Identification and Purification of Calcium Ion Dependent Modulators of Actin Polymerization from Bovine Thyroid†

Ryoji Kobayashi, William A. Bradley,‡ Joseph Bryan,§ and James B. Field*

ABSTRACT: We describe the purification of Ca^{2+}-dependent actin modulator proteins from bovine thyroid using DNase I affinity chromatography and diethylaminoethylcellulose chromatography. The 40K actin modulator has been purified to 98% homogeneity. It is a single polypeptide chain with a molecular weight of approximately 40 000 and an isoelectric point of 8.1. Its amino acid composition is different from previously described actin-associated proteins and thyroid actin. On the basis of the centrifugation assay and the DNase I inhibition assay, the actin complexed with the 40K protein is G-actin in its conformation rather than F-actin oligomers. Substoichiometric concentrations of the 40K protein rapidly inhibit actin polymerization in the presence of physiological concentrations of Ca^{2+} and Mg^{2+}. An 80K actin modulator also has been purified to 98% homogeneity. It is a single polypeptide chain with a molecular weight of approximately 80 000 and an isoelectric point of 6.35–7.0. Its amino acid composition is different from those of villin, gelsolin, and leukocyte actin polymerization inhibitor. On the basis of the DNase inhibition assay and the centrifugation assay, the nonprecipitable actin associated with the 80K protein was F-actin in its conformation. The 80K protein acts very efficiently as a Ca^{2+}-dependent nucleator for actin assembly and reduces its viscosity. In addition to the 40K and 80K actin modulators, 91K and 95K actin-associated proteins were partially purified. The 91K–95K fraction has similar activity to the 80K protein regarding precipitation of F-actin. The ^{125}I-G-actin polyacrylamide gel overlay technique [Snabes, M. C., Boyd, A. E., & Bryan, J. (1981) *J. Cell Biol. 90*, 809–812] revealed that both the 91K and 95K proteins bind ^{125}I-actin after sodium dodecyl sulfate ($NaDodSO_4$) electrophoresis while the 80K and 40K proteins do not. Thyroid 91K protein comigrated with a human platelet 91K actin binding protein on $NaDodSO_4$ gels and may be similar to macrophage gelsolin. The 95K protein may be similar to villin, the intestinal cytoskeletal protein.

Unlike most other hormones, thyroid hormones are stored extracellularly as components of thyroglobulin in the lumen of the thyroid follicle. Secretion of thyroid hormones initially involves transport of the thyroglobulin into the follicular cell as colloid droplets by the process of endocytosis. Subsequently, the colloid droplets fuse with lysosomes, and thyroxine and triiodothyronine are released following hydrolysis of thyroglobulin. It has been proposed that endocytosis may require contractile events, and contractile proteins have been tentatively implicated in the mechanism of transport and fusion of colloid droplets and lysomones in thyroid (Neve et al., 1972; Williams & Wolf, 1971). Morphological studies have revealed abundant microfilaments in microvilli, pseudopods, and the apical area of thyroid follicle cells (Fujita, 1975; Zelig & Wollman, 1977). A possible involvement of microfilaments in the thyroid endocytotic processes has been demonstrated since cytochalasin B inhibited thyroglobulin endocytosis in vitro (Neve et al., 1972; Williams & Wolf, 1971). Direct involvement of the actin-filament system in transporting coated vesicles during micropinocytosis of luminal colloid has been reported (Miyagawa et al., 1982). In addition thyrotropin induced disintegration of actin-filament bundles (Westermark & Porter, 1982; Tramontano et al., 1982). Furthermore the presence of actin (Kobayashi et al., 1982a), myosin (Kobayashi et al., 1977), tropomyosin (Kobayashi et al., 1982b), and profilin (Kobayashi et al., 1982c) supports an important role for contractile proteins in thyroid function.

These observations prompted us to purify and characterize actin modulator proteins for a detailed understanding of the molecular basis of thyroid hormone secretion and cellular motility. The present report describes the identification and purification of Ca^{2+}-dependent actin modulator proteins from bovine thyroid.

Experimental Procedures

Materials

Pancreatic DNase I (DNCL), cyanogen bromide activated Sepharose 4B, and Coomassie brilliant blue G-250 were obtained from Sigma Chemical Co. Sepharose 6B was a product of Pharmacia Fine Chemicals. Acrylamide, bis(acrylamide), $NaDodSO_4$,[1] TEMED, ammonium persulfate, Coomassie brilliant blue R-250, and Biolyte were purchased from Bio-Rad Laboratories. DEAE-cellulose (DE-52) was a product of Whatman.

Methods

Bovine thyroids were obtained from a local abattoir and transported to the laboratory packed in ice. After trimming, the tissue was used immediately or stored at −20 °C.

Purification of Bovine Thyroid "Actin Modulators". Bovine thyroid glands (450 g) were homogenized in 1000 mL of 2 mM Tris-HCl, 0.1 mM $CaCl_2$, 0.5 mM ATP, 6 mM 2-mercaptoethanol, and 0.02% NaN_3, pH 8.0 (G buffer), in a Waring blender for 30 s at 4 °C. All subsequent steps were performed

† From the Diabetes Research Center, St. Luke's Episcopal Hospital, and Department of Medicine, Baylor College of Medicine, Houston, Texas 77025. *Received April 5, 1982; revised manuscript received November 19, 1982.* This research was supported by U.S. Public Health Service Grant AM 26088 (J.B.F.) and Grant HL 26973 (J.B.) from the National Institutes of Health.

* Address correspondence to this author at the Diabetes Research Center, St. Luke's Episcopal Hospital, Houston, TX 77025.

‡ Present address: Department of Medicine, Division of Atherosclerosis and Lipoprotein Research, Baylor College of Medicine, Houston, TX 77030.

§ Present address: Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030.

[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; TEMED, *N,N,N',N'*-tetramethylethylenediamine; Tris, tris(hydroxymethyl)aminomethane.

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at 4 °C unless stated otherwise. The homogenate was filtered through two layers of cheesecloth. The filtered homogenate was centrifuged for 30 min with a Sorvall GSA rotor at 12000 rpm. The supernatant obtained is referred to as G-buffer extract.

The G-buffer extract was applied to a Sepharose affinity column of approximately 150 mL of resin, to which 500 mg of pancreatic DNase I was covalently bound according to previous procedures (Lazarides & Lindberg, 1974). After the column was washed with 600 mL of G buffer, proteins were eluted in a stepwise manner with (a) 0.2 M NaCl in buffer G, (b) 10 mM EGTA[1] in buffer G, (c) 1 M KI in buffer G, and (d) 3 M guanidine hydrochloride buffer of Lazarides & Lindberg (1974). The Coomassie blue binding assay for protein (Bradford, 1976) and $NaDodSO_4$/polyacrylamide gel electrophoresis (Laemmli, 1970) were used to monitor the elution profile. A representative chromatographic profile is shown in Figure 1.

Active fractions of the EGTA eluate (see Results) were pooled and dialyzed exhaustively against G buffer. The dialyzed sample was applied to a column (2.0 cm × 8 cm) of DEAE-cellulose (DE-52, Whatman) which had been equilibrated with G buffer. The column was eluted with a linear NaCl gradient (0–0.4 M). Fractions of 4.2 mL were collected at a rate of approximately 100 mL/h. A representative chromatographic profile is shown in Figure 3.

Gel Electrophoresis. $NaDodSO_4$/polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970) with 9% polyacrylamide slab gels. Two-dimensional gel electrophoresis was performed as described by O'Farrell (1975). Quantitative densitometry of the Coomassie stained gels was performed as described by Kobayashi et al. (1982b).

Other Procedures. Actin gel overlay was carried out as described previously (Snabes et al., 1981). Amino acid analyses were performed with a Beckman amino acid analyzer as described previously (Kobayashi et al., 1979). Samples were hydrolyzed with 6 N HCl in evacuated sealed tubes for 24 h at 110 °C. Protein concentrations were determined by the method of Lowry et al. (1951). In some experiments, protein concentrations were estimated by the dye binding assay as described by Bradford (1976). Bovine γ-globulin was used as a standard. Rabbit skeletal muscle actin was purified according to the method of Spudich & Watt (1971). The viscosity of F-actin solutions was measured at high shear rates in a Cannon-Manning semimicroviscometer, size 100, with buffer flow times of about 53–58 s at 25 °C. The concentration of monomeric actin and total actin was determined by the method of DNase I inhibition assay as described by Blikstad et al. (1978).

Stokes Radius Determination by Gel Filtration. The elution of thyroid actin modulator proteins (see Results) from a Sepharose 6B column (0.9 cm × 90 cm) equilibrated with a solution containing 0.2 M KCl, 6 mM 2-mercaptoethanol, and 20 mM Tris-HCl, pH 7.5, was compared with that of standard proteins of known Stokes radius. The following proteins were used as standards: aldolase (46 Å), bovine serum albumin (35 Å), ovalbumin (30 Å), and chymotrypsinogen A (22 Å). The void and column volumes were identified with blue dextran and ^{125}I, respectively.

Assay of F-Actin Pelleting Inhibition Activity. At each step of purification, the activity of thyroid actin moduiators was examined in the following way. The proteins were dialyzed against G buffer for 16 h. At the concentration given in the text, aliquots of the proteins were mixed with known amounts

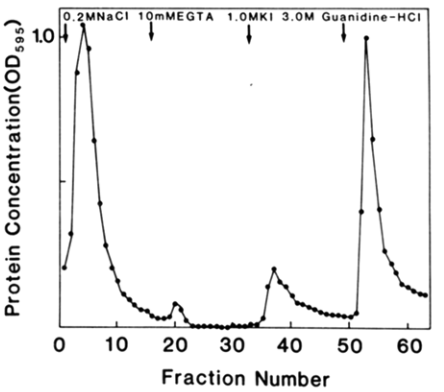

FIGURE 1: Chromatogram of G-buffer extract on DNase I–Sepharose column. After the column was washed with G buffer, elution was done with 0.2 M NaCl, 10 mM EGTA, 1.0 M KI, and 3 M guanidine hydrochloride. Protein concentrations were determined by the method of Bradford (1976).

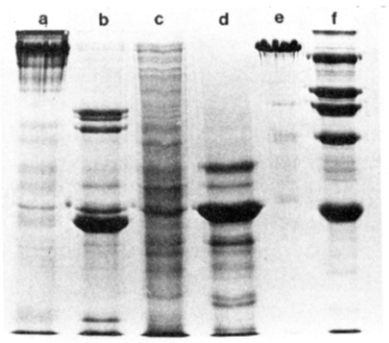

FIGURE 2: Electrophoretic analysis (9.0% gel) of fractions obtained by DNase I–Sepharose chromatography. (a) 0.2 M NaCl eluate; (b) 10 mM EGTA eluate; (c) 1.0 M KI eluate; (d) 3 M guanidine hydrochloride eluate; (e) chicken gizzard filamin as a standard; (f) molecular weight standards, myosin heavy chain (M_r 200000), β-galactosidase (M_r 116500), phosphorylase *b* (M_r 94000) bovine serum albumin (M_r 68000), and ovalbumin (M_r 43000).

of skeletal muscle actin, and KCl and $MgCl_2$ were added at final concentrations of 100 and 2 mM, respectively. After incubation at 25 °C for 60 min, tubes were centrifuged at 100000*g* for either 60 min or 12 h. Supernatants were removed and the pellets were resuspended in sample buffer for $NaDodSO_4$/polyacrylamide gel electrophoresis. After $NaDodSO_4$/polyacrylamide gel electrophoresis of the F-actin pellets, inhibition of the formation of the F-actin pellet was determined by quantitative densitometry (Kobayashi et al., 1982b).

Results

Purification of Thyroid Actin Modulator Proteins by DNase I Affinity Chromatography. The calcium-dependent interaction of actin modulator proteins with monomeric actin has been exploited as a basis for a rapid and simple purification procedure. Figure 1 shows the elution pattern of DNase I affinity chromatography. Figure 2 demonstrates the protein compositions of the fractions obtained during the isolation of thyroid actin modulator proteins. Analysis of the materials obtained by the 0.2 M NaCl elution revealed the high molecular weight doublet (M_r 250000 and 220000) bands with other contaminating proteins (Figure 2a). In $NaDodSO_4$ gel electrophoresis, the relative mobilities of the high molecular weight doublet was not the same as the relative mobilities of myosin heavy chain, chicken gizzard filamin, human blood cell spectrin, or bovine 19S thyroglobulin (data not shown). The 10 mM EGTA eluate contained six major components (Figure

Table I: Inhibition of F-Actin Pellet Formation by DNase I Column Eluates[a]

actin modulators	pelleted actin (μg)
none	24.0
0.2 M NaCl eluate	23.4
10 mM EGTA eluate	1.3
1.0 M KI eluate	18.2

[a] Actin (0.1 mg/mL) was incubated with 0.2 mg/mL DNase I column eluates in a total volume of 0.25 mL. The centrifugation assay and quantitative densitometry were done as described under Methods.

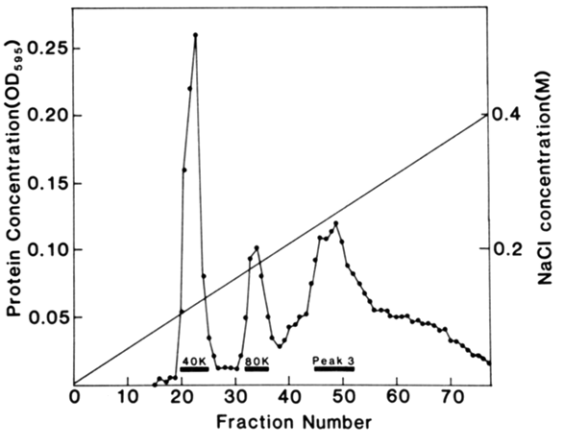

FIGURE 3: Elution pattern of the 10 mM EGTA fraction from the DEAE-cellulose column. Details of the conditions are described under Methods.

2b). These have relative mobilities corresponding to 95K-, 91K-, 80K-, 42K-, 40K-, and 20K-dalton polypeptides. The 42K polypeptide and actin have the same relative mobilities. Following elution with 1.0 M KI (Figure 2c), thyroid actin was recovered with 3 M guanidine hydrochloride buffer (Figure 2d) which removes actin from DNase I columns (Lazarides & Lindberg, 1974).

The interaction between these eluates and F-actin was studied by a centrifugation technique (Table I). The proteins were mixed to give a final concentration of 0.1 mg/mL actin and 0.2 mg/mL DNAse I eluates, brought to 25 °C, made 100 mM in KCl and 2 mM in $MgCl_2$, and incubated for 1 h. Under these conditions actin alone polymerized, and approximately 95% could be pelleted at 100000*g* for 60 min. The DNase I column eluates alone could not be pelleted. The 0.2 M NaCl eluate did not inhibit F-actin pellet formation. However, in the presence of 0.1 mM calcium, the EGTA eluate almost completely inhibited F-actin pelleting. The 1.0 M KI eluate had only a slight effect on formation of the F-actin pellet. Thus in the presence of 10^{-4} M calcium the 10 mM EGTA eluate inhibits actin polymerization or restricts actin from forming long sedimentable polymers which could be pelleted.

DEAE-cellulose Chromatography. The dialyzed EGTA eluate (see Methods) was applied at 4 °C to DEAE-cellulose. Following a wash with G buffer, the column was eluted with a 300-mL linear gradient of NaCl (0–0.4 M NaCl) in G buffer. Three protein peaks were obtained (Figure 3) which were characterized by $NaDodSO_4$/polyacrylamide gel electrophoresis (Figure 4). In some experiments the 40K protein eluted both in the flow through volume and as a peak eluted with approximately 0.12 M NaCl. Despite the fact that the 40K protein elutes as separate peaks from DEAE-cellulose, the specific activity is essentially constant. Purified 40K protein and 80K protein appear to be at least 98% homogeneous by quantitative densitometry of the $NaDodSO_4$/polyacrylamide gels (Figure 4). In some experiments, small amounts of contaminants were not removed from the 80K preparation. In such cases, complete separation of 80K protein from other contaminants was achieved by use of a second DEAE-cellulose linear gradient chromatography.

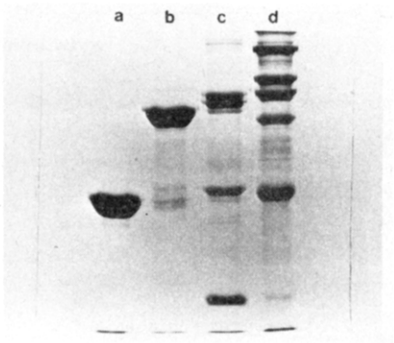

FIGURE 4: $NaDodSO_4$/polyacrylamide gel electrophoresis (9% gel) of DEAE-cellulose eluates. (a) 40K actin modulator; (b) 80K actin modulator; (c) peak 3 fraction; (d) molecular weight standards as in Figure 2.

Table II: Purification of Thyroid Actin Modulator Proteins

step	concn (mg/mL)	volume (mL)	total protein (mg)
1. G-buffer extract	32	1340	42880
2. DNase I-Sepharose chromatography	0.182	101	18.4
3. DEAE-cellulose chromatography			
a. peak 1 (40K protein)	0.145	20	2.9
b. peak 2 (80K protein)	0.093	28	2.6
c. peak 3	0.160	40	6.4

Preliminary Experiment for Separating the 91K and 95K Proteins and Other Contaminants. The peak 3 fraction from DEAE-cellulose contained the 91K, 95K, 42K, and variable amounts of the 20K proteins (Figure 4). For the separation of these proteins, we tried several different chromatographic procedures including DEAE-cellulose chromatography in the presence of EGTA, hydroxylapatite chromatography, and gel filtration in the presence of 1 M KI. However, the 91K and 95K proteins were not separated from each other or from the other contaminants by these procedures.

The peak 3 fraction was dialyzed against 8 M urea in buffer G at room temperature for 12 h. After dialysis, the sample was applied to a 2 × 5 cm DEAE-cellulose column which had been equilibrated with the same buffer. The column was eluted with a 200-mL linear gradient of NaCl (0–0.4 M NaCl) in urea/G buffer. As shown in Figure 5, 91K and 95K proteins were partially separated from the 42K proteins.

A summary of the purification of thyroid actin modulator proteins in presented in Table II. We did not calculate the fold purification and yield of the 40K and 80K proteins for two reasons. One, we did not have a quantitative assay for each protein. Two, the purification could not be followed by densitometry of $NaDodSO_4$/polyacrylamide gels because of the presence of a large amount of thyroglobulin. However, the data in Table I allow us to calculate the minimum concentrations of 40K, 80K, and peak 3 proteins to be 0.007%, 0.006%, and 0.015%, respectively.

F-Actin Pelleting Assay. The interaction of thyroid actin modulator proteins with F-actin was examined by the pelleting assay as described under Methods (Table III). The precipitation of F-actin was not influenced by 1 mM EGTA but was

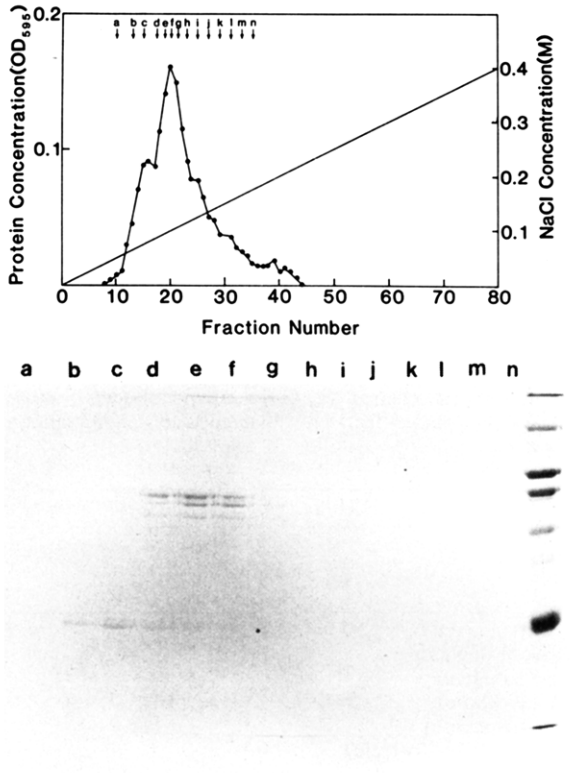

FIGURE 5: Chromatogram of peak 3 fraction on urea/DEAE-cellulose column (top panel). Details of the conditions are described in the text. NaDodSO$_4$/polyacrylamide gel (9%) electrophoresis of the fraction is shown in the bottom panel.

Table III: Ca^{2+}-Dependent Interaction between Thyroid Actin Modulators and F-Actin[a]

conditions	EGTA	pelleted actin (μg)
actin	−	28.4
	+	28.0
actin + 40K protein	−	8.3
	+	26.8
actin + 80K protein	−	4.0
	+	27.0
actin + peak 3 protein	−	2.5
	+	20.0

[a] F-Actin (0.15 mg/mL) was incubated with the various actin modulators as indicated in the table (0.06 mg/mL 40K protein, 0.03 mg/mL 80K protein, or 0.1 mg/mL peak 3 protein) in a final volume of 0.2 mL in the presence and absence of 1 mM EGTA. Other conditions are described in Table I.

significantly reduced by the 40K and the 80K proteins. Precipitation was almost completely inhibited by the peak 3 proteins. In the presence of 1 mM EGTA, however, the effects of the 40K and 80K proteins on F-actin pelleting were minimal while the effects of peak 3 proteins were reduced.

Although the above experiments demonstrated Ca^{2+}-sensitive interactions between thyroid actin modulators and F-actin, the actin in the supernatant could be in the form of monomers or of short oligomers. To test this possibility, the mixtures of F-actin and thyroid actin modulator proteins were subjected to short-term (60 min) and long-term (12 h) centrifugation in an attempt to pellet any oligomeric actin. In this experiment, we used relatively large amounts of the 40K protein relative to the actin concentration. Under these conditions, only the critical concentration of actin would be expected to remain in the supernatant (Oosawa & Asakura, 1975). As demonstrated in the above experiments, the 40K protein increased the nonprecipitable actin in both the short-term and long-term centrifugations (Table IV). Thus, it seems likely that the nonprecipitable actin was in the form of a 40K–G-actin complex rather than short oligomers.

Table IV: Short-Term and Long-Term Centrifugation Analysis of the Interaction between Thyroid Actin Modulators and F-Actin[a]

conditions	centrifugation (h)	pelleted actin (μg)	actin in supernatant (μg)
actin	1	382	14
	12	390	10
actin + 40K protein	1	57	330
	12	58	324
actin + 80K protein	1	36	350
	12	280	114
actin + peak 3 protein	1	30	362
	12	300	98

[a] Assay conditions were described in the text and Table I. F-Actin (2 mg/mL) was incubated with the various actin modulators indicated in the table (1.2 mg/mL 40K protein, 0.6 mg/mL 80K protein, or 0.6 mg/mL peak 3 protein) in a final volume of 0.2 mL.

Table V: Effects of a High Mg^{2+} Concentration of the Interaction between the Actin Modulators and F-Actin[a]

protein	salt condition (centrifugation condition)	pelleted actin (μg)
actin	0.1 M KCl + 2 mM $MgCl_2$ (at 100000g for 60 min)	27.8
actin + 40K protein		8.0
actin + 80K protein		4.0
actin + peak 3 protein		2.4
actin	20 mM $MgCl_2$ (at 12000g for 10 min)	28.0
actin + 40K protein		3.8
actin + 80K protein		2.2
actin + peak 3 protein		1.0

[a] Details were described in the text. Actin (0.1 mg/mL) was incubated with 0.08 mg/mL 40K protein, 0.02 mg/mL 80K protein, or 0.02 mg/mL peak 3 protein.

In contrast to the 40K protein, the 80K and peak 3 proteins have different effects on F-actin precipitability. In both cases, with short-term centrifugation, over 87% of the actin remained in the supernatant (Table IV). However, after 12 h of centrifugation, over 70% of the actin and actin modulator proteins were pelleted, presumably as bound complexes. We conclude that the actin pelleted during the long-term centrifugation represents short oligomers rather than actin monomers (Mooseker et al., 1980). Since Mg^{2+}-induced actin paracrystals have a different conformation from F- and G-actin (Harwell et al., 1980), the effects of the thyroid actin modulator proteins on the paracrystals were also examined. Mg^{2+}-induced actin paracrystals were pelleted by low-speed centrifugation (12000g for 10 min). Under these conditions, all three fractions reduced the amounts of actin which were pelleted (Table V).

Effects of the 40K, 80K, and Peak 3 Proteins on Actin Assembly Assessed by the DNase I Inhibition Assay. The effects of the 40K, 80K, and peak 3 proteins on actin polymerization were evaluated by a combination of the DNase I inhibition assay (Blikstad et al., 1978) and centrifugation techniques. As summarized in Table VI, the amount of nonprecipitable actin increased as a function of the 40K protein

Table VI: Effects of Thyroid Actin Modulators on Actin Assembly Assessed by DNase I Inhibition Assay[a]

actin modulators (μg/mL)		nonpelleted actin (μg/mL)	actin monomer (μg/mL)
none		46	15
40K protein:	33	121	105
	66	200	160
	99	285	248
	132	306	268
80K protein:	4	66	26
	12	154	31
	36	220	15
	108	294	46
P3 protein:	5	58	22
	15	138	43
	45	256	16
	135	288	43

[a] Actin (0.32 mg/mL) was incubated with various amounts of thyroid actin modulator proteins in G buffer containing 0.1 M KCl and 2 mM $MgCl_2$ at 25 °C for 1 h. After the incubation, the mixtures were centrifuged at 100000*g* for 1 h. The supernatants were removed and assayed for total and monomer actin concentrations by the DNase I inhibition assay (Blikstad et al., 1978).

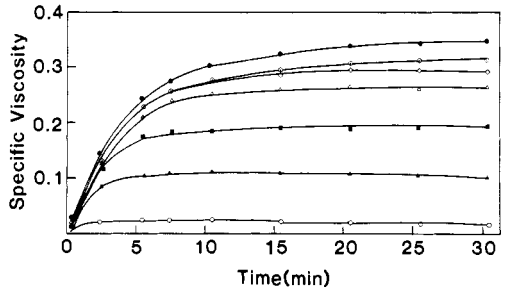

FIGURE 6: Effects of 40K protein on the polymerization kinetics of actin. Polymerization was followed by measuring viscosity in an Ostwald viscometer. G-Actin (318 μg/mL) was mixed with various amounts of 40K protein and polymerized at 25 °C with 0.1 M KCl and 2 mM $MgCl_2$ in G buffer. Actin alone (●), 3.3 μg/mL 40K protein (⊙), 8.3 μg/mL 40K protein (◇), 16.7 μg/mL 40K protein (△), 33.3 μg/mL 40K protein (■), 66.7 μg/mL 40K protein (▲), and 100 μg/mL 40K protein (○).

included in the mixture. A major portion of the nonprecipitable actin exists in the G-actin form as estimated by the DNase I inhibition assay. The amount of nonprecipitable actin also increased as a function of the amount of 80K protein or peak 3 proteins included in the mixture. However, in both cases, the changes in the actin monomer level cannot account for the observed increase in nonprecipitable actin. Thus, it seemed likely that the nonprecipitable actin was in the form of short filaments.

Effects of Purified 40K Actin Modulator Protein on Actin. Figure 6 demonstrates the effect of increasing amounts of thyroid 40K actin modulator on the viscosity of actin polymerized by the addition of 100 mM KCl and 2 mM $MgCl_2$ as a function of time. The 40K actin modulator decreased the final extent of the viscosity. The effects were dose dependent. The decreased final viscosity of G-actin polymerized in the presence of the 40K actin modulator remained relatively constant for up to 16 h (data not shown).

Effect of the 80K Actin Modulator Protein on Polymerization of Actin. The 80K actin modulator reduced the steady-state viscosity of F-actin in a dose-dependent manner (Figure 7). A molar ratio of the 80K actin modulator:actin of 1:257 results in 53% reduction in the final viscosity. Significant effects can be detected at ratios as low as 1:1200. Since the actin concentration in thyroid was estimated to be

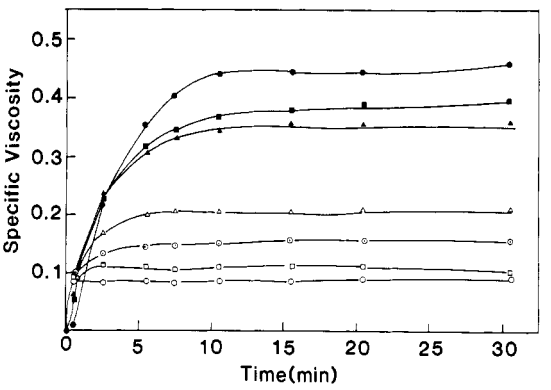

FIGURE 7: Effects of 80K protein on the polymerization kinetics of actin. G-Actin (438 μg/mL) was mixed with various amounts of 80K protein and polymerized with 0.1 M KCl and 2 mM $MgCl_2$ in G buffer. Actin alone (●), 0.65 μg/mL 80K protein (■), 1.3 μg/mL 80K protein (▲), 3.25 μg/mL 80K protein (△), 6.5 μg/mL 80K protein (⊙), 9.75 μg/mL 80K protein (□), and 13 μg/mL 80K protein (○).

0.2% of the total protein and the minimal 80K modulator concentration was estimated to be 0.006%, the ratios employed in the present in vitro experiment are likely to be physiologically meaningful. In addition to its effects on the final extent of the viscosity, the 80K modulator also acts in a dose-dependent manner to shorten the lag phase preceding a detectable change in viscosity after the addition of 0.1 M KCl and 2 mM $MgCl_2$.

Actin Overlay Technique. Recently, Snabes et al. (1981) showed that ^{125}I-G-actin can be used to detect specific actin binding proteins in platelets after separation by NaDodSO$_4$/polyacrylamide gel electrophoresis. We applied this technique to analyze the ability of thyroid actin modulator proteins to bind ^{125}I-actin. Although the 40K and 80K proteins interact with F- and G-actin under native conditions, neither protein binds ^{125}I-G-actin after electrophoresis (data not shown). However, in the thyroid peak 3 fractions, three major binding activities were detected at the 95K and 91K protein bands and in a fast migrating edge of the 42K protein (data not shown). Since Snabes et al. (1981) reported that the major actin binding proteins of platelets are 91K and 40K daltons, the thyroid peak 3 proteins and a hot NaDodSO$_4$ extract of platelets were directly compared by this technique. As shown in Figure 8, thyroid 91K protein comigrated with platelet 91K actin binding activity; the thyroid 95K protein was distinct from the platelet actin binding protein.

Physical and Chemical Properties of Thyroid Actin Modulator Proteins. Two-dimensional gel electrophoresis of the EGTA eluate from the DNase I affinity column revealed that the 95K protein consists of three isoelectric variants present in unequal amounts (Figure 9). The approximate isoelectric point is 6.7. The 91K protein consists of two isoelectric variants with an approximate isoelectric point of 6.3. The 80K protein is not well focused and has a range of isoelectric points from 7.0 to 6.35. The 40K protein was a more basic protein; the isoelectric point of the major spot is 8.1.

The purified 80K protein has the properties of a monomeric protein with a molecular weight of approximately 80 000. NaDodSO$_4$/polyacrylamide gel electrophoresis reveals a single polypeptide. The Stokes radius of the 80K protein was estimated by gel filtration on a calibrated Sepharose 6B column and was found to be approximately 35 Å (data not shown). The purified 40K protein also had the properties of a monomeric protein with a Stokes radius of approximately 27 Å by gel filtration on a Sepharose 6B column (data not shown).

Table VII indicates the amino acid composition of the thyroid 40K and 80K proteins with the compositions of other

Table VII: Amino Acid Composition of Thyroid 40K Protein and 80K Protein (mol %)

	thyroid 40K protein	thyroid actin[a]	fragmin[b]	DNase binding protein[c]	eu-actin[d]	thyroid 80K protein	gelsolin[e]	villin[f]	actin inhibitor[g]
aspartic acid	10.55	8.45	9.73	8.96	8.94	9.48	10.5	9.84	13.2
threonine	3.25	7.08	5.14	6.10	7.33	5.11	5.2	6.15	4.5
serine	7.00	4.90	4.59	5.67	6.22	8.62	6.4	6.14	6.2
glutamic acid	13.00	11.44	13.51	10.81	10.18	12.86	12.8	12.89	12.2
proline	6.31	6.27	3.78	4.32	4.18	8.60	6.8	6.76	4.4
glycine	9.09	8.17	10.27	8.68	7.45	9.50	8.7	7.65	8.0
alanine	8.71	7.90	10.00	11.89	8.34	8.74	8.9	8.03	7.8
$^1/_2$-cystine		0.82	0		0.82				
valine	4.79	5.45	5.41	6.41	5.33	7.33	7.2	6.67	5.1
methionine	2.16	4.09	0.81	1.49	4.04	1.64	1.9	2.17	2.1
isoleucine	4.78	7.08	4.05	4.79	7.74	4.21	3.5	3.16	5.5
leucine	9.34	7.36	9.73	9.01	7.92	8.34	9.2	7.82	10.9
tyrosine	2.16	4.09	3.78	3.68	4.59	3.05	3.1	4.17	2.9
phenylalanine	4.44	3.27	5.14	3.19	3.55	2.20	4.3	4.38	2.6
lysine	6.90	5.18	7.84	6.94	6.44	2.94	5.8	7.52	8.1
histidine	2.73	3.54	2.70	2.87	2.02	2.35	2.2	2.32	1.6
arginine	4.35	4.90	3.51	5.20	4.97	5.00	5.5	4.32	4.9

[a] Data taken from Kobayashi et al. (1982a). [b] Data taken from Hasegawa et al. (1980). [c] Data taken from Kohama & Holtzer (1981). [d] Data taken from Kuroda et al. (1981). [e] Data taken from Yin & Stossel (1980). [f] Data taken from Bretscher & Weber (1980). [g] Data taken from Southwick & Stossel (1981).

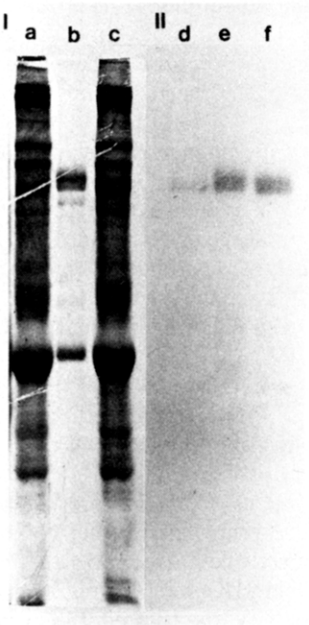

FIGURE 8: Coomassie blue staining pattern and autoradiograph of the ^{125}I-actin overlay of thyroid peak 3 proteins and hot $NaDodSO_4$ extract of human platelet extract. (I) Coomassie blue staining pattern: (a) platelet extract, (b) thyroid peak 3 proteins, and (c) mixture of (a) and (b). (II) Autoradiograph: (d) platelet extract, (e) thyroid peak 3 proteins, and (f) mixture of (d) and (e).

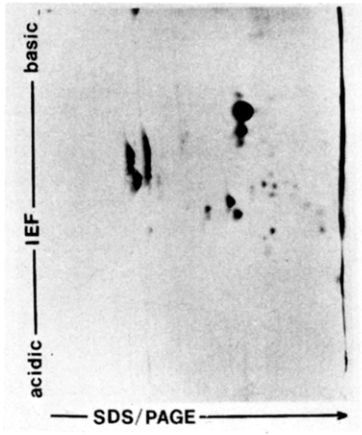

FIGURE 9: Two-dimensional electrophoresis of 10 mM EGTA eluate from DNase I column. Details of the conditions are described under Methods.

actin-associated proteins described in other tissues for comparison. The amino acid composition of the 40K protein shows no particularly striking features but is distinct from those of fragmin (Hasegawa et al., 1980), eu-actinin (Kuroda et al., 1981), DNase binding protein (Kohama & Holtzer, 1981), and thyroid actin (Kobayashi et al., 1982a). The amino acid composition of the 80K protein also shows no particular features but is distinct from those of gelsolin (Yin & Stossel, 1980), villin (Bretscher & Weber, 1980), and actin inhibitor (Southwick & Stossel, 1981).

Discussion

Cell motility, as well as more specialized cell functions such as pseudopod formation, exocytosis, endocytosis, and granular movements, appears to be mediated by contractile proteins in a variety of nonmuscle cells (Korn, 1978). Many, if not all, of these processes are essential in the synthesis, storage, and secretion of thyroid hormones. A detailed understanding of the molecular basis of cellular motility and hormone secretory processes requires the purification of the proteins which interact with actin. The isolation and characterization of the Ca^{2+}-dependent actin modulator proteins is a part of our endeavor to understand the molecular mechanism of thyroid hormone secretion.

DNase I affinity chromatogrpaphy was introduced by Lindberg & Eriksson (1971) for the isolation of a DNase I inhibitor which was later recognized as actin (Lazarides & Lindberg, 1974). The procedure is a powerful method for the rapid isolation of pure actin in a single step from cell and tissue extracts. The strong Ca^{2+}-dependent interaction of thyroid actin modulator proteins with monomeric actin has been exploited as a basis for a rapid and convenient purification of thyroid actin modulator proteins with a DNase I affinity column. A similar strategy was used for the isolation of a microvilli core protein, villin (Bretscher & Weber, 1980), and platelet actin binding proteins (Wang & Bryan, 1981). This purification procedure using a DNase I column developed for villin, platelet actin binding protein, thyroid profilin (Kobayashi et al., 1982c), and thyroid actin modulators may also be useful for the isolation of other factors which form a complex with monomeric actin. In addition, the method can be readily

scaled down to utilize small amounts of material which should make it particularly useful for isolating such proteins from tissue in which they are present in relatively low concentrations.

The ^{125}I-G-actin/polyacrylamide gel overlay technique revealed that both the 91K and 95K proteins can bind ^{125}I-actin after sodium dodecyl sulfate gel electrophoresis. However, we have been unsuccessful in renaturing the 40K and 80K proteins which bind to G- and F-actin in their native condition. The thyroid 91K protein comigrated with the human platelet 91K actin binding protein which is thought to be a gelsolin-like protein (Wang & Bryan, 1981). The thyroid 95K protein was distinct from the thyroid 91K protein and platelet 91K actin binding protein. Since thyroid contains abundant microvilli at the apical membrane surface and villin is thought to be distributed only in microvilli, it is possible that the thyroid 95K protein is a villin-like protein.

Although it is possible that the 40K, 80K, and 91K proteins are proteolytic fragments of the larger 95K protein of thyroid, there are several reasons to think this is not the case. The purification of thyroid actin modulator proteins was done with and without several protease inhibitors which included leupeptin, pepstatin, antipain, trasylol, and PMSF. In these experiments, the 40K, 80K, and 91K proteins are always observed (data not shown). In addition, since the 95K and 91K binding activity is observed by the actin overlay technique in samples prepared by putting fresh thyroid into boiling Na-DodSO$_4$ sample buffer, it seems unlikely that the 91K protein is a proteolytic fragment of the 95K protein.

Although the precise physiological role of thyroid actin modulator proteins remains to be elucidated, their presence in the thyroid with actin, tropomyosin, profilin, and myosin could provide the cell with a contractile and structural system essential for thyroid hormone synthesis and secretion.

References

Blikstad, I., Markey, F., Carlsson, L., Persson, T., & Lindberg, U. (1978) *Cell (Cambridge, Mass.) 15*, 935–943.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Bretscher, A., & Weber, K. (1980) *Cell (Cambridge, Mass.) 20*, 839–847.

Fujita, H. (1975) *Int. Rev. Cytol. 40*, 197–280.

Harwell, O. D., Sweeney, M. L., & Kirkpatrick, F. H. (1980) *J. Biol. Chem. 255*, 1210–1220.

Hasegawa, T., Takahashi, S., Hayashi, H., & Hatano, S. (1980) *Biochemistry 19*, 2677–2683.

Kobayashi, R., Goldman, R. D., Hartshorne, D. J., & Field, J. B. (1977) *J. Biol. Chem. 252*, 8285–8291.

Kobayashi, R., Kuo, I. C. Y., Coffee, C. J., & Field, J. B. (1979) *Metab. Clin. Exp. 28*, 169–182.

Kobayashi, R., Tawata, M., & Field, J. B. (1982a) *Metab. Clin. Exp. 31*, 133–138.

Kobayashi, R., Tawata, M., Mace, M. L., Jr., Bradley, W. A., & Field, J. B. (1982b) *Biochim. Biophys. Acta 702*, 220–232.

Kobayashi, R., Bradley, W. A., & Field, J. B. (1982c) *Anal. Biochem. 120*, 106–110.

Kohama, K., & Holtzer, H. (1981) *J. Biochem. (Tokyo) 89*, 341–349.

Korn, E. D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 588–599.

Kuroda, M., Tanaka, T., & Masaki, T. (1981) *J. Biochem. (Tokyo) 89*, 297–310.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lazarides, E., & Lindberg, U. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4742–4746.

Lindberg, U., & Eriksson, S. (1971) *Eur. J. Biochem. 18*, 474–479.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Miyagawa, J., Ishimura, K., & Fujita, H. (1982) *Cell Tissue Res. 223*, 519–532.

Mooseker, M. S., Graves, T. A., Wharton, K. A., Falco, N., & Howe, C. L. (1980) *J. Cell Biol. 87*, 809–822.

Neve, P. E., Ketelbant-Balasse, P., Willems, C., & Dumont, J. (1972) *Exp. Cell Res. 74*, 227–244.

O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.

Oosawa, F., & Asakura, S. (1975) *Thermodynamics of Polymerization of Protein*, Academic Press, London.

Snabes, M. C., Boyd, A. E., & Bryan, J. (1981) *J. Cell Biol. 90*, 809–812.

Southwick, F. S., & Stossel, T. P. (1981) *J. Biol. Chem. 256*, 3030–3036.

Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866–4871.

Tramontano, D., Avivi, A., Ambesi-Impiombato, F. S., Barak, L., Geiger, B., & Schlessinger, J. (1982) *Exp. Cell Res. 137*, 269–275.

Wang, L. L., & Bryan, J. (1981) *Cell (Cambridge, Mass.) 25*, 637–649.

Westermark, B., & Porter, K. R. (1982) *J. Cell Biol. 94*, 42–50.

Yin, H. L., & Stossel, T. P. (1980) *J. Biol. Chem. 255*, 9490–9493.

Zelig, J. D., & Wollman, S. H. (1977) *J. Cell Biol. 72*, 584–594.