Detection And Expression of a cDNA Clone That Encodes a Polypeptide Containing Two Inhibitory Domains of Human Calpastatin and its Recognition by Rheumatoid Arthritis Sera

Normand Després, Guylaine Talbot, Bertrand Plouffe, Gilles Boire, and Henri A. Ménard
Rheumatic Diseases Unit, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, Québec, Canada, J1H 5N4

Abstract
RA is the most frequent and most destructive inflammatory arthropathy. Rheumatoid factors, in spite of their lack of disease specificity, are important serological markers for RA and appear important in its immunopathogenesis as well. In search of more disease-specific autoimmune systems, we have screened a human placenta λgt11 cDNA expression library using selected sera from patients with classical erosive RA. We have identified one clone (RA-1) that is recognized by three of five screening sera. The 950-bp insert shows a complete nucleotide sequence homology to the cDNA encoding the two COOH-terminal domains of calpastatin. The deduced open reading frame of the RA-1 cDNA predicts a 284-amino acid protein, with a calculated mol wt of 35.9 kD. Calpastatin is the natural inhibitor of calpains, which are members of the cysteine proteinases recently implicated in joint destruction in rheumatic diseases. The two domains encoded by the RA-1 clone each contain the structural features associated with the inhibitory activity of human calpastatin. By Western blotting, 45.5% or 21/44 RA sera specifically recognized both the fusion and the cleaved recombinant protein. This is in contrast to 4.7% (2/43) in nonrheumatoid sera and 0/10 in normal sera. Anticalpastatin autoantibodies could represent a disease-associated marker in chronic erosive arthritis of the rheumatoid type and could hypothetically play a dual pathogenic role, directly via an immune interference and indirectly through an immune complex mechanism. (J. Clin. Invest. 1995. 95:1891-1896.) Key words: autoantibody • autoimmune • molecular cloning • cysteine proteinase inhibitor • autoimmunity

Introduction
Many manifestations of autoimmune diseases are characterized by their association with specific autoantibodies. In systemic lupus erythematosus, anti-dsDNA and anti-Sm autoantibodies can both serve as disease markers while only anti-dsDNA autoantibodies seem to have a pathogenic implication (1). In Graves' disease and myasthenia gravis, antibodies respectively targeting the thyroid-stimulating hormone and the acetylcholine receptors may actually drive the disease process (2, 3). Conversely, antithyroglobulin, antimicrosomal thyroid peroxidase, and anti-striated muscle autoantibodies appear to act only as disease markers.

Several autoantibodies have been reported to be more closely associated with RA (4-8). With the exception of rheumatoid factors, which are implicated in local immune complex formation and deposition, there is no evidence for a pathogenic role for these autoantibodies which may represent mere disease markers. A search for antigen–antibody systems more relevant to RA pathogenesis is thus warranted.

Isolation of cDNA clones encoding human proteins and peptides have been extensively used to identify new autoantigens (9). We undertook the immunoscreening of a human placenta λgt11 expression library with a pool of sera from five chronic erosive rheumatoid factor positive RA patients. In this study, we report that these sera recognized a cDNA clone (RA-1)1 encoding a protein corresponding to the two most COOH-terminal (residues 425-708) of the four inhibitory domains of calpastatin, the natural inhibitor of calpains (10). Calpains are calcium-dependent cysteine proteinases which are important intracellular activators in the signaling pathways and in triggering apoptosis (11, 12). They may also play a direct extracellular role in the destruction of connective tissue matrix and cartilage occurring in arthritic synovial joints (13). We also demonstrate that a significant proportion (45.5%) of RA patients produce antibodies against the purified recombinant protein encoded by RA-1. These observations allow us to hypothesize that immune interference with the natural inhibition of these proteinases may have implications in the severe erosive features observed in a subgroup of RA patients.

Methods
Sera and antibodies. Sera were obtained from 44 patients who fulfilled the American College of Rheumatology criteria for RA (14) and were seen at the Rheumatic Diseases Unit of the Université de Sherbrooke, Sherbrooke, Québec, Canada. We used 53 control sera: 11 from systemic lupus erythematosus patients, 27 from osteoarthritic patients, 5 from patients with miscellaneous rheumatic diseases, and 10 from normal subjects. A pool of five RA sera positive for rheumatoid factor, antiperi-

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1. Abbreviations used in this paper: GST, glutathione S-transferase; GST-RA-1, fusion protein; IPTG, isopropyl-β-D-thiogalactopyranoside; RA-1, cloned cDNA insert; rRA-1, recombinant protein.
nuclear factor, and anti-Sa autoantibodies while negative for other RA-associated autoantibodies (against SS-A (Ro), SS-B (La), U1RNP, A2 hmRNP, histones, and vimentin), were selected for cDNA library screening. Six Sa autoantibodies were detected by Western blotting using a semi-purified human placenta extract, as described (8). These pooled sera were absorbed with *Escherichia coli* strain Y1090 lysates to deplete natural antibodies to bacteria, some of which are capable of cross-reacting with autoantigens. A polyclonal goat antiserum specific for glutathione S-transferase (GST) (Pharmacia LKB Biotechnology, Inc., Uppsala, Sweden) was used to analyze the expressed fusion proteins.

Screening of cDNA library with patient sera. A human placenta *E1n1* oligo(dt)-primed expression library of 2.4 × 10^6 colonies (Clontech Laboratories Inc., Palo Alto, CA) was screened with the serum pool diluted 1/200 in Tris-buffered saline containing 0.05% Tween 20 (15). This library was selected for its high number of recombinant clones and because placenta is a good source of Sa autoantigen, a specific disease marker for RA (8). Duplicate nitrocellulose filters (Hybond; Amersham Corp., Arlington Heights, IL) were used and only plaques positive on both sheets were further purified. Bound antibodies were detected with goat anti-human IgG (H+L) alkaline phosphatase-conjugated antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and developed with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrates (Promega Corp., Madison, WI). Positive clones were finally tested with the five individual RA sera from the pool of serum used for library screening and five sera from normal subjects.

**DNA sequencing.** Positive *E1n1* phages were amplified by plaque lysis (16) and DNA was isolated using a lambda purification kit (QIA-GEN Inc., Chatsworth, CA). cDNA fragments were subcloned into the pUC19 vector at the EcoRI cloning site (New England Biolabs Inc., Beverly, MA). DNA sequencing was carried out with an A.L.F.™ automated sequencer (Pharmacia LKB Biotechnology Inc.), according to the dideoxy technique of Sanger et al. (17), using reverse antisense and fluorescein oligonucleotide primers (Pharmacia LKB Biotechnology Inc.). Sequences were aligned and analyzed using the GenBank/EMBL databases (NCBI/BLAST network service).

**Expression and purification of the fusion and recombinant proteins.** The cDNA fragments were subcloned from pUC19 into pGEX-4T-1 (Pharmacia LKB Biotechnology Inc.). The pGEX-4T-1 expression vector is constructed to give fusion polypeptides with an NH2-terminal GST carrier (27 kD) and a thrombin cleavage site (18). The *E. coli* PR745 lon* strain (New England Biolabs Inc.) was transformed with these constructs and the correct size and orientation of the cDNA insert was confirmed by restriction analysis with PstI digestion. Parental or recombinant pGEX-4T-1 transformants were grown at 37°C until OD600 reached 0.5–1.0 and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (90 min). Cells were lysed and fusion proteins purified as GST-RA-1 affinity-purified using glutathione Sepharose-4B (Pharmacia LKB Biotechnology Inc.), as described (18). Recombinant proteins (rRA-1) were obtained by removing the GST carrier using 10 cleavage units of thrombin per milligram of fusion protein (Sigma Chemical Co., St. Louis, MO).

**SDS-PAGE and immunoblotting.** Bacterial lysates, fusion, and recombinant proteins were subjected to electrophoresis on reducing SDS-polyacrylamide gels (10%) and the protein bands were Coomassie blue stained or electrophoblotted onto nitrocellulose filters (8). The blots were blocked with 5% dry milk in PBS, pH 7.3 and probed with an appropriate dilution of nonabsorbed serum in PBS-5% milk. Bound antibodies were detected with Protein A-horseradish peroxidase (ICN Biomedicals, Inc., Costa Mesa, CA) diluted in PBS-0.5% milk. Filters were developed using the enhanced chemiluminescence detection system (ECL; Amersham Corp.). Sera were coded and the immunoblots read blind. All sera were tested at least twice on each substrate; when the results were discordant for a given serum, the results of a third test was the one recorded. Sera giving indeterminate results (i.e., very faint bands) were read as negative.

**Results**

**Isolation of the RA-1 cDNA clone.** Approximately 2.5 × 10^6 plaques were immunoscreened and one strongly positive clone (RA-1) was identified and further characterized. The RA-1-isolated cDNA insert was shown to contain ~950 bp by agarose gel electrophoresis. At this stage, we verified if the expressed β-galactosidase fusion protein from the purified *E1n1* clone induced by IPTG reacted with individual sera. Three of the five rheumatoid sera recognized the positive plaque, whereas no reaction was detected with five nonrheumatoid control sera (data not shown). The cDNA insert was subsequently subcloned into pUC19 vector and sequenced. The 950-bp sequence of RA-1 was analyzed using the NCBI/BLAST network service and found to encode a 284-amino acid polypeptide (Fig. 1). The search for homologous sequences in the GenBank/EMBL databases showed an almost complete identity with the COOH-terminal portion of human liver calpastatin at both the nucleo-
Figure 2. Purification of pGEX-4T-1—expressed recombinant proteins analyzed by SDS-PAGE (A) and Western blotting (B). Recombinant proteins from E. coli PR745 lon™ containing pGEX-4T-1/RA-1 or parental vector were induced, purified on glutathione Sepharose 4B, and digested with thrombin as described in Methods. The Western blot was performed using a pool of five nonabsorbed rheumatoid sera diluted 100-fold. Proteins were run separately in each lane: pGEX-4T-1/RA-1 transformant cell lysates before (lane 1) and after (lane 2) induction of GST-RA-1; parental pGEX-4T-1 transformant cell lysates before (lane 3) and after (lane 4) induction of GST alone; purified GST-RA-1 (lane 5) and parental GST (lane 6) fractions eluted from glutathione Sepharose 4B; rRA-1 from the flow-through after thrombin cleavage of GST-RA-1 bound to glutathione Sepharose 4B (lane 7); and control thrombin cleavage of bound GST alone (lane 8).

Figure 3. Recognition of GST-RA-1 (A) and rRA-1 (B) polypeptides using rheumatoid and nonrheumatoid sera. Affinity-purified GST-RA-1 and cleaved rRA-1 polypeptide were separated on SDS-PAGE and transferred onto nitrocellulose sheets. These filters were probed with sera diluted at 1:100 for GST-RA-1 or at 1:50 for rRA-1. Lanes 1–19: serum from 20 patients with RA. Lanes 20–22: patients with systemic lupus erythematosus. Lanes 23–25: normal human sera. In A, sera detecting the two upper bands were considered positive. The strong signal below RA-1 bands likely represent E. coli contaminant proteins detected by natural antibodies. In B, the detection of the two upper RA-1 bands was considered a positive result. The results obtained with both fusion and recombinant RA-1 proteins were similar. Lanes 1, 2, 6–9, 12, 14 and 15 are strongly positive while lanes 3, 4, 11 and 17–19 are weakly positive.

tide (position 1435–2384) and the predicted amino acid (position 425–708) levels (Accession number D16217, total nucleotides: 2,493 and total amino acids: 708). The RA-1 cDNA ends at position 2384 of the published sequence of human calpastatin, 95 bp downstream from the stop codon, rather than at position 2,493, 204 bp downstream from the stop codon (19). This has no effect on homology since the lacking base pairs are located in a noncoding region. The coding sequence of RA-1 cDNA contains a nucleotide substitution (G→A) at position 1937 of calpastatin cDNA; it would result in a glycine to glutamate replacement at position 592 of the calpastatin protein. The same nucleotide difference was found in a cDNA encoding domain 4 and all the 3', noncoding region, including the polyadenylation signal and the poly-A tail, that we recently cloned using a RA-1 cDNA-derived probe (data not shown). Interestingly, the deduced amino acid sequences of pig and rabbit calpastatins, aligned with human calpastatin sequence, also have a glutamate residue at position 592. This residue is situated in a highly conserved region (19).

Expression of RA-1 cDNA and purification of the fusion and recombinant proteins. The RA-1 cDNA was subcloned in the pGEX-4T-1 vector to express a recombinant protein in the accurate reading frame. GST-RA-1 expressed in E. coli PR745 lon™ was affinity purified, eluted with glutathione (GST-RA-1), or the recombinant protein cleaved from its carrier protein (rRA-1). Both antigens were identified by SDS-PAGE and immunoblotting with the pool of RA sera (Fig. 2). GST-RA-1 was of the expected M, of 65 kD (Fig. 2 A, lane 2). Two major polypeptide bands were affinity purified using IPTG-induced RA-1 bacterial lysates using a glutathione Sepharose column (Fig. 2 A, lane 5). Only the upper of these two bands was strongly recognized by the serum pool (Fig. 2 B, lane 5). A few lower mol wt bands were also immunoreactive and these may represent proteolytic degradation products. A 65-kD band detected in both induced and uninduced parental pGEX-4T-1 (Fig. 2 B, lanes 3 and 4) transformant cell lysates likely represented a bacterial protein recognized by patient sera. This protein was not induced by IPTG (Fig. 2 A and B, lane 4) and it was not affinity purified from glutathione Sepharose-4B (Fig. 2 A and B, lane 6). Thrombin cleavage of rRA-1 from its GST carrier resulted in a few 40–47-kD bands (Figure 2 A, lane 7), two of which were strongly recognized by the pool of RA sera (Fig. 2 B, lane 7) and by its individual component sera (e.g., Fig. 3 B, lanes 1 and 2). Although, the predicted mol wt of rRA-1 is 36 kD, the two major antigenic bands migrated as proteins of 43 and 47 kD (Fig. 2 B, lane 7).

The 75-kD polypeptidic band (Fig. 2 B, lane 7) is an E. coli heat-shock protein produced by the dnaK gene that copurifies with the recombinant protein (20) and is recognized by natural antibodies in some nonabsorbed patient sera. The reactivity against this protein could be abolished after E. coli absorption of the sera without depletion of anti-rRA-1 antibodies (data not shown). A protein with an apparent M, of 40 kD was recognized by some sera (Fig. 3 B), independently of the recognition of the two rRA-1 bands. For example, in Fig. 3 B, the serum in lane 1 only recognized the 43- and 47-kD rRA-1 doublet, the serum in lane 10 recognized only the lower 40-kD band, while the sera in lanes 6–9 recognized all three bands. This suggests that the 40-kD band is not antigenically related to rRA-1. The absence of recognition of GST-RA-1 by sera recognizing only the 40-kD band in the rRA-1 preparation further supports this interpretation (Fig. 3 A and B, lane 10).
Finally, the pool of sera failed to react with GST (Fig. 2 B, lanes 4 and 6). Taken together, these results showed a strong reactivity of RA sera against the RA-1 polypeptides with a lack of detection of the GST moiety.

Reactivity of rheumatoid and control patients with fusion and recombinant RA-1. To determine whether individual sera from RA patients reacted with the RA-1 polypeptides, Western blots were performed using purified GST-RA-1 and rRA-1 (Fig. 3). The results with both proteins were similar. The two rRA-1 polypeptidic bands (43 kD and 47 kD) were recognized by 21 of 44 RA sera (45.5%) vs two of 43 (4.7%) nonrheumatoid sera and none of 10 normal sera. The two nonrheumatoid sera had the clinical diagnosis of osteoarthritis. These data show that patients with rheumatic diseases produce autoantibodies recognizing a polypeptide (rRA-1) that corresponds to two functional domains of calpastatin. They also suggest that the production of anti-rRA-1 antibodies may be preferentially, but not exclusively, associated with RA.

Because the library screening sera were anti-Sa positive and because the prevalence of anti-RA-1 and anti-Sa antibodies (8) in RA were similar, the 97 sera were also tested for anti-Sa antibodies by Western blot (Table I). 31 RA sera were concordant (18 both positive and 13 both negative) and 13 were discordant (3 anti-RA-1 positive only and 10 anti-Sa positive only). The 53 control sera were all anti-Sa negative. Because these studies did not use full length calpastatin or pure Sa as antigens, it would be premature to conclude on the presence or absence of a molecular relationship between the two autoimmune systems.

Discussion

Autoantibodies to intracellular components expressed on cell surfaces or secreted in the extracellular environment are potentially pathogenic (1–3, 21). There is substantial evidence that many disease-associated autoantibodies have the ability to interact directly with enzymes and downregulate their activity (1, 21). Recent reports further suggest that autoantibodies can also upregulate enzymatic activity through interaction with proteinase inhibitors. Thus, anti-C1 inhibitor antibodies have been detected in patients with severe episodes of angioneurotic edema and acquired C1 deficiency (22). A subpopulation of antiproteinase 3 antibodies from patients with Wegener’s granulomatosis can block the interaction of this serine proteinase with its natural inhibitor alpha 1-antitrypsin (23, 24). The present report provides another example of autoantibodies targeting a proteinase inhibitor.

Calpastatin is composed of five domains of about 140 amino acid residues each. The NH2-terminal domain (domain L) is nonhomologous to the four others and per se has no inhibitory activity. Each of the four COOH-terminal domains (domains 1–4) is repetitive and contains one TIPPPXYR consensus sequence associated with the inhibitory activity of calpastatin. Indeed, using subcloned calpastatin cDNA fragments, it has been shown that each of the four homologous domains can block the proteinase activity of calpains (25). The RA-1 clone encodes two of the inhibitory sequences, those of domains 3 and 4 of calpastatin (Fig. 1). Strong evidence suggest that RA-1 cDNA is derived from the calpastatin mRNA. First, only 1 out of 950 consecutive bp (i.e., a 99.89% identity) differentiates RA-1 cDNA from the published 3’ end of the human liver calpastatin cDNA; this identity spans both the coding and an adjacent 95-bp noncoding sequence. Second, the same difference was found in an additional cDNA encoding domain 4 of calpastatin and all the 3’ noncoding region, including the polyadenylation signal and the poly-A tail, that we recently cloned using a RA-1 cDNA-derived probe (data not shown). Third, this single nucleotide difference is not found when pig and rabbit calpastatin cDNAs are looked at. These data suggest either a polymorphism of the calpastatin mRNA or the existence of a sequencing mistake in the GenBank entry for human calpastatin. Thus, the immunoreactivity against RA-1 raises the hypothesis of specific immune interference with the interaction between calpastatin and calpains. However, logical, this proposition remains to be demonstrated.

Proteolytic enzymes are thought to play an important role in joint destruction. These enzymes belong to three major families: the matrix metalloproteinases, the serine proteinases, and the cysteine proteinases (26). The relative importance of these proteinases and their respective inhibitors in matrix destruction remains to be definitely established in vivo. However, recent data suggest that cysteine proteinases, particularly the calpains and their natural inhibitor calpastatin, could be important (27–29). First, calpains are present in increased quantity in the synovial fluid (30–34) and membrane (13). Second, they are secreted in vitro by TNFα and IL-1–stimulated synovial fibroblast-like cells (35). Third, calpains are capable of degrading matrix components of articular cartilage and calpastatin can inhibit this degradation (29, 31, 32). Fourth, a role for synthetic cysteine proteinase inhibitors as disease-modifying antirheumatic agents has been recently proposed in adjuvant-induced arthritis of rats and in collagen-induced arthritis of mice (36).

Under normal and pathological conditions, proteinases from each major family are inactivated by their natural inhibitors: tissue inhibitors of metalloproteinases for matrix metalloproteinases (37), serpins for serine proteinases (38), and the cysteine proteinase inhibitors. The natural cysteine proteinase inhibitors are members of a superfamily including the cystatin, the stefin, and the kininogen families. Calpastatin does not belong to this superfamily and is unique in its inhibitory specificity for calpains (10). It has been suggested that an imbalance between matrix metalloproteinases and tissue inhibitors of metalloproteinases could be responsible for cartilage breakdown in osteoarthritis (39). Calpastatin having been shown to be less abundant than calpains in the synovial fluid of RA patients (13), a disadvantageous enzyme/inhibitor ratio in some RA patients could also be associated with a more destructive potential of the arthri-
tis. Antibodies to calpastatin could be entirely or partly responsible for this imbalance by binding to and somehow inactivating the inhibitor following its synthesis, expression on the plasma membrane, and/or secretion in vivo. This would result in both an immune complex-mediated inflammation and an uncontrolled activity of calpains.

One interesting observation is the discrepancy between the immunoreactivity of biochemically purified placental Sa antigen and of rRA-1. Indeed, absorption of sera with rRA-1 did not completely deplete their anti-Sa reactivity (data not shown). A number of explanations are possible. First, rRA-1 may lack epitopes found on the corresponding in vivo antigen: some epitopes may be situated on the NH2-terminal region of the protein that is not encoded by the RA-1 clone, or rRA-1 may lack antigenic posttranscriptional modifications. Second, some renaturation of the antigen blotted on nitrocellulose sheets is possible during immunoblotting; rRA-1 may thus lack some conformational epitopes found on the corresponding in vivo antigen. Third, anti-rRA-1 and anti-Sa antibodies may represent linked, but not identical, autoantibody systems, similar to anti-Ro and anti-La or to anti-U1 RNP and anti-Sm (1). The existence of anti-rRA-1 antibodies within sera of some patients without anti-Sa antibodies supports this last hypothesis. Careful definition of the epitope(s) recognized by anti-Sa and anti-RA-1 antibodies will be required to answer these questions.

The identification of calpastatin as an autoantigen in almost half of RA sera provide new research avenues. Studies are required to demonstrate the capacity of these antibodies to block the inhibitory activity of calpastatin on calpains. Furthermore, the presence of calpastatin-containing immune complexes should be demonstrated at the site of tissue damage in erosive RA. Finally, clinical correlation between the presence of anticalpastatin antibody and erosive disease will need to be systemically verified in RA and other rheumatic diseases, especially if an eventual therapeutic window is opened by synthetic cysteine proteinase inhibitors.

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References


