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NATIVE SPECIES OF HELIX DESTABILIZING PROTEIN-1 IN MOUSE MYELOMA IDENTIFIED BY ANTIBODY PROBING OF WESTERN BLOTS

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Antibody probing of Western blots is a method for analyzing the apparent M_r of a protein in any given preparation (Renart, J., Reiser, J., and Stark, G., Proc. Natl. Acad. Sci. USA 76: 3116, 1979). We prepared a rabbit antiserum to purified mouse myeloma helix destabilizing protein-1 and used this antiserum in Western blotting experiments with a crude homogenate of mouse myeloma. The results indicated that the native species of helix destabilizing protein-1 can be degraded during purification. This in vitro proteolysis results in complete loss of the native species and accumulation of lower M_r proteins that represent limit digestion products. These findings have identified the true native form of mouse myeloma HDP-1 as a protein of apparent M_r=36,000 to 38,000, instead of the M_r=24,000 and 27,000 proteins obtained by routine purification. © 1985 Academic Press, Inc.

Mammalian helix destabilizing proteins (HDP) or single-stranded DNA binding proteins are typified by calf thymus UP1 and mouse myeloma HDP-1 known as UP1 and HDP-1, respectively (1, 2). Usual preparations of each of these proteins exhibit polypeptide size heterogeneity. With preparations of mouse myeloma HDP-1 for example, the main species was 27 KDa. Yet, tryptic peptide mapping revealed that polypeptide species ranging in size from ~24 to ~33 KDa shared extensive primary structure homology with each other and with the 27 KDa protein (2). The basis of the size heterogeneity of HDP-1 could not be determined, although in vitro proteolysis during purification was considered as an obvious possibility (2).

In the present work, a new rabbit antiserum to mouse myeloma HDP-1 was prepared and then used to probe Western blots of the mouse myeloma crude extract and two of the intermediate fractions during the usual purification of HDP-1. The results suggest that limited proteolysis of 36 to 38 KDa native species of HDP-1 occurs during purification. This limited proteolysis appears to account for most, if not

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all, of the size heterogeneity observed previously with preparations of mouse myeloma HDP-1.

MATERIALS

Mouse myeloma solid tumor (MOPC 104E) was obtained as previously described (3). Nonidet P-40 (NP-40), bovine serum albumin, and Ponceau S concentrate were from Sigma. Nitrocellulose papers (BA 83) were from Schleicher and Schuell. Horseradish peroxidase (HRP) conjugated IgG and normal goat serum were obtained from Cappel Laboratories. 3,3'diaminobenzidine was from Polysciences, Inc. The calf thymus helix destabilizing protein, UP1, was kindly provided by R. Karpel; this sample was an essentially homogeneous preparation of the 24,000-M_r UP1. DNA binding proteins, histones, P8, and HMG proteins were purified from mouse myeloma as described previously (2). Helix destabilizing protein-1 fractions were prepared from mouse myeloma as described (2). Briefly, fraction II was the material obtained after dsDNA-cellulose: ssDNA-cellulose column chromatography and Fraction III was the material obtained by chromatography of Fraction II on a column of ssDNA-cellulose.

METHODS

Immunization Protocol. Female, young adult New Zealand white rabbits were injected in multiple sites intradermally with an emulsion of 300µg glutaraldehyde-crosslinked mouse myeloma HDP-1 fraction IV in PBS and complete Freund's adjuvant. The rabbits were reinjected with glutaraldehyde-crosslinked HDP-1 emulsified in incomplete Freund's adjuvant on days 14 (200µg), 29 (200µg), 68 (50µg), 146 (200µg), and 230 (100µg).

Solid Phase Radioimmunoassay. All steps were conducted at 25°C using 96-well polyvinylchloride flat-bottom microtiter dishes (Dyna Tech). Each well was exposed to 50µl of a solution of 2µg/ml mouse myeloma HDP-1 Fraction IV in PBS for 1 hr. This solution was removed and the well was treated with 1% BSA in PBS for 30 min, then the well was washed with 200µl PBS four times. The antibody samples to be tested were diluted to the desired concentration with 1% BSA in PBS, and 50µl of this solution was added to the well. This liquid was removed after 30 min, and the well was washed 4 times with PBS. Finally, 50µl of a solution of 20 ng/ml [^{125}I]Protein-A (~50,000 cpm: specific activity 25-30µCi/mg), 1% BSA in PBS was added to the well. This liquid was removed after 30 min, and the well was washed 4 times with PBS. The well then was cut from the plate and radioactivity was measured.

Electrophoretic Transfer to Nitrocellulose and Immunobinding. After SDS-polyacrylamide gel electrophoresis, fractionated polypeptides in the gel were electrotransferred onto nitrocellulose paper (0.20µM pore size) according to the method described by Towbin et al. (4) for 3 hr at 60 V using a Bio-Rad Trans-Blot cell. The nitrocellulose sheets could be air dried and stored at 4°C, if desired, or immediately processed.

For visualization of blotted polypeptide markers, the sheet was rehydrated in distilled water with the protein side up and stained with 0.2% Ponceau S and 3% sulfosalicylic acid in a solution of 3% TCA. The stained sheets were destained by soaking in 5% acetic acid for 2 min with 3 changes of this destaining solution.

In immunobinding experiments, all incubations were conducted for 2 hr at 37°C. The nitrocellulose sheets were incubated in 3% BSA in PBS. This was followed by washing the sheets with water, and then incubating them in 1% normal goat serum and 1% BSA in PBS. After extensive washing with water, the sheets were incubated with the first antibody solution in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.25% gelatin and 0.05% NP-40 (TGN buffer) according to the method described by Renart et al. (5). The nitrocellulose sheets were washed 3 times with TGN buffer. Finally,

the sheets were incubated with a second antibody (peroxidase conjugated) in TGN buffer and then washed three times with TGN buffer. Horseradish peroxidase was localized by incubating the sheets for 30-45 seconds in a freshly prepared solution of 0.6 mg/ml 3,3' diaminobenzidine, 0.02% H202 in PBS. The reaction was stopped by rinsing the sheets in water and air drying. In a typical experiment, the protein concentrations of the second antibody was 50μg/ml goat IgG fraction against rabbit IgG, and the HDP-1 antiserum was diluted 1 to 25.

RESULTS

Preparation and Properties of HDP-1 Antiserum. Three rabbits were immunized with ~1mg protein each of Fraction IV HDP-1 from mouse myeloma. Samples of serum were obtained periodically and examined for HDP-1 antibody by solid phase radioimmunoassay (RIA). Two of the rabbits failed to develop antibody and this was consistent with our experience during previous attempts to raise antibody in rabbits against purified mouse HDP-1. One rabbit, however, developed a weak immune reaction two months after the original immunization. This rabbit was further immunized with HDP-1 and serum was collected as described under Methods. The HDP-1 antibody titer in this rabbit remained constant for about one month, but then diminished in spite of further injections with HDP-1.

Routine RIA of the HDP-1 antiserum is shown in Figure 1. The immunobinding reaction was dependent upon both serum dilution and amount of immobilized HDP-1. Ir the experiment shown in Table I, immunobinding to homogenous preparations of mouse myeloma HDP-1 (Fraction V) and the analogous helix destabilizing protein from calf thymus, UP1, was examined. There was roughly equal binding to the two homogenous helix destabilizing proteins. There was no cross-reaction with three other nucleic

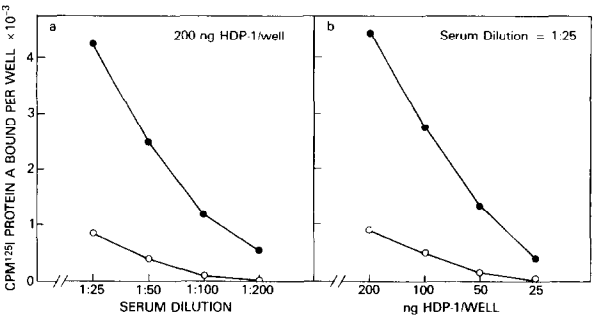

Figure 1: Solid phase radioimmunoassay with HDP-1 antiserum and immobilized mouse myeloma HDP-1 Fraction IV. Results with pre-immune rabbit serum (o) and antiserum (o) are shown. In panel b, ng HDP-1/well indicates protein amount in the solution used during the step of protein adsorption to the well.

TABLE I: Specificity of HDP-1 antiserum by radioimmunoassay

Immobilized Highly Purified Protein	CPM ^{125}I Protein A Bound[a]
Mouse HDP-1 (Fraction V)	6,000
Calf Thymus UP1	2,100
Mouse Histones	0
Mouse HMG Protein	0
Mouse "low salt" DNA binding protein (P8)	0
Mouse HDP-1 Fraction IV	3,100

[a]Each well of the microtiter dish was treated with 50µl of protein solution at a concentration of 4µg/ml. Antiserum and pre-immune serum were used at a dilution of 1 to 25. Values obtained with pre-immune serum were subtracted (average = 500 cpm). For a description of the proteins see text and reference 2.

acid binding proteins from mouse myeloma, as expected from earlier results of comparative tryptic peptide mapping of these proteins (2).

The HDP-1 antiserum was evaluated for use in probing Western blots of DNA binding proteins. A sample of Fraction IV HDP-1 from mouse myeloma was electrophoresed and proteins were electrophoretically transferred to nitrocellulose paper. The paper was incubated with the antiserum and the immunobound rabbit IgG was detected with horseradish peroxidase conjugated-goat anti-rabbit IgG. The pattern of immunobinding by rabbit IgG with the Fraction IV sample was identical with earlier results (2) obtained by simple Coomassie blue staining of the gel (c.f. Fig. 1, ref. 2). Thus, the lowest M_r and highest M_r epitope-containing polypeptides migrated at ~24,000-and ~33,000-M_r, respectively, and the intensity of signal for the 27,000 and 24,000-M_r species was higher than for the 5 or 6 other polypeptides. Similar experiments with homogeneous preparations of calf thymus UP-1 and mouse myeloma HDP-1 revealed one signal in the blot coinciding precisely with the single Coomassie blue stained protein band in each sample at 24,000 and 27,000-M_r, respectively.

Native HDP-1 in the Mouse Myeloma: Typical results of HDP-1 antiserum probing of Western blots of the initial mouse myeloma crude homogenate and of two

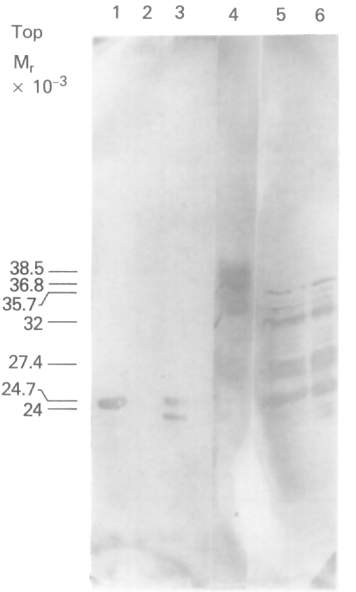

Figure 2: Photograph of results of immunological probing of Western blots using HDP-1 antiserum. Lane 1, 1.2µg purified calf thymus UP1; Lane 2, 0.8µg purified 36 KDa "low salt" DNA binding protein of mouse myeloma; Lane 3, 0.4µg mouse myeloma HDP-1 Fraction V; Lane 4, 750µg mouse myeloma crude extract; Lane 5, 5µg mouse myeloma HDP-1 Fraction II; Lane 6, 2µg mouse myeloma HDP-1 Fraction III (prior to concentration). The amount of material applied to Lanes 4, 5 and 6 corresponded to 0.02g wet wt. of starting tissue. HDP-1 antiserum was used at a dilution of 1 to 50. Proteins used as molecular weight standards were ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor.

intermediate fractions during the HDP-1 purification are shown in Figure 2. The amount of protein material electrophoresed in each lane corresponded to the same amount of starting tissue (0.02g), such that the recovery of individual immunobinding peptides could be compared directly from lane to lane. Samples of calf UP1, mouse HDP-1, and the mouse "low salt" or P8 DNA binding protein (2) were included as references. The results in Figure 2 reveal that immunobinding polypeptides in the crude homogenate were between ~36,000 and ~38,500-M_r, but not in the 24,000- to 27,000-M_r region or in higher M_r regions. By contrast, Fractions II and III had relatively little ~36,000- to ~38,500-M_r material. These fractions exhibited notable size heterogeneity in immunobinding polypeptides, and the strongest signals corresponded to the 24,000- and 27,000-M_r polypeptides that are abundant by Coomassie blue staining of gels (c.f. Figure 1, ref. 2). Immunobinding to proteins in the homogeneous preparations of UP1 and HDP-1 was in the 25,000-M_r

range; the signals observed coincided with the Coomassie blue staining pattern of these particular samples (not shown). For this experiment, the sample of HDP-1 had been stored at -20° for several years and had undergone degradation from a single polypeptide of 27,000-M_r. Finally, no immunobinding was observed in the lane containing the P8 protein.

The absence of signals at 24,000- and 27,000-M_r in the crude extract blot was not due to a failure to detect these proteins in the crude sample, since mixing of the 24,000-M_r protein with the homogenate lead to a strong signal at 24,000-M_r (not shown).

DISCUSSION

The data presented here show that antibodies raised against mouse myeloma HDP-1 reacted with protein in crude homogenates having apparent M_r of ~36,000 to ~38,000 and not the apparent M_r characteristic of purified HDP-1, 24,000 to 33,000.

Epitopes were detected in the smaller sized proteins after the homogenates had been fractionated or when they were supplemented with the smaller sized proteins. The results, taken together, suggest that native HDP-1 in mouse myeloma is a ~36,000 to 38,000-M_r protein and that this protein is degraded during the preparation of Fraction II.

In addition to the observations described here, other groups have reported higher M_r helix destabilizing proteins or ssDNA binding proteins in mammalian cells. Although Williams et al. (7) found that the ~24,000-M_r UP1 was an abundant ssDNA binding protein in calf thymus, a second species of ssDNA binding protein of M_r = ~39,000 was found also. Amino acid sequencing revealed that this 39,000-M_r protein and UP1 share partial primary structure homology; however, a precursor-product relationship appeared inconsistent with the sequence data (7). Sapp et al. (8) recently reported isolation of 48,000 and 61,000 KDa ssDNA binding proteins from calf thymus. These two proteins were found to be immunologically related to each other and to a third calf thymus ssDNA binding protein of M_r ~24,000. This latter protein was obtained in considerably higher yield than the 48,000 and 61,000 KDa proteins and corresponded to UP1. On the basis of this and other data, Sapp et al.

suggested that the 24,000-M_r species was an in vitro degradation product of the higher M_r proteins (8). Our present results with the mouse myeloma system support this idea of in vitro degradation, but not the size of the precursor. Rather, we conclude that the predominant native form of helix destabilizing protein is apparent M_r=36,000 to 38,000. The antibody probe used here did not reveal epitope containing polypeptides of M_r>40,000; our results, however, do not exclude the possibility that the amounts of putative 48,000- and 61,000-M_r HDP polypeptides in mouse myeloma were below the level of detection. Recently, Riva and coworkers (9) reported the preparation of a polyclonal antibody to calf thymus UP1 and use of this antibody in Western blotting analysis of a crude extract from calf thymus. Their results were similar to the results shown in Figure 2, lane 4; epitope-containing calf thymus polypeptides formed a set of hands of M_r ~33,000 to ~38,000, and almost no immunobinding to polypeptides of other M_r was observed (9).

The study of cDNAs corresponding to helix destabilizing protein mRNAs (10) will yield further information on the relationship between the ~36,000- to ~38,000-M_r protein and the higher M_r structurally related proteins (8). Concerning the ~36,000 to ~38,000-M_r protein described here, Cobianchi et al. (10) have isolated a rodent cDNA corresponding to a mRNA with a single long open reading frame that predicts a protein of about this same M_r and purification experiments with cultured mouse myeloma cells as the source of protein also indicated that at least 90% of the material in the HDP fraction had about the same M_r as the epitope-containing polypeptide seen here by Western blotting (10,11).

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REFERENCES

1. Herrick, G., & Alberts, B.M. (1976) J. Biol. Chem. 251, 2124-2132.
2. Planck, S.R., & Wilson, S.H. (1980) J. Biol. Chem. 255, 11547-11556.
3. Karawya, E.M., & Wilson, S.H. (1982) J. Biol. Chem. 257, 13129-13134.
4. Towbin, H., Staehelim, T., & Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350-4354.
5. Renart, J., Reiser, J., & Stark, G.R. (1979) Proc. Natl. Acad. Sci. USA 76, 3116-3120.

6. Williams, K.R., Stone, K.L., LoPresti, M.B., Merrill, B.M., & Planck, S.R. (1985) Proc. Natl. Acad. Sci. USA, In press.
7. Merrill, B.M., LoPresti, M.B., Stone, K.L., & Williams, K.R. (1985) J. Biol. Chem., In press.
8. Sapp, M., Konig, H., Riedel, H.D., Richter, A., & Knippers, R. (1985) J. Biol. Chem. 260, 1550-1556.
9. Valentini, O., Biamonti, G., Pandolfo, M., Morandi, C. and Wilson, S.H. (1985) Nuc. Acids Res. 13, 337-346.
10. Cobianchi, F., Williams, K.R., & Wilson, S.H. (1985) Fed. Proc. 44, 856.
11. Cobianchi, F., SenGupta, D., Zmudzka, B. and Wilson, S.H. (1985) J. Biol. Chem., Submitted.