A BRANCHED, SYNTHETIC OCTAPEPTIDE OF UBIQUITINATED HISTONE H2A AS TARGET OF AUTOANTIBODIES

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Stress proteins have recently been shown to be implicated in a variety of diseases, such as nonviral infections (1) and certain autoimmune diseases (2). In particular, ubiquitin, a heat shock protein of 76 amino acid residues found in every eukaryotic cell, has been found to be a major antigenic target in SLE (3). There is also evidence that the ubiquitin system is involved in diseases of the nervous system, such as Alzheimer and Parkinson diseases (4), and that heat shock proteins may play a central role in all cases of inflammation where heat is a major clinical sign (5).

The precise way in which ubiquitin is involved in these various diseases has not yet been clarified. It is well known that ubiquitin may be present in cells both as a free molecule and conjugated to a number of nuclear, cytoplasmic, and membrane acceptor proteins (6–8), and that it may also be associated to the microtubule network (9).

Cytosolic ubiquitin conjugates are known to be involved in mediating selective degradation of damaged or abnormal proteins (10). The role of ubiquitin conjugates involving the histones H2A and H2B remains poorly understood. Histone ubiquitination is a selective, reversible post-synthetic modification that occurs mainly in actively transcribed regions of chromatin (11, 12) and may be involved in mechanisms of chromatin relaxation. Ubiquitin may also indirectly modulate chromatin structure through stimulation of histone deacetylase activity (13). Compared with cytosolic conjugates, histone-ubiquitin conjugates are more slowly degraded (14).

In the nucleus, ubiquitin was found to be enzymatically conjugated via an isopeptide bond between its COOH-terminal glycine 76 (α-COOH group) and the ϵ-NH_2 groups of lysine 119 of H2A and lysine 120 of H2B, respectively, thus forming branched molecules (15, 16). In mammalian cells, ubiquitinated H2A (U-H2A) and H2B (U-H2B) are usually present at levels of 10% and 1%, respectively (17, 18); levels of U-H2B exceed that of U-H2A within "active" H1-depleted regions of chromatin (19). In Physarum, ~6% of histones H2A and H2B are ubiquitinated (20).

In a previous report (3), we described the presence in SLE of autoantibodies able to react in an ELISA with ubiquitin and with a synthetic fragment corresponding

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to residues 22–45 of the molecule. In addition, the SLE sera also reacted in immunoblotting with two polypeptides of M_r ~52,000 and ~43,000, found to be present as contaminants in a commercial preparation of ubiquitin. Using antisera specific for H2A and H2B, we demonstrated that these P43 and P52 components correspond to ubiquitinated complexes containing H2A and H2A-H2B, respectively. To further characterize these complexes, we prepared antisera against two synthetic peptides corresponding to the branched moiety of U-H2A and used them in immunoblotting experiments. The results reported here confirm the presence of covalent U-H2A in polypeptides P43 and P52. The fine specificity of autoantibodies was defined using the branched peptides of U-H2A as antigens. Close to 95% of SLE sera able to react with ubiquitin were found to also contain antibodies specific for the branched peptide of U-H2A, suggesting that this latter conjugate may play a role in the appearance of autoantibodies in SLE.

Materials and Methods

Histone Fractions and Ubiquitin. Chicken histones H2A and H2B were extracted and purified by the acid method of Johns (21). Ubiquitin was a commercial preparation from Sigma Chemical Co., St. Louis, MO (U 6253, batch 106F-9355).

Synthetic Peptides. Synthetic peptides 22–45 and CYS 22–45 of ubiquitin have been previously described (3). Two synthetic peptides, T1 and T2, corresponding to the branched region of U-H2A were prepared by classical solid-phase methods using terbutyloxycarbonyl (Boc) for N-α protection. The synthesis was performed on a manual system starting from Boc-Glu (OcHx) Pam resin prepared as described elsewhere (22). For trifunctional amino acids, the following side chain protecting groups were used: cyclohexyl for Glu, benzyl for Thr, 2-chlorobenzyloxycarbonyl for Lys 118, Tosyl for Arg, paramethylbenzyl for Cys, 2,6-dichlorobenzyl for Tyr, and fluorenylmethyloxycarbonyl (Fmoc)[1] for Lys 119 (Fig. 1).

The Boc groups were removed using 65% trifluoracetic acid in dichloromethane for 13 min. Coupling reactions were performed in dimethylformamide (DMF) with a threefold excess of hydroxybenzotriazol active ester prepared just before use. All amino acids gave a negative ninhydrin test after 15 min of coupling. The peptides corresponding to fragment 116–121 (T1) and 118–121 (T2) were synthesized at first; after deprotection of the last residues (Leu for T1 and Cys for T2) acetylation was performed using 10 molar excess of acetic anhydride in the presence of the same amount of diisopropylethylamine for 10 min. The Fmoc group of Lys 119 was deprotected by two treatments of 50% piperidine in DMF (2 min each). Residues 73–76 of ubiquitin were then assembled to the Lys 119 as described above. Cleavage of the peptide from the solid support was achieved using hydrogen fluoride in the presence of 10% (vol/vol) of *p*-cresol as scavenger for 45 min at 0°C (in the case of peptide T2, 1% of 1,2-ethanedithiol was added). The crude product was then lyophilized and purified by medium pressure liquid chromatography. The purified products were assessed by HPLC, amino acid analysis (6 N HCl, 110°C, 20 h), and fast atom bombardment (FAB) mass spectrometry measurements.

An additional tyrosine residue was added at the NH_2-terminal Leu-73 of T1 and T2 to allow the peptide to be conjugated to carrier protein by means of bis-diazotized benzidine (23). Before conjugation, internal lysines of peptides were protected by citraconylation. The yield of coupling was checked by amino acid composition. An NH_2-terminal cysteine residue was added in the case of peptide T2 in order to allow subsequent coupling of the peptide through its SH group.

Rabbit Antisera to Histones, Ubiquitin, Synthetic Peptides, and Human Sera. Antisera to purified histones H2A, H2B, H3, and H4 were those used in previous works (24–27). Antiubiquitin antiserum and antisera to peptides 22–45 and CYS 22–45 were raised in rabbits as previously

[1] *Abbreviations used in this paper:* DMF, dimethylformamide; FAB, fast atom bombardment; Fmoc, fluorenylmethyloxycarbonyl; RA, rheumatoid arthritis.

described (3). Antisera to peptides T1 and T2 were obtained by immunizing rabbits (two animals for each peptide) by means of eight intramuscular injections of 50 μg (expressed in peptide) of OVA-conjugated peptide (molar ratio carrier/peptide, 1:14 and 1:16, respectively, for T1 and T2). For the first injection, the conjugated peptide was emulsified with an equal volume of CFA and for the subsequent injections with IFA. Rabbits were bled 1 wk after each injection from the third. Human sera from patients with SLE and related autoimmune diseases were those used previously (3).

Immunological Tests. The ELISA used to measure the binding of rabbit and human antibodies towards histones and ubiquitin was described previously (3, 27). Respectively, 200 ng/ml H2A and 500 ng/ml ubiquitin were used for coating microtiter plates. The antigenic activity of peptides T1 and T2 was measured in microtiter plates directly coated with 2 μM peptide in 0.05 M carbonate buffer, pH 9.6. Immunoblotting experiments were performed as described previously (3), after separation of polypeptides by SDS 12-20% PAGE.

Affinity Chromatography. Peptide T2 was coupled either through its SH group to activated thiol Sepharose 4B as described by the supplier (Pharmacia Fine Chemicals, Piscataway, NJ) or through its amino groups to Act-Ultrogel AcA 22 (IBF, Villeneuve La Garenne, France) preactivated with glutaraldehyde. 500 μl of rabbit T2 antiserum or 200-500 μl of SLE serum was allowed to react with peptide adsorbant equilibrated in 10 mM PBS, pH 7.5. After 3 h incubation at room temperature, the flow-through was collected for characterization. The peptide-adsorbant column was extensively washed with 10 mM PBS, pH 7.5, and antibody was eluted from the column with 0.2 M glycine, pH 3.0. The fractions (0.5 ml) were immediately neutralized with 0.1 M Tris, pH 8.8 (vol/vol). The eluted fractions were characterized by ELISA.

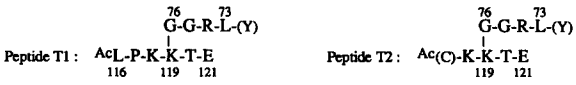

FIGURE 1. Structure of peptides T1 and T2.

Results

Branched Synthetic Peptides T1 and T2. The sequence of the two peptides, T1 and T2, is shown in Materials and Methods. The classical solid phase methodology used for the synthesis of the two peptides gave excellent results since both crude peptides T1 and T2 had a purity >70%. In this case, the Fmoc group was ideally suited for the side chain protection of Lys and this approach seems to be well adapted to the synthesis of branched peptides. The final products used in the immunochemical studies had a purity of 95% (see Fig. 2 for HPLC analysis). Amino acid analysis of the

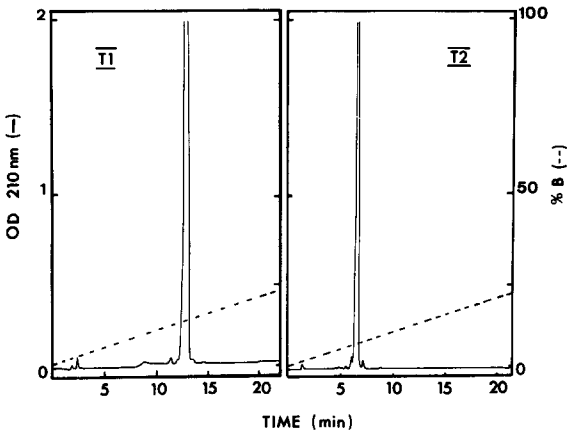

FIGURE 2. HPLC profile of purified peptides T1 and T2. The peptides were detected by UV absorbance at 210 nm. A 4.6 × 250-mm aquapore RP 300 C_8 column was used. Mobile phase A was triethylamine phosphate buffer, pH 2.5, and mobile phase B was acetonitrile. Elution was performed with a linear gradient of 1-21% over 21 min at a flow rate of 2 ml/min.

peptides gave the following composition (expected values indicated in parentheses): for peptide T1: Glx 1.00 (1), Gly 2.00 (2), Arg 0.95 (1), Thr 1.15 (1), Pro 0.95 (1), Tyr 1.02 (1), Leu 1.82 (2) Lys 2.11 (2); for peptide T2: Glx 1.01 (1), Gly 1.97 (2), Arg 0.97 (1), Thr 1.08 (1), Tyr 1.03 (1), Cys 0.87 (1), Leu 1.06 (1), Lys 1.87 (2). Molecular weights of the peptides were confirmed by FAB mass spectrometry, which gave the following values for the quasi molecular ion MH^+ (calculated values are given in parentheses): for T1, 1,303.8 (1,303.5); for T2, 1,196.6 (1,196.6).

The stability of lyophilized peptides T1 and T2 was controlled by HPLC analysis. After several months of storage at room temperature in the dark, no degradation was observed; dimerization of the T2 peptide by its terminal cysteine was <9%. The percentage of dimerized T2 fraction did not change when the peptide was incubated overnight in the carbonate buffer used for coating plates in ELISA, or in bidistilled water.

Reactivity of Antisera to Peptides T1 and T2 in ELISA. Antibodies were raised against OVA-conjugated peptides T1 and T2. Their reactivity was measured in ELISA using plates coated with either homologous nonconjugated peptides, ubiquitin, or H2A. A high antipeptide immune response was observed in the four immunized rabbits after three injections of conjugates. None of the antisera from the four rabbits was able to react with ubiquitin immobilized on the solid phase. Antisera to peptide T1 obtained from both immunized rabbits reacted with H2A in ELISA, whereas the antisera to peptide T2 did not (Fig. 3).

Reactivity of Antisera to Peptides T1 and T2 in Immunoblotting Experiments. The ability of the peptide antisera to react with the different components found to be present in commercial ubiquitin preparations (3) was tested by immunoblotting. When ubiquitin was analyzed by PAGE, immunostaining with antiubiquitin antibodies revealed the expected M_r 8.7 band as well as the P43 and P52 components (Fig. 4, lanes *7–10*); the latter two components were also revealed with anti-T1 and anti-T2 antibodies (Fig. 4, lanes *16* and *17*), anti-H2A and anti-H2B antibodies (lanes *11* and *12*), and by a majority of SLE sera (lanes *3–6*). Because of the sequence homology of five residues in the branched domain, it is possible that anti-T2 antibodies recognize both U-H2A and U-H2B. Anti-T2 antibodies, as well as anti-T1 antibodies,

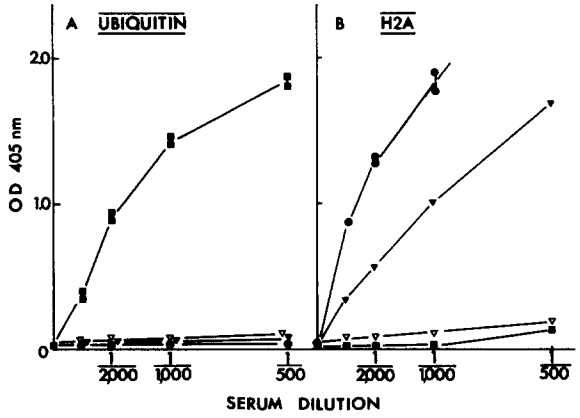

FIGURE 3. Binding in ELISA of ubiquitin (*A*) and histone H2A (*B*) to antisera against peptides T1 (▼) and T2 (▽), H2A (●), and KLH-conjugated ubiquitin (■). Absorbance values were measured after substrate hydrolysis time of 60 min.

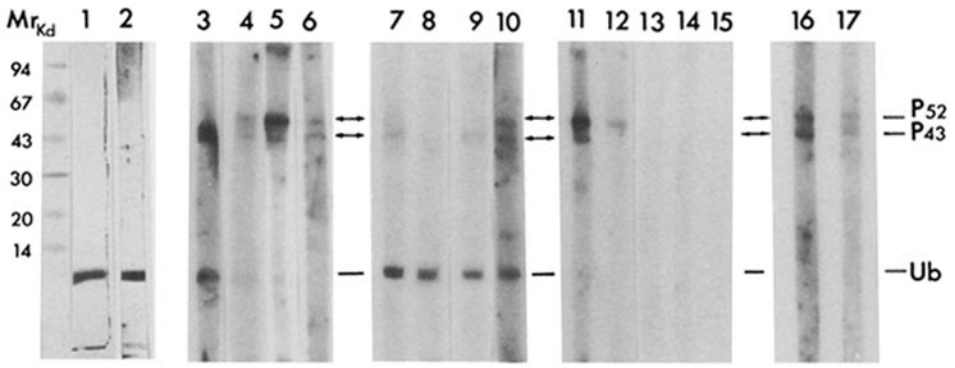

FIGURE 4. Immunoblotting of components present in ubiquitin preparations revealed with antisera directed against ubiquitin, peptide 22–45 of ubiquitin, peptides T1 and T2, and individual histones, as well as with SLE sera. Ubiquitin (10 μg/cm) was resolved in SDS 12–20% polyacrylamide gel (lane *1*, Coomassie staining) and electrophoretically transferred on hydrophobic Immobilon membrane (lane *2*, blot stained with amino black 0.1% in the mixture methanol-acetic acid-water 9:9:2 vol/vol/vol). For immunoblotting, all sera were diluted 1:1,000 in PBS-T. Sera were as follows: lanes *3–6*, SLE sera; lane *7*, anti-KLH ubiquitin conjugate; lane *8*, antipeptide CYS 22–45; lane *9*, antipeptide 22–45; lane *10*, antipeptide 22–45 coupled to OVA; lane *11*, anti-H2A; lane *12*, anti-H2B; lane *13*, anti-H3; lane *14*, anti-H4; lane *15*, anti-H1; lane *16*, antipeptide T1; lane *17*, antipeptide T2. After incubation with sera and ^{125}I-protein A, immunoblots were revealed by autoradiography at −70°C with an intensifying screen.

did not react in immunoblotting (serum dilution 1:100) with pure H2A and H2B or with other individual histones, H3, H4, and H1 (data not shown). Thus, the results obtained with anti-T1 and anti-T2 antibodies confirm the previous finding that the P52 and P43 polypeptides correspond to covalent conjugates of U-H2A and/or U-H2B.

Binding in ELISA of Autoantibodies from SLE Patients to Peptides T1 and T2 of U-H2A. From the results presented above, it clearly appears that P52 and P43 containing U-H2A (U-H2B) exhibit a stronger reactivity with SLE sera than does ubiquitin itself. Therefore, in order to know whether autoimmune sera contained autoantibodies with a narrow specificity for U-H2A, we examined a number of patients' sera for the presence of antibodies able to react in ELISA with the branched peptides T2 and T1 of U-H2A. The threshold for considering a serum positive was an arbitrary cut-off line corresponding to 0.3 OD units (3). As shown in Table I, 96% of the SLE sera (diluted 1:500) that recognized ubiquitin and DNA also reacted with peptide T2. In the case of SLE sera that reacted with ubiquitin but not with native DNA, the frequency of anti-T2 antibodies was slightly less (85%), although it remained high. On the other hand, only 13% of SLE sera that did not react with ubiquitin possessed antibodies able to bind peptide T2. The reactivity of the different sera with peptides T1 and T2 was very similar (data not shown). Among the SLE study group, 51.8% of sera contained autoantibodies to H2A; 62.1% of these sera also reacted with T2 and 17.9% did not. In the group of sera negative with H2A, 53.8% of sera reacted with T2 and 46.1% did not. Experiments are currently in progress to analyze with T2 a larger population of SLE sera with respect to anti-T2 activity in an attempt to correlate the presence of such antibodies with particular clinical features.

TABLE I

Incidence of Autoantibodies Reacting in ELISA with Peptide T2 of U-H2A in Sera of Patients with Rheumatic Disease

Type of sera	Known antigen reactivity*	Number of sera	Frequency of binding to T2 peptide	OD range	$\overline{OD}$‡
			%		
SLE	DNA +; ubiquitin +	26	96	(0.3;2.00)§	-
	DNA −; ubiquitin +	13	85	(0.3;1.52)	0.69
	DNA +; ubiquitin −	15	13	(0.3;0.32)	0.31
RA	Ubiquitin +	7	0	-	-
	Ubiquitin −	14	0	-	-
Scleroderma	Ubiquitin +	6	33	(0.3;0.62)	0.50
	Ubiquitin −	5	0	-	-

The sera were considered positive when OD values were >0.3 after 1 h incubation of anti-human Ig enzyme conjugate with substrate.

* Reactivity with native DNA and ubiquitin was measured as described previously (3) except that all sera were diluted 1:500.

‡ Values represent OD units measured with T2 peptide, using patients' sera diluted 1:500.

§ Upper limit ≥ 2.0 OD units.

Since in a previous study we observed that 16% of 43 sera from patients with rheumatoid arthritis (RA) and 15% of 48 patients with scleroderma reacted with ubiquitin (3), the reactivity of these sera with peptides T1 and T2 was also analyzed by ELISA. In contrast to SLE sera, none of the RA sera that reacted with ubiquitin or with peptide 22–45 of ubiquitin contained antibodies able to bind peptides T1 and T2 (Table I). In the case of scleroderma patients, two of six sera possessed autoantibodies specific for ubiquitin and peptide T2. Control experiments showed that rabbit antisera to ubiquitin and to H2A did not bind in ELISA with peptides T1 and T2.

Affinity Chromatography Purification and Characterization of Anti-T2 Autoantibodies. To establish if it was the same population of autoantibodies in SLE sera that recognize both the P43 and P52 components and the ubiquitin and histone molecules, affinity columns were prepared with the T2 peptide. When thiol Sepharose 4B was used to immobilize peptide T2 through its additional cysteine, no detectable antibodies recognizing T2 in ELISA were found to be present in SLE sera and were retained on the T2-adsorbent column. In contrast with this result, control rabbit anti-T2 antibodies reacted with the T2 adsorbent in these conditions and were eluted with 0.2 M glycine, pH 3, as expected.

Another immunoadsorbent was prepared by coupling peptide T2 to Act-Ultrogel AcA 22 support. In this case, antibodies from the seven different SLE sera that were tested were specifically bound (examples are shown in Fig. 5) and subsequently eluted at pH 3. The purified antibody fractions were found to recognize peptide T2 in ELISA, and depending on the particular SLE serum, the antibodies also bound in ELISA to H2A, and in three cases out of seven, also to ubiquitin (Fig. 5). In comparison, rabbit anti-T2 antibodies specifically eluted from the column reacted with T2 but not with isolated ubiquitin and H2A.

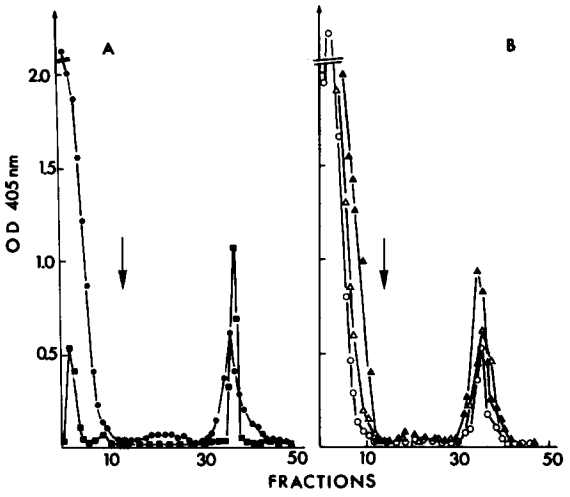

FIGURE 5. Affinity chromatography of SLE sera on T2 peptide conjugated to Act-Ultrogel AcA22; 500 μl undiluted SLE serum was loaded onto a 5 × 1.3-cm column equilibrated in 10 mM PBS, pH 7.5. After 3 h incubation at room temperature, elution with the same buffer was used to wash unbound material. Fractions of 2 ml were collected. Antibodies were eluted with 0.2 M glycine, pH 3.0 (*arrow*). The fractions (0.5 ml) were immediately neutralized with 0.1 M Tris, pH 8.8 (vol/vol). The eluted fractions were tested after dilution 1:2 in PBS-T-BSA. (*A*) Fractionation profiles of two SLE sera (●, ■) tested in ELISA with 2 μM T2 as solid phase antigen. (*B*) Fractionation profile of a third SLE serum tested in ELISA with T2 (△), H2A (▲), or ubiquitin (○) as antigens. Absorbance values were measured after hydrolysis time of 60 min.

Discussion

Autoantibodies to a wide variety of cellular constituents have been reported in different rheumatic diseases (28). In SLE sera, antiubiquitin antibodies have been identified by immunoblotting and by ELISA using ubiquitin and a synthetic peptide of ubiquitin (residues 22–45), as solid phase antigens (3). SLE sera also reacted in immunoblotting with ubiquitinated H2A and H2B complexes of apparent M_r 43 and 52 kD. In the present paper, these conjugates were further studied by means of antibodies directed against a synthetic peptide corresponding to a branched region of U-H2A. In view of the unusual structure of the branched peptide T2, it seemed possible that antibodies raised against it would constitute a specific probe for U-H2A unable to bind to either ubiquitin or H2A alone. Antibodies raised against the T2 octapeptide of U-H2A were found to react with the P43 and P52 components. It is noteworthy that when two additional amino acid residues located in the H2A sequence were present as in peptide T1, the antibodies elicited by this longer peptide did recognize H2A in ELISA (although not in immunoblotting). The use of antibodies raised against a synthetic peptide corresponding to a covalent-linked region between two individual proteins makes it possible to identify such conjugates unequivocally. This type of antibody probe should thus be useful for analyzing such complexes that at present are difficult to study in vitro and in vivo. Experiments are currently in progress in our laboratory to identify the presence of ubiquitinated histone complexes in different types of cellular extracts.

The existence of ubiquitinated histone species has been reported (29, 30). Although some of these molecules were found to accumulate in decondensed, possibly transcribed chromatin regions, the precise role of these conjugates has not yet been elucidated. One possibility is that polyubiquitination of H2A and H2B may function in tagging histones for degradation. Alternatively, ubiquitinated conjugates P53 and P42 may be related to ubiquitin-labeled forms of labile H2A-H2B dimers displaced

from nucleosomes in "active" genes (16, 31, 32). In native chromatin fibers, the two respective primary branching points in H2A and H2B have been found to be exposed for ubiquitination (33, Hacques, M. F., S. Muller, and C. Marion, manuscript submitted for publication).

Despite intensive efforts, no etiology has yet been found for SLE. Very probably, the development of SLE is multifactorial and requires contact with a pathogen or other factors, together with a particular predisposition of the host. In the present paper, we confirm that SLE patients possess autoantibodies able to bind ubiquitinated histone species. The specificity of the antibodies was clearly established by using branched peptides of U-H2A. The precise delineation of the region recognized by autoantibodies by means of the octapeptide T2 corroborate the presumptive evidence that U-H2A may play a role in the SLE autoimmune response (3). Interestingly, the specificity of antiubiquitin autoantibodies found in SLE, RA, and scleroderma appears to be different with regard to their reactivity with peptide T2. This implies that different mechanisms may be involved in the induction of antiubiquitin autoantibodies in these rheumatic diseases and that additional ubiquitinated conjugates may be implicated.

As far as the specificity of autoantibodies to U-H2A is concerned, our results show that some of the antibodies affinity purified on T2 adsorbent still react with H2A. Although a larger systematic survey of patient sera would be required to establish the generality of this phenomenon, our results suggest that it is possible for autoantibodies directed to U-H2A to be classified as "anti-H2A" antibodies as a result of their reactivity with isolated H2A in ELISA. Since the same could be true for other antibodies directed to various ubiquitinated receptor proteins, the development of improved diagnostic tests for autoimmune disease may require fine specificity studies at the epitope level.

The data presented here agree with the hypothesis of an antigen-driven immune response where the "autoantigen" could represent subcellular particles or a multicomponent molecular assembly rather than isolated proteins of the nucleus, nucleolus, or cytoplasm (34). Since ubiquitin is particularly abundant in the cell and appears to be a multifunctional protein affecting chromatin structure, intracellular proteolysis, cellular interactions, and the stress response, it is tempting to link this molecule with various manifestations of the autoimmune response. The mechanism through which histone ubiquitination could be involved in SLE remains unknown. It is conceivable that transient ubiquitin binding to self components could break tolerance and lead to autoimmunity either by modifying the receptor proteins themselves or by altering their interactions with other cellular components. It is also possible that in certain susceptible individuals, a strong immune response to ubiquitinated histones occurs when the DNA repair system becomes strongly stimulated. Other post-translational modifications of proteins have also been described as possible mediators for autoimmune response. These include phosphorylation (35), poly (ADP ribosyl)ation (36), and defective glycosylation of IgG in rheumatoid arthritis (37). All these findings are in agreement with the hypothesis that transitorily modified cellular components play a triggering role in autoimmune disorders.

Summary

Two peptides of eight (T2) and 10 (T1) residues corresponding to the branched moiety of ubiquitinated histone H2A have been synthesized and used for raising

specific antibodies in rabbits. Antisera to peptide T1 reacted in ELISA with T1 and with H2A but not with ubiquitin; antisera to peptide T2 reacted with T2 but not with H2A or ubiquitin. When tested in immunoblotting, both peptide antisera reacted with ubiquitinated H2A but not with unconjugated H2A or with ubiquitin.

Sera from patients with systemic lupus erythematosus (SLE) were shown previously to react with ubiquitin in ELISA and immunoblotting. When tested for their ability to react in ELISA with synthetic peptides T1 and T2, 96% of the SLE sera (diluted 1:500) that recognized ubiquitin also reacted with peptide T2. Of the SLE sera that did not react with ubiquitin, only 13% possessed antibodies able to bind peptide T2. Antibodies from seven SLE sera, purified on a T2-immunoadsorbent column, were also able to react either with H2A, and in three cases also with ubiquitin.

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