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Structural differences between the human and mouse 52-kD Ro autoantigens associated with poorly conserved autoantibody activity across species

C. L. KEECH, T. P. GORDON & J. McCLUSKEY *Centre for Transfusion Medicine and Immunology, Flinders Medical Centre, Bedford Park, Australia*

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SUMMARY

Anti-nuclear autoantibodies found in human autoimmune diseases frequently cross-react with homologous autoantigens in distant species, supporting the notion that autoantibodies target conserved functional domains. However, the 52-kD Ro(SS-A) protein is an exception, in that human autoantibodies are not known to recognize any equivalent antigen in the cells of rodents and other non-primate species. To understand this lack of cross-reactivity we have isolated cDNAs encoding the mouse 52-kD Ro molecule. The cDNA encoding mouse 52-kD Ro revealed an open reading frame of 470 amino acids, with 70% sequence identity to the human 52-kD Ro antigen. The putative leucine-zipper and zinc-finger motifs present in human Ro52 were conserved in the mouse protein. Recombinant mouse 52-kD Ro protein reacted with human autoantibodies by ELISA and immunoblot, but with approximately 10-fold lower reactivity than recombinant human 52-kD Ro protein under the same conditions. Detection of both human and mouse 52-kD Ro by immunoblot was dependent on antigen concentration which was limiting in the cell equivalents generally used in immunoblot assays. Differential chaotropic disruption of antibody binding suggested a lower avidity of human autoantibody binding to the mouse 52-kD Ro protein compared with the human antigen. Thus the poor reactivity of native mouse 52-kD Ro with human autoantibodies is associated with species divergence diffusely distributed throughout the primary structure of the 52-kD Ro molecule.

Keywords autoantigens Ro/SS-A 52-kD Ro

INTRODUCTION

Clusters of autoantibodies recognizing nuclear/cytoplasmic antigens are produced in patients with many rheumatic diseases and are used as clinical markers of these disorders [1]. In systemic lupus erythematosus (SLE) and primary Sjögren's syndrome (pSS), autoantibodies to the components of the Ro/LaRNP are common and include antibodies recognizing 60-kD Ro(SS-A) (referred to as Ro60), 52-kD Ro(SS-A) (referred to as Ro52), La(SS-B) polypeptides and hY RNAs.

Disease-associated autoantibodies often recognize functional domains of target autoantigens which are conserved across species [1], although exceptions to this generalization have been reported [2]. Human autoantibodies specific for the Ro60 and La autoantigens cross-react with the homologous proteins in many species, including mouse [3–5]. By contrast, autoantibodies reactive with the human Ro52 antigen do not readily identify a Ro52 homologue in mouse cells or in cells from other non-primate species [4]. Nonetheless, in mouse models of systemic autoimmunity, murine autoantibodies have been reported to react with human Ro52 antigen as well as Ro60 and La autoantigens, suggesting a cross-reactive mouse homologue of Ro52 antigen does exist [6]. Identification of the mouse Ro52 gene and protein would be of great value in understanding immune models of systemic autoimmunity and in defining autoantibody specificities. Therefore we have cloned, sequenced and expressed cDNAs encoding mouse Ro52 autoantigen. The mouse Ro52 cDNA predicted the complete protein to be 70% identical to the human Ro52 antigen, and putative functional sites were even more conserved than this. Human autoantibodies were found to react with recombinant mouse Ro52 antigen, but only at a 10-fold lower antibody titre or at significantly higher antigen concentrations than required for reactivity with human Ro52. Human cellular Ro52 autoantigen was limiting for immunoblot detection by patient autoantibodies. When mouse cellular protein lysates from spleen cells corresponding to very high cell equivalents (5×10^6 cells per lane) were analysed, murine Ro52 was weakly detectable by immunoblot as a 52-kD band co-migrating with human Ro52. These data demonstrate that the previous inability to detect mouse Ro52 antigen by human autoantibodies is associated with diffuse species divergence of their protein

Correspondence: James McCluskey, Centre for Transfusion Medicine and Immunology, Flinders Medical Centre, Bedford Park, South Australia 5042.

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sequences and the relatively low abundance of cellular Ro52 antigen.

MATERIAL AND METHODS

Isolation and sequencing of mouse and human Ro52 cDNA clones
Mouse Ro52 cDNA clones were isolated from a cDNA library made from normal mouse macrophages taken from bone marrow. The cDNA was size fractionated to obtain products of more than 1·4 kb which were then cloned into the λZAP II vector [7] kindly supplied by Jane Visvader (Walter and Elisa Hall Institute of Medical Research, Melbourne, Australia). Human Ro52 cDNA clones were isolated from a cDNA library derived from a human T cell line (Hutt-78). Libraries were screened by DNA hybridization to a 1·43 kb radiolabelled polymerase chain reaction (PCR) product corresponding to the human Ro52 coding region [8]. Hybridization was carried out at 42°C in 50% formamide, 5 × Denhardt's solution, 5 × SSPE, 0·1% SDS, and 100 μg/ml herring sperm DNA [9]. Hybridizing phage were plaque purified and the phage DNA isolated on a caesium chloride gradient [10]. After purification of the phage DNA the mouse Ro52 cDNA inserts were subcloned into pBluescript II KS (Stratagene, La Jolla, CA) for mapping of endonuclease sites and sequencing. Automatic dideoxy chain termination sequencing (Applied Biosystems Model 373A;

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                    -140                 -120                 -100
                       TCCGTTGCTG TCGAAAGCGA AGCTGGGAGC ACATAACTAA AGCTGAGGAC
          -80                  -60                  -40
 CCTGGTTAGA AACTCGGTTT CCCCTTCTGC CTGGCTTGCT CAGTGGGTGA GGCCGGAGGA GAGGCTCCTG

-20                     1                15                30
  TCATTCAGAT TCCACAGCAG ATG TCA CCC TCT ACA ACC TCA AAA ATG TCT CTG GAA AAG ATG
                        met ser pro ser thr thr ser lys met ser leu glu lys met

 45                 60                  75                  90
TGG GAG GAG GTC ACC TGT TCT ATC TGC CTG GAT CCC ATG GTG GAG CCT ATG AGT ATC GAA
trp glu glu val thr cys ser ile cys leu asp pro met val glu pro met ser ile glu

105                 120                 135                 150
TGT GGC CAT TGC TTT TGC AAG GAA TGC ATT TTT GAA GTT GGG AAG AAT GGG GGC AGT TCA
cys gly his cys phe cys lys glu cys ile phe glu val gly lys asn gly gly ser ser

165                 180                 195                 210
TGT CCC GAG TGC CGG CAA CAG TTT CTG CTC CGA AAC CTC AGG CCC AAT AGA CAT ATA GCC
cys pro glu cys arg gln gln phe leu leu arg asn leu arg pro asn arg his ile ala

225                 240                 255                 270
AAC ATG GTG GAA AAC CTT AAA CAG ATA GCC CAG AAT ACC AAG AAG AGT ACC CAG GAA ACG
asn met val glu asn leu lys gln ile ala gln asn thr lys lys ser thr gln glu thr

285                 00                  315                 330
CAC TGC ATG AAG CAT GGA GAG AAG CTT CAC CTA TTC TGT GAG GAA GAT GGG CAG GCC CTT
his cys met lys his gly glu lys leu his leu phe cys glu glu asp gly gln ala leu

345                 360                 375                 390
TGC TGG GTG TGT GCC CAG TCT GGG AAA CAC CGG GAC CAC ACC AGG GTC CCT ATT GAA GAG
cys trp val cys ala gln ser gly lys his arg asp his thr arg val pro ile glu glu

405                 420                 435                 450
GCT GCT AAG GTA TAC CAG GAG AAG ATC CAC GTG GTT TTA GAA AAA CTG AGA AAG GGG AAA
ala ala lys val tyr gln glu lys ile his val val leu glu lys leu arg lys gly lys

465                 480                 495                 510
GAG TTG GCC GAG AAG ATG GAA ATG GAT CTC ACG ATG CAA AGA ACA GAC TGG AAG AGG AAC
glu leu ala glu lys met glu met asp leu thr met gln arg thr asp trp lys arg asn

525                 540                 555                 570
ATT GAC ACC CAG AAG TCG AGG ATT CAC GCA GAG TTC GCA CTT CAG AAT AGC TTG CTG GCT
ile asp thr gln lys ser arg ile his ala glu phe ala leu gln asn ser leu leu ala

585                 600                 615                 630
CAG GAG GAG CAG AGG CAG CTG CAG AGG CTG GAG AAG GAT CAA AGG GAG TAC CTG AGA CTC
gln glu glu gln arg gln leu gln arg leu glu lys asp gln arg glu tyr leu arg leu

645                 660                 675                 690
CTG GGG AAG AAG GAG GCT GAG CTG GCT GAG AAG AAC CAG GCC CTG CAG GAG CTG ATC TCA
leu gly lys lys glu ala glu leu ala glu lys asn gln ala leu gln glu leu ile ser

705                 720                 735                 750
GAG CTG GAG AGG AGG ATT CGT GGT TCA GAG CTG GAG CTA CTG CAG GAG GTG AGG ATC ATC
glu leu glu arg arg ile arg gly ser glu leu glu leu leu gln glu val arg ile ile

765                 780                 795                 810
CTG GAA AGG AGT GGA TCC TGG AAC CTG GAC ACG TTA GAT ATT GAC GCC CCA GAC CTA ACA
leu glu arg ser gly ser trp asn leu asp thr leu asp ile asp ala pro asp leu thr
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Fig. 1.

Foster City, CA) of mouse Ro52 or human Ro52 cDNA inserts was performed using commercial universal primers for M13 (Promega Biotec, Madison, WI) and internal primers derived from the resulting mouse Ro52 sequence or from the published human Ro52 sequence (Macromolecular Resource, Fort Collins, CO). Specific internal primers used for sequencing were as follows: 5′-ATGTCACCCTCTACAACC-3′ (mRo52, bp1–18); 5′-CTCTTCAATAGGGACCCT-3′ (mRo52, bp385–402); 5′-CCTATTGAAGAGGCTGCT-3′ (mRo52, bp391–408); 5′-AGTGGATCCTGGAACCTG-3′ (mRo52, bp772–289, hRo52, bp760–777); 5′-ACCTAGCACCATGGGGTA-3′ (mRo52, bp994–1011); 5′-GCCCAGAGATTCTCCTCT-3′ (mRo52, bp1012–1029); 5′-TCAGTAGGCACTCGCTCC-3′ (mRo52, bp1420–1437); 5′-ATGGCTTCAGCAGCAGCG-3′ (hRo52, bp1–18); 5′-AAGCTCCAGGTGGCATTAGGG-3′ (hRo52, bp412–432); and 5′-TCAATAGTCAGTGGATCCTTGTG-3′ (hRo52, bp1406–1428). Both strands of the coding region were sequenced by this method. Sequences were translated and aligned using the computer programs DNA Strider [11], MacVector (IBI, New Haven, CT) and the FASTA align program [12].

Expression of recombinant protein

The coding regions of mouse and human Ro52 cDNAs were amplified by PCR with specific primers, mRo52, bp1–18 and mRo52, bp1420–1437, containing Sal I restriction sites, and subcloned into the expression vector pQE-9 (QIAgen Inc., Chatsworth, CA). The subsequent gene product was expressed as an in-frame hexa-his-fusion protein with six histidines at the NH_2 terminus. Recombinant protein was purified under denaturing conditions as described [13]. Estimations of protein concentration were determined by the Bradford assay [14].

Immunoblotting

Whole cell extracts were prepared by lysis of cells in sample buffer containing SDS and dithiothreitol. After boiling for 3 min, proteins were separated by standard SDS–PAGE [15] on 10% gels. Cell extracts or purified recombinant proteins were transferred to nitrocellulose (Amersham, Little Chalfont, UK) using a semi-dry transfer apparatus (Pharmacia, Uppsala, Sweden). Nitrocellulose filters were blocked for 1 h in PBS containing 3% low-fat powdered milk, then incubated for 1 h in sera diluted in wash

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825                 840                 855                 870
AGC ACA TGC CCT GTT CCA GGG CGG AAG AAG ATG CTG AGG ACG TGT TGG GTT CAT ATT ACT
ser thr cys pro val pro gly arg lys lys met leu arg thr cys trp val his ile thr

885                 900                 915                 930
CTG GAT CGC AAC ACC GCC AAC TCA TGG CTC ATC ATC TCA AAG GAT CGG AGA CAA GTG AGG
leu asp arg asn thr ala asn ser trp leu ile ile ser lys asp arg arg gln val arg

945                 960                 975                 990
ATG GGA GAC ACC CAT CAG AAC GTG TCT GAC AAT AAG GAG AGG TTT AGT AAT TAC CCC ATG
met gly asp thr his gln asn val ser asp asn lys glu arg phe ser asn tyr pro met

1005               1020                1035                1050
GTG CTA GGT GCC CAG AGA TTC TCC TCT GGG AAG ATG TAC TGG GAG GTA GAT GTG ACT CAA
val leu gly ala gln arg phe ser ser gly lys met tyr trp glu val asp val thr gln

1065               1080                1095                1110
AAG GAG GCC TGG GAT CTG GGG GTT TGC AGA GAT TCT GTT CAG AGG AAA GGG CAG TTT TCA
lys glu ala trp asp leu gly val cys arg asp ser val gln arg lys gly gln phe ser

1125               1140                1155                1170
CTC AGT CCC GAG AAT GGC TTC TGG ACC ATT TGG TTA TGG CAA GAC AGC TAT GAG GCT GGT
leu ser pro glu asn gly phe trp thr ile trp leu trp gln asp ser tyr glu ala gly

1185               1200                1215                1230
ACC AGT CCT CAG ACC ACC CTC CAC ATT CAA GTA CCT CCA TGC CAA ATT GGG ATC TTT GTG
thr ser pro gln thr thr leu his ile gln val pro pro cys gln ile gly ile phe val

1245               1260                1275                1290
GAC TAT GAG GCT GGC GTT GTC TCC TTC TAC AAC ATA ACT GAC CAT GGC TCC CTC ATT TAC
asp tyr glu ala gly val val ser phe tyr asn ile thr asp his gly ser leu ile tyr

1305               1320                1335                1350
ACC TTC TCG GAG TGT GTT TTT GCT GGA CCT CTG CGA CCT TTC TTC AAT GTT GGT TTC AAT
thr phe ser glu cys val phe ala gly pro leu arg pro phe phe asn val gly phe asn

1365               1380                1395                1410                1423
TAT AGT GGG GGA AAT GCA GCG CCT CTA AAG CTC TGT CCA CTA AAG ATG TGA  TGGTCAGGAG
tyr ser gly gly asn ala ala pro leu lys leu cys pro leu lys met ***

                  1443                 1463                 1483
  CCAGTGCCTA CTGACGGTAC TTCCCGGACA CTTACCTCCT CCTGTCCTGA TCAAGATCCA GTGACTCCTG
        1503                 1523                 1543                 1563
  GGGACCATTT CTGGATACTG CTGCTCACTT CCTGTTGGTG TTCTTCAGCC ATGCACTTTG TCATTTTGAC
                  1583                 1603                 1623
  CCATCTAGTT GGAACTACCC TAAACCCTCA TCTCTTTAAA GGAGTCAGGG TCCCAGAATG AAGGCATCAG
        1643                 1663                 1683
  CTGGACATAA CTGAAAATCA ATGTCAAATG ACCTTCACCA TCAATATACC TGACATAGAT TCCGCCGA
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Fig. 1. Nucleotide and predicted amino acid sequence of a mouse 52-kD Ro (SS-A) cDNA (Genebank accession no. L27990). The sequence of coding and untranslated regions were identical in two independent cDNA clones.

buffer (PBS, 3% powdered milk and 0·5% Tween 20). The nitrocellulose filters were washed five times in wash buffer, then probed with horseradish peroxidase (HRP)-conjugated, sheep anti-human immunoglobulin (Amersham) or sheep anti-mouse immunoglobulin (Silenus, Melbourne, Australia). Antibody binding was detected using enhanced chemiluminescence (Amersham). Prestained molecular weight markers (BioRad, Richmond, CA) were used to estimate molecular mass.

ELISA and antibody avidity determination

Binding of titrated human anti-Ro sera to recombinant human and mouse Ro52 protein was determined by ELISA as previously described [16]. The estimation of relative avidity of antibody binding to recombinant human or mouse Ro52 was determined by thiocyanate elution as described [17]. Briefly, in a variation of the standard ELISA method, replicate wells containing antibody bound to antigen were exposed to increasing concentrations of the chaotropic thiocyanate ion. Remaining bound antibody was detected with alkaline phosphatase-conjugated anti-human IgG (γ-chain) (Sigma, St Louis, MO). Resistance to thiocyanate elution was utilized as a relative measure of avidity by comparing the concentration of thiocyanate ions required to dissociate 50% of antibody binding.

RESULTS

Identification of the mouse Ro52 homologue

A human Ro52 cDNA clone was sequenced and found to be identical to that published by Itoh *et al*. [8], which also encodes a proline at residue 52 rather than an alanine as found in a cDNA reported by Chan *et al*. [18].

Two independent λZAP II clones, clone 4 ($\sim$ 1·8 kb) and clone 7 ($\sim$ 2·8 kb), were obtained from screening 1×10^6 recombinants of a normal mouse macrophage cDNA library with radiolabelled full-length human Ro52 PCR-derived probe. The complete nucleotide sequence of clone 4 was determined from pBluescript II KS subclones by the automated dideoxy chain termination method and was found to contain a single open reading frame of 470 codons (Genebank accession no. L27990, Fig. 1). No ribosomal binding motif was identified and the putative initiation site was deduced by comparison of the sequence with the human homologue. cDNA clone 4 also contained 5′ and 3′ untranslated regions of 140 and 381 bp, respectively. The coding region of clone 7 was also sequenced and found to be identical to clone 4.

The predicted amino acid (aa) sequences of human and mouse Ro52 were aligned as shown in Fig. 2. Mouse Ro52 contains 470 aa with a predicted molecular mass of 54·17 kD, whereas human Ro52 contains 475 aa and has a molecular mass of 54·14 kD. The mouse sequence is predicted to start four aa earlier, end eight aa earlier and contain a single aa deletion (human Ro52 aa 384) relative to the human Ro52 sequence. When these differences are taken into account 69·9% sequence identity is observed between the mouse and human Ro52. However, some of the changes are conservative in nature, and allowing for this, 81·5% similarity was determined by the FASTA.align program [12].

The differences in primary sequence between human and mouse Ro52 proteins are distributed throughout the entire molecule. The carboxy terminal half of the molecule is sightly more conserved between the human and mouse proteins (73·6%) compared with the amino terminal half (65·1%). The putative leucine zipper and zing-finger motifs identified in the human Ro52 sequence [8,18] are also present in the mouse Ro52 protein (Fig. 2), and are relatively more conserved than other regions of the molecule. Thus the proposed zinc-finger contains complete conservation of the cysteine residues with 78% (21/27 aa) of all positions being identical in the two species. Similarly, the four leucine residues comprising the leucine zipper motif are conserved so that each is spaced eight residues apart with 77% (17/22 aa) identity between species in this region.

Immunoreactivity of mouse Ro52

It has previously been reported that human autoantisera do not detect Ro52 from non-primate species, including mouse [4]. Considering the 70% identity in the predicted amino acid sequence of human and mouse Ro52, we analysed recombinant mouse Ro52 for immunoreactivity with human autoantisera containing anti-Ro52 antibodies under conditions where Ro52 antigen was not limiting. A microgram of purified recombinant human and mouse Ro52 was fractionated by SDS–PAGE, transferred to nitrocellulose and immunoblotted for anti-Ro52 reactivity (Fig. 3a). Filters were probed with normal human serum, a standard anti-Ro60 serum (CDC-7; CDC, Atlanta, GA) and four autoantisera (A–D). Antisera A–D were previously shown to contain mixed anti-Ro60 and anti-Ro52 antibodies as determined by (i) immunofluorescence on transfected HEp-2 cells over-expressing either human Ro52 [19] or human Ro60 [20], (ii) counter immunoelectrophoresis, and (iii) recombinant human Ro52 ELISA. The results shown in Fig. 3a revealed that recombinant mouse Ro52 was indeed immunoreactive with human anti-Ro52 autoantibodies under these conditions. Serum D did not appear to detect mouse Ro52, although this serum had lower reactivity against human Ro52, and it is probable that the reason for non-reactivity was quantitative under these conditions.

These observations suggested that the detection of mouse Ro52 was critically dependent on a threshold concentration of antigen easily obtained with recombinant protein, but below which antibody recognition was not evident. Therefore we tested whether such a threshold was evident when either mouse or human cell lysates were used as a source of Ro52 antigen. For this purpose, graded amounts of whole cell lysates ranging from 5×10^4 to 10^6 cell equivalents from human (Jurkat) and mouse (LTA-5) cell lines were immunoblotted with an anti-Ro serum, an anti-70-kD U1RNP serum and NHS (Fig. 3b). The 70-kD U1 RNP protein was readily detected at all cell equivalents loaded in both human and mouse cell lysates. The immunoblot revealed that cellular human Ro52 was detectable only when antigen from 10^5 cell equivalents was loaded into a single lane, whereas cellular mouse Ro52 from a fibroblast cell line was not detectable even when 10^6 cell equivalents were loaded per lane. The failure to detect mouse Ro52 in murine cell lysates was presumed to be due to the poor recognition of limiting amounts of mouse Ro52 by human autoantibodies rather than a complete lack of Ro52 expression in mouse cells. To test whether mouse Ro52 could be detected in mouse cell lysates, fresh spleen cells were isolated, lysed, fractionated on SDS–PAGE, and immunoblotted with an anti-Ro52 serum (Fig. 3c). When lysates from 5×10^6 spleen cells were loaded per lane, a 52-kD band co-migrating with human Ro52 was detected, albeit weakly when compared with the signal corresponding to one tenth the number of human cells. A faint band corresponding to mouse Ro52 antigen was also evident in the lane containing 10^6 spleen cell equivalents, suggesting variable expression of Ro52 in different cell types (compare with Fig. 3b).

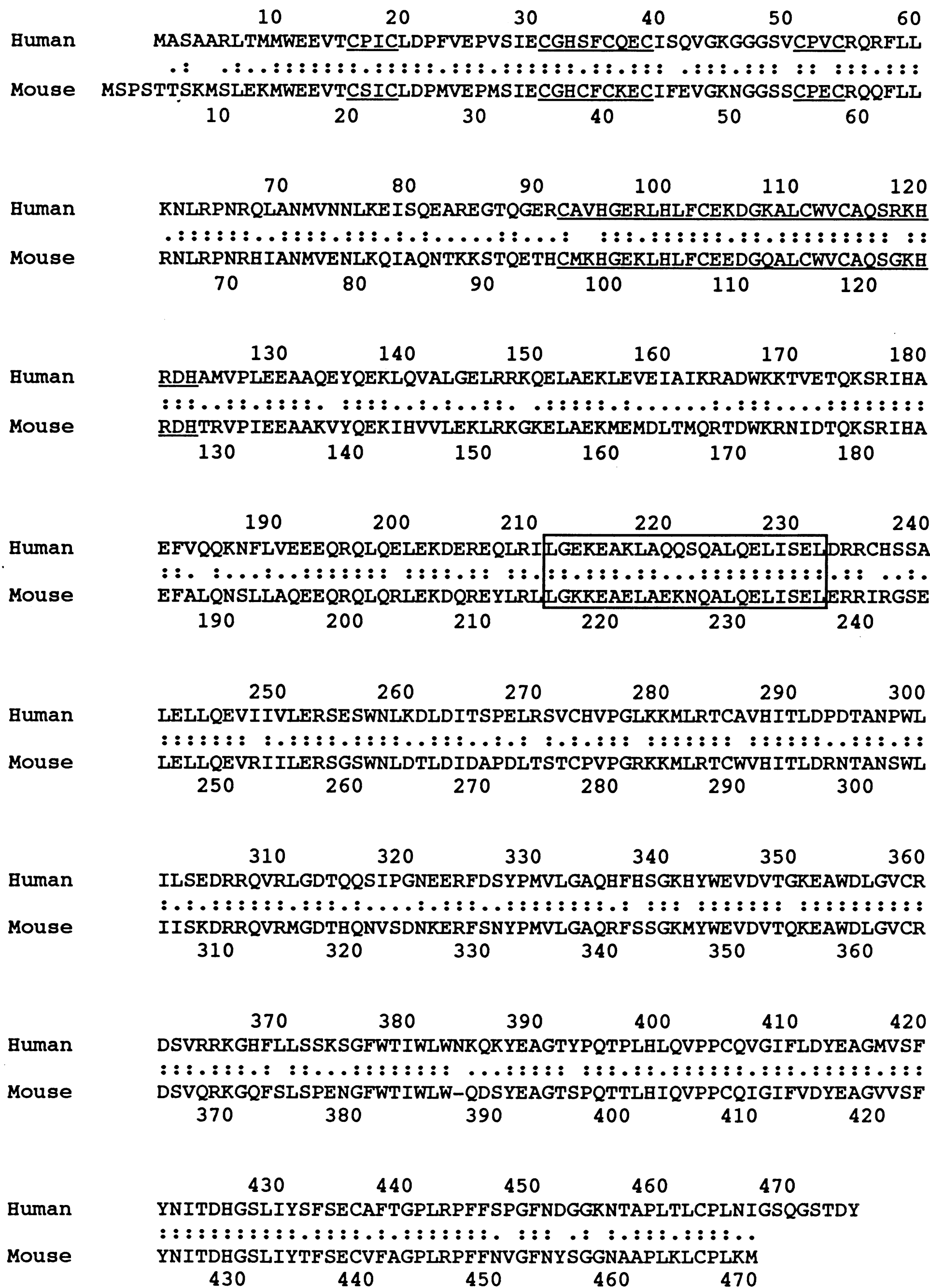

Fig. 2. Alignment of the predicted amino acid sequence of human and mouse Ro52. Amino acids are shown in single-letter code. Alignment was established using the FAST.P align program [12]. –, Sequence gaps introduced to facilitate alignment; :, identical amino acids; · , conservative amino acid changes (as determined by FAST.P align program); underlined regions, putative zinc finger motif; boxed region, putative leucine zipper motif.

The difference in autoantibody reactivity of human and mouse Ro52 was further examined by titrating the quantity of recombinant antigen required for autoantibody recognition in immunoblots (Fig. 4a). Graded amounts of human and mouse Ro52 antigen were immunoblotted with serum A, revealing a threshold for detection of 60 ng of human Ro52 with this serum. Under equivalent conditions 500 ng of mouse Ro52 were barely detected, demonstrating an approximate 10-fold difference in immunoreactivity of human and mouse Ro52 by immunoblot. To confirm this preferential autoantibody binding of human Ro52 *versus* mouse Ro52, the reactivity of autoantibodies with recombinant human and mouse Ro52 was tested by titrating antiserum against recombinant antigen in an ELISA assay (Fig. 4b). Five-fold dilutions of an anti-Ro serum (serum A) and NHS were tested on recombinant human and mouse Ro52 antigen coated at 1 μg/ml. An eight-fold lower dilution (higher concentration) of autoantiserum was required to give an $OD_{405} = 1$ with mouse Ro52 antigen compared with human Ro52 antigen. Thus, the relative quantity of antigen and

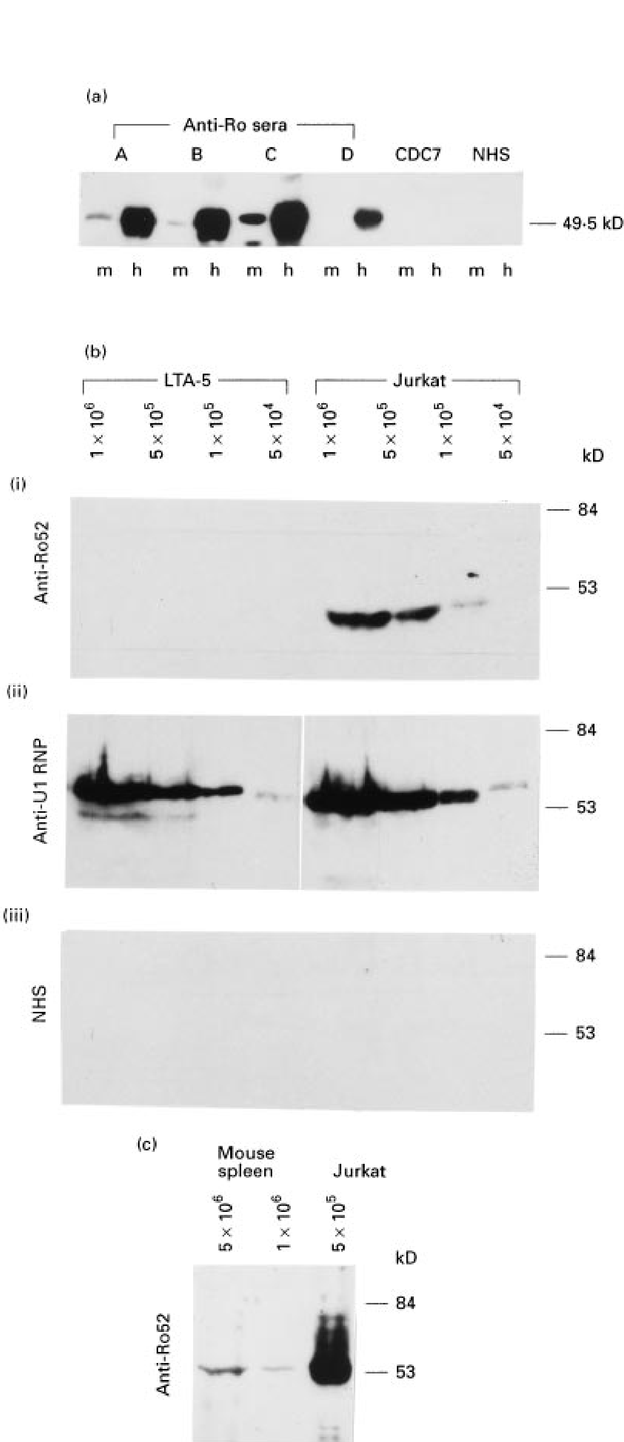

Fig. 3. Human anti-Ro52 sera react preferentially with the human Ro52 protein (a) Immunoblot analysis of four human anti-Ro52 sera (sera A–D), an anti-Ro60 serum (CDC7) and a normal human serum (NHS) with 1 μg recombinant mouse (m) or human (h) Ro52 protein. (b) Immunoblot analysis of (i) a human anti-Ro52 serum (serum C, anti-Ro52); (ii) a human anti-70-kD U1 RNP serum (anti-U1RNP); and (iii) a NHS on whole cell lysates representing 10^6, 5×10^5, 10^5 or 5×10^4 cell equivalents per lane of mouse (LTA-5), or human (Jurkat) cells. (c) Mouse Ro52 is weakly detectable in immunoblots of spleen cell lysates. Spleen cell lysates equivalent to 5×10^6 or 1×10^6 cells per lane, and 5×10^5 human cells (Jurkat) were immunoblotted with an anti-Ro52 serum (serum C). The position of molecular weight standards is shown.

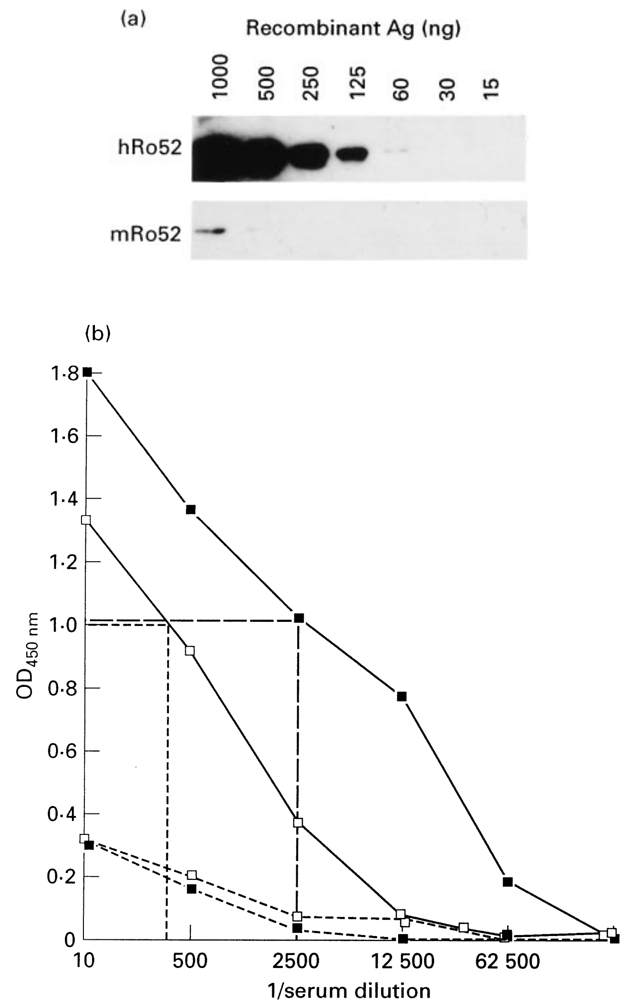

Fig. 4. Human autoantibodies preferentially react with human Ro52 compared with mouse Ro52. (a) A human antiserum containing anti-Ro autoantibodies (serum A, Fig. 3a) was used to immunoblot graded concentrations (15–1000 ng) of recombinant human Ro52 (hRo52) or recombinant mouse Ro52 (mRo52). (b) Immunoreactivity by ELISA of serial dilutions of a human anti-Ro serum (——), and a normal human serum (- - - -) against recombinant human Ro52 (■) or mouse Ro52 (□).

the antibody titration required for recognition of mouse Ro52 by human autoantisera indicated ~ 10-fold more efficient reactivity of human autoantisera with human Ro52 compared with mouse Ro52.

Human autoantibodies recognize mouse Ro52 antigen with lower relative avidity than human Ro52

The diminished binding of human autoantisera with mouse Ro52 antigen could be due to a generally lower antibody affinity for the mouse antigen or a reduced number of antigen epitopes recognized with the same affinity as human Ro52. The relative avidity of autoantibodies for human and mouse Ro52 antigen was therefore determined based on the susceptibility of the antigen–antibody complex to dissociation by the chaotropic thiocyanate ion. The concentration of thiocyanate ions required to dissociate 50% of antibody binding was used as an avidity-related index to compare the interaction between autoantibodies and human or mouse Ro52 antigen. Patient autoantibodies eluted from mouse Ro52 at lower concentrations of thiocyanate ion (50% elution at 4 M) compared with

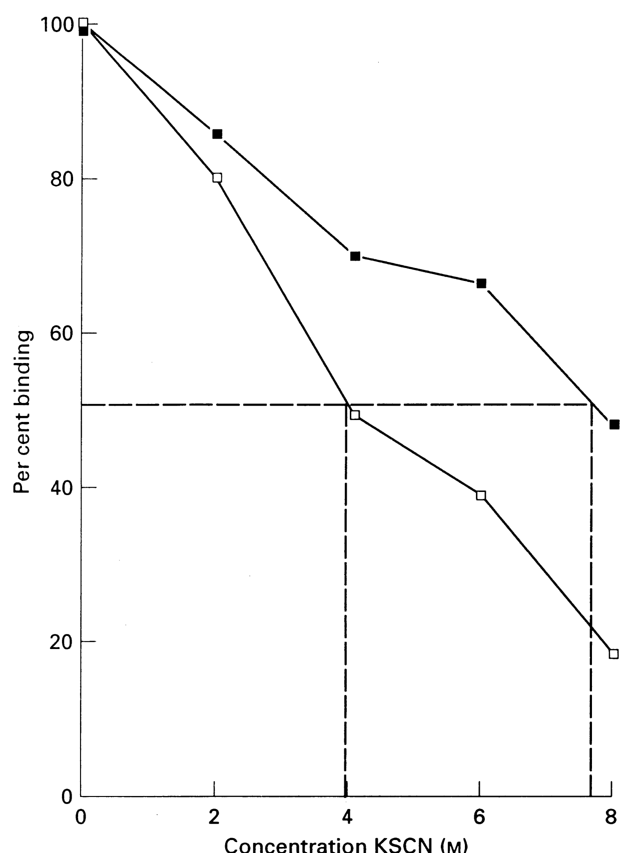

Fig. 5. Differential avidity of human autoantibodies for human *versus* mouse Ro52 autoantigen. Potassium thiocyanate was used to elute human anti-Ro serum bound to human Ro52 (■) or mouse Ro52 (□). Remaining bound antibody was detected with alkaline phosphatase-conjugated anti-human IgG (γ-chain). The concentration of the chaotropic thiocyanate agent required to give 50% elution of bound antibody is shown by the dashed lines.

human Ro52 (50% elution at 7·8 M) (Fig. 5). This finding suggests that although there may be fewer recognized epitopes on mouse Ro52, those antibodies that recognize mouse Ro52 do so with lower relative affinity, presumably due to the primary structural divergence between the human and mouse homologues.

DISCUSSION

Autoantibodies associated with systemic autoimmune diseases generally react with conserved functional domains which tend to be evolutionarily conserved within autoantigens [1]. Moreover, autoantigens targeted in human systemic autoimmune diseases tend to be similar to those recognized in murine models of systemic autoimmunity. One puzzling exception to these generalizations is the apparent lack of cross-reactivity of human autoantibodies with a mouse homologue of the 52-kD Ro antigen. The existence of a mouse Ro52 protein seemed obvious, since MLR/lpr mice develop autoantibodies which react with human Ro52 antigen, consistent with a self antigen-driven mechanism leading to autoimmune antibodies [6]. However, some autoantibodies reacting with denatured human Ro52 antigen are believed to cross-react with native Ro60 antigen, suggesting they may be driven by Ro60 antigen [21]. Here we have shown that the gene encoding a mouse Ro52 antigen homologue does indeed exist and that it is ~ 70% identical to its human counterpart. The data demonstrate that under appropriate conditions human autoantibodies specifically cross-react with recombinant mouse Ro52, but a given level of antibody binding requires higher concentrations of mouse Ro52 antigen than human Ro52 antigen and involves lower titre, lower affinity binding than is observed with human Ro52 antigen. The previous failure of human autoantibodies to detect mouse Ro52 by immunoblot or by indirect immunofluorescence is therefore explicable quantitatively.

The fine specificity of the human autoantibody response to Ro52 has been examined by a number of investigators using synthetic peptides [22,23], fragments and truncated forms of the human Ro52 protein produced as recombinant protein [24–27] or *in vitro* translated protein [28–30] (Fig. 6). However, epitope mapping by different investigators has not given a complete consensus map of the immunodominant regions of the human Ro52 protein, with most groups demonstrating a heterogeneous response dependent on the source and disease condition of the patient. Notwithstanding these results, the data suggest that most sera have strongest reactivity with epitopes in the central third of the human Ro52 protein (aa 136–292). The exact number of epitopes in this region is uncertain, and conformationally dependent epitope(s) requiring this central region and the amino terminus of the Ro52 protein have been identified [27]. In this region there is 65·6% identity between the human and mouse Ro52 proteins. The level of identity between human and mouse Ro52 in other fragments or peptides mapped as autoantibody epitopes ranges from 36% identity (aa 1–11) to 88% identity (aa 107–122 and aa 277–292) [22]. Larger fragments previously identified as autoantibody epitopes ranged in identity from 62% (aa 136–227) to 75% (aa 55–70). Notably the centrally located putative leucine zipper motif reacted with patient autoantibodies in four of the six studies, whereas the amino-terminal region containing the proposed C3H4 zinc finger motif reacted with patient autoantibodies in half of the studies. These proposed functional sites on Ro52 are relatively more conserved between human and mouse Ro52 in comparison with the rest of the molecule, yet the human and mouse Ro52 proteins remain poorly cross-reactive with human autoantibodies. Indeed, all of the Ro52 epitopes proposed from mapping studies diverge significantly from the homologous region in the mouse Ro52. Therefore it seems likely that the limited cross-reactivity of the human and mouse Ro52 antigen is due to the diffuse differences in their primary amino acid sequence which may still disturb recognition of relatively conserved putative functional sites as well as the less conserved parts of this molecule.

The availability of recombinant mouse Ro52 antigen should facilitate studies of systemic autoimmunity in mouse models and help unravel the immunological relationship of the Ro52 and Ro60 autoantigens.

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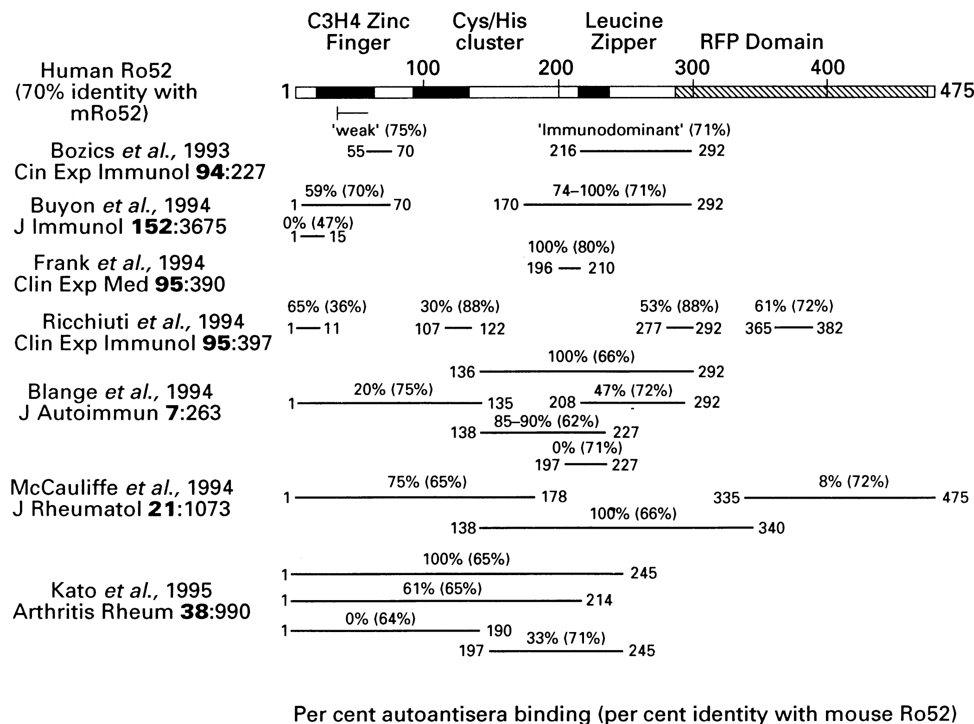

Fig. 6. Autoantibody epitope divergence between human and mouse Ro52 autoantigen. The previously identified epitopes of human Ro52 are shown with the per cent of sera which detect the fragment and in parentheses the per cent identity between human and mouse Ro52.

REFERENCES

1 Tan EM. Antinuclear antibodies: diagnostic markers for autoimmune diseases and probes for cell biology. Adv Immunol 1989; **44**:93–151.

2 Weng Y-M, McNeilage J, Topfer F, McCluskey J, Gordon T. Identification of a human-specific epitope in a conserved region of the La/SS-B autoantigen. J Clin Invest 1993; **92**:1104–8.

3 Harley JB, Rossario MO, Yamagata H, Fox OF, Koren E. Immunologic and structural studies of the lupus/Sjögren's syndrome autoantigen, La/SSB, with a monoclonal antibody. J Clin Invest 1985; **76**: 801–6.

4 Slobbe RL, Pruijn GJM, Damen WGM, Van Der Kemp JWCM, Van Venrooij WJ. The detection and occurence of the 60- and 52-kD Ro (SS-A) antigens and of autoantibodies against these proteins. Clin Exp Immunol 1991; **86**:99–105.

5 Reichlin M. Molecular definition of the Ro(SS-A) particles: a frequent target of autoimmunity in systemic lupus erythematosus and Sjögren's syndrome. Brit J Rheum 1991; **30** (Suppl. 1):58–62.

6 Wahren M, Skarstein K, Blange I, Pettersson I, Jonsson R. MLR/*lpr* mice produce anti-Ro 52 000MW antibodies: detection, analysis of specificity and site of production. Immunol 1994; **83**:9–15.

7 Visvader J, Begley CG, Adams JM. Differential expression of the *LYL*, *SCL* and *E2A* helix-loop-helix genes within the hemopoietic system. Oncogene 1991; **6**:187–94.

8 Itoh K, Itoh Y, Frank MB. Protein heterogeneity in the human Ro/SSA ribonucleoproteins: the 52- and 60-kD Ro/SSA autoantigens are encoded by separate genes. J Clin Invest 1991; **87**:177–86.

9 Maniatis T, Sambrook J, Fritsch EF. Molecular cloning; a laboratory manual. Cold Spring Harbor: Cold Spring Harbor Press, 1989.

10 Buckley MF, Gould JW. Preparation of bacteriophage lambda DNA using the TL-100 ultracentrifuge. Anal Biochem 1988; **175**:281–3.

11 Marck C. 'DNA Strider': a 'C' program for the fast analysis of DNA and protein sequences on the Apple Macintosh family of computers. Nucleic Acids Res 1988; **16**:1829.

12 Pearson WR, Lipman DJ. Improved tools for biological sequence analysis. Proc Natl Acad Sci USA 1988; **85**:2444.

13 Gordon T, Grove B, Loftus JC, O'Toole T, McMillan R, Lindstrom J, Ginsberg MH. Molecular cloning and preliminary characterization of a novel cytoplasmic antigen recognised by myasthenia gravis sera. J Clin Invest 1992; **90**:992–9.

14 Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; **72**:248–5.

15 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bateriophage T4. Nature 1970; **227**:680–5.

16 Gordon T, Mavrangelos C, McCluskey J. Restricted epitope recognition by precipitin-negative anti-La/SS-B-positive sera. Arthritis Rheum 1992; **35**:663–6.

17 Pullen GR, Fitzgerald MG, Hosking CS. Antibody avidity determination by ELISA using thiocyanate elution. J Immunol Methods 1986; **86**:83–87.

18 Chan EKL, Hamel JC, Buyon JP, Tan EM. Molecular definition and sequence motifs of the 52-kD component of human SS-A/Ro autoantigen. J Clin Invest 1991; **87**:68–76.

19 Keech CL, Gordon TP, McCluskey J. Cytoplasmic accumulation of the 52 kDa Ro(SS-A) nuclear autoantigen in transfected cell lines. J Autoimmun 1995;**8**:699–712.

20 Keech CL, McCluskey J, Gordon TP. Transfection and over-expression of the human 60 kDA Ro/SS-A autoantigen in HEp-2 cells. Clin Immunol Immunopathol 1994; **73**:146–51.

21 Itoh Y, Itoh K, Frank MB, Reichlin M. Autoantibodies to the Ro/SSA autoantigen are conformation dependent. II. Antibodies to the denatured form of 52 kD Ro/SSA are a cross reacting subset of antibodies to the native 60 kD Ro/SSA molecule. Autoimmunity 1992; **14**:89–95.

22 Ricchiuti V, Briand JP, Meyer O, Isenberg DA, Pruijn G, Muller S. Epitope mapping with synthetic peptides of 52-kD SSA/Ro protein reveals heterogeneous antibody profiles in human autoimmune sera. Clin Exp Immunol 1994; **95**:397–407.

23 Frank MB, Itoh K, Fujisaku A, Pontarotti P, Mattei M-G, Neas BR. The mapping of the human 52-kD Ro/SSA autoantigen gene to human chromosome 11, and its polymorphisms. Am J Hum Genet 1993; **52**:183–91.

24 Blange I, Ringertz NR, Pettersson I. Identification of antigenic regions of the human 52 kD Ro/SS-A protein recognised by patient sera. J Autoimmun 1994; **7**:263–74.

25 Frank MB, Itoh K, McCubbin V. Epitope mapping of the 52-kD Ro/SSA autoantigen. Clin Exp Immunol 1994; **95**:390–6.

26 McCauliffe DP, Yin H, Wang L, Lucas L. Autoimmune sera react with multiple epitopes on recombinant 52 and 60 kDa Ro(SSA) proteins. J Rheumatol 1994; **21**:1073–80.

27 Kato T, Sasakawa H, Suzuki S, Shirako M, Tashiro F, Nixhioka K, Yamamoto K. Autoepitopes of the 52-kD SS-A/Ro molecule. Arthritis Rheum 1995; **38**:990–8.

28 Bozic B, Pruijn GJM, Rozman B, Van Venrooij WJ. Sera from patients with rheumatic diseases recognize different epitope regions on the 52-kD Ro/SS-A protein. Clin Exp Immunol 1993; **94**: 227–35.

29 Buyon JP, Slade SG, Reville JD, Hamel JC, Chan EKL. Autoantibody responses to the 'native' 52-kDA SS-A/Ro protein in neonatal lupus syndromes, systemic lupus erythematosus, and Sjögren's syndrome. J Immunol 1994; **152**:3675–84.

30 Peek R, Pruijn GJM, Van Venrooij WJ. Epitope specificity determines the ability of anti-Ro52 autoantibodies to precipitate Ro RNPs. J Immunol 1994; **153**:4321–9.