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Immunology

Immunological crossreaction between alpha-fetoprotein and albumin

(carcinoembryonic antigens/amino acid sequence homology/fetal serum/immunological properties of unfolded polypeptide chains)

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ABSTRACT **Alpha-fetoprotein (AFP), a fetal protein associated with certain tumors, and serum albumin did not crossreact immunologically in native form. Unfolding of their polypeptide chains by reduction of the disulfide bonds followed by carboxamidomethylation produced derivatives with immunochemical properties different from those of the native proteins. Precipitating reactions and radioimmunoassays preformed with antibodies to such unfolded derivatives showed strong crossreactions between albumin and AFP. These assays were found to lack species specificity: AFPs and albumins from different species were equally active. Several other proteins unrelated to AFP or albumin did not react or reacted much less in these assays. These results support the conclusions derived from recent sequence data that AFP and albumin are structurally related and have a common ancestral gene.**

Alpha-fetoprotein (AFP) is a fetal plasma protein produced in large quantities by the fetal liver and yolk sac (1–3). In adults the concentration of AFP is extremely low (4) except in patients with primary liver cancer or teratoma (5, 6). AFP has a molecular weight of 70,000, consists of a single polypeptide chain, and contains about 4% carbohydrate (7, 8). The similarity of the chemical properties of AFP and albumin and the fact that their concentrations in fetal and normal serum, respectively, are high and inversely related (9) suggested that AFP may be evolutionarily related to albumin. Our recent demonstration (10) of homologies between amino acid sequences of fragments of AFP and the known sequence of serum albumin (11) strongly supports this hypothesis.

We provide here further evidence for the relatedness of AFP and albumin by showing that the two proteins crossreact immunologically when they are modified by reduction and alkylation, a method which has been shown to reveal existing sequence homologies (12, 13). Our data also provide validation for this method of showing homology between proteins.

MATERIALS AND METHODS

Proteins. Human AFP (HAFP) was purified using anti-AFP immunoadsorbents (14). Traces of normal human serum proteins contaminating the AFP obtained from the anti-AFP adsorbent were removed with Sepharose-conjugated antibodies to normal human serum. The purity of AFP was checked with gel electrophoresis and immunological tests. The preparations used did not react with antibodies to normal human serum (Behringwerke) in immunodiffusion when tested at a concentration of 5 mg ml^{-1}. This excludes the presence of more than 0.1% albumin in the AFP samples used. Rat AFP (RAFP) was purified by similar techniques. These procedures and characterization of the AFP preparations have been reviewed in detail (15). The human serum albumin (HSA, Sigma, grade III) was shown by radioimmunoassay to contain less than 0.0001% AFP. Human gamma globulin came from Lederle (Grade II) and purified human α_1-acid glycoprotein was kindly provided by M. Egan. They did not contain detectable albumin or AFP at the 0.1% level. Carcinoembryonic antigen (CEA) was purified from colon cancer metastases as described (16). Bovine serum albumin (BSA) was from Pentex, bovine hemoglobin from Sigma, and bovine fetuin from Gibco.

The samples to be reduced and alkylated (17) were dissolved at a concentration of about 10 mg/ml in 0.1 M Tris·HCl buffer (pH 8.2) containing 8 M urea. After deaeration with nitrogen, dithiothreitol was added to a final concentration of 10 mM. After 3 hr at room temperature under nitrogen, the reaction mixture was cooled on ice and recrystallized iodoacetamide was added to make the final concentration 20 mM. After another 15 min the sample was dialyzed against distilled water and lyophilized. Phosphate-buffered saline containing 0.05% gelatin, 0.02% sodium azide, and 0.1% Triton X-100 (PBS-GTX) with urea added to it to give a 2 M solution was used to dissolve the dry proteins, some of which were poorly soluble in ordinary buffers. In some cases the reduced and carboxamidomethylated (RC-) protein was diluted directly into this solution for testing without dialysis and lyophilization. HAFP labeled with ^{125}I was reduced and alkylated similarly and freed of reactants by gel filtration on Sephadex G-15 equilibriated with PBS-GTX.

Antisera. Anti-HAFP sera have been described (6, 8). Using a similar schedule, we produced one antiserum each to RC-HSA and RC-BSA and two to RC-HAFP in rabbits. The dose used per injection was 5 mg (RC-HSA, RC-BSA) or 0.5–2 mg (RC-HAFP) of protein suspended in PBS and given in complete Freund's adjuvant. A precipitating antiserum to RC-CEA was obtained similarly, using 0.25 mg of RC-CEA per injection.

Immunological Tests. Immunodiffusion was performed on plates cast in a conventional buffer and soaked overnight in PBS-GTX prior to use. Radioimmunoassays were performed using the double antibody technique as described (4). The first and second antibody were added in the form of an IgG fraction in assays where native albumin was tested for crossreactivity with AFP. The diluent buffer used throughout was PBS-GTX.

RESULTS

Lack of Crossreaction between Native AFP and Albumin. Antisera produced against highly purified HAFP do not react with albumin in immunodiffusion (ref. 15, our unpublished results). Radioimmunoassay also failed to reveal any crossreactivity between native HAFP and native HSA or RC-HSA. RC-HAFP was also almost completely devoid of activity in the usual AFP assay (Fig. 1).

Abbreviations: AFP, alpha-fetoprotein; HAFP and RAFP, human and rat AFPs, respectively; HSA, human serum albumin; BSA, bovine serum albumin; CEA, carcinoembryonic antigen; RC-, reduced and S-carboxamidomethylated protein derivative; PBS, phosphate-buffered saline; PBS-GTX, PBS containing gelatin and Triton X-100.

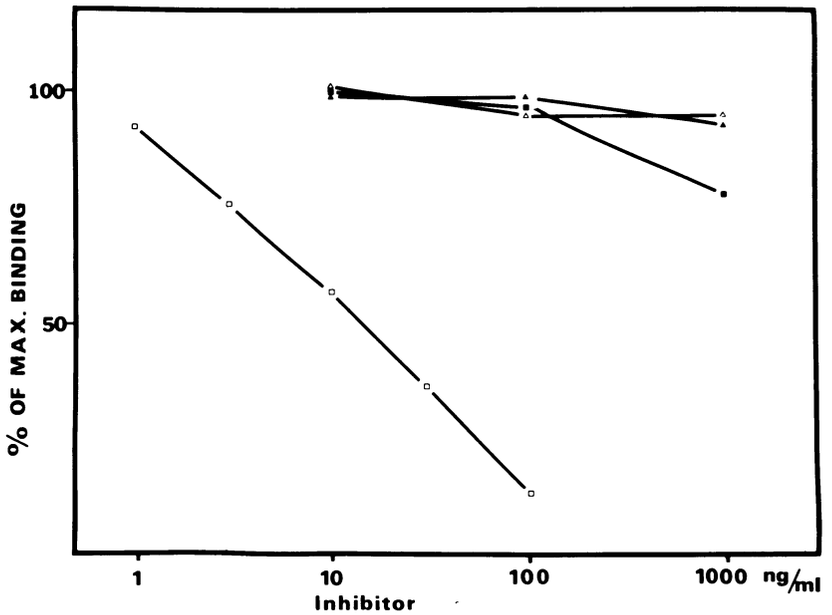

FIG. 1. Conventional AFP radioimmunoassay using inhibition of binding of ^{125}I-labeled HAFP to anti-HAFP. Inhibition curves were obtained with native HAFP (□), native HSA (△), RC-HAFP (■), and RC-HSA (▲). The assays were carried out using IgG fractions of anti-HAFP and, as the second antibody, antibody to rabbit IgG.

Crossreactions between RC-AFP and RC-Albumin. Anti-RC-HSA and anti-RC-BSA sera contained antibodies demonstrable at the immunodiffusion level. Strong precipitates were obtained with RC-HSA, RC-BSA, and RC-HAFP. RC-IgG produced a weak line close to the antibody well. In subsequent tests RC-IgG (200 μg/ml) was added to the antisera. RC-HSA, RC-BSA, and RC-AFP reacted with the absorbed antisera, while RC-IgG (Fig. 2), RC-CEA, RC-α_1-acid glycoprotein, and RC-hemoglobin failed to react. Native BSA (Fig. 2) and native HSA reacted weakly with a line distinct from that produced by the RC-proteins. The RC-BSA and RC-HSA lines fused completely. RC-AFP also seemed to react identically with the RC-albumins, but this was difficult to judge because the precipitates given by the RC-proteins were less distinct than is usually seen in immunodiffusion. Addition of 200 μg/ml of either RC-AFP, RC-HSA, or RC-BSA to the antisera completely abolished these immunodiffusion reactions. The anti-RC-HAFP sera did not precipitate any of the antigens used, presumably because of low titer of antibodies.

Radioimmunoassays confirmed the crossreactivity between RC-AFP and RC-albumins. In binding assays, reduction and alkylation of ^{125}I-labeled HAFP diminished the binding of the label to anti-HAFP, with a concomitant appearance of binding to anti-RC-HSA and anti-RC-HAFP (Fig. 3). Anti-RC-HAFP sera, which, unlike anti-RC-HSA, did not have high enough antibody titers to be demonstrable in immunodiffusion, had a lower titer in this assay. Anti-RC-CEA, which was used as a control, showed only weak binding at the lowest dilution.

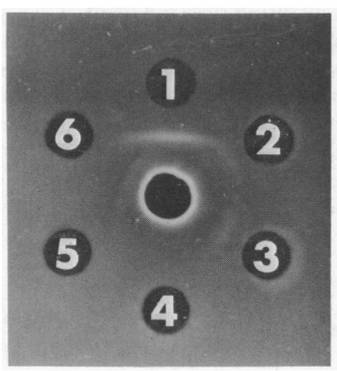

FIG. 2. Immunodiffusion in agarose. RC-derivatives of BSA, 1; HSA, 2; HAFP, 3; human IgG, 4; native HAFP, 5; and native BSA, 6; tested against anti-RC-BSA (center well). The proteins tested were used at a concentration of 1 mg/ml and the plate contained PBS-GTX.

We then used assays based on inhibition of binding of ^{125}I-labeled RC-HAFP to anti-RC-HSA. RC-HAFP and RC-HSA were equally effective inhibitors in this assay (Fig. 4). Native HAFP inhibited slightly at the highest concentration used, but was 1000 times less inhibitory than RC-HAFP. Native HSA did not inhibit in this assay. Reduced and carboxamidomethylated forms of bovine albumin and rat AFP gave inhibition curves similar to those of RC-HAFP and RC-HSA, while various control proteins, RC-IgG, RC-α_1-acid glycoprotein, and RC-hemoglobin, were 100–300 times less efficient inhibitors (Fig. 5). Two further control proteins, also expressed in the fetus, but distinct from AFP, RC-CEA and RC-fetuin, were less active than RC-IgG. Nanogram amounts of the RC-AFPs and RC-albumins were inhibitory in these assays. This sensitivity is comparable to that of usual radioimmunoassays for proteins and tends to exclude nonspecific inhibition as the cause of the crossreactions observed.

When similar assays were carried out using binding of ^{125}I-labeled RC-HAFP to the anti-RC-HAFP sera, RC-HSA was as inhibitory as RC-HAFP with one antiserum. However, only the homologous antigen, RC-HAFP, inhibited the other serum, suggesting that it may be more difficult to detect crossreactivity in a homologous system than it is in an assay where the label and antibody are crossreactive.

DISCUSSION

We have shown here immunological crossreactivity between AFP and albumin. This crossreactivity could be demonstrated when the proteins were unfolded by reduction and carboxamidomethylation, but not when AFP and albumin were in their native conformations. Arnon and Maron (12) have reported

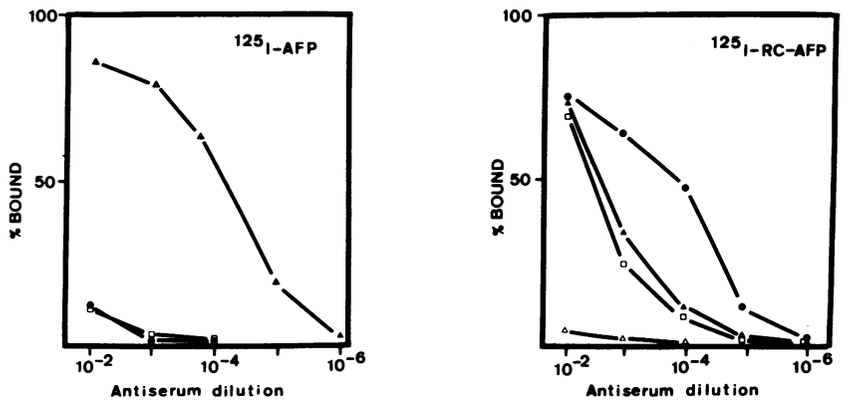

FIG. 3. Antibody binding curves obtained with serial dilutions of various antisera and native ^{125}I-labeled HAFP (left) and reduced and carboxamidomethylated ^{125}I-labeled HAFP (right). Anti-HAFP (▲), anti-RC-HSA (●), anti-RC-HAFP (□), and anti-RC-CEA (△).

similar results with lysozyme and lactalbumin, which have a sequence homology of about 40%. These proteins do not crossreact in native form, but become crossreactive after reduction and carboxymethylation. Lysozymes from human leukemia cells and from hen egg white also crossreact mainly as their reduced carboxymethyl derivatives (13).

Antibodies to native proteins have been shown to be directed mainly against conformational rather than sequential determinants (reviewed in ref. 18). Unfolded polypeptide chains of proteins elicit an antibody response specific for the unfolded form of the protein (12, 13, 19). In agreement with the earlier results, our assays also showed specificity to the unfolded derivatives of the proteins used.

The crossreaction we found between the unfolded derivatives of AFP and albumin lacked species specificity. Bovine albumin and rat AFP were as effectve as the corresponding human proteins, while five different control proteins, three of which are known to have no significant sequence homology with albumin (IgG, α_1-acid glycoprotein, hemoglobin) (20, 21), showed only weak reactivity in the assays. Also, an antiserum against RC-CEA that precipitated RC-CEA in immunodiffusion and bound ^{125}I-labeled RC-CEA up to a dilution of 10^{-6}, bound ^{125}I-labeled RC-HAFP only weakly in the lowest dilution tested. These results indicate that our antisera against RC-proteins do show specificity for the respective immunogens.

However, antibodies to unfolded proteins may not be as highly specific as antibodies against native proteins. This was suggested by the weak activity shown, especially by RC-IgG. While this may partly be due to the presence of trace amounts of albumin in IgG, it could also result from chance similarities in the sequences of the various proteins used. The clearcut quantitative differences (100- to 300-fold) between the reactivities of RC-AFPs and RC-albumins as opposed to the control proteins nevertheless strongly suggest that the crossreactivity can be regarded as indicative of similarities in the primary structures.

Further support for this argument comes from the finding that the RC derivative of chicken AFP (22) is reactive in the assay with ^{125}I-labeled RC-HAFP and anti-RC-HSA. Twice the amount of RC chicken AFP gave the same inhibition as the mammalian AFPs (unpublished). Chicken AFP does not crossreact with the mammalian AFPs in usual immunoassays, but has a molecular weight and expression in the fetus similar to that of the mammalian AFPs (22) and is presumably structurally homologous with them.

Our results from immunological assays with unfolded AFP and albumin provide a new example of a successful demonstration of immunological crossreactivity between two related proteins, and tend to validate this method of obtaining evidence for sequence homology. With the use of surfactants to solubilize

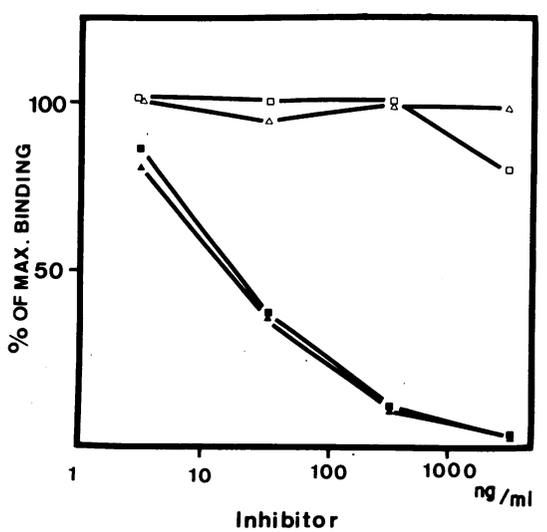

FIG. 4. Radioimmunoassay using binding of ^{125}I-labeled RC-HAFP to anti-RC-HSA. Inhibition curves were obtained with RC-HAFP (■), RC-HSA (▲), native HAFP (□), and native HSA (△).

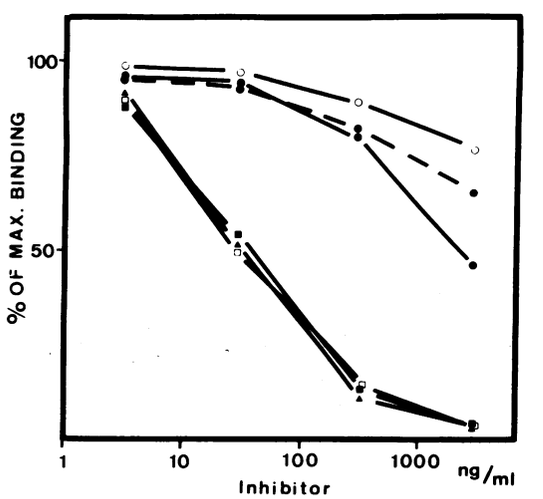

FIG. 5. Radioimmunoassay using binding of ^{125}I-labeled RC-HAFP to anti-RC-HSA. Inhibition curves obtained with RC-HAFP (■—■), RC-BSA (□—□), RC-RAFP (▲—▲), human RC-IgG (●—●), RC-α_1 acid glycoprotein (● - - - ●), and RC-bovine hemoglobin (○—○).

the unfolded protein derivatives as in this study, the method may have general applicability to demonstration of relatedness between proteins.

Our recent amino acid sequence data on human AFP have shown that AFP and albumin have homologous regions. This information is based on the sequence of a total of 70 residues contained in three cyanogen bromide fragments of AFP (ref. 10, and unpublished results) and their comparison with the complete albumin sequence (11). An average homology of 45% was found between the AFP fragments and corresponding regions in albumin. Computer comparison of the AFP sequences with all other known protein sequences did not reveal any further significant homologies and established the uniqueness of the similarity with albumin (10). The extent of homology between AFP and albumin is of the same order as that of, e.g., the α and β chains of hemologobin, but less than that between β chain and γ chain of fetal hemoglobin. Proteins showing such sequence homology are believed to have originated as a result of duplications of a common ancestral gene with subsequent divergent development (21). The immunological crossreactions and the earlier sequence work suggest that this is the case with AFP and albumin.

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