Natural Mutations in a 2′−5′ Oligoadenylate Synthetase Transgene Revealed Residues Essential for Enzyme Activity†

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ABSTRACT: Unlike other RNA polymerases, 2′−5′ oligoadenylate synthetases, a family of interferon-induced enzymes, catalyze the formation of 2′−5′, not 3′−5′, phosphodiester bonds. Moreover, to be active, these proteins require double-stranded RNA as a cofactor. We have been identifying the specific residues of these proteins that impart their novel properties. Here, we report the identity of three such residues that underwent natural mutations in a transgenic mouse line. When deliberately introduced into recombinant proteins, each of these mutations rendered the protein enzymatically inactive. In an effort to understand the roles of these residues in enzyme activity, new mutants carrying other residues in one of these three sites were generated. Detailed characterization of the properties of the mutant proteins revealed that Lys 404 is needed for proper binding of the acceptor substrate, Pro 500 provides structural flexibility to the protein, and Ser 471 is probably required for its proper folding. This study illustrates the power of using natural mutations in transgenes as guides for studying structure−function relationships of proteins.

The 2′−5′ oligoadenylate [2−5 (A)][1] synthetases (OAS) are a family of interferon-induced proteins that, upon activation by double-stranded (ds) RNA, polymerize ATP to 2−5 (A) (*1*−*4*). The 2−5 (A)s, in turn, activate a latent ribonuclease, RNase L. Activated RNase L can degrade cellular and viral RNAs and inhibit protein synthesis (*5*). In humans, three sets of genes on chromosome 12 encode three size classes of these proteins: OAS1−OAS3 (*6*). Within each size class, multiple members arise as a result of alternate splicing of the primary transcript. Recently, it has been shown that one of the alternatively spliced isoforms of OAS1, E17 (previously known as 9-2 isozyme), can act as a pro-apoptotic protein of the Bcl-2 family (*7*). The enzymatic properties of the members of three size classes vary considerably in terms of the length of 2−5 (A) oligomers that are synthesized. The OAS1 isozymes synthesize only up to hexamers of 2−5 (A) (*8*), whereas the OAS2 isozyme, P69, can synthesize up to 30-mers of 2−5 (A) (*9*). The OAS3 isozyme, P100, makes mostly dimers of 2−5 (A) (*10*). Since dimeric 2−5 (A)s cannot activate RNase L, the biological role of P100 remains elusive. Another difference between the three size classes of isozymes is their oligomeric protein compositions: the OAS1 isozymes are functional only as tetrameric proteins; on the other hand, the OAS2 isozyme must dimerize for enzymatic activity and the OAS3 isozyme functions as a monomer (*11*, *12*).

We are interested in understanding the structure−function relationship of 2−5 (A) synthetases and have focused on the OAS2, P69 isozyme. Previously, we have identified the catalytic site, the acceptor binding site, and the donor binding site of P69 (*13*). These sites are highly conserved in other isozymes, and mutations in any of these three sites inactivate the enzyme. We have also identified the dimerization domain of P69 and reported that its mutation inactivates the protein. P69 is active only as a dimer because it catalyzes the joining of the acceptor substrate bound to one subunit to the donor substrate bound to the other subunit. Many of the general conclusions drawn from our mutagenesis studies have been supported by the information obtained from the recently determined crystal structure of an OAS1 isozyme (*14*).

Here we report the identities and the function of three additional residues that are essential for OAS activity. These residues were naturally mutated in a transgenic mouse line that we generated in the course of testing the biological activities of OAS1 isozyme E17 (*15*). Because the mutant protein, expressed in transgenic mice, was enzymatically inactive, we investigated the nature of its defect. From the results presented in this report, we conclude that each of these mutated residues, Lys 404, Pro 500, and Ser 471, serves an essential function in maintaining the enzyme activity of the protein.

EXPERIMENTAL PROCEDURES

Isolation of the OAS Mutants from a Transgenic Mouse. Production of transgenic mice expressing E17 isozyme has been described by Gomos et al. (*15*). The 2−5 (A) synthetase activity assays were performed on testes extracts after partial purification of the FLAG-tagged E17 by anti-FLAG antibody agarose (Sigma, St. Louis, MO) binding. The expressed

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[1] Abbreviations: 2−5 (A), 2′−5′ oligoadenylate; poly(I)·poly(C), polyinosinic acid·polycytidilic acid; dsRNA, double-stranded RNA; Wt, wild-type; moi, multiplicity of infection.

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Table 1: Kinetic Constants for All Mutants (kinetic data are means from three experiments)

protein	K_m (ATP) (mM)	k_{cat} (s^{-1})	activity (% of Wt)
Wt	1.9	6.4	100.0
K404G	4.9	0.4	6.3
K404A	5.4	5.2	81.3
K404E	no data	no data	<0.1
S471L	no data	no data	<0.1
S471A	2.6	5.6	87.5
P500L	1.6	0.5	7.8
P500A	3.1	3.2	50.0
P500G	1.6	1.1	17.2
S499A	1.7	0.3	4.7

transgenes were cloned by RT-PCR from line 5 and line 9. Briefly, total RNA from testes of two mouse lines was extracted using RNA-B (Tel-Test Inc., Friendswood, TX) according to the manufacturer's instructions. Reverse transcription was performed with random hexamers using the Superscript First Strand Synthesis for RT-PCR kit (Invitrogen, Carlsbad, CA). The entire coding region of the E17 transgene, including a portion of the PGKII promoter and the bovine growth hormone poly(A) tail, was amplified by polymerase chain reaction using Hi-fidelity Taq and then subcloned into the EcoRI and XbaI sites in pBluescriptII KS (Stratagene, La Jolla, CA). The entire coding region of each PCR product was sequenced in both directions by Cleveland Genomics (Cleveland, OH). The sequences obtained from the coding region of both line 5 and line 9 were then compared to the published E17 sequence to identify mutations.

Preparation, Purification, and Characterization of Wild-Type and Mutant P69 Proteins. The K404G, S471L, P500L, and other mutants (Table 1) were constructed by introducing individual mutations into the N-terminally His-tagged wild-type P69 construct by megaprimer PCR using appropriate primers. Production of corresponding baculovirus and purification of mutant proteins have been described previously (*16*). The acceptor (A*), donor (D*) and catalytic (C*) site mutants have been used from a previous study (*13*). All proteins were purified by affinity chromatography using Ni−NTA agarose for His-tagged proteins or anti-FLAG antibody agarose for FLAG-tagged proteins. Methods for enzyme activity measurements, enzyme kinetic parameter calculations, and substrate binding determinations have been described previously (*11*−*13*, *16*).

Complementation Experiments. Heterodimers containing different mutant or wild-type proteins were expressed by simultaneously infecting High Five (H5) cells with two recombinant baculoviruses carrying expression cassettes for different proteins. In the case of single infections, we used virus at a moi of 20, whereas for co-infection experiments, a moi of each virus of 10 was used. Depending on experiments, proteins were either purified by Ni−NTA chromatography or anti-FLAG antibody agarose immunoprecipitation. At the end of the washing, instead of elution of the proteins, beads were finally washed and resuspended in 20 mM Tris-HCl (pH 7.5), 20 mM magnesium acetate, and 2.5 mM DTT. Part of the bead suspension was then boiled in SDS−PAGE loading buffer and analyzed by Western blotting with the appropriate antibody. The quantities of proteins were determined by densitometric scanning, normalized in all samples, and the 2−5 (A) synthetase assay was performed with equivalent amounts of proteins as described previously (*17*).

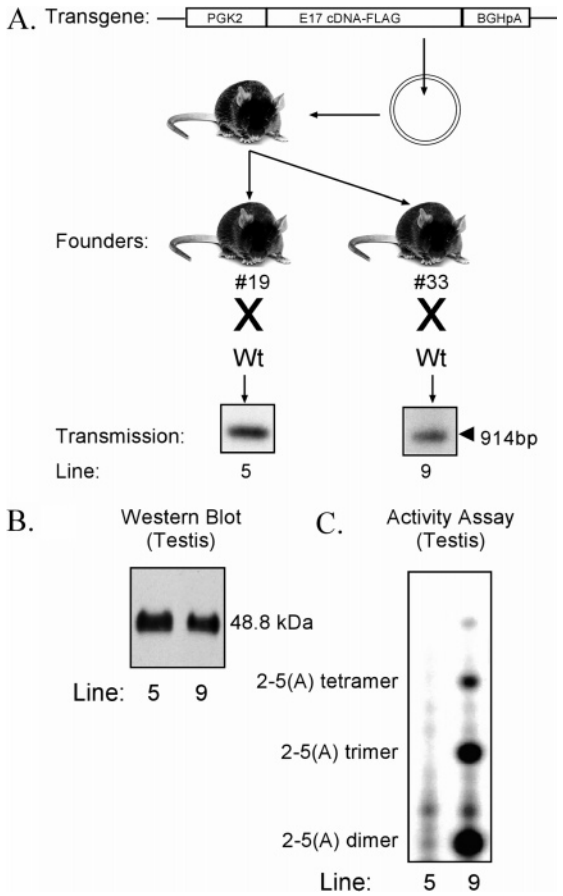

FIGURE 1: Inactive 2−5 (A) synthetase mutant generated from transgenic studies. (A) The establishment of E17 transgenic mouse lines is shown schematically. The 2.45 kb transgene, consisting of the 515 bp human phosphoglycerate kinase II promoter driving expression of the E17 isoform of OAS1, followed by a C-terminal flag epitope tag and bovine growth hormone poly(A) tail, was microinjected into pronuclei of C57Bl6/CBA zygotes. Founder transmission, confirmed by Southern hybridization utilizing the 914 bp E17 cDNA probe, is shown for the two lines of interest: line 9 with Wt 2−5 (A) synthetase activity and line 5 with inactive 2−5 (A) synthetase. (B) Western blot of transgenic line 5 and line 9. The C-terminal FLAG-tagged E17 isozyme expressed in the testes extracts was analyzed with the anti-FLAG M2 antibody. (C) In the activity assay, E17 proteins expressed in these two lines were partially purified with M2 antibody agarose and 2−5 (A) synthetase activity was measured.

RESULTS

Characterization of the Mutations in the OAS1 Transgene. We generated several transgenic mouse lines expressing the E17 (9-2) isoform of OAS1 (*15*). The transgene was driven by the promoter of the PGK2 gene, which is highly expressed in maturing sperm cells (Figure 1A) (*18*). Twelve founder mice were established, among which only nine transmitted the transgenes to their progeny. Five of the nine lines expressed detectable levels of the transgenic proteins in their testes. From those five, lines 5 and 9 were characterized further for this study. Although both expressed the transgenic proteins (Figure 1B), there was no detectable enzyme activity of the protein expressed in line 5 (Figure 1C). The proteins expressed in line 9 (Figure 1C) and the other three transgene-expressing lines were enzymatically active (*15*). We sus-

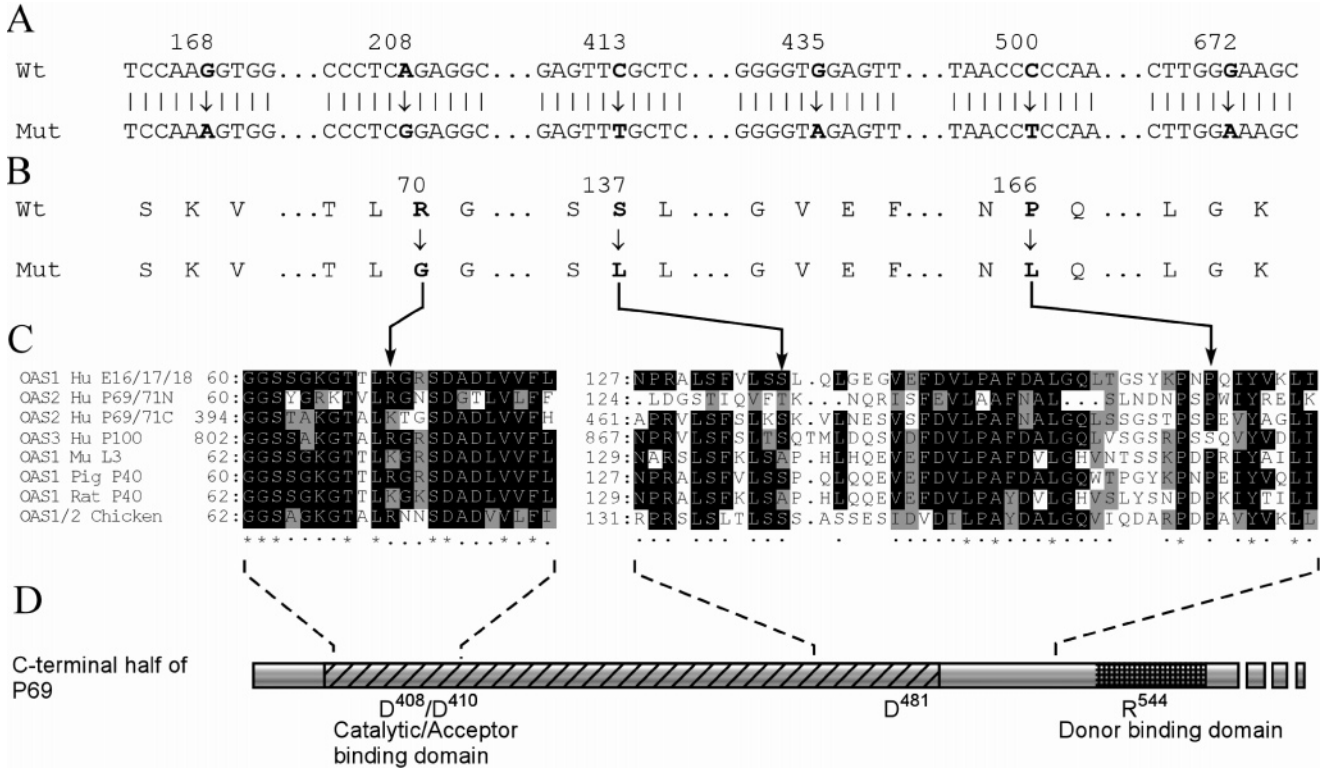

FIGURE 2: Mutations generated in the line 5 transgenic mouse. (A) DNA sequence around the six mutation sites in the line 5 mouse along with the wild-type E17 isozyme. Base numbering was done according to the E17 cDNA coding sequence. (B) The changed amino acid residues in the mutant protein are shown below. (C) Protein sequence alignments of several 2−5 (A) synthetase isozymes around mutated residues showing the conserved nature of these residues. (D) Schematic linear representation of the carboxy-terminal half of P69 indicating functionally important domains.

pected that the OAS1 protein in the testes of the line 5 mice was inactive because it carried unknown mutations. To examine this possibility, the coding regions of the corresponding mRNAs were cloned from line 5 and line 9 mice, using reverse transcriptase polymerase chain reactions. Sequencing of the cDNAs revealed that the line 5 transgene, but not the line 9 transgene, had accumulated six transition mutations (Figure 2A). Three of these mutations were silent, while the other three caused substitution mutations: R70 to G, S137 to L, and P166 to L. As expected, the cloned mutant protein, when expressed in vitro, was enzymatically inactive (data not shown). To examine the effect of each mutation, we introduced the mutations individually to Wt E17 cDNA, expressed the proteins in vitro, and measured their enzyme activities. All three mutants were enzymatically inactive (data not shown). To evaluate their importance, we examined the conservation of the mutated residues in other isozymes of OAS (Figure 2C). In several OAS1 isozymes, in both halves of OAS2, and in OAS3, the residues corresponding to R70 in E17 were basic residues, either R or K. S137 was either S or T in most of the isozymes, except in mouse L3 and rat P40 where the corresponding residue was A. P166 was uniformly conserved, except in OAS3. In the C-terminal half of the P69 OAS2 isozyme, all three mutations were in the same linear stretch of the protein that contained the catalytic center, the acceptor binding domain, and the donor binding domain (Figure 2D). Previously, we have extensively used the P69 OAS2 isozyme as the prototype for studying structure−function relationships of this family of enzymes. Because the important residues required for enzymatic activity all reside in the C-terminal half (*12*, *13*), we decided to introduce the new mutations individually into the P69 protein. Thus, three mutants of P69 OAS2, K404G, S471L, and P500L, were generated for probing the nature of the defects in their enzymatic properties. To study the role of the side chains of these three residues, we also replaced K404 with A and E, S471 with A, and P500 with A and G.

Properties of the P69 Mutants. The Wt and P69 mutants were expressed as hexahistidine-tagged proteins in insect cells using the baculovirus expression system and subsequently purified by affinity chromatography. The enzymatic activities of all three mutants, carrying the mutations generated from the transgene, were reduced by almost 20-fold. The K404G mutant had 6.3% of the Wt activity, and the P500L mutants had 7.8% of the Wt activity; on the other hand, the S471L mutant was completely inactive (Figure 3A and Table 1). Activity measurements using increasing concentrations of ATP established that the P500L mutant had the same K_m as the Wt protein, but this parameter was increased by 2.5-fold for the K404G mutant (Figure 3B and Table 1). These results indicated that the K404G mutant might have a lower affinity for substrates. ApppA can serve as the acceptor substrate of these enzymes, and its affinity for the proteins can be conveniently measured by fluorescence quenching of the protein (*12*). Such measurements revealed that the Wt and P500L proteins had very similar dissociation constants for ApppA, but the K404G mutant had a higher value (Figure 4A), indicating that it was defective in acceptor binding.[2] Acceptor binding properties could also be monitered by cross-linking radiolabeled 2−5 (A) to the proteins (*13*). Although this assay is not as quantitative as the fluorescence quenching assay, it confirmed the conclusion

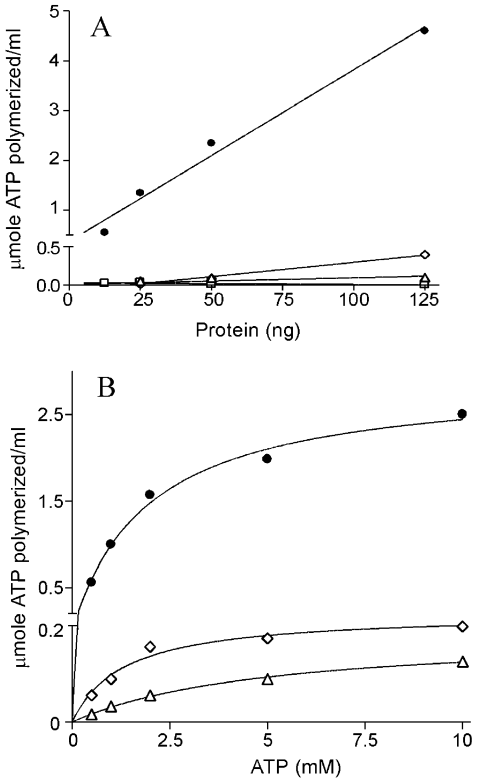

FIGURE 3: Kinetic properties of the mutants. (A) 2−5 (A) synthetase activity vs enzyme concentrations. 2−5 (A) synthetase assays were performed in the presence of 5 mM ATP, 50 μg/mL poly(I)·poly(C), and the indicated amounts of proteins for 3 h using purified K404G (△), S471L (□), and P500L (◇) mutants along with wild-type P69 (●). 2−5 (A) products were analyzed by TLC. (B) ATP concentration curves for wild-type P69 (●), K404G (△), and P500L (◇); 50 ng of proteins for each sample was assayed, as described above, for 3 h. Products were analyzed by gel electrophoresis for more accurate quantitation.

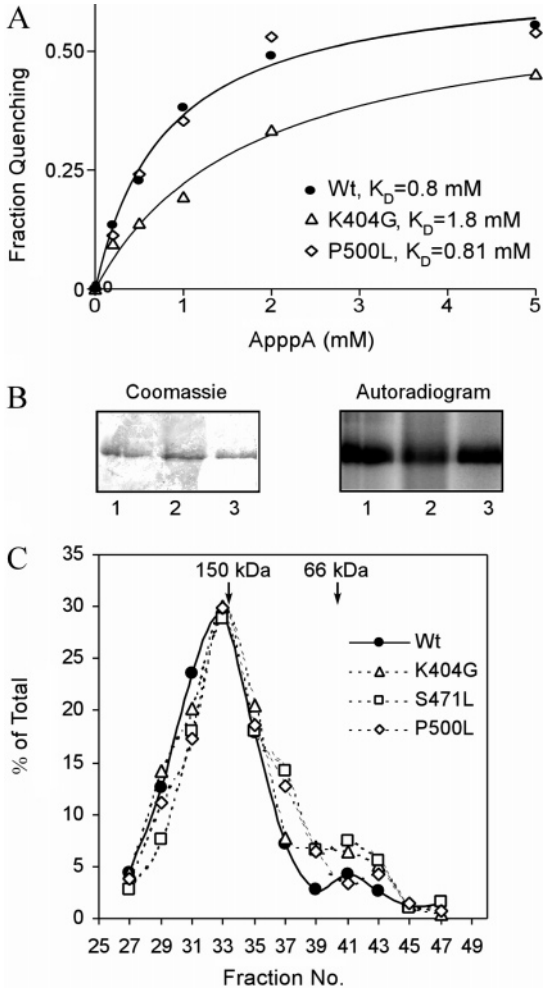

FIGURE 4: Substrate binding properties of the mutants. (A) ApppA binding of the wild type (●), the K404G mutant (△), and the P500L mutant (◇) was assessed by fluorescence quenching. Samples were excited at 290 nm, and the emissions were monitored at 322 nm. Corrected fluorescence intensities were plotted as fraction quenched. Data were fitted with a single-site hyperbolic binding equation to determine the dissociation constants (K_D). (B) 2−5 (A) cross-linking of the mutants. Radiolabeled azido 2−5 (A) dimers (0.8 mM) were UV cross-linked to each mutant protein (0.07 mg/mL) in a volume of 10 μL as detailed in Materials and Methods. Cross-linked samples were electrophoresed, stained with Coomassie blue (left panel), and autoradiographed (right panel): lane 1, Wt; lane 2, K404G; and lane 3, P500L. (C) Size fractionation analysis of three mutants in which 40 μg of purified proteins for Wt (●), K404G (△), S471L (□), and P500L (◇) was analyzed by gel filtration chromatography on a Sephacryl S300 column as previously described (*9*).

that the K404G mutant, but not the P500L mutant, was defective in acceptor binding (Figure 4B). Like the Wt protein, the K404G and the P500L mutant proteins were primarily dimers (Figure 4C). The S471L protein was also dimeric, but its solubility was very low, making it impossible to measure its substrate or acceptor binding properties. This indicated that the S471L mutation might have affected the folding properties of the protein. As expected, from the sequence of the murine OAS1 isozyme where the corresponding residue of S471 is an alamine, the P69 S471A mutant was active with slightly reduced activity (87% of that of the Wt, Table 1). Neither the K404G nor the P500L mutant exhibited any difference from the Wt protein with respect to the dsRNA concentration required for optimum activation (data not shown).

Defects of the K404G Mutant. In addition to the weakened acceptor binding of K404G, two other mutations made at the same residue, K404E and K404A, were also defective in substrate binding. K404E was completely inactive (we were unable to determine any kinetic parameter for this mutant), whereas K404A had activity but a high K_m, indicating defective substrate binding (Table 1). This defective K_m and the weakened ApppA binding by the K404G mutant indicated that the Lys residue at position 404 of P69 may be part of the acceptor binding site. In addition to the biochemical testing described above, we could test the nature of its defects using a functional complementation assay. Because the two subunits of P69 participate in a criss-cross enzymatic reaction, a heterodimer consisting of one subunit mutated in the acceptor site and another subunit mutated in the donor site is enzymatically active, whereas homodimers of either mutant are inactive (*18*). Hence, the assay described above can be used to distinguish between an acceptor site

[2] The ApppA and ATP K_D values for the wild-type protein determined from the fluorescence quenching experiments were lower than what we previously reported (*12*). This was because in the previous case, we used protein preparations that contained hydrogenated Triton X-100. The protein preparations used in experiments described in this paper did not have any Triton X-100, thus giving rise to lower K_D values showing better binding. Upon addition of the same amount of hydrogenated Triton X-100 to the new preparations, we observed a similar increase in K_D values as reported previously (data not shown).

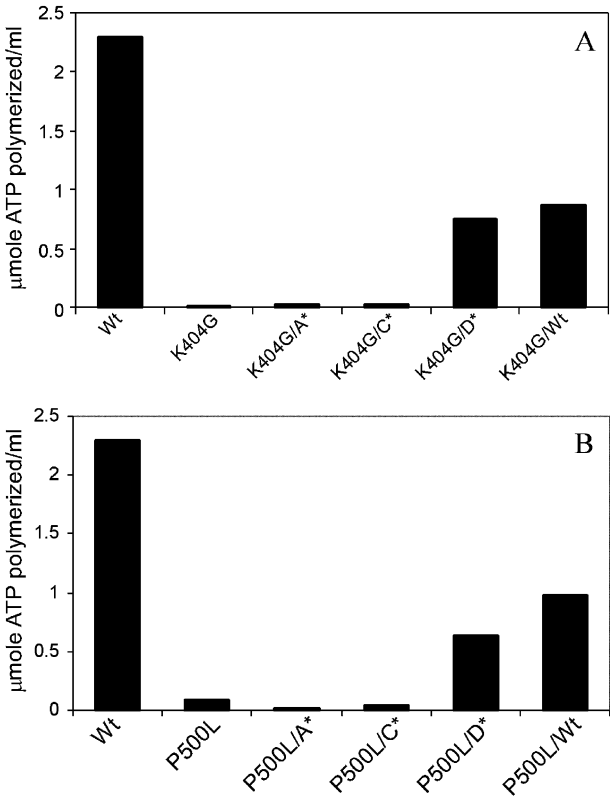

FIGURE 5: Complementation assay of the mutants. (A) Activity assays for the K404G homo- and heterodimer with acceptor (A*), donor (D*), and catalytic (C*) site mutants and Wt P69. Heterodimers were expressed by co-infection of appropriate amounts of two recombinant baculoviruses, partially purified by tandem affinity chromatography and assayed for 2−5 (A) activity as described previously (*17*). (B) Complementation of the activity assay for the P500L mutant.

mutant and a donor site mutant. For this purpose, the differentially tagged K404G mutant and a known donor site mutant, R544A (*13*, *17*), were coexpressed in insect cells and the heterodimer (K404G/D*) was purified by sequential affinity chromatography. The heterodimer was enzymatically active with a specific activity close to half of that of Wt homodimers (Figure 5A). A K404G/Wt heterodimer was similarly active. However, heterodimers of K404G and a known acceptor site mutant, Y421P, and heterodimers of K404G and a known catalytic site mutant, D408A/D410A, were completely inactive (Figure 5A). These results strongly indicate that K404G is an acceptor site mutant and hence Lys 404 is a part of the acceptor site of P69. Similarly, experiments with the S471L mutant showed that it could not form active heterodimers with known acceptor, donor, or catalytic site mutants (data not shown).

Defects of the P500L Mutant. Biochemical assays (Figures 2 and 3) indicated that the P500L mutant has no defect in acceptor binding. Compared to P500L, when we changed P500 to G or A, the enzyme did recover some activity: 50% of the Wt activity for P500A with a higher than normal K_m and 17% of the Wt activity for P500G (Table 1). This indicated that the leucine side chain at position 500 caused more disturbances in the enzyme structure than A or G. Our complementation assay showed that it could complement a donor site mutant, but not an acceptor site or a catalytic site mutant (Figure 5B). Because these are the properties of C*, a catalytic site mutant (*12*), our results suggested that the

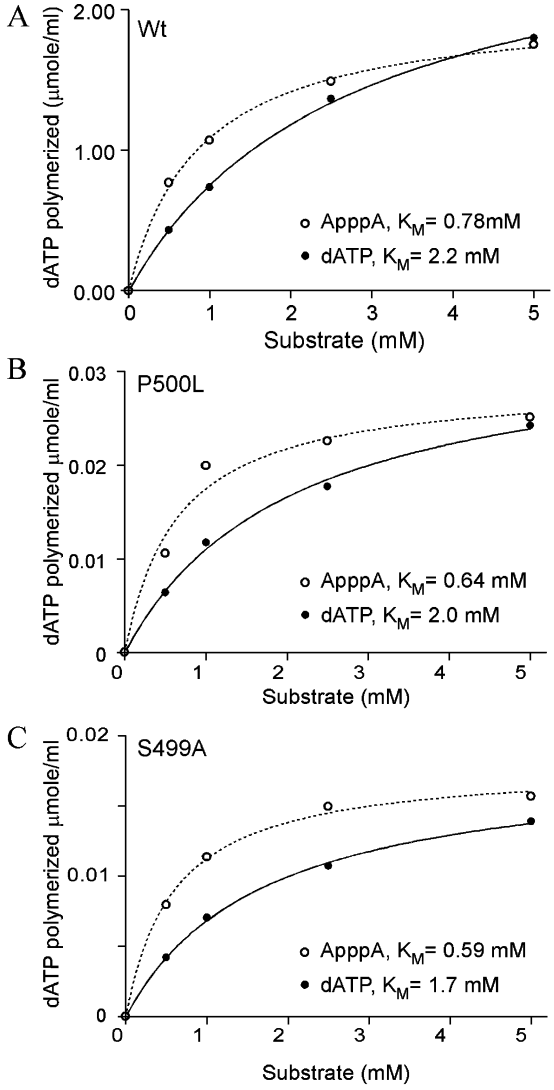

FIGURE 6: Determination of ApppA and dATP K_M values of P500L and S499A mutants. ApppA and dATP kinetics experiments were carried out with 50 ng of purified P500L (B) and S499A (C) mutants along with the Wt (A) in the presence of 50 μg/mL poly(I)·poly(C). The ApppA K_M value was determined in the presence of 4 mM dATP and increasing concentrations of ApppA (○), whereas the dATP K_M value was determined in the presence of a fixed ApppA concentration (4 mM) and increasing concentrations of dATP (●).

P500L mutant could bind both acceptor and donor normally, but remain catalytically inactive. We wondered whether this inactivity could be due to its improper structure since Pro residues, preceded by Asn or Ser residues, are known to create a cap for α-helices. Indeed, P500 is preceded by Asn in the OAS1 isozyme and by Ser in the P69 OAS2 isozyme. Moreover, it sits on the top of an α-helix of OAS1 (*14*). If this putative role of P500 were true, mutating S499 in P69 should have the same effect because both residues are needed to cap the helix (*19*). Consistent with this prediction, the P69 S499A mutant was as enzymatically inactive as the P500L mutant (Table 1). The substrate binding properties of these two mutants were compared further with those of the Wt protein. In the experiments shown in Figure 6, ApppA and dATP were used as substrates. The former can function as only an acceptor, whereas the latter can be only a donor. Thus, the K_m value for one, in the presence of a large excess of the other, reflects its binding constant for the enzyme. As

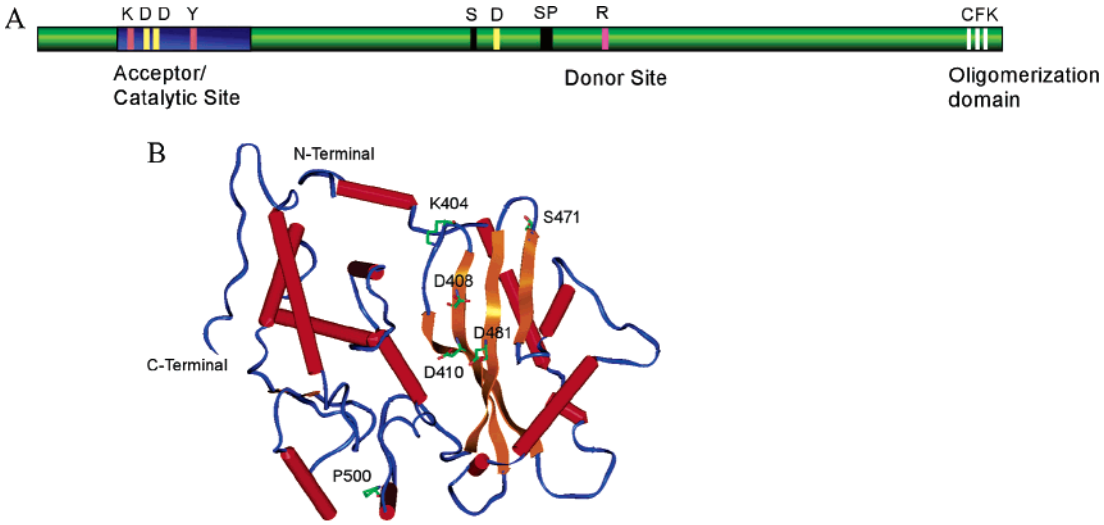

FIGURE 7: Locations of functionally important residues in the three-dimensional structure of the C-terminal half of P69. (A) Different newly and previously identified domains and essential residues in the C-terminal half of the P69 primary structure are shown. (B) The available crystal structure of porcine small 2−5 (A) synthetase isozymes (*14*) was used as a template to generate a three-dimensional model of the C-terminal half of P69 using automated modeling server SWISS-MODEL (http://swissmodel.expasy.org/). Catalytic Asp and the mutations described in this paper are marked. The α-helices are colored red, the *β*-sheets yellow, and the unstructured regions blue.

shown in Figure 6, the Wt, P500L, and S499A proteins had similar affinities for both the acceptor and the donor, although their catalytic capacities were very different (note the difference in the scale in the *Y*-axis in panels A−C of Figure 6). Thus, it appears that S499 and P500 provide a structural integrity to the protein that is essential for its enzyme activity.

DISCUSSION

This study has identified new residues that are required for enzymatic activity of OAS1, OAS2, and probably other isozymes of this family. These residues add to the list of residues previously identified through biochemical studies introducing deliberate mutations into sites suspected to be important. In contrast, the mutations identified in this study were made and selected naturally in vivo. All our transgenic lines were made at the same time from the same Wt cDNA stock, and four of five expressing lines produced the active Wt protein. It was surprising that the fifth line produced an inactive protein. It had accumulated six mutations in the 5′-half of its coding region, three of which caused amino acid substitution mutations, each of which could individually inactivate the protein. It is unclear why in this particular mouse line so many mutations accumulated and why every substitution mutation was inactivating. It is possible that in some other transgenic lines the coding region of the transgenes had mutated as well, but because those putative mutations did not affect enzymatic activity, they escaped our attention. Similarly, for the four nonexpressing lines that transmitted the transgene, mutations in the regulatory regions of the transgenes might have prevented their expression. Irrespective of the exact mechanism of accumulation of the three substitution mutations in the coding region of the transgene of the line 5 mouse, it provided us with a novel opportunity to analyze the roles of the mutated residues in OAS enzyme activity. We introduced each of these mutations separately into the P69 OAS2 isozyme, which had been the main focus of our past structure−function investigations. Not only did we know most about the enzymatic characteristic of that isozyme, we also had powerful experimental systems and reagents to study it.

Of the three mutated residues, the specific function of Ser 471 remains elusive. The S471L mutant was inactive and could not form active heterodimer with either the acceptor/catalytic site or the donor site mutant. Although the protein formed dimers, its low solubility prevented us from carrying out fluorescence quenching or 2−5 (A) cross-linking experiments. Thus, experimentally, we could not learn much about the specific role of Ser 471. It is notable that the corresponding residue is Ala in Wt murine OAS1 and rat OAS1 (Figure 2), making it unlikely that the Ser residue is a target of phosphorylation. In fact, when we introduced the S471A mutation into P69, it was active (Table 1). In the crystal structure of OAS1 and in the corresponding modeled structure of the C-terminal half of OAS2 (Figure 7B), Ser 471 is located in a loop region between *β*-strands 4 and 5. Thus, a possible structural role of this residue is also not obvious. Given the observations described above and the low solubility of the S471L mutant protein, we tentatively concluded that S471 is involved in proper folding of this protein.

Lys 404 was clearly identified as a functional component of the acceptor binding site. The K404G mutant protein bound ApppA and 2−5 (A) poorly and had a higher K_m for ATP. Moreover, it formed active heterodimers with a donor site mutant. In contrast, as expected for an acceptor site mutant, it could not complement another acceptor site mutant or a catalytic site mutant. In all isozymes, this residue is either Lys or Arg, indicating that a basic residue is required in that location. To test this hypothesis, we changed K404 to A and E. The K404E mutant was completely inactive. However, to our surprise, the K404A mutation resulted in a partially active protein with a much higher K_m, signifying a low substrate binding efficiency. This is consistent with the function ascribed to K404, namely, that it contributes to substrate binding, but there are other residues as well that can partially compensate for the positive charge. Thus, Lys

404 is important for the enhancement of substrate binding. In the linear sequence, Lys 404 is located near the two Asp residues of the catalytic center and the Tyr residue of the acceptor binding site (Figure 7A). All of these residues reside in the P-loop of the protein (Figure 7B), which mediates both acceptor binding and the nucleotidyl transfer reaction. Thus, it appears, by all measures, that Lys 404 is an integral part of these enzymes' acceptor binding site.

Pro 500 and Ser 499 were identified as residues with roles that are distinct from acceptor or donor binding and catalysis. Their roles are clearly structural. The recently determined crystal structure of OAS1 (*14*) and the derived modeled structure of OAS2 (Figure 7B) guided us to identify the function of P500. In the sequence database, we observed that this residue was often preceded by Asn or Ser (Figure 2C), and in the three-dimensional structure, the Ser-Pro sequence sits at the top of an α-helix (Figure 7B). For transmembrane proteins, Pro residues play crucial roles in proper folding (*20*). In globular proteins, these residues are known to generate kinks in α-helical regions (*21*, *22*). In a more novel situation, an Asn-Pro sequence creates a cap for an α-helix, permitting more flexibility in the region (*19*). Thus, it is highly likely that Pro 500 serves a similar role in OAS proteins. This conclusion was strongly supported by the fact that as predicted, the S499A mutant behaved like the P500L mutant. In OAS, the Ser-Pro sequence probably caps the helix and prevents it from extending further. Such an extension may restrict the flexibility required for the presumed conformational change associated with dsRNA-mediated activation of the enzyme. On the other hand, when the P500 was changed to either A or G, the mutant enzymes had partial activity. This finding reemphasizes not only the contribution of P500 as a helix cap residue but also its role as a residue with a short nonpolar side chain. In the structural model of P69 (Figure 7B), P500 is located at the end of the helix and in the beginning of the next solvent accessible loop region, and replacement of this residue with leucine, which has a large nonpolar side chain, most probably causes a severe distortion in the loop region structure and disrupts its interaction with other parts of the protein. Consequently, a L at position 500 may prevent the protein from attaining the right conformation required for catalytic activity, whereas A and G substitutions at position 500, with shorter side chains, may be more tolerant.

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