Production and Purification of Recombinant 2′-5′ Oligoadenylate Synthetase and Its Mutants Using the Baculovirus System[†]

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ABSTRACT: Investigation of the structure−function relationship of the 2′-5′ oligoadenylate [2-5 (A)] synthetases has been hampered by the lack of an efficient expression system for a recombinant enzyme. Here, we report that the 9-2 isozyme of murine 2-5 (A) synthetase can be efficiently expressed in insect cells using the baculovirus system. The recombinant protein was purified to apparent homogeneity, and its enzymatic activity was characterized. It had a high specific activity, required double-stranded RNA as a cofactor, and synthesized dimers to hexamers of 2-5 (A). The utility of our expression system was demonstrated by studying the properties of two previously reported mutant proteins. Both of these mutants, when produced in bacteria, are enzymatically inactive, although similarly produced wild-type protein is active. Unexpectedly, when expressed in insect cells, both mutant proteins were enzymatically as active as the wild-type protein. These results suggest that in the eukaryotic expression system described here, the mutant proteins can undergo appropriate modifications or folding that is required for attaining an enzymatically active conformation.

2′-5′ Oligoadenylate [2-5 (A)][1] synthetases are a family of enzymes that polymerize ATP into a series of 2′-5′-linked oligoadenylates (*1*, *2*). All of these enzymes require double-stranded (ds) RNA as a cofactor, and their synthesis is induced by treatment of cells with interferons. The 2-5 (A) molecules can activate a latent ribonuclease, RNase L, by causing its dimerization (*3*). In an interferon-treated cell, virus infection causes the production of dsRNA, consequent production of 2-5 (A), activation of RNase L, and degradation of RNA (*4*). Thus, the 2-5 (A) synthetase/RNase L pathway can prevent viral replication by activating a cellular RNA-degradation pathway.

2-5 (A) synthetases fall into three size classes: large, medium, and small (*2*). The different isozymes are structurally related. In humans, there is one large synthetase of 100 kDa, two medium synthetases of 69 and 71 kDa, and two small synthetases of 46 and 40 kDa (*5*). The two members of each of the small and medium family are the products of alternatively spliced mRNAs transcribed from the same gene. Similarly, in mouse, 9-2 and 3-9 are two small synthetases originating from the same gene, whereas L-3 is a different small synthetase encoded by a separate gene (*6*, *7*). There is very high sequence conservation across the species (*6*). For example, the human small synthetases and the murine 9-2/3-9 synthetases have almost identical sequences except for the carboxyl terminal regions that are encoded by the alternatively spliced regions. The different isozymes of 2-5 (A) synthetase reside in different subcellular compartments. This differential distribution is dictated by isozyme-specific posttranslational modifications in some cases. The small synthetases exist as tetramers, the medium as dimers, and the large as monomers (*8*).

We have been studying the structure−function relationship of the murine 9-2 synthetase whose cDNA we cloned (*6*, *9*). For these studies, we have used in vitro reticulocyte lysate system and *Escherichia coli* for expressing wild-type and mutant synthetases. We have shown that a mutant, P7Q, of this isozyme is enzymatically inactive although it can tetramerize and bind ATP and dsRNA (*10*). Similarly, Kon and Suhadolnik (*11*) have reported that mutation of the lysine residue at position 199 of the human small synthetase inactivates the protein. This was presumably because of the requirement of the lysine residue for formation of the active enzyme complex.

Structural and mutational studies of 2-5 (A) synthetase have been hampered by the lack of a good expression system of the recombinant enzymes. Although expression of the human small isozyme in *E. coli* was reported 9 years ago (*12*), only recently was it purified to apparent homogeneity using an affinity tag (*11*). The bacterial expression system is, however, not well-suited for studying this family of enzymes for a variety of reasons. High-level expression and purification from the bacterial system are difficult, important posttranslational modifications of the recombinant proteins are not achieved, and, as we report here, spurious conclusions are reached about the functional abilities of some mutants.

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[1] Abbreviations: 2-5 (A), 2′-5′ oligoadenylate; dsRNA, double-stranded RNA; PKR, protein kinase, RNA-activated; poly(I)·poly(C), polyinosinic acid·polycytidilic acid; PCR, polymerase chain reaction; Wt, wild-type; bp, base pair.

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Expression in mammalian cells has, however, been ineffective because of the activation of the RNase L pathway and consequent anticellular actions. Here we report that the 9-2 isozyme can be expressed to high levels in insect cells using the baculovirus system. The recombinant enzyme was purified to homogeneity. It had a high specific activity and was totally dependent on dsRNA. Using the insect cell expression system, we demonstrated that two mutant proteins are as active as the wild-type protein although the same mutants are inactive when expressed in bacteria.

MATERIALS AND METHODS

Reagents. Reagents for preparing recombinant baculovirus and for expression of insect cells were purchased from Gibco-BRL. Immobilon PVDF membrane was from Millipore, peroxidase-conjugated antibody was from Boehringer Mannheim, and enhanced chemiluminescence kit was purchased from Amersham. The 2-5 (A) antibody was a gift from Dr. Yoshihiro Sokawa (*13*). Radiolabeled ATP was from New England Nuclear Labs, PEI cellulose plates were from EM Scientific, poly(I)·poly(C) and Sephacryl S-300 were from Pharmacia, and phosphocellulose was from Whatman.

Cells and Viruses. Spodoptera frugiperda cells were used for propagation of viruses and *Tricoplusia ni* cells were used for protein expression. The cells were cultured at 27 °C in Grace's supplemented insect media containing 10% heat-inactivated fetal bovine serum. Wild-type baculovirus (Ac-MnPV) was purchased from Gibco-BRL.

Expression of 9-2 Protein in Insect Cells. The Gibco-BRL protocol was followed for recombinant baculovirus production. The hexahistidine-tagged 9-2 cDNA (*10*) was cloned at the *Xba*I-*Xho*I sites in PFastBac plasmid. The recombinant plasmid DNA was used to transform DH10 Bac cells. Colonies containing the recombinant plasmids were identified by the loss of β-galactosidase activity. High-molecular-weight DNA was prepared from the bacterial cells harboring the recombinant plasmid. *S. frugiperda* (Sf 21) cells were transfected with that DNA using CellFectin reagent. The culture media were collected as a source of the recombinant virus. The virus stock was maintained at −70 °C following virus titer measurement using plaque assay technique. For protein production, monolayer cultures of *T. ni* (High Five) cells were infected with the recombinant virus at a multiplicity of infection of 10. The cells were further incubated at 27 °C in Grace's supplemented insect cell media (Gibco-BRL) containing 10% fetal bovine serum before harvesting at 40 h. The cells could be stored at this stage at −70 °C for several months. The cells (10^8) were sonicated at 4 °C for 6 min using four 30-s pulses in 4 mL of buffer A (25 mM Hepes, pH 7.4, 10% glycerol, 2 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.5% NP-40, 1 mM PMSF, 2 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin) containing 450 mM NaCl. The cell extract was centrifuged at 30000*g* for 15 min, and the supernatant was stored at −70 °C for use as a starting material for enzyme purification.

Enzyme Purification. The 9-2 protein was precipitated by dialyzing the cell extract (prepared from 10^8 cells, 10 mg of protein) against buffer B (buffer A containing 25 mM NaCl). When the precipitate was resuspended in buffer A containing 450 mM NaCl, more than 80% of the protein became solubilized. This process eliminated some contaminating proteins. The enriched extract was then loaded onto a Sephacryl S-300 column (1.2 cm × 100 cm) equilibrated with buffer A containing 450 mM NaCl; 2-mL fractions were collected after the void volume and assayed for protein and for 2-5 (A) synthetase activity. The 9-2 protein was eluted right after the void volume. The active fractions were pooled and dialyzed against buffer A containing 200 mM NaCl. The dialyzed supernatant was then loaded onto a phosphocellulose column (1 cm × 4 cm) equilibrated with the same buffer and eluted with a linear salt gradient of 0.2−1.0 M NaCl, in buffer A. The 9-2 protein was eluted between 0.3 and 0.4 M NaCl as judged from activity assay and SDS−PAGE analysis. For further purification, active fractions were pooled, dialyzed against buffer A containing 200 mM NaCl, and chromatographed on a smaller phosphocellulose (1 cm × 2 cm) column in a similar fashion as done above. Fractions were assayed for 2-5 (A) synthetase activity and checked for homogeneity by SDS−PAGE analysis. The purified fractions were pooled, dialyzed against buffer A containing 0.3 M NaCl and 25% glycerol, and stored in 1.5 mL of buffer at −20 °C. The enzyme remained fully active even after 6 months under this condition.

Enzyme Assay. The 2-5 (A) synthetase assay was performed following an earlier method (*6*, *10*) with minor modifications. In a typical 10-μL reaction mixture, unless otherwise stated, the enzyme (1.0 μg/mL) was incubated with 20 mM Tris-HCl, pH 7.4, 20 mM magnesium acetate, 2.5 mM DTT, 5 mM ATP, 5 μCi of [α-^{32}P]ATP, and 50 μg/mL poly(I)·poly(C) for 1 h at 30 °C. The reaction was stopped by heating (95 °C, 5 min), and the mixture was treated with calf intestinal alkaline phosphatase and analyzed by thin-layer chromatography as described previously (*6*). For analysis of the different oligomeric forms of 2-5 (A), either [α-^{32}P]- or [γ-^{32}P]ATP (5 μCi/sample) was used in the reaction mixture and the reaction products were analyzed directly by high-voltage electrophoresis (1600 V) in 20% polyacrylamide gel in the presence of 7 M urea as described earlier (*14*). In all cases, 2-5 (A) oligomers synthesized were quantitated by Phosphorimager analysis and are expressed either as nmol of ATP polymerized/mL or as arbitrary units.

SDS−PAGE and Immunodetection. SDS−polyacrylamide gel electrophoresis was performed under reducing conditions on 10% gel as described previously (*6*). For the immunodetections of 9-2 protein in crude extracts as well as in purified preparations, the method described earlier (*9*, *10*) was used with minor modifications. The blotted membrane was washed with Tris-buffered saline containing 0.1% Tween 20 (TBST). The peroxidase-conjugated goat anti-mouse IgG was used at a 1:2000 dilution.

Site-Directed Mutagenesis. Generation of the P7Q mutant has already been described (*10*). The K199R mutant was generated as follows by polymerase chain reaction (PCR) using four oligonucleotide primers. Primer 1 was a sense primer spanning nucleotides 542−559 upstream of the *Pst*I site at 561 (*6*). Primer 2 was an antisense primer corresponding to nucleotides 604−637. This primer carried the mutation from AAG to AGG for converting the lysine residue encoded by nucleotides 618−620 to an arginine residue. Primer 3 was a sense primer spanning nucleotides 621−637, and primer 4 was an antisense primer spanning nucleotides 731−747 downstream of a *Hin*dIII site at nucleotide 687.

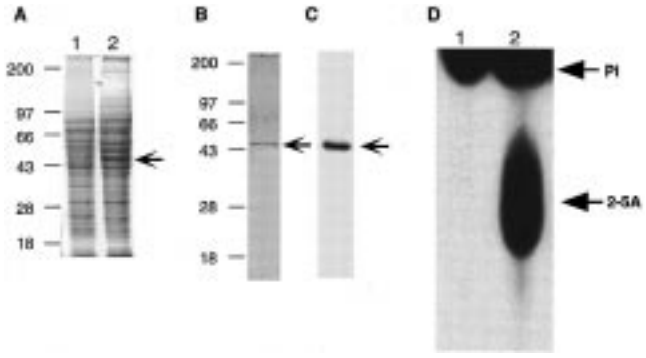

FIGURE 1: Purification of recombinant 9-2 protein. The 9-2 protein was expressed using the baculovirus expression system and purified as described. (A) Coomassie brilliant blue staining pattern of proteins present in crude extracts (~5 μg) of insect cells infected with control vector AcMnPV (lane 1) and with vector containing the 9-2 cDNA (lane 2). The arrow on the right indicates the position of the 9-2 protein. The numbers on the left show the mobilities of molecular weight markers in kDa. (B) Silver staining pattern of purified protein. The arrow on the right indicates the position of the protein. The numbers on the left show the mobilities of molecular weight markers in kDa. (C) Western blot analysis of purified 2-5 (A) synthetase using a monoclonal antibody (*13*). The arrow on the right indicates the position of the protein. (D) Enzyme activity of the purified protein: lane 1 no protein; lane 2 purified protein. The arrows indicate the positions of 2-5 (A) oligomers and phosphates.

The first round of PCR was performed in two separate tubes using the Wt 9-2 cDNA as the template and primers 1 and 2 in one tube and primers 3 and 4 in the other. The incubation mixtures were heated to 95 °C for 5 min followed by 30 cycles of 95 °C for 1 min, 42 °C for 1 min, and 72 °C for 1 min followed by 72 °C for 10 min. The products (94 and 124 bp) were purified by gel electrophoresis followed by extraction using the QIAEX gel extraction kit (QIAGEN). Equal amounts of the two products were mixed and used for the second PCR using primers 1 and 4 and the same incubation conditions as described above. The final PCR product (208 bp) was purified by gel electrophoresis and cloned in the TA vector (Invitrogen). The recombinant clones were digested with *Pst*I and *Hin*dIII, and the 122-bp insert was cloned in the corresponding sites of Wt 9-2 in PET15b that had been digested with the same enzyme to eliminate the corresponding Wt fragment. The final recombinant clone was sequenced to confirm the presence of the desired mutation. Both P7Q and K199R mutants were subsequently cloned into PFastBac and expressed as hexahistidine-tagged proteins.

RESULTS

Production and Purification of the Recombinant 9-2 Isozyme. Hexahistidine-tagged 9-2 cDNA was cloned in the genome of the baculovirus and the recombinant virus was used to infect insect cells for protein production. When the infected cell extract was analyzed by gel electrophoresis followed by Coomassie blue staining, we observed a major polypeptide of approximately 46 kDa, the expected size of the recombinant 9-2 product (Figure 1A). This band was absent in the extract of uninfected cells and it specifically reacted with a monoclonal antibody to 2-5 (A) synthetase (*13*). The infected, but not the uninfected, cell extract also had a high 2-5 (A) synthetase enzyme activity. For monitoring purification of the recombinant protein, we used both enzyme activity and protein staining assays.

Table 1: Purification of 9-2 Protein

fraction	total protein (μg)	sp activity (nmol of ATP polymerized/ μg/h)	purification (-fold)	yield (%)
crude extract	10000	0.85	1	100
Sephacryl S-300	550	10.63	12.5	68
phosphocellulose (first)	35	100	125	41
phosphocellulose (second)	15	170	200	29

We put the hexahistidine tag on the amino terminus of the 9-2 protein with the purpose of using the tag for affinity purification of the recombinant protein. Unfortunately, affinity purification of the protein on Ni columns was not efficient: only a small fraction of the native protein bound to the affinity resin. However, the binding was very efficient when the protein was denatured in the presence of urea. These results suggest that the amino terminus of the 9-2 protein may be buried in the native enzyme which is known to be tetrameric. In view of the failure of affinity purification, we resorted to purifying the recombinant protein using conventional chromatographic procedures. The infected cell extract was dialyzed against a buffer containing a low NaCl concentration (25 mM). A precipitate containing the 9-2 protein was recovered from the dialysis tube and dissolved in the high-salt buffer. The enriched extract was chromatographed on a Sephacryl S-300 column. Fractions containing the recombinant protein were pooled, dialyzed, and absorbed to a phosphocellulose column, which was eluted with a linear gradient of 0.2−1 M NaCl. The recombinant protein was eluted between 0.3 and 0.4 M NaCl. For further purification, fractions containing the 9-2 protein were pooled, dialzyed, and rechromatographed on a smaller phosphocellulose column. Fractions containing the 9-2 protein were pooled and stored as purified recombinant 9-2 protein. A 200-fold purification of the activity was obtained (Table 1). As shown in Figure 1B, silver staining revealed the presence of only one polypeptide of 46 kDa which reacted strongly with monoclonal antibody to synthetase (Figure 1C). Thus, we were able to purify the recombinant protein to apparent homogeneity. The purified protein was highly active as shown in Figure 1D. The specific activity of the purified protein was 170 nmol of ATP polymerized/μg/h.

Characterization of Purified Recombinant Enzyme. In the next series of experiments we examined the enzymatic characteristics of the purified 9-2 protein. Linear enzyme reaction was observed over 200 min (Figure 2A), and the amount of product accumulation increased with increasing enzyme concentration (Figure 2B). There was no appreciable change in enzyme activity over the pH range of 6.5−8.0 (data not shown). For determining the substrate affinity and its utilization rate, reactions were performed with increasing ATP concentrations (Figure 3A). By appropriate analysis of the data, we calculated a K_m value of 1.1 mM, a V_{max} value of 170 nmol/h/mL, and a k_{cat} value of 9.5/s. 2-5 (A) synthetases require dsRNA as a coactivator which presumably changes the conformation of the protein to an active form. To ensure that the recombinant 9-2 protein, purified from insect cells, was similar to the native enzyme in terms of its requirement of dsRNA for activation, the experiment

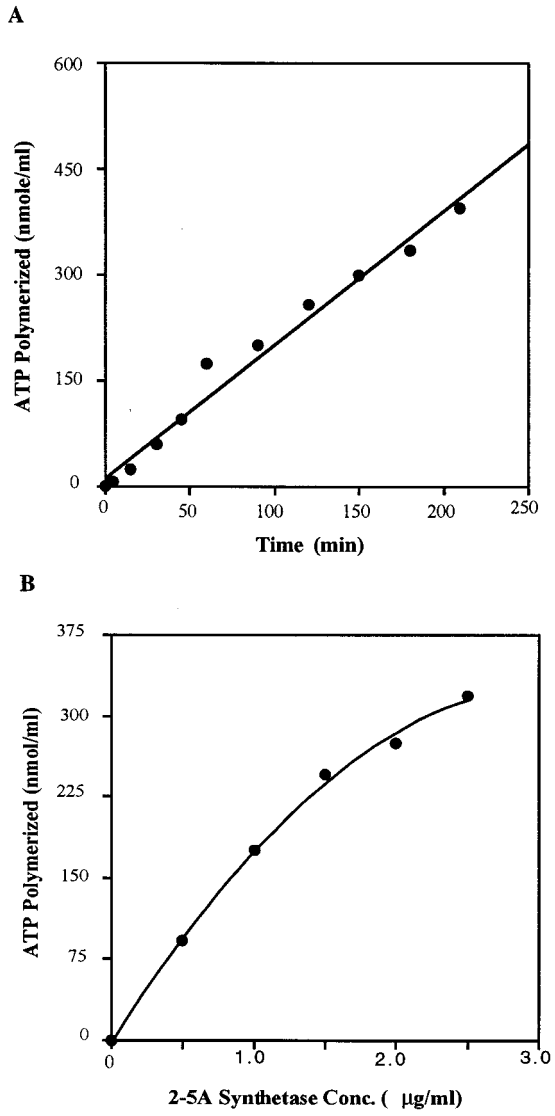

FIGURE 2: (A) Kinetics of 2-5 (A) synthesis; 1.0 μg/mL enzyme was used in this experiment. The results are expressed in nmol of ATP polymerized/mL. (B) Enzyme concentration curve. Different concentrations of 9-2 synthetase were used for this study. Each assay was performed for 1 h.

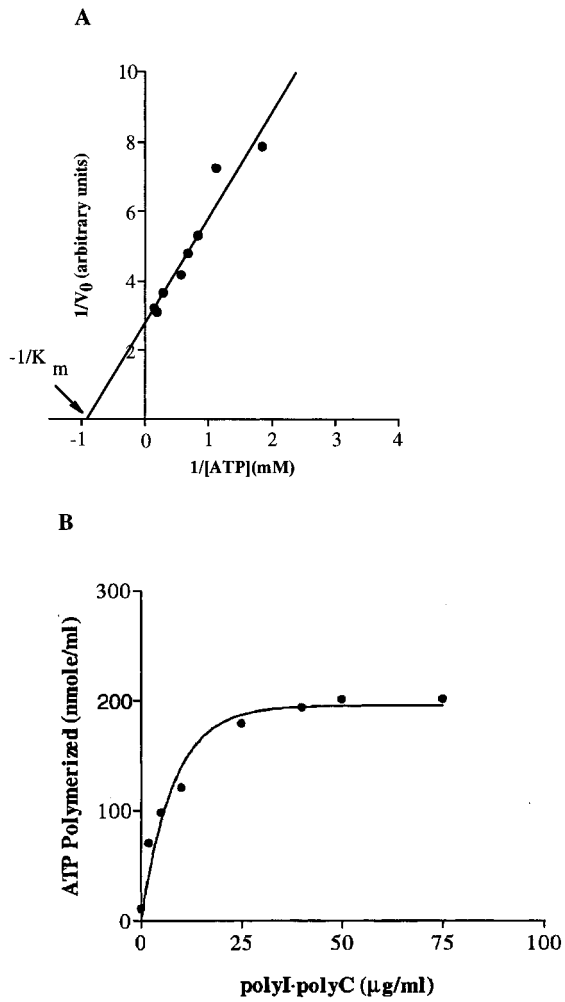

FIGURE 3: (A) Determination of catalytic characteristics. The K_m value for ATP was evaluated from Lineweaver−Burk plot. The synthetase activity was assayed at different concentrations of ATP for 1 h. The enzyme concentration was 1.0 μg/mL for each point. The initial velocity (V_0) is expressed as arbitrary units. The maximum velocity (V_{max}) of 9-2 synthetase is 170 nmol/h/mL, the Km value for the enzyme is 1.1×10^{-3} M and the K_{cat} value is 9.5/s. (B) Effects of increasing dsRNA concentrations. 2-5 (A) synthetase activity at an enzyme concentration of 1.0 μg/mL was assayed in the presence of increasing concentrations of poly(I). poly(C).

shown in Figure 3B was performed. The enzyme was inactive without dsRNA, and 25 μg/mL poly(I)·poly(C) was saturating in activating the enzyme although even 1 μg/mL was enough to observe substantial activation of the enzyme.

The 2-5 (A) synthetases are known to oligomerize a series of 2-5 (A) molecules with increasing chain lengths. For determining the sizes of the products, we analyzed them by denaturing gel electrophoresis (Figure 4). During the relatively short incubation times used for experiments shown so far, the predominant product was a dimer (Figure 4B). However, with increasing time, higher oligomers started accumulating. After 18 h of incubation, up to hexamers of 2-5 (A) could be observed. That all three phosphates of the acceptor ATP are retained in the products was confirmed by the experiment shown in Figure 4A: similar product profiles were observed using either [α-^{32}P]-ATP or [γ-^{32}P]-ATP.

Properties of the P7Q and the K199R Mutants Produced in Insect Cells. We have previously reported that deletion of residues 1−9 inactivates the 9-2 isozyme (*10*). Within this deleted region, residue 7 is the critical one. Replacement of the proline residues at that position by any of a number of other residues partially or totally abolished the enzymatic activity. The P7Q mutant produced by in vitro translation or by expression in *E. coli* is enzymatically inactive. However, the mutant protein is otherwise normal: it can bind ATP and dsRNA, and it can oligomerize. In the experiment shown in Figure 5, hexahistidine-tagged P7Q mutant was produced in insect cells. As shown in Figure 5A, equal amounts of Wt and P7Q proteins were expressed in cells infected with the respective recombinant viruses whereas there was no 9-2 protein in cells infected with the natural virus without any cDNA insert. Surprisingly, the mutant protein was as active as the Wt protein (Figure 5B,C). To rule out the possibility that the viral insert might have reverted back to the Wt sequence, DNA from the mutant virus was directly sequenced and it was confirmed that it still carried the P7Q mutation (data not shown). Thus, we demonstrated that the mutant protein, P7Q, was enzymati-

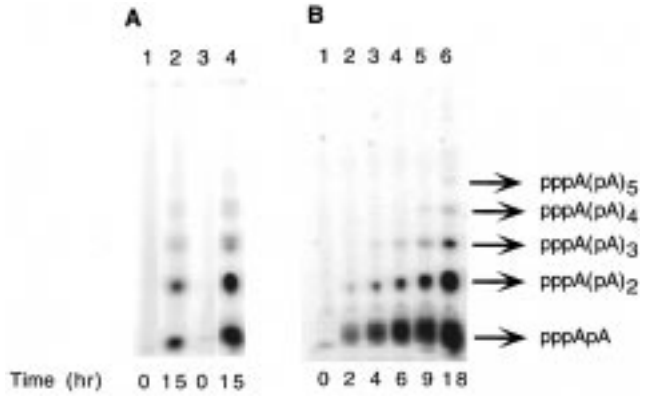

FIGURE 4: Determination of product size. The 2-5 (A) oligomers were directly analyzed by polyacrylamide gel electrophoresis. (A) Autoradiogram of activity assay for 0 h (lanes 1 and 3) or 15 h (lanes 2 and 4) using either [γ-^{32}P]ATP (lanes 1 and 2) or [α-^{32}P]-ATP (lanes 3 and 4). B. An autoradiogram showing the synthesis of 2-5(A) products at different times. The position of each product is indicated on the right.

cally active when produced in insect cells, although the same protein is inactive when expressed in *E. coli*.

Kon and Suhadolink (*11*) have identified the ATP-binding domain of an human isozyme of 2-5 (A) synthetase which is virtually identical in sequence to the murine 9-2 isozyme that we have studied here. They demonstrated that 8-azido-ATP can cross-link to the lysine residue present at position 199, thus establishing it as a crucial residue for enzyme activity. When the K199R mutant protein was expressed in *E. coli*, it was enzymatically inactive although it still bound ATP. We expressed the same mutant, K199R, in the context of the 9-2 isozyme, in insect cells (Figure 6). Similar amounts of hexahistidine-tagged Wt and K199R proteins were synthesized (Figure 6A), and both proteins were equally active in synthesizing 2-5 (A) (Figure 6B,C). These results support our conclusion that mutants that are inactive when produced in *E. coli* can be active when produced in higher eukaryotic cells such as the insect cells.

DISCUSSION

2-5 (A) synthetases are interesting enzymes for a variety of reasons: in vivo their actions initiate a cascade of cellular actions, they require dsRNA as a cofactor, and they are the only known nucleotide polymerases which catalyze 2′-5′ phosphodiester bond formation (*1*, *2*). Despite these novel enzymatic features, structural information about these proteins is scanty. Earlier we identified the amino terminal region to be responsible for dsRNA binding (*6*). This region, however, does not contain the dsRNA-binding motif present in another class of dsRNA-binding proteins represented by the interferon-induced protein kinase, PKR (*15*−*18*). Thus, 2-5 (A) synthetases belong to a separate and distinct class of dsRNA-binding proteins whose dsRNA-binding domain remains to be critically defined. Similarly, the ATP-binding domain of these enzymes is also not easily discernible. They do not contain the ATP-binding motif present in various proteins kinases and ATPases. Recently, using photoaffinity labeling, Kon and Suhadolnik (*11*) have identified the ATP-binding residue of the human 40-kDa isozyme. Surprisingly, mutation of the critical lysine residue, however, did not abolish ATP binding by the mutant protein.

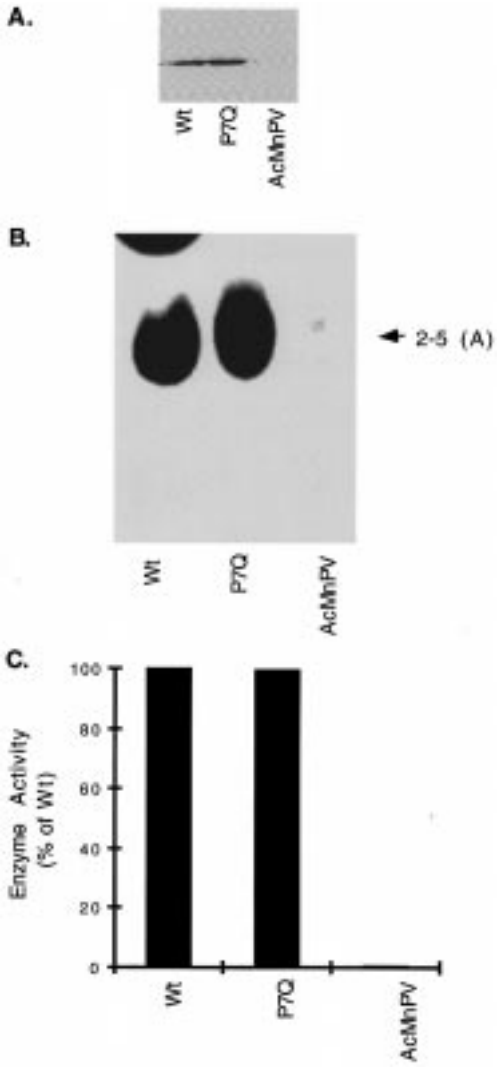

FIGURE 5: Enzyme activity of the P7Q mutant expressed in insect cells. The Wt and P7Q mutant were expressed in the insect cell. (A) Equal amounts of the two proteins were expressed as shown by Western analysis. There was no cross-reacting protein in the extract of cells infected with the empty virus. (B) Enzyme activity assay using extracts from cells infected with Wt virus (Wt), mutant virus (P7Q), and empty virus (AcMnPv). (C) Quantitation of the activity assay shown in B.

One of the main impediments in studying the structure−function relationship of 2-5 (A) synthetase has been the absence of an expression system for the recombinant enzymes. The natural enzymes are difficult to produce in large quantities because they need to be induced by interferon treatment. Once induced, they are difficult to purify because many isozymes are induced in the cell at the same time and the activity assay cannot distinguish between them easily. Thus, production of a recombinant enzyme is ideal for its purification in large quantities. Moreover, specific mutant proteins can only be produced by expressing a cDNA clone. In the past, we and others have used *E. coli* for expressing these enzymes (*10*−*12*). However, the yields are poor, the specific activity of the protein is low, and as discussed below, properties of mutant proteins are affected negatively. Expression of these enzymes in transfected mammalian cells, on the other hand, has been difficult. In contrast, the insect cells used in the current study could produce a high level of active 9-2 protein. Since the baculovirus/insect cell system

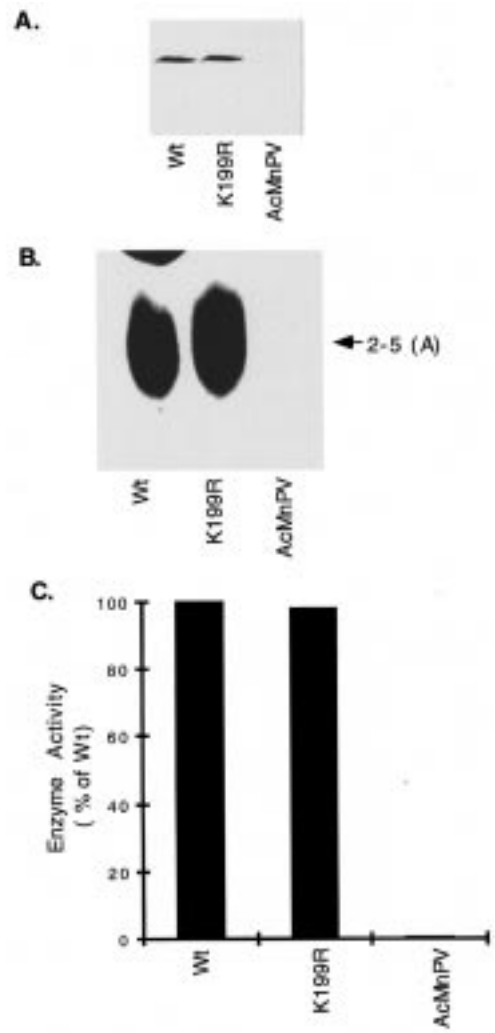

FIGURE 6: Enzyme activity of the K199R mutant expressed in insect cells. The analyses were done and the results are presented as described for Figure 5.

is a highly efficient system for the production of recombinant proteins, it should be an ideal source for 2-5 (A) synthetases and their mutants. Enough recombinant proteins can be produced by this system to initiate structural studies using X-ray crystallography or NMR analysis.

Purified 9-2 protein produced in insect cells had appropriate enzyme characteristics, thus ensuring its usefulness. The specific activity of this enzyme was at least 6-fold higher than that reported for the human 40-kDa protein produced in *E. coli* (*11*). The recombinant protein was not constitutively active, and it still required dsRNA for activation. In contrast, PKR produced in *E. coli* is often constitutively active and does not require dsRNA. Our conclusion that 25 μg/mL poly(I)·poly(C) was optimum for activating the recombinant enzyme is consistent with previous reports (*11*, *12*). The K_m value obtained for the reaction was also consistent with the corresponding published values for other isozymes (*19*, *20*). The product profile was quite distinct from those of the medium and large isozymes (*5*, *8*). Even with prolonged incubation, the recombinant 9-2 enzyme synthesized mostly dimers and trimers, and the largest product detected was a hexamer. In contrast, the medium synthetases can synthesize up to 22-mers of 2-5 (A) under identical incubation conditions (our unpublished data). Whether the different product profiles of the two classes of isozymes affect their cellular actions, is an open question. For RNase L activation in vitro, all products from trimer onward are equally active.

The properties of the two mutant proteins produced in insect cells were unexpected. They were enzymatically as active as the wild-type protein, suggesting that the introduced mutations did not affect the structure of the protein in a way that destroys its catalytic property. Since we did not determine all the enzymatic characteristics of purified mutant proteins, it remains possible, however, that there are subtle differences between the properties of the wild-type and the mutants. When we studied the P7Q mutant synthesized in vitro or in *E. coli*, although it was enzymatically inactive, it retained the properties of binding dsRNA and ATP and it could oligomerize (*10*). Similarly, the K199R mutant, produced in *E. coli*, although inactive, could bind ATP (*11*). These observations suggest that the mutant proteins have subtle differences when produced in eukaryotic cells, such as the system described here, and in bacteria. The putative difference could be in the proper folding of the protein to acquire an active conformation. The difference could also be in the potential posttranslational modifications of the protein which are known to be different in eukaryotic and prokaryotic cells. Sequence analysis reveals potential sites for phosphorylation, glycosylation, and isoprenylation of the 9-2 protein. Whether these modifications occur, however, remains to be examined. The critical role of the expression system on the activity of the mutant protein was confirmed by the expression of the P7Q mutant in mammalian cells. The P7Q mutant expressed in COS cells was active (data not shown). Further investigations will be needed to identify the crucial difference between the prokaryotic and eukaryotic expression systems that affects the activity of the mutants.

It appears that the difference between the two expression systems, be it protein folding or posttranslational modification, affects the mutants only and not the wild-type protein. The above conclusion could, however, be misleading since the recombinant enzyme purified from *E. coli* has a much lower specific activity than the protein purified from insect cells (*11*). Thus, it is possible that the putative lack of a posttranslational modification also affects the activity of the wild-type protein produced in bacteria, and the introduced mutations could somehow exaggerate this effect. Irrespective of the exact nature of the underlying biochemical mechanism responsible for active enzyme production, the current study clearly shows that, for expressing 2-5 (A) synthetases, the baculovirus system is preferable to the bacterial system, because it produced wild-type enzyme with a higher specific activity. But more importantly, this study showed that mutations identified as deleterious using the bacterial system are indeed innocuous in eukaryotic cells, the natural environment for these enzymes. Thus, in retrospect, the inadvertent but erroneous conclusion made that K199 is absolutely required for catalysis could have been avoided if the mutant had been tested in a eukaryotic cell. The insect cell system, coupled with the bacterial expression system, could be extremely useful, in the future, in uncovering as yet unknown modification of this important class of proteins and in elucidating how these putative modifications affect the enzyme activity.

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