RESEARCH COMMUNICATION

Expression and DNA binding of the human 52 kDa Ro/SSA autoantigen

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The 52 kDa Ro/SSA protein is an intracellular autoantigen that is frequently recognized by antibodies in sera of patients with systemic lupus erythematosus or Sjogren's syndrome. While the function of this molecule is not known, zinc finger and leucine zipper motifs have been identified in its predicted amino acid sequence which suggest that it may interact with nucleic acids. To test this hypothesis, the human gene which encodes this protein was cloned in a baculovirus and expressed in *Spodoptera frugiperda* cells. Extracts from these infected insect cells were used as a source of protein for this study. The protein is similar in size

and antigenicity to that expressed in human cells. This protein binds to DNA at physiological temperature and is eluted with high concentrations of sodium chloride. Striking similarities were found between the sequence in, and adjacent to, the nucleic acid-binding motifs of 52 kDa Ro/SSA and a growing family of zinc finger proteins which have been shown to bind to DNA or regulate gene expression. The findings presented here place this protein structurally and functionally in this family and demonstrate a biochemical assay which can be used to study its function.

INTRODUCTION

A characteristic autoimmune feature of sera from systemic lupus erythematosus (SLE) patients is the presence of high titres of circulating autoantibodies which bind to a number of intracellular antigens. These antibodies are directed against chromosomal constituents such as double-stranded DNA and histones, ribonucleoprotein complexes including Ro/SSA, La/SSB and RNA splicing complexes, and a variety of intracellular proteins. The presence of these autoantibodies is frequently correlated with particular clinical manifestations of this disease [1–3].

Antibodies to Ro/SSA were first described in patients with lupus and Sjogren's syndrome [4,5]. Analysis of the immunoprecipitated Ro/SSA particle showed that it was composed of a 60 kDa protein bound non-covalently to a group of small RNAs [6–9]. More recently, Western blots typed with anti-Ro/SSA-positive sera from these patients demonstrated additional immunoreactivity with a smaller 52 kDa protein [10]. The absence of cross-reactivity between the 60 and 52 kDa antigens, suggesting that they consisted of separate polypeptides [11], was confirmed by the cloning and sequencing of distinct cDNAs encoding these proteins [12,13] and the identification of each locus on separate human chromosomes [14]. Thus, anti-Ro/SSA-positive sera contain autoantibodies to at least two biochemically distinct 'Ro/SSA' proteins.

Despite extensive biochemical studies of these proteins and the autoimmune responses directed against them, the function of the 60 kDa Ro/SSA complex and the 52 kDa Ro/SSA protein is unknown. The 60 kDa protein has a zinc finger and an ribonucleoprotein-binding site, both of which are consistent with its binding to the hY RNAs [15]. Zinc finger and leucine zipper motifs were identified on the 52 kDa Ro/SSA protein, suggesting that it also interacts with nucleic acids [12,13]. However, this protein fails to bind to the hY RNAs in the particle containing

60 kDa Ro/SSA [16]. Zinc fingers are composed of pairs of cysteine and/or histidine residues which coordinate binding to a zinc atom. These structures occur in certain transcriptional factors and other proteins which bind to DNA or RNA [17]. Leucine zippers consist of heptad repeats of leucine residues present on one plane of α -helical loops. Proteins with this motif may form dimers by non-covalent bonding to leucine zippers of other proteins. These dimers may then bind to nucleic acids and have been implicated in differential gene expression [17]. The zinc finger and leucine zipper regions of 52 kDa Ro/SSA are similar in sequence to a family of proteins which bind DNA and regulate gene expression.

Because of these findings, we tested the hypothesis that the 52 kDa Ro/SSA protein binds to DNA. Because of the low quantities of this protein in human cells, a eukaryotic expression system was developed to obtain sufficient quantities for use in these experiments. In this report, we provide experimental evidence that the 52 kDa Ro/SSA protein does indeed bind to DNA, as predicted by its sequence and localization within the cell. These findings place it structurally as well as functionally in a subfamily of zinc finger proteins which interact with DNA.

MATERIALS AND METHODS

Baculoviral cloning of the human 52 kDa Ro/SSA protein

A pUC19 clone containing the human 52 kDa Ro/SSA cDNA [12] was digested with *Eco*RI and *Bgl*II. A 1781 bp fragment which contained the entire coding region for the protein was isolated and ligated to the *Eco*RI and *Bgl*II sites downstream of the polyhedrin promoter (*polh*) of the pBacPAK9 transfer vector (Clontech). The resulting construct was electroporated into *Escherichia coli* strain JM103, and a clone containing this insert, designated BP9XW3, was identified by restriction enzyme di-

gestion. *Bsu*36I digested BacPAK6 DNA, a recombinant variant of *Autographa californica* nuclear polyhedrosis virus, was co-transfected with BP9XW3 plasmid DNA by calcium phosphate precipitation into the *Spodoptera frugiperda* insect cell line ILPB-SF-21 (SF-21) [18]. Cells were grown until plaques were observed visually. Immunological screening for plaques which expressed the 52 kDa Ro/SSA protein was performed *in situ* with serum from an SLE patient with a high titre of autoantibodies to the 52 kDa Ro/SSA protein [19]. Six positive plaques were identified and one was plaque purified to a titre of 1.25×10^7 /ml for use in this study.

Baculoviral expression of the human 52 kDa Ro/SSA protein

SF-21 insect cells were maintained in suspension culture at 25–27 °C in TNM-FH media (Gibco) supplemented with 10% fetal bovine serum and 50 µg/ml of gentamicin sulphate (Life Technologies). These cells were infected with the recombinant 52 kDa Ro/SSA viral stock at a multiplicity of infection of 0.1 and grown for 4 days. Cells were counted, washed twice in PBS and lysed by sonication. Following centrifugation at 10 500 g, the supernatant was used as the source of this protein.

An e.i.s.a. was developed using this cell extract as a source of antigen. The extract was diluted in 0.1 M Tris/HCl, pH 7.6, and a 50 µl volume containing extract from 500 cells was used to coat each well on a microtitre plate (Costar). Sera from five SLE patients with anti-52 kDa Ro/SSA autoantibodies, five patients whose serum lacks these autoantibodies, and five healthy, unrelated controls were tested as previously described [7]. Absorbance values at 405 nm were adjusted by subtraction of results obtained with extract from uninfected SF-21 cells.

Binding of the 52 kDa Ro/SSA protein to DNA

HeLa cells were maintained in RPMI-1640 media supplemented with 2 mM L-glutamine and 10% fetal calf serum. DNA was isolated from these cells as described [20]. DEAE-Sephadex A-50 (Pharmacia Fine Chemicals) was equilibrated in binding buffer (75 mM NaCl/20 mM phosphate buffer, pH 7.4). DNA was mechanically sheared in binding, and saturating amounts were bound to the DEAE-Sephadex resin at room temperature. Baculoviral extracts containing human 52 kDa Ro/SSA protein were brought to 75 mM NaCl in 20 mM phosphate buffer, pH 7.4, gently mixed and then incubated for 30 min with the bound DNA. DEAE-Sephadex without DNA was treated identically and used as a negative control. Proteins were eluted with increasing concentrations of NaCl in 40 mM phosphate buffer, pH 6.7. Eluates were dialysed in 80 mM NH_4HCO_3 at 4 °C and concentrated by freeze-drying. Aliquots were prepared for electrophoresis in 10% SDS/polyacrylamide gels and electroblotted onto a nitrocellulose membrane [21]. These membranes were incubated with sera diluted in TBST [50 mM Tris/HCl (pH 8.0)/150 mM NaCl/0.05% Tween-20] from SLE patients or healthy controls. The membranes were then washed four times in TBST at room temperature and antibody binding was detected with 5-bromo-4-chloro-3-indolyl phosphate and Nitroblue Tetrazolium, after incubation with a goat anti-(human IgG) alkaline phosphatase-conjugated antiserum (Sigma Chemical Co.).

RESULTS AND DISCUSSION

A eukaryotic system for the expression of the 52 kDa Ro/SSA protein

Previous experiments in our laboratory used *E. coli* to express the human 52 kDa Ro/SSA protein. Although the protein was

Figure 1 Western blot of the baculovirus-expressed human 52 kDa Ro/SSA protein

SF-21 cells were either infected with a recombinant baculovirus containing the human 52 kDa Ro/SSA gene (lanes 1 and 2) or were uninfected (lanes 3 and 4). Sonicates from these SF-21 cells and from the human MOLT-4 T-cell line (lanes 5–7) were separated on 10% SDS/polyacrylamide gels and transferred to nitrocellulose membranes. The 52 kDa protein was screened with 1:100 (lanes 1, 3, 5), 1:1000 (lane 6) or 1:10000 (lane 7) dilutions of an SLE serum which contained these autoantibodies, and with serum from a healthy individual diluted 1:100 (lanes 2 and 4). M_r protein standards are shown to the left.

readily expressed and strongly antigenic [12,21], its sequestration in bacterial inclusion bodies required solubilization with 7 M urea and the conformation of the resulting molecules following removal of the urea was not known. Partial degradation of the protein, a problem common to bacterially expressed eukaryotic proteins, was detected in Western blots. No binding of this bacterially expressed product to DNA was detected.

Because of this and potential problems related to the lack of appropriate post-translational modification in *E. coli*, the human 52 kDa Ro/SSA cDNA was subcloned into the pBakPAK9 transfer vector and co-transfected in SF-21 cells with a linear baculovirus. Allelic replacement of the human gene into the baculovirus occurred in a portion of these cells via double recombination. Positive plaques were screened *in situ* by the method of Capone [19] with an SLE serum that was positive for anti-52 kDa Ro/SSA autoantibodies (Figure 1, lanes 5–7) and amplified to high titre. When SF-21 cells were infected with this virus, an antigenic protein with an M_r of 52000 was identified with the patient serum (Figure 1, lane 1). No immunoreactivity was detected in these cells using normal human sera (lane 2), or when uninfected cells were analysed with patient (lane 3) or normal serum (lane 4). No degradation of this protein was detected on Western blots, in contrast to earlier observations with bacterially expressed molecules. These results indicate that intact human 52 kDa Ro/SSA was expressed in insect cells.

The baculovirus-expressed 52 kDa Ro/SSA protein was studied in e.i.s.a. with sera from additional patients who produce and others who do not produce anti-52 kDa Ro/SSA autoantibodies, as well as with sera from healthy controls. Absorbances at 405 nm are plotted as a function of serum dilutions in Figure 2. All patient sera with anti-52 kDa Ro/SSA antibodies reacted strongly in a dose-dependent manner with antigen produced by infected cells (open symbols). Sera from SLE patients which lack anti-52 kDa Ro/SSA autoantibodies, as determined by Western blots of human cell extracts and bacterially expressed 52 kDa Ro/SSA protein and in e.i.s.a. with the bacterially expressed recombinant protein, did not react

Figure 2 Immunoreactivity of 52 kDa Ro/SSA protein in e.i.s.a.

Sera from five lupus patients containing anti-52 kDa Ro/SSA autoantibodies (open symbols), one of five lupus sera lacking these autoantibodies ($\blacktriangle$) and one of five sera from healthy individuals ($\bullet$) are shown with the baculovirus-expressed protein as antigen.

Figure 3 Western blots of proteins eluted from DNA

Extracts of SF-21 cells infected with the recombinant baculovirus containing the human 52 kDa Ro/SSA gene were incubated in the presence or absence of DNA and eluted with increasing concentrations of NaCl. The 75 mM fractions show unbound protein (left) and a 75 mM NaCl eluate (right). The first two fractions eluted at higher salt concentrations are shown. Additional fractions were negative. M_r protein standards are shown to the right.

with the baculovirus-expressed protein in e.i.s.a. (Figure 2, filled triangle). Similarly, no normal human serum has been found to react with this material (Figure 2, filled circle).

The 52 kDa Ro/SSA protein binds human DNA

The baculovirus-expressed 52 kDa Ro/SSA protein was used to test the hypothesis that it binds to DNA. SF-21 cells infected with the recombinant baculovirus containing the human 52 kDa Ro/SSA gene were washed and lysed by sonication. Cell extracts were incubated in the presence or absence of human DNA bound to a Sephadex matrix. Unbound protein was removed and the matrix was washed in binding buffer. Multiple aliquots were collected from elutions with increasing salt concentrations. Each aliquot was individually analysed for the presence of the 52 kDa Ro/SSA protein using Western blots. For each salt concentration, two aliquots are shown in Figure 3. In the absence of DNA, nearly all of this protein failed to bind to the solid-phase matrix. However, in the presence of DNA, only a small proportion of this protein failed to bind. (This protein is not always observed in this fraction, suggesting that it may represent a portion of the protein which is denatured in the course of

preparation.) Instead, the majority bound to the DNA and was detected in the 600 mM NaCl eluate. Very little protein was detected in the 3.0 M NaCl eluate. No binding was detected when human DNA was replaced with salmon DNA or with either linear or supercoiled plasmid DNA. These findings support the hypothesis that the binding of 52 kDa Ro/SSA is dependent on sequences which are present in the human genome.

The binding of 52 kDa Ro/SSA to human DNA occurred at physiological temperatures. This protein was detected in the higher salt fraction following incubation with the DNA at 37 °C (Figure 3). No binding to DNA was detected at 4 °C. As noted above, the human 52 kDa Ro/SSA protein expressed by *E. coli* failed to show DNA binding, suggesting that the bacterial expression product remained denatured after removal of the urea used for its solubilization. No reduction in binding was detected when 5 mM EGTA or 5 mM 1,10-phenanthroline was added to the protein before incubation with DNA. These observations suggest that zinc may not be a critical cofactor for this interaction. These experiments are the first to demonstrate that 52 kDa Ro/SSA can interact with DNA.

The zinc finger and leucine zipper motifs on the predicted sequence of the 52 kDa Ro/SSA protein suggested that this molecule functions as a DNA-binding protein. In fact, the similarities between these portions of this protein and others was so striking that it led us to speculate that these motifs represent a novel zinc finger family [12]. This concept has similarly been recognized by investigators studying other proteins with these characteristics. Common features of these proteins include conservation of the spacing of cysteine and histidine residues, the primary amino acid sequences between them and their relative positions in the primary amino acid sequences. The majority of proteins within this family are now known to bind to DNA or to regulate gene expression. For example, mouse rpt-1 decreases levels of the interleukin-2 α receptor and HIV-1 long terminal repeat-directed gene expression [22]. The *RAG-1* gene, important for immunoglobulin and T-cell receptor gene rearrangements, is a member of this family [23], as are *RING-1* [24], a *Xenopus* transcription factor [25], a class of yeast DNA repair proteins [26–28], a photoregulatory gene in *Arabidopsis* [29] and various viral proteins [30,31]. Some of the genes in this family have been shown to undergo rearrangements in certain tumours such as *Rfp/Ret* [32,33] and *PML-RAR α* [34], while others such as *b-rat* [35] or *MEL-18* [36] are preferentially expressed in tumour cells. Sequence similarities extend in some cases beyond the zinc finger and leucine zipper regions of this family. For example, the C-terminal half of the 52 kDa Ro/SSA protein, which lacks motifs suggesting a particular role for this portion of the molecule, is similar to portions of the Rfp protein mentioned earlier, a lampbrush chromosome-binding protein and bovine butyrophilin [37,38]. These common features do not necessarily imply antigenic similarity, as shown by the lack of cross-reactivity between 52 kDa Ro/SSA and Rfp by Chan et al. [13]. The findings presented here indicate that the 52 kDa Ro/SSA protein is structurally as well as functionally a member of this family, and are consistent with the nuclear localization of this protein (K. Rayno, M. B. Frank and M. Reichlin, unpublished work).

Data from other laboratories have reported that some of this protein may bind to the 60 kDa Ro/SSA ribonucleoprotein complex [39]. However, it is not clear whether this represents a functional intracellular complex, an artifact of cross-reactivity or biochemical co-purification of these molecules following cell lysis. If an intracellular complex does indeed form within cells, it does not do so by interaction with its leucine zipper, since the 60 kDa Ro/SSA protein lacks this motif. Furthermore, the 52 kDa Ro/SSA does not bind to hY RNAs [16]. No other

human proteins are expressed in these insect cells, suggesting either that the 52 kDa Ro/SSA protein's ability to bind to DNA is an inherent property contained within this molecule or is mediated by interactions with a highly conserved eukaryotic protein. Additional experiments are needed to determine if a homodimer of this protein is formed via its leucine zipper before binding to DNA.

The significance of these findings to autoimmune diseases is currently under study. Although genetic contributions to lupus have been described, it is not clear how the broad range of clinical diversity seen within these patients is accounted for by a limited number of disease susceptibility genes [40,41]. Nosologic models for lupus have been proposed whereby this single disease is diversified by expression of particular autoantibodies which differ between patients [3]. However, associations between these antibodies and disease manifestations are not absolute and suggest that genes other than those involved in antigen recognition by the immune system may also be important. Of interest to this work was the finding of a polymorphism within the 52 kDa Ro/SSA gene which was associated with SLE [42]. This polymorphism may be a genetic marker for a disease-related variant of this protein. If so, such a variant could alter its ability to bind to DNA, which in turn could modify the expression of genes influencing disease susceptibility or clinical manifestations in patients. Future research is needed to test this model and understand the physiological importance of these DNA interactions and their relationship to the disease process. The findings presented here demonstrate a biochemical assay which will be useful to assess the function of this protein in cells.

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