Characterization of novel antigens recognized by serum autoantibodies from anti-CD1 TCR-transgenic lupus mice

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In this study, we further characterize the humoral autoimmune response in the recently described anti-CD1 autoreactive T cell receptor-transgenic mouse lupus model (CD1 lupus model). We discovered and characterized novel autoantigens, comprising a protein of 105 kDa (p105) and a novel RNA molecule of 140 base pairs (bp) that is likely associated with p105, and several additional factors with distinct biochemical properties. In the CD1 lupus model, lethally irradiated BALB/c/nu/nu mice were injected intravenously with sorted bone marrow cells and sorted splenic T cells from donor BALB/c mice expressing TCR α and β transgenes that encode autoreactivity for CD1d. Adoptive hosts injected with the single-positive ($CD4^+$ and $CD8^+$) subset of transgenic cells developed anti-double-stranded DNA antibodies and a lupus-like illness. Sera were analyzed by Western blotting and immunoprecipitation. Antigens were characterized by biochemical and serological methods. Serum autoantibodies from 5 of 12 (42%) CD1 lupus mice immunoprecipitated a 105-kDa protein, termed p105. p105 was associated with a small RNA of $\sim$140 bp. Anti-p105 autoantibodies appeared early in the course of disease. Serological and biochemical characterization suggested that p105 was distinct from known lupus autoantigens of similar molecular masses, indicating that p105 represents a novel autoantigen in lupus.

Received	7/10/03
Revised	12/3/04
Accepted	6/4/04

Key words: Lupus / Animal model / Autoantigen / Autoantibodies

1 Introduction

Animal models have provided multiple insights into the pathogenesis of systemic lupus erythematosus (SLE) [1]. Among rationales to develop and study new models for SLE, a better understanding of mechanisms governing the selection of autoantigens ranks high. Identification of novel antigens may provide important clues to early events leading to the breakage of tolerance against key antigens and subsequent epitope spreading [2]. In the CD1 lupus model, autoreactive anti-CD1-transgenic T cell subsets are adoptively transferred into syngeneic BALB/c/nu/nu hosts, leading to the production of anti-double-stranded (ds) DNA antibodies and the development of a severe lupus-like disease in recipients of sorted $CD4^+$ and $CD8^+$ (single-positive, SP), but not in recipients of $CD4^-CD8^-$ (double-negative, DN) transgenic T cells [3]. Whereas disease-preventing DN transgenic T cells produce large amounts of IL-4 and little IFN-γ, disease-inducing SP transgenic T cells secrete large amounts of IFN-γ and little IL-4 [3]. The activation of B cells via anti-CD1 T cell cross-linking of their CD1 receptors, and the influence of IFN-γ and other cytokines, have been proposed to contribute to the production of autoantibodies, IgG isotype switching, and the formation of pathogenic autoantibodies in this model [3].

We studied the spectrum of autoantibodies produced in the CD1 lupus model, identifying two sets of autoantibodies directed against (1) a member of the IFN-inducible 202 (Ifi202) family; and (2) a member of the heat shock protein (HSP)70 family, which is also weakly IFN-inducible (W.H., D.Z., S.S., P.J.U., manuscript submitted). Importantly, subsets of antinuclear antibodies

[DOI 10.1002/eji.200324201]

Abbreviations: ds: Double-stranded **SP:** Single-positive ($CD4^+$, $CD8^+$) **DN:** Double-negative ($CD4^-CD8^-$) **Ifi202:** IFN-inducible 202 **HSP:** Heat shock protein **RNP:** Ribonucleoprotein **snRNP:** Small nuclear RNP **hnRNP:** Heterogeneous nuclear RNP **Sm:** Smith proteins of the snRNP complex **PARP:** Poly(ADP-ribose) polymerase **SRP:** Signal recognition particle

that recognize Ifi16, the human homologue of Ifi202, were previously described in ~30% of SLE patients [4]. Similarly, HSP70 is a known candidate antigen in several human autoimmune diseases, including SLE [5, 6]. We speculated that other autoantigens, which may have human homologues, might be targeted in the CD1 lupus model. Herein, we report the discovery and characterization of a novel autoantigen, composed of a 105-kDa protein that was co-precipitated together with a small RNA of ~140 base pairs (bp), and several additional factors.

2 Results

2.1 Serum autoantibodies from a subset of CD1 lupus mice precipitate a 105-kDa protein

Lethally irradiated wild-type BALB/c/nu/nu host mice were injected with 5×10^6 bone marrow (BM) cells and 2×10^5 sorted $CD4^+$ and $CD8^+$ SP splenic T cells from TCR α and β SP transgenic BALB/c donor mice. Of 14 adoptive hosts that were injected in two separate experiments (group A, 6 animals, and group B, 8 animals), 12 developed anti-dsDNA antibodies and proteinuria; sera from these mice were used for subsequent serological studies. Immunoprecipitations of proteins from mouse cell extracts were performed using sera from adoptive hosts that had established lupus-like disease at the time of collection. NIH/3T3 cells were metabolically labeled with [^{35}S]methionine prior to detergent lysis, and proteins were immunoprecipitated using mouse sera and controls. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and the membrane was exposed for autoradiography.

A representative experiment is shown in Fig. 1. We used serum samples obtained at day 60 from four CD1 lupus host mice with proteinuria (CD1 lupus 4, group A, and CD1 lupus 1–3, group B), anti-Smith (anti-Sm) reference serum, and serum from a normal BALB/c mouse. Anti-Sm reference serum immunoprecipitated several Sm proteins (Sm-BB', Sm-C, and Sm-D) and the U1A component of the small nuclear ribonucleoprotein (snRNP) complex (Fig. 1, lane 1). Serum autoantibodies from CD1 lupus mice 2–4 immunoprecipitated a 105-kDa protein (Fig. 1, lanes 4–6). Sera from CD1 lupus mice 1–3 immunoprecipitated a second protein migrating as a doublet at 44/46 kDa (p44/46; Fig. 1, lanes 3–5). Serum from CD1 lupus host 4 recognized two additional proteins of 70 kDa and 50 kDa (Fig. 1, lane 6, arrowheads). We recently reported the identification of these two antigens, demonstrating that p70 is a member of the HSP70 family, and p50 is a member of the Ifi202 family of proteins (W.H., D.Z., S.S., P.J.U., manuscript submitted).

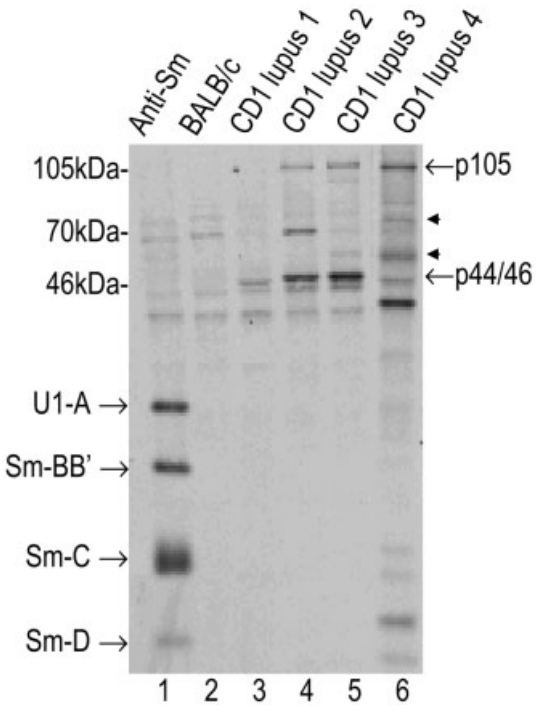

Fig. 1. Novel autoantigens are recognized by some CD1 lupus sera. Radiolabeled NIH/3T3 fibroblasts were lysed in NP40 lysis buffer, and proteins were immunoprecipitated from the lysate using sera from four different CD1 lupus mice (lanes 3–6), a normal BALB/c mouse (lane 2), and a human CDC reference serum containing antibodies against Sm and U1A (lane 1). Proteins precipitated by CD1 sera are indicated on the right side of the figure. Relative masses of molecular markers, and the U snRNP proteins precipitated by CDC reference serum, are indicated on the left side of the figure.

In summary, results shown in Fig. 1, and from additional immunoprecipitation experiments using sera from the remaining CD1 lupus mice of groups A and B, demonstrate that 5 of 12 (42%) CD1 host mice with lupus developed anti-p105 autoantibodies, and serum from 3 of these 12 (25%) mice contained anti-p44/46 autoantibodies (Fig. 1, lanes 3–5, and data not shown). Neither p105 nor p44/46 was precipitated by serum from five normal BALB/c mice (Fig. 1, lane 2, and data not shown). P50 and p70 were co-precipitated together with p105 (but not with p44/46) by serum from CD1 lupus mouse 4 (Fig. 1, lane 6). Co-precipitation of p105 and p44/46 was observed using CD1 lupus sera 2 and 3 (Fig. 1, lanes 4, 5).

2.2 Nucleolin and p105 are different proteins

To establish whether p105 might be a known antigen, we used reference sera containing antibodies directed against the Su complex (100/102 kDa, [7]), the RNA- and DNA-binding phosphoprotein nucleolin (110 kDa [8]), and poly(ADP-ribose) polymerase (PARP, 113 kDa [9]) to precipitate cognate antigens in co-migration experiments. In the case of PARP, we failed to immunoprecipitate the proteins from 3T3 or EL-4 cells. In the case of the 100/102-kDa Su antigen complex, p105 did not co-migrate with either of the two proteins (data not shown).

Using a reference polyclonal antibody directed against nucleolin, we did not detect the 110-kDa nucleolin when immunoprecipitation was attempted using 3T3 cells metabolically labeled with ^{35}S, probably due to incomplete labeling of nucleolin (data not shown). However, when we used a larger quantity of 3T3 cells to prepare a lysate from which to immunoprecipitate nucleolin, we detected nucleolin by enhanced chemoluminescence (ECL, Fig. 2A). To investigate the possibility that p105 was identical to nucleolin, p105 and nucleolin were immunoprecipitated and subjected to two-dimensional electrophoresis. As shown in Fig. 2B, C, migration of the two proteins was different in the first dimension: nucleolin migrated at an isoelectric focusing point (IF) of 5–5.5, whereas p105 migrated at an IF of approximately 3.5, indicating that p105 is distinct from nucleolin. Moreover,

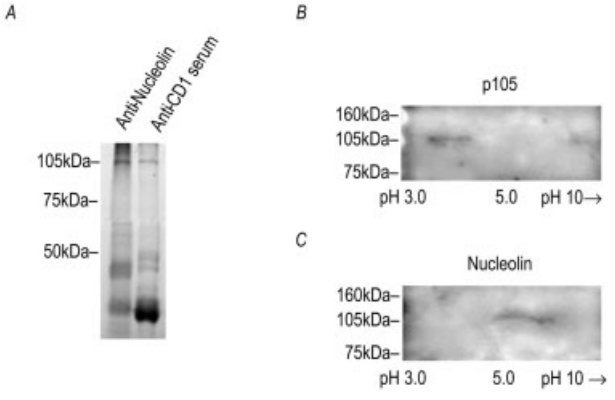

Fig. 2. Two-dimensional electrophoresis of nucleolin and p105. Nucleolin and p105 were immunoprecipitated from unlabeled 3T3 cell lysates (~1.6 mg total protein), followed by separation of precipitated proteins in two dimensions. (A) Coomassie blue-stained 1D gel of proteins immunoprecipitated from unlabeled 3T3 cell lysates. Antisera are indicated at the top of the image. (B, C) Migration of p105 and nucleolin, respectively. The non-linear pH gradient is indicated across the bottom of each panel, molecular mass markers in kDa are shown on the left side of each panel.

CD1 lupus serum did not recognize immunoprecipitated nucleolin on Western blot (data not shown).

2.3 Autoantibodies directed against p105 appear early in the course of disease

Sera from CD1 lupus mice of group A were collected at six time points after induction of disease, and analyzed by immunoprecipitation for the appearance of autoantibodies. A representative experiment using serial serum samples from two CD1 lupus animals that developed proteinuria is shown in Fig. 3. Antibodies against p105 were detectable in CD1 lupus serum 6 as early as 3 weeks after disease induction, and thereafter throughout the observation period (Fig. 3, lanes 6–10). In CD1

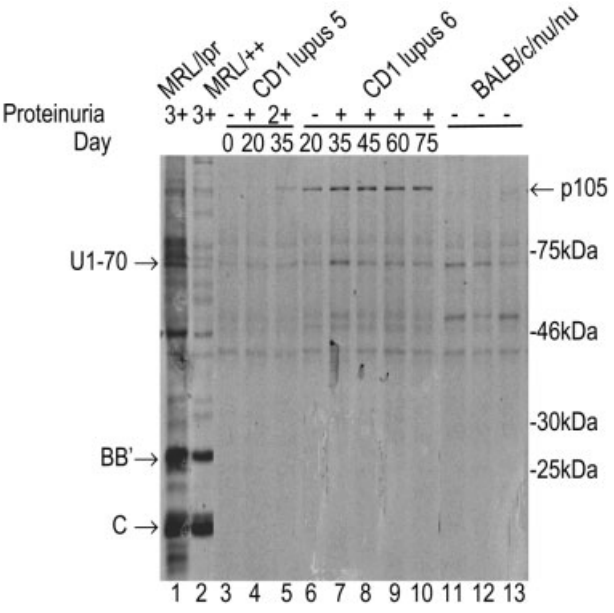

Fig. 3. Autoantibodies directed against p105 appear as early as 20 days after induction of disease. NIH/3T3 cells were metabolically labeled with [^{35}S]methionine, lysed, and proteins were immunoprecipitated with sera from the following animals: MRL/lpr mice (lane 1), MRL$^{+/+}$ mice (lane 2), two different CD1 lupus mice that received disease-inducing SP transgenic T cells (CD1 lupus 5, lanes 3–5, and CD1 lupus 6, lanes 6–10), BALB/c/nu/nu host mice that received SP transgenic cells but failed to develop lupus (lanes 11, 12) and an unmanipulated BALB/c/nu/nu mouse (lane 13). Sera from CD1 lupus 5 and CD1 lupus 6 mice were obtained at various time points as indicated across the top of the panel. For CD1 lupus 5 mouse, only three time points are shown. U snRNP proteins, which were precipitated by MRL/lpr and MRL$^{+/+}$ sera, are indicated on the left side of the figure. Relative masses of molecular markers, and the 105-kDa protein recognized by CD1 lupus sera, are indicated on the right side of the panel.

lupus serum 5, autoantibodies were first detectable at day 35 (Fig. 3, lanes 3–5).

Sera collected at day 60 from two BALB/c/nu/nu adoptive hosts that had received SP transgenic cells but did not develop anti-dsDNA antibodies or proteinuria (Fig. 3, lanes 11, 12), and serum from an unmanipulated BALB/c/nu/nu mouse (Fig. 3, lane 13) did not precipitate p105, p44/46, or any of the other proteins. No proteins were precipitated using sera from CD1 adoptive hosts that had received DN transgenic T cells, which did not induce lupus (data not shown). p44/46 was not co-precipitated by the two CD1 lupus sera used in the experiment shown in Fig. 3; samples from mice whose sera were capable of precipitating p44/46 were not available at time points earlier than day 45. Thus, we were unable to confirm that autoantibodies against the p44/46 antigen were already present at days 20 or 35, as observed for anti-p105 antibodies. We screened sera from aged NZB/NZW mice, $MRL^{+/+}$ and MRL/lpr mice (five sera per strain) for autoantibodies, but neither p105 nor p44/46 were precipitated (data not shown).

2.4 p90 is a phosphoprotein that is co-immunoprecipitated by some CD1 lupus sera

Many autoantigens are components of macromolecular complexes containing phosphoproteins. To investigate the phosphorylation status of p105 and p44/46, we labeled 3T3 cells with [^{32}P]orthophophate or [^{35}S]methionine, respectively. ^{32}P labeling enables direct identification of phosphorylated antigens, because only phosphoproteins are labeled and detectable by autoradiography. One of several experiments is shown in Fig. 4. After lysis of ^{32}P-labeled and ^{35}S-labeled 3T3 cells, respectively, antigens were immunoprecipitated using a prototypic CD1 lupus serum, or human anti-signal recognition particle (SRP)54 reference serum, which precipitates the phosphoprotein SRP72. CD1 lupus serum precipitated a single phosphoprotein migrating at 90 kDa. This protein was detectable on 7% gels (Fig. 4) rather than 12% gels (Fig. 1, 3), due to better protein separation on 7% gels in the respective molecular range. No antigens, including p105 and p44/46, were precipitated from lysates prepared from ^{32}P-labeled cells. p90 co-migrated with a similar ^{35}S-labeled antigen recognized by anti-SRP54 reference serum (Fig. 4, lane 2). Anti-SRP54 serum did not precipitate the co-migrating protein from ^{32}P-labeled extracts, indicating that these two proteins were distinct. This experiment demonstrates that the 90-kDa factor is the only phosphorylated protein immunoprecipitated by this CD1 lupus serum.

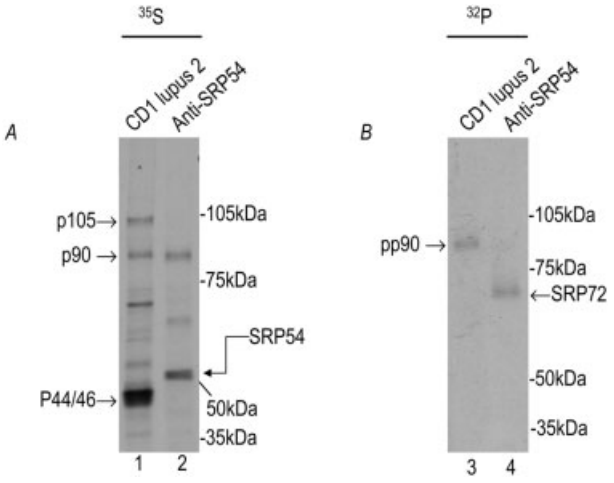

Fig. 4. p90 is a phoshoprotein that is co-precipitated with p105 and p44 using some CD1 sera. NIH/3T3 cells were radiolabeled with [^{35}S]methionine (A) or [^{32}P]orthophosphate (B). Lysates were prepared and labeled proteins were immunoprecipitated with CD1 lupus serum (lanes 1, 3) and human anti-SRP54 reference serum (lanes 2, 4), respectively. Unknown proteins precipitated by CD1 lupus serum are labeled on the left side of each panel. The relative migration of molecular mass markers is indicated on the right side of the panel.

2.5 p44 is cleaved during apoptosis

Since many antigens are cleaved following induction of apoptosis by various stimuli [10], we asked whether any antigens immunoprecipitated by CD1 lupus sera might be altered during apoptosis. 3T3 cells were grown to 70% confluency, incubated with anisomycin for 12 h, labeled with [^{35}S]methionine for 6 h, and a lysate was prepared as described. Proteins were precipitated with CD1 lupus serum recognizing the spectrum of antigens described above, and separated by SDS-PAGE.

p44 was no longer precipitated from protein extracts derived from anisomycin-treated cells, suggesting that p44 undergoes proteolysis during apoptosis (Fig. 5, lane 3). A protein migrating at 37 kDa in the lane representing antigens precipitated from apoptotic cells, suggestive of a p44 cleavage product, was reproducibly observed. No different precipitation patterns using lysates prepared from treated cells as compared with lysates prepared from untreated cells were observed for p50 (Fig. 5, lanes 1, 2) and the La protein (Fig. 5, lanes 5, 6), indicating that these proteins are not cleaved after induction of apoptosis by anisomycin. Several proteins in the molecular range 30–60 kDa were previously shown to be modified during apoptosis [11], including heterogeneous nuclear RNP (hnRNP) C and lamins A, B and C.

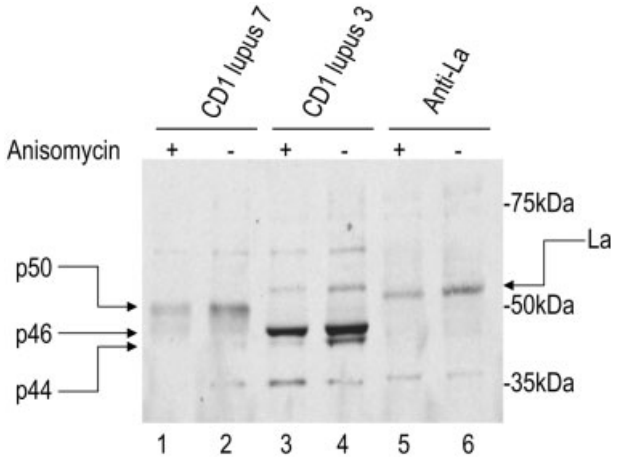

Fig. 5. p44 is cleaved during apoptosis. NIH/3T3 cells were metabolically labeled with [^{35}S]methionine, followed by incubation in the presence (+) or absence (–) of anisomycin for 12 h. After lysis, proteins were precipitated with two different CD1 lupus sera (lanes 1–4) and human anti-La/CDC reference serum (lanes 5, 6), from lysates prepared from treated (+) and untreated (–) cells, respectively. Proteins precipitated by CD1 lupus sera are labeled on the left side of the figure; the La protein precipitated by anti-La reference serum is labeled on the right side of the figure.

Using reference antibodies against these proteins, we failed to precipitate the cognate proteins from 3T3 cells or EL-4 cells for a direct comparison of their migration with p44 (data not shown).

2.6 A small RNA of ~140 bp is co-precipitated with p105 by some CD1 lupus sera

Nuclear protein antigens are frequently associated with nucleic acids, *e.g.* several proteins of the U1 snRNP complex that binds mature RNA, and proteins of the Ro-RNP complex that associate with small Y-RNA molecules. Some cytoplasmic antigens such as histidyl-transfer RNA synthetase (Jo-1) are complexed with transfer RNA. We tested the hypothesis that p105 is associated with an RNA molecule. Briefly, proteins were precipitated by CD1 lupus sera or by several autoimmune sera recognizing known protein-RNA complexes (including anti-SRP54 serum, which precipitates 7S RNA, and anti-Jo-1 serum, which precipitates histidyl-transfer RNA), followed by digestion of the protein component of the protein-RNA complexes by proteinase K. Next, the RNA molecules were extracted with phenol/chloroform/isoamyl alcohol, precipitated with ethanol, and separated on 8% sequencing gels.

A representative experiment is shown in Fig. 6. Four different CD1 sera that recognized p105 co-precipitated an RNA molecule of approximately 140 bp (Fig. 6, lanes 7, 8, and data not shown). This molecule differed in length from all known U snRNP-associated RNA molecules, since it did not co-migrate with any of the RNA species recognized by the reference sera and antibodies used in our experiments (Fig. 6), including the U1 RNA (164 bp) and Y-RNA molecules of 80–100 bp (data not shown). In summary, the RNA species co-precipitated by CD1 lupus sera appears to be a novel RNA component of an unidentified RNP complex. Although p105 is precipitated from lysates prepared from EL-4 cells by CD1 lupus sera, the 140-bp RNA species was not co-precipitated when EL-4 cells were used for the preparation of the protein-RNA extracts (data not shown), indicating that the association of p105 and the 140-bp RNA is cell type-specific.

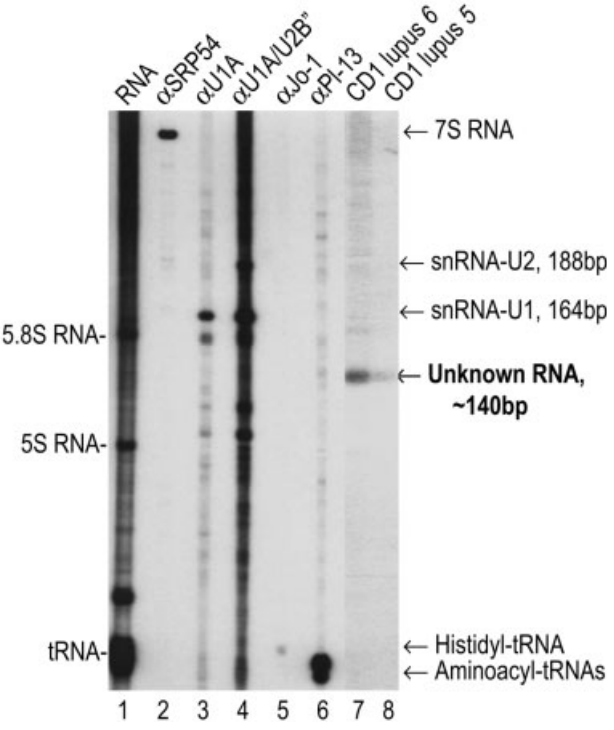

Fig. 6. A novel RNA of ~140 bp is co-precipitated together with p105. 3T3 cells were labeled with [^{32}P]orthophosphate for 14 h and lysed in 1% NP40 lysis buffer. After immunoprecipitation with CD1 lupus sera, human reference sera, or polyclonal antibodies, respectively, RNA was extracted and separated on 8% sequencing gels, followed by drying and autoradiographic exposure. A preparation of total RNA was included (lane 1). Specificities of antisera and antibodies are indicated across the top of the figure: lanes 2, 5, 6, human reference sera; lanes 3, 4, mouse polyclonal antibodies; lanes 7, 8, two different anti-p105-positive CD1 lupus sera. Known RNA moieties are labeled on the right side of the figure.

3 Discussion

In the present investigation, we extended our studies on the characterization of the B cell autoimmune response in the novel anti-CD1 autoreactive TCR-transgenic lupus model. We report the discovery of novel autoantigens, including a 105-kDa protein, termed p105, and a novel small RNA of ~140 bp that is co-precipitated together with p105. We present the biochemical and immunoserological characterization of these and additional antigens that are targeted in the CD1 model, comprising a 90-kDa phosphoprotein, and two proteins of 44 kDa and 46 kDa that are co-precipitated together with p105 by some sera.

Antibodies against p105 were detectable in 42% of diseased animals, and occurred as soon as 20 days after adoptive transfer of disease-inducing SP transgenic T cells. In contrast to anti-Ifi202 antibodies, which we observed to be associated with progressive kidney disease (W.H., D.Z., S.S., PJ.U., manuscript submitted), anti-p105 antibodies were found in sera from animals that developed severe kidney disease, and from animals with less severe lupus.

Not all animals with severe kidney disease developed anti-p105 antibodies, and several animals with mild proteinuria were also found to be anti-p105-positive. Anti-p105 autoantibodies were absent in sera from adoptive host mice that did not develop anti-dsDNA antibodies and proteinuria, and in sera from adoptive hosts receiving the DN transgenic subset of T cells. Although these observations do not rule out a pathogenic role for anti-p105 autoantibodies, a larger group of animals with kidney disease will need to be studied to detect a statistically significant relationship between anti-p105 autoantibodies and nephritis, and to elucidate the role of p105 in the chronology of autoantibody epitope spreading in this model.

The molecular mass of the p105 protein is similar to that of three known lupus candidate antigens: the DNA- and RNA-binding protein nucleolin (110 kDa), the nuclear Su protein complex (100/102 kDa), and PARP (113 kDa). However, the proteins precipitated by anti-Su reference serum did not co-migrate with p105. Moreover, we repeatedly failed to immunoprecipitate PARP and nucleolin from lysates prepared from radiolabeled 3T3 and EL-4 cells, respectively. In the case of PARP, this was not surprising, since anti-PARP antibodies were raised against human PARP, and lack of cross-reactivity between human and mouse proteins is not unusual. However, our experiments support the notion that p105 is an unphosphorylated RNA-binding protein, in contrast to PARP, a chromatin-associated DNA-binding phosphoprotein that is not known to associate with RNA [12]. Together with the considerable difference in molecular mass, we can rule out that the 113-kDa PARP protein is identical to p105.

Nucleolin yet represents another candidate antigen in mouse lupus. Comparison of p105 and nucleolin by two-dimensional electrophoresis clearly demonstrated that these proteins migrate at different isoelectric points, indicating that they are different proteins (Fig. 2). Additional immunoprecipitation/Western blotting experiments confirmed that CD1 lupus serum did not recognize the 110-kDa protein that was precipitated by anti-nucleolin polyclonal antibodies (data not shown). Also, we show that p105 is not a phosphoprotein (Fig. 4), in contrast to nucleolin, which is a highly phosphorylated nuclear protein [13]. We conclude that p105 is not nucleolin. No other lupus autoantigens within the range 100–115 kDa have previously been reported in the literature, supporting the conclusion that p105 and the 140-bp RNA component represent a truly novel autoantigen entity in mouse lupus. Experiments to definitively identify p105 and the 140-bp RNA molecule are under way.

In summary, our data suggest an association of p105 with an unknown RNA molecule of 140 bp. It is tempting to infer that p105 and the RNA molecule are part of a novel RNP complex. RNP complexes are common targets of autoantibodies in connective tissue diseases, for example the U1 snRNP complex, containing the U1 RNA molecules of 170–190 bp, and the Ro-RNP complex, containing the small Y-RNA molecules of ~100 bp (reviewed in [14]). Autoantibodies may be directed against the protein component, the RNA molecule, or both. It will be interesting to elucidate the exact epitopes within this putative macromolecular antigen complex that are targeted by the immune response in the CD1 lupus model.

Proteins cleaved during apoptosis are frequently targeted by autoantibodies found in sera from patients with autoimmune diseases. It has been proposed that presentation of autoantigens on or within membrane-bound apoptotic blebs accounts for the escape of self-antigens from immunological tolerance [15]. Our results presented in Fig. 5 demonstrate that the p44 antigen was almost completely cleaved following induction of apoptosis by anisomycin. A protein migrating at 37 kDa was weakly precipitated from the apoptotic cell lysate, and could potentially represent a cleavage product derived from p44. However, a co-migrating nonspecific band, seen in all experiments, rendered the interpretation of this finding difficult (Fig. 5, lanes 2–6). Since specific cleavage fragments of certain proteins may be detectable only weakly by immunoprecipitation analysis, *e.g.* the unique

cleavage fragments of the autoantigens Jo-1, RNA polymerase II, and PM-Scl [16], p44 might fall into this category.

Our attempts to establish the identity of p44 in co-migration experiments, using antibodies recognizing proteins of similar molecular masses that are known to be cleaved during apoptosis (*e.g.* the spliceosomal proteins hnRNP C1 and C2 [17], and the cytoskeletal proteins lamin A and B1 [18]), were unsuccessful. While lamin B1 is known to be cleaved completely, lamin A is only partially cleaved during apoptosis [19]. Because lamins A, B1 and C are known phosphoproteins, and since we did not observe phosphorylation of p44 (Fig. 4), all lamin proteins are unlikely to be candidate antigens.

To address whether anti-p105 or anti-p44/46 autoreactivity is present in human SLE, we performed preliminary experiments using well-characterized human sera from ten patients with SLE for their ability to immunoprecipitate proteins from mouse (EL-4) and human (Jurkat) cell lysates. We did not observe the presence of anti-p105 or anti-p44/46 autoantibodies in these sera (data not shown). However, the frequency of these antibodies might be low in SLE, or autoreactivity against these antigens might be restricted to subsets of SLE patients. Therefore, a larger, heterogeneous panel of sera from patients with SLE needs to be investigated. Moreover, human sera may not immunoprecipitate p105 from mouse cell lysates, and the human p105 protein may have different biochemical properties.

Several aspects of the CD1 mouse lupus model suggest that this model is an interesting addition to the list of existing animal models of lupus: (a) the array and type of antibodies produced, including (1) anti-Ifi202 and anti-HSP70 (W.H., D.Z., S.S., P.J.U., manuscript submitted), (2) antibodies directed against p105, a novel lupus autoantigen likely associated or complexed with a small RNA of 140 bp, and (3) anti-p44 autoantibodies, directed against an antigen which is cleaved during apoptosis and potentially represents the discovery of another autoantigen caspase substrate; (b) the rapid onset of disease after adoptive transfer of lupus-inducing transgenic T cell subsets, and the cytokine pattern produced by these lupus-inducing T cells [3]; and (c) a potential role for CD1 in the pathogenesis of hereditary mouse lupus [20] and human SLE [21], suggesting that CD1 might be a potential target molecule for drug discovery in lupus.

4 Materials and methods

4.1 Transgenic mice

Development of SP and DN lines of TCR α and β chain gene-transgenic mice was described in detail previously [22]. CD1 TCR-transgenic mice used in this study were backcrossed to BALB/c mice for at least 12 generations, and 2–3-month-old males were used as cell donors. All animal studies were approved by Stanford's Administrative Panel on Laboratory Animal Care (APLAC).

4.2 Induction of autoimmune disease

Lethally irradiated (800 cGy total body irradiation) 2–3-month-old male host BALB/c/nu/nu mice received 5×10^6 SP transgenic BM cells with 2×10^5 sorted SP transgenic splenic T cells intravenously within 12 h after irradiation. BM and spleen cells were stained with PE-conjugated anti-CD4 (GK1.5) and PE-conjugated anti-CD8 (anti-Lyt2) monoclonal antibodies obtained from CALTAG Laboratories (Burlingame, CA). Combined $CD4^+$ and $CD8^+$ T cells (>98% purity) were obtained from spleens of SP transgenic mice by sorting cells stained with a FITC-conjugated monoclonal anti-Thy1.2 (53–2.1) antibody obtained from CALTAG Laboratories [3]. Sera from two separate groups of host mice receiving SP transgenic cells were used for the experiments described herein: group A (six animals, all developing lupus as judged by anti-dsDNA antibodies and proteinuria), and group B (eight animals, six developing lupus).

4.3 Monitoring of autoimmune disease

Anti-DNA antibodies were measured using two-stage immunofluorescent staining of *Crithidia luciliae* fixed onto glass slides (Immunoconcepts, Sacramento, CA). Counterstaining was performed with rabbit anti-mouse IgG antibody conjugated with FITC (DAKO, San Diego, CA). Titers above 1:40 were considered positive. Proteinuria was measured on a 1–4+ scale using a colorimetric assay for albumin (Albustix; Miles, Inc., Elkhart, IN). Mice were considered to have proteinuria if at least two consecutive samples were 2+ (100 mg/dl) or greater. At least 75% of recipients of disease-inducing T cells developed kidney disease within 3 months after adoptive transfer, and all mice developing kidney disease died from severe lupus nephritis or were sacrificed due to severe illness.

4.4 Sera

Sera from animals of group A were obtained serially at days 0, 20, 35, 45, 60, and 75 after adoptive transfer. Sera from animals of group B were obtained at days 0, 45, and 60. Sera from aged NZB/NZW, $MRL^{+/+}$, MRL/lpr, and five normal BALB/c and unmanipulated BALB/c/nu/nu mice served as controls. All sera were stored at –80°C until used.

4.5 Antibodies

Human sera were provided by the Arthritis Foundation/Center for Disease Control (CDC) reference sera: anti-Jo-1, anti-La, anti-Ro, anti-Sm. Mouse polyclonal antibodies were gifts from Dr. W. J. van Venrooij, University of Nijmegen, Nijmegen, The Netherlands (anti U1A/4G3, anti-U1A/U2B"/9A9); from Dr. D. Choubey and Dr. P. Lengyel, Yale University, New Haven, CT (rabbit anti-mouse Ifi202); and from Dr. T. Medsger and Dr. N. Fertig, University of Pittsburgh, Pittsburgh, PA (anti-PL-13). Mouse monoclonal anti-Ro60 (2G10) and anti-Ro52 (2E7) were gifts from Dr. W. J. van Venrooij. Anti-hnRNP C1/C2 (4F4) was provided by Dr. G. Dreyfuss, University of Pennsylvania, Philadelphia, PA. Rabbit anti-nucleolin antibody was provided by Dr. D. Hirata and Dr. S. Minota, Jichi Medical School, Tochigi, Japan. Human anti-Su reference serum was a gift from Dr. M. Satoh, University of Florida, Gainesville, FL. Anti-SRP54 reference serum was from a dermatomyositis patient seen in Stanford's Arthritis Clinic.

4.6 Cell culture

NIH/3T3 cells and EL-4 cells were grown in 5% CO_2 at 37°C using RPMI 1640 for EL-4 cells and Dulbecco's modified Eagle's medium for 3T3 cells, respectively (GIBCO BRL, Grand Island, NY), supplemented with 9% fetal bovine serum (BioWhittaker, Inc., Walkersville, MD), penicillin, and streptomycin (GIBCO BRL). Cells were grown and harvested at mid-log phase (EL-4 cells) or at 80% confluency (3T3 cells).

4.7 Metabolic labeling

3T3 cells were incubated at 80% confluency, and EL-4 cells were incubated at a density of 1×10^6 cells/ml in labeling medium containing the following: 90% RPMI 1640 lacking either phosphate, or methionine and cysteine (GIBCO BRL), and 9% heat-inactivated fetal calf serum. [^{32}P]orthophosphate or [^{35}S]methionine and cysteine (NEN, Boston, MA) were added at a concentration of 0.15 mCi/ml. Cells were incubated at 37°C for 5 h unless stated otherwise.

4.8 Cell lysis

Lysis of cells was performed using Nonidet-P40 (NP40) lysis buffer (1% NP40, 150 mM NaCl, 50 mM Tris pH 7.8, and 1 mM EDTA). NP40 lysis buffer was supplemented immediately before use with 100× protease inhibitor cocktail, prepared as described [23]. After addition of 1 ml lysis buffer, the lysate was incubated on ice for 30 min, centrifuged at 13,000 rpm for 15 min, and the supernatant was used immediately.

4.9 Immunoprecipitation

Lysates were precleared three times for 15 min with 15 μl of a 50% solution of protein A-Sepharose (Pharmacia, Uppsala, Sweden) in PBS and 2.5 μg rabbit-anti mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Immunoprecipitations were performed in 1% NP40 buffer overnight. Precipitates were harvested and electrophoresed on 12% or 7% SDS-PAGE gels, as indicated. Proteins were transferred to nitrocellulose (Schleicher & Schuell, Keene, NH) or polyvinylidene difluoride (PVDF) membranes and exposed for autoradiography [10, 24].

4.10 Two-dimensional gel electrophoresis

A lysate was prepared as described before, except for using unlabeled instead of metabolically labeled 3T3 cells. Total protein content of the protein extract was 8 μg/μl as measured by the Bradford method (Bio-Rad, Hercules, CA). Nucleolin and p105 were immunoprecipitated on protein G-Sepharose beads using anti-nucleolin polyclonal antibodies and CD1 lupus serum, respectively, using 200 μl of cell lysate per sample (~1.6 mg total protein per sample), with overhead rotation at 4°C for 14 h. Protein G-Sepharose beads were recovered and resuspended in a sample solubilization buffer containing 7 M urea, 2 M thiourea, 2% CHAPS (all Sigma Chemicals Co.), 65 mM dithiothreitol (Bio-Rad) and 1.25% ampholyte (Bio-Rad). Protein loading, sample rehydration, isoelectric focusing, equilibration, and electrophoresis were performed as described previously [25], except for loading proteins to 3–10NL Immobiline DryStrips (Bio-Rad) with 3 μl of bromophenol blue, and performing isoelectric focusing at 80,000 V for 12 h. After transfer to PVDF membranes, proteins were visualized by ECL (Amersham Biosciences, Little Chalfont, GB).

4.11 RNA isolation and purification

Immunoprecipitates from lysates prepared from ^{32}P-labeled 3T3 cells or EL-4 cells were prepared as described above. After the third NP40 lysis buffer wash, the immunoprecipitate was digested, and RNA extracted, precipitated, and subjected to SDS-PAGE on 8% sequencing gels as described [26]. A small amount of whole-cell lysate was also processed as above and included as an internal standard on each gel.

4.12 Induction of apoptosis

NIH/3T3 cells were incubated in the presence or absence of 10 μg/ml anisomycin for 12 h before harvesting. The cells were lysed as described above and used in immunoprecipitation experiments.

Acknowledgements: We are grateful to Dr. Walther van Venrooij, Dr. Gideon Dreyfuss, Dr. Peter Lengyel, Dr. Daisuke Hirata, and Dr. Minoru Satoh for generously providing antibodies. We thank Truc Le for his help with RNA separation experiments, and Yin Ping Liu for excellent technical assistance. We would also like to thank members of the Utz laboratory for encouragement and critical review of the manuscript. These studies were supported by a James Klinenberg Fellowship from the Arthritis National Research Foundation to W.H.; by an Investigator Award from the Arthritis Foundation to D.Z.; by NIH grant AI-40093 to S.S.; and by NIH grants 1R01 AI0151614–01, K08 AI01521, and U19-DK61934, and an Investigator Award from the Arthritis Foundation to P.J.U. P.J.U. is the recipient of a Donald E. and Delia B. Baxter Foundation Career Development Award and a Dana Foundation Grant. W.H.R. was supported by NIH grant K08 AR02133, an Arthritis Investigator Award and an Arthritis Foundation Northern California Chapter Grants. P.J.U. and W.H.R. were supported by NIH Proteomics Contract N01-HV-28183 from the NHLBI.

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