Antisera Directed against Anti-Histone H4 Antibodies Recognize Linker Histones

NOVEL IMMUNOLOGICAL PROBES TO DETECT HISTONE INTERACTIONS*

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We introduce a novel immunological approach to detect structural interactions between chromosomal proteins. Antigenically pure core histone H4 was prepared from chicken erythrocytes and used to produce anti-histone H4 antisera. IgG fractions were isolated from purified anti-H4 antibodies and used as antigens to produce "second generation" antisera. Epitopes cross-reacting with the second generation antisera were then identified within chromosomal proteins. These epitopes were presumed to mimic the complementary molecular surface of the original anti-H4 antibodies, and thus proteins containing these epitopes were putatively identified as specific ligands of H4 in chromatin. Surprisingly, we found this immunoreactivity was predominantly directed against H1 compared with H5 from chicken erythrocytes. Further, the immunoreactive epitopes were located within the C-terminal tail domain of the linker histones. These results suggest similar complementary interactions occur between H4 and the C-terminal tail domain of H1s in native chromatin. This could occur either within a single nucleosome or as suggested by a previous report (Banères, J.-L., Essalouh, L., Jariel-Encontre, I., Mesnier, D., Garrod, S., and Parello, J. (1994) J. Mol. Biol., 243, 48–59) or between neighboring nucleosomes within the condensed chromatin fiber. The implications of these results with regard to the structure of the chromatin fiber and the future utility of this technique are discussed.

The primary repeating subunit of chromatin is a protein-DNA complex composed of a histone protein octamer, (H2A/H2B-H4/H3-H3/H4-H2B/H2A), a single linker histone protein, and approximately 200 base pairs of DNA (1, 2). Adjacent nucleosomes are connected together by a DNA linker of variable length depending on the species (3). Nucleosomal structures are generally considered to have a fundamental role in many nuclear activities which involve DNA, such as transcription, replication, recombination, and DNA repair (2). Modifications of histone proteins may modulate the condensation/decondensation of the chromatin fiber, which in turn changes the accessibility of the DNA to enzymes or factors involved in these nuclear activities (2, 4–8).

The histone octamer is comprised of four heterotypic dimers comprised of either H2A and H2B or H3 and H4. The majority of each histone protein is contained within an evolutionarily conserved structure known as the “histone fold,” which is comprised of a long, central α-helix flanked on each side by a short loop and a short α-helix (9–11). Two histone fold proteins interlock via the “handshake” motif with extensive complementary surfaces between heterodimerization partners (10). Histone dimers associate in an end-to-end fashion to form essentially a H2A/H2B-H4/H3-H3/H4-H2B/H2A helical tetramer of dimers onto which the DNA is wrapped (9, 10, 12).

Despite the well characterized nature of intra-core histone interactions, other histone-histone interactions likely to be found in chromatin have not been as well described. For example, in the compacted chromatin fiber it is likely that core histone proteins from nearby nucleosomes are brought into close proximity, and it has been proposed that inter-nucleosomal core or even histone-histone interactions are instrumental in directing the condensation of the fiber (13, 14). One manifestation of these interactions may be the propensity with which core histone octamers “close-pack” together when reconstituted with long DNA in vitro in the absence of linker histones (15). In addition, the interactions of many non-histone chromosomal proteins such as HMGs 1/2 or 14/17, which are likely to interact with multiple proteins within chromatin (16), have not been well defined.

Immunological approaches for analyzing the structural relationships between chromatin proteins have been extremely useful in the investigation of protein structures within chromatin (17). Indeed, since Stollar and Ward (18) first demonstrated that histones are adequate immunogens when complexed with RNA, numerous laboratories have obtained anti-histone antisera. The use of such antisera and anti-histone antibodies as probes has allowed the detection of many details of the surface of chromatin subunits, despite the limited amount of information concerning the molecular structure of epitopes found at the surface of these structures (19, 20). Further, Muller and colleagues have demonstrated that the production of antisera to synthetic peptides that mimic linear histone regions (21) could be used to detect the histone regions accessible to anti-peptide antibodies inside chromatin (4, 19–21). Anti-histone immunochemical reagents also have been instrumental in the elucidation of the role of histone posttranslational modifications in nuclear processes such as transcription and replication (6, 22, 23).

In the present report, we present a novel immunological approach to detect potential protein-protein interactions within chromatin. This approach is based on the complementarity of surfaces that are in contact within interacting systems. This phenomenon is exemplified by hormone/hormone receptor systems in which it has been demonstrated that antibodies to anti-hormone antibodies are able to mimic physiological effects of hormones (reviewed in Ref. 24; see "Discussion"). This effect is due to the complementary structures of the receptor and ligand within these systems and the fact that anti-
anti-hormone antibodies recapitulate many of the key facets of the original hormone structure (see Fig. 1). Here we demonstrate that IgG fractions from anti-H4 antibodies are able to induce production of antibodies that react with the original IgG fractions and also demonstrate specific immunoreactivity against the C-terminal tail domain of linker histones.

MATERIALS AND METHODS

Preparation of Histone H4—Chicken erythrocyte nuclei were isolated according to the procedure of Nethacker and Hildebrandt (25) with the modifications described by Loidl and Grohner (26). Histones were extracted from nuclei with H2SO4 as described by Helliger et al. (27). Histone H4 was purified by chromatography on a Bio-Gel P-60 (Bio-Rad) column (28), followed by a series of chromatographic fractionations on Sephadex G-100 column (Pharmacia Biotech Inc.) equilibrated in 50 mM sodium acetate successively at pH 5 and 4 as reported by Muller and Van Regenmortel (29). Rabbit antisera immunoreactive against all histone proteins (RAAHC)1 was prepared using acid extractable proteins from chicken erythrocyte nuclei. The purity of histone H4 was then assessed by Western blot probed the RAAHC antisem.

Preparation of Antibodies—Antiserum against purified histone H4 of chicken erythrocyte were obtained by immunizing mice biweekly with H4/RNA complexes (3.1, w/w) as previously reported by Stollar and Ward (18). For each vaccination, each animal received by subcutaneous injection 10 μg of histone H4 emulsified in complete Freund’s adjuvant for the first injection or in incomplete Freund’s adjuvant for the subsequent injections. Antiserum against IgG fractions either from anti-H4 antibodies or from antibodies recognizing no histone (control) were obtained by immunizing mice biweekly by subcutaneous injections with 5 μg of IgG molecules emulsified in complete Freund’s adjuvant for the first injection or in incomplete Freund’s adjuvant for subsequent injections. Antiserum was collected at regular intervals over a period of 20 weeks.

Preparation of Immunoglobulin G Fractions—Specific antibodies against H4 were isolated from anti-H4 antisemum by immunoaffinity on a nitrocellulose sheet according to the procedure described by Olimsted (30), with minor modifications. Briefly, chicken erythrocyte histones are resolved by preparative SDS-PAGE, electroblotted onto nitrocellulose sheets (31). The edge of the membrane was stained with Amido Black to localize histone H4 on the nitrocellulose sheet. After cutting out the band corresponding to H4, the nitrocellulose strip is saturated with 1% ovalbumin in PBS-T (phosphate buffered saline with 0.05% Tween 20). Then the strip was incubated with anti-H4 antiserum diluted 1:10 in PBS-T for 1 h under gentle agitation. Unbound components from the antiserum were removed by washing the nitrocellulose strip three times in PBS-T for 5 min each with gentle agitation. Specific H4-antibody complexes were then eluted by washing the strip in 0.1 M glycine-HCl, pH 2.5, three times for 5 min each at room temperature with gentle agitation. The three acid washes were combined, the pH of the solution was adjusted to 7 by addition of 1 M Tris-HCl, pH 9, and the solution was dialyzed overnight against PBS. Control serum was prepared from mice immunized with mouse IgG molecules depleted of any trace antibody immunoreactivity by exhaustive immunoaffinity on nitrocellulose sheets onto which all histone proteins had been adsorbed. This procedure was repeated three times, and then fractions were dialyzed overnight against PBS. Control and anti-H4 IgG fractions were then isolated by affinity chromatography on Sepharose CL-4B covalently linked with Staphylococcus aureus protein A (Pharmacia). Protein A-antibody complexes were separated as described above, the IgG fractions were finally dialyzed overnight against PBS, and the absence of detectable amounts of histones was confirmed by Western blot.

Immunoblotting—Protein samples were resolved by SDS-PAGE (32) and then electroblotted onto nitrocellulose sheets (31). The nitrocellulose sheets were blocked in (PBS-T) and 1% ovalbumin for 30 min at room temperature. The filters were then incubated with diluted primary antisem for 1 h, washed three times for 5 min with PBS-T, and incubated for 1 h with diluted peroxidase-conjugated anti-IgG (Jackson, Interchim, France) or with peroxidase-coupled protein A. The membranes were finally washed thoroughly in PBS-T, and peroxidase was detected as described by Harlow and Lane (33). The same procedure was followed for the Dot Blot assay. For inhibition experiments, non-immune serum, anti-H4 antisem, or purified histones were incubated overnight at 4 °C with antiserum directed against anti-H4 IgG fractions. After centrifugation, the supernatant was used to immunoprobe histones resolved by SDS-PAGE. The reactivity of immune response was monitored by measurement of the optical density with a gel scan analyzer (Vilber Lourmat, France).

Preparation of Linker Histones—Calf thymus and chicken erythrocyte linker histones were prepared as described previously (34). Bacterially expressed H1°a and the C-terminal deletion mutant of this protein containing residues 1–101 were prepared as described elsewhere (35). Purified Tetrathyamina H1 was a gift from Dr. David Allis, University of Rochester. H1 globular domain was prepared by treating 50 μg of total acid-extracted calf thymus H1 with 100 ng of trypsin in 10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl for 45–60 min at room temperature. The reaction was terminated by the addition of an excess of soybean trypsin inhibitor (Worthington) and used directly for SDS-PAGE.

RESULTS

Production of Specific Anti-H4 Antiserum—Our approach was to raise antibodies against purified anti-H4 antibodies and then to determine which chromatin proteins cross-react with this antisem (Fig. 1). Thus, we required antigenically pure H4. To ensure the purity of our H4 preparation we first isolated this histone by chromatographic methods and detected the protein by Western blot of fractions resolved on SDS-PAGE with antiserum directed against all histone proteins (RAAHC, see “Materials and Methods”). We obtained a fraction that clearly yielded a single band corresponding to histone H4 (data not shown but see Fig. 2). We then immunized three mice with the highly purified H4 fraction and monitored the resulting immune response over an extremely long immunization period. The results obtained with one animal are shown in Fig. 2A and correspond to the intensity of the immune response 1 week after each vaccination with the antigenic mixture. We observed that the immune response remained specific to histone H4 throughout the immunization period, although insignificant responses against H3 were observed at the beginning of the series of immunizations with this animal. Moreover, from the 5th to the 10th and final vaccination, the intensity of the immune response against H4 did not increase, but the antisem remained specific to histone H4. It is clear from the Western blot presented in Fig. 2B corresponding to the analysis of antisem obtained after the 10th immunization that this antisem is specific only to histone H4 when it is tested against all acid-extractable nuclear proteins. Thus, we have obtained even after a long immunization period specific antisem directed against histone H4, which is considered to be the least immunogenic chromosomal protein (17). These results show

1 The abbreviations used are: RAAHC, rabbit antisem raised against all histone proteins; MAH4, mouse antisem raised against the anti-H4; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.
that our H4 preparation is highly antigenically pure and raises an immune response only against H4 protein within the mice. The antiserum produced against this preparation of H4 was selected as a source of anti-H4 antibodies for further studies and will be referred to as MAH4 antiserum.

IgG Fractions from Anti-histone H4 Antiserum Mimic Histone Epitopes—To easily determine whether the IgG fractions from anti-H4 antiserum were able to mimic any histone epitopes, we carried out the Dot Blot shown in Fig. 3. Different amounts of IgG fractions purified from MAH4 antiserum or mouse preimmune serum were deposited on a nitrocellulose sheet. The IgG fractions were then probed with RAAHC. Note that the use of the IgG fractions from two different species was required to ensure that secondary antibodies only will detect interactions between the IgG from MAH4 antiserum (mouse) and the RAAHC antiserum (rabbit) used as primary antibodies. We observed significant immunoreactions with the IgG fractions from MAH4 but not those prepared from preimmune serum. Clearly IgG molecules from anti-histone H4 antiserum are capable of mimicking histone epitopes. As a control we also determined that the anti-H4 IgG preparation exhibited no detectable immunoreaction against the co-injected RNA (results not shown).

Antisera against IgG Fractions from Anti-H4 Antibodies Recognize Linker Histones—We wished to determine by immunochromal means which histone proteins contained the epitopes mimicked by the anti-H4 IgG fractions. To this end, we first purified the antibody fraction directed against H4 by nitrocellulose immunoaffinity fractionation of the antiserum as described previously by Olmsted (30). The purified antibody fraction was then deposited on a protein A column, and the IgG fractions were purified from other anti-H4 antibodies. The purity of the IgG fractions was finally assessed with RAAHC antiserum. The results presented in Fig. 4 show that there is no evidence of any histone protein contaminating these purified anti-H4 IgG fractions. Interestingly, in this Western blot, we observe immunodetection of the heavy chains of the IgG fractions, even when the RAAHC antiserum was omitted (data not shown). It seems therefore that denaturation of mouse IgG molecules in SDS-polyacrylamide gel exposes common epitopes between mouse IgG and rabbit IgG molecules, which are not revealed when the IgG molecules are folded in their native state (compare the IgG from preimmune serum in Fig. 3 and the IgG lane in Fig. 4).

The purified anti-H4 IgG fractions were then used as antigens to induce antibody production in mice. Three animals were immunized by a series of subcutaneous injections of 5 μg each of anti-H4 IgG molecules over a period of 5 months. After a series of immunizations one mouse failed to respond against histone, but the other two mice induced similar and satisfactory immune responses. As a control for any immune response against histones due to the IgG molecules themselves, several mice were immunized with mouse IgG molecules that had been exhaustively depleted of immunoreactivity against any of the histone proteins (see “Materials and Methods”).

Sera from mice immunized with the purified anti-H4 IgG fractions were tested for immunoreactivity against all histone subtypes. Western blots of acid-extracted chromatin proteins were probed with antiserum from the two mice that developed antibodies against histones and from two control mice immunized with IgG fractions depleted of anti-H4 immunoreactivity.
A time profile of the immunological response from the serum obtained from these mice is shown in Fig. 5. After one immunization with purified anti-H4 IgG an unusually rapid and very weak response was detected against histone H3 (Fig. 5). In contrast, after seven to eight immunizations with the purified anti-H4 IgG fractions, a much stronger and more typical response was detected against linker histones (Fig. 5). Moreover, the intensity of immune response against linker histones increased to much higher levels than the response against histone H3 (Fig. 5B). No reactivity against either linker histone or H3 is detected with antisera raised against IgG molecules that were depleted of anti-histone immunoreactivity (Fig. 5A). Furthermore, no such reactivity was observed after similar numbers of immunizations with purified H4 (Fig. 4) or with over 50 mice immunized with various histone antigens.2

To confirm that the specific immunoreactivity we detected was actually induced by the H4-complementary portions of the anti-H4 antibodies, we carried out inhibition assays. Increasing quantities of either control serum or anti-H4 antiserum (MAH4 antisera) were added to the mouse antiserum raised against the anti-H4 IgG fractions, and the effects on the immunoreactivity of the latter serum were determined. When increasing amounts of the control serum were added, there was no evidence of any inhibition of the immunoreactivity of the mouse antiserum raised against anti-H4 IgG to either immobilized linker histones (Fig. 6A) or H3 (results not shown). In contrast, increasing quantities of MAH4 antiserum clearly inhibited the binding of anti-anti-H4 IgG fractions to these immobilized proteins. Moreover, Western blots of IgG fractions purified from anti-H4 antiserum and probed with antiserum from mice immunized with these antisera showed no evidence of any specific recognition of denatured IgG molecules and the antiserum (results not shown). This result strongly suggests that only native IgG purified from anti-H4 antibodies and not the IgG molecules themselves are able to induce immune responses against histones and that antibodies raised against anti-H4 IgG fractions are directed against the idiotopes of the anti-H4 antibodies. We thus conclude from these experiments that IgG fractions from anti-H4 antibodies induced the production of antibodies that are directed against linker histones, and that this effect is dependent upon the original H4 epitopes.

We wished to further isolate the epitopes responsible for the observed specific immunoreaction. Because of the atypical and weak profile of reactivity detected against H3, we chose to further characterize only the immunoreactivity detected against linker histone. A blot of H1s from calf thymus, bacterially expressed *Xenopus* H1*, and linker histones isolated from chicken erythrocytes and *Tetrahymena* revealed strong reactiv-

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2 C. Thiriet, unpublished results.
bodies also specifically recognized linker histones and, perhaps, of antibodies against the antigen but, significantly, these anti-IgG fractions from anti-H4 antibodies induced the production of antibodies directed against other histone proteins and against histone H4 can serve as antigens to induce the production of antibodies against the antigen but, significantly, these antibodies also specifically recognized linker histones and, perhaps, to lesser extent histone H3. However, the kinetics of these two responses were quite different (Fig. 6), and it is uncertain why the response against H3 was so unexpectedly rapid and weak in nature, and why it did not increase with subsequent injections. Nonetheless, the response against linker histones followed a kinetic profile typical for an immunogenic reaction and yielded a much higher antibody titer. For these reasons we further characterized only the anti-linker histone immunoreactivity.

Several lines of evidence suggest that the specific response observed is most likely due to antibodies produced specifically against anti-H4 IgG. First, immunization with IgG fractions depleted of anti-H4 immunoreactivity did not give rise to antisera that exhibit immunoreactivity against any histone proteins. Second, we found the response is specific, such that either linker histones or the purified anti-H4 IgG fraction can effectively inhibit the anti-linker histone reactivity of antisera raised against anti-H4 IgG. Finally, similar specific responses were obtained with two mice against only certain histone proteins and were not obtained with control mice immunized with control IgG fractions. Interestingly, the immunoreactivity is largely directed against the H1-type histones relative to the H5 or H1° subtypes (Fig. 6). Thus, anti-idiotypic reactivity is predominantly against the least-represented subtype of linker histone found in the erythrocytes from which the H4 was prepared, arguing against contamination of the H4 preparation by linker histone.

Previous work has demonstrated that H1 exists in close proximity to the N-terminal tail of histone H4 within chromatin (36). We detected cross-species H1 reactivity, suggesting the putative H4-H1 interaction is a general feature of H1-containing chromatin. We have demonstrated that the simultaneous recognition of IgG fractions from anti-H4 antibodies and the histone proteins occurs via the same immunoreactive entity. Thus both the IgG fractions and linker histones possessed elements of complementary structures with the anti-anti H4 antibodies (summarized in Fig. 1). Interestingly, the immunoreactive epitopes existed within the C-terminal tail domain of H1 (Fig. 7). We conclude that the C-terminal tail domain of H1s must possess surfaces or structures that are complementary with histone H4 and that possibly the H4 N-terminal tail...
domain specifically interacts with linker histones in vivo.

A recent model of the binding of linker histone to the nucleosome suggests that the globular domain of this protein is situated near a point where the H4 N-terminal tail cross-links to the DNA (37, 38). This would bring the H1 C-terminal domain into close proximity to the H4 tail. Alternatively, it is possible that H4 makes contact with linker histone internucleosomally, i.e. between neighboring nucleosomes, which are made when the chromatin fiber is compacted. Experiments to determine the availability of linker histone epitopes in extended and condensed chromatin fiber are presently underway. Further characterization of the epitopes which exhibit complementary structures with H4 can be made by employing peptides from identified proteins (21). Preliminary results with the mouse antisera raised against anti-H4 IgG used in the present work suggested that the epitopes within linker histone are located near the end of the C-terminal tail domain. It is interesting to note that antibodies induced by immunization with anti-histone antibodies possesses a high degree of specificity for histones. The use of monoclonal antibodies may also allow a finer determination of exactly what epitopes are complementary but the production of monoclonal antibodies against histones is somewhat problematic (39–42).

Clearly, our approach does not detect all species interacting with the substrate in question. Within the histone octamer, H4 makes some contacts with histones H2B at the dimerization interface between the H4/H1 and H2A/H2B dimers (9). There are several possibilities for absence of reactivity of anti-anti-H4 with H2B. All regions of H4 are not immunogenic (4), and it is possible that the anti-H4 IgG fraction may not contain antibodies specific for the region of H4 that interacts with H2B. It is possible that this surface within H4 may be somewhat masked within the H4-RNA complex used to raise the initial anti-H4 antisera. The conformation of H4 within the H4-RNA complex is probably very different from that when this protein is complexed with histone H3 (43). Thus, the H2B-interacting surface of H4 may be more properly presented within the (H3/H4)2 complex. Experiments to test this possibility are underway.

We have investigated whether the histones interacting with H4 could be detected by antibodies by using the strategy successfully applied to hormone/hormone receptor systems (reviewed in Ref. 24). The novel immunological probes described in the present work demonstrate that the interactions between H4 and the linker histone are probably due to the recognition of complementary motifs found at the surface of these histones. Future applications of this approach could detect other interactions between histone proteins which may be relevant to contacts made between nucleosomes within the chromatin fiber as well as shed light on the poorly defined protein-protein interactions made by the many non-histone chromosomal proteins within chromatin.

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