A novel centromere monospecific serum to a human autoepitope on the histone H3-like protein CENP-A

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Abstract Centromere autoantibodies are commonly found in the serum of patients with some systemic autoimmune diseases. Previous studies have shown that a major human centromere autoantigen is the histone H3-like protein CENP-A. Although the human cDNA has been cloned, native CENP-A has been neither isolated nor expressed in *Escherichia coli*, and specific antibodies to this chromatin-associated centromere protein are not available yet. In this report, a highly charged peptide on CENP-A (residues 3–17) was used to generate a monospecific antibody that reacts by immunoblots with the 17 kDa centromeric protein. Immunofluorescence analysis showed reactivity of this anti-CENP-A serum in several but not all mammalian culture cells analyzed, suggesting that the sequence of this histone-like centromere protein could be more variable throughout evolution than originally thought. Selective extractions of human placenta nuclear proteins and immunoblot analysis indicated that CENP-A behaves in a similar way to the core histone polypeptides after nuclease digestion of chromatin. Also, immunoblot analysis demonstrated that the CENP-A peptide used as immunogen is a target region on the CENP-A molecule in several but not all CREST patients analyzed with high titers of autoantibodies to the centromere. Lastly, we found that in Jurkat cells induced to apoptosis, CENP-A remains associated with the centromere, in contrast to other human autoantigens studied during apoptosis.

Key words: Centromere; CENP-A; Autoantigen; Anti-peptide serum

1. Introduction

The discovery of centromere-specific autoantibodies in patients with limited systemic sclerosis (CREST syndrome) and the characterization of the antigens recognized by these sera have provided tools for the analysis of centromere structure and function in mammalian cells [1–4]. Three major antigens, the centromere proteins CENP-A, CENP-B and CENP-C, were initially identified, with CENP-B being the centromere autoantigen best characterized so far. CENP-B is a 80 kDa protein that has been cloned in human [5], mouse [6] and hamster [7]. It is located in the central domain of the centromere, where the protein binds to a subset of the alphoid satellite DNA that comprises the major DNA component of the human centromere, and may play a role in the higher order folding of alphoid chromatin through self-assembly [8]. However, several reports have presented evidence both for and against a direct role of CENP-B in centromere function [9]. On the other hand, CENP-C is a highly basic 140 kDa protein that has been cloned in human [10], mouse [11] and sheep [12]. It is located in the inner kinetochore plate, playing a critical role in the assembly of the kinetochore. Several classes of antibodies produced in vitro have contributed to the specific analysis of the putative function of the centromeric proteins CENP-B and CENP-C, in studies such as microinjection of cell cultures. In addition, CENP-A is a 17 kDa protein that has only been cloned in human, although evidence for its expression and putative binding to the centromere of other mammalian cells has been presented [13,14]. CENP-A shares a similar organization with the histone H3 protein [6,15,16]. The high degree of sequence identity seen between CENP-A and histone H3 suggests that CENP-A acts in a certain specific set of centromeric nucleosomes [9]. However, sequence analysis revealed that CENP-A possesses an N-terminal domain divergent from that of histone H3.

In contrast to CENP-B and CENP-C, to our knowledge CENP-A cDNA has not been expressed in *Escherichia coli* systems, and monospecific antibodies to this protein are not available yet. This type of monospecific probe could be valuable for studying the role of CENP-A in chromatin assembly at the centromere and for a better understanding of the association of the protein with the active kinetochore in mitosis and meiosis. Recently a human autoepitope on the CENP-A molecule has been indicated in a large number of CREST patients showing anti-centromere antibodies [17].

In this report, we describe for the first time a novel centromere serum to CENP-A generated in vitro against an N-terminal peptide of the CENP-A molecule. Our results indicate conservation of a human CENP-A autoepitope in other mammalian centromeres analyzed. Thus, CENP-A seems to play a universal role at the structure of mammalian centromeric chromatin organization.

2. Materials and methods

2.1. Reagents

Peroxidase-conjugated goat anti-rabbit IgG and fluorescein-conjugated goat anti-rabbit IgG were from Boehringer Mannheim Biochemicals (Germany); anti-Fas serum was kindly provided by Dr. José Brieva of the Puerta del Mar Hospital, in Cádiz; nitrocellulose blotting membranes (0.45 μm) were from Millipore (Bedford, MA, USA). DMEM and fetal calf serum were from BIO-Whitakker (Belgium). All other reagents were of analytical grade from Sigma.

2.2. Cell culture

HeLa, Indian muntjac and Jurkat cells were grown in DMEM/10% fetal calf serum in coverslips until 75% confluence before being used for immunofluorescence. When needed, anti-FAS/APOI serum was used to induce apoptosis in Jurkat cells for 3–6 h before immunofluorescence analysis.

2.3. Peptide synthesis and antibody production

Rabbit polyclonal antibodies were raised against the synthetic pep-

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tide PRRRSRKPEAPRRRS which corresponds to the N-terminal amino acid sequence (residues 3–17) of human CENP-A. This peptide was described by Muro et al. as a human autoepitope in centromere reactive sera [17]. A carboxy-terminal cysteine was added to the peptide for sulfhydryl coupling. Conjugation of the peptide to keyhole limpet hemocyanin (KLH) was performed as described [18]. The rabbit antibodies were generated by Eurogentec (Belgium).

2.4. Immunofluorescence

Culture cells were fixed and permeabilized in pure methanol at −20°C for 10 min and incubated with the primary anti-CENP-A antibody (dilution 1:400) in PBS for 30 min at 37°C. Thereafter, cells were washed three times with PBS and incubated for 30 min at 37°C with the secondary antibody fluorescein-conjugated goat anti-rabbit IgG (dilution 1:80) in PBS. Cells were washed three times with PBS, counterstained with DAPI (4′,6-diamidino-2-phenylindole, Sigma) (0.5 μg/ml) for DNA and mounted with PBS:glycerol (1:9). Images were taken using a Zeiss Axiophot fluorescence microscope. Jurkat cells were used for immunofluorescence, at time 0 and 6 h after induction to apoptosis with anti-Fas serum (100 ng/ml) [19].

2.5. Immunoblot analysis

Preparation of human placenta nuclear proteins was a modification of a previous method [4]. Briefly, human placenta tissue was cut with scissors into small pieces in TBS solution (10 mM Tris-HCl pH 8.0, 100 mM NaCl) containing a protease inhibitor cocktail (Boehringer Mannheim, Germany) (TBS buffer). Nuclei were isolated after a brief homogenization in a Dounce, by using 1% Triton X-100 (Sigma) in TBS buffer, followed by a centrifugation step at 3000 rpm for 10 min at 4°C. Isolated nuclei were incubated with micrococcal nuclease (Boehringer Mannheim) (200 units/ml) in TBS buffer containing 1 mM $CaCl_2$ for 30 min at 4°C. After a short centrifugation at 10 000 rpm for 10 min at 4°C, the nucleus material was incubated with heparin (1 mg/ml) (Sigma) in TBS buffer containing 2 mM EDTA for 30 min at 4°C. After a further centrifugation step the digested nucleus material was sonicated and stored like the rest of extracted protein samples until used, at −80°C. The extracts were resolved in a 15% SDS-polyacrylamide gel [20] and then transferred to nitrocellulose membrane in 25 mM Tris base, 180 mM glycine containing 10% methanol (v/v) for 90 min with a constant current of 250 mA [21]. Membranes were blocked with 5% non-fat dry milk in TBS for 3 h at room temperature, and incubated with antibodies overnight at room temperature. Human CREST serum was used at 1:400 dilution and rabbit anti-CENP-A at 1:500 dilution in PBS.

Detection of peroxidase-labelled secondary antibodies was done with chloronaphthol.

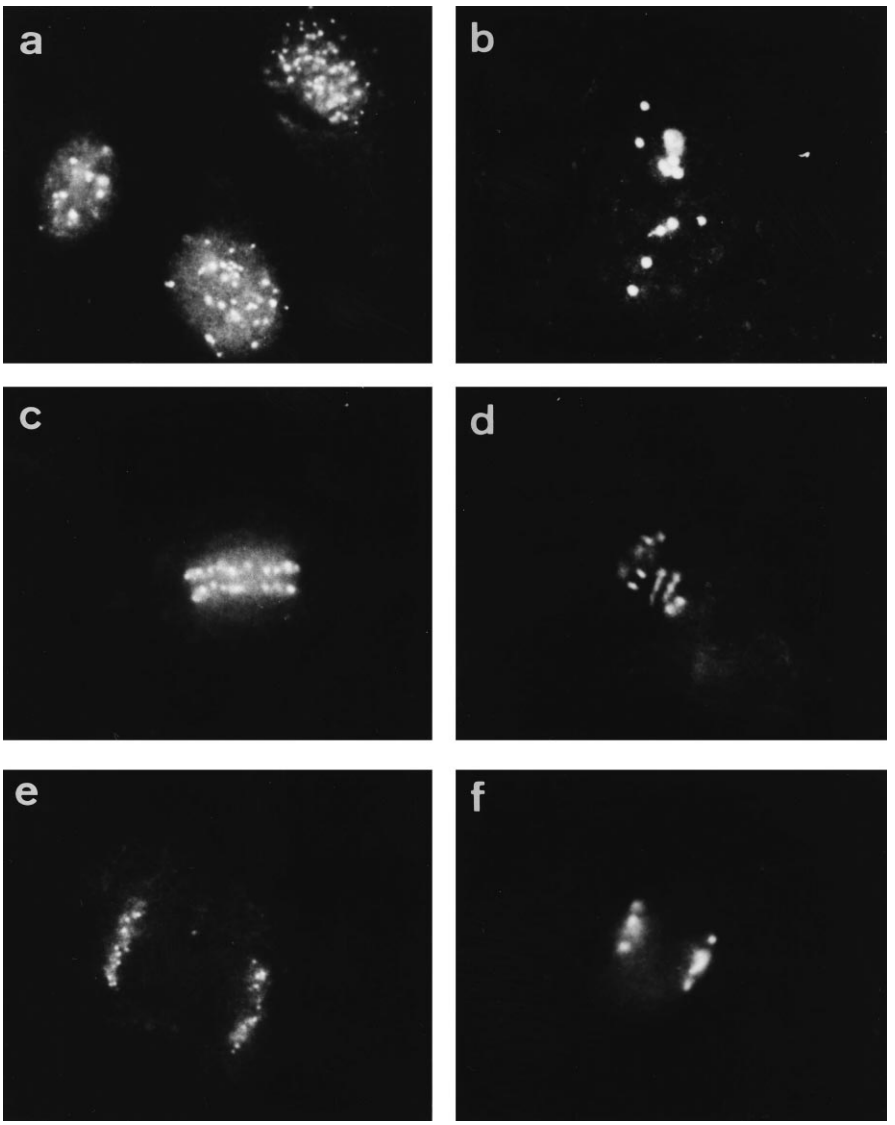

Fig. 1. Immunofluorescence staining of HeLa and Indian muntjac culture cells with specific anti-peptide CENP-A serum. Staining is restricted in human and deer cells to uniform dots at the centromeres. HeLa cells are stained in the interphase (a), metaphase (c), and telophase (e) stages of the cell cycle. The human CENP-A epitope is conserved in deer centromeres, as shown by staining with anti-peptide CENP-A antibody in interphase (b), metaphase (d) and anaphase Indian muntjac cells (f). Magnification 100×.

3. Results and discussion

3.1. Production of monospecific anti-CENP-A antibody

Although human CREST autoantibodies have been useful tools for centromere studies, monospecific immunoglobulins to each centromeric component are recommended to assign specific functions to each centromere antigen. Anti-CENP-B and anti-CENP-C sera are available from several laboratories [5,22–24]. However, monospecific anti-CENP-A antibodies are not available yet. Probably the main obstacles for raising antibodies to CENP-A are the difficulty in achieving a significant expression of the cDNA in *E. coli* systems and, additionally, the difficulty in purifying the native protein in sufficient quantity for immunization. Also, the high sequence conservation of the protein could be an extra impediment to obtaining a high and monospecific immune response in immunized animals. We decided to used the strategy of making anti-peptide antibodies to selected regions of the known human amino acid sequence of CENP-A. Previously, following a similar strategy, we had made monospecific antibodies to several regions of the highly conserved protein CENP-B including a human specific centromere serum [23]. Thus, this time we obtained a monospecific serum to the N-terminal region (residues 3–17) of the human centromeric histone H3-like protein CENP-A. This N-terminal motif was recently identified by Muro et al. as a target site for human autoimmune response in CREST patients [17]. As shown in Fig. 1 the rabbit anti-CENP-A serum stained centromeres of interphase and mitotic HeLa cells (Fig. 1a,c,e). The staining showed a uniform punctate focus in each centromere, similar to that described previously for human autoantibodies [1]. However, when analyzed more precisely, the CENP-A staining pattern clearly showed a different distribution over the surface area of the centromeric region from that described for other centromeric autoantigens. In particular, CENP-B is usually distributed in heterogeneous areas of the centromere, with array-like appearance, by immunofluorescence, reflecting the centromeric heterochromatin organization in each particular chromosome ([23,24] and data not shown). Double immunofluorescence analysis with human serum confirmed that CENP-A distribution covers a more restricted area on the centromere than other autoantigens (data not shown). This result should be clarified by more precise electron microscopy studies. Although the anti-CENP-A serum was generated against a human peptide antigen, reactivity was found in other mammalian cells analyzed, such as the deer culture cells from Indian muntjac (Fig. 1b,d,f). The staining pattern in this case was as predicted, specific to the centromere region of the unique chromosome set of this species in both interphase and mitotic cells. Surprisingly, this anti-CENP-A serum did not stain centromeres of mouse origin (3T3 cells) and only reacted very slightly with those from hamster (CHO cells), by immunofluorescence (data not shown). This agrees with a report by Sullivan et al. [13] in which it was reported that human affinity-purified autoantibodies to CENP-A did not recognize rat or chicken centromeres, by immunofluorescence or immunoblots. These previous data and our observations raise the possibility that CENP-A as a histone-specific protein associated with the centromeres may not be as highly conserved throughout evolution as originally predicted [15].

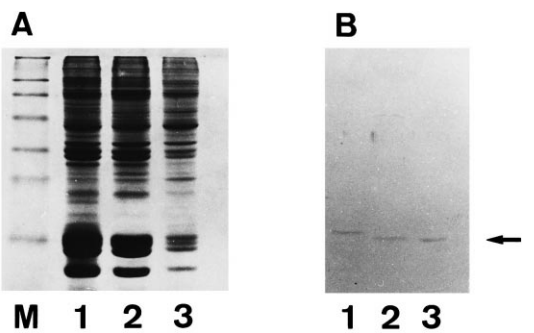

Fig. 2. Solubilization of CENP-A from human placenta nuclei. Coomassie blue staining of protein extracts of human nuclei after successive incubation with micrococcal nuclease (A, lane 1), heparin (A, lane 2) and a final sonication step (A, lane 3) are shown. B: Immunoblot analysis of the protein extracts shown in A with anti-CENP-A-specific antibody. The arrow in B indicates the 17 kDa centromere CENP-A polypeptide which is released from placenta nuclei, together with the core histones shown in A. Lane M in A indicates molecular weight markers, 96, 68, 50, 36, 30 and 16 kDa.

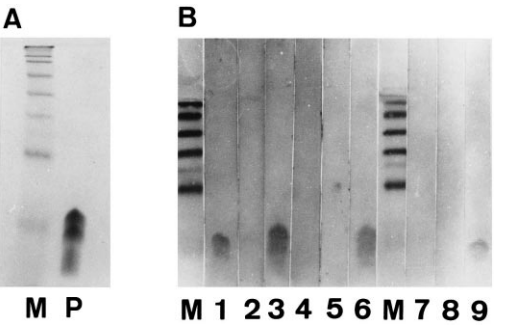

Fig. 3. The N-terminal residues 3–17 of CENP-A represents a human autoepitope recognized by some CREST sera. The CENP-A peptide conjugated to KLH (Coomassie blue in A, lane P) was tested by immunoblot against several human autoimmnune sera. B: CREST sera numbers 1, 3 and 6 react with the CENP-A epitope but not CREST sera 2, 4 and 5. Reactivity of rabbit monospecific serum to CENP-A is shown in lane 9. Two non-CREST human sera are shown in B, lanes 7 and 8. Molecular weight protein markers are shown in lane M.

Our immunoblot analysis in Fig. 2 shows that anti-CENP-A monospecific serum recognizes a 17 kDa polypeptide solubilized after chromatin extraction. These results indicate that CENP-A behaves like the core histones after the extraction of placenta nuclear proteins with nuclease and heparin, conditions known to free chromatin-associated components. Detailed biochemical studies centered on the role of CENP-A in the assembly of centromeric nucleosomes could now be undertaken by purifying native CENP-A from extracted chromatin, using a purification scheme including specific CENP-A affinity columns.

3.2. PRRRSRKPEAPRRRS is a major CENP-A autoepitope in humans

To demonstrate that the anti-peptide CENP-A serum specifically recognizes a human autoepitope, we did immunoblot analysis on KLH-conjugated CENP-A peptide with several human CREST sera. As shown in Fig. 3, three different human anti-centromere sera react with the KLH-conjugated CENP-A peptide (Fig. 3B, lanes 1,3,6) as did the rabbit anti-CENP-A monospecific serum (Fig. 3B, lane 9). However, other high titer human centromere sera apparently do not recognize the N-terminal epitope of CENP-A tested (Fig. 3B, lanes 2,4,5). Because these sera react with the 17 kDa

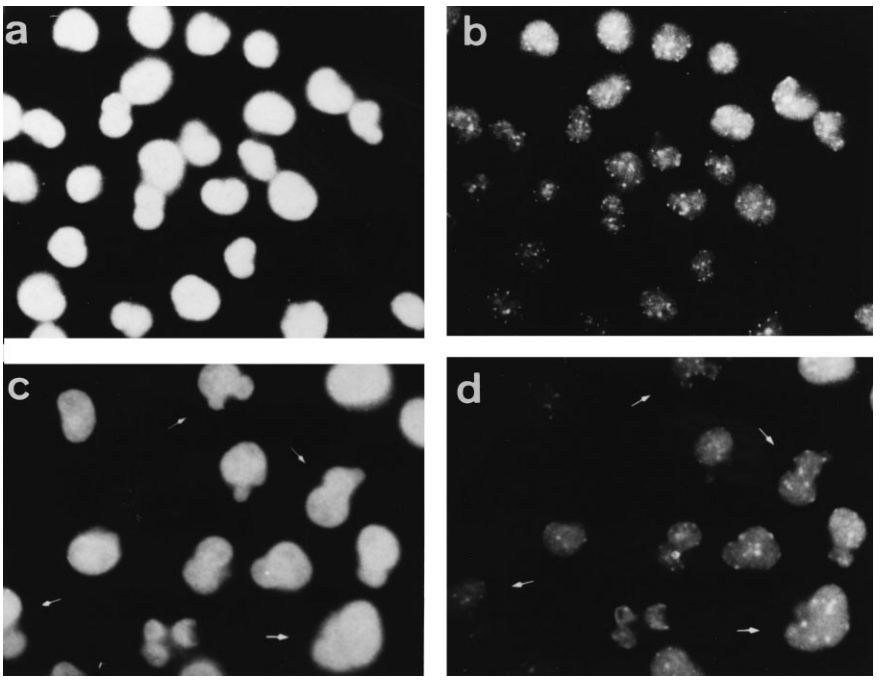

Fig. 4. Localization of CENP-A in Jurkat cells induced to apoptosis with anti-FAS/APO serum. Immunostaining with monospecific anti-CENP-A antibody on uninduced (b) and induced cells to apoptosis for 6 h (d) are shown. The typical round dots on centromeres are observed with a similar appearance in both uninduced and induced cells. Apoptotic cells (arrows in d) retain the standard pattern of multiple dots for centromeres, and localization of CENP-A autoantigen during apoptosis is not apparently modified. DAPI staining for DNA is shown in a and c. Magnification 40×.

polypeptide on immunoblots, it could be concluded from these data that the immune response to CENP-A in humans includes the 3–17 epitope residues, but not exclusively. Controls with other human autoantibodies demonstrated the specificity of the immunoblot assay for CENP-A (Fig. 3B, lanes 7,8). This result unequivocally demonstrated that the residues 3–17 on the N-terminal region of CENP-A constituted a putative linear epitope on the molecule, and represent a target of the human autoimmune response to the centromere in a large number of CREST patients, as indicated recently [17].

3.3. Localization of CENP-A in apoptotic cells

The mechanisms of autoimmunity are not known. A new hypothesis for autoimmune responses to cellular damage and apoptosis was proposed recently [25]. As has been reported, in some cases, human autoimmune antigens are described as having been relocated or proteolytically cleaved during apoptosis. This phenomenon on cell antigens could play some role in the autoimmune response [25]. In contrast, other autoantigens do not show any observable effect regarding their localization during in vitro induced apoptosis. To find out the distribution of CENP-A in apoptotic cells, we did immunolocalization of this histone-like centromere protein on Jurkat-induced cells. Our results in Fig. 4 clearly indicate that specific staining of CENP-A on uninduced and 6 h induced cells showed a similar pattern at the centromere. This result suggests that CENP-A belongs to the group of those autoantigens with unmodified localization during apoptosis, in contrast with other nuclear components such as UBF and RNA polymerase I [25]. These and other specific autoantigens are apparently target substrates for several caspases activated during early stages of apoptosis [26–28]. This observation indicates that the involvement of endogenous cleavage of some antigens in specific stages of the mechanisms for autoimmune response in humans may be variable among different autoantigens.

In conclusion, we report an anti-CENP-A antibody against an epitope on the histone-like centromere human autoantigen. Our study by immunofluorescence demonstrates epitope conservation of this antigen throughout evolution in some mammals, but not all.

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