Staikou EV, **Routsias JG**, Makri AA, Terzoglou A, Sakarellos-Daitsiotis M, Sakarellos C, Panayotou G, Moutsopoulos HM, Tzioufas AG. ***¢ əŬəɟ ŮŰəɞɡəɶɖ ŬəəɖəŮ ɚŭɟ Ÿ ŮəəŮəŰəŸ ɛ ŮŰɜɡʃ ɔɟ Ŭɛ ɛ ɚəɞɨ ʃ ɖ-Ů ɚŰɧ´ ɞɡʃ Űɜɡ ŬɡŰɜŬɜŰɞɧɜɞɡ Ro60, kD, ενισχύοντας την αναγνωρισή τους από τα αντι-Ro60 kD αυτοαντισώματα.*** Clin Exp Immunol. 2003 Oct;134(1):143-50.

Η ασβεστιοδικτίνη αποτελεί μοριακή συνοδό των νεοσυντεθέντων πολυπεπτιδίων και πιθανό μέλος του συμπλόκου Ro/La RNP. Στη παρούσα μελέτη εξετάστηκε η πιθανότητα σύνδεσης του μορίου με Β-κυτταρικούς επιτόπους του Ro/La RNP συμπλόκου, καθώς και η ειδική αναγνώριση του συμπλέγματος πεπτιδίου-ασβεστιοδικτίνης από αντι-Ro αυτοντισώματα. Κεκαθαρμένη από ανθρώπινο σπλήνα ασβεστιοδικτίνη βρέθηκε να αλληλεπιδρά ειδικά με βιοτινυλιωμένα πεπτιδικά ανάλογα των επιτόπων του Ro60 175-184 aa(10p) και 216-232 aa(17p), όχι όμως και με αντίστοιχα πεπτιδικά ανάλογα των La/SSB και Sm μορίων, ενώ η αλληλεπίδραση αυτή ευνοήθηκε από το συνδυασμό θέρμανσης, δισθενών κατιόντων και ATP. Ακολούθως τα συμπλέγματα ασβεστιοδικτίνης με τα πεπτίδια 10p και 17p αναγνωρίστηκαν και από τους τριάντα-οκτώ αντι- Ro60 θετικούς ορούς σε ποσοστά 95% και 100%, αντιστοίχως, ενώ μεμονωμένα τα πεπτίδια και η πρωτεΐνη αναγνωρίστηκαν ελάχιστα από τους ίδιους ορούς. Οι είκοσι-τρεις αντι-Ro60 αρνητικοί οροί που δοκιμάστηκαν δεν παρουσίασαν αξιοσημείωτη δραστικότητα. Με αυτά τα δεδομένα παρουσιάζεται για πρώτη φορά μια τέτοιου είδους συσχέτιση ανάμεσα σε ένα συνοδό μόριο και συστατικών ενός ενδοκυττάριου αυτοαντιγόνου, συντελώντας στην καλύτερη κατανόηση των μηχανισμών παραγωγής των αυτοαντισωμάτων.

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Calreticulin binds preferentially with B cell linear epitopes of Ro60 kD autoantigen, enhancing recognition by anti-Ro60 kD autoantibodies

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SUMMARY

Calreticulin is a molecular chaperone to newly synthesized polypeptides. Previous studies suggested that calreticulin is probably a protein member of the Ro/La RNP complex. The aims of this study were (a) to investigate whether linear B cell epitopes of the Ro/La RNP complex are bound to calreticulin and (b) if the complex peptide–calreticulin is recognized specifically by anti-Ro autoantibodies. Calreticulin was isolated from either human or pig spleen using a multi-step purification method and found to interact preferentially with biotinylated peptides derived from the sequence of the Ro60 kD 175–184aa(10p) and 216–232aa(17p). The interaction of the peptide–calreticulin complex was favoured by the combination of heat treatment, divalent cations and ATP. La/SSB epitopes did not react with calreticulin. Peptides corresponding to La/SSB epitopes as well as the common epitope of Sm did not interact with calreticulin. Thirty-eight anti-Ro60 KD positive and 23 anti-Ro60 kD negative sera of patients with systemic lupus erythematosus (SLE) and primary Sjögren's syndrome (pSS) were tested. All anti-Ro60 kD positive sera bound the complex calreticulin-17p, while 95% of the same sera had activity against the complex calreticulin −10p. Tested individually, calreticulin, pep10p and pep17p presented very low reactivity (8%, 11% and 29%, respectively) against anti-Ro60 kD positive sera. Anti-Ro60 KD negative sera did not exhibit significant reactivity either with calreticulin, 10ρ and 17ρ or with the complexes calreticulin −10p and calreticulin-17p (<5%). These results suggest that calreticulin can induce conformation-dependent recognition of the Ro60 kD epitopes, leading eventually to their recognition by autoantibodies. This is the first time that such a relationship is shown between a chaperone protein and fragments of an intracellular autoantigen. This work also provides insights into the understanding of mechanisms for autoantibody production. Furthermore, this association can be proved useful for the development of new sensitive assays for autoantibody detection.

Keywords anti-Ro/ SSA B cell epitopes calreticulin Sjögren's syndrome systemic lupus erythematosus

INTRODUCTION

Antibodies targeting the Ro60 kDa protein are a prominent feature of the autoantibody profile in patients with autoimmune diseases such as primary Sjögren's syndrome (pSS) and systemic lupus erythematosus (SLE). The Ro60 kDa protein forms part of the RoRNP complex. The latter is composed of one small cytoplasmic RNA associated non-covalently with at least two immunologically distinct proteins, the Ro60 kDa protein and the 48 kDa phosphoprotein La/SSB. Additional proteins such as the Ro52 kDa protein and calreticulin are thought to participate in the complex, but this association has not been definitely proved [1–6].

Over the last decade, several investigators focused on identifying the antigenic determinants of the Ro/SSA autoantigen. In 1988, Lieu *et al*. isolated a 60 kDa protein from the Wil 2 lymphoblastoid cell line and prepared a synthetic peptide corresponding to the aminoterminal sequence of this protein. This peptide was found to react specifically with autoimmune sera containing anti-Ro/SSA antibodies [7]. Based on this finding, the authors proposed that this protein was the 60 kDRo/SSA autoantigen and that its major antigenic epitope resided within the N-terminal region of the protein. In 1989, two independent groups isolated the cDNA clones of the Ro60 kDa autoantigen [8,9], but the

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N-terminal sequence of the corresponding recombinant protein did not match the amino acid sequence of the 60 kDa protein isolated previously by Lieu. In 1990 McCauliffe *et al*. isolated the cDNA of the '60 kDRo/SSA' autoantigen published by Lieu and concluded that it encoded a human homologue of calreticulin, a calcium-binding protein of the endoplasmic reticulum [10]. In agreement with McCauliffe, Rocheach *et al*. [6] pointed out that Ro60 kDa and calreticulin were two distinct proteins. Antibodies against the N-terminal domain of calreticulin were detected in sera of patients with systemic lupus erythematosus (SLE), Sjögren's syndrome, rheumatoid arthritis and mixed connective tissue disease [11]. Recently, autoantibodies against calreticulin have been described also in complete congenital heart block [12], parasitic diseases [13], coeliac disease [14], primary biliary cirrhosis [15] and halothane induced hepatitis [16].

Calreticulin is an ER-resident protein of approximately 46 kDa molecular weight with multiple functions, including the control of cellular adhesiveness and steroid-sensitive gene expression, regulation of calcium homeostasis and molecular chaperoning (reviewed in [17]).

Based on these observations, we evaluated the interaction of calreticulin with the linear B cell epitopes of the autoantigen Ro60 kDa and the capacity of the complex to be recognized by human anti-Ro60 kDa autoantibodies. We demonstrated that linear epitopes of Ro60 kDa bind specifically to native calreticulin and that the vast majority of anti-Ro60 kD positive sera react with the calreticulin–Ro60 kD epitope complexes.

MATERIALS AND METHODS

Patients and sera

Thirty-eight anti-Ro/SSA positive, anti-La/SSB negative sera of patients with systemic autoimmune diseases (20 SLE, 18 pSS) were tested. All patients fulfilled the revised criteria for the classification of SLE [18] and pSS [19]. Twenty-three sera negative for autoantibodies from patients with systemic autoimmune diseases were used as disease controls, and 30 sera of healthy donors were used as normal controls. The evaluation of the serum samples for anti-Ro/SSA and anti-La/SSB antibodies was performed with counter-immunoelectrophoresis and immunoblot after SDS PAGE electrophoresis of HeLa cells extract, as described previously [11]. All sera had been taken for diagnostic purposes. Ethical approval for the study was obtained.

Peptide synthesis

Biotinylated peptide analogues corresponding to the linear epitopes of Ro60 kD, spanning the sequences 216–232aa (KALSVETEKLLKYLEAV), and 175–184aa (NGWSHKDLLR) [20,21], La/SSB: 147–154aa (HKAFKGSI), 291–302aa (NGNLQLRNKEVT), 301–318aa (VTWEVLEGEVEKEALKKI) and 349–364aa (GSGKGKVQFQGKKTKF) [22–24], as well as the common epitope of the Sm 192–198aa (PPGMRPP) [25] were synthesized by the stepwise solid phase procedure (SPPS) on a phenylacetamidomethyl (PAM) resin according to standard methods. A control dipeptide, biotin-Leu-Leu-OH was also prepared and tested. The identity and purity of the peptides was confirmed by mass spectroscopy (MS) and two-dimensional [^{1}H]-nuclear magnetic resonance (NMR). Previous studies have shown that these sequences represent major linear B cell epitopes of the autoantigens Ro60 kD [21,26], La/SSB [22,24] and Sm [27].

Purification of calreticulin

The amino acid sequences of human and pig calreticulin are highly similar, because they possess 93% homology. One hundred g of either human or pig spleen were homogenized in 100 ml of phosphate buffered saline (PBS) in the presence of 0·67 mM phenylmethylsulphonylfluoride (PMSF) as protease inhibitor. The supernatant was subjected to differential ammonium sulphate precipitation and the 65–90% fraction of saturation was obtained. This fraction was dialysed overnight against a 10-mM Tris-HCl buffer (pH = 8·0) containing 0·6 M NaCl. $ZnCl_2$ (0·5 mM) was added and this fraction applied to hydrophobic interaction chromatography using a phenyl sepharose column (Sigma Chemical Co., St Louis, USA). The column was pre-equilibrated with a Tris-HCl buffer (pH = 8·0) containing 0·5 mM $ZnCl_2$ and 0·6 M NaCl. The elution of the impure protein was performed with 3 mM EDTA in 0·6 M NaCl/Tris-HCl buffer [28]. The EDTA eluate was desalted by overnight dialysis in 20 mM Tris-HCl buffer, 2 mM EDTA (pH = 8·0), subjected to anion exchange chromatography in fast pressure liquid chromatography (FPLC) using a mono-Q column (Akta-Purifier, Pharmacia Biotech, Uppsala, Sweden) and eluted with 0–1·0 M NaCl gradient in Tris-HCl buffer (pH = 8·0). The identity and purity of calreticulin was confirmed using SDS-PAGE, enzymatic digestion and nanospray mass spectrometry as well as immunoblot using a monoclonal antibody to human calreticulin (Stressgen, Victoria, BC, Canada). Before its use the calreticulin preparation was desalted extensively in Tris 0·05 M, NaCl 0·15 M, pH = 7·5.

Interaction of calreticulin with biotinylated peptides

Costar microtitre plates were coated with 100 μl of calreticulin (5 μg/ml) for 2 h at 25°C and blocked with 200 μl of bovine serum albumin (BSA) 2% for 1 h. Following two washes, peptides were added at 15 μg/ml concentration in a total volume 100 μl/well and incubated at 40°C or 4°C for 2 h. After three washes with Tris 0·05 M, NaCl 0·15 M pH:7·5, streptavidine conjugated to horseradish peroxidase was added in a dilution of 1 : 2500 in 2% BSA/Tris per well for an 1 h. Colour development was read at 405 nm after the addition of 100 μl ABTS substrate. Coating of plates with albumin, followed with incubation with biotinylated peptides, without calreticulin did not produce any reaction.

Effect of different ions on the calreticulin–peptide interaction

ELISA experiments were carried out as described previously. The incubation buffer used for both calreticulin and peptides (Tris 0·05 mM, NaCl 0·15 mM, pH:7·5) contained different concentrations of the divalent cations Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+} (ranging from 0·1 mM to 10 mM) or 1 mM EDTA plus 3 mM ATP, in all possible associations.

Detection of antibodies against individual linear peptides of Ro, La or Sm protein or calreticulin

Costar microtitre plates were coated with 100 μl of peptide solution (15 μg/ml) or calreticulin (5 μg/ml) and kept at 25°C for 2 h. After blocking the remaining binding sites with 2% BSA in Tris 0·05 M, NaCl 0·15 M, pH:7·5 at room temperature for 1 h, the plates were incubated with human sera (1 : 100 in blocking buffer) at 4°C overnight. Subsequently, alkaline phosphatase conjugated antihuman IgG-Fc (diluted 1 : 1500) was added (100 μl) for 1 h and the enzyme reaction was developed using p-nitrophenyl as a substrate. Optical density (OD) was determined at 405 nm.

Detection of antibodies against complexes of biotinylated peptides and calreticulin

Costar microtitre plates were coated with 100 μl of calreticulin at a final concentration of 5 μg/ml and kept at room temperature for 2 h. Non-specific binding was blocked subsequently with BSA 2% (200 μl) for 1 h. The plates were washed and incubated with biotinylated peptides (15 μg/ml) for 1·5 h at 40°C and then at room temperature for 30 min. The next steps were performed as described previously. The incubation buffer used for both calreticulin and synthetic peptides was Tris 0·05 M, NaCl 0·15 M, ATP 3 mM, 1 mM Mg^{2+}, pH: 7·5. In all experiments positive reactions were considered to be those that gave OD greater than the mean OD of the negative control plus three standard deviations (s.d.).

RESULTS

Development of a new purification procedure of calreticulin

A new procedure for calreticulin purification was developed by the combination of ammonium sulphate precipitation, hydrophobic interaction chromatography and FPLC refinement. This method was based on the conformational changes induced in calreticulin by Zn^{2+} binding [28]. More specifically, Zn^{2+} bound four of the histidine residues located in the N-terminal domain of calreticulin, enabling calreticulin to interact with the hydrophobic matrix of the phenyl sepharose column. Once Zn^{2+} is removed by the addition of 3 mM EDTA, the conformation of calreticulin is altered and the protein no longer interacts with the hydrophobic matrix and is eluted. The calreticulin preparation was subjected to further purification using MonoQ-FPLC and eluted with a 0–1·0 M NaCl gradient (Fig. 1). Peak 1 (eluted at 0·24 M) contained a 66-kDa band, which was later identified by enzymatic digestion–nanospray mass spectrometry as albumin (data not shown). Peak 2 was eluted at 0·42 M NaCl and contained two bands, the 66 kDa (albumin) and one 60 kDa band. Peak 3 (eluted at 0·65 M NaCl) contained only one band at 60 kDa. The 60 kDa band was recognized specifically by a monoclonal anticalreticulin antibody (Fig. 1, lane 4). More precise characterization was performed by nanospray mass spectrometry. After visualization by Coomassie blue, the 60 kDa band was excised and subjected to in-gel digestion with trypsin (modified sequence grade trypsin, Promega). Tryptic fragments were extracted from gel pieces and a final peptide preparation in 2% acetonitrile/0·1%. Formic acid was applied to a C-18 HPLC column (75 µm × 15 cm) on an UltiMate nano-HPLC system (LC-Packings, the Netherlands). Peptides were separated with an acetonitrile gradient at 150 nl/min and analysed on an ion trap mass-spectrometer (LCQ-Deca, Thermo-Finnigan, USA) with a nanospray source. MS and MS/MS data were used to search the SwissProt database with the TurboSEQUEST search engine. The best match was with human calreticulin (P27797) (Fig. 2).

Calreticulin interacts specifically with the biotinylated B cell epitope analogues of Ro/SSA autoantigen

In order to evaluate the binding of calreticulin to the B cell epitopes of the 60kDRo/SSA, La/SSB and Sm autoantigens a solid phase immunoassay (ELISA) was performed, as described in Materials and methods. These experiments revealed that the two linear determinants of the Ro60 kDa autoantigen N^{175}GWSHKDLLR^{184} and K^{216}ALSVETEKLLKYLEAV^{232} bind to purified calreticulin (Fig. 3). The differences in binding between Ro and control epitopes ranged from threefold (for the epitope 301–318 of La/SSB) to 20-fold (for the epitopePPGMRPP of Sm). Because both Ro peptides contain a common dileucine moiety, we tested a biotinylated control peptide (biotin-Leu-Leu-OH) for possible interaction with calreticulin following the same procedure. This control peptide did not react with calreticulin (data not shown).

The effect of temperature and ions on the peptide binding to calreticulin

Costar microtitre plates were coated with calreticulin as described in Materials and methods. Incubation of calreticulin with the biotinylated epitope analogues Ro/SSA 175–184, Ro/SSA 216–232, La/SSB 147–154 and La/SSB 301–318 (the two later peptides were used as controls) took place at either 4°C or 40°C, keeping unchanged the rest of the coating conditions. It was observed that heating at 40°C favoured the calreticulin–peptide interaction in the presence of divalent cations, most probably reflecting the increased exchange between the naturally bound and exogenously added peptides into the calreticulin [29,30] (Fig. 4).

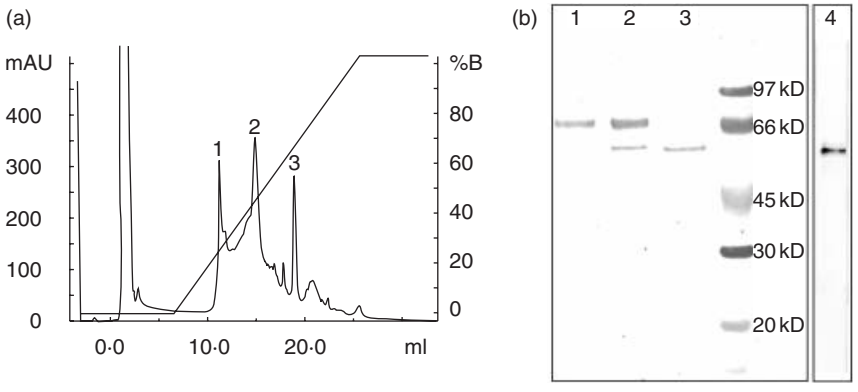


Fig. 1. Purification of calreticulinin with FPLC and analysis on SDS-PAGE and immunoblot. (a) Elution of FPLC-monoQ column; peak 1 : 0·24 M NaCl, peak 2 : 0,42 M NaCl and peak 3 : 0,65 M NaCl. (b) Peaks 1, 2 and 3 were resolved on SDS-PAGE; peak 2 contained albumin and calreticulin (lane 2) while peak 3 had only calreticulin (lane 3). Identification of calreticulin in peak 3 was performed by immunoblot using a monoclonal anticalreticulin antibody (lane 4).

(a)

Seq	#	b	y	#
E	1	130·1	–	12
Q	2	258·2	1282·3	11
F	3	405·4	1154·2	10
L	4	518·6	1007·0	9
D	5	633·7	893·9	8
G	6	690·7	778·8	7
D	7	805·8	721·7	6
G	8	862·9	606·6	5
W	9	1049·1	549·6	4
T	10	1150·2	363·4	3
S	11	1237·3	262·3	2
R	12	–	175·2	1

(b)

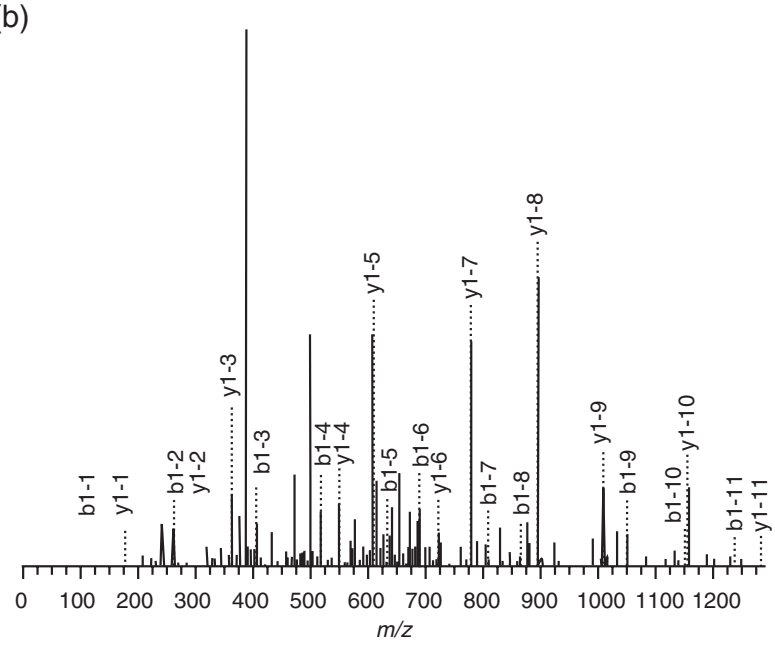
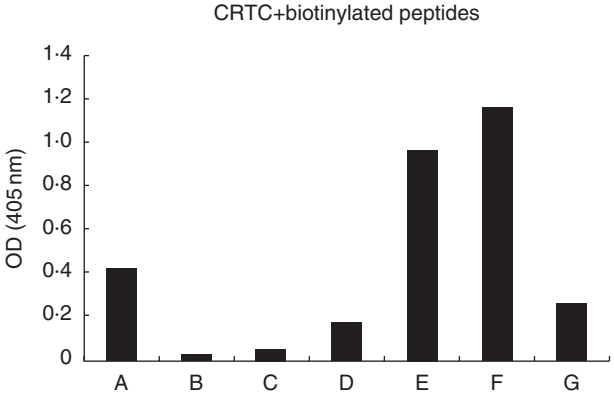


Fig. 2. An example of calreticulin peptide identification by mass spectrometry. Numbers in bold (a) show b′ and y′ fragments present in the MS/MS spectrum (b).



A: VTWEVLEGEVEKEALKKI (La/SSB: 301–318)
B: PPGMRPP (Sm: 192–198)
C: GSGKGKVQFQGKKTKF (La/SSB: 349–364)
D: ANNGNLQLRNKEVTWEVLEG (La/SSB: 289–308)
E: KALSVETEKLLKYLEAV (Ro/SSA: 216–232)
F: NGWSHKDLLR (Ro/SSA: 175–184)
G: TLHKAFKGSIFVVFDSIESA (La/SSB: 145–164)

Fig. 3. Interaction of calreticulin with biotinylated peptides corresponding to B cell epitopes of Ro60 kD, La/SSB and Sm. Purified calreticulin interacted preferentially with the epitopes, spanning the regions 175–184aa and 216–232aa of the autoantigen Ro60 kD (lanes E and F), but not with the other peptides.

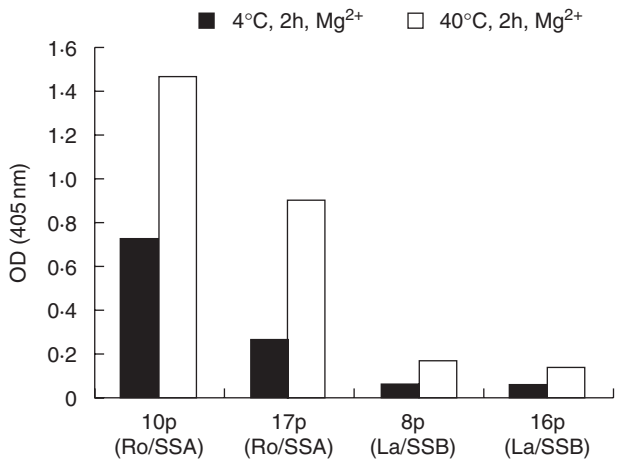


Fig. 4. The effect of temperature on peptide binding to calreticulin. Elevation of the temperature from 4°C (black bars) to 40°C (grey bars), in the presence of Mg^{2+}, resulted in increased binding of peptides 10p (175–184aa) and 17p (216–232aa) of Ro60 kD onto calreticulin by two- and fivefold, respectively.

In order to investigate the influence of divalent cations in peptide binding [31,32] a series of experiments was performed, utilizing varying concentrations (10 mM, 5 mM, 1 mM, 0·5 mM, 0·1 mM) of four divalent cations (Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}) as well as 1 mM EDTA. Divalent cations or EDTA were added in both coating buffer and the biotinylated peptide incubation buffer. It was found that for both Ro/SSA epitopes (175–184aa and 216–232aa) the optimum concentration of Mg^{2+} and Ca^{2+}, corresponding to the highest binding, was estimated at 1 mM, whereas the optimum concentration of Zn^{2+} was at 1 mM for the Ro epitope 216–232 (Fig. 5a) and at 0·1 mM for the Ro epitope 175–184 (Fig. 5b). These results also show that in the presence of divalent cations at optimum concentration, calreticulin exhibited

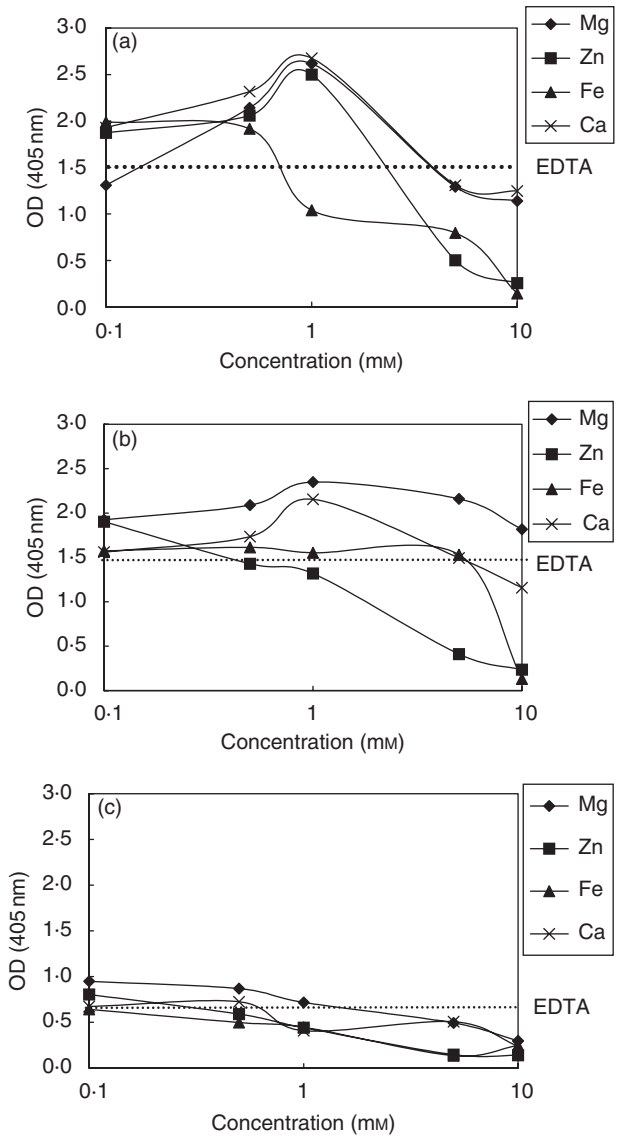


Fig. 5. Analysis of the effect of divalent cations on peptide binding to calreticulin, using different concentrations of the ions Mg^{2+}, Zn^{2+}, Fe^{2+} and Ca^{2+}, ranging from 0·1 mM to 10 mM, as well as 1 mM EDTA. (a) pep10p (Ro epitope175–184aa)–calreticulin. (b) pep17p (Ro epitope 216–232aa)–calreticulin interactions. (c) La epitope 349–364aa was used as control.

a stronger binding capacity compared to that observed in the presence of 1 mM EDTA (approximately 50% increase in OD measured). On the other hand, the control epitope 349–364 La/SSB exhibited diminished interaction with calreticulin in the presence of the same divalent cations at 1 mM concentration (approximately 50% decrease in OD measured). (Fig. 5c). The presence of iron negatively affected the binding of the peptides to calreticulin. Among the three cations (Mg^{2+}, Ca^{2+} and Zn^{2+}) tested, the strongest interaction was detected at the concentration of 1 mM Mg^{2+}.

Recognition of the calreticulin/Ro60 kD epitopes complex by anti Ro positive autoimmune sera

Thirty-eight anti-Ro60 kDa positive and 23 anti-Ro60 kDa negative sera of patients with systemic autoimmune rheumatic disorders were examined in solid phase immunoassays, in order to detect the presence of circulating autoantibodies recognizing the calreticulin/Ro60 kD complex. Calreticulin–peptide interaction took place in the presence of divalent cations (1 mM Mg^{2+}) and 3 mM ATP at 40°C, as described above. All Ro60 kDa positive sera bound on the complex CRT-17p, while 95% of the same sera specifically recognized the complex CRT-10p. Tested individually, calreticulin, pep10p and pep17p presented very low reactivity (8%, 11% and 29% of the anti-Ro60 kDa positive sera, respectively). (Fig. 6a). Interestingly, inhibition experiments using as inhibitors soluble 10p and 17p did not affect the binding of antibodies to calreticulin-peptide complex, indicating that the antibodies are bound to a conformation depended moiety within the complex. In homologous inhibition experiments using the calreticulin–peptide complex as inhibitor, the binding of antibodies onto the immobilized complex was only partially (55%) blocked.

Anti-Ro60 kDa negative sera (Fig. 6b) as well as normal controls (Fig. 6c) did not exhibit significant reactivity against calreticulin, 10p and 17p or with the complexes calreticulin-10p and calreticulin-17p (<5%).

DISCUSSION

Human autoimmune sera often contain autoantibodies to Ro/SSA and La/SSB as well as to calreticulin. The later is considered to be a putative member of the Ro/La RNP complex, although convincing evidence for this does not yet exist [4–6]. A large body of recently reported experimental data regarding the biological and immunological properties of calreticulin has refocused the interest on its association with the Ro/La RNP complex and on the autoantibodies that are produced against it in systemic autoimmune rheumatic diseases.

In summary, calreticulin has been shown to: (i) serve as a molecular chaperone, binding reversibly to hydrophobic surfaces of polypeptide segments of unfolded (glyco)proteins, assisting them in adopting a native conformation [33]; (ii) it has the ability to bind antigenic peptides, transporting them to the professional antigen presenting cells in lymph nodes. For this function, calreticulin utilizes the CD91 molecule, a specific receptor for heat-shock proteins [30,34]. Subsequently, calreticulin escorts the peptides into either exogenous or endogenous antigen processing pathways, delivering them to MHC-II and MHC-I molecules, respectively; (iii) calreticulin is co-localized with the Ro/SSA autoantigen in apoptotic blebs [35], supporting the theory of their close association.

Prompted by these diverse functions of calreticulin, we sought to study first of all whether chemically synthesized epitope peptide analogues of the Ro60 kDa autoantigen interact with calreticulin; and secondly, whether calreticulin can enhance the antigenicity and the conformation depended recognition of these epitopes, hence being involved in several important immunological processes of the Ro/La RNP complex. Native calreticulin was highly purified from either human or pig spleen, using a procedure which is a combination of different chromatographic methods. Previous purification approaches have shown that calreticulin can be eluted from a MonoQ column at 0·42 M NaCl [36]. In our study, using a gradient of 0–1·0 M NaCl, calreticulin was detected in both 0·42 and 0·65 M NaCl. However, the single peak at 0·42 M NaCl was found to contain both calreticulin and albumin, while the peak at 0·65 M contained pure calreticulin. The

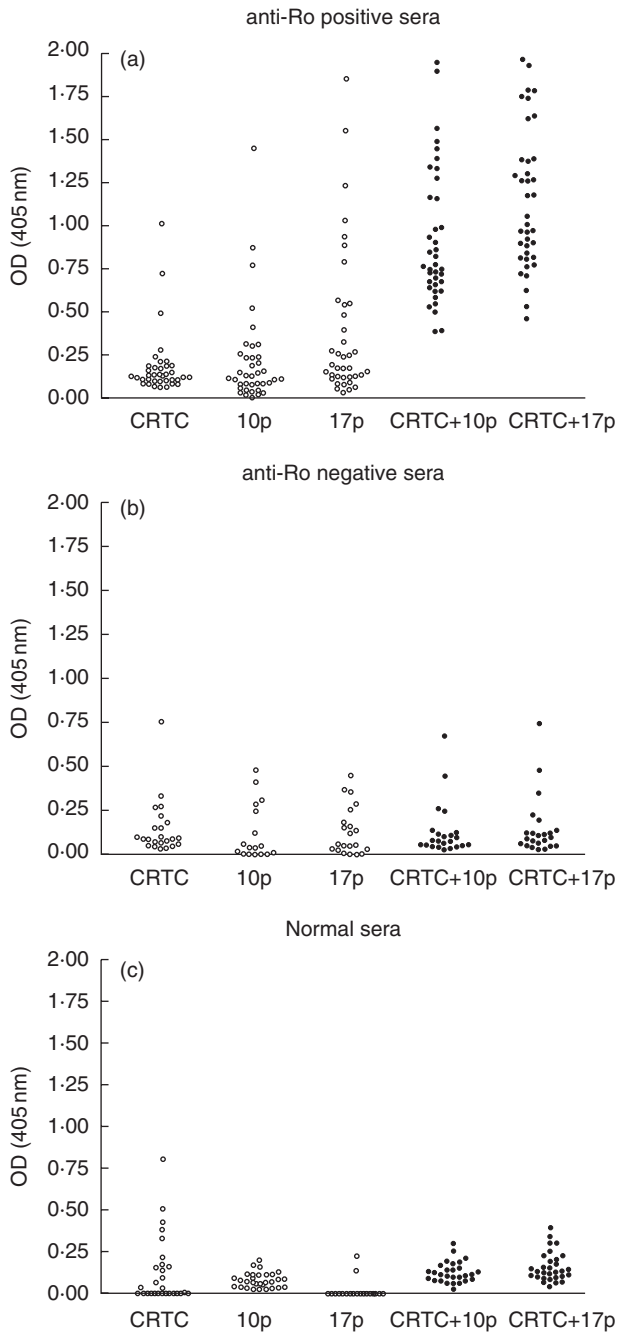


Fig. 6. Reaction of the complexes biotinylated peptides–calreticulin with anti-Ro60 kDa positive and anti-Ro60 kDa negative sera from patients with autoimmune diseases. (a) All anti-Ro60 kDa positive sera bound on the complex calreticulin–epitope 216–232 (17p), while 95% of the same sera had activity against the complex calreticulin–epitope 175–184 (10p).Tested individually, calreticulin, 10p and 17p had very low reactivity (8%, 11% and 29% of the anti-Ro60 kDa (+) sera, respectively). (b,c) Both anti-Ro60 kDa negative and normal sera did not exhibit significant reactivity against either calreticulin, 10p and 17p, or against the complexes calreticulin–10p and calreticulin–17p (<5%). CRTC: calreticulin, 10p: Ro epitope175–184aa, 17p: Ro epitope 216–232aa.

lower salt concentration required for the elution of calreticulin in peak 2 can probably be attributed to an interaction between calreticulin and albumin which weakens the binding onto the anion exchange matrix.

Among the seven synthetic peptides tested, only the two epitopes of Ro60 kDa autoantigen, spanning the sequences 175–184aa and 216–232aa, exhibited a substantial binding to calreticulin. The four peptides, derived from the sequence of La/SSB as well as the Sm analogue, failed to interact with purified calreticulin. Subsequently, the factors affecting the association of calreticulin with the two Ro antigenic peptides were determined. According to the literature, the conditions that may potentially influence peptide binding onto calreticulin are the presence of divalent cations and ATP as well as the temperature [29–32].

Different investigators have demonstrated clearly the peptide binding ability of native calreticulin [33,37]. Furthermore, it has been demonstrated that exposure at high temperatures permits the exchange of naturally bound peptides to calreticulin with those added exogenously [29,30]. Therefore, it is highly likely that, despite the high performance purification procedure followed in our study, the purified calreticulin might carry endogenous peptides that should be displaced by Ro60 kD linear epitopes administered exogenously. Our results indicated that heating at 40°C favours the calreticulin–Ro60 kD peptide interaction. A similar effect, through a different mechanism, may also be produced in the presence of ATP. ATP binding to calreticulin increases its hydrophobicity, resulting in significant conformational alterations of the molecule [32]. Finally, the interaction of peptides with calreticulin is strongly dependent on the presence of divalent cations [31,32]. In fact, because calreticulin is rich in negatively charged residues, the divalent cations appear to play a regulatory role on its activity as chaperone, most probably by neutralizing the negative charges, thereby inducing specific conformational changes. Taking into account these observations, we tested four divalent cations (Ca^{2+}, Mg^{2+}, Zn^{2+} and Fe^{2+}) in order to determine the optimum concentration required for the strongest binding of the Ro60 kD linear epitopes to calreticulin. The data presented herein also raise the possibility that the specific interaction of calreticulin with the peptide analogues of the B cell epitopes of the Ro60 kDa autoantigen could be attributed to their specific physicochemical properties. To address this question, the charge and hydrophobicity of all peptides tested were defined and matched with those of calreticulin. A summary of the results is shown in Table 1 [38]. Despite its highly negative charge [-59 at

Table 1. The PI, charges and aliphatic index [38] of calreticulin and the epitopes of Ro60 kD, La/SSB and Sm

Molecule	pI	Charge at pH = 7	Aliphatic index
Calreticulin	4·29	–59	52
La/SSB:301–318	4·65	–2	119
La/SSB:349–364	10·60	+5	18
La/SSB:291–302	8·75	+1	89
La/SSB:147–154	10·00	+2	61
Sm:192–198	9·47	+1	0
Ro/SSA:216–232	6·23	0	138
Ro/SSA:175–184	8·75	+1	78

pH = 7 (pI = 4·29)], calreticulin does not interact preferentially with positively charged peptides [as, for example, the 349–364aa peptide derived from La/SSB sequence with a charge + 5 at pH = 7 (pI = 10·6)]. Thus, it seems that the binding of peptides onto calreticulin is not dependent upon ionic forces but is achieved most probably via hydrophobic interactions. In agreement with this observation are previously published reports that describe the preference of calreticulin to bind with sequences bearing hydrophobic aminoacid residues [39].

The combination of the presence of divalent cations and simultaneous heat treatment not only increased the interaction of calreticulin with Ro60 kDa epitopes, but also the antigenicity of the complex which exhibited a much more stronger antiRo60 kDa reactivity, compared to that observed when calreticulin and peptides were tested individually. In fact, all 38 anti-Ro60 kDa positive sera of patients with autoimmune rheumatic diseases recognized the complex calreticulin–17p, while 95% of the same sera also bound the calreticulin–10p complex. On the other hand, antiRo60 kDa negative sera did not react with either calreticulin, 10p and 17p or with the complexes calreticulin–10p and calreticulin–17p (< 5%). These results are consistent with our previous report, which showed a relatively limited recognition of the two epitope analogues of the Ro60 kDa autoantigen (174–185aa and 216–232aa) by Ro60 kD positive sera [21] (ranging from 33% to 55%). The conformation-dependent epitope recognition by anti-Ro60 kDa positive sera is widely accepted [40,41]. The enhanced recognition of the Ro/60 kD linear epitopes by autoantibodies when they are bound to calreticulin might be due to the ability of calreticulin to act as a molecular chaperone converting the extended conformation of the peptide to a more compact, partially folded structure. This would provide new conformational epitopes capable of being recognized by human anti-Ro60 kD autoantibodies. In this regard, the results presented in this report are promising for the development of new sensitive assays for autoantibody detection, increasing the sensitivity and specificity of Ro60 kDa linear epitopes for autoantibody detection.

As mentioned previously, calreticulin, when completed with peptides, can prime specific $CD8^+$ CTL responses against the peptides. From this point of view, it is possible that calreticulin may play a more active role for the generation of autoimmune response against Ro60 kDa. Thus, calreticulin can be released into the extracellular space, together with Ro fragments after apoptosis [35], necrosis [42] or cell lysis by cytotoxic T-cells [43]. Under certain physicochemical conditions favoured by the microenviromental millieau, calreticulin could bind Ro peptides producing conformational changes and thereby increasing their antigenicity. The complex is then transported to professional APCs and the peptides are presented to autoreactive T cells. If this hypothesis is correct then calreticulin or other chaperones could be used as vehicles to deliver peptides in the immune system, modulating specifically the autoimmune response.

In summary, our findings indicate that calreticulin can be complexed with Ro60 kD epitopes, inducing conformation-dependent recognition by autoantibodies from autoimmune human sera. Such a relationship between a chaperone protein and fragments of an intracellular autoantigen is shown for the first time and provides potential insights into the understanding of mechanisms for autoantibody production. Furthermore, this association may prove useful for the development of new sensitive assays for autoantibody detection.

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