Biochemical Evidence for a Novel Low Molecular Weight 2-5A-Dependent RNase L in Chronic Fatigue Syndrome

ABSTRACT

Previous studies from this laboratory have demonstrated a statistically significant dysregulation in several key components of the 2',5'-oligoadenylate (2-5A) synthetase-RNase L-RNase L and PKR antiviral pathways in chronic fatigue syndrome (CFS) (Suhadolnik et al. Clin Infect Dis 18, 596–104, 1994; Suhadolnik et al. In Vivo 8, 599–04, 1994). Two methodologies have been developed to further examine the upregulated RNase L activity in CFS. First, photoaffinity labeling of extracts of peripheral blood mononuclear cells (PBMC) with the azido 2-5A photoaffinity probe, $[^{32}P]$ApAp(8-azidoA), followed by immunoprecipitation with a polyclonal antibody against recombinant, human 80-kDa RNase L and analysis under denaturing conditions. A subset of individuals with CFS was identified with only one 2-5A binding protein at 37 kDa, whereas in extracts of PBMC from a second subset of CFS PBMC and from healthy controls, photolabeled/immunoreactive 2-5A binding proteins were detected at 80, 42, and 37 kDa. Second, analytic gel permeation HPLC was completed under native conditions. Extracts of healthy control PBMC revealed 2-5A binding and 2-5A-dependent RNase L enzyme activity at 80 and 42 kDa as determined by hydrolysis of poly(U)-3'-$[^{32}P]$pCp. A subset of CFS PBMC contained 2-5A binding proteins with 2-5A-dependent RNase L enzyme activity at 80, 42, and 30 kDa. However, a second subset of CFS PBMC contained 2-5A binding and 2-5A-dependent RNase L enzyme...
activity only at 30 kDa. Evidence is provided indicating that the RNase L enzyme dysfunction in CFS is more complex than previously reported.

INTRODUCTION

CHRONIC FATIGUE SYNDROME (CFS) is an illness of unknown etiology, often associated with sudden onset, flu-like symptoms, debilitating fatigue, low-grade fever, myalgia, and neurocognitive dysfunction(1,2). Diagnosis of CFS remains one of exclusion. An accumulating body of evidence suggests that CFS is associated with dysregulation of both humoral and cellular immunity, including mitogen response, reactivation of viruses, abnormal cytokine production, diminished natural killer (NK) cell function, and changes in intermediary metabolites.(3-13) On the basis of these observations, we reasoned that the clinical and immunologic abnormalities observed in CFS might include defects in the double-stranded RNA (dsRNA) dependent, interferon (IFN)-inducible pathways, that is, the 2',5'-oligoadenylate (2-5A) synthetase/RNase L and PKR antiviral defense pathways.(14,15) The 2-5A synthetase/RNase L pathway is part of the antiviral defense mechanism in mammalian cells and has also been implicated in the regulation of cell growth and differentiation.(16-19) When activated by dsRNA, 2-5A synthetase converts ATP to 2',5'-linked oligoadenylates. Biologically active 2-5A binds to and activates a latent endoribonuclease, RNase L, which hydrolyzes single stranded viral and cellular RNA primarily after UpNp sequences, thereby inhibiting protein synthesis.

Initial studies on the 2-5A synthetase/RNase L pathway CFS in this laboratory revealed a statistically significant dysregulation in which the 2-5A synthetase is present predominantly in its activated form, bioactive 2-5A levels are elevated and RNase L activity is upregulated compared with healthy controls.(14-15) We have also reported that expression of the serine-threonine kinase, PKR, is downregulated in CFS.(15) PKR controls initiation of protein translation through phosphorylation of eIF-2. In the current study, we have applied two methodologies. to further examine the 2-5A binding and 2-5A-dependent RNase-L enzyme activity in extracts of peripheral blood mononuclear cells (PBMC) from individuals with CFS and healthy controls. In the first methodology, 2-5A binding proteins have been identified by photoaffinity labeling with an azido photoprobe of 2-5A, followed by immunoprecipitation with a highly purified...
recombinant human 80-kDa RNase L-specific polyclonal antibody and electrophoretic analysis under denaturing conditions. In the second methodology, 2-5A binding and 2-5A-dependent RNase L enzyme activity have been determined following fractionation by analytic gel permeation HPLC under native (nondenaturing) conditions. Marked differences have been observed in the molecular mass and RNase L enzyme activity of 2-5A binding proteins in extracts of PBMC from individuals with CFS compared with healthy controls. Biochemical evidence is presented for an RNase L enzyme dysfunction in CFS.

MATERIALS AND METHODS

Study subjects and controls

Study subjects were individuals who had previously fulfilled the diagnostic criteria for CFS per the 1994 CDC guidelines(2) and healthy controls. Patients and controls were selected from the medical practices of Dr. Daniel L. Peterson, Incline Village, NV, and Dr. Paul R. Cheney, Charlotte, NC. Criteria for selection of patients and controls and clinical variables at initiation of the study were as described from this laboratory.\(^{(14,15)}\) At the time of blood sampling, selected symptoms were evaluated on a self-graded symptom checklist level of disability was assessed using the Karnofsky Performance Score (KPS) (mean KPS = 56). Ten age-matched and gender-matched control subjects were recruited. Each CFS patient and healthy control underwent a medical history taking and physical examination. The age distribution of the controls was not significantly different from that of the individuals with CFS (CFS mean age = 46 years; control mean age = 41.7 years). All controls were interviewed and specifically denied having chronic fatigue or any other significant symptoms, and the results of physical examinations were normal. Approval for the study was obtained from local institutional review boards (IRB). Informed consent was obtained from each patient and control.

Peripheral blood mononuclear cells and cell extracts

PBMC were separated from heparinized blood (50 ml) by Ficoll-Hypaque density gradient centrifugation within 4 h of blood drawn.\(^{(14)}\) Cytoplasmic extracts were prepared in the presence or absence of protease inhibitors according to the manufacturer's directions (Mini-Complete protease inhibitor cocktail tablets, Boehringer/Mannheim, containing aprotinin, leupeptin, Pefabloc SC, and EDTA).\(^{(14)}\)

Production of recombinant human RNase L polyclonal antibody

A polyclonal antibody against recombinant human 80 kDa RNase L was elicited in New Zealand white rabbits by immunization with highly purified recombinant human 80-kDa RNase L as described.\(^{(20,21)}\) Immunization was performed by Cocalico Biologicals, Inc. Serum was prepared before immunization and retained as a control (preimmune serum). Initial inoculation was performed on day 1 with 100 µg of glutathione 5-
transferase (GST)-RNase L mixed with an equal volume of complete Freund's adjuvant. Boosters with 50 µg of (GST-RNase L (50% native and 50% heat-denatured protein) mixed with incomplete Freund's adjuvant were given at 14, 21, 49, and 84 days. Blood samples for antibody production were drawn at 120, 150, and 180 days, preceded by additional boosters. Following hydrolysis of GST-RNase L fusion protein with human thrombin, RNase L was covalently coupled to a glutaraldehyde-activated cartridge (Whatman) according to the manufacturer's specifications. Sodium borohydride was circulated through the column to reduce the glutaraldehyde that was not coupled to RNase L. The antiserum containing polyclonal antibody to RNase L was circulated through the glutaraldehyde cartridge for 1 h at ambient temperature and eluted according to the manufacturer's specifications. The RNase L polyclonal antibody was characterized by Western blotting using cytoplasmic extracts of human 293 cells (ATCC CRL 1573) and an Escherichia coli-expressed recombinant GST-RNase L fusion protein. (21)

Photoaffinity labeling and immunoprecipitation of RNase L in human 293 cell extracts with the 2-5A photoprobe, [$^{32}$P]pApAp(8-azidoA)

Human 293 cell extracts (100 µg protein) were incubated with 5'-O-phosphoryladenylyl-(2' → 5')-adenyllyl-(2' → 5')-8-azidoadenosine, or [$^{32}$P]pApAp(8-azidoA) (5 µCi) at 4°C for 30 min (15 µl final volume), followed by photolysis for 30 sec. (22) An aliquot of serum (20-40 µl) or highly purified recombinant human 80-kDa RNase L polyclonal antibody (5 µg protein) was added to the photolabeled mixture with 30 µl of Protein A-agarose (Sigma Chemical Co.) and 100 µl phosphate-buffered saline (PBS). The mixture was rotated slowly at 4°C for 1 h. The Protein A-agarose was washed with 0.5 µl PBS three times. The resin was mixed with 40 µl of protein solubilization solution and boiled for 5 min. The supernatant was fractionated by 10% SDS-PAGE. The immunoprecipitated protein was visualized by autoradiography of the dried gel.

Western blot analysis

Cytoplasmic extracts of human 293 cells or PBMC were fractionated by 10% SDS-PAGE, electrophoretically transferred to nitrocellulose, and probed with a 1:250 dilution of RNase L polyclonal antibody. Following incubation with a 1:2500 dilution of an antirabbit horseradish peroxidase-labeled secondary antibody and washing per the manufacturer's specifications, blots were developed with the enhanced chemiluminescent (ECL) Western blotting detection Systems (Amersham). Western blot analyses determined that 50 µg of PBMC extract protein provided the optimal signal/background ratio with the highly purified polyclonal antibody against recombinant human 80-kDa RNase L.

Azido photoaffinity labeling and immunoprecipitation of 2-5A binding proteins in PBMC extracts under denaturing conditions

Chemical synthesis of the 2-5A azido photoprobe, ApAp(8-azidoA), 5'-monophosphorylation with [$^{32}$P] ATP and polynucleotide kinase to produce [$^{32}$P]pApAp(8-azidoA), and photolabeling of 2-5A binding proteins in PBMC extracts were as described previously from this laboratory. (22) Photolabeling of the 2-5A binding proteins was accomplished by incubation of PBMC extracts (100 µg protein) prepared
in the presence or absence of protease inhibitors with the 2-5A photoprobe, $[^{32}P]p$A-pAp(8-azidoA) (60 µCi/nmol, 5 µCi) (30 min at 4°C), followed by UV irradiation (8000 µ W/cm², for 30 sec, 0°C). The photolabeling mixture was combined with affinity-purified RNase L polyclonal antibody (24 µg protein), Protein A-Sepharose (30 µl), and 100 µl PBS, and the mixture was rotated for 1 h at 4°C. After three PBS washes, the resin was mixed with 40 µl of protein solubilization solution, boiled for 5 min, and centrifuged (10,600 x g for 5 min at ambient temperature). The entire supernatant was fractionated by 10% SDS-PAGE. The azido photo-labeled/immunoprecipitated 2-5A binding proteins were visualized by autoradiography and phosphorimaging analysis (Fuji BAS 2000 Phosphorimager) of the dried gel.

**Molecular mass determination of 2-5A binding proteins in PBMC extracts by analytic gel permeation HPLC under native conditions**

Extracts of PBMC (200 µg protein) from individuals with CFS or healthy controls were incubated for 30 min at 4°C in the presence of $[^{32}P]p$ApAp(S-azidoA) (110 µCi/nmol, 10 µCi) in the presence or absence of protease inhibitors, UV irradiated (8000 µW/cm² for 30 sec, 0°C), loaded onto a Superdex 200 (Hiload 16/60) gel filtration column (Pharmacia Biotech Inc.), and eluted at ambient temperature with 25 mM Tris-Cl (pH 7.4), 80 mM KCl, 1 mM EDTA, 5 mM MgCl₂, 0.1 mM ATP, and 14 mM β-mercaptoethanol at a flow rate of 0.5 ml/min. Then, 0.5-mi fractions were collected (20,22). The $[^{32}P]$azido 2-5A photoprobe that was covalently linked to the 2-5A binding protein(s) in each fraction was quantitated by Cerenkov radiation (50% efficiency) scintillation spectrometry in a Tm Aalalytics model 6895 scintillation spectrometer. The Superdex 200 column was calibrated with myosin, β-galactosidase, phosphorylase b, bovine serum albumin (BSA), ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor (220, 116, 97A, 68, 44, 31, and 21.5 kDa, respectively).

**2-5A-dependent RNase L enzyme activity in PBMC extracts following analytic gel permeation HPLC under native conditions**

Extracts of PBMC (200 µg protein) from healthy controls and individuals with CFS were purified under native conditions on a Superdex 200 gel filtration column as described in the preceding paragraph. RNase L activity in an aliquot (5-20 µl) of each fraction was determined by the hydrolysis of poly(U)-3'-$[^{32}P]$Cp (20,000 dpm) in reaction mixtures (15-30 µl) containing 5-O-triphosphoryl-adenylyl-(2'→5')-adenylyl-(2'→5')-adenosine (p₃A₃) (1 x 10⁻⁸ M to 1 x 10⁻⁷ M). (21) Nonspecific RNase activity was measured by hydrolysis of poly(C) 3'-$[^{32}P]$Cp (14,000 dpm) in reaction mixtures (15-50 µl) in the absence of p₃A₃. Radioactive measurements were accomplished using Scintiverse I (Fisher) (>99% efficiency).

**Ribosomal RNA cleavage assays**

Ribosomal RNA cleavage assays were used to measure total RNase L activity in unfractionated PBMC extracts, as previously described from this laboratory (14,15). The formation of specific cleavage products (SCP) due to 2-5A-dependent RNase L activity was quantitated by densitometric tracings of photographs of gels and was expressed as
the ratio of the products of the reaction (SCP) to the substrate (28S and 18S rRNA), multiplied by 100.

RESULTS

Production, characterization, and specificity of a polyclonal antibody to recombinant human 80-kDa RNase L

The GST fusion protein strategy was used to obtain purified recombinant human 80-kDa RNase L required for production of the RNase L polyclonal antibody.\(^{(21)}\) The specificity of the polyclonal antibody was determined by photoaffinity labeling/immunoprecipitation and Western blot analysis. One of two rabbits produced polyclonal antibody to recombinant human 80-kDa RNase L 50 days after the initial injection of antigen (GST-RNase L) as determined by Western blot analysis (data not shown). Antibody production reached a maximum at 120 days. The second rabbit failed to respond to the antigen. These observations were confirmed by photoaffinity labeling/immunoprecipitation experiments. Immune complex formation between photolabeled RNase L in extracts of human 293 cells and the recombinant human 80-kDa RNase L polyclonal antibody was detected by SDS-PAGE fractionation and autoradiography. Using antiserum from the rabbit that responded to the antigen, an 80-kDa radiolabeled protein was observed (Fig. 1A, lane 2). The recombinant human 80-kDa RNase L polyclonal antibody did not block 2-5A binding as evidenced by the photoaffinity labeling of RNase L after binding to the antibody. The same phenomenon has been observed with antimurine RNase L polyclonal antibody.\(^{(23)}\) No radiolabeled protein was observed in either preimmune serum or antiserum from the rabbit that failed to respond to the antigen (Fig. 1A, lanes 1 and 3).

The RNase L polyclonal antibody was purified from antiserum using an RNase L-GTA cartridge (covalently crosslinked) as an affinity column. The purified recombinant human RNase L polyclonal antibody specifically recognized only one protein in human 293 cell extracts that migrated at 80 kDa (Fig. 1B).

Identification of 2-5A binding proteins in extracts of CFS PBMC and healthy control PBMC by azido photoaffinity labeling and immunoprecipitation under denaturing conditions

Previous studies from this laboratory reported a statistically significant upregulation in RNase L activity in a pilot study of individuals with CFS who were severely disabled \(p < 0.0001\).\(^{(14)}\) A subsequent placebo-controlled, multicenter study confirmed the upregulation of RNase L activity in individuals with CFS as measured by ribosomal RNA cleavage assays \(p = 0.001\).\(^{(15)}\) Consistent with these observations, ribosomal RNA cleavage assays on the current study subjects demonstrated up-regulated RNase L activity in extracts of PBMC from individuals with CFS compared with healthy controls (CFS mean ± SE = 195 ± 47 \(n = 7\); healthy control mean ± SE 85 ± 17 \(n 7\) \(p < 0.02\)).

Further characterization of the upregulated RNase L in CFS PBMC was accomplished with an azido photolabeling/immunoprecipitation methodology that specifically identifies 2-5A binding, RNase L immunoreactive proteins in extracts of PBMC. This
methodology eliminates proteins that immunoreact with the polyclonal antibody to recombinant human 80-kDa RNase L but are not 2-5A binding proteins and also eliminates 2-5A binding proteins that are not immunoreactive to the polyclonal antibody to RNase L. Under denaturing conditions, three 2-5A binding proteins with molecular masses of 80, 42, and 37 kDa were observed in healthy control PBMC (Fig. 2, lanes 2,3) and in a subset of extracts of CFS PBMC prepared in the absence of protease inhibitors (Fig. 2, lanes 1,4,5). However, photoaffinity labeling/immunoprecipitation revealed a second subset of CFS PBMC in which only one 2-5A binding protein with an estimated molecular mass of 37 kDa was observed. No 80-kDa or 42-kDa immunoreactive 2-5A binding proteins were observed (Fig. 2, lanes 6,7,8,9).

**Purification of human RNase L and molecular mass determination in healthy control and CFS PBMC extracts under native conditions**

Characterization of the 2-5A binding proteins detected in CFS PBMC extracts by photoaffinity labeling/immunoprecipitation by SDS-PAGE (denaturing conditions) was continued by inalytic gel permeation HPLC and assay of 2-5A binding and 2-5A-dependent RNase L enzyme activity as determined by the hydrolysis of poly(U)-3'-[^32P]pCp under native (nondenaturng) conditions. In healthy control PBMC extracts (prepared in lie absence of protease inhibitors) analyzed under native conditions, 2-5A binding and 2-5A-dependent RNase L enzyme activities were observed at 80 and 42 kDa (Fig. 3A, D). In one subset of CFS PBMC extracts, three 2-5A binding proteins with 2-5A-dependent RNase L enzyme activity were observed at 80, 42, and 30 kDa (Fig. 3B, E).

In a second subset of CFS PBMC extracts (as shown in Fig. 2 lanes 6,7,8,9), no 2-5A binding or 2-5A-dependent RNase L enzyme activity was observed at 80 or 42 kDa. However, 2-5A binding and 2-5A-dependent RNase L enzyme activity was observed at 30 kDa (Fig. 3C, F). The 30-kDa protein is subsequently referred to as the low molecular weight (LMW) RNase L. Poly(C)-3'-[^32P]pCp was used in control assays to show that the 2-5A-dependent RNase L enzyme activity observed in PBMC extracts was not due to nonspecific RNase activity. No hydrolysis of poly(C)-3'-[^32P]pCp was observed in any fraction, which is taken as additional evidence for the presence of 2-5A-dependent RNase L. The specificity of binding of the[^32P]pApAp-(8-azidoA) photoprobe was confirmed by competition experiments with authentic p3A3 (data not shown). Furthermore, no hydrolysis of poly(U)-3'-[^32P]pCp was observed in the absence of 2-5A, the allosteric activator of RNase L. The 2-5A binding protein observed at 37 kDa under denaturing conditions is in reasonable agreement with the 30-kDa protein observed under native conditions, based on literature precedents accounting for differences in molecular mass observed under denaturing and native conditions. No difference was observed in the RNase L activity profile of PBMC extracts prepared in the presence or absence of protease inhibitors.

**Stability of immunoreactive 2-5A binding proteins to proteolysis**

PBMC extracts were prepared in the presence and absence of protease inhibitors to assess the possible effect of proteolytic degradation during the preparation and processing of the extracts. The azido photoprobe,[^32P]pApAp-(8-azidoA), was covalently bound to its 2-5A binding proteins and further purified by immunoprecipitation with recombinant human 80-kDa RNase L polyclonal antibody.
The photoaffinity-labeled, immunoreactive 2-5A binding proteins were quantitated by phosphorimaging analysis after SDS-PAGE. CFS PBMC extract prepared in the presence of protease inhibitors contained an LMW immunoreactive 2-5A binding protein at 37 kDa in addition to the 80-kDa 2-5A-dependent RNase L (Fig. 4, lane 1). Photoaffinity labeling and immunoprecipitation of the same CFS PBMC extract, prepared in the absence of protease inhibitors, revealed immunoreactive 2-5A binding proteins at 37 and 42 kDa in addition to the 80 kDa RNase L (Fig. 4, lane 2). Quantitation by phosphorimaging analysis demonstrated that the protein bands observed at 37 kDa in extracts of CFS PBMC were of equal intensity in the presence and absence of protease inhibitors, indicating that the 37-kDa protein is stable (Fig. 4, lanes 1 and 2).

A 40-kDa protein from mouse spleen extract has been reported to be stable under similar experimental conditions. In extracts of healthy control PBMC prepared in the presence of protease inhibitors, only the 80-kDa RNase L was detected (Fig. 4, lane 3). In the same extract prepared in the absence of protease inhibitors, there was a 70% decrease in the 80-kDa RNase L (Fig. 4, compare lanes 3 and 4). However, no 37-kDa immunoreactive 2-5A binding protein was detected in this healthy control PBMC extract prepared in the presence or absence of protease inhibitors (Fig. 4, lanes 3 and 4). Proteolytic cleavage of 80-kDa RNase L and accumulation of a 42-kDa 2-5A binding protein has been reported in murine liver. In 2 of 7 healthy control PBMC extracts tested to date, an immunoreactive 2-5A binding protein was observed at 50 kDa but not at 37 kDa (Fig. 4, lane 4). However, the 50-kDa 2-5A binding protein did not exhibit 2-5A-dependent RNase L activity as measured by the hydrolysis of poly(U)-3'-[^32P]pCp (data not shown).

**DISCUSSION**

The working hypothesis of this research is that the characteristic signs and symptoms of CFS are associated with a dysregulation of the 2-5A synthetase/RNase L pathway. The 2-5A synthetase/RNase L pathway in mammalian cells is involved in hydrolysis of cellular and viral RNA and is tightly regulated by dsRNA, 2-5A, and RLI. Induction of the 2-5A synthetase/RNase L pathway by IFN has been implicated in the maintenance of RNA metabolism and control of cell growth and differentiation. Previous reports from this laboratory have demonstrated a statistically significant upregulation of the 2-5A synthetase/RNase L pathway in CFS; 2-5A synthetase is present predominantly in the activated form, bioactive 2-5A levels are elevated, and total RNase L enzyme activity is upregulated. Two new methodologies have been developed in continuing studies designed to further characterize the upregulated RNase L activity in extracts of PBMC from individuals with CFS compared with healthy controls. The first methodology identifies 2-5A binding proteins by azido photoaffinity-labeling, followed by immunoprecipitation analysis under denaturing conditions. The second methodology involves fractionation by analytic gel permeation HPLC under native (nondenaturing) conditions to identify 2-5A binding proteins, their molecular masses, and 2-5A-dependent RNase L enzyme activity. These methodologies have revealed several striking differences in 2-5A binding and 2-5A-dependent RNase L enzyme activity profiles in extracts of PBMC from individuals with CFS compared with extracts of PBMC from healthy controls.
The results of this study confirm and extend our previous reports of upregulated RNase L activity associated with CFS. The azido photolabeling/immunoprecipitation analyses under denaturing conditions have revealed marked differences in the 2-5A binding proteins in extracts of PBMC from individuals with CFS compared with healthy controls. In healthy controls, three immunoreactive 2-5A binding proteins are observed with molecular masses of 80, 42, and 37 kDa (representative healthy controls are shown in Fig. 2, lanes 2 and 3). Three 2-5A binding proteins are also detected in a subset of individuals with CFS (Fig. 2, lanes 1, 4, 5). However, in a second subset of individuals with CFS, only one 2-5A binding protein is observed with a molecular mass of 37 kDa. The 80-kDa and 42-kDa 2-5A binding proteins are absent (Fig. 2, lanes 6, 7, 8, 9).

To determine if the 2-5A binding proteins identified by azido photoaffinity labeling/immunoprecipitation under denaturing conditions had 2-5A-dependent RNase L enzyme activity, further analysis was completed under native (nondenaturing) conditions by analytic gel permeation HPLC. Extracts of PBMC from all healthy controls revealed 2-5A binding and RNase L enzyme activity only at 80 and 42 kDa, as determined by hydrolysis of poly(U)-3'-[32P]pCp but not poly(C)-3'-[32P]pCp (Fig. 3D). Even though 2-5A binding proteins were observed at 50 and 37 kDa in some extracts of healthy control PBMC under denaturing conditions, there was no evidence of 2-5A-dependent RNase L activity at 50 kDa or at 37 kDa. In all CFS PBMC analyzed under native conditions, a 30-kDa protein with 2-5A-dependent RNase L enzyme activity was observed. However, in one subset of CFS PBMC, three immunoreactive 2-5A binding proteins with 2-5A-dependent RNase L enzyme activity were observed (Fig. 3E). In these PBMC, 2-5A-dependent RNase L enzyme activity was detected in fractions equivalent to 80, 42, and 30 kDa. Forms of 2-5A-dependent RNase L with molecular masses between 70-90 kDa and 40-42 kDa have been reported. Further, 2-5A binding proteins at 40-42 and 38 kDa have been reported in differentiated marine cells in culture and marine liver and were proposed to be the result of proteolytic degradation. In a second subset of CFS PBMC, 2-5A binding and 2-5A-dependent RNase L enzyme activity were observed only at 30 kDa (Fig. 3C, F). This phenomenon has not been observed in any healthy control PBMC.

Several possible biochemical mechanisms can be proposed to account for the presence of the LMW (30 kDa) RNase L and absence of the 80-kDa and 42-kDa RNase L in CFS. First, the LMW RNase L may be the result of proteolytic degradation of the 80 or 42 kDa 2-5A-dependent RNase L by a cellular or virus-encoded protease. Numerous proteases have been demonstrated to have functional impact in normal and virus-infected cells. For example, PKR is hydrolyzed by a protease encoded by the poliovirus genome. CFS PBMC extracts prepared in the presence and absence of protease inhibitors show a 37-kDa immunoreactive 2-5A-dependent RNase L that does not arise from protease-mediated degradation of the 80 kDa RNase L at the time of PBMC extract preparation and processing (Fig. 4, lanes 1 and 2). In addition, no protease-mediated degradation of the 37-kDa RNase L was evident at the time of extract preparation and processing.

A second possible mechanism is that the LMW 2-5A-dependent RNase L in CFS is regulated by 2-5A in a manner distinct from the 80-kDa and 42-kDa forms of RNase L.
The upregulated RNase L activity observed in CFS PBMC extracts (as determined by ribosomal RNA cleavage assay) might be due solely to the LMW RNase L, particularly in view of the observation that the LMW RNase L is the only immunoreactive 2-5A binding protein detected in some PBMC extracts (Fig. 2, lanes 6-9, Fig. 3C, F). This laboratory has reported previously that PBMC extracts from individuals with CFS have elevated levels of bioactive 2-5A. (14,15) The 2-5A molecule may preferentially activate the LMW protein over the 80 kDa and 42 kDa forms of RNase L. Steady-state kinetics and changes in $K_m$ with catalysis of the 2-5A-activated 80-kDa RNase L ligand have been quantitated by precise cleavage of oligoribonucleotide substrates containing unknown dyad sequences. (34) Kinetic characterization and amino acid sequencing of the LMW RNase L observed in CFS PBMC extracts is under way in this laboratory.

A third possibility is that regulation of the LMW 2-5A-dependent RNase L enzyme activity may require protein-protein interactions with the 80-kDa or 42-kDa RNase L (or both). In the absence of the 80-kDa and 42-kDa isoforms, the LMW RNase L may be unregulated. A loss of regulation of RNase L could result in a constitutively active LMW RNase L. The resulting continuous turnover of cellular RNA might contribute to the marked decrease in ATP pools that has been observed in CFS. (35) It is also possible that the recently identified RNase L inhibitor (RLI) may be involved in the regulation of RNase L in CFS. (19,30,31) RLI is a negative regulator of RNase L that functions by inhibiting the binding of the allosteric activator, 2-5A, to RNase L. The role of RLI in regulation of the LMW RNase L in CFS remains to be established. The biochemical and immunologic data presented here have identified a potential subgroup of individuals with CFS with an RNase L enzyme dysfunction that is more profound than previously observed. CFS, as currently defined, may well represent a heterogeneous group of disorders. No unifying pathophysiology for CFS has been established. Analysis of the clinical status of the individuals with CFS in this study failed to reveal any subgroup variables to account for the RNase L enzyme dysfunction, including circulating IFN level, type of onset (acute vs. gradual), and length of illness. Expanded studies are underway to determine if the results presented in this study are representative of all patients with CFS and if these findings can distinguish individuals with CFS from those with clinically similar illnesses. Longitudinal studies also are underway in this laboratory to establish if the RNase L enzyme dysfunction observed in CFS is characteristic of a particular stage in the course of the illness or if the dysfunction fluctuates with time.

Whatever the biochemical mechanism responsible for the LMW RNase L, it exhibits the biochemical properties of an authentic 2-5A-dependent RNase L. Based on the differences in 2-5A binding and 2-5A-dependent RNase L activity observed in the CFS PBMC in this study, it is tempting to speculate that the presence of the LMW RNase L and absence of the 80-kDa and 42-kDa RNase L are related to the severity of CFS symptoms. It is noteworthy that extracts of PBMC from the two most severely disabled individuals with CFS in this study (KPS = 40) contained only the LMW RNase L (Fig. 2, lanes 7 and 8). This is consistent with the possibility that absence of the 80-kDa and 40-kDa RNase L and presence of the LMW RNase L correlate with the severity of CFS clinical presentation.

It will be of interest to determine the extent to which the LMW RNase L may be used in the diagnosis of CFS and if control of the RNase L enzyme dysfunction could provide therapeutic benefit. For example, this laboratory has reported on the inhibition of 2-5A-
dependent RNase L activation by several stereochemically modified phosphorothioate derivatives of 2-5A.\textsuperscript{(21,36,37)} The possibility that such 2-5A derivatives can function as antagonists of the 2-5A-mediated activation of RNase L in CFS is under investigation in this laboratory.

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