The Journal of Immunology

α-Actinin Is a Cross-Reactive Renal Target for Pathogenic Anti-DNA Antibodies

Bisram Deocharan, Xiaoping Qing, Juan Lichauco and Chaim Putterman

This information is current as of October 21, 2021.

J Immunol 2002; 168:3072-3078; ;
doi: 10.4049/jimmunol.168.6.3072
http://www.jimmunol.org/content/168/6/3072

References This article **cites 19 articles**, 5 of which you can access for free at:
http://www.jimmunol.org/content/168/6/3072.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2002 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Downloaded from http://www.jimmunol.org/ by guest on October 21, 2021

α-Actinin Is a Cross-Reactive Renal Target for Pathogenic Anti-DNA Antibodies[1]

Bisram Deocharan,* Xiaoping Qing,† Juan Lichauco,† and Chaim Putterman[2]*†

Anti-DNA Abs commonly found in patients with systemic lupus erythematosus are thought to play an important pathogenic role in lupus nephritis. Anti-DNA Abs may contribute to renal disease by cross-reactivity with renal Ags, the identity of which remain elusive. To identify a target Ag for pathogenic anti-DNA Abs, we performed Western blotting and immunoprecipitations of mesangial cell lysates from the lupus-prone MRL-*lpr/lpr* mouse and a nonautoimmune BALB/c mouse with the pathogenic anti-DNA Ab R4A. We found that R4A (but not a nonpathogenic Ab mutant of R4A) binds to and immunoprecipitates a 100-kDa protein expressed on the cell surface and in lysates of MRL-*lpr/lpr* mesangial cells. DNase treatment of the lysate and of the R4A Ab did not effect binding, indicating that the binding of R4A to the 100-kDa protein was direct and not mediated by an antigenic bridge containing DNA. Binding was greatly diminished in BALB/c lysates, suggesting that Ag expression or availability at the level of the target organ may be a factor in determining susceptibility to lupus nephritis. Following identification of this 100-kDa protein as nonmuscle α-actinin, binding of R4A to α-actinin was confirmed by Western blot, ELISA, inhibition studies, and immunofluorescence. High titers of anti-α-actinin Abs were present in sera and kidney eluates of lupus mice with active nephritis. These results indicate that the nephritogenicity of some anti-DNA Abs may be mediated via cross-reactivity with α-actinin. Furthermore, variations in target Ag display between individuals may underlie differential susceptibility to anti-DNA Ab-induced renal disease. ***The Journal of Immunology*****, 2002, 168: 3072–3078.**

Antibody-mediated glomerulonephritis is a major determinant in the prognosis of patients with systemic lupus erythematosus (SLE).[3] Serologically, SLE is characterized by the presence of Abs to a wide variety of nuclear Ags, including Abs to dsDNA. Although anti-dsDNA Abs are widely used in the serologic diagnosis of lupus, it has become clear that anti-dsDNA Abs also play a crucial role in the pathogenesis of lupus nephritis (1). Current data suggest that anti-DNA Abs may deposit in the kidney by binding to DNA deposited nonspecifically on the glomerular basement membrane (2). Alternatively, we and others have provided evidence that anti-dsDNA Abs may trigger kidney damage by direct cross-reactivity with kidney Ags (3). However, the exact nature of the cross-reactive target(s) for anti-dsDNA Abs in renal tissue remains elusive.

To identify a cross-reactive renal Ag bound by nephritogenic anti-DNA Abs, we performed Western blotting of mesangial cell (MC) lysates with R4A, a pathogenic anti-DNA Ab, and with 95, a closely related but nonpathogenic Ab. We found that R4A bound to and immunoprecipitated a 100-kDa protein identified as α-actinin from MC lysates. Binding was more pronounced using MRL-*lpr/lpr* (*lpr*) than nonautoimmune BALB/c MC lysates, suggesting that Ag expression in the target organ plays a role in anti-DNA Ab binding to renal tissue. Furthermore, sera and kidney eluates from lupus mice with active nephritis displayed high titers of anti-α-actinin Abs. We conclude that α-actinin is a cross-reactive target for pathogenic anti-dsDNA Abs in renal tissue, and that genetic variability in Ag expression and/or accessibility may contribute to the susceptibility to anti-dsDNA Ab-induced nephritis.

Materials and Methods

Antibodies

R4A is a pathogenic murine IgG2b anti-dsDNA Ab. When administered i.p. in the form of an ascites-producing hybridoma to SCID mice, R4A deposits in renal glomeruli and induces significant proteinuria (4). Ab 95, which has a single aspartic acid to glycine substitution in complementarity determining region 3 of the R4A H chain, no longer binds DNA and is nonpathogenic (4). MOPC 141 is an isotype-matched IgG2b mAb (Sigma-Aldrich, St. Louis, MO) that also does not bind DNA or deposit in glomeruli. BM-75.2 is an IgM anti-α-actinin mAb, and TEPC 183 is an isotype-matched purified myeloma Ig (Sigma-Aldrich).

Mice

Six- to 8-wk-old female BALB/c, (NZB × NZW)F_1, and MRL-*lpr*/*lpr* mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in the animal facility of the Albert Einstein College of Medicine (Bronx, NY).

Cell lines

MCs were isolated according to a protocol obtained from G. Gilkeson (Medical University of South Carolina, Charleston, SC). Briefly, the outer cortexes of 10 kidneys were minced with a razor, and the tissue was forced through progressively smaller stainless steel sieves (180, 150, and 90 μm). Glomeruli were then caught on a 75-μm sieve, washed twice with PBS, and centrifuged for 5 min at 220 × *g*. Washed glomeruli were treated with collagenase for 10 min at 37°C and washed again as above. Cells were plated out in RPMI 1640 supplemented with amino acids, insulin, and 20% FCS, and maintained at 37°C/5% CO_2. After 30 days, the cultures consist of virtually pure MCs. MCs derived from BALB/c mice were transformed using an SV40 vector received from the laboratory of Dr. R. Pestell (Bronx, NY). Transformed *lpr* mesangial and tubular cells were a kind gift from Dr.

*Department of Microbiology and Immunology, and †Division of Rheumatology, Department of Medicine, Albert Einstein College of Medicine, Bronx, NY 10461

Received for publication August 21, 2001. Accepted for publication January 10, 2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Institutes of Health Grant KO8AR02015-02, and a Lupus Research Institute (SLE Foundation) Novel Research Project in Lupus Award (to C.P.).

[2] Address correspondence and reprint requests to Dr. Chaim Putterman, Division of Rheumatology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461. E-mail address: putterma@aecom.yu.edu

[3] Abbreviations used in this paper: SLE, systemic lupus erythematosus; RT, room temperature; MALDI-MS, mass spectrometry matrix-assisted laser desorption ionization; *lpr*, MRL-*lpr/lpr*; MC, mesangial cell; *lpr*-mc, *lpr* derived MC; TC, tubular cell; *lpr*-tc, *lpr* derived TC; TBM, tubular basement membrane.

Copyright © 2002 by The American Association of Immunologists

0022-1767/02/$02.00

Downloaded from http://www.jimmunol.org/ by guest on October 21, 2021

M. Madaio (University of Pennsylvania, Philadelphia, PA). An immortalized kidney podocyte cell line was a kind gift from Dr. P. Mundel (Bronx, NY).

Flow cytometry

MCs were detached from tissue-culture plates using 2 mM EDTA, pelleted, and washed twice with DMEM. Cells were resuspended at 2×10^6 cells/ml in HBSS, pelleted, and blocked with 1% BSA/PBS and anti-mouse CD16/CD32 (FcγIII/IIR; BD PharMingen, San Diego, CA) for 1 h at 4°C. Primary Abs were incubated with the cells at a concentration of 10 μg/ml for 1 h at 4°C, followed by biotin-labeled goat anti-mouse IgG2b (BD PharMingen) for 45 min at 4°C, and streptavidin-allophycocyanin (BD PharMingen) for 30 min at 4°C. Cells were washed, resuspended in 1% paraformaldehyde, and analyzed by flow cytometry (BD Immunocytometry Systems, Mountain View, CA).

Western blotting and immunoprecipitation

Cells were detached from tissue-culture plates, washed, pelleted, and resuspended to 2×10^8 cells/ml in lysis buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% NaN3, 1% Triton X-100, and protease inhibitors) for 25 min on ice. The cell lysis mixture was centrifuged at 14,000 rpm for 20 min at 4°C. The supernatant was removed, aliquoted, and kept at −70°C until used. Protein concentration was assayed by spectrophotometry, using the Protein Assay ESL kit from Boehringer Mannheim (Indianapolis, IN). For Western blotting, 20 μg of protein lysates were combined with reducing or nonreducing sample buffer. For some experiments, protein samples were first incubated with 100 μg/ml of DNase I (Sigma-Aldrich) for 1 h at 37°C. Samples were loaded into 4–15% gradient polyacrylamide gels (Bio-Rad, Hercules, CA), and electrophoresed at 150 V for 1 h. Proteins were transferred to a polyvinylidene difluoride membrane using Mini Protean 3 cell apparatus (Bio-Rad) at 200 mAmp for 1 h. The membrane was blocked in 5% nonfat milk and incubated with primary Ab at 1 μg/ml for 30 min at room temperature (RT). The membrane was repeatedly washed with PBS-Tween, and incubated with the appropriate HRP-conjugated secondary Ab diluted 1/5,000 for 30 min at RT. The membrane was developed with the ECL Plus kit, and exposed to Hyperfilm (Amersham, Aylesbury, U.K.). The intensity of the developed bands was quantitated by densitometry and the ImageQuant software program (Amersham Biosciences, Sunnyvale, CA). Loading of equivalent amounts of protein and of adequate membrane transfer was confirmed by staining the polyvinylidene difluoride membrane with Ponceau Red and by Western blotting with an anti-tubulin Ab in parallel to the test Abs. For assay of inhibition of binding by Western blot, serial dilutions of chicken α-actinin (Sigma-Aldrich), salmon sperm dsDNA (Calbiochem, La Jolla, CA), or peptide (DWEYSVWLSN on a polylysine backbone from Research Genetics, Huntsville, AL) were incubated with R4A for 2 h at 37°C. The assay was then continued as described above.

Immunoprecipitations were performed using protein G-Sepharose beads (Pharmacia, Piscataway, NJ). Protein G beads prewashed with radioimmunoprecipitation assay buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% NaN3, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, and protease inhibitors) were incubated with 75 μg of mAb for 2 h at 4°C with constant mixing. Three milligrams of precleared protein lysate were combined with the Ab-loaded protein G beads and incubated overnight at 4°C with gentle shaking. The mixture was centrifuged at 10,600 rpm for 20 s and the pellet was washed three times with 1% radioimmunoprecipitation assay buffer and once with 50 mM Tris-HCl (pH 8.0). The pellet was resuspended in sample reducing buffer and heated to 100°C for 5 min. The supernatant was removed and the proteins were separated by SDS-PAGE and Western blotted as above.

On-dish membrane biotinylation of MC surface proteins was performed using EZ link Sulfo-NHS-L-biotin (Pierce, Rockford, IL) and the manufacturer's instructions. Only external surface proteins are labeled by this method. Briefly, cell culture medium was removed and the cells were washed twice to remove any contaminating proteins. The biotinylation reagent was added and incubated for 30 min at 4°C. Cells were removed, washed, pelleted, and resuspended in extraction buffer (50 mM boric acid, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 2.5 mM PMSF). The mixture was passed twice through a 21G needle, incubated on ice for 30 min, and spun at 12,000 rpm for 20 min at 4°C. To immunoprecipitate biotin-labeled cell surface proteins, 3 mg of biotinylated proteins were combined with prewashed streptavidin-conjugated agarose beads and incubated overnight at 4°C. Isolation of the precipitated proteins, electrophoresis, and Western blotting were continued as described above.

Protein analysis

SDS-PAGE of anti-DNA Ab-immunoprecipitated proteins was conducted as described above. The appropriate-sized protein band was cut from the gel and transferred to a prewashed microfuge tube. Protein identification using mass spectrometry:matrix-assisted laser desorption ionization (MALDI-MS) and nanospray MS/MS was performed at the Howard Hughes Medical Institute Biopolymer/Keck Foundation Biotechnology Resource Laboratory at the Yale University School of Medicine (New Haven, CT).

Immunocytochemistry

MCs grown to confluence on tissue culture-treated cover slips were washed twice with 1% BSA/PBS and incubated with 10 μg/ml of R4A, 50 μg/ml of BM-75.2, or isotype-matched Abs diluted in block for 4 h at 4°C. The cells were then washed with PBS and fixed in 1% paraformaldehyde/PBS for 30 min. The cells were rinsed and washed three times with PBS. The appropriate fluorochrome-conjugated secondary Ab was applied and the slides were incubated for 60 min in the dark. Coverslips were mounted following several washes and the slides were allowed to dry overnight at 4°C. A Bio-Rad Radiance 2000 scanning confocal microscope with a Kr/Ar laser for excitation at 488 and 568 nm was used for visualization with Nikon 60X NA 1.4 planapo infinity corrected optics (Melville, NY).

ELISAs

Anti-dsDNA ELISAs were performed as previously described (5). For the α-actinin ELISA, α-actinin (Sigma-Aldrich) at a concentration of 20 μg/ml was coated onto Immulon II 96-well microtiter plates (Dynatech Laboratories, Chantilly, VA) overnight at 4°C. Plates were blocked with 3% FCS for 1 h at 37°C and incubated with mAb or serum at a 1/200 dilution for 2 h at RT. Plates were washed five times with PBS-Tween, and alkaline phosphatase-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL) diluted 1/1000 in 3% FCS was added for 1 h at 37°C, followed by substrate. For inhibition ELISAs, Abs were preincubated with serial dilutions of DNA or α-actinin for 1 h at 37°C before transfer to the preblocked Ag-coated plate. The assay was then continued as described above. The value for the percentage of inhibition was calculated as ((OD without inhibitor − OD with inhibitor)/(OD without inhibitor)) × 100.

Cell surface ELISA

MCs at a concentration of 2×10^6 cells/ml were plated onto sterile 96-well tissue culture-treated plates and incubated at 37°C for 72 h. The supernatant was removed and the plates were washed three times with cold wash buffer (PBS with 1% FCS and 0.05% NaN3). Plates were blocked with PBS/3% FCS for 1 h at 4°C and washed again. Primary Ab diluted in cold PBS was added and incubated for 2 h at 4°C. The plates were centrifuged at 2000 rpm for 5 min, washed three times, and the appropriate alkaline-phosphatase-linked goat anti-mouse Ab diluted 1/1000 in PBS was added for 90 min at 4°C. The plates were again centrifuged, washed three times, and developed for reading at 405 nm.

Results

R4A binds to the cell surface of MCs

To determine whether the anti-dsDNA Ab R4A is cross-reactive with kidney cell surface proteins, we studied whether R4A binds to *lpr*-derived mesangial cells (*lpr*-mc) by flow cytometry. As shown in Fig. 1*A*, >95% of *lpr*-mc are bound by R4A, but not by an isotype-matched IgG2b Ab. This experiment also demonstrates that the binding of R4A to MC is not mediated by the Fc portion of the Ab, which is shared with other Abs of the same isotype. To exclude the possibility that EDTA treatment of the adherent cells resulted in nonphysiological exposure of the Ag on the membrane, we repeated the flow cytometry studies on MC not exposed to EDTA. MCs were either grown on tissue-culture plates and removed by a sterile cell scraper, or the cells were grown on non-tissue culture-treated petri dishes and removed easily by gentle pipetting. In non-EDTA-treated cells, we found comparably strong Ab binding, indicating that the observed binding of R4A to MC by flow cytometry was not an artifact of EDTA treatment.

Downloaded from http://www.jimmunol.org/ by guest on October 21, 2021

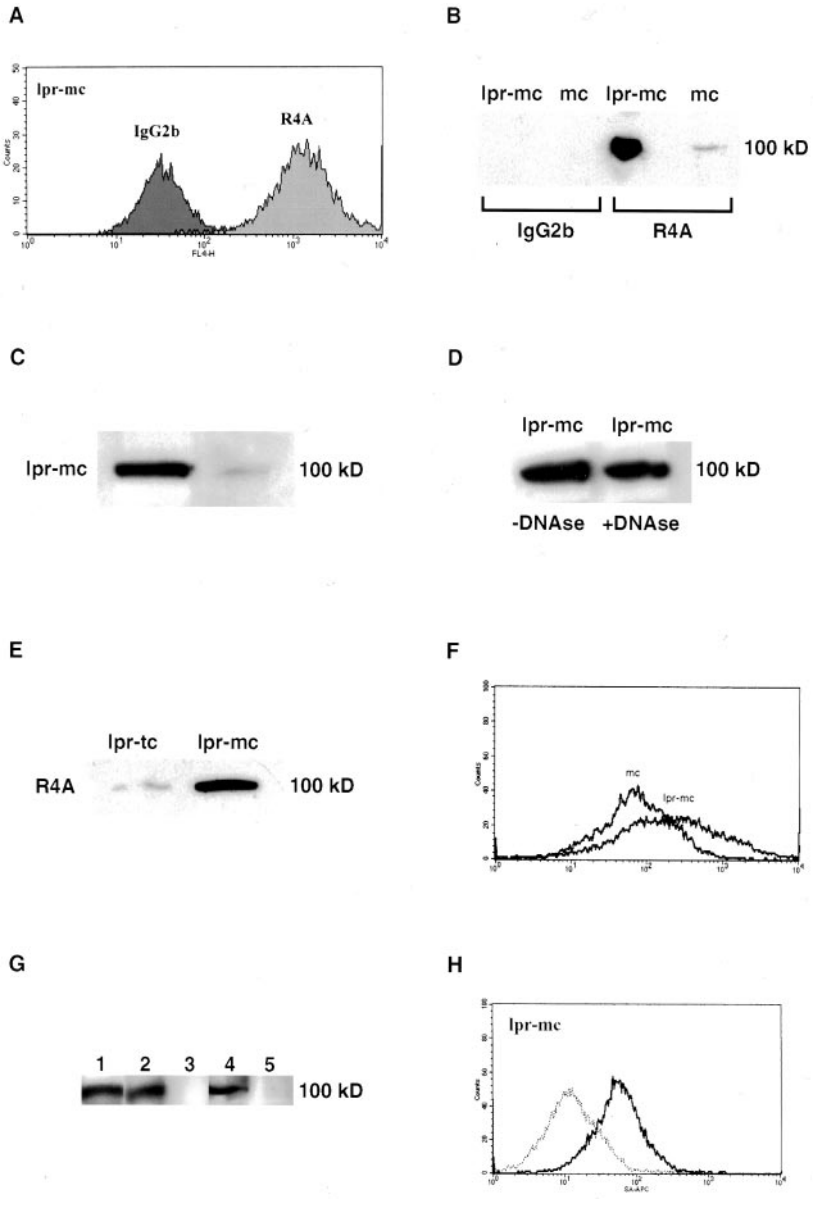

FIGURE 1. R4A binds to a differentially expressed MC protein. *A*, R4A or the isotype-matched mAb MOPC 141 (IgG2b) at 10 μg/ml were incubated with *lpr*-mc cells, followed by biotin-conjugated goat anti-mouse IgG2b and streptavidin-allophycocyanin. *B*, Total MC lysates from *lpr* (lpr-mc) and BALB/c mice (mc) were run on a SDS-PAGE gel, and Western blotted with 1 μg/ml R4A and MOPC 141. *C*, *lpr*-mc lysate was incubated with Protein G beads bound to R4A, and the immunoprecipitate was separated by SDS-PAGE. *Left*, Total cell lysate blotted with R4A. *Right*, R4A immunoprecipitate blotted with R4A. *D*, *lpr*-mc lysate pretreated (*right*) or not (*left*) with DNase I was blotted with 1 μg/ml R4A. *E*, Western blotting with 1 μg/ml R4A was performed on *lpr*-mc (*right*) and *lpr*-tc (*left*) lysates. *F*, R4A binding to the *lpr*-mc and the BALB/c MC was compared by flow cytometry. *G*, Biotinylated cell surface proteins from MC lysates were Western blotted with serum from BALB/c mice immunized with α-actinin in adjuvant (*lane 1* and *2*); serum from a control mouse immunized with adjuvant alone (*lane 3*); an IgM anti-α-actinin mAb (*lane 4*); and an IgM control mAb (*lane 5*). Sera were used at a dilution of 1/50 and mAbs at 1 μg/ml. *H*, Flow cytometry was performed on *lpr*-mc stained with serum from a BALB/c mouse immunized with α-actinin in adjuvant (*right*, continuous tracing) and serum from a control mouse immunized with adjuvant alone (*left*, dotted tracing). Sera were used at a dilution of 1/50.

R4A binds to and immunoprecipitates a 100-kDa protein in lpr-*mc*

Western blotting and immunoprecipitations were performed to identify the cross-reactive kidney Ag bound by R4A. As shown in Fig. 1*B*, R4A, but not the isotype-matched control Ab, binds strongly to a 100-kDa protein present in *lpr*-mc lysate. Similarly, binding by R4A to a 100-kDa protein was also observed using lysates of a podocyte cell line. Treatment of cell lysate with proteinase K completely abrogated the observed binding of R4A to MC lysate. Fig. 1*C* shows that R4A also immunoprecipitated the 100-kDa protein from solution.

It has been shown that the binding of certain anti-DNA Abs to kidney Ag may be mediated by a bridge of nuclear Ag (DNA or nucleosome) (2). We repeated the Western blotting experiments with R4A on *lpr*-mc, using a lysate and an Ab that were pretreated with DNase. Binding of R4A to the 100-kDa protein was not strongly affected by DNase treatment of the lysate (Fig. 1*D*) or of the R4A Ab (data not shown). To confirm that the binding of R4A to the 100-kDa MC protein is mediated by the Ag-binding site, we performed inhibition studies. DWEYSVWLSN, a peptide DNA mimetope previously shown to bind in the Ag-binding site of R4A (Ref. 5; Fig. 2*A*), as well as dsDNA (Fig. 2*B*), inhibited the binding of R4A to the 100-kDa protein. Taken together, these experiments indicate that R4A binds to a MC 100-kDa protein Ag via the Ab Ag-binding site. This binding is direct and not mediated by an Ag bridge containing DNA.

Anti-100-kDa Abs with a titer that increased with age were found in sera of lupus-prone (NZB × NZW)F_1 (Fig. 2*C*) and *lpr* mice. The very similar time course for the spontaneous development of IgG anti-dsDNA and IgG anti-100-kDa Abs suggests that there is a significant degree of cross-reactivity between Abs of these specificities in lupus mice.

The subcellular localization of the 100-kDa protein was studied by separate Western blotting by R4A of membrane, cytoskeletal, and cytosolic fractions of total MC lysates (6). Consistent with the flow cytometry data, the 100-kDa protein bound by R4A was present in the plasma membrane fraction. Furthermore, we repeated the Western blotting, using only biotinylated cell surface proteins from the total MC lysate, as the substrate for Ab binding. R4A bound to a biotinylated 100-kDa cell surface protein,indicating that the 100-kDa protein bound by R4A was externally located on the cell membrane.

Downloaded from http://www.jimmunol.org/ by guest on October 21, 2021

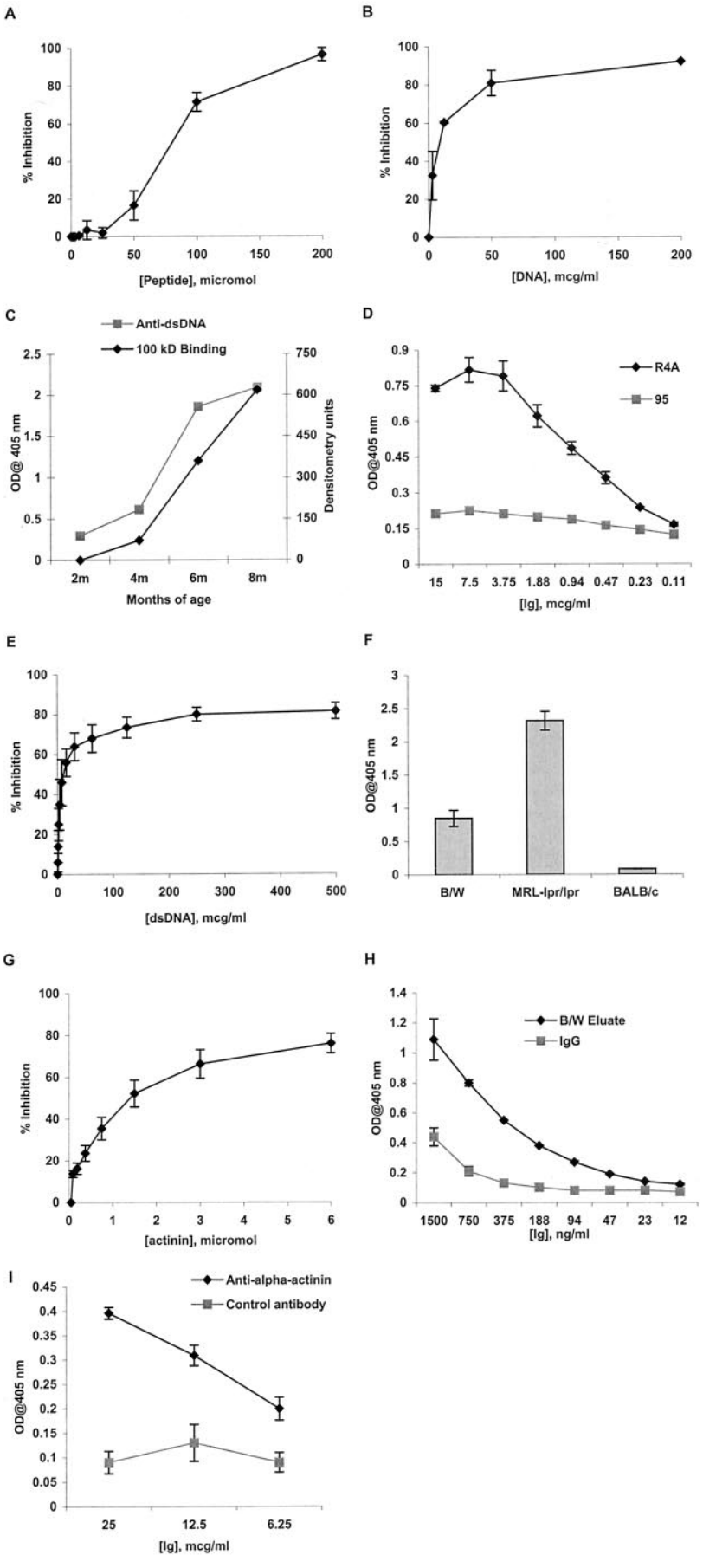

FIGURE 2. Ab binding to 100-kDa protein and to α-actinin. *A*, R4A (1 μg/ml) was incubated with serial dilutions of DWEYSVWLSN, followed by Western blotting of the *lpr*-mc lysate. *B*, R4A (1 μg/ml) was incubated with serial dilutions of salmon sperm dsDNA followed by Western blotting of *lpr*-mc lysate. *C*, Sera from (NZB $\times$ NZW)F_1 mice ($n = 9$) at a dilution of 1/100 were examined for binding to dsDNA (by ELISA) and to 100-kDa *lpr*-mc protein (by Western blot). *D*, Titration curves of R4A and 95 binding to α-actinin by ELISA. *E*, dsDNA inhibition of R4A binding to α-actinin. Serial dilutions of dsDNA were incubated with R4A, followed by an α-actinin ELISA. *F*, IgG anti-α-actinin Abs in sera of lupus mice with active nephritis ((NZB $\times$ NZW)F_1: $n = 10$, MRL-*lpr*/*lpr*: $n = 8$) and control BALB/c mice ($n = 10$). *G*, α-actinin inhibition of *lpr* sera binding to dsDNA. Serial dilutions of α-actinin were incubated with sera from 5-mo-old *lpr* mice ($n = 8$), followed by a dsDNA ELISA. *H*, A titration curve of kidney eluted Igs from nephritic (NZB $\times$ NZW)F_1 mice binding to α-actinin. IgG are polyclonal IgG Igs from nonautoimmune mice. *I*, Serial dilutions of an IgM anti-α-actinin mAb and an isotype-matched control Ab were reacted with *lpr*-mc in a cell surface ELISA.

Downloaded from http://www.jimmunol.org/ by guest on October 21, 2021

Localization of anti-DNA Ab deposition in the kidney is dependent on the presence of a cognate renal Ag

When administered to SCID mice, R4A deposits in renal glomeruli, but not in renal tubules (4). To understand the basis for this differential renal Ig deposition, we assayed the binding of R4A to the lysate of a tubular cell (TC) line derived from the *lpr* mouse (*lpr*-tc) by Western blotting. Fig. 1*E* demonstrates that when compared with R4A binding to *lpr*-mc, binding of R4A to the 100-kDa protein in *lpr*-tc is much decreased. Similarly, no binding of R4A to *lpr*-tc was found by immunofluorescence. Therefore, the lack of R4A deposition in renal tubules is likely due to reduced Ag expression and/or availability in renal tubules.

The 100-kDa protein bound by R4A is differentially expressed in an autoimmune mouse strain

To study a possible role for variations in Ag display in determining the susceptibility to anti-dsDNA Ab-induced nephritis, we compared the binding of R4A to the 100-kDa protein in *lpr*-mc and in lysates of a MC line derived from the nonautoimmune BALB/c mouse strain (mc). Fig. 1*B* (*right*) demonstrates that R4A displays much stronger binding to the 100-kDa protein in *lpr* than in BALB/c MC lysates. A significant difference in 100-kDa protein expression between these cell lines could also be demonstrated by immunofluorescence (data not shown). Similarly, binding by R4A was significantly more pronounced to *lpr*- than to BALB/c-derived MC by flow cytometry (Fig. 1*F*).

The 100-kDa MC protein bound by R4A is α-actinin

MALDI-MS data was obtained on tryptic digests of the 100-kDa MC protein band immunoprecipitated by R4A. Peptide masses obtained matched 41 and 46% of the known sequences of nonmuscle α-actinin (isoform 1 and 4, respectively). After deleting the peptide masses which were matched to known α-actinin sequences, no additional proteins were identified. Nanospray MS/MS analysis of tryptic digests of the immunoprecipitated MC proteins confirmed the MALDI-MS data, namely that the 100-kDa protein bound to and immunoprecipitated by R4A is nonmuscle α-actinin.

Binding of R4A to purified α-actinin was studied by ELISA and Western blotting. As shown in Fig. 2*D*, R4A, but not the 95 Ab, binds to α-actinin. Furthermore, dsDNA inhibited the binding of R4A to α-actinin (Fig. 2*E*). Importantly, sera from (NZB × NZW)F_1 and *lpr* mice with active disease bound strongly to α-actinin (Fig. 2*F*). Inhibition studies (Fig. 2*G*) demonstrated that up to 75% of the binding of lupus mice sera to dsDNA could be inhibited by α-actinin, thus confirming the large degree of cross-reactivity between the anti-dsDNA and anti-α-actinin Ab responses. Igs eluted from the kidneys of nephritic (NZB × NZW)F_1 mice were found to contain high titers of IgG anti-α-actinin Abs (Fig. 2*H*), indicating that anti-α-actinin Abs are deposited in renal tissue with active lupus glomerulonephritis. Binding of R4A and lupus sera to purified α-actinin and inhibition of binding to MC lysates by α-actinin were confirmed by Western blotting. Moreover, dsDNA and α-actinin significantly inhibited cell surface binding of R4A to *lpr*-mc by flow cytometry. By Western blotting, an anti-α-actinin mAb (Sigma-Aldrich) stained the same 100-kDa size band in *lpr*-mc and the MC lysate as R4A (data not shown).

The cell surface localization of α-actinin was confirmed by Western blot, flow cytometry, cell surface ELISA, and immunofluorescence studies. Using biotinylated cell surface proteins from MC as a substrate in a Western blot, an anti-α-actinin mAb and sera from α-actinin immunized BALB/c mice (but not an isotype-matched Ab or control sera, respectively) bound the same size 100-kDa band as R4A (Fig. 1*G*). Similarly, sera from α-actinin immunized mice displayed significant binding to the cell surface of *lpr*-mc by flow cytometry, as compared with sera from control mice (Fig. 1*H*). Further confirmation of the cell surface localization of α-actinin was found in cell surface ELISA experiments. An anti-α-actinin mAb (Fig. 2*I*) as well as R4A, but not isotype-matched control Abs, bound to the cell surface of *lpr*-mc. Finally, immunofluorescence studies were undertaken to confirm the identity and localization of the kidney Ag bound by R4A. As shown in Fig. 3, an anti-α-actinin mAb bound to the cell surface of live, nonfixed MC. A similar membrane immunofluorescence pattern was observed with R4A binding to *lpr*-mc. Cell surface staining of R4A was also present in podocytes. When MC were first incubated with an anti-α-actinin mAb, subsequent binding by R4A was significantly diminished (Fig. 4). Taken together, these results indicate that the Ag in MC bound by R4A is α-actinin, and that α-actinin is expressed on the cell surface of MC.

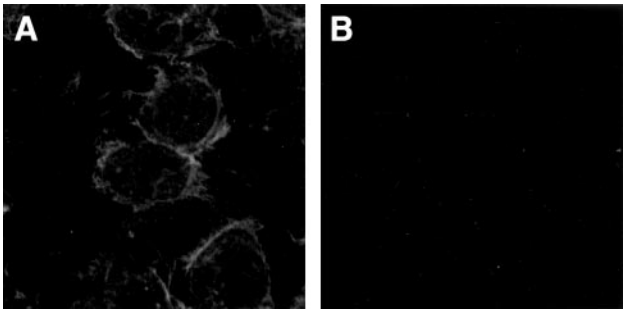

FIGURE 3. An anti-α-actinin Ab binds to the cell surface of *lpr*-mc. *lpr*-mc were incubated with Ab at 4°C, followed by fixation with paraformaldehyde and a FITC-labeled secondary Ab. Stained cells were visualized by confocal microscopy. *A, lpr*-mc stained with an IgM anti-α-actinin mAb. *B, lpr*-mc stained with an isotype-matched control Ab.

Discussion

We found that the pathogenic anti-dsDNA Ab R4A, but not a closely related nonpathogenic Ab, binds directly to α-actinin in renal MC. Furthermore, kidney Ig eluates and sera from lupus mice contained high titers of IgG anti-α-actinin Abs. The titer of anti-α-actinin Abs increased with disease activity in lupus mice and paralleled the time course for the development of anti-dsDNA Abs. Binding of R4A to α-actinin was much more prominent in *lpr*-mc than in *lpr*-tc lysates. Our results are consistent with the hypothesis that cross-reactivity of anti-DNA Abs with renal Ag is

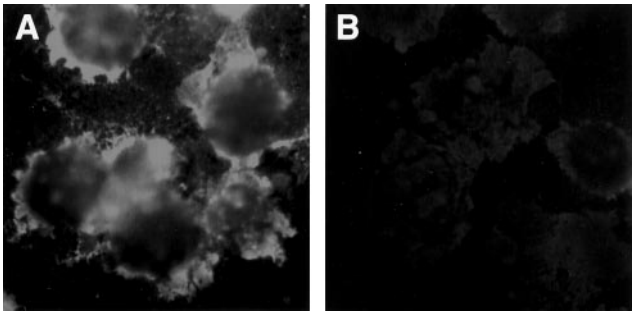

FIGURE 4. Prior incubation with an anti-α-actinin mAb inhibits subsequent binding of R4A to MCs. *A, lpr*-mc were incubated with R4A at 4°C, followed by fixation with paraformaldehyde and a Texas Red-labeled secondary Ab. *B, lpr*-mc were preincubated with an anti-α-actinin mAb for 2 h at 4°C, followed by staining with R4A as above.

Downloaded from http://www.jimmunol.org/ by guest on October 21, 2021

an important determinant of their nephritogenic potential and determines, as well, the anatomic localization of Ab deposition in the kidney.

We demonstrated a significant difference in binding of R4A to MC derived from autoimmune *lpr* and nonautoimmune BALB/c mice. We believe that this increased binding of R4A to *lpr*-mc lysates reflects a true difference in MC expression of α-actinin, as we demonstrated differences in surface binding to α-actinin also by immunofluorescence and flow cytometry. It has been shown in animal models that susceptibility to autoimmune disease is determined not only by the presence of Ag-specific autoreactive lymphocytes, but also by the availability of Ag at the level of the target organ. BN rats immunized with heterologous tubular basement membrane (TBM) develop autoimmune tubulointerstitial nephritis, with circulating anti-TBM Abs, linear IgG deposition along the TBM, and tubular abnormalities. Lewis rats also develop circulating anti-TBM Abs following immunization; however, no disease develops in this strain because they lack the anti-TBM target Ag (7). Therefore, in this model, susceptibility to Ab-induced nephritis is determined by genetically regulated expression of the target Ag. Similarly, it has been demonstrated by Diamond and coworkers (8) that the susceptibility to anti-myosin Ab-induced autoimmune myocarditis is dependent on genetically determined expression of myosin in cardiac extracellular matrix. These models and others demonstrate that for some autoimmune diseases, genetically determined expression of, or accessibility to, a target Ag is necessary for disease expression in addition to pathogenic Abs with the appropriate specificity.

Based on our results, we propose that expression of the appropriate renal Ag may be a susceptibility factor for anti-DNA Ab-induced nephritis in lupus. Some lupus patients display consistently increased serum anti-dsDNA Ab titers yet do not develop nephritis; one explanation may be that the target Ag for cross-reactive Abs is not appropriately expressed. It is important to note that although after intravenous injection, R4A deposits in the glomeruli of BALB/c mice, histological glomerular disease at the light microscopy resolution is not seen. In patients with lupus with borderline concentrations or affinities of serum anti-dsDNA Abs, it seems reasonable to propose that Ab deposition leading to disease will preferentially occur in those patients with suprathreshold expression of the relevant target Ag. Therefore, although R4A does bind also to kidneys of nonautoimmune mice, we believe that the up-regulated expression of α-actinin in autoimmune-, as compared with nonautoimmune-derived MCs may be potentially important in contributing to differential susceptibility to anti-dsDNA Ab-induced renal injury. Susceptibility to anti-dsDNA Ab-induced nephritis may be influenced by genetically regulated expression and availability of α-actinin in kidney cells. As the genes encoding each of the four α-actinin isoforms are located on different chromosomes, any genetically restricted variability in α-actinin expression is likely to be specific for a particular isoform.

α-Actinin is a dimeric actin-bundling protein, composed of two 100-kDa monomers with an anti-parallel structure that forms an actin-binding region at either end of the molecule. In the kidney, α-actinin is present in MCs, podocytes, capillaries, and larger blood vessels (9). Alterations in the distribution and expression of α-actinin have been described in experimental models of renal disease. Puromycin aminonucleoside injection leads to a significant induction in glomerular α-actinin that clearly occurs prior to effacement of foot processes and proteinuria, suggesting a pathogenic role for α-actinin in disease (10). Recently, it has been demonstrated that mutations in the human *α-actinin-4* gene cause familial focal and segmental glomerulosclerosis (9). Several pathways may lead from the binding of anti-DNA Abs to α-actinin to renal disease. Binding to α-actinin may interfere with normal actin filament assembly, thus altering the mechanical characteristics of α-actinin containing cells. Alternatively, other cellular proteins besides actin bind to α-actinin and interference with these interactions by the bound Ab may lead to functional disturbances. Finally, renal hemodynamics may also be affected due to the presence of α-actinin in the renal vasculature (9). Although the pathogenic mechanisms in the two diseases described above (puromycin aminonucleoside injection and focal and segmental glomerulosclerosis) are likely to be different from those operative in lupus nephritis, these studies demonstrate that alterations in α-actinin can be associated with significant renal disease.

How does α-actinin become accessible for binding with anti-DNA Abs? By flow cytometry, cell surface ELISA, Western blotting, and immunofluorescence, we demonstrated that R4A binds to α-actinin on the cell surface of MC. Although α-actinin is clearly an important component of the cytoskeleton, others have confirmed that α-actinin is also membrane associated (6, 11). Furthermore, recent studies have demonstrated that certain anti-dsDNA and other autoantibodies display the capability of penetration into living cells (12). We found that R4A, but not 95, could penetrate through the membrane of living *lpr*-mc and reach the nucleus (data not shown), suggesting that R4A (and similar Abs) may also be able to bind to and interact with intracellular α-actinin.

Cytokines play an important modulatory role in the expression and progression of lupus nephritis. Several of these cytokines present in lupus kidneys can specifically modulate expression and localization of α-actinin. In (NZB $\times$ NZW)F_1 mice, kidney mRNA levels of TGF-β, insulin-like growth factor-1 and basic fibroblast growth factor increase 8- to 11-fold with the progression of lupus nephritis, as compared with little change in the kidneys of control NZW mice (13). Brooks (14) demonstrated that cell stimulation with insulin-like growth factor-1 causes a specific redistribution of α-actinin at the cell interface, while Hsu (15) showed that TGF-β and basic fibroblast growth factor significantly induce α-actinin mRNA expression. We postulate that cytokines may contribute to lupus nephritis by affecting the local kidney expression of α-actinin, a hypothesis consistent with the report that α-actinin was readily detected in a 7-mo-old (NZB $\times$ NZW)F_1 mouse with nephritis, but not in a 3-mo-old (NZB $\times$ NZW)F_1 mouse (6). We are undertaking studies to directly examine the effects of cytokines on α-actinin expression. If proved correct, our hypothesis would raise the intriguing possibility of the therapeutic use of cytokines to manipulate end-organ expression of the target Ag for pathogenic anti-dsDNA Abs.

It is of interest to compare our results to several previous attempts to identify anti-dsDNA reactive proteins by immunoblotting. Minota (16) described a 110-kDa protein from PBMCs that reacted with the sera of lupus patients. However, binding was low titer and mostly IgM, and present in several other autoimmune diseases as well as in infectious mononucleosis and acute hepatitis. Viard et al. (17) isolated a murine anti-dsDNA Ab that bound a 94-kDa protein on the cell surface of fibroblasts; however, binding was detected only in the presence of nucleosome or DNA-histone complexes and no further identification of the protein was conducted. Madaio and colleagues (18) described a 110-kDa protein identified as myosin 1 that was immunoprecipitated by a murine anti-DNA mAb from rat hepatoma cells. Seddiki (19) reported that a 50-kDa receptor on human lymphocyte cell lines identified as calreticulin may mediate the cellular penetration of some anti-DNA Abs. Most recently, Eilat and coworkers (6) demonstrated that five pathogenic anti-dsDNA Abs isolated from (NZB $\times$ NZW)F_1 mice cross-reacted with α-actinin, while two mAbs which were nonpathogenic did not.

Downloaded from http://www.jimmunol.org/ by guest on October 21, 2021

Identification of the cross-reactive kidney Ag bound by pathogenic anti-dsDNA Abs as α-actinin has important implications in understanding the pathogenesis of lupus nephritis. It has been recently demonstrated by Mathis and coworkers (20) that T cell and Ig recognition of a ubiquitously expressed Ag (glucose-6-phosphate isomerase) can lead to an inflammatory autoimmune process localized to joints. Similarly, we hypothesize that while α-actinin is expressed in several organs, it is the specific binding to α-actinin in the kidney that underlies the nephritogenic effects of anti-dsDNA Abs. Whether α-actinin is also targeted by human anti-DNA Abs is currently under investigation. Preliminary studies show that R4A binds to α-actinin in human MCs, and that sera from active lupus patients have high titers of IgG anti-α-actinin Abs. Conclusive identification of the renal target for pathogenic lupus autoantibodies may lead to the development of improved methods for prognostication and serological monitoring of patients with lupus, as well as to new therapeutic approaches intended to modulate Ag expression and interfere with the deposition of cross-reactive anti-dsDNA Abs in the kidney.

Acknowledgments

We thank our colleagues Drs. Anne Davidson and Betty Diamond for many helpful suggestions and discussions, and Dr. Peter Mundel for assistance with the immunofluorescence studies.

References

1. Hahn, B. H. 1998. Antibodies to DNA. *N. Engl. J. Med. 338:1359.*
2. Lefkowith, J. B., and G. S. Gilkeson. 1996. Nephritogenic autoantibodies in lupus: current concepts and continuing controversies. *Arthritis Rheum. 39:894.*
3. Putterman, C., W. Limpanasithikul, M. Edelman, and B. Diamond. 1996. The double-edged sword of the immune response: mutational analysis of an anti-pneumococcal, anti-DNA antibody. *J. Clin. Invest. 97:2251.*
4. Katz, J. B., W. Limpanasithikul, and B. Diamond. 1994. Mutational analysis of an autoantibody: differential binding and pathogenicity. *J. Exp. Med. 180:925.*
5. Gaynor, B., C. Putterman, P. Valadon, L. Spatz, M. D. Scharff, and B. Diamond. 1997. *Proc. Natl. Acad. Sci. USA 94:1955.*
6. Mostoslavsky, G., R. Fischel, N. Yachimovich, Y. Yarkoni, E. Rosenmann, M. Monestier, M. Baniyash, and D. Eilat. 2001. Lupus anti-DNA antibodies cross-react with a glomerular structural protein: a case for tissue injury by molecular mimicry. *Eur. J. Immunol. 31:1221.*
7. Yoshida, H., Y. Wakashin, S. Ueda, R. Azemoto, K. Iesato, S. Yamamoto, T. Mori, M. Ogawa, Y. Mori, and M. Wakashin. 1990. Detection of nephritogenic antigen from the Lewis rat renal tubular basement membrane. *Kidney Int. 37:1286.*
8. Liao, L., R. Sindhwani, M. Rojkind, S. Factor, L. Leinwand, and B. Diamond. 1995. Antibody-mediated autoimmune myocarditis depends on genetically determined target organ sensitivity. *J. Exp. Med. 181:1123.*
9. Kaplan, J. M., S. H. Kim, K. N. North, H. Rennke, L. A. Correia, H. Q. Tong, B. J. Mathis, J. C. Rodriguez-Perez, P. G. Allen, A. H. Beggs, and M. R. Pollak. 2000. Mutations in ACTN4, encoding α-actinin-4, cause familial focal segmental glomerulosclerosis. *Nat. Genet. 24:251.*
10. Smoyer, W. E., P. Mundel, A. Gupta, and M. J. Welsh. 1997. Podocyte α-actinin induction precedes foot process effacement in experimental nephrotic syndrome. *Am. J. Physiol. 273:F150.*
11. Spehar, T., and M. Strand. 1995. Molecular mimicry between HIV-1 gp41 and an astrocyte isoform of α-actinin. *J. Neurovirol. 1:381.*
12. Alarcon-Segovia, D., A. Ruiz-Arguelles, and L. Llorente. 1996. Broken dogma: penetration of autoantibodies into living cells. *Immunol. Today 17:163.*
13. Nakamura, T., I. Ebihara, I. Nagaoka, S. Osada, Y. Tomino, and H. Koide. 1993. Effect of methylprednisolone on TGF-β, insulin-like growth factor-I, and basic fibroblast growth factor gene expression in the kidneys of NZB/W F_1 mice. *Ren. Physiol. Biochem. 16:105.*
14. Brooks, P. C., R. L. Klemke, S. Schon, J. M. Lewis, M. A. Schwartz, and D. A. Cheresh. 1997. Insulin-like growth factor receptor cooperates with integrin $\alpha_V\beta_5$ to promote tumor cell dissemination in-vivo. *J. Clin. Invest. 99:1390.*
15. Hsu, D. K., Y. Guo, G. F. Alberts, K. A. Peifley, and J. A. Winkles. 1996. Fibroblast growth factor-1-inducible gene *FR-17* encodes a nonmuscle α-actinin isoform. *J. Cell. Physiol. 167:261.*
16. Minota, S., W. N. Jarjour, R. A. Roubey, T. Mimura, and J. B. Winfield. 1990. Reactivity of autoantibodies and DNA/anti-DNA complexes with a novel 110-kilodalton phosphoprotein in SLE and other diseases. *J. Immunol. 144:1263.*
17. Viard, J. P., J. F. Bach, and L. Jacob. 1987. Splenocytes from MRL/Mp-*lpr/lpr* mice spontaneously produce antibodies against a cell-surface protein cross-reacting with DNA. *Clin. Immunol. Immunopathol. 45:516.*
18. Yanase, K., R. M. Smith, A. Puccetti, L. Jarett, and M. P. Madaio. 1997. Receptor-mediated cellular entry of nuclear localizing anti-DNA antibodies via myosin 1. *J. Clin. Invest. 100:25.*
19. Seddiki, N., N. Farida, P. Lafaye, Z. Amoura, J. C. Piette, and J. C. Mazié. 2001. Calreticulin, a potential cell surface receptor involved in cell penetration of anti-DNA antibodies. *J. Immunol. 166:6423.*
20. Matsumoto, I., A. Staub, C. Benoist, and D. Mathis. 1999. Arthritis provoked by linked T and B cell recognition of a glycolytic enzyme. *Science 286:1732.*

Downloaded from http://www.jimmunol.org/ by guest on October 21, 2021