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Calreticulin, a Potential Cell Surface Receptor Involved in Cell Penetration of Anti-DNA Antibodies

Nabila Seddiki,* Farida Nato,* Pierre Lafaye,† Zahir Amoura, † Jean Charles Piette, † and Jean Claude Maziét

A 50-kDa protein was purified as a potential receptor, using an affinity matrix containing biotinylated F14.6 or H9.3 anti-DNA mAbs derived from autoimmune (New Zealand Black × New Zealand White)F1 mouse and membrane extracts from cells. This protein was identified as calreticulin (CRT) by microsequencing. Confocal microscopy and FACS analysis showed that CRT was present on the surface of various cells. CRT protein was recognized by a panel of anti-DNA mAbs in ELISA. The binding of F14.6 to lymphocytes and Chinese hamster ovary cells was inhibited by soluble CRT or SPA-600. Thus, the anti-DNA mAbs used in this study bound to CRT, suggesting that CRT may mediate their penetration into the cells and play an important role in lupus pathogenesis. The Journal of Immunology, 2001, 166: 6423–6429.

Systéme lupus erythématosus (SLE) is an autoimmune disease typified by the presence of Abs directed against nuclear Ags (1). These lupus antinuclear Abs, include anti-dsDNA Abs, which are considered to be the hallmark of SLE and are thought to be pathogenic. The pathogenicity of lupus anti-dsDNA Abs is complex and involves several mechanisms such as the binding of extracellular Ags directly or via immune complex formation and/or Ab penetration into living cells (2). Alarcon-Segovia et al. (3, 4) first suggested that SLE autoantibodies can penetrate the membranes of living cells, react with intracellular targets in the nucleus, and induce functional abnormalities or even cell death.

The mechanism of anti-dsDNA Ab penetration into cells is unclear but seems to involve binding to cell surface Ags (2). Heparan sulfate (5–7), collagen type IV (8), fibronectin (9), and myosin I (10) have been proposed as cell surface Ags for anti-dsDNA Ab binding. However, it is unclear whether these molecules are able to mediate the penetration of autoantibodies and to drive them to the nucleus.

Autoantibodies have been reported to interact with cell surface Ags if the Ag is of intracellular origin. This Ag may be accessible after translocation to the cell membrane (11). Binding to intracellular Ags may not be a primary event in SLE, and activation, inflammation, apoptosis, or other cellular processes seem to be required. This may result in the translocation of Ags from the nucleus to the cell surface, and these Ags may then become accessible for interaction with circulating Abs (12, 13). Calreticulin (CRT) is one such Ag capable of translocation to the cell surface. This protein has been found to be intimately and transiently associated with the Ro/SSA and La/SSB Ags in the nucleus (14–16). However, CRT may be expressed at the cell surface without stimulation (17, 18). Zhu et al. (19) immunocaptured a surface complex containing αβ1 integrin, two molecular forms of CRT (ecto- and endo-CRT), and carboxyl-terminal endoplasmic reticulum retention signal sequence (KDEL) docking protein from B16 mouse melanoma cells (19, 20). CRT has also been detected in the cytoplasm and nucleus (21–23).

In previous studies, it has been shown that some anti-DNA mAbs derived from autoimmune (New Zealand (NZ) Black × NZ White)F1 mice penetrate and accumulate in the nuclei of a variety of cultured cells (24, 25). These results suggested that the penetration of these anti-DNA mAbs into the cells is mediated by binding to cell surface structures (24, 25).

In this study, the aim was to identify the potential receptor(s) and cofactor(s) involved in the entry of some of those anti-DNA mAbs into cells. By using an affinity matrix containing biotinylated anti-DNA F14.6 or H9.3 mAbs and membrane cell extracts, we purified a protein of 50 kDa, which was identified as CRT.

Materials and Methods

Abs and cells

Spleen cells from a 9-mo-old nonimmunized (NZ Black × NZ White)F1 mouse were purified using a 9-mo-old nonimmunized (NZ Black × NZ White)F1 mouse were fused with P3 × 63Ag8 myeloma cells as previously described (24). IgG mAbs isotypes were determined using mouse alkaline-phosphatase conjugated anti-IgG1, IgG2a, IgG2b, and IgG3 Abs. Anti-DNA Ab-positive hybridomas were cloned and expanded. IgG2a mAb: F14.6, F4.1, J20.8, and H9.3 were purified using protein A/G-agarose (Boehringer Mannheim GmbH, Mannheim, Germany). Purified Abs were biotinylated according to the manufacturer’s (IBF Biotechnologies, Villeneuve-la-Garenne, France) instructions. BAS is a polyclonal human anti-DNA Ab purified on a dsDNA-cellulose column. This Ab was obtained from Z. Amoura (Hôpital Pitié-Salpêtrière, Paris, France).

UPC mAb, the IgG2a isotype control, was obtained from Sigma (St. Louis, MO). Anti-CRT Abs: C-17, SPA-600, and SPA-601 were obtained from ABT (StressGen Biotechnologies, Le Perray-en-Yvelines, France). Anti-cJun mAb (26) IgG1 isotype was produced in the Laboratoire d’Ingénierie des Anticorps (Institut Pasteur, Paris, France). Nucleosomes were prepared as described by Lutter (27). 6E5 mAb (anti-nucleosome Ab) was obtained from Z. Amoura.

Human CEM and Jurkat T cell lines, human K562 erythrocytes, and human Raji and Daudi lymphocytic B cell lines, were cultured in RPMI
1640 medium (Life Technologies, Paisley, U.K.) with 10% FCS (Bayer Diagnostics, Puteaux, France), 1% glutamine, and penicillin-streptomycin medium (Life Technologies). K41 mouse embryonic fibroblasts immortalized with SV40 (provided by M. Michalak, Department of Biochemistry, University of Alberta, Canada), COS (saimian ovary), and CHO (Chinese hamster ovary) cells. Primary T lymphocytes derived from human tonsils (Hôpital Necker Enfants Malades, Paris, France) were obtained as described by Lafaye et al. (28). These cells were stimulated with 2 µg/ml PHA (Life Technologies) for 72 h before use; they were maintained in RPMI 1640 medium supplemented with 10% FCS, 1% glutamine, and penicillin-streptomycin. Cells were maintained in culture at 37°C in 5% CO2.

Analysis of the binding of anti-DNA Abs to cells
We used flow cytometry to measure anti-DNA mAb binding to the cell as follows: 5 × 10⁵ cells were incubated for 30 min at 4°C in 100 µl of PBS-supplemented BSA (1%) and azide (0.02%) (PBS/1% BSA/0.02% NaN₃) in the presence of 10 µg/ml anti-DNA mAbs, UPC, or anti-cJun in the presence or not of 0.05% saponin, depending on the aim of analysis (extra- or intracytoplasmic labeling). The permeabilized cells were washed and incubated with anti-mouse mAb conjugated to FITC (StressGen; TEBU) or to streptavidin-FITC (TEBU) (30 min). Cells were fixed in 1% paraformaldehyde before analysis by flow cytometry (FACSScan, BD Biosciences, Mountain View, CA).

Confocal microscopy
Cells (5 × 10⁵/well) were placed in the wells of a six-well plate containing a 12-mm glass coverslip and grown until 50% confluent. Cells were then fixed by incubation for 15 min at room temperature in PBS/3.7% paraformaldehyde/0.03 M sucrose. The cells were washed in PBS-BSA (1 mg/ml), and incubated with FITC-labeled H9.3 or F14.6 mAbs for 20 min at 4°C in PBS containing 1% BSA and 0.05% NaN₃ (PBS-BSA). After washing with PBS-BSA buffer, the complex (H9.3 or F14.6 ligands) was extracted from cells using 20 M Tris buffer pH 7.6 containing 150 mM NaCl, 2.5 mM MgCl₂, 0.2 mM PMSF, 1000 U/ml aprotinin, and 0.5% Triton X-100, as previously described (29). After centrifugation, the supernatant was incubated with avidin-garosor (ImmunoPure Immobilized Avidin; Pierce, Rockford, IL) in PBS for 2 h or overnight at 4°C. The samples were washed extensively with PBS-BSA, and the purified proteins were eluted from avidin-garosor by heating for 5 min at 100°C in 1 M NaCl solution containing 20 M Tris-HCl, pH 7.6, 50 mM KCl, 1 mM EDTA, 1 mM PMSF, 5 mM 2-ME, and 20% (v/v) glycerol. Purified extracts were analyzed by SDS-PAGE (7.5%).

Analysis of the purified complex by Western blotting
Total and purified extracts from CHO cells were subjected to electrophoresis in 10% polyacrylamide gels containing SDS. The proteins were transferred to nitrocellulose membrane (Hybond; Amersham Pharmacia Biotech, Piscataway, NJ) previously saturated by incubation for 2 h in PBS-BSA 3% at room temperature. The membrane was incubated with FITC-labeled (10 µg/ml), C-17 (1/200) (anti-CRT rabbit polyclonal Ab; TEBU) or with SPA-601 (10 µg/ml) (mAb anti-CRT; TEBU) for 2 h at room temperature. The membrane was thoroughly washed and incubated with alkaline phosphatase-conjugated anti-mouse or anti-goat Abs (1/1000) and detected using buffered substrate tablet (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium tablets; Sigma).

Microsequencing of the purified ligand of anti-DNA mAb
After migration of the purified proteins in SDS-PAGE (7.5%) polyacrylamide gel, the gel was fixed by incubating twice for 20 min in 50% methanol, 10% acetic acid, and was stained overnight with Amido Black reagent (3% Amido Black in 45% methanol, 10% acetic acid, Sigma). The gel was washed several times with H₂O, and the band was excised from the gel digested with endoproteinase Lys-C enzyme (Roche Diagnostics, Mannheim, Germany), which cleaves peptides adjacent to lysine residues. The peptides were purified by HPLC (DEAE-C18), using a gradient of acetonitrile in 0.1% trifluoroacetic acid. Microsequencing was conducted by the Microsequencing Laboratory at Institut Pasteur.

Interaction between CRT and anti-DNA mAbs in ELISA
We used ELISA to measure the binding of mAbs to CRT. The microtiter plates (Nunc-Immuno Modules; Nunc, Roskilde, Denmark) were coated with soluble CRT (sCRT; 500 ng/ml or 1 µg/ml, C-4714; Sigma) by incubating overnight at 4°C. The plates were washed several times and incubated for 2 h at 37°C with F14.6, H9.3, UPC (0.1–100 µg/ml), or with 10 µg/ml J20.8, BAS, 6E5, or UPC mAbs. The plates were washed five times, peroxidase-conjugated anti-mouse or anti-human Abs were added, and the plates were incubated for 1 h at 37°C. The plates were then washed, and binding was detected (Sanofi Diagnostics Pasteur, Marnes-la-Cochette, France).

Evaluation of CRT expression on the surface of lymphocytes and CHO
We used flow cytometry to detect CRT on the surface of activated human lymphocytes. Cells were incubated for 30 min at 4°C with SPA-600 (1/200) (anti-CRT rabbit polyclonal Ab; TEBU). Unbound IgG was removed by several washes, FITC-conjugated anti-rabbit Abs were added, and the cells were incubated for 30 min at 4°C. In some experiments, SPA-600 was used as competitor of F14.6 mAb binding to the cell surface. It was incubated with the cells for 30 min at 4°C, and F14.6 mAb was added after several washes. sCRT (50 µg/ml) was also used as a competitor; in this case, F14.6 was incubated with CRT for 30 min at 37°C before being added to the cells. FITC-conjugated anti-mouse Abs were added to cells and incubated for 30 min at 4°C. The cells were then analyzed by flow cytometry.

Results
Purity of monoclonal anti-DNA Abs
Anti-DNA Abs secreted into the culture supernatant of hybridomas were purified on protein A-agarose columns and their isotype determined.

One of the major problems with the anti-DNA mAbs is that these IgG may be complexed with nucleosomes (DNA complexed with histones) released from dead cells in the culture supernatant of hybridomas and may give false positive results in assays of cross-reactive binding to several Ags (7, 30, 31). To assess the purity of the mouse mAbs used, we estimated the possible extent of nucleosome contamination. Large amounts of various purified mAbs, corresponding to 5 and 25 µg of IgG were separated by SDS-PAGE (Fig. 1). Heavy and light chain Abs, with molecular mass of ~53 and 23 kDa gave clear signals. The purity of anti-DNA mAbs (F14.6, H9.3, J20.8, and F4.1) was compared with that of UPC and 6E5. No migration of low-molecular-mass proteins corresponding to nucleosome-derived histone contaminants was observed (Fig. 1).

Anti-DNA mAbs bind extra- and intracytoplasmic Ags
We investigated the effect of the anti-DNA mAbs on cells by first studying their capacity to bind to the surface of various cells. In FACS analysis and in the absence of saponin, all the anti-DNA mAbs gave significant labeling on the surface of the erythrocytes (K562), T (CEM and Jurkat), and B (Raji) cell lines used (Fig. 2).
F14.6 mAb binding was the strongest; for this reason, most of the subsequent experiments were performed using this mAb.

We measured the binding of F14.6 mAb to intracytoplasmic Ags. We found that if cells were permeabilized with saponin during incubation with the F14.6 mAb, more anti-DNA mAb binding was observed, indicating that these Abs bound intracytoplasmic Ags, whereas the negative control, UPC, had no effect, and the positive control, anti-cJun mAb, gave a positive signal (data not shown).

Internalization of the anti-DNA mAbs into cells

We evaluated, in the same experiment, the cell surface binding of H9.3 and F14.6 mAbs before internalization. To study penetration and to detect intracytoplasmic mAbs, saponin was added to the cells in the presence of FITC-conjugated anti-mouse IgG. We assessed the level of anti-DNA mAbs in the cytoplasm (Fig. 3, + saponin). No fluorescence was observed if unpermeabilized cells were used (Fig. 3, − saponin). The decline in the amount of IgG at the surface was associated with an increase in intracellular IgG levels. Over the next 2 h of incubation at 37°C, a rapid increase in nuclear labeling was observed with F14.6 and J20.8, but not with H9.3, in COS cells (data not shown).

Thus, F14.6 and H9.3 anti-DNA mAbs are able to pass through the cell membrane and to enter the cytoplasm. We observed no intracytoplasmic fluorescence with the conjugated-FITC mAb alone or with the UPC mAb control, and the cells were well visualized in the phase contrast system.

Purification and identification of a cell surface protein that bound the anti-DNA mAbs

In these experiments, we recovered cell surface ligands independently from their cytoplasmic counterparts. As described in Materials and Methods, CEM or Jurkat cells were incubated with biotinylated F14.6 or H9.3 mAbs, lysed, and then submitted to an avidin-agarose column. A major protein of ~50 kDa was immunopurified (Fig. 4, lanes 3–6). In lanes 1 and 2, F14.6 and H9.3 mAbs were used as controls, and the heavy and light chains of these mAbs are visible. The heavy and light chains of the mAbs
used for capture and purification gave bands at ~53 and 23 kDa, respectively.

We used microsequencing to identify the purified 50-kDa ligand and confirmed the results by Western blot assay. The 50-kDa protein (p50) purified using an affinity matrix containing biotinylated F14.6, was digested with endoproteinase Lys-C enzyme. The peptides were purified by HPLC, and the three main peaks isolated were processed for microsequencing (see Materials and Methods). The amino acid sequences obtained in this analysis correspond to positions 25–41, 65–71, and 287–301 and were found to be 100% identical with that of human CRT (32, 33) (Table I). Amino acid sequences were compared using the GenBank database and the FASTA and PSI-BLAST programs (34, 35).

We used the C-17 anti-CRT Ab to confirm that the purified protein was indeed CRT. Total and purified CHO extracts were incubated separately with C-17 and F14.6 and compared. A 50-kDa protein was detected with F14.6 and C-17 in crude (Fig. 5, lanes 1 and 3) and purified (lanes 2 and 4) extracts. These results were confirmed using another anti-CRT mAb (SPA-601) (data not shown).

**Anti-DNA mAbs and CRT interactions**

We used ELISA to measure the direct binding of mouse and human anti-DNA mAbs to the coated CRT protein. F14.6, J20.8, H9.3, and BAS mAbs used at concentrations of 10 μg/ml gave a significant signal. UPC and 6E5 had no effect (Fig. 6). F14.6 mAb gave a dose-dependent ELISA signal, significantly higher than that with H9.3 mAb (data not shown). No ELISA signal was observed if these mAbs were incubated with BSA coated at the same concentration as CRT (data not shown).

CRT is classically thought to be a resident endoplasmic reticulum protein, but it is also found on the surface of cells. FACS demonstrated that CRT was present on the surface of activated lymphocytes. The SPA-600 Ab labeled the surface of lymphocytes indicating the presence of CRT (Fig. 7A). The specificity of this binding was demonstrated using the SPA-600 Ab in inhibition experiments. The F14.6 mAb interaction with human lymphocytes was inhibited by ~70% (Fig. 7A). In addition, sCRT used as competitor inhibited the binding of F14.6 mAb to CHO cells by ~82% (Fig. 7B). No inhibition was observed with PHA or isotype control. We confirmed these results by confocal microscopy, where we observed strong labeling of K41 cells by SPA-600 indicating the presence of CRT. Significantly less binding was observed if K41 cells were preincubated with F14.6 before adding SPA-600 (data not shown).

**Discussion**

In this study, we aimed to identify potential cell surface receptors responsible for the penetration of murine anti-DNA mAbs into cells. Using several approaches, including the use of an affinity matrix containing biotinylated F14.6 or H9.3 mAbs with membrane extracts obtained from various cells, we isolated a 50-kDa protein identified by microsequencing as CRT. The 50-kDa protein was confirmed to be CRT in Western blot experiments with C-17 Ab in total or purified cell extracts. Flow cytometry analysis, using SPA-600 Ab or sCRT, showed that the CRT was present on the surface of activated human lymphocytes and CHO cells, and that the binding of F14.6 mAb to these cells was specifically inhibited by SPA-600 or sCRT. In ELISA experiments, four different mouse
or human anti-DNA mAbs bound to CRT. F14.6 mAb gave a significant dose-dependent ELISA signal (data not shown).

The function of CRT on the cell surface is not well understood. In human fibroblasts, surface CRT appears to function as a receptor for fibrinogen and is essential for its mitogenic effect (36). Surface CRT may also be a receptor for specific ligands, via its ability to bind to other proteins or by binding to the extracellular matrix or other glycoproteins via its lectin site (18). CRT has also been found to be associated with the Ro/SSA, La/SSB ribonucleoprotein complex (37). These three proteins are well characterized. The human 48-kDa CRT gene is located on chromosome 19 (32, 33), whereas the human genes encoding the 60- and 52-kDa protein kinases (PKC), which has key regulatory roles in a wide spectrum of signal transduction pathways, interacts with CRT in vivo (45); these two proteins may operate in common signaling pathways. There are similarities, in both function and structure, between CRT and RACKs (receptors for activated C-kinase) (45). RACK1 binds directly to the cytoplasmic domain of the integrin subunit, suggesting a link between integrins and PKC via RACK1.

Autoantibodies against cell surface cC1qR/CRT may lead to the direct activation of the cells (37). It is not entirely clear how cC1qR/CRT is involved in signal transduction. It may be that protein kinase C (PKC), which has key regulatory roles in a wide spectrum of signal transduction pathways, interacts with CRT in vivo (45); these two proteins may operate in common signaling pathways. There are similarities, in both function and structure, between CRT and RACKs (receptors for activated C-kinase) (45).

Table I. Amino acid sequence similarity of the three main peptides from the p50 to CRT

<table>
<thead>
<tr>
<th>Protein</th>
<th>HPLC Fraction</th>
<th>Amino Acid Sequence</th>
<th>Sequence Similarity (aa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p50</td>
<td>Peak25</td>
<td>(K) EQLDGDGWTSRWIES (K)</td>
<td>CRT (25–40)</td>
</tr>
<tr>
<td></td>
<td>Peak26</td>
<td>(K) GLQTSQ</td>
<td>CRT (65–70)</td>
</tr>
<tr>
<td></td>
<td>Peak35</td>
<td>(K) GTWIPEIDNPYES</td>
<td>CRT (287–300)</td>
</tr>
</tbody>
</table>

The 50-kDa (p50) protein purified from cell extracts using the affinity matrix containing biotinylated F14.6 was recovered from the SDS-PAGE gel, digested with endolysin C, and the peptides were purified by HPLC. Three main peptides were microsequenced. The similarity of the amino acid (aa) sequences obtained to that deduced from the nucleotide sequence of the cDNA corresponding to the known protein is given. As endolysin C cleaves peptide bonds following lysine residues, the (K) at the beginning of the sequences of the various peptides, and at the end of some peptides, indicates that in the homologous protein sequence, these peptides are indeed adjacent to a lysine residue. The results show that p50 is homologous to human CRT (32, 33). Amino acid sequences were compared using the GenBank database and the FASTA and PSI-BLAST programs (34, 35).
and further implicating PKC in integrin-mediated cell signaling (46). There is evidence of similarities consistent with our results, because CRT may exert such effects via its interaction with a putative membrane protein with a transmembrane domain (44). The putative membrane protein in question may be integrin \( \alpha_6 \beta_1 \), which is intimately associated with CRT (19, 47). We investigated this possibility using CD49 mAb (anti-\( \alpha_6 \beta_1 \) integrin) to inhibit the interaction of F14.6 mAb with human lymphocytes. CD49 mAb inhibited this interaction by 96% (data not shown). The degree of this inhibition was similar if integrin was used in combination with sCRT. These results suggest that these two proteins cooperate and that integrin \( \alpha_6 \beta_1 \) may be involved in the postbinding events in the process of entry of anti-DNA mAbs into cells. The CRT/\( \alpha_6 \beta_1 \) integrin complex may transmit information in both directions across the plasma membrane (48–50) for anti-DNA mAb penetration. All these results suggest the existence of multiple receptors for the anti-DNA mAb penetration into cells.

Another mechanism for the entry of anti-DNA mAbs into cells was proposed by Koutouzov et al. (51), who reported that anti-DNA Abs could not penetrate into cells unless they were associated with nucleosomes (51). We investigated this and checked for the possible entry of an “anti-DNA Ab/nucleosome” complex via cell surface CRT, using soluble nucleosome in FACS and ELISA binding experiments. CRT did not react with the nucleosome, and the penetration of F14.6 did not increase whether nucleosome was added (data not shown). The binding of F14.6 mAb to the surface of various cells used appeared to be direct and specific, consistent with the absence of nucleosome in our purified mAb preparation.

This study demonstrates that CRT may act as a cell surface receptor for penetrating anti-DNA mAbs. In addition to its role as a chaperone with putative isoforms, and its known multiple functions (19, 52), there is other evidence supporting the key role of this protein in the pathogenesis of SLE. CRT is also the major intracytoplasmic reservoir of calcium, and changes in cellular calcium flux is one of several possible mechanisms by which auto-antisbodies may exert pathogenic effects after penetrating cells (2).


