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Molecular Recognition Patterns of Serum Anti-DNA Topoisomerase I Antibody in Systemic Sclerosis

Paul Q. Hu,²† Noreen Fertig, † Thomas A. Medsger, Jr., † and Timothy M. Wright³‡‡

Autoreactive anti-DNA topoisomerase I (anti-Topo I) Abs are commonly detected in sera of systemic sclerosis (SSc) patients. Our studies have established a positive correlation between the levels of serum anti-Topo I Abs and both disease severity and activity of SSc. The molecular targets of anti-Topo I Ab on Topo I domains remain to be further defined. In this report, we studied the molecular recognition pattern of serum anti-Topo I Ab in 52 SSc patients. The highest reactivity of serum anti-Topo I Abs was against the core subdomains I and II (aa 207–441) and, to a lesser extent, against the core subdomain III (aa 433–636) of Topo I. The linker domain (aa 636–712) and the C-terminal domain (aa 713–765) had much less reactivity than the core domain (aa 207–636). Strikingly, very little reactivity was directed against the N-terminal domain (aa 1–213) by serum anti-Topo I Ab. This molecular recognition pattern was consistent among all SSc serum samples studied. Results from patients with serial serum samples indicated that this pattern remained unchanged over time. Interestingly, some naive B cells from healthy controls, upon transformation by EBV, produced IgM Abs against Topo I. These Abs had low affinity for Topo I and reacted equally to all domains of Topo I. The molecular recognition pattern of serum anti-Topo I Ab in SSc suggests the presence of a unique antigenic stimulation in vivo in this disease. The Journal of Immunology, 2004, 173: 2834–2841.

SSc, systemic sclerosis (scleroderma) is a generalized disease of connective tissue characterized by fibrosis and vascular obliteration which affects the skin and certain internal organs, including the lung, heart, gastrointestinal tract, and kidney (1). Anti-nuclear Abs (ANAs) are found in the sera of >95% of all SSc patients (2). A specific ANA type is often associated with a homogeneous clinical subset of SSc (2–4). Antitopoisomerase I (Topo I) Ab is considered a specific marker for SSc (5). In the past two decades, clinical studies have established the association of anti-Topo I Ab with the diffuse cutaneous involvement variant of SSc and with pulmonary fibrosis (3). Recent studies by our group (6) and others (7, 8) have demonstrated a positive correlation between serum levels of anti-Topo I Abs and both disease severity and activity. However, the role of anti-Topo I Ab in the pathogenesis of SSc remains to be elucidated.

The molecular target of serum anti-Topo I Ab from SSc patients was initially identified as a truncated form of Topo I, which produced a 70-kDa band on SDS-PAGE (9, 10). The full-length cDNA sequence of Topo I was subsequently cloned (11). The availability of Topo I cDNA has stimulated a number of investigators to fine-map Ab epitopes on the Topo I molecule (12–17). Using Western blot with recombinant Topo I fragments as Ags, several anti-Topo I Ab epitopes distributed from the N-terminal to the C-terminal portion of Topo I molecule have been reported (12, 17, 18). “Major” or “immunodominant” epitopes were also identified on Topo I using synthetic peptides (19), an epitope expression library (20), or a combination of Western blot and immunoprecipitation (IP) techniques (21–24). It has been appreciated for some time that autoreactive Abs recognize both linear and conformational epitopes (25, 26). A previous study by our group suggests that a major epitope of anti-Topo I Ab is conformational in nature (22–24). Although anti-Topo I Abs recognize multiple epitopes on the Topo I molecule, the overall molecular recognition pattern of these Abs in relation to the domain structure of Topo I remains unclear. In this manuscript, the Topo I domains were produced as recombinant fusion proteins and the reactivity of anti-Topo I Ab on different Topo I domains was measured by ELISA and confirmed by competitive ELISA, Western blot, and IP. We have established a molecular recognition pattern of anti-Topo I Ab based on the domain structure of the Topo I molecule (27) and analyzed this pattern in SSc patients with serial serum samples. In addition, the molecular recognition pattern of anti-Topo I Abs produced by naive B cells from healthy controls infected by EBV was also analyzed.

Materials and Methods

SSc patients

Fifty-one anti-Topo I Ab-positive SSc patients, including 40 patients with a single serum sample available and 12 SSc patients with multiple serum samples (a total of 30 serial samples), were included in this study. Anti-Topo I Abs were determined by immunofluorescence and double immunodiffusion. All clinical information and serum samples were from the University of Pittsburgh Scleroderma Databank and Serumbank. Serum samples were stored at −80°C before use.

Engineering of baculoviruses expressing full-length rDNA (r)Topo I and individual Topo I domains

The rTopo I was produced by recombinant baculovirus in the S99 insect cells as described previously (6). Since the N-terminal domain of Topo I is

Abbreviations used in this paper: ANA, antinuclear Ab; Topo I, topoisomerase I; IP, immunoprecipitation; MaBP, maltose-binding protein; rTopo I, full-length rDNA topoisomerase I; SSc, systemic sclerosis; TSS, total skin score.
prone to protease digestion and the core domain of Topo I is resistant to protease cleavage (27, 28), intact Topo I domains or subdomains could not be produced by protease digestion of rTopo I. We produced Topo I domains as recombinant fusion proteins in the Sf9 insect cell protein expression system.

According to the published domain structure of Topo I determined by x-ray crystallographic analysis (28), we designed four fusion protein constructs to encompass the entire Topo I sequence. The structures of Topo I and Topo I fusion proteins are summarized in Fig. 1. Topo I A/α encodes aa 1–213 on Topo I, which comprises the entire N-terminal domain. Topo I/B consists of aa 207–441, which includes the core subdomains I and II. Topo I/C contains aa 433–636, which is the core subdomain III of Topo I. Topo I/D contains aa 625–765, which has both the linker domain and the C-terminal domain.

To express maltose-binding protein (MaBP)-tagged fusion protein in the Sf9 insect cell culture system, we modified the “shuttling” vector pFast-BacHTa (Life Technologies, Rockville, MD) by removing the 6-histidine tag sequence and replacing it with the open reading frame of MaBP. The sequence of MaBP was derived from the Escherichia coli protein expression vector pMalC2 (New England Biolabs, Beverly, MA) by PCR amplification using an upstream primer with an overhanging RsrII site and a MalE primer (New England Biolabs) as downstream primer. DNA sequences of Topo I fusion proteins were derived from Topo I cDNA (17) by PCR with proper restriction enzymes in the overhangs of these amplified sequences. All DNA sequences and orientations of fusion protein constructs were confirmed either by automated DNA sequencing or by restriction enzyme digestion.

The recombinant baculoviruses expressing Topo I domains were produced according to the manufacturer’s instructions (Life Technologies). The expression of Topo I domains by Sf9 cells after infection with recombinant baculoviruses were examined by SDS-PAGE.

**Purification of MaBP-tagged Topo I domains**

The purification of MaBP-tagged fusion proteins was performed as described previously (29), with minor modifications. All purification procedures were performed either in a cold room or on ice. At 48 h postinfection with recombinant baculovirus at an estimated multiplicity of infection of 3:1, 1 L of Sf9 cells was harvested by centrifugation at 400 × g in a tabletop centrifuge and rinsed twice with 250 ml of ice-cold PBS (pH 7.4). After the final wash, Sf9 cells were collected and resuspended by vigorously shaking in 50 ml of cell lysis buffer containing 50 mM Tris (pH 7.4), 5 mM MgCl2, 0.5 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM NaCl, 0.2 mM PMSF, and 10 mM DTT (PMSF and DTT were added immediately before use). The genomic DNA of Sf9 cell was precipitated by dropwise addition of 18% polyethylene glycol (18% polyethylene glycol, 1 M NaCl, and 10% glycerol) to the lysate with a continual stirring for 30 min. The lysates were clarified by centrifugation at 15,000 × g for 30 min in a superspeed centrifuge.

The supernatant was collected and diluted with 4 volumes of Tris-HCl buffer (20 mM, pH 7.4). The diluted supernatant was passed through a 30-ml bed volume amyllose resin column, which was pre-equilibrated with column buffer containing 200 mM NaCl, 0.2 mM Tris (pH 7.4), 5 mM EDTA and 1/1000 volume freshly added 2-ME at a flow rate of 3 ml/min. The column was then washed with 300 ml of column buffer and eluted with column buffer containing 10 mM maltose. Fractions containing eluted protein were identified by analyzing 10 μl of each fraction with 100 μl of diluted Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA). The identified fractions were pooled and concentrated in a Centriprep (Millipore, Bedford, MA). The concentrated protein was then dialyzed against 1/1000 volume of PBS, which was replaced every 4 h for a total of four times.

**ELISA using purified individual Topo I domains as coating Ags**

The ELISA procedures were described previously (6), with slight modifications. Briefly, ELISA plates were coated with 100 μl of each of the purified rTopo I domains at a concentration of 2.5 μg/ml overnight. ELISA plates were washed with ELISA buffer and blocked ELISA buffer containing 3% BSA overnight. Serum samples were diluted to 1/1000 and diluted samples were incubated in ELISA plates at a volume of 100 μl for each well. After serum incubation, the plates were washed five times with ELISA buffer and incubated with 1/4000 diluted HRP-conjugated Ab against human Ig (ICN Cappel, Aurora, OH). After five washes with ELISA buffer, the ELISA plates were incubated with 100 μl of substrate Sigma 104 (Sigma-Aldrich, St. Louis, MO) for 30 min at room temperature. To stop the enzymatic reaction, 50 μl of 3 N NaOH was added to each well. All serum samples and secondary Abs were diluted in TBS plus 3% BSA. Only data obtained within the same experiment were compared.

The ELISA for supernatants of EBV-transformed B cell culture was essentially the same, except that the supernatant was incubated overnight in a cold room. The secondary Abs were specific for human IgG, IgA, or IgM, respectively.

**Competitive ELISA with individual Topo I domains**

The reactivity of serum samples to each Topo I domain was also measured by the competitive ELISAs. In competitive ELISAs, serum samples were titrated and diluted to a concentration, which was ~10 U or equivalent to the 1/10,000 dilution of the reference serum (6). The competing Abs were added to serum samples at a final concentration of 0.2 μg/ml or at increasing concentrations in the titration experiment. The serum samples with competing Abs were then rocked overnight in a cold room. The decreased anti-Topo I Ab reactivity of each sample was then measured on an ELISA plate coated with rTopo I. The reactivity of each serum sample to each Topo I domain was recorded either as the OD reading or as the percentage inhibition of reactivity, which is: percent inhibition of activity = 100 × (Abs of control − Abs with competing Ab)/Abs of control.

**Western blot**

rTopo I and Topo I domains were produced in Sf9 insect cells infected with recombinant viruses. After 48 h of infection, two million Sf9 insect cells were harvested, washed, and lysed directly in 500 μl of 2× SDS-PAGE sample buffer by boiling for 5 min. Total proteins were resolved by electrophoresis and transferred onto 0.45-μm nitrocellulose membranes, which were blocked in TBS (pH 7.4) containing 5% nonfat milk powder overnight. Membranes were incubated with 1/2000 serum samples in TBS containing 1% BSA for 1 h at room temperature followed by five washes with TBS plus 0.05% Tween 20. Next, membranes were incubated with 1/4000 diluted HRP-conjugated sheep anti-human Ig for 1 h at room temperature. After five washes with TBS plus 0.05% Tween 20, the membranes were developed with chemiluminescence reagent (Renaiusence Plus II; NEN Life Science, Boston, MA) and exposed to x-ray films immediately. As a control for the detection of the fusion protein tag MaBP, membrane strips were incubated with a 1/10,000 diluted rabbit anti-MaBP serum as the primary Ab and 1/5,000 diluted HRP-conjugated goat anti-rabbit antisera as the secondary Ab.

**Immunoprecipitation**

35S-labeled rTopo I or the Topo I domains were produced by metabolic labeling in Sf9 insect cells infected with recombinant baculovirus. Briefly, 20 million Sf9 cells were allowed to attach to a T75 flasks in 10 ml of complete Grace’s insect culture medium for 1 h before being infected with recombinant baculovirus at a multiplicity of infection of 1:3. After infection, complete Grace’s insect culture medium was replaced with methionine-deficient Grace’s insect medium (Life Technologies) plus 500 μCi [35S]methionine/cysteine (NEN Life Science). After 2 days, Sf9 cells were harvested, washed with PBS, and suspended in IP buffer. The cells were lysed by sonication and the lysate was stored at ~80°C until use.

Before adding 35S labeled Ags to each serum sample, serum Abs or Abs in EBV transformed B cell culture supernatants were absorbed to either protein A Sepharose beads or protein L Sepharose beads (Life Technologies, Rockville, MD) by preincubation in 1.5-ml Eppendorf tubes. The final volume of each tube containing 25 μl of beads was adjusted to 0.5 ml. The protein A or protein L beads were rocked with Ab in a cold room overnight followed by three washes with 1 ml of IP buffer containing 10 mM Tris (pH 8.0), 0.5 M NaCl, and 0.1% Igepal (Sigma-Aldrich). The 35S-labeled lysate was thawed and clarified by 1 min spin at 14,000 rpm at 4°C. One hundred microliters of lysate was added to each 1.5-ml Eppendorf tube and the tubes were rocked in a cold room for 2 h. The beads were then washed five times with IP buffer. After the final wash, the beads were collected and boiled in 20 μl of 2× SDS-PAGE sample buffer. After a 10-min spin, the samples were resolved by SDS-PAGE on a 10% polyacrylamide gel. The dried gel was exposed to x-ray film to detect precipitated radiolabeled proteins.

**EBV transformation of B cell and analyses of EBV culture supernatant**

In our preliminary experiments, we noticed that transformation efficiency of B cells by the ELISAs was low. It was likely that this low efficiency was due to the activation of EBV-specific CTLs. To increase the transformation efficiency of EBV, T cells were depleted from PBMC using Dynal Pan T beads (Dynal, Oslo, Norway), which have bead-bound anti-CD3 Ab and are designed for T cell depletion. T cell depletions were performed essentially according to the supplier (Dynal). The T cell-depleted PBMC were plated at 0.2 million PBMC (before the depletion of T cells) per well in a flat 96-well culture plate in a volume of 0.2 ml in RPMI.
The domain structure of Topo I and the constructs of rTopo I domains and constructs of Topo I domain fusion proteins. Topo I consists of the N-terminal domain (aa 1–214), the core subdomains I (aa 215–232, 320–433), II (aa 233–319), and III (aa 434–635), the linker domain (aa 636–712), and the C-terminal domain (713–765). A, Topo I/A (aa 1–213) includes the entire N-terminal domain (B). Topo I/B (aa 207–441) comprises the core subdomains I and II (C). Topo I/C (aa 433–636) has the core subdomain III (D). Topo I/D (aa 625–765) consists of the linker domain and the C-terminal domain (E). MaBP is the affinity tag of all Topo I domain fusion proteins (F).

**Results**

**rTopo I domain fusion proteins**

The domain structure of Topo I and the constructs of rTopo I domains are schematically shown in Fig. 1. We engineered recombinant baculoviruses to express the Topo I domains and subdomains as MaBP-tagged fusion proteins. Fusion protein Topo I/A encodes aa 1–213, which is the entire N-terminal domain. Topo I/B is comprised of aa 207–441, which includes the core subdomains I and II. Topo I/C runs through aa 433–636, which is the entire core subdomain III. Topo I/D contains aa 625–765 and includes both the linker domain and the C-terminal domain.

The rTopo I domains were expressed at high levels (lanes 2, 4, 6, and 8 in Fig. 2A) in Sf9 insect cells upon infection by recombinant baculoviruses and migrated at the expected molecular masses on SDS-PAGE. After purification through an amylose resin column (NEB, Boston, MA), each rTopo I domain showed a single major band on SDS gel (lanes 3, 5, 7, and 9 in Fig. 2A). All four rTopo I domain fusion proteins were positive on Western blot using an antiserum specific for MaBP, indicating that the recombinant fusion proteins contain MaBP (Fig. 2B).

**Molecular recognition pattern of anti-Topo I Abs by ELISAs**

We previously showed that serum samples from healthy controls and SSc patients did not have any detectable Ab against MaBP, which was also used as the affinity tag of fusion proteins in this study (29). Therefore, the purified rTopo I domains were used as coating Ags for ELISA plates without removing the MaBP. The ELISA cutoff value for positive reactivity of each individual rTopo I domain was set as the mean \( A_{405} \) value plus 3 SDs of 20 healthy control serum samples. Twenty healthy control serum samples produced very low absorption as measured by the \( A_{405} \) value on an ELISA plate coated with each individual rTopo I domain. The cutoff values were 0.024, 0.027, 0.025, and 0.029 for Topo I/A, Topo I/B, Topo I/C, and Topo I/D, respectively.

Anti-Topo I Ab reactivity to Topo I domains was determined by ELISA in a group of 40 anti-Topo I Ab-positive SSc patients, each with a single serum sample. The results are summarized in Fig. 3A. All 40 serum samples were positive for rTopo I. rTopo I produced a mean \( A_{405} \) value of 0.829, which was much higher than that for any individual Topo I domain. Topo I/B had a mean \( A_{405} \) of 0.252, which was the highest for all of the Topo I domains. Thirty-eight (95%) of the 40 serum samples were positive for Topo I/B (Table I). Topo I/C had the second highest reactivity with a mean \( A_{405} \) value of 0.084 and 77.5% of the serum samples were positive. The positive reactivity frequency of Topo I/D was 45%, which was lower than that of Topo I/B and Topo I/C, and the mean \( A_{405} \) value of Topo I/D was 0.044. Surprisingly, Topo I/A, the entire N-terminal domain, was recognized by only one serum sample. The mean \( A_{405} \) value of Topo I/A was 0.006, which was below the cutoff of 0.024.

### Table I

<table>
<thead>
<tr>
<th>Domain</th>
<th>Mean ( A_{405} ) Value (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Topo I/A</td>
<td>0.252 (0.000)</td>
</tr>
<tr>
<td>Topo I/B</td>
<td>0.084 (0.000)</td>
</tr>
<tr>
<td>Topo I/C</td>
<td>0.044 (0.000)</td>
</tr>
<tr>
<td>Topo I/D</td>
<td>0.006 (0.000)</td>
</tr>
</tbody>
</table>

**Figure 2.** The expression, purification, and characterization of Topo I domain fusion proteins. A, Topo I domains were produced as fusion proteins in Sf9 cells infected by recombinant baculoviruses. After 48 h of infection, Sf9 cells were harvested, lysed directly in SDS sample buffer, resolved by SDS-PAGE, and stained with Coomassie brilliant blue. MW, molecular weight standards. Lane 1, Uninfected Sf9 cells; lanes 2, 4, 6, and 8, Sf9 cells infected by recombinant baculoviruses expressing Topo I/A, Topo I/B, Topo I/C, or Topo I/D, respectively; lanes 3, 5, 7, and 9, purified Topo I/A, Topo I/B, Topo I/C, and Topo I/D, respectively. B, Topo I domains were recognized by an anti-MaBP serum on Western blot. Topo I/A, Topo I/B, Topo I/C, and Topo I/D are indicated as I/A, I/B, I/C, and I/D, respectively.
SSc patients (core domain of Topo I than for the N-terminal domain and the anti-Topo I Abs of all serum samples had a higher reactivity for the Since Topo I/B and Topo I/C represent the core domain of Topo I, "tivity after incubation with that Topo I domain. A higher Ab reactivity of serum samples to a par-
ticular Topo I domain resulted in a greater inhibition of the reac-
tivity of anti-Topo I Abs. The remaining re-
activity of anti-Topo I Ab was measured on an ELISA plate coated competing Ags. Error bars represent the SD. C. The anti-Topo I Ab reactivity of the same 40 serum samples to rTopo I and Topo I domains were measured by competitive ELISA. Ab reactivity of each serum sample is shown as the percentage of inhibition, which is the decreased A405 value in the presence of competing Ags divided by the A405 value without any competing Ags. Each filled circle in A and B represents the A405 value from a single serum sample.

Among all 40 patients, 33 (82.5%) had the dominant anti-Topo I Ab reactivity pattern of Topo I/B > Topo I/C > Topo I/D > Topo I/A. Four additional patients (10%) had a pattern of Topo I/B > Topo I/D > Topo I/C > Topo I/A and three patients (7.5%) had a pattern of Topo I/C > Topo I/B > Topo I/D > Topo I/A. Since Topo I/B and Topo I/C represent the core domain of Topo I, anti-Topo I Abs of all serum samples had a higher reactivity for the core domain of Topo I than for the N-terminal domain and the linker and C-terminal domains. This molecular recognition pattern of anti-Topo I Ab was the same as that of another large group of SSc patients (n = 55; data not shown).

To confirm the ELISA results, we set up a competitive ELISA by including each rTopo I domain as a competing Ag to "remove" Abs specific for that particular Topo I domain. The remaining re-
activity of anti-Topo I Ab was measured on an ELISA plate coated with rTopo I. A higher Ab reactivity of serum samples to a particular Topo I domain resulted in a greater inhibition of the reactiv-
ty after incubation with that Topo I domain.

We first tested whether preincubation of each rTopo I domain with reference serum produced a concentration-dependent inhibition of anti-Topo I Ab reactivity. The reference serum, a pooled serum from five SSc patients, was diluted to 1/1000 with ELISA buffer and preincubated with increasing concentrations of each Topo I domain and rTopo I. Preincubation with either rTopo I, Topo I/B, Topo I/C, or Topo I/D showed dose-dependent inhibitions of anti-Topo I Ab reactivity (Fig. 3B). As expected, the incubation of rTopo I produced the greatest inhibition of anti-Topo I Ab reactivity. The incubation with Topo I/B also efficiently decreased the A405 value significantly. As the concentration of Topo I/B increased to 1 μg/ml, the A405 value decreased to 0.15, as compared with 0.3, which was the A405 value in the absence of any competing Ag. Preincubation with both Topo I/C and Topo I/D also slightly decreased Ab reactivity, although not as dramatically as that with Topo I/B. As expected, preincubation of Topo I/A with reference serum to a concentration of 1 μg/ml (Fig. 3B), or even to a concentration of 20 μg/ml (data not shown), had no effect on anti-Topo I Ab reactivity.

Since the competing Topo I domains at a concentration of 0.2 μg/ml showed a significant inhibition of anti-Topo I Ab reactivity (Fig. 3B), we chose to add 0.2 μg/ml competing Ags in all subsequent experiments. Ab reactivity measured by competitive ELISA was converted to percentage inhibition, which was the ratio of the decreased A405 value of each sample with competing Ag divided by the A405 value of the same sample without competing Ag. The mean inhibition of rTopo I was 71.8%, which was much higher than that of any single Topo I domain (Table I). The mean inhibition of the Topo I domains was 50.1, 33.5, and 23.5% for Topo I/B, Topo I/C, and Topo I/D, respectively. Topo I/A did not inhibit the anti-Topo I Ab reactivity significantly, although it produced a mean inhibition of 7.9%. The results of these competitive ELISAs were consistent with the results obtained from ELISAs.

### Table 1. Serum anti-Topo I Ab reactivity to rTopo I and Topo I domains determined by ELISA and competitive ELISA

<table>
<thead>
<tr>
<th>Ags</th>
<th>ELISA Mean A405 Values (n = 40)</th>
<th>% Positive (n = 40)</th>
<th>Competitive ELISA Mean % inhibition (n = 38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rTopo I</td>
<td>0.829 ± 0.358</td>
<td>100</td>
<td>71.8 ± 11.4</td>
</tr>
<tr>
<td>Topo I/A</td>
<td>0.006 ± 0.014</td>
<td>2.5</td>
<td>7.9 ± 5.3</td>
</tr>
<tr>
<td>Topo I/B</td>
<td>0.252 ± 0.209</td>
<td>95</td>
<td>50.1 ± 19.6</td>
</tr>
<tr>
<td>Topo I/C</td>
<td>0.084 ± 0.101</td>
<td>77.5</td>
<td>33.5 ± 12.8</td>
</tr>
<tr>
<td>Topo I/D</td>
<td>0.044 ± 0.063</td>
<td>45</td>
<td>23.5 ± 12.9</td>
</tr>
</tbody>
</table>

Western blots and IPs

The established molecular recognition pattern of anti-Topo I Ab was further confirmed by Western blot and IPs. Western blots were set up by using Topo I domains in the lysate of SF9 cells infected with recombinant viruses. According to the results from ELISA and competitive ELISA, all Topo I domains except Topo I/A should be positive on Western blots for most serum samples. The results of eight representative Western blots are shown in Fig. 4. As expected, rTopo I was positive for all serum samples. Topo I/B,

![Figure 3](http://www.jimmunol.org/Downloadedfrom/linked-file/resource/image.png)
Topo I/C, and Topo I/D were all positive in seven of eight selected serum samples. In contrast, Topo I/A was positive in only two of the eight serum samples tested. These two positive samples (4G and 4H) had the highest A0.05 values (0.0473 and 0.045) of the 40 serum samples on the ELISA plate coated with rTopo I. Thus, the results from Western blot were largely consistent with the results from ELISA testing.

IP is a less sensitive, but a more definitive assay for Ab detection. Since our reference serum consisted of the pooled sera from five SSc patients and represented the overall reactivity pattern of anti-Topo I Ab, we chose this serum for IP experiments. We expected a negative correlation between the anti-Topo I Ab reactivity to a Topo I domain and the amount of serum sample required to precipitate the Topo I domain. Sf9 cell lysates containing [35S]-labeled rTopo I or Topo I domains were incubated with serial dilutions of the reference serum. An anti-MaBP serum was incubated with Sf9 cell lysates and used as a positive control for the detection of Topo I domains as indicated by arrows in lanes 1 and 2. The rTopo I and Topo I/B were precipitated almost equally well and were detected by as little as 0.5 μl of reference serum (Fig. 5, lane 6). Topo I/C was clearly detected by 5, 2.5, and 1 μl and was barely detectable with 0.5 μl of reference serum (Fig. 5, lane 6). Both Topo I/A and Topo I/D were positive with the anti-MaBP serum, indicating that these two domains were produced in infected Sf9 cells (Fig. 5, lane 2), but they were not detected at any concentration of reference serum (Fig. 5, lanes 3–6), indicating a low Ab reactivity against these two Topo I fusion proteins.

The results of Western blot and IP further supported the reactivity pattern of anti-Topo I Ab established by ELISA and competitive ELISA.

Anti-Topo I Ab reactivity pattern remained the same over time in patients with serial serum samples

Serum anti-Topo I Abs primarily target the core domain of Topo I. Despite varying disease durations and different HLA genes in SSc patients (clinical records not shown), all serum anti-Topo I Abs had virtually the same molecular recognition pattern. To more accurately evaluate the possibility that the molecular recognition pattern of serum anti-Topo I Abs remains the same over time, we examined Ab reactivity patterns by ELISA in 12 anti-Topo I Ab-positive patients with available serial serum samples (a total of 50 serum samples). The overall levels of Ab reactivity to Topo I/B, Topo I/C, and Topo I/D fluctuated, but all largely paralleled one another over time, as illustrated in three representative patients shown in Fig. 6. The levels of Ab reactivity to Topo I domains either paralleled (Fig. 6, B and C) or preceded (Fig. 6A) the change of total skin thickness score (TSS) as well. In an earlier study (6), we demonstrated that the levels of Ab reactivity to Topo I either paralleled or preceded the change of total TSS. Topo I/B had the highest overall level of reactivity in 10 of 12 patients, followed by Topo I/C and Topo I/D. In the additional two patients, Topo I/C had the highest reactivity, followed by Topo I/B and Topo I/D. The reactivity to Topo I/A was not detectable at any time in these 12 patients (Fig. 6 and data not shown).

Molecular recognition pattern of anti-Topo I Abs produced by naive B cells from healthy controls

It is a remarkable feature of serum anti-Topo I Ab in SSc patients that the N-terminal domain of Topo I was not recognized. One possibility is the lack of B cells specific for the N-terminal domain.
of Topo I in the naive B cell repertoire of humans. Since EBV is an efficient polyclonal B cell activator and is able to promote both naive and mature B cells to secrete a wide spectrum of Abs (30), including anti-DNA Abs in healthy controls (31), we took advantage of this system and determined Ab reactivity against Topo I domains in the culture supernatants of EBV-transformed B cells derived from healthy controls.

We screened Abs produced in EBV-transformed B cell culture supernatants specific for Topo I using MaBP as a control Ag in ELISA, since all Topo I domain fusion proteins are MaBP tagged. On average ~20% culture supernatant of all EBV-transformed PBMC wells (200,000 T cell-depleted PBMCs per well) were significantly reactive for Topo I with an A<sub>405</sub> value above 0.05 on ELISA plates specific for anti-Topo I Abs, based on the results of samples from a small group of four healthy controls and more than five SSc patients. This would indicate that approximately one of five wells (0.2 million PBMC per well before T cell depletion) of healthy control EBV-transformed B cells had the potential capability to produce anti-Topo I Ab. There was no difference in the percentage of wells with A<sub>405</sub> reading above 0.05 in four healthy controls and five SSc patients (data not shown).

The finding of Abs specific for Topo I in EBV-transformed PBMC from healthy controls was surprising because we were unable to detect any anti-Topo I Ab in culture supernatants of EBV-transformed B cells from healthy controls in a previous study (32). We performed additional experiments to confirm that the Abs produced in EBV culture supernatants were indeed specific for Topo I. We prepared Western blots using purified rTopo I as Ag and MaBP as a control Ag. As shown in Fig. 7, an EBV culture supernatant detected only rTopo I (top band), whereas a MaBP-specific

![FIGURE 6. Changes of anti-Topo I Ab reactivity to Topo I domains over time. Three selected patients are shown in A–C. Anti-Topo I Ab reactivity (left y-axis) to Topo I/A (●), Topo I/B (▲), Topo I/C (■), and Topo I/D (○) was measured over time (months) by ELISA in SSc patients with multiple serum samples. The changes of TSS (○, right y-axis) in these patients over time are also shown.](image-url)
antisera (anti-Ma BP) detected only MaBP (bottom band) but not the serial dilutions of EBV-transformed B cell culture supernatants. Even at lower dilutions of supernatant (as low as 1/5), the diluted supernatant did not show any reactivity to MaBP.

The Abs produced by EBV-transformed B cells had a low affinity for Topo I, as these EBV-transformed B cell culture supernatants with a relatively higher A405 reading on an anti-topo I Ab plate failed to precipitate 35S-labeled rTopo I by IP (data not shown). As shown in Fig. 8A, the mean A405 readings of 33 anti-topo I EBV culture supernatants that produced an A405 >0.05 from a representative healthy control in the group of four healthy individuals were 0.01, 0.005, and 0.42 for IgG, IgA, and IgM, respectively, indicating that the IgM Ab was the only anti-topo I Ab isotype present in healthy controls. We also detected anti-topo I IgG Abs and IgA Abs from PBMC culture supernatants from SSc patients (data not shown), which was consistent with our previous publication (32). Fig. 8B shows the mean reactivity to each topo I domain from the same 33 anti-topo I wells of EBV-transformed PBMC with an A405 reading >0.05 from the same representative healthy control. In contrast to the molecular recognition pattern by serum anti-topo I Ab from SSc patients, all topo I domains were recognized by Abs from EBV-transformed B cells of healthy controls.

Discussion

We have shown that, in all anti-topo I Ab-positive SSc patients, serum anti-topo I Abs have the same molecular recognition pattern. Anti-topo I Abs primarily target the core domain of topo I (aa 207–636), although the C-terminal domain is also targeted in most of the serum samples examined in this study. The entire N-terminal domain was unrecognized. For the first time, we have identified the major antigenic domain recognized by the anti-topo I Ab in SSc. In addition, although results from SSc patients with serial serum samples also clearly demonstrate that the levels of Ab reactivity fluctuate over time, the anti-topo I Ab molecular recognition pattern remains unchanged.

Since topo I was originally identified as the Ag for Abs specific for Scl-70 (old name for anti-topo I Ab), attempts have been made in the past 10 years to identify epitopes on topo I. An 11-aa epitope on the C-terminal of topo I was identified by Maul et al. (16) and was found to share 6 of 11 sequential amino acids with the group-specific (p30066) Ag of some mammalian retroviruses. Muryoi et al. (33) reported an epitope, which recognizes the N-terminal portion of topo I, in both SSc patients and in a SSc animal model, the tight skin mouse. This epitope appears to be cross-reactive with other portions of topo I and shares a certain degree of homology with UL70 protein of CMV (33). Based on this observation, a possible molecular mimicry mechanism was suggested for the pathogenesis of SSc. In contrast to the finding of a single epitope on topo I, Verheijen et al. (13) showed the reactivity of anti-topo I sera against three different epitope regions toward the C-terminal of topo I. D’Arpa et al. (14) expressed six portions of topo I and demonstrated that most of anti-topo I-positive sera recognize multiple epitopes. Kuwana et al. (17) detected four epitope regions on topo I from the N-terminal to C-terminal. Piccinini et al. (15) identified a “dominant” epitope region between amino acid residues 405 and 484 and showed that this region has at least two distinct epitopes. In contrast, Kato et al. reported a “universal” B cell epitope between amino acid residues 547 and 565 on topo I. Kuwana et al. (23) further analyzed this “universal” epitope and suggested that it is conformational in nature.

Our results do not necessarily contradict previous studies in which the N-terminal domain was included as an epitope region (12, 13, 17). First, in two of these studies (12, 17), the fusion protein constructs included the N-terminal domain and part of the core domain, the latter of which has the highest anti-topo I reactivity as we have demonstrated in this study. It is likely that the reactivity identified in those studies was directed against the core domain rather than the N-terminal domain, which is adjacent to the core domain. Second, some serum samples were able to recognize the N-terminal domain by Western blots, as shown in this and one other publication (13). In a more recent investigation of anti-topo I Ab epitopes using synthetic peptides (19), four “major epitopes” were identified. All four epitopes were on the core domain and three of them were on the core subdomains I and II (Topo I/B or aa 207–441) and one on core subdomain III (Topo I/C or aa 433–636). These results are consistent with our finding that the core subdomains I and II have the highest reactivity and core subdomain III has the next highest reactivity. However, we were able to detect a relative low reactivity toward the C-terminal domain of topo I in almost all serum samples.

Based on early studies, it was clear that although multiple anti-topo I Ab epitopes were identified, the overall picture of the anti-topo I Ab reactivity pattern on domains of topo I was still not clear. We designed topo I fusion constructs based on the domain structure of topo I and a combination of different techniques including ELISA, competitive ELISA, IP, and Western blot. Our results demonstrated that the core domain of topo I is the primary target of anti-topo I Ab, whereas much less reactivity was directed against the C-terminal domain and the N-terminal domain. The reactivity of anti-topo I Ab determined in this study should be considered as an extension of, complementary or summary of previous studies on anti-topo I Ab.

With regard to epitope changes on topo I during the course of disease, our results argue against the suggestion in a recent study which concludes that the titers and immunodominant epitopes of anti-topo I Abs vary over time (34). Our results clearly show that the relative levels of anti-topo I Ab reactivity against each topo I domain (and thus the molecular recognition pattern of anti-topo I Ab) are similar over time. This indicates that no significant change of topo I epitopes occur in SSc patients or in the same patient over time. The persistent of anti-topo I Ab is in consistent with other longitudinal studies of anti-topo I Ab (7, 8) and anti-fibrillin-1 Ab in mixed connective tissue disease and calcinosis, Raynaud’s esophageal dismottility, sclerodactyly, and telangectasis syndrome (35). It would be very interesting to study the domain reactivity pattern of anti-topo I Abs in those early anti-topo I Ab-positive patients.

One striking finding in this study is that the N-terminal domain of topo I is not recognized by anti-topo I Ab. It has been known for some time that, in early stages of many autoimmune diseases, autoantibodies recognize a few epitopes or only one subunit of a particular autoantigen. Autoantibody reactivity can subsequently “spread” to the whole molecule or additional physically associated subunits of the autoantigen during the course of disease (36). Although the mechanism underlying this observation remains to be further investigated, it suggests that the autoimmune response against topo I in SSc is not initiated from the N-terminal domain. It is reasonable to assume that the N-terminal domain, which is the nuclear localization domain, is not important for the initiation of anti-topo I autoimmune response. It is also possible is that the N-terminal domain is removed from the whole molecule before topo I is taken up and processed by APC. This mechanism could explain the lack of epitope spreading to the N-terminal domain from the adjacent core domain of topo I. The N-terminal of topo I is susceptible to proteases and was indeed frequently removed in the Ag preparations of some early studies. In fact, anti-topo I Ab was described as Scl-70 in the early literature (5). The N-terminal
domain has been shown to be frequently cleaved; one study demonstrated that multiple sites on the N-terminal domain of Topo I are the targets of caspase-3 and caspase-6, two proteases involved in the process of apoptosis (37).

In this study, we also analyzed the domain reactivity pattern of "naive" B cell-specific for Topo I in healthy controls. According to previous publications, EVB is very efficient in transforming B cells to promote the secretion of the membrane-bound Abs specific for different cellular and protein targets, including erythrocytes, a mouse mammary carcinoma, DNA, and sperm Ags, without deliberate vaccination with these Ags and cells in healthy controls (30). In fact EVB has been shown to be the most efficient polyclonal stimulator of B cells (38). The detected Abs, albeit with low affinity ($K_d \approx 10^{-3} - 10^{-7}$ mol/L), are reactive to a variety of self-Ags and exogenous Ags from both healthy controls and Hashimoto’s disease and systemic lupus erythematosus patients (38). Anti-DNA Ab production by B cells in healthy controls upon EVB transformation was also reported (31). In addition, the Leu-1-positive B cells from healthy controls constitute $\approx 17\%$ of total B cells shown to produce Abs reactive to rheumatoid factor and anti-ssDNA (39). We took advantage of this system and analyzed the domain reactivity pattern of Ab against Topo I in healthy controls. We were indeed able to detect low-affinity IgM Ab against all portions of Topo I. These results were consistent with previous findings showing the presence of low-affinity autoreactive Abs in healthy controls. Our results further demonstrated that it is unlikely that lack of reactivity of anti-Topo I Ab to the N-terminal domain of Topo I is due to lack of B cells specific for the N-terminal, since the naive B cells appear to be able to recognize the N-terminal domain as well as other domains. Our results support the possibility that an N-terminal truncated form of Topo I is presented to APC and initiates a response to Topo I in vivo.

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