Enzymatic Activity of 2′–5′-Oligoadenylate Synthetase Is Impaired by Specific Mutations that Affect Oligomerization of the Protein*

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Previous studies from our laboratory have shown that deletion of residues 321 to 344 of the 9-2 isozyme of 2′–5′-oligoadenylate (2–5(A)) synthetase causes a loss of its enzyme activity (Ghosh, S. K., Kusari, J., Bandypadhyay, S. K., Samanta, H., Kumar, R., and Sen, G. C. (1991) J. Biol. Chem. 266, 15293–15299). Sequence comparison of this region among the different isoforms of 2–5(A) synthetases revealed that the residues at positions 330 to 333 are highly conserved. Alanine-scanning mutagenesis of these residues demonstrated that the residues present at 331, 332, and 333 are important for activity but the proline at position 330 was dispensable. The triple mutant containing Ala residues at 331, 332, and 333 was completely inactive. Different double mutants were slightly active, and the three single mutants were partially active. The triple mutant was further characterized for delineating the nature of its defect. The mutant protein was enzymatically inactive irrespective of whether it was synthesized in rabbit reticulocyte lysate, Escherichia coli or Trichoplusia ni insect cells. It could bind double-stranded RNA and ATP as efficiently as the wild type protein. It was, however, defective in oligomerization. Gel filtration and sedimentation velocity analyses of in vitro synthesized proteins revealed that the wild type protein, but not the triple mutant, formed tetramers. The tetrameric fraction, but not the monomeric fraction of the wild type protein was enzymatically active. The failure of the triple mutant to participate in homomeric protein-protein interaction was confirmed by in vivo assays in insect cells. These results indicate that tetramerization of the protein is required for the enzymatic activity of the small 2–5(A) synthetases.

2′–5′-Oligoadenylate (2–5(A)) synthetases are a family of enzymes that are induced by interferons (1, 2). These enzymes require double-stranded RNA as a co-factor and they polymerize ATP into 2–5(A), which activates the latent ribonuclease, RNase L. In an interferon-treated cell, virus infection often activates ATP into 2–5(A), which activates the latent ribonuclease, RNase L. The 2–5(A) synthetases leading to the production of 2–5(A), activation of RNase L, and degradation of cellular RNA. The 2–5(A) synthetase/RNase L pathway is responsible for mediating interferon’s antiviral effect against picornavirus, and the same pathway has also been implicated in the antitumor effects of interferon. Another interferon-induced enzyme that requires dsRNA as a co-factor is the protein kinase, PKR (1, 3, 4). The dsRNA-binding domain of PKR overlaps with its dimerization domain (5). This domain contains two dsRNA-binding motifs that are also present in a number of other dsRNA-binding proteins (6–11). 2–5(A) synthetases, however, do not contain this motif and the critical residues responsible for dsRNA binding by these proteins have not yet been identified.

There are three size classes of 2–5(A) synthetases: large, medium, and small (2). The different isoforms are structurally related, and there is strong sequence conservation across the species (12). The large human synthetase is 100 kDa, and the medium synthetase is 69 kDa. The small synthetases have molecular weights ranging from 40 to 46 kDa (13). There are two human small synthetases, E16 and E18, that are the products of alternatively spliced mRNAs of the same gene (14). There are three murine small synthetases: 9-2 and 3-9 isoforms are the products of two alternatively spliced mRNA (12), whereas L3 is encoded by a separate gene (15). The different isoforms reside in different subcellular compartments. Their specific subcellular locations are sometimes dictated by their specific post-translational modifications. The small synthetases can form tetramers, whereas the medium synthetase exists as a dimer (16).

We cloned the cDNA of the murine 9-2 isozyme and have been investigating its structure-function relationship (12, 17). We have reported that the loss of as little as 9 residues from the amino terminus of the protein inactivates it (18). The crucial residue within this region is proline at the 7th position. A mutant, P7Q, is enzymatically inactive. However, it can bind dsRNA and ATP, and the mutant protein can tetramerize like the wild type protein. In this report, we have investigated the properties of another class of 9-2 mutants which carry mutations near the carboxyl terminus. We have previously shown that a C-terminally truncated protein containing residues 1–344 is enzymatically active, but another deletion mutant containing residues 1–320 is inactive (12). In this study, we have identified specific residues present between residues 320 and 344 that are required for enzymatic activity. A triple mutant containing alanine substitutions at position 331, 332, and 333 was enzymatically inactive. The mutant protein could bind dsRNA and ATP, but it failed to oligomerize. These results indicate that tetramerization of the protein is needed for its enzyme activity.

MATERIALS AND METHODS

Reagents—Poly(l)-poly(C)-agarose was from Pharmacia Biotech Inc., polyethyleneimine cellulose plates were from EM Scientific and [α-32P]ATP and [35S]cysteine were from NEN Life Science Products. ATP-agarose containing adenosine 5′-y-4-aminophenyl triphosphate

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1 The abbreviations used are: 2–5(A), 2′–5′-oligoadenylate; dsRNA, double-stranded RNA; PKR, protein kinase, RNA-activated; poly(l)-poly(c), polyinosinic acid-polyribidylic acid; TM, triple mutant; WT, wild type.
was from Sigma and RNase inhibitor, restriction enzymes, and other fine chemicals were from Boehringer Mannheim. TNT, a DNA-driven in vitro translation system was purchased from Promega, and anti-FLAG M2 monoclonal antibody Sepharose was purchased from Eastman Kodak Co. Sepharocry S300 was a product of Pharmacia. Reagents for protein precipitation and recombinant baculoviruses and for expression of proteins in insect cells were purchased from Life Technologies, Inc. Immobilon polyvinylidene difluoride membrane was from Millipore, peroxidase-conjugated antibody was from Boehringer Mannheim. Enhanced chemiluminescence kit was purchased from Amersham.

Site-directed Mutagenesis—The 9-2 cDNA clone of 2–5(A) synthetase was used to prepare primers to replace residues 330, 331, 332, and 334 with alanines singly or in combination. The mutations were introduced in antisense primers used for polymerase chain reactions. The antisense primer also contained a DraII site present 3’ to the mutation sites in the 9-2 cDNA. The sense primer was located 5’ to an upstream HindIII site. Products from polymerase chain reactions were filled in using Klenow DNA polymerase and digested with HindIII and DraII. The fragments containing the mutations replaced the corresponding HindIII-DraII fragment of wild type 9-2 cDNA clone in pGem7Zf. The newly synthesized portions of the mutant cDNAs were completely sequenced to confirm the presence of the desired mutations and the absence of any other mutation.

Synthesis of 9-2 Proteins—Wild type and mutant hexahistidine-tagged 2–5(A) synthetases were expressed in vitro using the TNT rabbit reticulocyte lysate and [35S]cysteine. The products were analyzed as described before (18). Bacterial expression of the hexahistidine-tagged triple mutant and its partial purification were done as described for the wild type protein (17).

Enzyme Assay—2–5(A) synthetase assay and analysis of the products were done as described before (18, 19) except that the incubations were for 45 min.

Affinity Chromatography—Poly1-poly(C)-agarose chromatography and ATP-agarose chromatography were done as before (18).

Velocity Gradient Sedimentation Analysis—The conditions for this analysis were the same as described before (18).

Gel Filtration—in vitro translated proteins were analyzed at room temperature on a 1 × 50-cm Sepharose 600 column. The column was equilibrated with a buffer containing 20 mM Tris-Cl, pH 7.5, 450 mM NaCl, 5 mM MgCl2, 5 mM β-mercaptoethanol, and 0.2% Triton X-100. 25 μl of in vitro translation product was diluted with 300 μl of the above buffer and applied to the column. 270-μl fractions were collected. Proteins in 200 μl of the fractions were precipitated by adding 1.0 ml of acetone in the presence of 50 μg of bovine serum albumin. The precipitates were dissolved in gel electrophoresis sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis. The 9-2 protein bands were quantitated by PhosphorImager analysis. The marker proteins were detected by Coomassie Blue staining.

Activity of Tetrameric and Monomeric Proteins—FLAG-tagged radiolabeled WT 9-2 protein was synthesized in vitro. It was size fractionated by gel filtration as described above and the tetrameric and the monomeric fractions were pooled separately. The pooled fractions were mixed with anti-FLAG antibody bound to Sepharose in the gel filtration buffer for 1 h at 4 °C. The Sepharose beads were centrifuged down, washed with 20 mM Tris-Cl, pH 7.5, and 10% glycerol and stored at 4 °C. Some portions of the beads were analyzed by gel electrophoresis for quantitating the amounts of bound proteins. Equal amounts of tetrameric and monomeric proteins, bound to the beads, were incubated for 2 h with frequent mixing for activity assay. The reaction products were analyzed and quantitated using methods described above.

Western Blot Analysis—Insect cell extracts were analyzed by SDS-polyacrylamide gel electrophoresis and blotted onto a polyvinylidene difluoride membrane. The membrane was soaked in 5% nonfat dried milk at 37 °C for 2 h followed by incubation overnight at 4 °C in 1% nonfat dried milk containing 1:150 dilution of a monoclonal antibody against mouse 2–5(A) synthetase (20). The membrane was washed for 15 min with three changes of phosphate-buffered saline containing 0.05% Tween 20. It was then incubated with goat anti-mouse IgG conjugated with peroxidase at a 1:1000 dilution for 1 h at 37 °C with orbital shaking. The membrane was washed for 30 min with five changes of phosphate-buffered saline containing 0.05% Tween 20 and incubated with enhanced chemiluminescence detection reagent for 1 min before exposing to a x-ray film.

Virus and Cell Culture—Wild type baculovirus (AcMNPV) stock was purchased from Life Technologies, Inc. WT and recombinant viruses were propagated in confluent monolayers of S. frugiperda cells (SF21), and high levels of proteins were expressed in T. ni cells (High five). The cells were cultured at 27 °C in Grace’s supplemented insect media containing 10% heat inactivated fetal bovine serum that has been tested for insect cell growth and antibiotic and antimiycotic reagent (Life Technologies, Inc.).

RESULTS

Identification of Specific Residues Needed for Activity—The murine 9-2 isozyme of 2–5(A) synthetase has 414 residues. By deletion analysis, we have previously shown (12) that the protein can be truncated from the carboxyl terminus removing residues 345–414 without a loss of enzyme activity. Further deletion of residues 321–344, however, inactivated the protein completely. To obtain indications of what specific residues might be important, we compared the amino acid sequence of this region with those of the corresponding regions of other isozymes of 2–5(A) synthetase (Fig. 1). Among the small synthetases, the residues are completely conserved among mouse 9-2 and 3-9 and human E16 and E18 proteins. The mouse isozyme L3, on the other hand, has only partial homology. The same is true for the medium synthetase P69. Since L3 and P69 were the most divergent isozymes and residues 330, 331, 332, and 334 were conserved even in these proteins, we decided to focus on the most divergent isozymes and residues 330, 331, and 332 were conserved even in these proteins, we decided to focus on the mutations replaced the corresponding residues of interest for this study are boxed.

Fig. 1. Sequence homology near the carboxyl termini of 2–5(A) synthetase isozymes. The top three synthetases are murine and the bottom three are human. P69 is a medium synthetase, and others are small isozymes. The conserved residues are indicated by dots. The conserved residues of interest for this study are boxed.

For protein expression, T. ni (High five) cells were infected with the appropriate recombinant virus at a multiplicity of infection of 10. Cells were harvested after 40 h, washed three times in cold phosphate-buffered saline and lysed at 4 °C by sonication for 6 min, using four 30-s pulses in a buffer containing 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM MgCl2, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 mM aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.1% Nonidet P-40. The cell extract was centrifuged at 30,000 × g for 15 min, and the supernatant was stored at −70 °C for further analysis.

In Vivo Interaction Assay—High five cells were infected with appropriate recombinant viruses either singly or in combinations. When two viruses were used, equal quantities of each (5 plaque-forming units/cell) were used. Cell extracts were made as described above. Immunoprecipitation of FLAG-tagged proteins present in these extracts was done as described before (18) with the following modifications. The anti-FLAG Sepharose beads were presoaked in uninfected cell extract (5 mg/ml protein) for 15 min at 4 °C with rotary shaking. This was followed by incubation with the test extract, at 15 mg/ml of protein, for 1 h at 4 °C with rotary shaking. The beads were washed five times before analysis of the bound proteins by SDS-polyacrylamide gel electrophoresis and Western blotting.
activity. Alanine-scanning mutagenesis was done to residues 330–333. The corresponding mutant proteins were synthesized by in vitro translation. Equal amounts of WT and mutant proteins were used for assaying enzyme activities, which are presented as percent of WT activity. Error bars are from three independent measurements. Activities of four single mutants, three double mutants, and one triple mutant are shown.

FIG. 2. Enzyme activity of mutants.
Alanine-scanning mutagenesis was done for residues 330–333. The corresponding mutant proteins were synthesized by in vitro translation. Equal amounts of WT and mutant proteins were used for assaying enzyme activities, which are presented as percent of WT activity. Error bars are from three independent measurements. Activities of four single mutants, three double mutants, and one triple mutant are shown.

Production of the Triple Mutant in Other Systems—We wanted to ensure that the observed loss of activity of the TM is not a consequence of its synthesis in vitro in the reticulocyte system. We have previously shown that the 9-2 protein can be expressed in E. coli, and the WT protein has high enzyme activity (17). The bacterial system was used to produce the TM protein as well, and equal amounts of partially purified WT and TM proteins were assayed for enzyme activity. The bacterially produced TM was also inactive (Fig. 3, A and B). We have recently developed a baculovirus expression system for producing the 9-2 protein in insect cells. Since large amounts of proteins can be synthesized in insect cells and their post-translational modifications are similar to those in mammalian cells, this system is sometimes preferable to the bacterial expression systems. As shown in Fig. 3C, both the WT and the TM 9-2 proteins were highly expressed in insect cells infected with the corresponding viruses carrying the 9-2 genes. Infection with the viral vector alone did not cause the production of any cross-reacting protein. Again, the WT protein was highly active (Fig. 3, D and E), but the TM protein had no enzymatic activity. These results demonstrated that the TM Protein was inactive irrespective of its source, which included rabbit reticulocyte lysate, E. coli, and insect cells.

Properties of the Triple Mutant—Once we established that the TM was inactive, we wanted to examine its other properties. We used dsRNA-agarose and ATP-agarose as affinity matrices to evaluate the ability of the TM to bind to its substrate and co-factor. As shown in Fig. 4A, the TM could bind to poly(I)-poly(C)-agarose as efficiently as the WT protein. Under our experimental conditions, about 30% of the input in vitro translated protein bound to the affinity resin. Similarly, both WT and TM proteins bound to ATP-agarose equally well (Fig. 4B). Thus, we could not detect any deficiency of the TM with respect to its interaction with the substrate and the co-factor.

Next, we investigated the oligomerization property of the TM. We have previously used sedimentation velocity gradient analysis to show that in vitro synthesized 9-2 protein can form tetramers (18). Under our experimental conditions, which use a high salt concentration for avoiding nonspecific aggregation of proteins, about 15% of the input WT 9-2 protein existed as a tetramer. However, for the TM, the tetrameric complex was not detectable (Fig. 5). This result suggested that the induced mutations could have affected the oligomerization property of the protein. To confirm this conclusion, we performed a gel filtration assay, similar to the one originally used by Hovanesian and his colleagues (16) to demonstrate that small synthetases exist as tetramers. In vitro translated radiolabeled proteins were chromatographed on a Sephacyr S300 column; proteins in different fractions were separated by gel electrophoresis, and the 9-2 bands were quantitated by PhosphorImager analysis. As shown in Fig. 6, the wild type protein was separated into several size classes: one peak was at the monomeric position, another was at the position of a dimer, and there was a distinct peak at the tetrameric position of 200 kDa. In contrast, the tetrameric peak was absent for the triple mutant; this protein was almost totally monomeric.

Since analysis of the in vitro synthesized proteins suggested that the TM is defective in multimerization, we wanted to examine this property further by using the mutant protein expressed in insect cells. In Fig. 7, we used a co-immunoprecipitation assay that detects in vivo homomeric interaction of the 9-2 protein. Cells were simultaneously infected with two viruses, one carrying a FLAG-tagged 9-2 gene and another carrying a non-tagged WT or TM 9-2 gene. Cell extracts were mixed with FLAG antibody bound to Sepharose; the Sepharose beads were washed, and the bound proteins were eluted, separated by gel electrophoresis, and detected by Western blotting using a synthetase antibody. The FLAG-tagged and the untagged proteins could be distinguished by their differences in mobility. Comparable amounts of the tagged and untagged proteins were synthesized in infected cells for both WT and TM viruses (Fig. 7, lanes 1–5). As expected, from cells infected with single viruses, the protein bound to the affinity matrix only if it contained the FLAG-tag (Fig. 7, lanes 6, 7, and 9). But with

S. Bandyopadhyay, A. Ghosh, S. N. Sarkas, and G. C. Sen, unpublished results.
co-infections, the WT-untagged protein co-purified with the FLAG-tagged protein because of protein-protein interactions (Fig. 7, lane 8). On the other hand, the triple mutant failed to interact with the FLAG-tagged protein (Fig. 7, lane 10), although they were present in the same cells (Fig. 7, lane 5). These results clearly demonstrate that the TM lacks the ability to interact with itself and thus form a tetramer.

Enzyme Activity of the Tetrameric and Monomeric Proteins—
The above results suggested that the lack of activity of the TM is due to its failure to form tetramers. One would expect, therefore, that monomeric WT 9-2 protein is also inactive, whereas the tetrameric form is active. This possibility was tested in the experiment shown in Fig. 8. A large quantity of FLAG-tagged radiolabeled WT 9-2 protein was synthesized in vitro, and the tetrameric and monomeric forms were separated by gel filtration. Fractions containing the two forms were pooled separately, and the proteins were recovered by binding to anti-FLAG antibody bound to Sepharose (Fig. 8A). Equal amounts of the tetrameric and monomeric forms of the protein were assayed for enzyme activity (Fig. 8B). For the tetramer, over a 6-fold range, enzyme activity increased linearly with increasing concentrations of the protein (Fig. 8C). The monomer was, however, totally inactive even at the highest concentration tested. These results provide biochemical evidence for the conclusion that the 9-2 protein requires tetramerization for its enzymatic activity.

DISCUSSION

Deletion analysis of the 9-2 protein in our previous studies had indicated the importance of the residues 321–344. A truncated protein containing the residues 1–320 could bind dsRNA, but it did not have enzyme activity (12). The addition of 24 more residues to the carboxyl terminus restored the activity fully. In the current study, we have focused on this region of the protein with the purpose of identifying the crucial residues. We took advantage of the strongly conserved sequences among the different synthetases for doing homology search. Comparison of the 9-2 sequence with those of the L3 and the P69 proteins was very useful. The longest contiguous stretch of conserved residues within this region spanned residues 330–334, and we performed an alanine-scanning mutagenesis to this region of the protein. Although we originally expected the proline at 330 to be the crucial residue, its mutation to alanine did not affect the enzyme activity (12). The addition of 24 more residues to the carboxyl terminus restored the activity fully. In the current study, we have focused on this region of the protein with the purpose of identifying the crucial residues. We took advantage of the strongly conserved sequences among the different synthetases for doing homology search. Comparison of the 9-2 sequence with those of the L3 and the P69 proteins was very useful. The longest contiguous stretch of conserved residues within this region spanned residues 330–334, and we performed an alanine-scanning mutagenesis to this region of the protein. Although we originally expected the proline at 330 to be the crucial residue, its mutation to alanine did not affect the activity of the 9-2 protein. In contrast, individual mutations of Cys at 331, Phe at 332, and Lys at 333 reduced the enzyme activity considerably. The double mutants were even less ac-
and the triple mutant was completely inactive. We, thus, identified a stretch of 3 residues, CFK, whose mutation to AAA completely destroyed the protein’s enzymatic activity.

We decided to express the mutant protein in three different systems, rabbit reticulocyte lysate, E. coli, and insect cells. The wild type protein expressed in all three systems was active, but the TM was inactive irrespective of its source. These results suggest that the introduced mutations affect a fundamental property of the protein and not its putative interaction with post-translational modification enzymes, which are different in the three different systems of expression used by us.

As expected from the previous knowledge of the location of the dsRNA (12) and ATP-binding sites on the protein (21), the TM bound dsRNA and ATP as efficiently as the wild type protein. The mutant protein, however, was defective in forming tetramers. This result strongly suggests that the tetrameric structure is the enzymatically active form. The above suggestion was vindicated by our biochemical analysis. Activity measurements of physically separated tetrameric and monomeric WT proteins showed that only the tetrameric fraction was enzymatically active. Hovanessian and his colleagues (16) observed that the small synthetases exist as tetramers, and the medium synthetase exists as a dimer. Since the medium synthetase has two homologous domains, each of which has strong homology with the small synthetases (13), they postulated that for both isozymes the active center is formed by the juxtaposition of four identical domains. The four domains are presumably supplied by four polypeptides in the case of the small synthetases, whereas two molecules of the medium synthetase, each containing two domains, are sufficient. Our mutational results clearly support this model. Consequently, we would expect that the same mutations, if introduced to the P69 protein, would also inactivate that protein by preventing its dimer-
This prediction remains to be experimentally tested. We do not have enough information yet to understand how the mutated residues affect oligomerization. Although a cysteine residue is included in the mutated region, disulfide bridge formation is probably not involved in the oligomerization process. The above conclusion is made because of the observations that oligomerization of the wild type protein, as measured by size fractionations and protein-protein interaction assay, is not affected by the presence of reducing agents such as β-mercaptoethanol or dithiothreitol. Moreover, the C331A mutant was partially active, thus suggesting that the cysteine residue is not indispensable. Even though the TM failed to score positive in the protein-protein interaction assay, it would be premature to conclude that the mutated residues actually reside at the interacting site. The introduced mutations could have altered the conformation of the protein in such a way that the contact point at a different location of the protein would be distorted. These issues will be resolved when the pathway to tetramer formation is better understood. Although several alternative scenarios can be envisioned, the simplest steps would be dimerization of the protein followed by further dimerization of the dimers. The existence of dimers as intermediates is indicated in the results shown in Fig. 6. For the wild type protein, there was clearly a peak at the expected position of a dimer. For the mutant, although there was no peak at the position of the tetramer, the putative dimer peak was there. Thus, it seems that the triple mutant can form dimers but not tetramers. In that case, why does it score negative in the protein-protein interaction assay? The apparent discrepancy could be due to the different conditions used for the two assays. The protein interaction assay used a more stringent condition to eliminate nonspecific interactions, and that might have been too harsh a condition for maintaining dimeric interaction of the mutant protein. Clearly, more definitive assays need to be developed in the future to gain further insights. The presence of intermediate dimeric peaks in the gel filtration analysis of the triple mutant (Fig. 6) suggests that it may be able to form unstable oligomers that dissociate at a faster rate as compared with the WT oligomers.

**Fig. 7.** Protein-protein interaction in vivo. High five cells were infected with recombinant baculoviruses encoding WT 9-2, WT 9-2 FLAG, or TM. Cell extracts were analyzed by Western blotting using synthetase antibody either directly (Total) or after immunoprecipitation with anti-FLAG-Sepharose (Bound). Lanes 1 and 6, infection with WT 9-2 FLAG virus; lanes 2 and 7, infection with WT 9-2 virus; lanes 3 and 8, co-infection with WT 9-2 FLAG and WT 9-2 viruses; lanes 4 and 9, infection with WT 9-2 FLAG and TM 9-2 viruses. Twice as much protein was used for immunoprecipitation as was used for direct electrophoresis.

**Fig. 8.** Enzyme activity of tetrameric and monomeric WT 9-2 proteins separated by gel filtration. FLAG-tagged 35S-labeled WT 9-2 protein was translated in vitro and analyzed by gel filtration (A). Fractions containing the tetrameric protein and monomeric protein were pooled separately and the FLAG-tagged protein was recovered by binding to anti-FLAG-Sepharose. Portions of the bound tetrameric and monomeric protein were analyzed by gel electrophoresis and quantitated by PhosphorImager analysis. Increasing amounts of monomeric and tetrameric proteins were tested for enzyme activity (B). The autoradiogram of the thin-layer chromatogram is shown. The amounts of 2–5(A) synthesized were quantitated and plotted against the amounts of protein used for the enzyme reaction (C). The activities are presented in PhosphorImager units obtained from analysis shown in panel B, and the amounts of protein are in PhosphorImager units obtained from analysis shown in panel A.
The enzymatically inactive triple mutant may be useful for exploring the cellular activities of 2–5(A) synthetases. Because it does not oligomerize with the wild type protein, it does not inhibit its activity in vitro. Thus, the mutant will probably not be useful as a trans-dominant inhibitor in vivo. It could, however, be useful as a sink for dsRNA in virus-infected cells. It would be interesting to examine if interferon can inhibit picornavirus replication in a cell overexpressing the triple mutant. Similarly, the triple mutant, being enzymatically inactive, will be a good tool for identifying cellular proteins that interact with 2–5(A) synthetases in vivo. The wild type protein is unsuited for that purpose because its expression causes cellular toxicity.

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