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Actin Is the Naturally Occurring Inhibitor of Deoxyribonuclease I

(affinity chromatography/immunofluorescence/sodium dodecyl sulfate–gel electrophoresis)

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ABSTRACT **Various tissues and cells in culture contain a specific inhibitor of DNase I (EC 3.1.4.5). In this paper evidence is presented that this inhibitor is actin, one of the major structural proteins of muscle and nonmuscle cells. (*a*) The inhibitor is a major cellular component constituting 5–10% of the soluble protein. (*b*) It migrates with actin on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, having a characteristic molecular weight of 42,000. (*c*) It has an amino-acid composition closely similar to that of actin. (*d*) The peptide maps of the two proteins are nearly identical. (*e*) Skeletal muscle actin inhibits the enzymatic activity of DNase I. (*f*) DNase I–agarose affinity chromatography quantitatively retains purified skeletal muscle actin, and actin, specifically, from high-speed supernatants of whole cell extracts. (*g*) An antibody to purified inhibitor protein from calf thymus, used in indirect immunofluorescence on cells grown in culture, stains a two-dimensional network of fibers similar to that seen with an actin-specific antibody.**

The observation that actin can be isolated by DNase–agarose affinity chromatography provides a useful tool for the biochemical study of actin under different physiological conditions.

Various eukaryotic cells contain a protein that strongly and specifically inhibits the activity of DNase I (EC 3.1.4.5) (1–6). The inhibitor from calf spleen has been purified to homogeneity (7, 8). The protein, which under certain conditions readily forms high-molecular-weight aggregates, acts in the monomeric form by binding tenaciously to the enzyme. This interaction gives rise to a complex containing one molecule each of DNase I and inhibitor (9). Analogous proteins from calf thymus (10) and rat serum (11) have also been extensively purified and shown to have properties similar to those of the spleen inhibitor. The inhibitor is present in remarkably large amounts in the cell, comprising 5–10% of the soluble protein of the crude extract (10, 12). Its physiological significance, however, has remained unknown.

DNase I was isolated and crystallized from bovine pancreas by Kunitz in 1948 (13). Recently this enzyme has been subjected to an extensive investigation (14–21), including the determination of its amino acid sequence (22). So far the only known enzymatic activity found associated with DNase I is its ability to degrade DNA, and the DNase inhibitor has been primarily thought of as a protein controlling or modifying this activity.

Actin is a major structural component of all cell types (23). It has been implied to be involved in a large number of cellular functions like cytokinesis, membrane ruffling, cell locomotion, attachment of cells to a substratum, pinocytosis, and maintenance of cell shape (24–30). Thus, actin and actin-containing structures appear to have a broad spectrum of activities within a cell, a number of which may still be unknown. Recently an antibody was obtained against mouse fibroblast actin. When this antibody was used in indirect immunofluorescence on cells grown in culture, it revealed a complex network of actin-containing fibers (31). These fibers were subsequently shown to be identical to submembraneous bundles of microfilaments (32).

This paper presents biochemical and immunological evidence that actin and the widely occurring DNase I inhibitor are identical proteins. The implications of this observation for the cellular function of actin and DNase I are discussed.

Abbreviations: DNase I, deoxyribonucleate 5′-oligonucleotidohydrolase; EC 3.1.4.5 (pancreatic deoxyribonuclease); NaDodSO$_4$, sodium dodecyl sulfate.

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MATERIALS AND METHODS

DNase I (1× crystallized; Schwartz/Mann) was used without further purification for DNase and DNase inhibitor assays and for the preparation of DNase–agarose (see below). DNase I inhibitor from calf thymus was purified as described by Lindberg and Skoog (10). In the experiments reported here, the second inhibitor peak from the DEAE–cellulose chromatography was used. DNase and inhibitor assays were performed as described (5); one inhibitor unit is defined as the amount of inhibitor that causes a 1% inhibition of the activity of 1 μg of DNase I.

Purified chicken skeletal muscle actin was a generous gift from Dr. Susan Lowey.

Affinity Chromatography on DNase–Agarose. DNase–agarose was prepared by the CNBr activation method (33), essentially according to the procedure described by Kato and Anfinsen (34). For this purpose, 25 g of Biogel A15 (BioRad) was washed with distilled water on a Büchner funnel and resuspended in 60 ml of water. Cyanogen bromide (4.5 g) was dissolved in 135 ml of water and added to the suspended agarose beads. The mixture was stirred with dropwise addition of 2 M NaOH to keep the pH at 11.0–11.5. After completion of the reaction (20 min), the resin was washed on a Büchner funnel with 0.1 M $NaHCO_3$ and resuspended in 25 ml of 0.1 M $NaHCO_3$, 0.1 mM $CaCl_2$. DNase I, 20 mg dissolved in 2 ml of 0.1 M $NaHCO_3$, 0.1 mM $CaCl_2$, was mixed with the activated agarose and stirred overnight. The whole procedure was carried out at 0–4°. The product DNase–agarose was washed free of unbound protein and used for purification of DNase I inhibitor as described by Lindberg and Eriksson (12), except for minor changes in the composition of the elution buffers as indicated in the figure legends. The peak fractions from the affinity chromatography (see Figs. 2 and 3) were pooled as

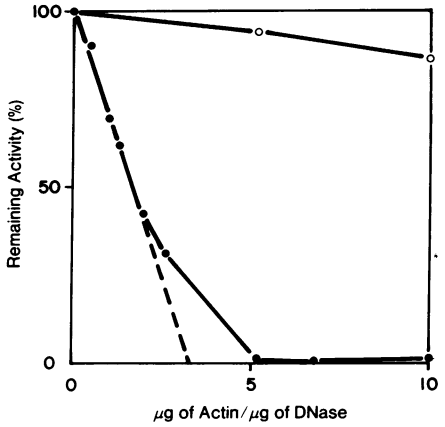

FIG. 1. Inhibition of DNase I by actin from chicken skeletal muscle. Purified actin from chicken skeletal muscle was lyophilized from a solution containing 10 mM Tris·HCl (pH 7.4), 0.1 mM $CaCl_2$, 0.1 mM ATP, 1 mM dithiothreitol. A stock solution was prepared by dissolving the protein in 50 mM Tris·HCl (pH 7.4), 1 mM $CaCl_2$ at a concentration of 1 mg/ml. The DNase assay was performed as described (5). In the above experiment, increasing amounts of actin were added to the standard amount of DNase (0.2 μg) immediately before the addition of the substrate. In the diagram, the results are expressed as percent remaining activity of the DNase against the amount of actin added per μg of enzyme. ●, Native actin; ○, actin treated for 2 min at 90°.

indicated and dialyzed against distilled water for 18 hr. The samples were lyophilized and redissolved in electrophoresis sample buffer [50 mM Tris·HCl (pH 6.8), 2% sodium dodecyl sulfate ($NaDodSO_4$), 10% glycerol, 0.002% bromphenol blue, 0.1 M dithiothreitol] and analyzed by $NaDodSO_4$–polyacrylamide slab-gel electrophoresis. Electrophoresis was performed according to the Tris-glycine system of Laemmli (35) as described by Anderson *et al.* (36). The gels contained 12.5% acrylamide and 0.067% bisacrylamide.

Tryptic peptide analysis was performed essentially as described by Leggett-Bailey (37) and Anderson *et al.* (36).

Antibody Preparation. Antibodies against the DNase I inhibitor from calf thymus were obtained by immunization of rabbits by a procedure similar to that previously used by Lazarides and Weber for preparation of actin antibodies (31). The immunization was kindly performed for use by Dr. F. Miller at the State University of New York at Stonybrook. The thymus inhibitor was purified as described (10). This preparation was about 95% pure (see Fig. 3C). The protein was freed from salt by dialysis against water and lyophilized. It was then dissolved in electrophoresis sample buffer (see above) at a concentration of 1–2 mg/ml by heating for 4 min in boiling water. For immunization, 0.5 mg of the denatured and reduced inhibitor protein was thoroughly mixed with complete Fruend's adjuvant and injected subcutaneously in rabbits. Three weeks later, the rabbits were injected intravenously with 0.1 ml of the denatured protein, and this step was repeated every third day. After a month of immunization the rabbits were bled and the serum was collected. The gamma globulins were partially purified by precipitation with 50% ammonium sulfate. After dialysis against 0.15 M NaCl, 10 mM Tris·HCl (pH 7.8), the antibody preparation was stored at −20°. Indirect immunofluorescence of cells grown on coverslips was performed as described for the actin-specific antibody (31).

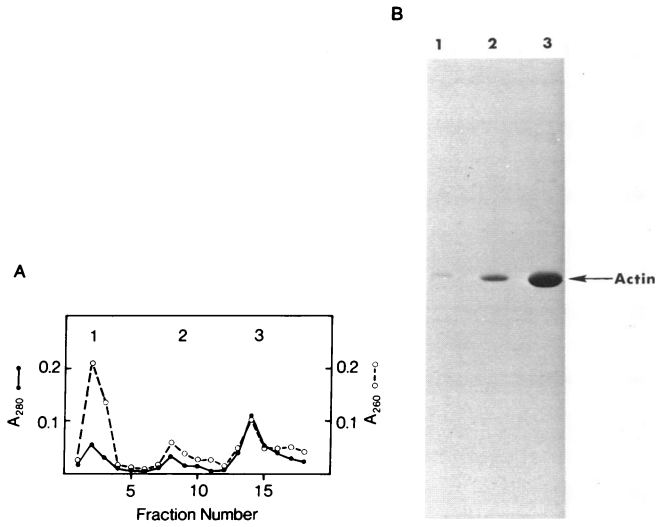

FIG. 2. Affinity chromatography of actin from chicken skeletal muscle on DNase–agarose. A stock solution of purified actin was prepared as described in the legend to Fig. 1. The protein (0.5 mg) was diluted with an equal volume of 0.5 M sodium acetate, 1 mM $CaCl_2$, 30% glycerol (buffer A), and applied to a DNase–agarose column (1.2 ml containing 1 mg of DNase) packed in a 5-ml plastic disposable syringe. (A) The protein was eluted stepwise from the affinity column with 6 ml each of the following buffers (1-ml fractions were collected): (*1*) buffer A, (*2*) buffer A plus 0.75 M guanidine·HCl (pH 6.5), (*3*) buffer A plus 3.0 M guanidine·HCl (pH 6.5). The peak fractions were pooled and processed for $NaDodSO_4$–polyacrylamide slab gel electrophoresis as described in *Materials and Methods*. (B) The lyophilized fractions (*1–3*) were dissolved in equal volumes of $NaDodSO_4$ sample buffer; and 10 μl of fractions *1* and *2* and 5 μl of fraction *3* were applied to the gel.

RESULTS

The DNase I inhibitor is a major soluble protein component in eukaryotic cells with a molecular weight in the range of 42,000 (10). This observation led to the idea of looking for a possible resemblance between this protein and actin, which also is a major cellular protein and characteristically migrates on $NaDodSO_4$–polyacrylamide gel electrophoresis with a molecular weight of 42,000. An indication that these two proteins could be related was obtained in the comparison of the amino-acid composition of the inhibitor from calf spleen (7) and actin from different sources, as reported (38–41). We recalculated the amino-acid residues, using the molecular weight of 41,785 calculated from the amino-acid sequence data reported for rabbit skeletal muscle actin (38). In the amino-acid composition of the inhibitor, 10 of the 18 listed amino acids did not deviate by more than ±1 residue and three by ±2 residues from the corresponding values of rabbit skeletal muscle actin. Most likely, this is within experimental error. Of the remaining five amino acids that showed slightly larger deviations, four constitute a group that, in general, show variations among actins from different species.

The close similarlity of the amino-acid composition of actin and the inhibitor prompted us to test the effect of purified actin on the enzymatic activity of DNase I. As shown in Fig. 1, purified actin from chicken skeletal muscle effectively inhibits the degradation of DNA by the enzyme. The kinetics of the inhibition implies that around 3 μg of actin are needed to inhibit 1 μg of DNase I. Assuming that actin causes this inhibition in the same manner as the calf spleen and thymus

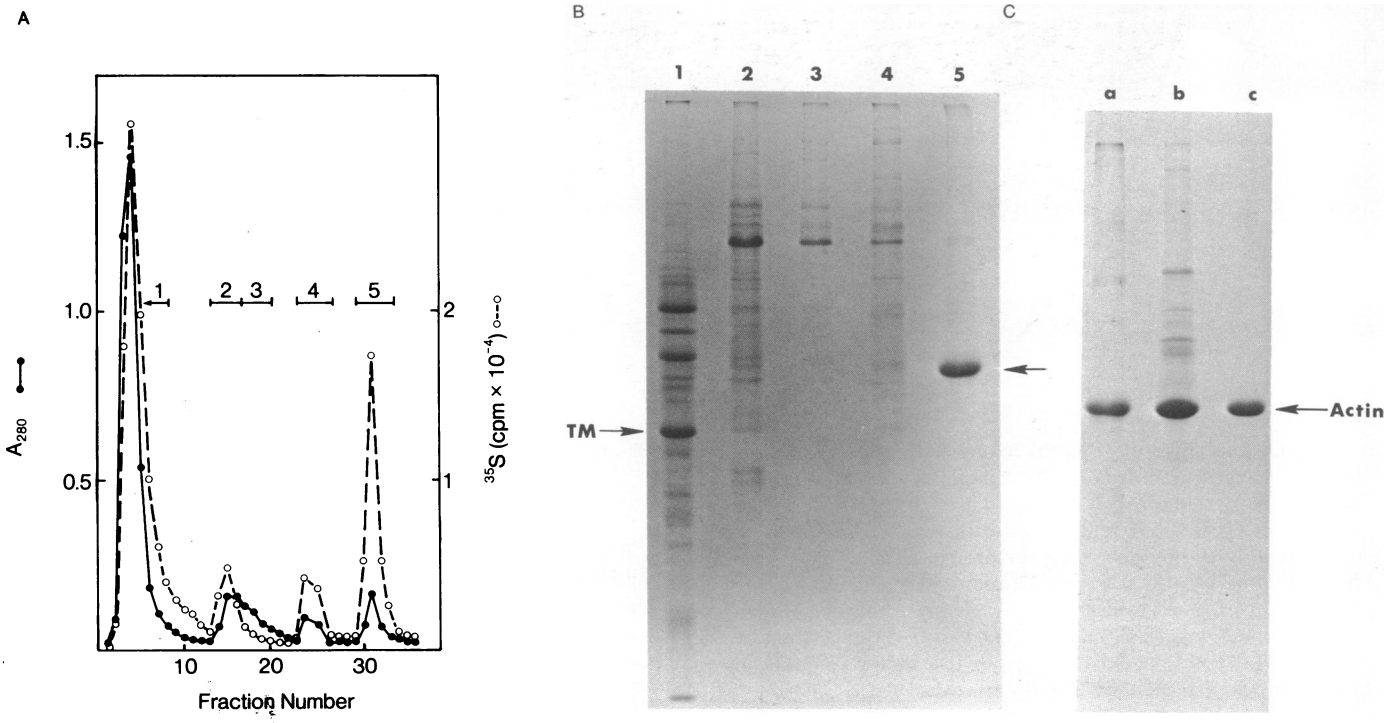

FIG. 3. Fractionation of a KB cell high-speed supernatant by affinity chromatography on DNase–agarose. KB cells grown in spinner culture (3×10^5 cells per ml) were labeled with [^{35}S]methionine (specific activity 100 Ci/mmole) at 2 μCi/ml. After 9 hr of labeling, a crude extract was prepared from the collected cells by homogenizing in 50 mM Tris·HCl (pH 7.5), 1 mM $CaCl_2$. A high-speed supernatant was obtained by centrifugation of the crude extract for 2 hr at 50,000 rpm (+4°) in a Spinco SW50.1 rotor. The DNase I inhibitory activity of the supernatant was 8000 units/ml (for definition see *Materials and Methods*). For affinity chromatography (panel A), 16 A_{280} units of the supernatant were applied to a column of DNase–agarose containing 1.7 mg of enzyme. Stepwise elution with 25 ml of each buffer (2.5-ml fractions) was performed as follows: (*1*) 0.15 M NaCl, 50 mM Tris·HCl (pH 7.4), 1 mM $CaCl_2$; (*2* and *3*) buffer A (see legend to Fig. 2); (*4*) buffer A plus 0.75 M guanidine·HCl; and (*5*) buffer A plus 3 M guanidine·HCl. The fractions were pooled as indicated in panel A and processed for $NaDodSO_4$–polyacrylamide slab-gel electrophoresis as described in *Materials and Methods*. The lyophilized samples were dissolved in equal volumes of $NaDodSO_4$ sample buffer, and 10 μl of each of the samples was loaded on the gel, except for sample 1 where 5 μl was used. The results of the electrophoresis are presented in panel B. TM refers to KB cell tropomyosin (unpublished observation). Panel C shows the coelectrophoresis of (a) DNase I inhibitor purified from calf thymus, (b) the protein from KB cells purified by affinity chromatography on DNase agarose, and (c) actin from chicken skeletal muscle.

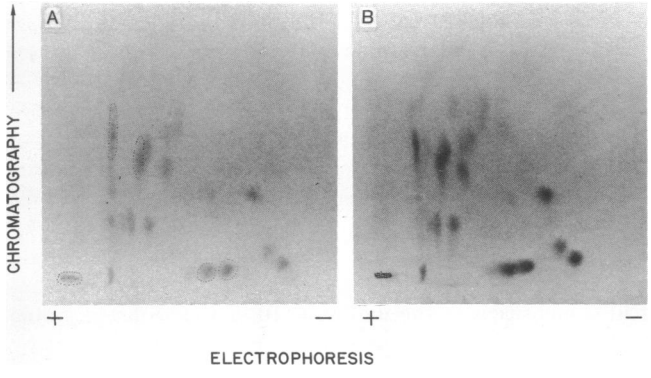

FIG. 4. Tryptic peptide map analysis of actin and DNase I inhibitor. For peptide mapping, protein samples (100–200 μg) were oxidized and digested with trypsin as described (36, 37). Panel A shows the analysis of a mixture of the tryptic peptides of actin from chicken skeletal muscle and the [^{35}S]methionine-labeled protein from KB cells purified on DNase–agarose (see Fig. 3A and B). Panel B shows the corresponding analysis of the DNase inhibitor from calf thymus. The peptides of skeletal muscle actin and the thymus inhibitor were stained with ninhydrin. The plate with the peptides of skeletal muscle actin was autoradiographed to reveal the [^{35}S]methionine-containing peptides of the KB cell protein (indicated by dotted lines).

inhibitors (formation of a 1:1 addition complex) (9, 10), then at least 50% of the actin molecules in this preparation was available for interaction with DNase. A 5-fold excess of actin inhibited the enzyme more than 99%.

Affinity chromatography on DNase–agarose columns was used to study further the interaction between purified skeletal muscle actin and DNase I. This method was successfully used previously to purify the DNase inhibitor from crude extracts of calf thymus (12). Only one major protein was shown to bind tenaciously to the DNase–agarose. It could be eluted with a buffer containing 3 M guanidine·HCl and was shown to be identical to the previously purified thymus inhibitor. In the experiments illustrated in Fig. 2, 0.5 mg of purified skeletal muscle actin was applied to a column containing approximately 1 mg of DNase I. The column was washed and eluted with the different salt solutions, and the peak fractions were pooled and processed for slab gel electrophoresis. The results (Fig. 2A and B) demonstrated that virtually all actin was retained by the DNase–agarose and eluted with 3 M guanidine·HCl. The material passing through the column unadsorbed and having a high A_{260}/A_{280} ratio was due to excess ATP present in the actin preparation.

The specificity and applicability of the affinity chromatography in the purification of the DNase I inhibitor (= actin) is further illustrated by the experiment shown in Fig. 3.

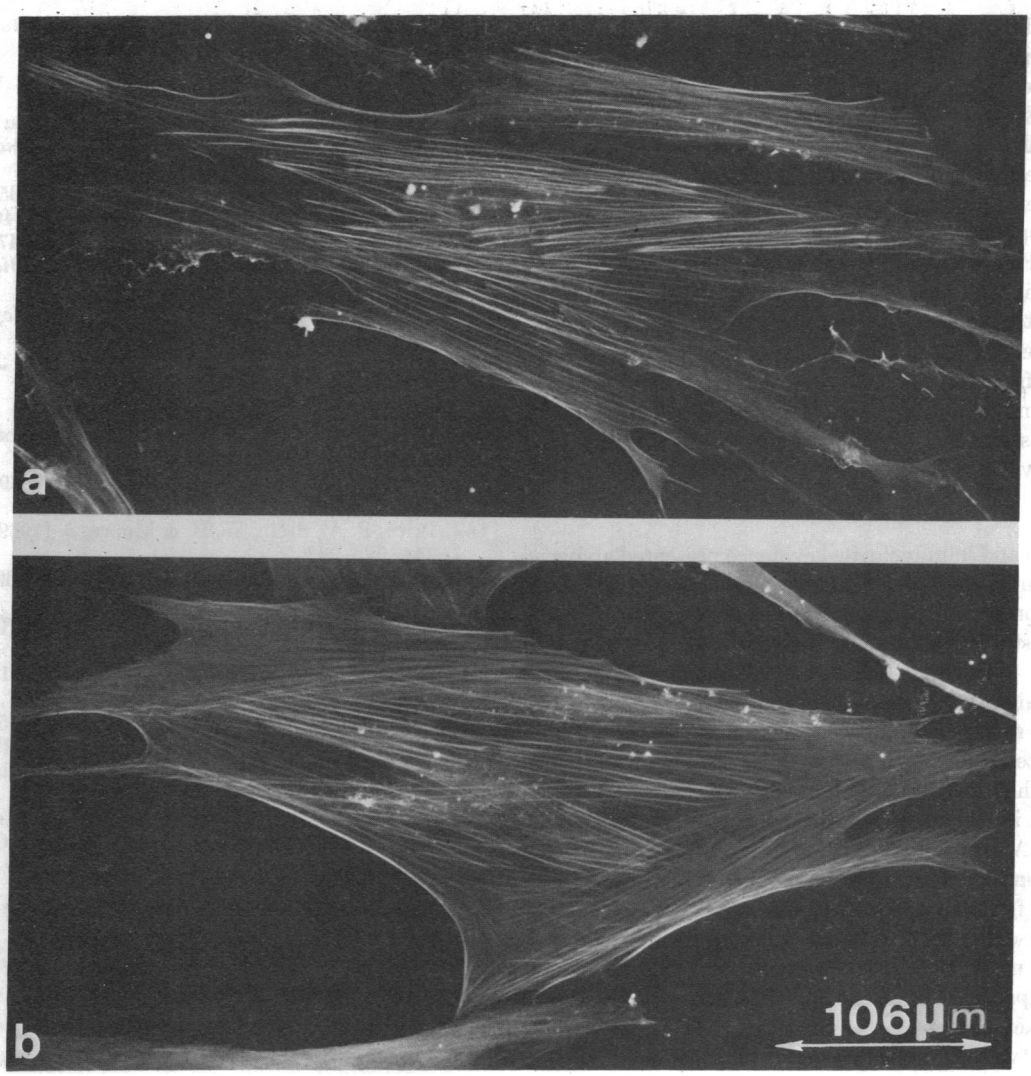

FIG. 5. Indirect immunofluorescence with actin and DNase inhibitor antibodies. Human skin fibroblasts (designated CRL 1139, EN SON, cystic fibrosis) were obtained from the American Tissue Culture Collection. The cells were grown in Dulbecco's modification of Eagle's medium containing 10% fetal calf serum, 1 mg/ml of gentamycin, 100 units/ml of penicillin, and 100 µg/ml of streptomycin. The actin-specific antibody was obtained as described (31). The antibody to purified thymus inhibitor was prepared as described in *Materials and Methods*. Photographs show immunofluorescent staining of human cells with the actin-specific antibody (panel a) and with antibody to the DNase inhibitor (panel b).

Here, a high-speed supernatant of an extract from [^{35}S]-methionine-labeled KB cells was passed through a column of DNase–agarose (Fig. 3A). The eluted fractions were pooled and processed as in the previous experiment. As shown by NaDodSO$_4$–gel electrophoresis (Fig. 3B), one major protein, which electrophoresed with actin, was quantitatively and specifically retained by the column and eluted with 3 M guanidine·HCl. It has previously been shown that when such high-speed supernatants of cultured cells are analyzed by NaDodSO$_4$ gel electrophoresis, the major band, which migrates with a molecular weight of 42,000, is actin (31). Twenty-five percent of the methionine label of the high-speed supernatant was recovered in the 3 M guanidine·HCl fraction; 95% of this radioactivity migrated with the actin band (data not shown). Fig. 3C shows the coelectrophoresis of chicken skeletal muscle actin, DNase I inhibitor from calf thymus, and actin from KB cells purified by affinity chromatography on DNase–agarose. All three proteins migrated identically. The nature of the minor components contaminating the actin eluted from the DNase–agarose is not known, but it is possible that they have some relationship to the actin-DNase I system.

To further substantiate the identification between the DNase I inhibitor and actin, we compared samples of the two proteins by tryptic peptide analysis. The DNase I inhibitor and actin used in this analysis had both been purified by conventional purification methods. As illustrated in Fig. 4A and B, the distribution of peptides in the two maps was closely similar. Furthermore, when the [^{35}S]methionine-labeled protein from KB cells purified by affinity chromatography on DNase–agarose (Fig. 3B) was subjected to peptide map analysis (Fig. 4A), four of the [^{35}S]methionine-containing peptides were well resolved and coincided with ninhydrin-stained spots from chicken skeletal muscle actin.

Actin purified by NaDodSO$_4$–polyacrylamide gel electrophoresis has been shown to be antigenic in rabbits and to give rise to an antibody, which in indirect immunofluorescence reveals actin-containing filaments in cells (31). A similar

method was used here to obtain an antibody against DNase I inhibitor. For this purpose, the inhibitor purified from calf thymus by conventional techniques was denatured with SDS and reduced with dithiothreitol and used for immunization in rabbits as described in *Materials and Methods*. The resulting antibody was tested on human skin fibroblasts by indirect immunofluorescence. These cells were chosen because they are large and well spread and contain a highly developed actin fiber network. Fig. 5a shows a cell stained with the actin antibody, and Fig. 5b a cell stained with the DNase inhibitor antibody. In both cases an array of fibers was revealed. The fibers mark the cell periphery, often converge to focal points, and are frequently seen to span the entire cell length. As judged by immunofluorescence, both antibody preparations show crossreactivity with actin-containing fibers in a variety of cell types.

DISCUSSION

The biochemical and immunological evidence presented above proves that actin is the widely occurring inhibitor of DNase I. The interaction between actin and the enzyme results in the formation of a stable complex. As shown earlier (7, 12) and corroborated in this work (Fig. 3), this association is characterized by a high degree of specificity.

DNase I has so far been thought of as a DNA degrading enzyme. The interaction of actin with DNase I may mean that actin controls the nucleolytic activity of this enzyme during the cell cycle. It is also possible that the complex has a specific function in DNA metabolism. A third alternative is that the primary function of DNase I is not related to DNA but to the formation and function of actin filaments. However, these possibilities are difficult to evaluate, since little information is available at present on the occurrence of DNase I type enzymes in nonpancreatic cells and on the existence of DNase I-actin complexes *in vivo*.

The specificity of the interaction of actin and DNase I makes the isolation of actin by affinity chromatography of considerable experimental value. Actin isolated by this method from high-speed supernatants of cellular extracts is both radiochemically and by staining more than 90% pure. Thus, this method can be conveniently applied to study actin modifications occurring throughout the cell cycle or under various physiological conditions.

Finally, it should be pointed out that the DNase inhibitory activity of actin provides a novel assay for this structural protein. The high specificity of this interaction makes it possible to determine the presence of actin in crude extracts of any type of cell.

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