The Structure and Amount of Actin in IMR-90 Fibroblasts

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Double-labelling and peptide isolation have been used to examine the homology between the actin of IMR-90 human embryo fibroblasts and muscle actin. After separation of mixtures of [^{14}C]carboxymethylated muscle actin and [^3H]carboxymethylated proteins of IMR-90 cells of electrophoresis on sodium dodecyl sulphate-containing polyacrylamide gels, peptides were generated from the material co-migrating with actin by digestion with chymotrypsin. Peptides homologous with peptides accounting for Cys-217, Cys-256, Cys-284 and Cys-373 of muscle actin are present in this material, but no peptide homologous with a Cys-10-containing peptide was detected. From the amount of actin-derived peptides present, the actin content of IMR-90 fibroblasts was calculated to be 4.2% of the total protein of these cells.

Microfilaments formed by the polymerization of a protein homologous with muscle actin have been found in a variety of eukaryotic cells (Pollard & Weihing, 1974). These structures are thought to function in cell processes requiring movement such as growth and division (Pollard & Weihing, 1974) and membrane-receptor mobility (Edelman & Yahara, 1976; Wang & Goldberg, 1976). The polymerization of cellular actin to form microfilaments appears to be an equilibrium process that can be altered, by as-yet-unknown mechanisms, by such diverse means as viral transformation (Pollack *et al.*, 1975) or treatment with chemicals such as cyclic AMP derivatives (Puck, 1977). From studies of such alterations, it appears that cells exhibiting regulated growth have large numbers of microfilaments compared with cells that are tumorigenic (Kopelovich *et al.*, 1977).

Necessary to an understanding of the function of actin in cellular processes is a knowledge of the structure and amount of this protein. Obtaining this information for cellular actin is difficult. Since the protein has no known enzymic properties, indirect means must be used for its detection. This may account for the low yields obtained when actin was purified from such non-muscle sources as chicken-embryo fibroblasts (Yang & Perdue, 1972) and rabbit alveolar macrophages (Hartwig & Stossel, 1975). To overcome these difficulties a chemical procedure that allows the quantification and structural examination of actin without the necessity of measuring its biological properties or of purifying the protein has been

described (Anderson 1976*a,b*). This method utilized double-labelling and peptide isolation.

A cell line to which application of these techniques might be expected to be of value is the human diploid cell line IMR-90 (Nichols *et al.*, 1977). This is a fibroblast-like cell type that has been well characterized cytologically and biochemically and that exhibits a limited population-doubling potential *in vitro*. The cells have been 'banked' in sufficient amounts so that long-term availability for research purposes is to be expected. Since the cells exhibit a normal phenotype in culture, it is relatively difficult to obtain sufficient amounts of materials to allow purification and characterization of individual proteins by normal procedures.

Accordingly, double-labelling and peptide isolation have been used to examine the actin of IMR-90 fibroblasts as a preliminary to the understanding of the role of this protein in these cells. Previously described methodology (Anderson, 1976*a,b*) has been both modified and extended. The modifications in procedure facilitate the application of double-labelling and peptide isolation to protein quantification. The number of peptides examined allows comparisons of IMR-90 actin sequence with peptides accounting for all the thiol groups of muscle actin.

Experimental

Materials

Actin was prepared from rabbit muscle and post-mortem human muscle as previously described (Anderson, 1976*a*). Iodo[2- ^{14}C]acetic acid was obtained from Amersham-Searle, Oakville, Ont., Canada, and diluted with carrier to a specific radio-

Abbreviations used: SDS, sodium dodecyl sulphate; CmCys, carboxymethylcysteine.

activity of 1 mCi/mmol before use. Iodo[2-³H]-acetic acid of specific radioactivity 86 mCi/mmol was obtained from the same source and used without dilution with carrier. Trypsin and α -chymotrypsin were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., as were reagents required for making polyacrylamide gels. Analytical-grade formic acid from BDH Chemicals, Ltd., Poole, Dorset, U.K., was diluted to 70% (v/v) with distilled water before use. Sequanal-grade reagents from Pierce Chemical Co., Rockford, IL, U.S.A., were used in chemical modifications and peptide isolations and characterizations. Materials for cell culture were obtained from Flow Laboratories, Rockville, MD, U.S.A.

Cell culture

IMR-90 cells (population-doubling level 14, passage no. 6) were obtained from the Institute for Medical Research, Camden, NJ, U.S.A. They were grown in Medium 199 (Flow Laboratories) supplemented with 10% (v/v) foetal-calf serum and containing penicillin (100 units/ml) and streptomycin (100 μ g/ml). Falcon 75 cm² plastic flasks containing cells and 25 ml of supplemented Medium 199 were gassed with CO₂/air (1:19) and incubated at 37°C. Confluent cultures were harvested by using trypsin and subcultivated at split ratios of 1:2. Actin was determined in cells harvested from confluent cultures by scraping with a 'rubber policeman'. Harvested cells were washed three times with 10 vol. of (Ca²⁺ and Mg²⁺)-free phosphate-buffered saline (Dulbecco & Vogt, 1954) and frozen at -20°C. Acetone-dried powders were prepared from the frozen cells as previously described (Anderson, 1976a).

Actin determinations

Acetone-dried powders and samples of purified actin and tubulin were dissolved in 8 M-urea containing 100 mM-Tris/HCl, pH 8.0, and 2 mM-dithiothreitol to a concentration of 10 mg/ml. Chemical modification of thiol groups was then carried out by the addition of iodo[2-³H]acetic acid to a final concentration of 10 mM. After 45 min in the dark, excess 2-mercaptoethanol was added and the samples were dialysed against distilled water and freeze-dried. Under these conditions, thiol groups are quantitatively modified (Anderson, 1974). Other samples of purified muscle actin were treated identically, except that iodo[2-¹⁴C]acetic acid was used to modify thiol groups.

Mixtures of [³H]carboxymethylated samples containing known amounts of protein (Itzaki & Gill, 1964) and known amounts of [¹⁴C]carboxymethylated muscle actin were dissolved in 6 M-urea containing 10 mM-sodium phosphate, pH 6.8, 1% SDS and 0.0001% Bromophenol Blue and applied to 7.5% (w/v) polyacrylamide gels containing 0.1% SDS (Weber & Osborn, 1969). Approx. 100 μ g of

protein was applied to each 4 mm $\times$ 90 mm gel and electrophoresis was carried out for 16 h at a current of 4 mA/gel. Gels were then stained with Coomassie Blue and destained in acetic acid/methanol/water (3:10:27, by vol.). To determine the distribution of radioactivity after proteins had been separated on acrylamide gels, the gels were cut into 1 mm slices and then extracted with 0.5 ml of 70% formic acid and 0.1 ml portions were counted for radioactivity in 10 ml of Aquasol (NEN Canada, Lachine, Que., Canada) in a Nuclear-Chicago Isocap 300 liquid-scintillation counter. ³H and ¹⁴C radioactivities were determined by using a double-isotope program. Corrections for spill-over were determined by counting for radioactivity samples containing only ¹⁴C- or ³H-modified protein under identical conditions. For actin quantification, bands corresponding in mobility to muscle actin were cut out and the protein was extracted with 70% formic acid. The formic acid was removed under a stream of N₂ and the extracted protein was suspended in 0.5% ammonium bicarbonate. Digestion with chymotrypsin (10 μ g/mg of protein applied to gel) of the extracted protein was carried out for 2 h at 37°C. The resulting chymotryptic peptides were separated by high-voltage paper electrophoresis at pH 6.5 and pH 2.1 and by paper chromatography in butanol/acidic acid/water/pyridine (15:3:12:10, by vol.). After location by radioautography, ¹⁴C-containing peptides were eluted from paper in 70% formic acid, and 0.1 ml portions were mixed with 10 ml of Aquasol and ³H and ¹⁴C radioactivities were determined. Peptides were identified by comparison of their mobilities on electrophoresis and chromatography with those of known actin-derived peptides.

Results

Fig. 1 shows the distribution of radioactivity obtained when mixtures of [³H]carboxymethylated proteins and [¹⁴C]carboxymethylated actin were separated in electrophoresis on SDS/polyacrylamide gels. From Fig. 1(a) it is apparent that the conditions used give reasonable resolution of proteins, since tubulin and actin are well separated. Fig. 1(b) shows the pattern obtained when a mixture of the [³H]carboxymethylated proteins of IMR-90 cells and [¹⁴C]carboxymethylated muscle actin was similarly separated. A large number of peaks are distributed throughout the gel. There are several peaks of similar mobility to actin, and the largest peak of ³H radioactivity has a mobility identical with that of actin.

Estimates of the actin content of IMR-90 cells based on the amount of protein co-migrating with actin on SDS/polyacrylamide-gel electrophoresis were made by separating mixtures containing known amounts of [¹⁴C]carboxymethylated muscle actin

Fig. 1. Distribution of radioactivity in SDS/polyacrylamide gels after electrophoresis

[^{14}C]Carboxymethylated muscle actin was mixed with (a) [^3H]carboxymethylated muscle actin and brain tubulin and with (b) the [^3H]carboxymethylated proteins of IMR-90 cells before separation on gels. ----, ^{14}C radioactivity $\times 10^{-3}$, and —, ^3H radioactivity $\times 10^{-4}$.

and known amounts of [^3H]carboxymethylated IMR-90 protein. The material co-migrating with actin was eluted from the gels with 70% formic acid and the $^3\text{H}/^{14}\text{C}$ ratio of the eluted material was determined.

From the $^3\text{H}/^{14}\text{C}$ ratio obtained when mixtures of known amounts of [^3H]carboxymethylated and [^{14}C]carboxymethylated muscle actins were similarly treated, the amount of actin that would account for the ^3H -labelled protein of IMR-90 cells co-migrating with muscle actin was calculated. Table 1 gives the

Table 1. Actin content of IMR-90 cells determined by the amount of material co-migrating with actin on polyacrylamide gels

An equimolar mixture of ^3H - and ^{14}C -labelled actin gave a $^3\text{H}/^{14}\text{C}$ ratio of 43.1 under the conditions of labelling and radioactivity determination used. By using this value and the known amounts of [^{14}C]carboxymethylated actin and [^3H]carboxymethylated proteins in the initial mixtures, the actin content was calculated from the $^3\text{H}/^{14}\text{C}$ ratio of material co-migrating with actin on gels.

Expt.	$^3\text{H}/^{14}\text{C}$ ratio		Actin content (%, w/w)
	Initial	After separation on gel	
1	193.2	9.5	7.2
2	137.1	9.6	10.3
3	81.4	4.2	7.6

results of three such experiments. Different preparations of labelled proteins and various relative amounts of ^3H -labelled proteins and ^{14}C -labelled actin were used in the different experiments. Similar, but not identical, values were obtained in each experiment. Variation between experiments might be expected to arise from slight differences in the size of the actin band taken. Overall accuracy of the estimates depends on whether all other labelled proteins are resolved from actin in the electrophoresis system used, and if the actin of the IMR-90 cells contains the same number of radioactive groups per mol as muscle actin. There is no simple way to eliminate these uncertainties. However, if actin-specific peptides were isolated from the material co-migrating with actin on gels, the likelihood of inaccurate estimates due to contaminating proteins would be greatly decreased. In addition, the isolation of peptides containing 1 mol of labelled group per mol of peptide would eliminate problems arising from different numbers of labelled groups in the standard protein and the protein to be measured.

Previous studies of the thiol groups (Lusty & Fasold, 1969) and of the amino acid sequence of muscle actin (Collins & Elzinga, 1975) indicated that digestion of carboxymethylated muscle actin with chymotrypsin should produce unique peptides accounting for the five modified cysteine residues. Accordingly, a sample of [^{14}C]carboxymethylated muscle actin was treated with chymotrypsin and radioactive peptides were isolated by using paper chromatography and electrophoresis. Only one radioactive peptide having a basic mobility on electrophoresis at pH 6.5 was observed. This peptide, designated peptide B, had the composition CmCys₁, Phe₁, Lys₁, Arg₁ and an *N*-terminal arginine residue. Comparison with the known amino acid sequence (Collins & Elzinga, 1975) indicated that this was the

peptide Arg-Lys-CmCys-Phe, accounting for residue Cys-373 of muscle actin. Four peptides having an acidic mobility on electrophoresis at pH 6.5 were isolated. These were designated peptides A1, A2, A3 and A4 in order of increasing acidic mobility. Peptide A1 had the composition CmCys₁, Asp₃, Ile₂, Leu₁, Tyr₁, Lys₂, Arg₁ and an *N*-terminal lysine residue. This is consistent with its being the peptide Lys-CmCys-Asp-Ile-Asp-Ile-Arg-Lys-Asp-Leu-Tyr, accounting for Cys-284. Peptide A2 had the composition CmCys₁, Thr₁, Glu₁, Pro₁, Leu₁, Arg₁ and an *N*-terminal arginine residue. This is consistent with its being peptide Arg-CmCys-Pro-Glu-Thr-Leu, accounting for Cys-256. Peptide A3 had the composition CmCys₁, Tyr₁ and an *N*-terminal carboxymethylcysteine residue, indicating that it was the peptide CmCys-Tyr, accounting for residue Cys-217. Peptide A4 had the composition CmCys₁, Asp₂, Ser₁, Gly₂, Val₁, Leu₁ and an *N*-terminal valine residue. This is consistent with its being the peptide Val-CmCys-Asp-Asp-Gly-Ser-Gly-Leu, accounting for residue Cys-10. Together these peptides account for all five cysteine residues of muscle actin. These peptides could be isolated from the carboxymethylated actin of either human or rabbit muscle.

To examine the homology between the actin of IMR-90 cells and muscle actin and to determine the actin content of the cells based on the amount of actin derived peptide they contained, the following procedure was developed. Mixtures of known amounts of [³H]carboxymethylated IMR-90 protein and [¹⁴C]carboxymethylated muscle actin were subjected to electrophoresis on SDS/polyacrylamide gels. Materials with the mobility of actin was eluted with 70% formic acid. Eluates from 10 gels, to each of which approx. 50 µg of muscle actin mixed with 50 µg of IMR-90 protein had been applied, were pooled. After removal of formic acid, peptides were generated from the eluted protein by digestion with chymotrypsin. The ¹⁴C-containing peptides were separated by paper chromatography and paper electrophoresis. Peptides were identified by comparison of their mobilities with those of purified peptides B, A1, A2, A3 and A4. After purification, peptides were eluted from paper with 70% formic acid. The radioactivity

was then determined by liquid-scintillation counting for 10 min of portions of the samples containing at least 200 ¹⁴C c.p.m. above background. Table 2 gives the ³H/¹⁴C ratios obtained when peptides were generated from the gel-separated actin and IMR-90 protein mixtures described in Table 1. The amount of ³H label in the isolated peptides and the similarity of the ³H/¹⁴C ratios for peptides B, A1, A2 and A3 indicate that IMR-90 cells contain a protein that co-migrates with muscle actin on SDS/polyacrylamide-gel electrophoresis that is homologous with muscle actin in the regions of amino acid sequence from which these peptides arise. Very little ³H label co-migrates with peptide A4. This suggests that muscle actin and the protein from IMR-90 cells are different in this region of the sequence. Table 2 also contains estimates of the amount of actin in IMR-90 cells calculated from the ³H/¹⁴C ratios of isolated peptides. Calculations using the average ³H/¹⁴C ratios of peptides B, A1, A2 and A3 were carried out as described for Table 1. A reproducible actin content of 4.2% of the total protein of IMR-90 cells was obtained.

Discussion

It is often difficult to obtain sufficient amounts of material from normal cells grown in culture to allow purification and structural determinations of individual protein components. Because it has no easily measured biological properties, the determination of the amounts and structure of the protein actin in small amounts of biological materials presents special difficulties. A method allowing the identification and quantification of actin based on double-labelling and the isolation of actin-derived peptides has been described (Anderson, 1976a,b). No preliminary protein separation before peptide generation is used in this procedure and difficulties arise in its application. Relatively large peptides must be isolated to increase the probability that a specific peptide is uniquely derived from a specific protein. Since contaminating peptides will result in overestimates of actin content and very large numbers of peptides are initially generated, many laborious and

Table 2. Actin content of IMR-90 fibroblasts calculated from the amount of actin-derived peptide that they contain. The material co-migrating with actin described in Table 1 was treated with chymotrypsin, and specific CmCys-containing peptides were isolated. The average ³H/¹⁴C ratio of the isolated peptides, excluding peptide A4, was then used to calculate the actin content as described in Table 1.

Expt.	Peptide	³ H/ ¹⁴ C ratio of isolated peptides					Average	Actin content (%, w/w)
		B	A1	A2	A3	A4		
1		5.7	5.2	5.6	5.5	0.3	5.5	4.2
2		3.7	4.0	4.1	3.5	0.3	3.8	4.1
3		2.4	2.1	2.4	2.7	0.2	2.3	4.2

time-consuming steps of peptide purification must be carried out.

Although several forms of actin have been distinguished on the basis of their isoelectric points (Whalen *et al.*, 1976; Rubenstein & Spudich, 1977), there is no indication that actins differ in either molecular weight or mobility on SDS/polyacrylamide gels. Estimation of the amount of material co-migrating with actin on such gels has been used as a measure of actin content (Bray & Thomas, 1975). However, the accuracy of such determinations is uncertain because of the possibility that actin is not resolved from all other components by the electrophoresis system and because of the difficulty in determining the relative response compared with other proteins of actin to the detecting system.

A method of actin quantification that combines both gel separation and peptide isolation has now been developed and applied to the determination of actin in IMR-90 cells. Mixtures of proteins are first separated on SDS/polyacrylamide gels. Because this step eliminates many proteins before peptide generation, relatively small peptides generated from the material co-migrating with actin are likely to be specifically derived from protein homologous with actin. By using only the 20 amino acids coded for by genes and the restrictions placed on neighbouring residues by the specificity of enzymic digestion, approx. 10^6 tetrapeptides could be produced from proteins by digestion with chymotrypsin. Assuming that a 40000-mol.wt. protein could give rise to a potential 400 tetrapeptides, less than 1 in a 1000 proteins might therefore be expected to give rise to a given tetrapeptide on a random basis. Not only does protein separation before peptide generation allow the use of small peptides for actin quantification, but because a much decreased number of proteins are present in the material from which the peptides are generated, fewer peptides are initially present and peptide isolation would be expected to be easier.

Accordingly, peptides accounting for all the thiol groups of muscle actin have been isolated from mixtures of [^{14}C]carboxymethylated muscle actin and [^3H]carboxymethylated proteins of IMR-90 cells after protein separation by SDS/polyacrylamide gel electrophoresis and digestion with chymotrypsin. From the $^3\text{H}/^{14}\text{C}$ ratio of these isolated peptides, it has been possible to conclude that IMR-90 cells contain a protein that co-migrates with muscle actin on SDS/polyacrylamide gel and is homologous with muscle actin in the regions of amino acid sequence around four of the five cysteine residues of muscle actin. The similarity of the $^3\text{H}/^{14}\text{C}$ ratios for each of these four peptides indicates that if multiple forms of actin are present in IMR-90 cells, they do not differ in these regions of actin sequence. The lack of ^3H label co-purifying with a chymotryptic peptide

accounting for Cys-10 of muscle actin indicated that such a peptide was not generated from the protein of IMR-90 cells co-migrating with muscle actin on gels. This finding indicates that the actin-like protein from IMR-90 cells differs from muscle actin in this region of amino acid sequence.

The amount of actin present in the cells calculated from the amount of homologous peptide produced is considerably less than the amount of actin determined on the basis of the amount of material co-migrating with actin on gels. The presence of radioactive contaminating proteins or peptides will lead to overestimates of actin content by the methods used. The results suggest that such contaminants are present after protein separation, but are removed by the peptide-isolation procedures. The similarity of $^3\text{H}/^{14}\text{C}$ ratios obtained for different peptides is consistent with pure peptides having been isolated in each case. However, as additional evidence of peptide purity, isolated samples of peptide B (Arg-Lys-CmCys-Phe) were digested with trypsin; peptide CmCys-Phe, which is acidic on pH6.5 electrophoresis, was subsequently isolated. The $^3\text{H}/^{14}\text{C}$ ratio of this peptide was unchanged from that of the sample of peptide B from which it was generated. The probability of peptide material of different amino acid sequence from peptide B co-purifying with it and subsequently with a peptide of different properties produced from it is remote. Thus it is likely that an accurate estimate of the actin content of IMR-90 cells has been made. It should now be possible to determine whether the actin content of such cells as IMR-90 human fibroblasts varies with such things as position in the cell cycle or time in culture. These determinations might be expected to be of value in defining the role of actin in cell processes. It is also possible to obtain additional structural information about cellular actin by using the types of procedures described by varying such things as the method of peptide generation or the type of chemical modification.

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References

- Anderson, P. J. (1974) *Biochem. J.* **140**, 341–343
- Anderson, P. J. (1976a) *Biochem. J.* **155**, 297–301
- Anderson, P. J. (1976b) *Biochem. J.* **159**, 185–187
- Bray, D. & Thomas, C. (1975) *Biochem. J.* **147**, 221–228
- Collins, J. H. & Elzinga, M. (1975) *J. Biol. Chem.* **250**, 5915–5920
- Dulbecco, R. & Vogt, M. (1954) *J. Exp. Med.* **99**, 167–182
- Edelman, G. M. & Yahara, I. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2047–2051
- Hartwig, J. H. & Stossel, T. P. (1975) *J. Biol. Chem.* **250**, 5696–5705

- Itzaki, R. T. & Gill, D. M. (1964) *Anal. Biochem.* **9**, 401–410
- Kopelovich, L., Conlon, S. & Pollack, R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3019–3022
- Lusty, C. J. & Fasold, H. (1969) *Biochemistry* **8**, 2933–2939
- Nichols, W., Murphy, D., Cristofalo, V., Toji, L., Greene, A. & Dwight, S. (1977) *Science* **196**, 60–63
- Pollack, R., Osborn, M. & Weber, K. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 120–123
- Pollard, T. D. & Weihing, R. R. (1974) *CRC Crit. Rev. Biochem.* **2**, 1–65
- Puck, T. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4491–4495
- Rubenstein, P. A. & Spudich, J. A. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 120–123
- Wang, E. & Goldberg, A. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 4065–4069
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4503–4509
- Whalen, R. G., Butler-Browne, G. S. & Gros, F. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2018–2022
- Yang, Y.-Z. & Perdue, J. F. (1972) *J. Biol. Chem.* **247**, 4503–4509