Analysis of anticentromere autoantibodies using cloned autoantigen CENP-B

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ABSTRACT A cDNA clone encoding CENP-B, the 80-kDa human centromere autoantigen, was used to construct a panel of hybrid proteins containing four different regions of CENP-B. These have allowed us to identify three independent epitopes on CENP-B that are targets of autoantibodies. Two of these are recognized concurrently in >90% of patient sera containing anticentromere autoantibodies (ACA), conclusively demonstrating that this autoimmune response is polyclonal. When present and previous data are combined, ACA are shown to recognize at least five independent epitopes on CENP-B. A radioimmunoassay based on cloned CENP-B has demonstrated that sera from >96% of patients with ACA recognize the cloned antigen, thus defining a region of the protein that is recognized by virtually all patients with ACA. These findings have significant implications for models that seek to explain the origin of ACA and for the future detection of this group of autoantibodies in the clinical setting.

The rheumatic diseases are characterized by the production of autoantibodies directed against nuclear and cytoplasmic autoantigens (reviewed in refs. 1–8). The reasons for autoimmune expression are generally unknown, and many theories seeking to explain the phenomenon are currently under consideration. In particular, it is not known if the autoimmune response results from a classical antigen-driven immunization or is a result of aberrations of the mechanisms that normally control the immune system.

We have chosen the anticentromere autoantibody (ACA) response for study since this involves the production of high-titer, high-affinity autoantibodies that recognize protein antigens. ACA were discovered in 1980, when it was found that certain patients with the calcinosis/Raynauds phenomenon/esophageal dysmotility/sclerodactyly/telangiectasias (CREST) variant of scleroderma produce autoantibodies that recognize the centromere region of chromosomes (9–11). Though ACA are closely associated with the CREST syndrome, the only clinical finding common to all ACA+ individuals is Raynauds phenomenon (12).

Our prior immunoblotting analysis revealed that >96% of the test group of 39 ACA+ sera recognized three chromosomal polypeptides of 17 kDa (CENP-A), 80 kDa (CENP-B), and 140 kDa (CENP-C) (refs. 12 and 13; see also refs. 14–19). Antibodies affinity purified from CENP-B cross-reacted with CENPs A and C, indicating that these antigens are structurally related (13). Antibodies to CENP-B are present at high titer in all ACA+ sera examined, whereas the titer of antibodies to CENPs A and C is occasionally lower (12).

We describe below a detailed examination of the binding of ACA to subdomains of CENP-B that have been cloned and expressed in bacteria. Our experiments reveal that the autoimmune response against this protein is multifocal: as many as five distinct determinants are recognized. Contrary to our prior expectations, the data suggest that ACA arise from a specific polyclonal immune response directed against centromeres.

MATERIALS AND METHODS

General Methods. All cloning methods and procedures have been described in detail elsewhere (20). NaDodSO4/PAGE was performed using the method of Lewis and Laemmli (21). Electrophoretic transfer of proteins to nitrocellulose (22) was performed at 340 mA for 6 hr at 4°C. The immunoblotting protocol has been described (12), as has the method for affinity purification of antibodies from nitrocellulose strips (13). The antibody-blocking experiments were described in ref. 20.

RIA. Fusion protein granules were isolated by differential centrifugation from induced (23, 24) cultures of lysogenic λ-CENP-B (20). Granules were then solubilized in hot urea and dialyzed into 10 mM Tris-HCl, pH 7.7/50 mM NaCl/2 mM EDTA. The yield was 1 mg of CtermCENP-B[β-gal] per liter of bacterial culture. CtermCENP-B[β-gal] in 10 mM imidazole buffer was adsorbed to microtiter plates (0.1 μg per well; Removawell strips, Immulon) that were then probed with a 1:50 dilution of patient serum followed by 125I-labeled protein A; radioactivity was determined in a γ counter as described (25). All assays were performed in triplicate and sera were used in random order.

RESULTS

Molecular Cloning of CENP-B. We previously obtained a series of overlapping cDNA clones corresponding to ~95% of the mRNA encoding CENP-B (20). The availability of the clones permitted us to produce the following series of chimeric proteins as fusions with the bacterial TrpE protein (using the pATH plasmid series of expression vectors, gift of T. J. Koerner, Duke University). With the exception of CtermCENP-B[β-gal] (described below), all chimeric proteins used in the studies presented here were TrpE fusions. (The relative sizes and locations of these proteins are presented in Fig. 5.)

CtermCENP-B[β-gal] consists of the amino-terminal 113-kDa portion of β-galactosidase linked to the carboxyterminal 147 amino acids of CENP-B. This hybrid gene was carried by bacteria lysogenic for the recombinant λ bacteriophage originally detected by autoantibody (20). CtermCENP-B[β-gal] was used to elicit production of two monoclonal ACA, m-ACA1 and m-ACA2, which recognize two nonoverlapping determinants on CENP-B (20).

Abbreviations: ACA, anticentromere autoantibody(ies); CREST, calcinosis/Raynauds phenomenon/esophageal dysmotility/sclerodactyly/telangiectasias.

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CtermCENP-B consists of the 147 carboxy-terminal amino acid residues of cloned CENP-B fused to the bacterial TrpE protein. CtermCENP-BL and CtermCENP-Br were produced by further subdividing the human portion of CtermCENP-B into segments of 104 amino-terminal and 43 carboxy-terminal amino acid residues, respectively, and expressing these as TrpE fusion proteins.

NproxCENP-B is comprised of TrpE linked to 347 amino acid residues from the amino-terminal region of CENP-B (20). (The cDNA encoding the ≈50 amino-terminal residues has not yet been isolated.) The human portion of the protein encoded by this clone is separated in the CENP-B sequence by 98 amino acid residues from that encoded by CtermCENP-B (Fig. 5).

Localization of Centromere Epitope 1 (CE1), a Major Autoepitope in the Carboxy-terminal Region of CENP-B. To identify the epitope(s) in CENP-B that are targets for autoantibodies, we have examined the interactions of a panel of 37 ACA+ patient sera and 3 ACA+ control sera with the cloned CENP-B polypeptide. (All patient sera were previously characterized by indirect immunofluorescence and immunoblotting against the proteins of isolated mitotic chromosomes (12).) Remarkably, all 37 ACA+ sera bound significantly to CtermCENP-B (Fig. 1A). Strong binding was evident for 86% (32 sera) and weaker binding characterized the remaining 14% (5 sera). Control sera (lanes 12, 18, and 40) did not bind the cloned antigen.

In our previous studies of cloned CENP-B, we isolated a murine monoclonal antibody, m-ACA1, that recognizes a site (CE1) present in CtermCENP-B (20). We now show that the site recognized by m-ACA1 overlaps a major autoepitope on CENP-B. Nitrocellulose blots containing CtermCENP-B were cut into 40 strips, each of which was incubated overnight with a 1:50 dilution of human serum. Each strip was then probed with m-ACA1, the binding of which was detected with 125I-labeled goat anti-mouse IgG (Fig. 1D). Pretreatment of the blot strips with any of the 37 ACA+ sera blocked the binding of m-ACA1 to some extent, with 19 sera showing ≥80% blocking (Fig. 1D). The ACA+ sera least effective at blocking the binding of m-ACA1 (sera 8, 20, and 33) were also 3 of the poorest at binding to CtermCENP-B (Fig. 1A), suggesting that the variability of blocking of m-ACA1 may be largely due to variations in the titer of the anti-CtermCENP-B autoantibodies.

CE2, a Second Autoepitope in the Carboxy-terminal Region of CENP-B. A minority of the panel of patient sera recognizes at least one other epitope (CE2) in CtermCENP-B (identified by immunoblotting of bacterial lysates expressing Cterm- CENP-BL, Fig. 1C). Sera 6 and 9 recognized this hybrid protein (and a proteolytic fragment) strongly, whereas weaker binding was exhibited by sera 3, 13, 19, 24, 32, and 38 (making 22% in all).

The location of CE2 was defined by a second murine monoclonal antibody, m-ACA2, previously shown to bind to CtermCENP-BL (20). Three patient sera showed significant blocking of the binding of m-ACA2 (Fig. 1E, lanes 6, 9, and 19). The other five sera that bound to CtermCENP-BL in immunoblots failed to block the binding of m-ACA2 to a significant degree, perhaps due to low titers of anti-CE2 antibodies.

Even though all ACA+ sera bound to CtermCENP-B, only a handful (22%) recognized CtermCENP-BL and none recognized CtermCENP-Br (not shown). Thus, most ACA+ sera apparently recognize only one epitope in CtermCENP-B.

CE3, an Epitope Present on N-proximal CENP-B. To determine whether additional autoantibody binding sites occur outside the carboxy-terminal region of CENP-B, we examined the binding of the panel of 40 sera to NproxCENP-B in immunoblots (Fig. 1B). Ninety percent (33) of the ACA+ sera showed significant binding, although the intensity was much more variable than that observed with CtermCENP-B (compare Fig. 1A and B). Thus, a third autoepitope(s), CE3, is localized within the amino-terminal 60% of CENP-B. The precise location and number of epitopes recognized in this region are not known.

Titer of ACA. We have measured the titers of antibodies against chromosomal CENPs A, B, and C against two regions of cloned CENP-B for one serum (KG). The titer of antibodies specific for each antigen was determined by probing parallel blot strips of chromosomal proteins with serial dilutions of the patient serum (Fig. 2). Positive signals were obtained in overnight exposures for the following serum dilutions: CENP-A, 1:1,638,400; CENP-B, ≥1:3,276,800; and CENP-C, 1:1,800 (lanes 1–9). Thus, while the titers of antibodies against CENPs A and B are comparable, antibodies to CENP-C are less abundant (roughly by a factor of 200). (Note, however, that the three chromosomal antigens may be present in differing amounts.) When the serum was titered against cloned CtermCENP-B, a positive signal was obtained at an antibody dilution of 1:4,096,000 (Fig. 2, lane 14). The titer of antibodies recognizing NproxCENP-B was also high, with a positive signal being observed at an antibody dilution

**Fig. 1.** Binding of a panel of 37 ACA+ sera and 3 control sera (lanes 12, 18, and 40, indicated by a *) to various regions of cloned CENP-B. (A–C) Binding of the sera to blots containing CtermCENP-B (A), NproxCENP-B (B), and CtermCENP-BL (C) (see Fig. 5 for a diagram of these polypeptides). In each case a nitrocellulose strip cut from a blot of the appropriate bacterial lysate was incubated with the indicated patient serum. Blots were processed for antibody detection as described (12). Only the region of antibody binding is shown. (D and E) Binding of the autoantibodies to sites on CtermCENP-B defined by monoclonal antibodies m-ACA1 (D) and m-ACA2 (E). In these experiments, a strong signal is seen when the human serum does not recognize the site (i.e., does not block the binding of the monoclonal antibody).
sera for binding to Cterm CENP-B[β-gal]. We also analyzed 20 sera from normal individuals and 92 ACA+ patient sera. The latter included all those available to us from patients with Raynaud phenomenon and scleroderma (with or without full or partial CREST) as well as randomly chosen sera from individuals with systemic lupus erythematosus and Sjögren syndrome. Virtually every ACA+ patient serum binds to this portion of CENP-B [Fig. 3, except for two sera, both characterized by the presence of substantial levels of anti-Cterm CENP-B of the IgG3 subtype, which is not recognized by protein A (R. A. Eisenberg, B.J.B., W.C.E., and N.F.R., unpublished data)]. The average values for the three serum classes shown were normal control sera, 195 ± 100 cpm; ACA+ patient sera, 284 ± 187 cpm; and ACA+ patient sera, 6013 ± 4809 cpm (a 22-fold stimulation for ACA+ sera over ACA− control sera).

In a control experiment, the binding of a number of randomly chosen ACA+ and ACA− sera to Cterm CENP-B[β-gal] and to β-galactosidase was examined (Fig. 4A). (The latter comprises 87% of the mass of Cterm CENP-B[β-gal]). A rabbit antiserum (elicited by injection with Cterm CENP-B[β-gal], ref. 20) was included as a positive control. This serum bound to both substrates in this assay (Fig. 4A, lane 4). However, none of the patient or control sera exhibited significant binding to β-galactosidase.

A quantitative immunoblotting assay also confirmed that the binding observed in the RIA was specific for the human portion of Cterm CENP-B[β-gal]. Bacterial lysates containing Cterm CENP-B (fused to TrpE and therefore containing no bacterial sequences in common with Cterm CENP-B[β-gal]) were subjected to NaDodSO4/PAGE and immunoblotting (Fig. 1A). The region of the nitrocellulose containing the fusion protein with its bound autoantibody and 125I-labeled protein A was excised and counted in a γ counter. The results for the panel of 40 sera are shown in Fig. 4B along with the values obtained for these same sera by RIA. The striking

Fig. 3. Use of Cterm CENP-B[β-gal] in a RIA to detect ACA. The RIA shows binding of various patient and control sera to Cterm CENP-B[β-gal]. Each dot is the average of three measurements for the serum from a different individual. (Inset) Isolation of partly purified Cterm CENP-B[β-gal] from induced lysogens. Lane 1, marker proteins (molecular masses indicated in kDa at the left of the gel). Lanes 2–4, Coomassie blue staining of proteins of whole cell lysate (lane 2), soluble protein fraction (lane 3), and final fraction (lane 4). Lanes 5–7, immunoblotting analysis of the samples of lanes 2–4 using patient serum GS (1:1000).

Fig. 4. Demonstration that binding in the RIA is specific for cloned human CENP-B. (A) Test for the binding of a random panel of control and patient sera to β-galactosidase and Cterm CENP-B[β-gal] by RIA. The sera used were normal controls (1–3), serum from a rabbit immunized with Cterm CENP-B[β-gal] (contains both anti-CENP-B and anti-β-galactosidase) (4), ACA+ patient control sera (5–9), and ACA+ patient sera (10–21). (B) Comparison of the RIA of Fig. 3 and the immunoblotting results of Fig. 1A. The relevant portions of the immunoblots shown in Fig. 1A were excised and counted in a γ counter. The two sets of results were normalized to give an equivalent average value.
correlation exhibited by the two data sets confirms that the RIA detects the binding of autoimmune sera to the human portion of CENP-B.

DISCUSSION

Classes of ACA. Our analysis of the binding of ACA to cloned portions of CENP-B expressed in bacteria identifies three epitopes, CE1, CE2, and CE3, that are targets for the ACA autoimmune response. These results, in conjunction with our previous work, define at least six independent CE1 binding observed with antibody, CE1 that Because of the close correspondence between the levels of CENP-B. It is strongly recognized CtermCENP-B. This epitope is recognized by all) of the remaining distinct structural determinants, since affinity-purified sera tested, and, even in the sera anti-CE3 do not cross-react sera, but lower titers of anti-CE3 ACA given determined by immunoblotting (Fig. 4B), it is likely that CE1 is the principal determinant being recognized in the RIA. CE1 is recognized by virtually all ACA+ patient sera previously identified by indirect immunofluorescence.

CE2 is located within a 104 amino acid stretch starting 43 amino acids upstream from the carboxyl terminus of CENP-B. This epitope is recognized by only ~20% of the patient sera tested, and, even in the sera that interact most significantly with it (Fig. 1C, lanes 6 and 9), anti-CE2 comprises only a small fraction of the antibodies that recognize CtermCENP-B.

CE3 is located somewhere within the amino-terminal 60% of CENP-B. It is strongly recognized by a minority of ACA+ sera, but lower titers of anti-CE3 ACA are present in most (or all) of the remaining ACA+ sera. Overall, ~90% of the ACA+ sera exhibit detectable binding to CE3, CE1 and CE2 are distinct structural determinants, since affinity-purified anti-CE1 and anti-CE3 do not cross-react (20).

Three additional autoepitopes recognized by ACA were previously identified by analysis of the binding of various affinity-purified patient sera to chromosomal antigens in immunoblots (13). The first of these, CE4, is present on CENPs A and B and is absent from CENP-C (defined by sera GS and SN, ref. 13). We demonstrated that ~97% (38/39) of a panel of 39 ACA+ patient sera had anti-CENP-A detectable by immunoblotting (12). We assume that all sera binding to CENP-A bind to CE4, although the structure of this antigen may be more complex. Epitope CE5, shared by CENPs B and C, was defined by antibodies from serum JR, affinity-purified from CENP-C, that were subsequently found to cross-react strongly with CENP-B (13). Finally, CE6, found solely on CENP-C, was defined when antibodies were affinity purified from CENP-C (using serum GS) that showed no rebinding to CENP-B (13). The pattern of binding observed using antibodies affinity purified from CE1, CE3, CE5, CE6, and CE1 indicates that all of these determinants are structurally independent (13, 20).

Solid-Phase Binding Assay for ACA. Antibodies to CENP-B appear to be diagnostic for ACA, since all ACA+ patient sera we have tested (105 sera, from Farmington, Baltimore, Montreal, Houston, La Jolla, Nijmegen, and Heidelberg), recognize chromosomal CENP-B in immunoblots. We have yet to observe anti-CENP-B in any ACA+ patient (365 tested) or normal control (32 tested) serum (W.C.E., B.J.B., and N.F.R., unpublished). Moreover, a RIA based on cloned CtermCENP-B[β-gal] provides a sensitive, reliable method for the detection of ACA. This assay may eventually provide an alternative method for screening large numbers of patient sera for ACA in the clinic.

Origin of ACA. Although much progress is being made in identifying and characterizing autoantigens, the origin of antinuclear autoantibodies remains obscure. It has been suggested that autoantibodies might arise as a result of fortuitous cross-reactions exhibited by normal antibodies (28, 29), as a result of chance mutations causing normal B-cell clones elicited by foreign antigen to change specificity and recognize self components (30, 31), or as idiotypes elicited during an immune response against a viral protein (7). Our data are inconsistent with all such models. These models could explain an autoimmune response against any single epitope, but they cannot account for the ACA response, since multiple structurally independent epitopes are targeted in virtually every affected individual (requiring multiple chance events).

Two of our observations presented above suggest that ACA might arise from an antigen-driven response. (i) Cloned CENP-B [ unlike double-stranded DNA (32)] is immunogenic in rabbits and mice (20). (The Sm antigen (a marker antigen for systemic lupus erythematosus) is also immunogenic in rabbits and mice (33).] (ii) ACA are polyclonal. Serum from single patients recognizes at least four independent epitopes on CENP-B. We have shown here that >90% of ACA+ sera recognize CE1 and CE2 and have shown elsewhere that ~95% of the sera also recognize CE4 and either CE5 or CE6 (12). Thus, CENP-B appears to be the target of a diversified polyclonal response. This was postulated earlier to be true for anti-Sm (33, 34) and antinucleoprotein (35) autoantibodies, but in neither case was the conclusion confirmed by isolating multiple noncross-reactive specificities from patient serum.

In a recent review, Hardin (6) noted that most major autoantibodies found in systemic lupus erythematosus recognize nucleoprotein structures (small nuclear ribonucleoproteins, cytoplasmic ribonucleoproteins, and nucleosomes). Our data suggest that ACA fit this pattern and that the ACA response may result from self immunization with centromeres. This is surprising, since centromeres are extremely minor cellular components and as such seem unlikely can-
candidates for the progenitors of a high-titer immune response (particularly since ACA occur in only a small fraction of the autoimmune patients who make antinuclear antibodies). Monoclonal antibody m-ACA1, which we have shown here to bind to a major autoepitope in CENP-B, should provide an important tool for future studies that attempt to locate the source of centromere antigen in affected individuals.

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