Characterization of a 2',5'-Oligoadenylate (2–5A)-dependent 37-kDa RNase L

AZIDO PHOTOCHEMISTRY LABELING AND 2–5A-DEPENDENT ACTIVATION

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Upregulation of key components of the 2',5'-oligoadenylate (2–5A) synthetase/RNase L pathway have been identified in extracts of peripheral blood mononuclear cells from individuals with chronic syndrome, including the presence of a low molecular weight form of RNase L. In this study, analysis of 2',5'-oligoadenylate (2–5A) binding and activation of the 80- and 37-kDa forms of RNase L has been completed utilizing photolabeling/immunoprecipitation and affinity assays, respectively. Saturation of photolabeling of the 80- and the 37-kDa RNase L with the 2–5A azido photoprobe, [32P]pApAp(8-azidoA), was achieved. Half-maximal photoinsertion of [32P]pApAp(8-azidoA) occurred at 3.7 × 10⁻⁸ M for the 80-kDa RNase L and at 6.3 × 10⁻⁸ M for the 37-kDa RNase L. Competition experiments using 100-fold excess unlabeled 2–5A photoaffinity probe, pApAp(8-azidoA), and authentic 2–5A (pA₅) resulted in complete protection against photolabeling, demonstrating that [32P]pApAp(8-azidoA) binds specifically to the 2–5A-binding site of the 80- and 37-kDa RNase L. The rate of RNA hydrolysis by the 37-kDa RNase L was three times faster than the 80-kDa RNase L. The data obtained from these 2–5A binding and 2–5A-dependent activation studies demonstrate the utility of [32P]pApAp(8-azidoA) for the detection of the 37-kDa RNase L in peripheral blood mononuclear cell extracts.

Previous studies from this laboratory (1) described structural similarities between two forms of 2',5'-oligoadenylate (2–5A)-dependent RNase L in extracts of peripheral blood mononuclear cells (PBMC). The 2–5A-dependent 80-kDa RNase L is the terminal enzyme in the 2–5A synthetase/RNase L antiviral defense pathway (2). RNase L is distinguished from other RNases in that it requires 2–5A for allosteric activation (reviewed in Refs. 1 and 3–6). The 80-kDa RNase L plays an essential role in the inhibition of viral mRNA and thus the inhibition of viral protein synthesis (3, 4). In addition to the 80-kDa form of RNase L, a 40/42-kDa form has been identified and is thought to be a proteolytic degradation product of the 80-kDa RNase L (7, 8).

As part of ongoing studies on the up-regulation of the 2–5OAS/RNase L antiviral pathway, a previously unknown 37-kDa form of RNase L has recently been identified in extracts of PBMC from individuals who met the diagnostic criteria for chronic fatigue syndrome (CFS) (9–12). We had previously demonstrated that the 2–5OAS/RNase L pathway is constitutively active in PBMC extracts from individuals with CFS; 2–5OAS is predominantly in its activated form and levels of bioactive 2–5A and RNase L enzyme activity are elevated in CFS PBMC extracts compared with healthy control PBMC extracts (13, 14). Continued analyses of CFS PBMC extracts have revealed the presence of a novel low molecular mass RNase L that binds 2–5A, exhibits 2–5A-dependent RNase L activity, and has a molecular mass of 37-kDa under denaturing conditions (1, 10). The 37-kDa RNase L has not been observed in PBMC extracts from individuals diagnosed with fibromyalgia or depression (15).

Two subsets of individuals with CFS have been identified in PBMC extracts using azido photoaffinity labeling with [32P]pApAp(8-azidoA) and immunoprecipitation with a polyclonal antibody specific to the human 80-kDa RNase L (1, 10). One subset contains only the 37-kDa RNase L and the second subset contains both the 80- and 37-kDa RNase L. The subset of CFS PBMC containing only the 37-kDa RNase L had the higher level of RNase L activity compared with the subset containing both the 80- and 37-kDa forms of RNase L (2). In a two-site randomized-coded study, the presence of the 37-kDa RNase L also correlated with the level of clinical disability experienced by the study subjects (10).

The elucidation of the molecular structure of the 37-kDa RNase L has been a major effort in this laboratory (1). We hypothesized that the observed differences in the levels of biologically active 2–5A and RNase L activity in healthy control and CFS PBMC extracts could be attributed to differences in the 2–5A binding affinity or enzyme activity of the 80- and 37-kDa forms of RNase L. In the current study, we have characterized the 2–5A binding and activation of the 80- and 37-kDa RNase L in healthy control and CFS PBMC extracts utilizing azido photochemical cross-linking and affinity binding assays. Saturation of 2–5A azido photolabeling was achieved for the 80- and 37-kDa forms of RNase L. Competition experiments revealed that the 2–5A azido photoaffinity probe,
[8-azidoA], binds specifically to the 80- and 37-kDa RNase L in healthy control and CFS PBMC extracts, respectively. The rate of RNA hydrolysis by the 37-kDa RNase L was three times faster than the 80-kDa RNase L. The data presented in this study also demonstrate the use of the 2–5A azido photoprobe for the detection of the 37-kDa RNase L in CFS PBMC extracts.

**EXPERIMENTAL PROCEDURES**

**Materials**—The 2–5A azido photoprobe, ApAp(8-azidoA), and 2–5A were generously supplied by Dr. Wolfgang Pfleiderer (Konstanz Universität, Konstanz, Germany). The fibrous cellulose powder CF11 used to prepare the core(2–5A)-cellulose was from Whatman. Healthy control and CFS PBMC extracts were used as source of the 80- and 37-kDa RNase L, respectively. PBMC extracts were prepared in the presence of protease inhibitors as described previously (1). All other materials were as described previously from this laboratory (1).

**Synthesis of [32P]pApAp(8-azidoA) Azido Photoprobe**—The chemical synthesis of ApAp(8-azidoA) was completed as described (16). Phosphorylation of the 5’ terminus of ApAp(8-azidoA) (0.15 mM; 6 × 10^6 Ci/mol; 10,000 dpm) and unlabeled pApAp(8-azidoA) (0–5 × 10^-6 M), UV-irradiated, immunoprecipitated, and analyzed as described under “Experimental Procedures.” Inset depicts the covalent photolabeling of the 80-kDa RNase L (B) and the 37-kDa RNase L (D) by [32P]pApAp(8-azidoA) when unlabeled pApAp(8-azidoA) was added to the assay mixture at concentrations less than 1 × 10^-6 M. Photolabeling of the 80- and 37-kDa RNase L by [32P]pApAp(8-azidoA) in the absence of unlabeled pApAp(8-azidoA) represents 100% photoinsertion. The data were analyzed by nonlinear regression using Sigma plot 5.0. Isotope competition experiments were completed for three healthy control PBMC extracts containing only the 80-kDa RNase L and for three CFS PBMC extracts containing only the 37-kDa RNase L. Results from a representative healthy control (A and B) and from a representative individual with CFS (C and D) are shown.

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**Synthesis of [32P]pApAp(8-azidoA) Azido Photoaffinity Probe**—The chemical synthesis of ApAp(8-azidoA) was completed as described (16). Phosphorylation of the 5’ terminus of ApAp(8-azidoA) (0.15 mM) was performed with [γ-32P]ATP (6000 Ci/mmol) in the absence and presence of ATP (1.6 mM) and T7 polynucleotide kinase according to the manufacturer’s directions. The yield of the radiolabel incorporation for [32P]pApAp(8-azidoA) in the absence of ATP was 30% with a specific activity of 6000 Ci/mmol and 5–10% in the presence of ATP with a specific activity of 60 Ci/mmol.

**Azido Photoaffinity Labeling and Immunoprecipitation of 2–5A-binding Proteins in PBMC Extracts**—Covalent photoaffinity labeling, immunoprecipitation, and quantitation of the 2–5A-binding immunoreactive proteins from healthy control and CFS PBMC extracts were as described previously (1). Isotope competition experiments were completed for three healthy control PBMC extracts containing only the 80-kDa RNase L and for three CFS PBMC extracts containing only the 37-kDa RNase L. Results from a representative healthy control (A and B) and from a representative individual with CFS (C and D) are shown.
Azido Photolabeling of 37-kDa RNase L in CFS PBMC

FIG. 3. Displacement of radiolabeled [32P]pApAp(8-azidoA) from RNase L in PBMC extracts by pA3. PBMC extracts were incubated with [32P]pApAp(8-azidoA) (1 × 10⁻⁸ M; 6 × 10⁶ Ci/mol, 60,000 dpm) and pA3 (1 × 10⁻⁸ to 1 × 10⁻⁷ M), UV-irradiated, immunoprecipitated, and analyzed as described under “Experimental Procedures.” Photolabeling of the 80- and 37-kDa RNase L by [32P]pApAp(8-azidoA) in the absence of pA3 represents 100% photoinsertion. The data were analyzed by nonlinear regression using Sigma plot 5.0. Competition experiments using [32P]pApAp(8-azidoA) and pA3 were completed for three healthy control PBMC extracts with only 80-kDa RNase L and for three CFS PBMC extracts with only 37-kDa RNase L. Results with PBMC extracts from a representative healthy control (A and C, open circles) and a representative individual with CFS (B and C, closed circles) are shown.

RESULTS AND DISCUSSION

In the current study, we utilized the methods of covalent photoaffinity labeling with the 2–5A azido photoprobe, [32P]pApAp(8-azidoA), and immunoprecipitation with a polyclonal antibody to human RNase L to determine the binding requirements for the interaction of 2–5A with 80- and 37-kDa RNase L in healthy control and CFS PBMC extracts, respectively. By using cell extracts prepared from PBMC, we have chosen to employ a system that contains all the components necessary to form the activated complexes for the 80- and 37-kDa RNase L proteins (22). In previous photolabeling studies, we have used 2–5A azido photoprobe to specifically identify the different forms of RNase L in interferon-treated L929 cell extracts (21). We have also demonstrated that the activation of the 80-kDa RNase L in interferon-treated L929 cells by an 8-azido–2–5A photoaffinity probe was comparable to the RNase L activity measured in the presence of pA3 using core (2–5A)-cellulose assays (16, 21). Core (2–5A)-cellulose assays were employed in the current study to determine 2–5A-dependent RNase activity in healthy control and CFS PBMC extracts.

Saturation of Photolabeling of RNase L in PBMC Extracts—We have demonstrated that the expression of the 37-kDa RNase L in CFS PBMC extracts correlates with elevated levels of bioactive 2–5A and RNase L activity (1, 10). Using the combined methods of covalent photoaffinity labeling with the 2–5A photoaffinity probe, [32P]pApAp(8-azidoA), and immunoprecipitation with a polyclonal antibody to human 80-kDa RNase L, the binding affinity of 2–5A to 80-kDa RNase L in healthy control PBMC extracts and to 37-kDa RNase L in CFS PBMC extracts was determined (Fig. 1). The healthy control PBMC extracts chosen for the study contained only the 80-kDa RNase L and the CFS PBMC extracts contained only the 37-kDa RNase L. Binding of [32P]pApAp(8-azidoA) to either 80- or 37-kDa RNase L in PBMC extracts reached equilibrium in 30 min at 0 °C and remained constant through 90 min prior to photolabeling/immunoprecipitation (data not shown). The extent of photolabeling at 0 °C of the 80- and 37-kDa RNase L by [32P]pApAp(8-azidoA) reached a maximum at 60 s and remained constant through 90 s (data not shown). Therefore, photolysis was conducted for 60 s to maximize the extent of [32P]pApAp(8-azidoA) photoinsertion into the 80- and 37-kDa RNase L. In the absence of UV irradiation, there was no cross-linking of the [32P]pApAp(8-azidoA) to RNase L. The amount of [32P]pApAp(8-azidoA) covalently bound to the 80- or 37-kDa RNase L was linear with respect to the amount of used PBMC extract or CFS PBMC extract up to 100 µg of total protein (data not shown).

The best curve fit for the binding of the 2–5A azido photoprobe to the 80- and 37-kDa forms of RNase L following nonlinear regression analysis was for a one-site model, which indicates that the molar ratio of 2–5A to RNase L is 1:1 (Fig. 1). Saturation of photolabeling by [32P]pApAp(8-azidoA) was approached at 3.0 × 10⁻⁷ M for the 80- and 37-kDa RNase L (Fig. 1, A and D). Based on earlier photolabeling studies (23), we performed similar experiments assuming that the efficiency of 2–5A azido photolabeling of the 80- and 37-kDa is constant at
Azido Photolabeling of 37-kDa RNase L in CFS PBMC

![Graph](image)

**Fig. 4. Time-dependent hydrolysis of poly(U)-3'-[32P]Cp by RNase L in PBMC extracts.** RNase L in PBMC extracts from healthy controls (A) and from individuals with CFS (B) were immobilized on core(2–5A)-cellulose as described under “Experimental Procedures.” Activation of 2–5A-dependent RNase L was measured at different time intervals following the addition of poly(U)-3'-[32P]Cp and 2–5A to the affinity purified RNase L. One hundred percent RNA remaining in panels A and B represents 2810 and 2352 dpm, respectively, of poly(U)-3'-[32P]Cp bound to glass fiber filters. The data shown represent the mean of three separate determinations completed for healthy control and CFS PBMC extracts.

all concentrations of [32P]pApAp(8-azidoA). These photolabeling data have been used to determine apparent dissociation constants, which are represented by the concentration at which 50% of the maximal photoinsertion is attained. The apparent mean $K_d$ for the 80-kDa RNase L in three healthy control PBMC extracts was determined to be 3.7 ± 2.8 × 10⁻⁸ M. Half-maximal 2–5A azido photolabeling for the 80-kDa RNase L from the representative healthy control PBMC extract shown in Fig. 1B is 6.9 × 10⁻⁸ M. The apparent mean $K_d$ for the 37-kDa RNase L in three CBS PBMC extracts was determined to be 6.3 ± 5.5 × 10⁻⁸ M. Half-maximal 2–5A azido photolabeling for the 37-kDa RNase L from the representative CBS PBMC extract shown in Fig. 1D is 1.7 × 10⁻⁸ M. Saturation of photolabeling studies revealed relatively similar apparent mean $K_d$ for the covalent binding of the [32P]pApAp(8-azidoA) to the 80- and 37-kDa RNase L.

Displacement of Radiolabeled [32P]pApAp(8-azidoA) from RNase L by Unlabeled pApAp(8-azidoA) in PBMC Extracts—To determine the specificity of [32P]pApAp(8-azidoA) for the 2–5A-binding site on the 80- and 37-kDa RNase L, two types of competition experiments were completed using unlabeled pApAp(8-azidoA) or pApAp(8-azidoA). In the first type, a concentration-dependent protection against photoaffinity labeling of the 80- and 37-kDa forms of RNase L was observed when healthy control and CBS PBMC extracts were incubated with a mixture of radiolabeled [32P]pApAp(8-azidoA) and unlabeled pApAp(8-azidoA), respectively (Fig. 2). The binding of the 2–5A photoaffinity probe to the 80-kDa RNase L was inhibited by 50% with 1.7 × 10⁻⁸ M unlabeled pApAp(8-azidoA) (Fig. 2, A and B, and D, inset), which is in good agreement with the apparent mean $K_d$ determined from the saturation of photolabeling studies. Fifty percent protection against photoinsertion of [32P]pApAp(8-azidoA) into the 2–5A-binding site of the 37-kDa RNase L was achieved with 3.9 × 10⁻⁸ M unlabeled pApAp(8-azidoA) (Fig. 2, C and D, and D, inset), which is consistent with the apparent mean $K_d$ determined from the saturation of photolabeling experiments (Fig. 1). An apparent increase in covalent photoincorporation of [32P]pApAp(8-azidoA) into the 2–5A-binding site of the 80-kDa RNase L was observed when photolabeling mixtures contained unlabeled photoprobe at concentrations less than 1.0 × 10⁻⁸ M (Fig. 2B, inset). Such an apparent increase in photoincorporation can be observed only in the presence of unlabeled photoprobe at concentrations below the apparent $K_d$ for ligand binding to a protein (24, 25). Under these conditions, a cooperative allosteric effect may occur, as has been demonstrated in isotope competition experiments with the photoprobe [α-32P]2-azido-NAD⁺ and ovine prolactin (24, 25). This precedent might account for the difference in requirements for formation of the activated complex for 80- and 37-kDa RNase L. Simultaneous incubation of 1.0 × 10⁻⁸ M [32P]pApAp(8-azidoA) with unlabeled pApAp(8-azidoA) at concentrations less than 1.0 × 10⁻⁸ M did not result in an increase in covalent photolabeling of [32P]pApAp(8-azidoA) to the 37-kDa RNase L (Fig. 2D, inset). Incubation of a 100-fold excess of unlabeled pApAp(8-azidoA) (1.0 × 10⁻⁸ M) with the 80-kDa RNase L resulted in complete inhibition of photolabeling (Fig. 2, B and D, inset). In contrast, complete inhibition of photolabeling of the 37-kDa RNase L was achieved by incubation with a 10-fold excess of unlabeled pApAp(8-azidoA) (1.0 × 10⁻⁷ M) (Fig. 2, D and D, inset). Therefore, protection of photoinsertion by [32P]pApAp(8-azidoA) into the 80- and 37-kDa RNase L could be achieved with 100-fold excess unlabeled pApAp(8-azidoA).

Displacement of Radiolabeled [32P]pApAp(8-azidoA) from RNase L by pApAp in PBMC Extracts—Based on the data presented in Figs. 1 and 2, a second type of competition experiment was performed to measure the specific photolabeling of the 2–5A allostERIC binding site of the 80- and 37-kDa forms of RNase L by [32P]pApAp(8-azidoA). Using authentic pApAp at concentrations ranging from 1 × 10⁻¹¹ to 1 × 10⁻⁶ M in the presence of 1 × 10⁻⁸ M [32P]pApAp(8-azidoA), a concentration-dependent protection of 2–5A azido photolabeling was observed (Fig. 3). Photoincorporation of [32P]pApAp(8-azidoA) into the 2–5A-binding site of the 80-kDa RNase L was inhibited 50% by pApAp at concentrations of 1 × 10⁻⁸ M (Fig. 3, A and C, ○), which is in agreement with 4 × 10⁻¹⁰ M pApAp required to inhibit 50% of the chemical cross-linking of 2–5A in L929 cell extracts (26). Photolabeling of 37-kDa RNase L by [32P]pApAp(8-azidoA) was inhibited by 50% at a concentration of 1.2 × 10⁻¹⁰ M pApAp (Fig. 3, B and D, ○). Incubation of [32P]pApAp(8-azidoA) and pApAp at equal concentrations (1 × 10⁻⁸ M) with 80- and 37-kDa RNase L resulted in complete protection against covalent photolabeling (Fig. 3C). These data indicate that [32P]pApAp(8-azidoA) binds specifically to the 2–5A allostERIC binding site of the 80- and 37-kDa RNase L.

Time-dependent Hydrolysis of Poly(U)-3'-[32P]pCp by RNase L in PBMC Extracts—In contrast to the photoaffinity labeling/immunoprecipitation method used to determine 2–5A binding activity, the core(2–5A)-cellulose method is an affinity binding assay used to measure 2–5A-mediated hydrolysis of single-stranded RNA in the absence of non-2–5A-dependent RNases and other factors which may cause modifications, synthesis, or degradation of 2–5A (20). We previously reported that RNase L activity as measured by the ribosomal RNA cleavage assay was elevated up to 45-fold in PBMC extracts from individuals with CFS compared with healthy controls (13, 14). In view of the elevated RNase L activity observed in CBS PBMC extracts, we hypothesized that, in the presence of 2–5A and poly(U)-3'-[32P]pCp, the 37-kDa RNase L would be more active than the 80-kDa RNase L. Time-dependent cleavage of poly(U)-3'-[32P]pCp by the 80- and 37-kDa forms of RNase L was observed (Fig. 4). Poly(C)-3'-[32P]pCp was also used to measure nonspe-
specific RNase activity; the results revealed that the observed 2–5A-dependent RNase L activity was not due to the activity from nonspecific RNases (data not shown). Maximum hydrolysis of poly(U)-3’-[^32P]pCp by the 80-kDa RNase L was attained at 60 min with half-maximal hydrolysis observed at 24 min (Fig. 4A). In PBMC extracts from healthy controls, we observed a maximum of 65% hydrolysis of poly(U)-3’-[^32P]pCp at 120 min (Fig. 4A), which is in reasonable agreement with the 80% hydrolysis observed in L929 cell extracts (20). Only 2% hydrolysis of poly(U)-3’-[^32P]pCp by the 80-kDa RNase L was observed at 10 min. The delay in the activation observed with the 80-kDa RNase L in healthy control PBMC extracts could be attributed to the time required for 80-kDa RNase L to dissociate from the core(2–5A)-cellulose and form its activated homodimer complex following the addition of poly(U)-3’-[^32P]pCp and 2–5A. A similar lag time for the activation of 80-kDa RNase L immobilized on core(2–5A)-cellulose was previously observed for the 80-kDa RNase L in mouse L929 cell extracts (20, 21, 26). In contrast, maximum hydrolysis of poly(U)-3’-[^32P]pCp by the 37-kDa RNase L was attained by 30 min with half-maximal hydrolysis at 8 min (Fig. 4B). Unlike the 80-kDa RNase L, the 37-kDa RNase L did not exhibit a lag time for activation. Taken together, these results strongly suggest that the 37-kDa RNase L hydrolyzes poly(U) at a rate three times faster than the 80-kDa RNase L. This increase in the hydrolysis of poly(U)-3’-[^32P]pCp by the 37-kDa RNase L compares with a 10-fold increase in RNase L activity in CFS PBMC extracts as measured by ribosomal cleavage assays (13, 14).

The aim of this study was to validate the specific interactions involved in the allosteric binding of 2–5A and activation of the 80- and 37-kDa forms of RNase L in PBMC extracts from healthy controls and individuals diagnosed with CFS, respectively. Further characterization of the molecular structure of the 37-kDa RNase L is underway in this laboratory (1). The results in this study demonstrate that[^32P]pApAp(8-azidoA) specifically binds to the 2–5A allosteric binding site of the 80- and 37-kDa RNase L with relatively the same affinity. However, time-dependent hydrolysis of poly(U)-3’-[^32P]pCp was three times faster with the 37-kDa RNase L than with the 80-kDa RNase L, which suggests that the elevated RNase L activity observed in CFS PBMC extracts may be attributed to the increased affinity of the RNA substrate and to the increased efficiency of RNA cleavage the 37-kDa form of RNase L.

Current efforts are aimed at extending these results to identify the amino acid sequence that comprises the 2–5A-binding site of the 37-kDa RNase L. Azido photoaffinity probes have been used to specifically identify ligand binding and active sites of enzymes (18, 27). Further examination of the parameters required for the formation of the activated complex for 37-kDa RNase L observed in CFS PBMC extracts is underway in this laboratory. The biochemical data presented in this study demonstrate the utility of[^32P]pApAp(8-azidoA) for the detection of the 37-kDa RNase L.