Cloning of follistatin-related protein as a novel autoantigen in systemic rheumatic diseases

Masao Tanaka, Shoichi Ozaki, Fumio Osakada\textsuperscript{1}, Kiyoshi Mori, Mitsuo Okubo\textsuperscript{2} and Kazuwa Nakao

Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan
\textsuperscript{1}Takasago Research Laboratories, Kaneka Corp., 1-8 Miyamae-machi, Takasago-cho, Takasago-shi, Hyogo 676-8688, Japan
\textsuperscript{2}Department of Internal Medicine II, Fukushima Medical College, 1 Hikarigaoka, Fukushima, 960-1295, Japan.

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Abstract

In an attempt to identify autoantigens of synovium in rheumatoid arthritis (RA), we constructed \textsuperscript{\lambda} phage expression cDNA libraries from synovium and screened them by IgG purified from synovial fluids, both of which were derived from RA patients. As a result of this unique combination of the libraries and probes, we cloned follistatin-related protein (FRP) as a novel autoantigen in systemic rheumatic diseases. FRP is a secreted protein containing a similar amino acid sequence to follistatin, an inhibitor of activin. FRP was first cloned as a transforming growth factor-\textbeta 1-inducible protein (called TSC-36) from a mouse osteoblastic cell line and was suggested to have some roles in the negative regulation of cellular growth. Immunoblotting analyses detected synovial fluid and serum anti-FRP antibodies of IgG class more frequently in RA than any other systemic rheumatic diseases and controls. Synovial fluid anti-FRP antibodies appeared in 44\% of RA (n = 18) and none of osteoarthritis (OA) (n = 15) patients. Serum antibodies were detected in 30\% of RA (n = 67), 17\% of systemic sclerosis (n = 18), 10\% of systemic lupus erythematosus (n = 51) and Sjogren's syndrome (n = 10), and none of polymyositis/dermatomyositis (n = 13) patients and healthy subjects (n = 30). These antibodies recognized an EC domain, an extracellular Ca\textsuperscript{2+} binding module. In anti-FRP antibody-positive RA patients, serum C-reactive protein level and erythrocyte sedimentation rate were more elevated than negative patients (P < 0.05 and P < 0.01, respectively). FRP gene expression was higher in RA than OA synovium (P < 0.05). However, there was no difference between these groups in the amount of synovial FRP, suggesting its elevated turnover in RA. As follistatin inhibits activin, FRP might inhibit some growth factor-like molecule. Detection of anti-FRP antibodies, possibly having disease-promoting effects as the blocking antibodies, could be one of the markers for clinical evaluation of systemic rheumatic diseases.

Introduction

Systemic rheumatic/autoimmune diseases, represented by systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), systemic sclerosis (SSc), Sjogren's syndrome (SjS) and polymyositis/dermatomyositis, are characterized by destruction of systemic organs due to autoimmunity, which is specific to each disease. Patients with these diseases often complain of arthralgia and myalgia, so-called 'rheumatism', where specific autoantibodies serve as diagnostic, therapeutic and prognostic indices. Detection and isolation of corresponding autoantigens have been performed using such autoantibody probes, which come from sera of selected patients with peculiar clinical profiles. For instance, detection of autoantigens in recent-onset RA (1) is an elegant work with well-selected probes. An expression cloning technique is an excellent screening method to identify autoantigens. Those cloned by this technique in RA are a 372 kDa protein of the...
Golgi complex (2), annexin XI (3) and calpastatin (4,5). In these cases, cDNA libraries were constructed from generally available tissues or cell lines, but not from rheumatoid synovium.

In the tissues inflamed by autoimmunity, it is constitutively expressed antigens that seem to play a major role to sustain the inflammation by activating B and T cells. Synovium may well be conceived as such tissue in RA. Our preliminary immunoblotting studies with synovial lysate and IgG purified from synovial fluids from RA patients showed unidentified antigens with unique molecular masses. Expecting to identify novel antigens more responsible for RA, we performed expression cloning of synovial antigens with the original combination of cDNA source and probes, i.e. λ phage expression cDNA libraries were constructed with RA patient-derived synovial cell mRNA and they were screened by synovial fluid IgG of RA patients. We cloned follistatin-related protein (FRP) as a novel autoantigen, and analyzed the significance of anti-FRP antibodies in RA and other related diseases.

Methods

Sera and synovial fluids

Sera were obtained from 67 patients who fulfilled the American College of Rheumatology criteria for RA (6). Control sera were from 51 patients with SLE, 18 with SSc, 10 with SJ, 13 with polymyositis/dermatomyositis and 30 normal healthy subjects. Synovial fluids were obtained from arthrocentesis from 18 patients with RA and 15 patients with osteoarthritis (OA) as controls. For immunological screening of cDNA libraries, we used synovial fluid IgG (SFIgG) purified with a Protein A column (Prosep A; bio PROCESSING, Durham, UK).

mRNA isolation from cultured synovial cells

Synovial membranes obtained at synovectomy were cut into small pieces and digested by 0.25% collagenase (Collagenase S-1; Nitta Gelatin, Osaka, Japan) in serum-free RPMI 1640 medium for 8 h. Cells were collected by centrifugation (200 g, 5 min), seeded at ~5 x 10^4 cells/cm^2 in 80 cm^2 flasks, and cultured in RPMI 1640 supplemented with 10% FCS, 100 units/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml amphotericin B. The medium was changed after 12 h when fixed cells began to extend processes. At this stage, non-adherent cells were washed away with PBS. Cells became confluent within 7 days, the medium was discarded and RNA was isolated by adding a TRIzol reagent (Gibco/BRL, Gaithersburg, MD) to flasks (5 ml/flask) according to the manufacturer's protocol.

Construction of cDNA libraries

From pooled total RNA (~1 mg) of six RA patients' synovial membranes, poly(A)^+ RNA (~10 µg) was purified with oligo(dT)-latex beads (Oligotex-dT30; Takara Shuzo, Otsu, Japan). Two kinds of cDNA were synthesized using a Time-o:lligo(dT)–latex beads (Oligotex-dT30; Takara Shuzo, Otsu, Japan), poly(A)^+ RNA, with random hexamers and NotI oligo(dT) primers. In both cases, cDNA products >200 bp were size-selected by Chroma spin-400 columns (Clontech, Palo Alto, CA) and ligated with EcoRI adapters. NotI oligo(dT)-primed cDNAs were digested with NotI. These cDNA fragments were purified by Chroma spin-400 columns again and ligated with λExCell EcoRI/CIP or NotI/EcoRI/CIP (Pharmacia), and packaged by Gigapack II Gold packaging extract (Stratagene, La Jolla, CA).

Quality check of cDNA libraries and SFIgG

cDNA inserts were cut out from λExCell DNA and electro- phoresed in agarose gels. Size distribution of cDNA inserts was evaluated by image analyzing tools (AE-6905C: ATTO, Tokyo, Japan, and Image Gauge; Fuji Photo Film, Tokyo, Japan). Cloned cDNA species were estimated by PCR analysis with the Gene Checker kit (Invitrogen, San Diego, CA). Purity of IgG probes was checked by SDS–PAGE under reducing conditions and silver staining.

Immunological screening of cDNA libraries

Prior to library screening, we selected SFIgG solutions from six RA patients that detected the most bands in synovial cell lysates in a preliminary immunoblotting experiment. The SFIgG solutions, containing 20–35 mg/ml IgG, were diluted 1:50–100 with PBS containing 5% skimmed milk (Difco, Detroit, MI) and one-third volume of Escherichia coli NM 522 (Pharmacia) lysate to remove anti-E. coli antibodies (7). With this SFIgG mixture, we screened random hexamer-primed and NotI oligo(dT)-primed cDNA libraries as described by Young and Davis (8). Plaque-transferred filters were washed sequentially with PBS containing 3, 0.5 and 0% Tween 20 for 5 min each, blocked with 5% skimmed milk in PBS, and incubated at 4°C overnight in the SFIgG mixture. The filters were washed again as described above and reacted with 1:1000 diluted horseradish peroxidase-conjugated goat anti-human IgG (Cappel #55252; Organon Teknika, Durham, NC) in PBS with 5% skimmed milk at room temperature for 1 h. After washing 3 times with PBS for 5 min each time, signals of the second antibody were detected with an ECL kit (Amersham, Amer- sham, UK). Positive clones were purified by repeating the screening of plaque progenies until all were positive. Cloned λ phage λExCell was converted to circular plasmid pExCell according to the manufacturer's protocol.

DNA sequencing and analysis

Sequencing was performed with a Prism ready reaction dyeideoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and a 373 DNA sequencing system (Applied Biosystems). The DNA Data Bank of Japan (DDBJ) was searched using fasta/ffasta programs to determine sequence similarities to known genes.

Construction of plasmid vectors expressing glutathione S-transferase (GST) fusion recombinant follistatin-related protein (fFRP) and truncated mutant proteins

Truncated FRP mutants, D1–D6, were designed as shown in Fig. 4(A). Figure 2(A) shows this construction process for truncated rFRP and truncated mutants, except for primer designs. Primers were designed in order to detect restriction enzymes (5’. SmaI or EcoRI; 3’. NotI or XhoI). For anti-sense primers, anti-sense sequences of double stop codons (-TCATTA-) were also added. The sense primers were 5’-AAGAACGGAGGAGAGGGTCAGGAGCAA-3’ for intact rFRP and D1–D5, and 5’-AAGAATTCCTCAAGAT-
CTGTGCCAATGT-3’ for D6. The anti-sense primers were 5’-TTGGGCGCCGTCGTGCCCCCTCATGAGTCT-3’ for intact rFRP, 5’-TTCCTGAGTCTACTTACATTTCACCAGGCCAGA-3’ for D1, 5’-TTCTGAGACTGCTGCTTGCATTCCCTCGAGG-3’ for D2, 5’-TTCCTGAGTCTAAAACCTGAGAACTGAGTGT-3’ for D3, 5’-TTCCTGAGTCTAGACAGGTTTACATG-3’ for D4, and 5’-TTCCTGAGTCTACTTGGCAGTCTCATGTT-3’ for D5 and D6. PCR was performed in 50 µl reaction solutions containing 15 pmol primers, 50 ng of plasmid template, 200 µM dNTPs, Pfu polymerase (Stratagene) and its buffer (94°C, 30 s; 55°C, 30 s; 72°C, 1.5 min; 25 cycles). After electrophoresis in agarose gels, PCR products were purified from gel slices using a Geneclean II kit (BIO101, La Jolla, CA) and subcloned in pCRII plasmid vector (Invitrogen). Cloned PCR products in pCRII and GST fusion protein expression vectors pGEX-4T-3 (Pharmacia) were digested with restriction enzymes and ligated with each other. The constructs were certified by sequencing. 

Expression and purification of recombinant proteins
The plasmid pExCell and pGEX-4T-3 encoding FRP or its truncated mutants, termed pExCell/rFRP, pGEX-4T-3/rFRP, pD1/-D6 respectively, were transferred into E. coli NMS22 (Pharmacia). Preparation of cultures of these transformants, induction of recombinant protein synthesis and subsequent affinity purification of GST fusion proteins by glutathione–Sepharose 4B (Pharmacia) were performed according to the manufacturer’s protocol.

SDS-PAGE and immunoblotting
Samples were boiled for 5 min in a buffer containing 40 mM Tris–HCl, 2% SDS, 5% 2-mercaptoethanol, 5% glycerol and 0.002% bromophenol blue, fractionated on SDS–polyacrylamide gels (10 or 12.5%), and electroblotted onto PVDF membranes (Immobilon IPVH00010; Millipore, Bedford, MA). The blots were blocked with 5% skimmed milk in PBS for 1 h at room temperature, and incubated at 4°C overnight in PBS containing 5% skimmed milk and primary antibody solutions. As primary antibody solutions, SFIgG and sera were diluted 1:50 or 1:10, and synovial fluids were diluted 1:2 or 1:10. Viscosity of synovial fluids was reduced by 300 U/ml hyaluronidase (Wako Pure Chemical Industries, Osaka, Japan). They were all preabsorbed with the original samples diluted 1:1.5 or 1:10 with non-recombinant E. coli lysate. The subsequent procedure of wash and detection of bound IgG was the same as described in ‘Immunological screening of cDNA libraries’.

Northern blotting
RNA was prepared from fresh synovial specimens with a TRIzol reagent (Gibco/BRL). Samples of 20 µg of total RNA were separated on 1.33% agarose gels containing 37% formaldehyde and transferred onto nylon membranes (GeneScreen Plus; NEN Research, Boston, MA). FRP cDNA probes (50 ng) were prepared from pGEX-4T-3/rFRP by SmaI and Nof digestion, and were labeled with 50 µCi [α-32P]dCTP (NEG-513H; NEN Research). After hybridization with these probes (~7.5×105 c.p.m./ml), blotted membranes were exposed to an imaging plate (LAS-III; Fuji Photo Film) for 12 or 24 h and analyzed with an image analysis system (LAS-2000 II and Bastation; Fuji Photo Film). Gene expression in multiple organs was studied using human multiple tissue Northern blot I and II, and human G3PDH cDNA control probe (Clontech).

Transient expression of eukaryotic recombinant FRP
FRP cDNA bearing SalI (5’) and XhoI (3’) ends was created with the primers, 5’-AAGTCGACACAGATGGTGGAAGCCGCCTG-3’ (5’) and 5’-TTCCTGAGTGTGCTCTCATTAGATCT-3’ (3’) by the same method described in ‘Construction of plasmid vectors’. The directional and inverted (mock) cDNA was ligated with mammalian expression vector pCXN2 (9) at the Xhol site and transferred into COS-7 cell with the CellPect Transfection Kit (Pharmacia). Cells were cultured in DMEM containing 10% FCS. Culture supernatant was collected after 96 h. pCXN2 vector was kindly provided by Dr Jun-ichi Miyazaki (Department of Nutrition, Osaka University).

Immunoprecipitation of FRP from synovial fluids and plasma
Rabbit antiserum (OCT9702) and mouse mAb (ANOC9701) to FRP were produced with GST fusion rFRP by standard methods. FRP in 1 ml of synovial fluids and plasma was immunoprecipitated with 1 µg of anti-FRP mAb (ANOC9701) and 10 µl bed volume of agarose coupled with goat anti-mouse IgG (A-6531; Sigma, St Louis, MO). After washing the agarose 3 times with PBS, the immunoprecipitates were eluted with 20 µl of 100 mM glycine–HCl (pH 2.5) containing 0.1% Triton X-100 and neutralized with 5 µl of 1 M Tris–HCl (pH 8.0). The eluted samples were subjected to SDS–PAGE under reducing conditions and immunoblotted with rabbit antiserum to FRP (OCT9702) and anti-rabbit Ig, peroxidase-linked species-specific whole antibody (NA934; Amersham). Signal densities were measured by an Image Gauge (Fuji Photo Film).

Results
Cloning of cDNA encoding full-length human follistatin-related protein (FRP)
Two cDNA libraries primed with random hexamer and Nof oligo(dT) contained 50,000 and 150,000 independent clones respectively. The mean length of cDNAs was ~1 kb in both libraries, and the two libraries appeared complementary to each other for the range and species of cloned cDNAs (Fig. 1A and B). Purity of SFIgG was ascertained (Fig. 1C). From the random hexamer-primed library, three positive clones were isolated, and shown to have 2.5, 2.0 and 1.5 kb cDNA inserts. The nucleotide sequence of the 2.5 kb cDNA was identical to human FRP cDNA except for part of the 3'-untranslated region and encoded a full-length protein (the accession number of this nucleotide sequence is D89937 in the DDBJ/EMBL/GenBank DNA databases). FRP cDNA was inserted in-frame into the EcoRI site of the β-galactosidase α-peptide gene. On translation, this fusion gene produced a protein with an extra 50 amino acid residues at its N-terminus. (40 kDa band; Fig. 2B, lane 2).

Preparation of recombinant FRP (rFRP) using the GST gene fusion system
We adopted the GST gene fusion system using the pGEX-4T-3 vector for easy purification and also for reconstruction of the
Follistatin-related protein as a novel autoantigen

Fig. 1. Check of library cDNA length (A) and species (B), and purity of IgG probe (C). (A) The random hexamer-primed cDNA library was digested by EcoRI (lane 1), and the NotI oligo(dT)-primed one was digested by EcoRI and NotI (lane 2). Arrows at 1 kbp show mean length of cDNA inserts evaluated by densitometry. Actual length seemed larger because some cDNA have EcoRI or NotI sites. (B) PCR analysis of library cDNA with various primer sets of the Gene Checker kit (Invitrogen) [top figure: random hexamer-primed library; bottom figure: NotI oligo(dT)-primed library; lane 1, 3’ region of β-actin 720 bp apart from 3’ end; lane 2, 5’ region of β-actin 1.7 kbp apart from 3’ end; lane 3, mid-region of clathrin 2 kbp apart from 3’ end; lane 4, 5’ region of clathrin 6 kbp apart from 3’ end; lane 5, mid-region of GAPDH 840 bp apart from 3’ end]. The random hexamer-primed library lacked only the 3’ region of β-actin cDNA; however, the Not oligo(dT)-primed library had only it besides the GAPDH cDNA. The two libraries appeared complementary to each other for the range and species of cloned cDNAs. (C) Silver staining of SFIgG probe after SDS–PAGE under reducing conditions showed two bands of heavy and light chains (55 and 25 kDa) without extra bands and proved its purity.

FRP cDNA without the 50 amino acid residues derived from a pExCell vector, which may have unexpected antigenicity. As shown in Fig. 2(A), plasmid pGEX-4T-3/FRP encoded a GST fusion protein composed of GST and rFRP polypeptides with a signal peptide replaced by six accessory amino acid residues (Gly-Ser-Pro-Asn-Ser-Arg-) including the thrombin recognition site. On immunoblots shown in Fig. 2(B), GST fusion rFRP and non-fusion rFRP were detected as 60 kDa (lanes 6 and 8) and 40 kDa (lane 10) bands respectively.

Detection of anti-FRP antibodies in synovial fluids and sera from patients with RA and control diseases

We screened for IgG class anti-FRP antibodies in sera from patients with RA, other systemic rheumatic diseases and healthy individuals. Synovial fluids from patients with RA and OA were also tested. Some of the representative results of immunoblotting are demonstrated in Fig. 3. The frequencies of anti-FRP antibodies are summarized in Tables 1 and 2. In synovial fluids, the frequency in RA was significantly higher than OA (P < 0.005). The mean ± SD value of synovial fluid IgG (mg/dl) was 702 ± 312 in RA and 596 ± 360 in OA, which showed no significant difference. Anti-FRP antibodies appeared in the sera from RA patients significantly more frequently, although not exclusively, than from SLE (P < 0.01), polymyositis/dermatomyositis (P < 0.05) and normal healthy controls (P < 0.001).

Epitope mapping of FRP molecule

We determined the approximate epitopes of the FRP molecule. This could also mitigate the possible artificial antigenicity caused by addition of several amino acid residues (Gly-Ser-Pro-Asn-Ser-Arg-) to the N-terminus of the molecule to facilitate thrombin cleavage. According to the procedure of Maurer et al. (10), we divided the FRP molecule into four domains: an FS domain (a follistatin-like module containing 10 conservative cysteine residues), an EC domain (an extracellular Ca\(^{2+}\)-binding module containing an EF-hand) and others (Fig. 4A, the top schema). We designed six truncated FRP molecules (D1–D6) based on these domains for rough epitope mapping (Fig. 4A). D1 was a FRP molecule lacking the MAP1B/neuraxin homologous sequence. D6 was the only molecule without the N-terminal domain. Some of the representative results of immunoblotting are demonstrated in Fig. 4(B). The reactivity of patients’ sera to these truncated FRP mutants is summarized in Table 2. These results indicate that most anti-FRP autoantibodies recognize the part of the EC domain between the FS domain and the EF-hand. This unidirectional epitope-mapping study could detect no other antibodies, if any, in the same sera reactive to the part closer to the C-terminus.

Surveys of relationship between clinical profiles of RA and anti-FRP antibodies

Among the serological tests examined, erythrocyte sedimentation rate (ESR) and serum C-reactive protein (CRP) were significantly higher in anti-FRP antibody-positive patients than negative patients (Fig. 5). There was no significant difference in sex ratio, age, disease duration and steroid or methotrexate dosages between the two groups (data not shown), as well as in rheumatoid factor (Fig. 5).

Distribution of FRP mRNA in various tissues and comparison of FRP gene expression in RA and OA synovial tissue

Northern blotting analysis showed that FRP mRNA was 4.4 kb and not restricted in the synovium but was expressed almost
ubiquitously in various tissues with the exception of the peripheral blood leukocytes. This transcript was strongly expressed in the heart, placenta, prostate, ovary and small intestine (Fig. 6A). In synovium, FRP gene expression was higher in the specimens from RA than OA patients ($P < 0.05$).

**Reactivity of rabbit antisemur and mouse mAb to rFRP and immunoprecipitation of eukaryotically expressed FRP**

Immunoblotting showed that rabbit antisemur (OCT9702) and mouse mAb (ANOC9701) reacted with E. coli-expressed FRP as immunogen (Fig. 7A). Eukaryotically expressed FRP, which was first immunoprecipitated by the mAb, reacted with the rabbit antisemur and RA patient serum (Fig. 7B). The molecular size of FRP under reduced conditions was estimated as 50–55 kDa, which differed from the value (40–48 kDa) reported by Zwijsen et al. (11). As their samples were derived from a tumor cell line, the difference seemed to result from the molecular conditions affected by post-translational modification.

**Immunoprecipitation of FRP from synovial fluids and plasma**

As shown in Fig. 8, FRP was detected in most of the synovial fluids tested. In synovial fluids, there was no difference in the
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Fig. 3. Representative results of immunoblotting analysis for the detection of anti-FRP antibodies in synovial fluids and sera from RA patients and non-RA controls. Antigens applied were affinity-purified and thrombin-cleaved rFRP. All of the synovial fluids and sera except controls reacted to rFRP (40 kDa bands). Primary antibodies used were SFIgG from RA and OA patients for lanes *1–*3 and *C, and sera from RA patients and a healthy control for lanes #1–#3 and #C. IgG in lanes *1 and #1, and *3 and #3 were derived from the same patients respectively. Signals in lanes *3 and #3 were weak. Extra bands seemed to originate from the same source as in Fig. 2. The amount of total IgG (mg/dl) in primary antibody solution is as follows: *1, 30.6; *2, 34.7; *3, 45.2; *C, 48.3; #1, 21.6; #2, 15.6; #3, 16.0; #C, 12.5 (values in parentheses are for undiluted sera).

Table 1. Detection by immunoblotting of synovial fluid IgG class anti-FRP antibodies in RA and OA

<table>
<thead>
<tr>
<th>Diagnostic group</th>
<th>No. tested</th>
<th>No. positive</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA</td>
<td>18</td>
<td>8</td>
<td>44</td>
</tr>
<tr>
<td>OA</td>
<td>15</td>
<td>0</td>
<td>0(^a)</td>
</tr>
</tbody>
</table>

Frequencies are significantly lower than RA: \(^aP < 0.005\) (Fisher's exact probability test).

Table 2. Detection by immunoblotting of serum IgG class anti-FRP antibodies in systemic rheumatic diseases

<table>
<thead>
<tr>
<th>Diagnostic group</th>
<th>No. tested</th>
<th>No. positive</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA</td>
<td>67</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>SLE</td>
<td>51</td>
<td>5</td>
<td>10(^a)</td>
</tr>
<tr>
<td>SSC</td>
<td>18</td>
<td>3</td>
<td>17</td>
</tr>
<tr>
<td>SJ S</td>
<td>10</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Polymyositis/dermatomyositis</td>
<td>13</td>
<td>0</td>
<td>0(^b)</td>
</tr>
<tr>
<td>Normal healthy controls</td>
<td>30</td>
<td>0</td>
<td>0(^c)</td>
</tr>
</tbody>
</table>

Frequencies are significantly lower than RA: \(^aP < 0.01\) (\(\chi^2\) test), \(^bP < 0.05\) (Fisher’s exact probability test), \(^cP < 0.001\) (\(\chi^2\) test).

Table 3. Reactivity to truncated rFRP peptides

<table>
<thead>
<tr>
<th>Antigens</th>
<th>RA</th>
<th>SLE</th>
<th>SSC</th>
<th>SJ S</th>
<th>NHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>20</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>D1</td>
<td>17</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>D2</td>
<td>17</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>D3</td>
<td>17</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>D4</td>
<td>17</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>D5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D6</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
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</table>

\(^a\)Detected in immunoblots using antigens indicated.

Discussion

We succeeded in cloning autoantigens from RA synovium by RA synovial fluid IgG probes. One of the isolated cDNA clones encoded a whole molecule of FRP.

FRP was first cloned by Shibanuma et al. (12) as a protein encoded by the TSC-36 gene from a mouse osteoblastic cell line. Rat and human homologues were cloned from glioma cell lines and termed FRP by Zwijsen et al. (11). A chick homologue termed Flik (follistatin-like) protein was also cloned to investigate its gene expression in the development of avian embryos (13,14). Human FRP was reported to have a diffuse molecular mass of 40–48 kDa under denaturing conditions. FRP is a secreted soluble protein. Although the function of FRP has not been clarified, it was suggested that FRP plays some roles in the negative regulation of cellular growth (12). Its name is derived from its follistatin-like sequence containing 10 conserved cysteine residues, termed a FS module. Follistatin is a specific inhibitor of activin, a cytokine with various activities (15–17). Since the FS module has also been detected in other proteins such as SPARC/BM40, QR1, SC1/hevin and testican (10), FRP should be renamed after its function is clarified.

As shown by Northern blotting analysis, FRP mRNA was expressed not only in synovial tissue, but was detected almost ubiquitously in various organs. Unidentified receptor(s) of FRP, however, might be expressed in specific organs. FRP mRNA was detected as a single species of ~4.4 kb with no variants. Thus, it is likely either our cloned FRP cDNA or that isolated by Zwijsen is the product of incompletely spliced mRNA, i.e. heteronuclear RNA, which would explain the difference in the nucleotide sequences in the 3'-untranslated region. FRP gene expression was higher in fresh synovial

levels (mean ± SD) of arbitrary unit analyzed from signal densities between RA and OA patients (135 ± 52 and 123 ± 41), or between anti-FRP antibody-positive and anti-FRP antibody-negative group including both RA and OA patients (154 ± 28 and 121 ± 48), or between those two groups only in RA patients (154 ± 28 and 117 ± 68) (Fig. 8, left panel). In plasma, however, FRP was detected in one RA patient with positive anti-FRP antibody and not in normal healthy controls (Fig. 8, right panel), though more samples need to be investigated.
Fig. 4. Schematic representation of truncated FRP molecules for crude epitope mapping (A) and their immunoblotting study (B). (A) Each domain was defined according to Maurer et al. using MacPattern version 3.2 (Rainer Fuchs EMBL Data Library, Heidelberg, Germany) and Blocks Database version 8.0 software (Fred Hutchinson Cancer Research Center, Seattle, WA). FS, a follistatin-like module; EC, an extracellular Ca\textsuperscript{2+}-binding module containing an EF-hand indicated by a filled bar. The N-terminus is at the left. D1–D5 are reduced patterns from the C-terminus. D6 is identical to the FS module. (B) Immunoblotting of GST fusion rFRP and its truncated mutants with goat anti-GST antibody (left panel) and anti-FRP antibody-positive sera from patients and normal healthy control (NHC) serum (right panel). Samples applied were non-digested GST fusion polypeptides of rFRP (lane ‘Intact’), truncated mutants (lane ‘D1–D6’) and a mixture (lane ‘Intact & D1–6 mix.’ and all lanes of the right panel). The two panels were aligned to have the same molecular scale. In lanes ‘D5’ and ‘D6’, each recombinant protein formed a wide strong band fused with those extra bands. In lane ‘Intact & D1–6 mix.’, D3 and D4 were detected weakly due to their reduced quantities. Extra bands seemed to originate from the same source as in Fig. 2.

tissues resected at synovectomy from RA than OA patients. However, our immunoprecipitation analysis showed that there was no correlation between the amount of FRP molecules in synovial fluids and the disease entities, i.e. OA and RA, or the presence of anti-FRP autoantibodies. This discrepancy could be attributed to the elevated consumption of FRP involved in RA-specific inflammatory processes. Therefore, anti-FRP autoantibodies might interfere with the supply of functional FRP molecules and make the augmented production of FRP not meet their demand.

Screening of synovial fluids and sera indicated that anti-FRP autoantibodies of the IgG class appeared most frequently in RA, although they were also detected at substantial frequencies in other systemic rheumatic diseases. The presence of those antibodies might result from the aberrant immune response to FRP itself or molecular mimicry. In this study, however, epitopes were so roughly determined that their similarity to other organism polypeptide sequences could not be estimated. Detailed epitopes are under investigation.

The clinical significance of anti-FRP antibodies remains to be clarified. The term ‘rheumatism’ has indicated general symptoms such as arthralgia and myalgia in systemic rheumatic diseases. Since anti-FRP antibodies are not specific to RA, they might represent some kinds of common pathophysiology of ‘rheumatism’ and could raise a new concept to subdivide a group of systemic rheumatic diseases.

It has been reported that some autoantibodies can modify or inhibit the activities of functional proteins by direct
Fig. 5. Relationship between detection of serum anti-FRP antibodies, on the one hand, and CRP, ESR and rheumatoid factor (RF), on the other, in patients with RA. CRP and ESR were significantly higher in anti-FRP antibody-positive patients than negative patients. RF in both groups was not significantly different. The mean ± SD value of CRP (mg/dl) was 4.4 ± 3.3 in the anti-FRP antibody-positive group (n = 20) and 2.3 ± 2.6 in the negative group (n = 47) (P < 0.05). That of ESR (mm/h) was 65 ± 35 in the former and 43 ± 25 in the latter (P < 0.01). P values were calculated by Fisher's Protected Least Significant Difference. Horizontal bars represent 10, 25, 50 (median), 75 and 90 percentiles. Values outside of the 10–90 percentile range were plotted by points.

Fig. 6. Northern blotting analysis of FRP gene expression in various human tissues (A) and synovium derived from RA and OA patients (B). (A) FRP mRNA was strongly expressed in the heart, placenta, prostate, ovary and small intestine. Each lane contained 2 µg of poly(A)+ RNA. GAPDH cDNA was used as a control. (B) FRP gene expression was higher in the synovium from RA than OA patients (P < 0.05 by t test). The mean ± SD arbitrary unit of the amount of transcript was 30.1 ± 4.6 in RA and 19.1 ± 3.2 in OA samples. Each lane contained 20 µg of total RNA. Gels were stained with ethidium bromide and densities of 28S rRNA bands were used for standardization in quantitative analysis.

Fig. 7. Immunoblotting of E. coli-expressed FRP with rabbit antiserum and mouse mAb (A) and immunoblotting of COS-7 cell-expressed FRP immunoprecipitates with those antibodies (B). (A) Antigens applied were thrombin-digested GST fusion FRP. Primary antibody solutions were RA patient serum (lane 1), pre-immune rabbit serum (lane 2), post-immune rabbit serum OCT9702 (lane 3) and mouse mAb ANOC9701 (lane 4). Extra bands (80 kDa in lane 1, 3 and 4) appeared to be indissoluble dimers peculiar to this sample because of its large-scale preparation. (B) Fractionated samples were immunoprecipitated cultures of inverted FRP cDNA (mock)-transferred COS-7 cells (lane 1) and FRP cDNA-transferred COS-7 cells (lanes 2 and 3). Primary antibody solutions were the rabbit antiserum (lanes 1 and 2) and RA patient serum (lane 3). The difference of band broadness between lanes 2 and 3 appeared to result from antibody reactivity to FRP molecules with different post-translational modification, e.g. glycosylation or phosphorylation.
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Fig. 8. FRP was detected in most of the tested synovial fluids (left panel) and some of the RA plasma (right panel). Aliquots of 1 ml of the samples were immunoprecipitated by mouse anti-FRP mAb (ANOC9701) and immunoblotted with rabbit antiserum to FRP (OCT9702). Antigens applied in the rFRP control lanes were immunoprecipitates from culture of transiently FRP-expressing COS-7 cells. It seemed that the larger the amount of non-specifically immunoprecipitated contaminants, the faster the bands of FRP moved.

interaction (18–22). Among the ones detected in RA, anti-IL-1α (18) and anti-calpastatin (5) antibodies have such activities. In this study, the presence of anti-FRP antibodies had a significant correlation with inflammatory signs such as elevation of ESR and serum CRP in RA patients. It is therefore possible that autoantibodies to FRP also could reduce the activity of FRP so as to promote tissue inflammation. To address this point, it is essential to clarify the function of FRP and to demonstrate if anti-FRP autoantibodies have such activities in vitro or in vivo.

In conclusion, FRP is a novel autoantigen in systemic rheumatic diseases. Our results suggest the possibility that the detection of anti-FRP antibodies could be one of the markers for clinical evaluation of systemic rheumatic diseases. Further studies of FRP in those diseases will give us a new approach to elucidating unknown process in their complex pathological mechanisms and to creating a new therapy for FRP-associated rheumatic diseases.

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Abbreviations

FRP follistatin-related protein

GST glutathione S-transferase
NHC normal healthy controls
OA osteoarthritis
RA rheumatoid arthritis
rFRP recombinant follistatin-related protein
SFlgG synovial fluid IgG
SjS Sjögren’s syndrome
SLE systemic lupus erythematosus
SSc systemic sclerosis

References

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