Sequenase™ II as polymerase.

Northern Blot Analysis—Total cytoplasmic RNA was prepared by the single-step method of extraction (RNAzol-Bliseprobe) according to a procedure recommended by the manufacturer. Polyadenylated RNAs were isolated from total cytoplasmic RNA using spin column (Amersham Pharmacia Biotech kit). For Northern blot analysis, total RNA or polyadenylated RNAs were size-fractionated in 1% agarose, 2.2 M formamide gels and transferred to nylon membrane (Hybond N°, Amersham Pharmacia Biotech). Membranes were hybridized in RBS buffer (Amersham Pharmacia Biotech) at 55 °C for 2 h in the presence of 106 cpm/ml of 32P-random-prime cDNA probe (Megaprime, Amersham Pharmacia Biotech). Membranes were washed in 0.1 × SSC at 55 °C. A 32P-labeled 5′-deoxy-5-phosphate dehydrogenase probe was used to rehybridize membranes and monitor that all lanes contained equivalent amounts of total RNA.

Constitution of Plasmids Expressing Tagged Proteins—Full-length cDNA encoding p100 was subcloned in pBS-SK (Stratagene) and then cloned in the vector pcDNAV53.1 (version A; Invitrogen) into the Hin-dIII and AapI sites. The resulting expression vector was composed of the open reading frame encoding p100 fused to the V5 epitope without 3′-noncoding region. The vector pNeoStrIII containing the full-length cDNA encoding p69 2′,5′-OAS fused to the FLAG epitope has been described previously (33).

2′,5′-OAS Activity Assay—The activity of the natural forms of p69/ p71 and p100 2′,5′-OAS could be efficiently assayed after immunoprecipitation with rabbit polyclonal antibodies and ATP (21, 22). For the recombinant proteins, the 2′,5′-OAS activity was monitored after immunoprecipitation of the tagged proteins p69FLAG or p100V5 using the monoclonal antibody M2 specific to the FLAG epitope or the monoclonal antibody specific to the V5 epitope, respectively. Extracts from transfected cells (5 × 106) were incubated (2 h, 4 °C) with the monoclonal antibody M2 or anti-V5 coupled to agarose. The binding was carried out for 2 h before washing (five times) with buffer A (each wash representing at least 150 times the volume of protein A-agarose) and buffer II (two washes): 10 mM Hepes, pH 7.6, 50 mM KCl, 1 mM MgCl2, 7 mM 2-mercaptoethanol, and 20% glycerol (v/v). Cell extract volumes were adjusted in order to obtain similar amounts of purified p69 and p100. The purified proteins, immunoprecipitated using 50 μl of the antibody-agarose, were incubated in the 2′,5′ reaction mixture (100 μl)
containing 20 mM Hepes, 50 mM KCl, 25 mM Mg(OAc)₂, 7 mM 2-mercaptoethanol, 5 mM ATP, 10 mM creatine phosphate, 0.16 mg/mL creatine kinase, 100 μg/mL poly(U)poly(C), and [α-³²P]ATP or [γ-³²P]ATP (10 μCi/sample). The pH of the reaction mixture was adjusted to 6.5 and 7.5 for p69/p71 and p100 activity assays, respectively. The reaction was stopped by heating (95 °C, 5 min), and the 2–5A products were analyzed by electrophoresis on 20% polyacrylamide gels containing 7 M urea as described (34, 43).

**Results**

**Purification and Peptide Sequencing of the Human Natural p100**—The human 100 kDa form of 2’,5’-OAS was purified from interferon α-treated Daudi cells by affinity chromatography using the monoclonal antibody 11/25 specific for this protein (31). The purified polypeptide was detected as a single Coomassie Blue-stained band of 100 kDa on SDS-polyacrylamide gel electrophoresis (data not shown). Polyclonal antibodies against purified p100 preparations were produced in mice as described (31). In addition, the band corresponding to p100 was excised from the polyacrylamide gel and digested with endo-lysine C, and the purified peptides were processed for microsequencing (see “Materials and Methods”). We were able to obtain the amino acid sequence of eight peptides composed of 6–14 residues derived from the p100 protein (shown below).

**Cloning of a Full-length cDNA Encoding p100**—Polyclonal antibodies raised against p100 were used to screen a Agt11 expression library made from mRNA from interferon-treated Daudi cells. Consistently, a cDNA clone was isolated (referred to as NT-15), the nucleotide sequence of which had no homology with the sequence of p40/p46 and p69/p71 (34, 43). Furthermore, this cDNA in Northern blot experiments did not reveal any interferon-induced mRNA (data not shown), and we concluded that NT-15 resulted from nonspecific binding of the polyclonal antibodies. We then included a denaturation-renaturation step of the proteins transferred onto the nitrocellulose before incubation with the polyclonal antibodies (see “Materials and Methods”). By this process, 10 cDNA clones were isolated, 2 of which were NT-15, 4 that matched p40/p46, 2 clones derived from a 56-kDa 2’,5’-OAS-related protein (46), and only 1 clone that was found to correspond to part of p100 (see results herein). This single clone was identified upon screening 600,000 phage plaques. Sequencing of this clone revealed a 590-nucleotide insert that had 55% homology with p40/p46 and p69/p71, and contained motifs conserved between these forms of 2’,5’-OAS. Moreover, the deduced amino acid sequence of this cDNA contained peptides that were homologous to the amino acid sequence of three peptides (MGDPVQSK, SGLGHPI, and SLNAGL), which could be considered as specific 2’,5’-OASs previously cloned. Structural analysis revealed that p100 is composed of three contiguous 2–5A synthetase-like domains (Fig. 2), each homologous to the domain previously described in one copy in p40/p46 and in two copies in p69/p71 (28). In p100, domain I is composed of 362 residues (aa 1–362), domain II consists of 339 residues (aa 404–742), and domain III comprises 345 residues (aa 743–1087). These domains share a strong identity with one another: domain I shares 60% identity with domain II and 44% identity with domain III, whereas domain II shares 49% identity with domain III. Domains II and III are strictly adjacent, whereas domains I and II are linked by a peptide of 42 amino acids (Fig. 2). As is the case for the linker peptide between the two domains in p69/p71 (28), the sequence of the linker peptide in p100 does not share homology with the 2’,5’-OAS unit.

Comparison of the amino acid sequence of domain I, II, and III of p100 with that of the first 364 residues conserved in human p40/p46 revealed the presence of highly conserved domains, which could be considered as specific 2’,5’-OAS signature sequences (Fig. 3). These sequences are represented by a stretch of conserved 15–20 amino acids, most probably corresponding to functional microdomains implicated in the catalytic activity of 2’,5’-OASs and in the binding capacity to the activator dsRNA (Fig. 3). The glycine-rich subdomain with the motif Lys/Arg-Gly-Gly-Ser-X-Gly/Ala-Lys/Arg-Gly-Thr-X-Leu-Lys/Arg at amino acid 60 in p40/p46 and 343 in p69/p71, and at amino acids 59, 458, and 801 in p100 (Fig. 3 and Ref. 28), could be part of the substrate ATP/GTP binding domain as we had suggested previously (28).

In vitro expression of the cDNA encoding p100, using the coupled transcription/translation system in rabbit reticulocyte...
lysates, generated a major product of 100 kDa (data not shown), as is the case for the natural p100 from interferon-treated human cells. This apparent molecular mass is in agreement with the theoretical molecular mass deduced from the predicted open reading frame encoded by the full-length cDNA encoding p100.

Expression of mRNA Encoding p100—In Northern blot analysis, cDNA REB2 hybridized to an unique interferon α-induced transcript of 7 kb. This transcript is almost nondetectable in control untreated Daudi cells, but its steady state level is increased significantly upon interferon α treatment (Fig. 4). Similarly, the induction of this transcript was observed following treatment of cells with β interferon; however, the level of induction was lower with γ interferon (Fig. 4). It should be noted that this 7-kb transcript was detectable by the different cDNA fragments corresponding to the full-length of p100: cDNA REB2, 3A1, and LC1 (data not shown).

Expression of p100 in Transfected Cells—An expression plasmid was constructed containing the complete cDNA encoding p100. We epitope-tagged the recombinant p100 at its COOH terminus using the V5 epitope in order to be able to detect the expression of the recombinant protein using an anti-V5 specific

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Fig. 1. Cloning the full-length cDNA encoding p100. A, the isolation of p100 related cDNA clones. Clone REB2 (1000 nucleotides) was isolated from an oligo(dT)-primed Agt10 cDNA library using the labeled cDNA probe (590 nucleotides), which was initially isolated from a random primed Agt11 expression library by screening with p100 specific polyclonal antibodies. These Agt10 and Agt11 cDNA libraries were constructed with mRNA from interferon-treated human cells (40). cDNA 3A1 (3867 nt) was isolated by screening a keratinocyte cDNA library with cosmid clones encompassing the cluster of 2',5'-OAS genes (41). The LC1 fragment between REB2 and 3A1 (1712 nt) was obtained by RT-PCR using mRNA from interferon-treated HeLa cells and GSP1, GSP2, and GSP3 primers. The 5' missing region of p100 cDNA (5TER) was isolated by screening a library constructed from a PAC DNA containing the 5' gene with oligonucleotides located in the 5' part of p100 cDNA as probe (see "Materials and Methods"). A complete cDNA encoding p100, was reconstructed using the isolated cDNA fragments REB2, 3A1, and LC1, and the genomic region encoding the missing 5'-coding region (STER). B, the deduced amino acid sequence corresponding to the sequence of peptides (numbered 1–8) obtained from the purified natural p100 are underlined.
monoclonal antibody. Subcellular distribution of the transiently expressed p100-V5 in HeLa cells was then examined by confocal immunofluorescence using such a V5 specific antibody. The recombinant p100 was found to be well distributed throughout the cytoplasm (Fig. 5). Experiments by confocal immunofluorescence using interferon α-treated HeLa cells and specific monoclonal antibody against p100 showed a similar subcellular localization as the recombinant p100 (Fig. 5). In order to recover p100-V5, the transfected cells were lysed using non-ionic detergents Triton X-100 or Nonidet P-40. Consistently, we were able to recover high amounts of recombinant p100-V5, which was identified by immunoblotting using either the monoclonal antibody against the V5 epitope or by the polyclonal antibody raised against the natural p100 (Fig. 6). These results confirm that recombinant p100 has structural features that are similar to the natural protein. The detection of p100 in HeLa cells transfected with the control pcDNA3.1 (without insert) demonstrates the constitutive expression of low levels of this protein in cultured cells. The endogenous natural p100 and the recombinant p100 showed a similar mobility in polyacrylamide gel.

Recombinant p100 Preferentially Synthesizes Dimeric Forms of 2–5A Like the Natural Protein—Using specific monoclonal antibodies to purify p69 and p100 from interferon-treated cells, we demonstrated previously that p100 preferentially synthesizes dimeric forms of 2–5A, whereas p69 synthesizes higher oligomers (34). Here we confirm that p100 indeed synthesizes preferentially dimeric molecules of 2–5A.

The recombinant p69-FLAG and p100-V5 proteins were purified by immunoprecipitation using anti-FLAG and V5 monoclonal antibodies, respectively. The immune complexes recovered with protein A-agarose were assayed for the synthesis of 2–5A. In the absence of dsRNA activator, no detectable 2–5A was observed in p100-V5 and p69-FLAG samples as with the corresponding natural proteins. However, in the presence of dsRNA, 2–5A molecules were synthesized. Interestingly, the recombinant p100 preferentially synthesized dimeric molecules of 2–5A compared with the recombinant p69. Furthermore, the profiles of 2–5A molecules generated by the recombinant proteins were similar to their respective natural counterpart (Fig. 7).

In the presence of increasing concentration of poly(I)poly(C), maximum activation of p69-FLAG occurred at 100 μg/ml, whereas maximum activation of p100-V5 was obtained at 10 μg/ml poly(I)poly(C), in accord with our previous reports using natural proteins (34). At such optimum activation of each enzyme, the proportion of 2–5A dimers per total oligomers of 2–5A molecules synthesized by the recombinant p100 and p69 was 70% and 5%, respectively (Fig. 8). It should be noted that p100 manifested a preference for the synthesis of 2–5A dimer molecules at any concentration of dsRNA, concentrations ranging from 1 to 100 μg/ml. On the other hand, the proportion of 2–5A dimers synthesized by p69 was significantly reduced at...
Higher concentrations of dsRNA, *i.e.* at optimum activation of the enzyme (Fig. 8). Thus, upon maximum activation of p69, higher oligomers of 2–5A become generated at the expense of the dimeric forms.

Kinetic experiments, carried out at optimal pH and dsRNA concentrations for p69 and p100, confirmed once again that the recombinant proteins behave as the natural proteins (data not shown). These findings illustrate that the full-length cDNA encoding the large form of 2–5A can generate a recombinant protein with enzymatic parameters similar to that of the natural p100 (34).

**DISCUSSION**

Here we describe the cloning and characterization of the full-length cDNA encoding the 100-kDa form of human 2’,5’-OAS (p100). This cDNA hybridizes to an interferon-induced 7-kb mRNA and encodes a protein that has an electrophoretic mobility similar to the natural p100 present in interferon-treated human cells. The deduced amino acid sequence of this cDNA contains the sequence of the seven peptides that were microsequenced from the purified p100, thus confirming the identity of the isolated cDNA. The identity between p100 and the recombinant protein produced by the expression of this cDNA was further demonstrated by positive reactivity with the recombinant protein produced by the expression of this cDNA.

Comparison of the sequence of p100 with international data banks like Swissprot revealed that this protein is homologous only to the previously cloned 2’,5’-OASs: human p40/p46 and p69/p71, and murine, rat, and chicken homologues of the small human 2’,5’-OAS (25–28). Recently, we and others have described the cloning of a cDNA encoding a 56-kDa protein (p56), which is highly homologous to the sequence of known 2’,5’-OASs (46, 48). This interferon-induced p56 binds DNA and dsRNA, but is devoid of catalytic activity typical of the 2’,5’-OAS activity of p40/p46, p69/p71, and p100. Accordingly, this p56 was referred to as OAS-related protein, which might have as yet unidentified function(s) (46). Interestingly, although the gene of this OAS-related protein maps on chromosome 12q24.2, it is localized outside the 2’,5’-OAS locus containing p40/p46, p69/p71, and p100.5

The homology between the different domains of p69/p71 and p100, with the first 346 amino acids common in p40/p46, is discontinuous and is characterized by the presence of highly conserved stretches containing 7–14 amino acids (Fig. 3). These conserved motifs probably represent structural motifs essential for the 2’,5’-OAS catalytic activity of these proteins, such as the capacity to bind substrates ATP/GTP and to become activated by dsRNA. The conservation of these motifs in the two domains of p69/p71, and in the three domains of p100, is consistent with their implication as specific signatures of 2’,5’-OAS. The pentapeptide DFLKD in p40 has been reported to represent a part of the ATP binding site, lysine 199 inside this pentapeptide being essential for catalytic activity (50). The sequences corresponding to the position of this pentapeptide in domains I, II, and III of p100 are NFVNI, NFVNI, and NFNI, respectively (Fig. 1B), and in domains I and II of p69/p71 are KFDNI and NFIRS, respectively (28). Therefore, apart from the phenylalanine residues, no other amino acid residue is conserved in comparison with the DFLKD sequence of the potential ATP binding site. Consequently, the role of this sequence as an ATP


![](Image)Fig. 4. Interferon-induced mRNA recognized by the p100 cDNA. Polyadenylated RNA (3 μg) extracted from untreated (lane C) or from interferon-treated (lane IFN α, β, or γ) Daudi cells were resolved on a 1.2% formaldehyde-agarose gel, transferred to a nylon membrane, and hybridized successively with p100 cDNA (cDNA REB2) and glyceraldehyde-3-phosphate dehydrogenase cDNA as αP-labeled probe. Treatment of Daudi cells was at 1000 units/ml for each type of interferon for 8 h. The molecular size profile of RNA markers (in kb) is shown on the left.

Isoforms of the middle-sized 2’,5’-OASs (p69/p71) share a common amino terminus of 683 residues composed of two highly homologous domains I and II, and which share 41 and 53% identity in amino acid sequence, respectively, with the first 346 amino acids common between the two isoforms of the small 2’,5’-OAS (p40/p46). The results presented here have demonstrated that p100 is composed of three adjacent domains that share 44–60% sequence similarity with each other, and each domain is homologous to the first 346 amino acids in p40/p46 (Figs. 2–4). In view of the observation that the human OAS forms p40/p46, p69/p71, and p100 contain 1, 2, and 3 conserved OAS domains or units, respectively, we have proposed designating these three forms of related enzymes as OAS1 for p40/p46, OAS2 for p69/p71, and OAS3 for p100. Recently, we showed that the genes encoding the three forms of 2’,5’-OASs are clustered on chromosome 12q24.2 within a region of 130 kb, which represents the 2’,5’-OAS locus. They share the same orientation of transcription and are arranged in the order centromere–5’–OAS1–OAS3–OAS2–3’–telomere (41). The clustering of these genes, and the demonstration that the small, middle and large forms of 2’,5’-OAS contain increased numbers of the 2–5A functional unit, suggest their evolutionary relationship, possibly through the duplication of the conserved functional domain, *i.e.* the conserved OAS unit. The characterization of the events leading to the emergence of the 2’,5’-OAS family requires detailed studies on the exon-intron organization of these genes, with the aim to better understand their evolutionary relationship.
binding domain is questionable. Indeed, recent evidence indicates that mutation of lysine 199 in the pentapeptide DFLK199Q in p40 generates an active enzyme when the mutant protein is expressed in insect cells but not when it is expressed in Escherichia coli (51). Thus, the mutant protein is generated as an active enzyme in higher eukaryotic cells, probably due to appropriate modifications or folding of p40.

A critical characteristic of human 2',5'-OAS is oligomerization. By gel filtration experiments, we have previously demonstrated that p40/p46 exist as tetramers, p69/p71 as dimers, and p100 as monomers. Recently, mutations in the tripeptide CFK in the murine analog of p40 have been reported to abolish the capacity of the enzyme to tetramerize along with loss of catalytic activity (52), thus pointing out that oligomerization of p40 OAS is essential for its enzymatic activity. In the p69/p71 sequence, such a tripeptide is conserved in domain II (position 668–671) but not in domain I (28), a situation that could be sufficient for the dimerization of p69/p71 OAS. Indeed, deletion of this conserved CFK motif in domain II of p69 results in enzyme inactivation and lack of dimerization (53). Search for the CFK motif in p100 revealed that the amino acid sequences at the corresponding position in domains I, II, and III are CFL, CFL, and CCM, respectively (Fig. 1). Thus, the CFK motif is not conserved in p100, and consequently this difference could account for its lack of oligomerization. As p40/p46 and p69/p71 exist as tetramers and dimers and manifest common catalytic parameters, we have postulated that the 2',5'-OAS activity requires the presence of four catalytic domains, which can be provided by four molecules of p40/p46 and two molecules of p69/p71. The presence of only three 2–5A domains in p100 and its lack of oligomerization could account for the differences observed when compared with the other two 2',5'-OASs. Indeed, unlike p40/p46 and p69/p71, which synthesize preferentially higher oligomers of 2–5A, p100 preferentially catalyzes the synthesis of 2–5A dimers. Moreover, p100 is activated at lower concentrations of dsRNA compared with p40/p46 and p69/p71. Furthermore, pH optimum for the activation of p100 is 7.5, whereas that for the other forms is 6.5 (34).

Besides a possible role in mediating resistance to virus infection (19, 20, 54–58), the 2–5A system (the OASs, 2–5A, and RNase L) has also been implicated in the control of cell growth,
The recombinant p69 (panel 3) and p100 (panel 4) from interferon α-treated HeLa cells were immunoprecipitated using monoclonal antibodies specific to the FLAG and V5 epitope, respectively. The natural p69-FLAG (panel 1) and p100-V5 (panel 2) of the 100-kDa 2′,5′-OAS reaction mixture containing [γ-32P]ATP in the absence (lanes −) or presence (lanes +) of 100 μg/ml poly(I)poly(C). The 32P-labeled 2′-5A products were then analyzed by 20% polyacrylamide gel electrophoresis containing 7 M urea. The positions of dimer and higher oligomers of 2′-5A produced by p100 or p69 were quantified as described previously (34). Spots corresponding to the dimer and tetramer were excised and counted by a PhosphorImager. The values are expressed as the percentage of the dimeric forms of 2′-5A.

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Tripartite Composition of the 100-kDa 2′,5′-OAS

The 100-kDa 2′,5′-Oligoadenylate Synthetase Catalyzing Preferentially the Synthesis of Dimeric pppA2 2′p5′A Molecules Is Composed of Three Homologous Domains
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