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Purification and Characterization of a New Mammalian Serum Protein with the Ability To Inhibit Actin Polymerization and Promote Depolymerization of Actin Filaments†

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ABSTRACT: A protein with capacity to bind G-actin and the ability to inhibit polymerization and promote depolymerization of actin filaments has been isolated from the serum of rabbit. The protein, SAIP (for serum actin inhibitory protein), has been purified by affinity chromatography of serum over actin–Sepharose followed by protein fractionation with ammonium sulfate and chromatography over DEAE-cellulose. Five milligrams of purified SAIP is obtained from 100 mL of serum. Rabbit SAIP is resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis into two closely related polypeptides of 60 000 and 56 000 daltons, respectively (ratio 5.7:1). Each of these polypeptides consists of two isoelectric variants. SAIP binds to monomeric actin with a stoichiometry of 1:1 and a K_d of 0.12 μM. The SAIP–actin complex binds to DNase I. Actin polymerization is completely inhibited by incubation of actin with an equal concentration of SAIP. At equimolar concentrations to F-actin, SAIP induces complete depolymerization of the actin filaments. SAIP is also present in calf serum.

Actin is a contractile–structural protein present in all eukaryotic cells. Upon polymerization, the monomeric form of actin (G-actin)[1] forms double-stranded helical filaments (F-actin). These filaments are a major component of the cell cytoskeleton and play an important role in cell motility [for reviews, see Clarke & Spudich (1977), Hitchcock (1977), Korn (1978), and Lindberg et al. (1979)]. The way cells polymerize actin depends on their function. Tissues specialized in contraction–relaxation, such as striated and possibly smooth muscle, organize the actin filaments in stable structures (i.e., thin filaments). On the other hand, the diversity and transient character of many of the structural and contractile functions in which actin plays a role in nonmuscle cells requires the frequent polymerization and depolymerization of actin in these cells. The assembly of actin can be regulated at different levels including filament nucleation and the addition and loss of actin monomers to elongating and steady-state filaments [for review, see Hitchcock-De Gregori (1980)].

The nucleation of actin filaments has been shown to be promoted by a complex of spectrin–actin–band 4.1 isolated from human erythrocytes (Lin & Lin, 1979; Ungewickell et al., 1979) and also by a complex of spectrin–actin obtained from sheep erythrocytes which blocks the slow-growing end of the actin filaments (Brenner & Korn, 1980). More recently several proteins capping the fast growing end of the actin filaments (i.e., capping proteins) have been isolated from sources as diverse as *Acanthamoeba* (Isenberg et al., 1980), *Physarum* (Hasegawa et al., 1980), and chicken intestinal epithelial cells (Craig & Powell, 1980; Glenney et al., 1980, 1981). This class of proteins also has the property to enhance nucleation of actin filaments (Isenberg et al., 1980; Hasegawa et al., 1980; Craig & Powell, 1980; Glenney et al., 1981), and some of them, like gelsolin, villin, and fragmin, show the ability to sever the actin filaments, producing short nonsedimentable oligomers of actin in the presence (Yin & Stossel, 1979; Hasegawa et al., 1980; Glenney et al., 1981) or absence of Ca^{2+} (Isenberg et al., 1980). Actin nucleation can also be controlled by proteins which bind to G-actin preventing polymerization. One of these proteins, profilin (Carlsson et al., 1977), seems to prevent actin nucleation specifically without inhibiting filament elongation (Reichstein & Korn, 1979). The inhibitory effect of profilin on actin nucleation can be reversed by α-actinin (Blikstad et al., 1980), short fragments of actin filaments (Reichstein & Korn, 1979), and a cytochalasin binding complex isolated from erythrocytes (Grumet & Lin, 1980). Other proteins binding to G-actin in addition to inhibiting the nucleation of actin also prevent filament elongation and cause depolymerization of the filaments. To this group of proteins belongs DNase I (Lazarides & Lindberg, 1974), which has been found complexed to G-actin in rat pancreatic juice (Mannherz & Rohr, 1978) and the 65K and 62K polypeptides isolated recently from human granulocytes (Southwick & Stossel, 1981).

Blood plasma and sera from various mammals have been shown to have the capacity to depolymerize actin filaments. Human serum contains a Ca^{2+}-dependent enzyme which promotes catalytically the depolymerization of actin filaments (Norberg et al., 1979). On the other hand a 92 000-dalton protein has been isolated from pig plasma which binds stoi-

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[1] Abbreviations: G-actin, globular actin; F-actin, filamentous actin; IEF, isoelectric focusing; SAIP, serum actin inhibitory protein; DNase I, deoxyribonuclease I; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; DEAE, diethylaminoethyl; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

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chiometrically to G-actin, inhibiting the polymerization and promoting the depolymerization of actin filaments (Harris & Gooch, 1981). Here we report the purification and partial characterization of a 60000-dalton protein from rabbit serum with the capacity to inhibit polymerization and to promote the depolymerization of actin filaments.

Materials and Methods

Sera. Rabbit serum was obtained from blood collected by heart puncture. Calf serum was purchased from Gibco (Belgium).

Actin Purification. Acetone powder of rabbit back and leg muscle was prepared according to the procedure of Straub (1942). Actin was purified from the acetone powder as described by Spudich & Watt (1971). Purified actin was stored as G-actin in 2 mM Tris-HCl, 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, and 0.2 mM $CaCl_2$, pH 7.8 (buffer G), at 4 °C for a maximum of 3 days.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was used to measure the content of actin and SAIP in the pellets and supernatants after polymerization. Polymerization pellets were prepared for electrophoresis by boiling in 100 μL of 13 mM Tris-HCl, 1% $NaDodSO_4$, 10% glycerol, 50 mM DTT, and 0.025% bromophenol blue, pH 6.8, for 5 min. Polymerization supernatants were mixed with 1 volume of the same buffer and also boiled for 5 min prior to electrophoresis. Electrophoresis was performed by the method of Laemmli (1970) on 1.5-mm thick slab gels consisting of 12.5% acrylamide–0.2% bis(acrylamide). The stacking gel contained 5% acrylamide–0.26% bis(acrylamide). Gels were stained for protein with 100 mL of 0.25% Coomassie blue in 45% methanol–9.2% acetic acid for 20 min and destained overnight with a mixture of 5% methanol–7.5% acetic acid. The actin and SAIP content of unknown samples was measured by densitometry. The absorbance values obtained were converted to moles by extrapolation of absorbance curves obtained from known concentrations of actin (M_r 43000) and rabbit SAIP (M_r 60000–56000) run in parallel with the unknown samples. The correlation between amount of protein and Coomassie blue staining of the protein was found to be linear only up to 1.5 nmol of actin and 1 nmol of SAIP per gel slot.

Studies on Actin Polymerization. The capacity of SAIP to inhibit actin polymerization was studied in samples containing 0.7–30.5 μM G-actin preincubated in the presence of 3.2 μM SAIP for 15 min at 20 °C in buffer G. Polymerization was initiated by addition of KCl to 0.1 M and $MgCl_2$ to 1 mM final concentration (buffer F). After incubation for 90 min at 20 °C, the actin polymers formed were harvested from 100-μL aliquots by centrifugation at 100000*g* for 30 min using a Beckman airfuge. Polymerization was measured by determining the actin content in the resulting pellets using $NaDodSO_4$–polyacrylamide gel electrophoresis (see above).

Studies on the Binding of SAIP to G-Actin. The binding of SAIP to monomeric actin was studied by the method of Scatchard (1949). For this purpose, 50 μL of actin–Sepharose, prepared in buffer G to 24 nmol of actin/mL of suspension, was mixed with 0.9–5 μM [^{125}I]SAIP (2.1×10^3–11.8×10^3 Ci/mol) prepared in 190 μL of 20 mM Tris-HCl and 150 mM NaCl, pH 7.0, containing 20 μg of ovalbumin. The mixture was incubated with continuous shaking for 30 min at 20 °C, and the free SAIP was then separated from that bound to G-actin–Sepharose by centrifugation for 30 s in an Eppendorf centrifuge. The concentration of free SAIP was measured by determining the amount of [^{125}I]SAIP in the resulting supernatants. SAIP bound to G-actin was calculated by subtracting the free SAIP from the total SAIP used in the binding assay. Because the biological activity of the G-actin bound to Sepharose was unknown, the method of Scatchard could not be used to measure the stoichiometry of binding of SAIP to G-actin. This was determined by polymerizing an excess of G-actin in the presence of SAIP, in buffer F, and measuring the molar concentration of SAIP and G-actin in the resulting polymerization supernatants (for experimental conditions, see Studies on Actin Polymerization). The concentrations of G-actin obtained in these experiments were corrected for the concentration of actin critical for polymerization (1.6 μM) measured in the absence of SAIP (Oosawa & Kasai, 1962).

Studies on F-Actin Depolymerization. These experiments were carried on in buffer F. The time course of F-actin depolymerization by SAIP was studied by incubating F-actin (5.9 μM monomers in filaments) with an equimolar amount of SAIP at 20 °C and analyzing the content of F-actin in aliquots withdrawn at various times of incubation. F-Actin was determined both by the capacity of the aliquots to inhibit DNase I activity (Blickstad et al., 1978) and by measuring the actin content of pellets obtained by centrifugation (100000*g*, 150 min, 20 °C) of the aliquots by $NaDodSO_4$–polyacrylamide gel electrophoresis (see above). For calibration of the inhibition of DNase I by the actin contained in unknown samples, standard curves of DNase I inhibition by different concentrations of G-actin (1.0–4.0 μM) and F-actin (5.9 μM) were used. The effect of the SAIP concentration on the depolymerization of F-actin was studied by incubating a fixed concentration of F-actin (6.2 μM actin in filaments) with various concentrations of SAIP (2.5–25 μM) in a final volume of 0.5 mL for 30 min at 20 °C. The F-actin remaining was quantified by $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of the pellets obtained by centrifugation at 100000*g* for 2 h at 4 °C using a Beckman L5-75 ultracentrifuge.

Studies on the Binding of SAIP to the G-Actin–DNase I Complex. Two aspects of the binding of SAIP to the G-actin–DNase I complex were studied. First, the abilities of affinity chromatography columns made on DNase I–Sepharose and of G-actin–DNase I–Sepharose to bind SAIP were compared. For this purpose equal amounts of SAIP prepared in 10 mM Tris-HCl and 5 mM $CaCl_2$, pH 7.5, were passed simultaneously over both columns equilibrated with the same buffer. The columns were washed with 2 bed volumes of 10 mM Tris-HCl, 0.5 M NaCl, and 5 mM $CaCl_2$, pH 7.5, and eluted, first with 0.5 mM sodium acetate (pH 6.5) containing 0.75 M guanidinium chloride and 30% glycerol and then with 1 M sodium acetate (pH 6.5) containing 3 M guanidinium chloride and 30% glycerol (Lindberg & Eriksson, 1971). The eluted fractions were collected and analyzed for SAIP by $NaDodSO_4$–polyacrylamide gel electrophoresis. Second, the capacities of G-actin, SAIP, and the G-actin–SAIP complex to inhibit the DNase I activity were studied. The assay was performed by using a 2-fold molar excess of G-actin, SAIP, or the G-actin–SAIP complex over the concentration of DNase I. DNase I activity was determined by using the hyperchromicity assay as described (Blickstad et al., 1978).

Peptide Mapping. The 60000-dalton and 56000-dalton SAIP polypeptides were separated by preparative $NaDodSO_4$–polyacrylamide gel electrophoresis (Lazarides, 1976). Polypeptides were electrophoretically eluted and free from $NaDodSO_4$ and Coomassie blue by dialysis against water followed by lyophilization and extraction (Henderson et al., 1979). Limited chymotryptic digestion of both proteins was carried out as described (Cleveland et al., 1977) by using an enzyme to substrate ratio of 1:30 (w/w) for 20 min at 20 °C.

Peptide mapping of the protein digests was by $NaDodSO_4$–polyacrylamide gel electrophoresis using the gel system described above.

Two-Dimensional Gel Electrophoresis. Samples for isoelectric focusing were prepared by precipitating the protein in 60% ethanol at −20 °C. The resulting pellets were dissolved in 3% $NaDodSO_4$ and 10% 2-mercaptoethanol, boiled for 5 min, and mixed with 10 volumes of 9.5 M urea containing 2% Nonidet P-40, 1.6% ampholine, pH 5–7, 0.4% ampholine, pH 3.5–10, and 5% 2-mercaptoethanol. Isoelectric focusing was carried out as reported (O'Farrell, 1975). The resulting gels were equilibrated for 20 min in 0.075 M Tris-HCl, 3% $NaDodSO_4$, and 50 mM DTT, pH 8.8 (Garrels, 1979), and layered over an $NaDodSO_4$–12.5% polyacrylamide–0.2% bis(acrylamide) gel for electrophoresis in the second dimension. Gels were stained and destained as indicated above. Protein isoelectric points were determined from the first-dimension isoelectric focusing gels as described (Yin & Stossel, 1980).

Amino Acid Analyses. Purified proteins were desalted on Sephadex G-10 equilibrated with pyridine–acetate buffer, pH 6.5 (10% pyridine), divided in aliquots of 0.2 mg, lyophilized, and hydrolyzed in 0.5 mL of 6 N HCl at 110 °C for 24, 48, and 72 h. Amino acid analyses were carried out by using a Bio-Cal 201 amino acid analyzer. The values for serine, threonine, methionine, and tyrosine were calculated by extrapolating the values obtained after 24-, 48-, and 72-h hydrolysis. The values of the other amino acids were calculated as the average values obtained from samples hydrolyzed for 24-, 48-, and 72-h hydrolysis. Cysteine was determined as cysteic acid (Hirs, 1956).

Other Methods. Protein was measured by the method of Lowry et al. (1951) by using bovine serum albumin (Armour) as a standard.

CNBr-activated Sepharose 4B was prepared as described (Cuatrecasas et al., 1968). DNase was coupled following the procedure of Lindberg & Eriksson (1971). Actin was coupled to CNBr-activated Sepharose 4B by mixing 2 volumes of G-actin in buffer G (0.5 mg/mL) with 1 volume of CNBr–Sepharose suspension in 0.1 M $NaHCO_3$ for 2 h at 4 °C. The actin–Sepharose was washed by filtration with several volumes of buffer G and then incubated for 15 min at 4 °C with 1 volume of 1 M ethanolamine, pH 8.5. Final washings were again with buffer G. The yield of the coupling was measured by amino acid analysis following acid hydrolysis of the protein bound to Sepharose with 6 N HCl. SAIP was iodinated with $Na^{125}I$ (New England Nuclear) by using the Chloramine T procedure (Greenwood et al., 1963). Guinea pig antibodies were raised against rabbit 60 000-dalton SAIP purified by $NaDodSO_4$–polyacrylamide gel electrophoresis following standard procedures (Chase, 1975).

Results

Purification of Serum Actin Inhibitory Protein (SAIP) from Rabbit Serum. All purification steps were performed at 4 °C; 100 mL of rabbit serum was passed over an actin–Sepharose 4B column (containing approximately 25 mg of actin) equilibrated with 20 mM Tris-HCl, 150 mM NaCl, and 1 mM $CaCl_2$, pH 7.0 (buffer A). The column was washed extensively with buffer A containing 0.57 M NaCl until an absorbance of less than 0.05 at 280 nm was obtained, followed by 2 bed volumes of 0.25 M glycine–HCl and 1 mM $CaCl_2$, pH 4.0. The proteins bound to actin were eluted with 0.25 M glycine–HCl and 1 mM $CaCl_2$, pH 2.75, and neutralized immediately to pH 7.0 by using 1 N NaOH. It was found that SAIP retained its full capacity to bind to monomeric actin and to depolymerize F-actin upon incubation in the pH 2.75 buffer

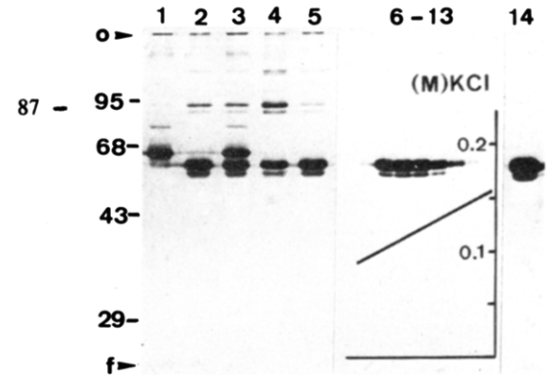

FIGURE 1: Purification of rabbit serum actin inhibiting protein. The protein fractions obtained during purification of SAIP were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. The gel origin (o) and front (f) are indicated. (Slot 1) Crude rabbit serum. (Slot 2) Proteins eluted from the actin–Sepharose column using 0.25 M glycine–HCl, pH 2.75. (Slot 3) Proteins shown in slot 2 mixed with rabbit serum. Note that the 60 000-dalton and 56 000-dalton polypeptides migrate ahead of rabbit serum albumin. (Slot 4) 20% ammonium sulfate cut of the proteins shown in slot 2. Note the high content of the 87 000-dalton protein. (Slot 5) 20–50% ammonium sulfate cut of the proteins shown in slot 2. Note the high content of the 60 000-dalton and 56 000-dalton proteins. Slots 6–13 show the elution profile of the 20–50% ammonium sulfate fraction passed over DEAE-cellulose and eluted with a linear gradient of KCl. SAIP is eluted between 0.1 M KCl and 0.15 M KCl. Slot 14 shows the polypeptide composition of the rabbit SAIP used in actin binding assays and actin polymerization experiments. Reference proteins used for molecular weight determination of the unknown proteins were muscle phosphorylase (95 000 daltons), bovine serum albumin (68 000 daltons), actin (43 000 daltons), and human erythrocyte carbonic anhydrase (29 000 daltons). The migration of the markers in the $NaDodSO_4$–polyacrylamide gel electrophoresis system is indicated by the first two digits of their molecular weight.

at 4 °C for more than 4 h (results not shown). $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of the eluted protein revealed the presence of three major polypeptides with molecular weights of 87 000, 60 000, and 56 000 (Figure 1, slot 2). Fractionation of these polypeptides was achieved by precipitation with a saturated solution of $(NH_4)_2SO_4$ prepared in 50 mM phosphate buffer, pH 6.8. The 60 000- and 56 000-dalton polypeptides were preferentially precipitated between 20% $(NH_4)_2SO_4$ and 50% $(NH_4)_2SO_4$ (Figure 1, compare slots 4 and 5). The resulting pellet of the 20–50% $(NH_4)_2SO_4$ fraction was dissolved in 3 mL of 10 mM imidazole, 0.1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 1 mM 2-mercaptoethanol, pH 6.8 (buffer B), and dialyzed extensively against the same buffer. Final purification was achieved by chromatography of the dialyzed protein over a DEAE-cellulose column (1.5 × 6 cm) equilibrated with buffer B. Following the sampling of the protein, the column was washed with 2 bed volumes of buffer B and 50 mL of the same buffer containing 50 mM KCl to wash out the remaining 87 000-dalton protein and other minor protein contaminants. The protein still retained was eluted with 60 mL of a 50–500 mM KCl linear gradient in buffer B. Protein analysis of the eluted fractions by $NaDodSO_4$–polyacrylamide gel electrophoresis showed that the 60 000- and 56 000-dalton polypeptides were eluted together between 100 and 150 mM KCl (Figure 1, slots 6–13). It is noteworthy that different preparations showed a constant ratio (5.7:1) of the 60 000- and 56 000-dalton polypeptides (Figure 1, slot 14). Amino acid analysis (Table I) and $NaDodSO_4$–polyacrylamide gel electrophoresis of partial chymotryptic digests of the 60 000- and 56 000-dalton polypeptides purified by preparative $NaDodSO_4$–polyacrylamide gel electrophoresis revealed extensive similarities between both proteins (Figure 2). Hence, no further attempts were made

Table I: Amino Acid Compositions of Serum Actin Inhibitory Proteins from Rabbit and Calf

	56 000-dalton rabbit SAIP		60 000-dalton rabbit SAIP		mixture of the 56 000- and 60 000-dalton rabbit SAIP[a]		mixture of the 53 000- and 57 000-dalton calf SAIP[b]	
amino acid	residues	mol (%)	residues	mol (%)	residues	mol (%)	residues	mol (%)
aspartic acid	45	8.8	48	8.7	47	8.7	48	9.4
threonine	33	6.5	34	6.3	34	6.3	32	6.3
serine	45	8.8	48	8.8	47	8.7	43	8.5
glutamic acid	65	12.8	68	12.5	67	12.4	59	11.6
proline	37	7.3	39	7.1	41	7.6	41	8.1
glycine	25	5.0	32	5.8	14	2.6	18	3.6
alanine	37	7.3	39	7.1	40	7.4	28	5.5
valine	24	4.7	26	4.8	28	5.2	26	5.1
methionine	8	1.6	9	1.6	9	1.7	10	2.0
isoleucine	15	3.0	16	2.9	17	3.1	18	3.6
leucine	46	9.1	50	9.2	54	10.0	49	9.7
tyrosine	19	3.7	20	3.7	21	3.9	20	3.9
phenylalanine	17	3.3	19	3.4	20	3.7	23	4.5
lysine	41	8.0	45	8.2	47	8.7	38	7.5
histidine	9	1.8	9	1.7	9	1.7	8	1.6
arginine	13	2.5	15	2.7	15	2.7	18	3.6
cysteine	28	5.6	30	5.5	30	5.5	28	5.5
total	507		547		540		507	

[a] Calculated assuming a total molecular weight of 60 000. [b] Calculated assuming a total molecular weight of 57 000.

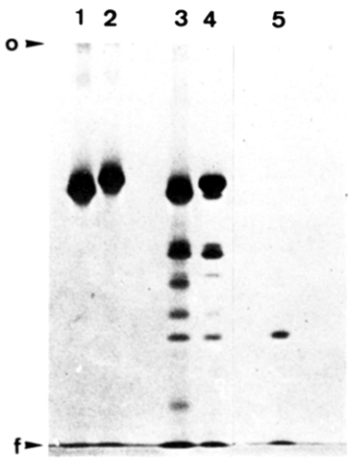

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis of partial chymotryptic digestion products of the 60 000- and 56 000-dalton rabbit SAIP polypeptides. The 60 000-dalton and 56 000-dalton SAIP polypeptides were purified by $NaDodSO_4$–polyacrylamide gel electrophoresis and digested with chymotrypsin as described under Materials and Methods. (Slot 1) 56 000-dalton SAIP. (Slot 2) 60 000-dalton SAIP. Slots 3 and 4 show respectively the 56 000-dalton and 60 000-dalton proteins partially digested with chymotrypsin. Slot 5 contains the chymotrypsin used for the digestion.

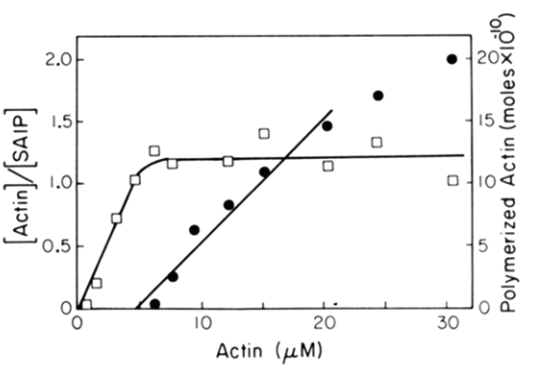

FIGURE 3: Effect of SAIP on the polyerization of G-actin. 3.2 μM SAIP was incubated with concentrations of G-actin varying from 0.7 to 30.5 μM. Polymerization was initiated by the addition of KCl to 0.1 M and $MgCl_2$ to 1 mM final concentration. After incubation for 90 min at 20 °C, the polymers of actin formed were harvested by centrifugation and quantified by $NaDodSO_4$–polyacrylamide gel electrophoresis (see Materials and Methods) (●). The last two points of the curve of polymerized actin were ignored due to the lack of correlation between amount of actin and Coomassie blue staining of the protein over 1.5 nmol of actin (see Materials and Methods). The corresponding polymerization supernatants were analyzed for actin and SAIP by $NaDodSO_4$–polyacrylamide gel electrophoresis to determine the molar ratio of G-actin/SAIP (□).

to separate the two polypeptides, and all the experiments were performed with the mixture of both. Henceforth we shall refer to this mixture as serum actin inhibitory protein (SAIP). An average of 5 mg of SAIP starting from 100 mL of serum was obtained. Purified SAIP showed no loss of its biological activity when stored in buffer B or in 20 mM Tris-HCl, 150 mM NaCl, and 1 mM $CaCl_2$, pH 7.0, for 2 months at −80 °C.

SAIP Capacity To Inhibit Actin Polymerization and To Bind to Monomeric Actin. The capacity of SAIP to inhibit actin polymerization was studied by incubating increasing concentrations of G-actin (0.7–30.5 μM) with a fixed concentration of SAIP (3.2 μM) under polymerization conditions (buffer F). As shown in Figure 3 under these conditions actin did not polymerize at concentrations lower than 4.7 μM. At higher G-actin concentrations, polymerization occurred in a concentration-dependent manner. Identical results were obtained when the free concentration of Ca^{2+} was reduced from 200 μM to 0.5 μM by using 2 mM EGTA in the polymerization mixture, indicating that the effect of SAIP on actin polymerization was independent of Ca^{2+} concentration. These results indicate that actin polymerization was inhibited by SAIP stoichiometrically rather than catalytically. The stoichiometry of the binding of SAIP to G-actin was determined by measuring the molar concentrations of G-actin and SAIP in the polymerization supernatants of the same experiment. As shown in Figure 3, the molar ratio of G-actin/SAIP was found to increase linearly between 0.75 μM actin and 4.7 μM actin and then to remain constant at an average of 1.2 mol of actin/mol of SAIP with higher concentrations of actin. It is important to note that the value of 1.2 for the actin/SAIP ratio was attained at the same concentration of actin (4.7 μM) required for minimal polymerization in the presence of SAIP. Further analysis of the binding of SAIP to G-actin by the method of Scatchard revealed one class of binding site with a K_d of 0.12 μM (Figure 4). The stoichiometry of binding could not be calculated directly by Scatchard's analysis as the

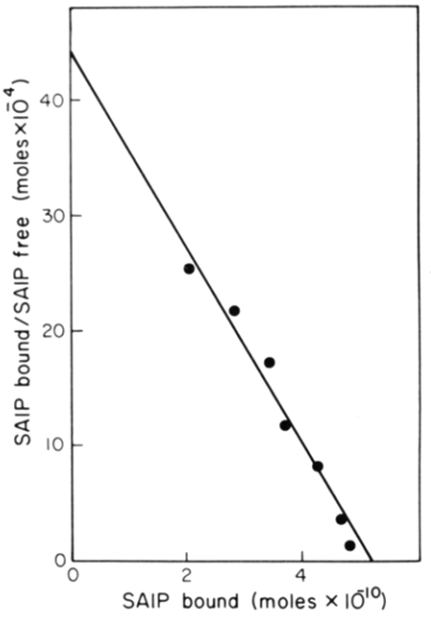

FIGURE 4: Scatchard's analysis of the binding of SAIP to actin. Increasing amounts of [^{125}I]SAIP (0.9–5 μM) were incubated with actin (5 μM) bound to Sepharose for 30 min at 20 °C. The Sepharose beads were then sedimented by centrifugation to remove the SAIP bound to actin and the supernatants counted for [^{125}I]SAIP to determine the concentrations of free SAIP and SAIP bound to actin. A K_d of 0.12 μM was obtained for the binding of SAIP to actin.

activity of the actin bound to Sepharose used in the assay was unknown (see Materials and Methods) and was thus measured as indicated above.

Effect of SAIP on F-Actin Depolymerization. The ability of SAIP to depolymerize the filaments of F-actin was studied by comparing the content in F-actin (sedimentation assay) and G-actin (DNase inhibition assay) of samples of F-actin incubated in the absence and the presence of SAIP for different times. Within the same experiment, the rates of F-actin depolymerization measured by both the sedimentation and the DNase inhibition assays were very similar, ruling out the possibility that SAIP severed the actin into short nonsedimentable oligomers. Figure 5 shows a typical experiment in which 50% of the F-actin was depolymerized by SAIP in 90 min. However, in similar experiments using different F-actin preparations, the time for half-depolymerization was found to vary between 1 h and 3 h. Depolymerization of F-actin by SAIP could result from either a direct effect of SAIP on the actin filament (i.e., destabilizing the entire actin filament, increasing the rate of loss of actin monomers, or decreasing the rate of incorporation of actin monomers to the filament ends) or an indirect effect by sequestering the actin monomers existing in equilibrium with the actin filaments. Two experiments were performed to distinguish between some of these possibilities. In a first experiment a fixed concentration of F-actin (6.2 μM monomers in filaments) was incubated with concentrations of SAIP ranging from 2.5 μM to 25 μM over a period of only 30 min at 20 °C (Figure 6). It is important to note that the lowest SAIP concentration assayed was higher than the actin critical concentration (1.6 μM). It was observed that, independent of both the SAIP concentration used and the amount of SAIP pelleted with the F-actin (Figure 6; see below), 2 nmol of actin was recovered in the pellet of 0.5 mL of F-actin–SAIP depolymerizing mixture; 2.8 nmol of actin/0.5 mL was recovered in the control containing F-actin incubated in the absence of SAIP, indicating that about 30% of the F-actin was rendered nonsedimentable by the action of SAIP. This result suggests that SAIP does not promote the

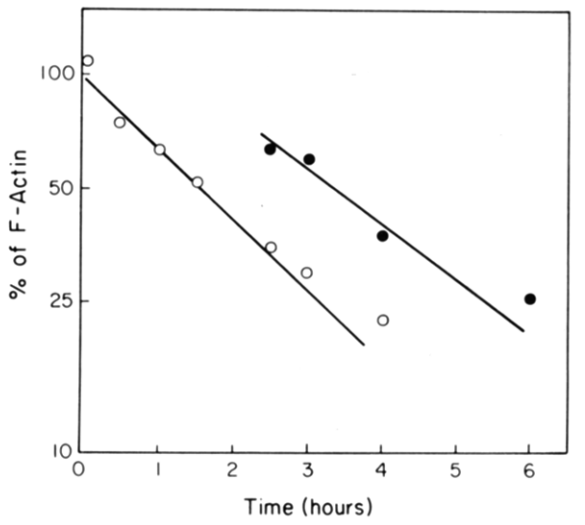

FIGURE 5: Effect of SAIP on F-actin depolymerization. 5.9 μM actin monomers in filaments was incubated in the absence or presence of an equal concentration of SAIP in buffer F at 20 °C. F-Actin was measured after different times of incubation by determining the amount of actin in the polymers harvested by centrifugation (●) and also by measuring the capacity of the reaction mixtures to inhibit the activity of DNase I (○). The values of F-actin measured by the sedimentation assay were computed after 150-min centrifugation at 100000g to harvest the actin polymers. Controls incubated in the absence of SAIP showed no decrease in F-actin during the time of the experiment. The amount of F-actin in the samples incubated with SAIP is expressed as a percentage of the total F-actin measured in the controls.

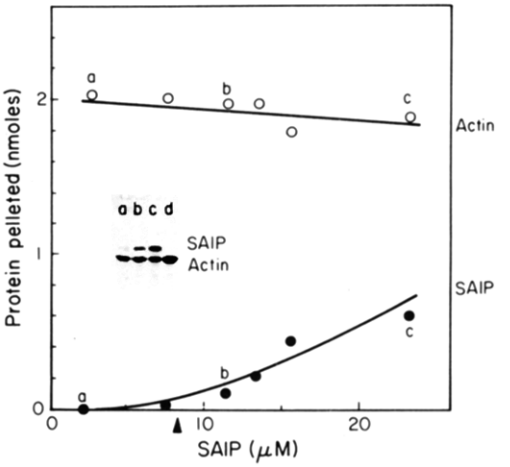

FIGURE 6: Independence of F-actin depolymerization on the concentration of SAIP. 6.2 μM actin monomers in filaments was incubated in the absence or presence of different concentrations of SAIP (2.5–25 μM) in a final volume of 0.5 mL for 30 min at 20 °C. The remaining polymers were harvested by centrifugation (2 h at 100000g), and their content in actin and SAIP was quantified by NaDodSO$_4$–polyacrylamide gel electrophoresis analysis. The pellet of the control sample incubated in the absence of SAIP contained 2.8 nmol of actin. Note that the pellets of the samples incubated with different concentrations of SAIP contained similar amounts of actin (2 nmol) (○) although increasing amounts of SAIP (●). Gel slots a, b, and c correspond to the points marked with the same letters in the concentration curves of actin and SAIP. Slot d corresponds to the F-actin pellet of the control sample.

depolymerization of the actin filaments by acting directly on the filaments. In a second experiment it was found that the rate of depolymerization of F-actin (5.6 μM actin monomers in filaments) by equimolar amounts of SAIP, measured by the centrifugation sedimentation assay, was not changed when the sample contained cytochalasin B (0.3 μM). Given the ability of cytochalasin B to cap the fast growing end of the filaments, this result suggests that SAIP does not increase the rate of

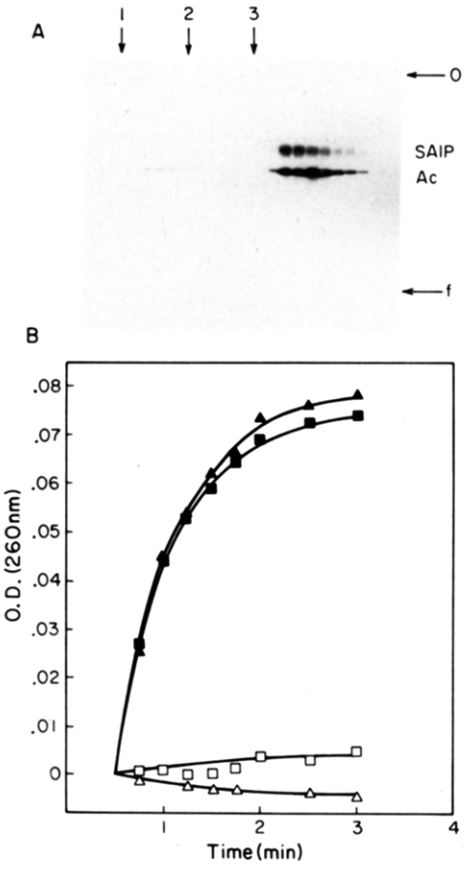

FIGURE 7: Binding of SAIP to the DNase I–G-actin complex. Inhibition of the DNase I activity by the SAIP–G-actin complex. (A) Elution profile of SAIP from a G-actin–DNase I–Sepharose column washed successively with 0.57 M NaCl (1), 0.75 M guanidinium chloride (2), and 3 M guanidinium chloride (3). The eluted fractions were analyzed for proteins by $NaDodSO_4$–polyacrylamide gel electrophoresis. The gel origin (o), gel front (f), and positions of SAIP and actin (Ac) in the electropherogram are indicated. (B) Time course of DNase I activity measured in the absence (■) or presence of a 2-fold molar excess of SAIP (▲), G-actin (□), and the G-actin–SAIP complex (△). Changes in absorbance were measured starting 30 s after adding the DNA to the protein mixtures.

actin monomer loss from the growing filament end. In conclusion, the first-order rate of F-actin depolymerization by SAIP (Figure 5), the independence of the rate of F-actin depolymerization on the concentration of SAIP (Figure 6), and the absence of any effect of cytochalasin B on the depolymerization rate by SAIP can probably be explained by assuming that SAIP binds and sequesters free actin monomers in equilibrium with the actin filaments (see Discussion).

Sedimentation analysis of samples of SAIP (2.5–25 μM) incubated in the absence and in the presence of actin (6.2 μM), in buffer G and buffer F, revealed that SAIP was only pelleted in the samples of actin incubated in buffer F (i.e., F-actin) (Figure 6). The amount of SAIP pelleted was dependent of the SAIP concentration and was always lower than 1 mol/mol of F-actin. The SAIP recovered from F-actin pellets was still fully active as shown by the capacity to prevent the polymerization of the actin pelleted following depolymerization in buffer G.

Studies on the Ability of SAIP To Bind to Actin–DNase I. The binding of SAIP and pancreatic DNase I to actin and their similar effects on actin polymerization and depolymerization made it interesting to study the binding of SAIP to actin in the presence of DNase I. For this purpose two different types of experiments were performed. In the first experiment SAIP was passed over identical columns of actin–

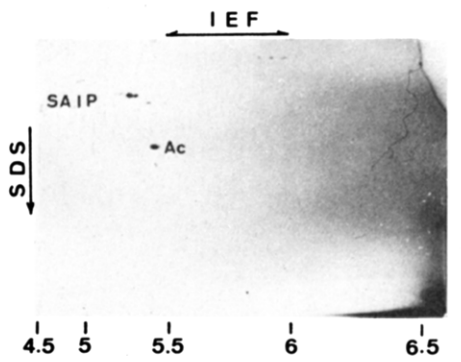

FIGURE 8: Two-dimensional IEF–$NaDodSO_4$–polyacrylamide gel electrophoresis analysis of SAIP. Purified rabbit SAIP was analyzed by isoelectric focusing in the first dimension and $NaDodSO_4$–polyacrylamide gel electrophoresis in the second dimension. Actin (Ac) was used as the marker. Numbers indicate the pH gradient in the isoelectric focusing gel.

DNase I–Sepharose and DNase I–Sepharose and the ability of each column to retain SAIP compared. SAIP was found to pass quantitatively through the DNase I–Sepharose and was recovered in the void volume of the column. On the other hand, the SAIP chromatographed over the actin–DNase I–Sepharose column was tightly retained. Washing of the column with 0.57 M NaCl and subsequently with 0.75 M guanidinium chloride failed to elute the bound SAIP. Elution of SAIP could only be achieved by washing the actin out of the column with 3.0 M guanidinium chloride (Figure 7A). In the second experiment the bindings of G-actin, SAIP, and the G-actin–SAIP complex to DNase I were compared by studying their capacity to inhibit the DNase I activity (Figure 7B). It was observed that, while SAIP alone had no effect on the DNase I activity, the inhibitory capacities of the G-actin and the G-actin–SAIP complex were comparable.

Physicochemical Properties of SAIP. As mentioned before, $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of rabbit SAIP revealed the presence of two polypeptides of 60 000 and 56 000 daltons. When reducing agents (i.e., dithiothreitol and 2-mercaptoethanol) were omitted from the electrophoresis sample buffer, both polypeptides migrated with an apparent lower molecular weight (M_r 51 000 and 47 000), and neither dimers nor other polymeric forms were observed (results not shown). Furthermore, the mobility of both SAIP polypeptides in $NaDodSO_4$–polyacrylamide gel electrophoresis was found to depend on the capacity of the reducing reagent used in the electrophoresis sample buffer. Thus, when SAIP was briefly incubated with 2-mercaptoethanol, both polypeptides gave less sharp bands than when incubated in the presence of dithiothreitol for the same period of time. Longer incubations with 2-mercaptoethanol resulted in sharpening of the SAIP bands and their migration to slightly higher molecular weight position (results not shown). Discrete bands of SAIP were obtained only when dithiothreitol was used as reducing agent. These results suggest that free SH groups were absent in SAIP and that the existing S–S bridges were highly stable. On the other hand the apparent lower molecular weights of the unreduced SAIP polypeptides could indicate that they form a compact tertiary structure which is unfolded upon reduction.

Isoelectric focusing of rabbit SAIP resolved the 60 000-dalton polypeptide (one pI = 5.2) and the 56 000-dalton polypeptide (one pI = 5.3) each into two major isoelectric variants (Figure 8).

Amino acid analysis of SAIP (Table I) showed a high cysteine content (5.5%) and high ratios of alanine/glycine,

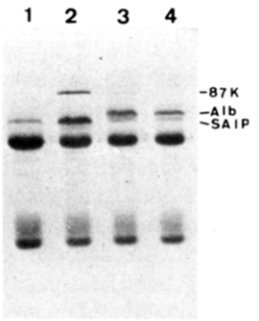

FIGURE 9: Specific immunoprecipitation of SAIP from rabbit serum by antibodies raised against the 60 000-dalton rabbit SAIP in guinea pig. Analysis of the immune precipitate was performed by NaDod-SO_4–polyacrylamide gel electrophoresis. (Slot 1) Immunoprecipitate harvested from rabbit serum incubated with anti-SAIP immune serum from guinea pig. (Slot 2) Mixture of the immunoprecipitated proteins shown in slot 1 and the 60 000-, 56 000-, and 87 000-dalton actin binding proteins retained from serum passed over an actin–Sepharose column. (Slot 3) Mixture of the immunoprecipitated proteins and rabbit serum. (Slot 4) Mixture of the immunoprecipitated proteins and immune serum from guinea pig.

leucine/isoleucine, and lysine/arginine. In accordance with its low isoelectric point, SAIP had a high content of aspartic acid plus asparagine and glutamic acid plus glutamine. As already indicated above, the amino acid composition of the 60 000- and 56 000-dalton components of SAIP was almost identical, the only noticeable difference being the glycine content (Table I). This variation is most probably due to glycine contamination during the preparative separation of the two polypeptides in Tris–glycine electrophoresis buffer, rather than to a real difference between the two SAIP components.

Antibodies Raised against Rabbit SAIP Do Not Cross-React with Other Actin Binding Proteins Present in Rabbit Serum. The capacity of the antibodies raised in guinea pig against rabbit 60 000-dalton SAIP to precipitate proteins from rabbit serum was studied by mixing equal volumes of guinea pig immune serum and rabbit serum. NaDodSO_4–polyacrylamide gel electrophoresis analysis of the resulting immunoprecipitates shows (Figure 9) that the only polypeptides contained were rabbit SAIP and the heavy and light chains of the immunoglobulins. The absence of both the 87 000-dalton protein retained by the actin–Sepharose column and serum albumin in the immunoprecipitates suggests that SAIP is unrelated to those two actin binding proteins (see Discussion).

Distribution of SAIP in the Sera of Mammals. Serum from calf was processed according to the procedure used to purify SAIP from rabbit serum, described above. A SAIP-like protein was purified from this serum in yields similar to that obtained from rabbit serum (5 mg/100 mL serum). Calf SAIP consists of two polypeptides with molecular weights of 57 000 and 53 000, present in a ratio of 13:1. It binds to monomeric actin in a 1:1 ratio and shows a capacity of depolymerizing F-actin similar to rabbit SAIP (result not shown here). Its amino acid composition (Table I) and isoelectric point (pI = 5.2) are very similar to those of rabbit SAIP. In spite of these similarities, antibodies raised against rabbit 60 000 SAIP did not cross-react with calf SAIP. This result suggests an evolutionary divergence of SAIP among different mammalian species.

Discussion

Both plasma and serum contain factors which promote the depolymerization of actin filaments (i.e., F-actin). A Ca^{2+}-dependent enzyme of molecular weight higher than albumin has been found in human serum (Norberg et al., 1979), and a 92 000-dalton protein which promotes actin depolymerization upon binding stoichiometrically to G-actin has been isolated from pig plasma (Harris & Gooch, 1981). In this paper we have reported the purification from rabbit serum of an actin binding protein which inhibits actin polymerization and depolymerizes actin filaments. On the basis of these properties we have named this protein serum actin inhibitory protein (SAIP).

SAIP isolated from rabbit serum consists of a mixture of two polypeptides of molecular weights 60 000 and 56 000 with a constant ratio of 5.7:1. Polypeptides with similar physicochemical properties and similar amino acid compositions were also found in calf serum, suggesting their general distribution in mammalian sera. The two rabbit SAIP polypeptides have very similar amino acid compositions and chymotryptic peptide maps, indicating that they only differ from each other in either a limited number of amino acid exchanges or that they are derived proteolytically from the same protein precursor. Resolution of each of the two SAIP components by isoelectric focusing into two isoelectric variants indicates further microheterogeneity (probably the result of posttranslational protein modification). SAIP is an acidic protein (pH 5.2–5.3) with a high cysteine content. This high cysteine content and the low number of free SH groups (see Results) are indicative of the existence of a high number of S–S bridges which could endow the molecule with a highly stable tertiary structure. This stable tertiary structure would explain the high stability of SAIP over a wide range of pHs (2.7–8 at 4 °C) and the isolation of SAIP in similar yields from both fresh serum and filter-sterilized serum stored for months at 4 °C.

SAIP shows the ability to prevent actin polymerization when added to solutions of G-actin. Inhibition of actin polymerization is partial at substoichiometric concentrations of SAIP to actin and complete when a molar excess of SAIP over actin is added. These results are in agreement with the binding data showing 1 mol of SAIP binding/mol of actin. Analysis of the binding of SAIP to monomer actin by the method of Scatchard shows that preparations of SAIP containing the four major polypeptide variants display a single type of binding site with a K_d of 0.12 μM. This result indicates that all four different forms of SAIP have the same ability to bind to actin.

Four different mechanisms can account for the ability of SAIP to depolymerize the actin filaments: (i) destabilization of the actin filaments following the binding of SAIP along the actin filaments; (ii) an increase in the rate of loss of actin subunits from actin filaments produced by the binding of SAIP to one or both ends of the actin filaments; (iii) prevention of the addition of G-actin monomers to the actin filaments by capping of the actin filaments by SAIP; (iv) displacement of the equilibrium between actin filaments and the G-actin monomers toward G-actin as the result of the binding of SAIP to G-actin.

The finding that the binding of SAIP to F-actin is dependent of the total concentration of SAIP but depolymerization of F-actin is not (see Figures 5 and 6) excludes the first mechanism. The fact that SAIP does not enhance the rate of depolymerization of actin filaments capped with cytochalasin B, at the assembly end (i.e., fast end), excludes the possibility that SAIP enhances the rate of actin loss from the disassembly end (second mechanism). The same result makes it also unlikely that SAIP enhances the rate of actin loss from the assembly end of the filaments.

The possibility that SAIP acts by capping the fast-growing end of the actin filaments cannot be excluded completely from

our experiments. However, proteins capping the fast growing end of the actin filaments are known to promote actin nucleation (Isenberg et al., 1980; Hasegawa et al., 1980; Craig & Powell, 1980; Glenney et al., 1981) and as a result to produce a large number of short filaments of actin which are not pelleted by centrifugation in the sedimentation assay. This results in the underestimation of the amount of F-actin when measured by using the sedimentation assay in samples of actin polymerized in the presence of capping proteins. However, the estimation of the F-actin content using the DNase I inhibition assay is independent of the length of the polymers formed, and the ability of a protein to cap the fast-growing end of the actin filaments can therefore be studied by comparing the values of F-actin obtained by the sedimentation and the DNase I inhibition assays. The very similar rates of actin depolymerization measured by the sedimentation and the DNase I assays suggest that SAIP does not cap the fast-growing end of the actin filaments. This conclusion is also supported by the observation that SAIP markedly decreases the initial rate of actin polymerization measured by turbidity (data not shown). On the basis of this observation, the ability of SAIP to bind to G-actin thus preventing actin polymerization and the constant ratio of 1 mol of actin/per mol of SAIP in supernatants of actin polymerized in the presence of SAIP, we favor the possibility that SAIP promotes depolymerization of actin filaments by binding to the monomers of G-actin in equilibrium with them.

In separate experiments performed under identical conditions we observed that the rate of F-actin depolymerization induced by SAIP could vary by a factor of 3. (Between 1 and 3 h was required to attain 50% depolymerization of F-actin when F-actin was incubated with an equivalent concentration of SAIP in buffer F.) So far, we have no definitive explanation for this variation, though this could be due to small amounts of capping proteins contaminating our actin preparations (MacLean-Fletcher & Pollard, 1980a,b) as these proteins would enhance the rate of filament depolymerization by decreasing the recycling of G-actin by the filaments. Though SAIP is pelleted by centrifugation only in the presence of F-actin and is fully active when recovered from the actin pellets, the possibility that SAIP is unspecifically bound to F-actin cannot be discarded. The lower ratio of SAIP to F-actin found in the polymerization pellets as compared to the one to one SAIP to G-actin ratio measured in the polymerization pellets indicates that SAIP has higher affinity for G-actin than for F-actin.

During the isolation of SAIP, we have observed that an 87 000-dalton polypeptide is retained by the actin–Sepharose column used in the first step of the purification. This polypeptide, which is poorly retained by DEAE at pH 6.8 and has a p*I* of 6.0, is similar in its properties to the 92 000-dalton, actin-depolymerizing factor recently isolated from pig plasma (Harris & Gooch, 1981). SAIP and this 87 000-dalton protein are probably unrelated as indicated by their different physicochemical properties (see above) and the failure of SAIP antibodies to precipitate the 87 000 dalton serum protein. It has recently been shown that albumin is very similar to, if not identical with, the actin binding protein β-actinin (Heizmann et al., 1981; Heizmann & Häuptle, 1977). However, the different migration of SAIP and serum albumin on NaDodSO$_4$–polyacrylamide gel electrophoresis, the different fingerprints of their [^{14}C]carboxymethylated tryptic peptides (data not shown), and the inability of the SAIP antibodies to precipitate serum albumin show that SAIP and albumin are different and distinct proteins. Among all the actin binding proteins described, the most similar to SAIP in the capacity to prevent actin polymerization and to promote actin depolymerization are the 65K and 62K polypeptides isolated from human granulocytes (Southwick & Stossel, 1981) and DNase I (Lazarides & Lindberg, 1974). SAIP and the granulocyte proteins differ in their molecular weight, their amino acid composition, and their capacities to bind to G-actin at high salt concentrations (0.6 M KCl abolishes the binding of the granulocyte proteins to actin while the SAIP–actin complex is stable in 1 M NaCl). In addition, the ability of actin–DNase columns to retain SAIP and the capacity of the actin–SAIP complex to inhibit the activity of DNase I indicate that SAIP and DNase I bind to different sites of actin and therefore are different.

Acknowledgments

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Syntheses and Modulations in the Chromatin Contents of Histones H1° and H1 during G_1 and S Phases in Chinese Hamster Cells†

Joseph A. D'Anna,* Lawrence R. Gurley, and Robert A. Tobey

ABSTRACT: Flow cytometry, conventional autoradiography, and autoradiography employing high concentrations of high specific activity [^{3}H]thymidine indicate that (1) treatment of Chinese hamster ovary (line CHO) cells with butyrate truly blocks cells in G_1 and (2) cells blocked in G_1 by isoleucine deprivation remain blocked in G_1 when they are released into complete medium containing butyrate. Measurements of H1° content relative to core histones and H1°:H1 ratios indicate that H1° is enhanced somewhat in G_1 cells arrested by isoleucine deprivation; however, (1) treatment with butyrate greatly increases the H1° content in G_1-blocked cells, and (2) the enhancement is very sensitive to butyrate concentration. Measurements of relative histone contents in the isolated chromatin of synchronized cultures also suggest that the acid-soluble content of histone H1 (relative to core histones) becomes greatly depleted in the isolated chromatin when synchronized cells are blocked in early S phase by sequential use of isoleucine deprivation and hydroxyurea blockade. We also have measured [^{3}H]lysine incorporation, various protein ratios, and relative rates of deposition of newly synthesized H1°, H1, and H4 onto chromatin during G_1 and S in the absence of butyrate. These measurements show that (1) H1° is synthesized and deposited onto chromatin during traverse of G_1 and S phases so that its specific activity during G_1 is 50–60% of its maximum value in S phase enriched (60–70%) cultures, (2) the ratio between rates of deposition of new histones H1° and H1 onto chromatin reaches a maximum during G_1 at 1.5–2.0 h after cells are released from the G_1 block, and it declines about 6-fold as cells enter S phase, and (3) the H1°:H1 molar ratio is modulated in the isolated chromatin of synchronized cultures so that it reaches a maximum near the G_1–S boundary. These results suggest a dynamic picture of chromatin organization in which (1) newly synthesized histone H1° binds to chromatin during traverse of G_1 and S phases and (2) histone H1 dissociates from (or becomes loosely bound to) chromatin during prolonged early S-phase block with hydroxyurea.

In 1969, Panyim & Chalkley (1969a) reported the isolation and amino acid analysis of a minor histone which has become known as histone H1°. The original observations of Panyim & Chalkley (1969a) and those of subsequent investigators [see D'Anna et al. (1981b) for a review] have led to the generalization that the cellular content of H1° is inversely proportional to the rate of DNA synthesis (Marsh & Fitzgerald, 1973). Although the inverse relationship between the cellular content of H1° and DNA synthesis indicates that the synthesis of H1° can be uncoupled from that of DNA, only a few studies of H1° synthesis or details of H1° enhancement have been reported. Gurley et al. (1972) observed that the rate of isotope incorporation into the H1° region of electrophoretic gels relative to that of the nucleosome core histones was greater in G_1-arrested and G_1-traversing cells than in exponentially growing cultures. More recently, Zlatanova (1980, 1981) has reported that (1) brief treatment of Friend erythroleukemia cells with hydroxyurea reduces synthesis of histone H1 and

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