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Distinct sites on the G-actin molecule bind group-specific component and deoxyribonuclease I

Pascal J. GOLDSCHMIDT-CLERMONT, Robert M. GALBRAITH, David L. EMERSON, Francois MARSOT, Andre E. NEL and Philippe ARNAUD
Departments of Basic and Clinical Immunology and Microbiology, and Medicine, Medical University of South Carolina, Charleston, SC, 29425, U.S.A.

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Addition of group-specific component (Gc) to G-actin with or without deoxyribonuclease I (DNAase) led to formation of binary complexes (Gc–G-actin) and ternary complexes (Gc–G-actin–DNAase) respectively. The electrophoretic mobility of ternary complexes, as shown by crossed and rocket immunoelectrophoresis, was slower than that of binary complexes, although both were faster than native Gc. In gradient polyacrylamide-gel electrophoresis, such complexes could again be resolved, apparently on the basis of relative molecular size: Gc–G-actin–DNAase (M_r approx. 131000), Gc–G-actin (M_r approx. 98000) and Gc (M_r approx. 56000). In contrast, the pI of ternary complex was indistinguishable by isoelectric focusing from that of binary complex, even though both were clearly more acidic than native Gc. The affinity of Gc for G-actin (affinity constant, K_a, $1.9 \times 10^8 M^{-1}$) was not significantly altered by additional interaction with DNAase (K_a, $1.5 \times 10^8 M^{-1}$), and both binary and ternary complexes still bound 25-hydroxycholecalciferol. In addition, the inhibitory effect of G-actin on DNAase activity was not discernibly affected by interaction with Gc. These results demonstrate that the various molecular forms of Gc can be distinguished by physicochemical parameters, and that Gc and DNAase bind to distinct sites on G-actin and can interact both independently and contemporaneously with this molecule.

Certain proteins are known to bind monomeric, or G-, actin. This group, which includes profilin, Gc (vitamin D-binding protein) and DNAase I, exhibits several properties in common (Korn, 1982; Weeds, 1982). Thus, all are of relatively small M_r [Gc, 56000; DNAase, 33000; profilin, 15000 (Cleve *et al.*, 1963; Lindberg, 1967; Carlsson *et al.*, 1976; Korn, 1982; Weeds, 1982)]. In addition, all three bind G-actin at a 1:1 molar ratio with relatively high affinity (Cooke *et al.*, 1979*a*; Mannherz *et al.*, 1980; Mockrin & Korn, 1980), a characteristic which may stabilize G-actin in monomeric form and thereby retard, or effectively prevent, polymerization. Both Gc and DNAase facilitate depolymerization of F-actin, in part by complexing the pool of monomeric G-actin and shifting the equilibrium in favour of F-actin disassembly (Mannherz *et al.*, 1975; Hitchcock *et al.*, 1976; Cooke *et al.*, 1979*a*; Korn, 1982; Weeds, 1982; Lees *et al.*, 1984). Since Gc and DNAase are present in a wide variety of cells and also in the circulation (Cooke *et al.*, 1979*b*; Chitrabamrung *et al.*, 1981), these proteins could play important physiological roles in the biology of actin. However, studies of interactions between these proteins and G-actin have been limited.

We have recently presented studies (Emerson *et al.*, 1984) which demonstrate that Gc complexed with G-actin (Gc–G-actin) can be differentiated from native Gc, and from Gc complexed with vitamin D_3 metabolites (Gc–D_3). To clarify further the interaction of G-actin with Gc and DNAase, we undertook analysis of interactions between these three proteins, with the use of both purified material and normal human serum.

Abbreviations used: DNAase, deoxyribonuclease I (EC 3.1.21.1); F-actin, filamentous actin; G-actin, globular actin; Gc, group-specific component (or vitamin D-binding protein); SDS, sodium dodecyl sulphate; 25-(OH)D_3, 25-hydroxycholecalciferol.

Materials and methods

Preparation of proteins

Gc and G-actin were purified as previously described (Goldschmidt-Clermont *et al.*, 1985) and radioiodinated by lactoperoxidase (Marchalonis, 1969) or Iodogen (Fraker & Speck, 1978). G-actin was maintained throughout in G-actin buffer [5mM-Tris (pH7.5)/0.1mM-$CaCl_2$/0.1mM-ATP/0.5mM-dithiothreitol]. DNAase was purified from crystalline material extracted from bovine pancreas (Sigma Chemical Co.) by using a method similar to that described by Lindberg (1967). Portions of up to 50 mg were subjected to gel filtration on Sephadex G-100 (column 2.5cm × 100cm) in 50mM-Tris buffer, pH7.5, containing 1mM-$CaCl_2$ and 1mM-phenylmethanesulphonyl fluoride (Price *et al.*, 1969; Blikstad *et al.*, 1978) at 4°C. As measured by A_{280}, three distinct peaks were eluted from the column and each was further studied by SDS/polyacrylamide-gel electrophoresis and for DNAase activity. This column was also used with G-actin buffer for gel filtration of mixtures containing purified Gc, G-actin and DNAase. Human serum was obtained with informed consent from healthy volunteers determined by isoelectric focusing (see below) to be of Gc1 or Gc1,2 phenotype by comparison with standards kindly supplied by Dr. Dale Dykes, Minnesota War Memorial Hospital, Minneapolis, MN, U.S.A.

Enzymic assay

Assays for pancreatic DNAase were performed following the approach described by Lindberg and colleagues (Lindberg, 1964; Blikstad *et al.*, 1978) based upon the increased A_{260} observed upon enzymic degradation of DNA. Reaction rates were calculated from the linear slope of the time curve obtained, and were directly proportional to the amount of the enzyme present.

Electrophoretic methods

Thin layer analytical isoelectric focusing (Emerson *et al.*, 1984) and rocket immunoelectrophoresis (Goldschmidt-Clermont *et al.*, 1985) were carried out as described previously. Crossed immunoelectrophoresis was performed according to Ganrot (1972). The heteroantiserum against Gc (Dakopatts) has been shown to be monospecific (Petrini *et al.*, 1984). Two-dimensional electrophoresis (isoelectric focusing followed by SDS/polyacrylamide-gel electrophoresis) was undertaken as previously reported (Petrini *et al.*, 1985). Polyacrylamide-gel electrophoresis was performed in linear gradients of polyacrylamide (5–20%) in Tris/glycine/HCl buffer (Laemmli, 1970) with and without 0.1% SDS, and gels were stained with either silver (Merril *et al.*, 1981) or Coomassie Blue. Measurements of affinity (K_a) were performed according to Cooke *et al.* (1979*b*) using polyacrylamide-gel electrophoresis and tracer amounts of ^{125}I-Gc, with analysis as described by Scatchard (1949).

Results

Preparation of proteins

Chromatography of commercial DNAase on Sephadex G-100 gave three major peaks of A_{280}. SDS/polyacrylamide-gel electrophoresis showed that the first peak contained proteins of M_r approx. 48000–66000, the second an apparently homogeneous major component of M_r 33000, and the third several components of $M_r < 25000$. Assay of individual fractions showed that the only fractions with detectable DNAase enzyme activity were those included in the second peak. Specific enzyme activity (Lindberg, 1964) was enhanced approx. 3-fold from 1.36×10^5 to 3.72×10^5 units/mg. Radioiodination of Gc with either lactoperoxidase or Iodogen did not lead to detectable alteration of the protein, although labelling was more efficient with lactoperoxidase (sp. radioactivity 0.8–1.2 μCi/μg). For G-actin, the lactoperoxidase method resulted in protein of very low specific radioactivity, whereas Iodogen gave adequately labelled protein with native properties (sp. radioactivity 3.0–3.5 μCi/μg). In contrast, DNAase treated with Iodogen showed clear evidence of degradation as judged by SDS/polyacrylamide-gel electrophoresis, while the lactoperoxidase procedure resulted in labelled material (sp. radioactivity 0.4–0.6 μCi/μg) with native physicochemical properties and no detectable loss of enzymic activity.

Inhibition of DNAase activity

The addition of increasing amounts of G-actin to purified DNAase preparations resulted, as anticipated (Lindberg, 1964; Blikstad *et al.*, 1978), in inhibition of DNAase activity in a dose-dependent fashion. The further addition of Gc in amounts sufficient to saturate G-actin (molar ratio 1:1) did not affect this inhibition. This observation, which has been noted previously (Haddad, 1982) could represent formation of a ternary complex between DNAase, G-actin and Gc in which the inhibitory action of G-actin for DNAase was not appreciably affected. A series of experiments was therefore performed to identify and characterize such a putative ternary complex.

Identification of ternary complex

Crossed immunoelectrophoresis showed two immunoreactive Gc peaks in samples to which subsaturating amounts of G-actin had been added, both in normal serum and with purified components

(Fig. 1). Experiments performed with ^{125}I-G-actin and autoradiography demonstrated that G-actin was present only in the faster anodal peak. Further addition of DNAase at concentrations slightly less than those of G-actin (Gc:G-actin:DNAase molar proportions 3:1.5:1) resulted in a third intermediate peak (Fig. 1*c*). Larger amounts of DNAase (Gc:G-actin:DNAase molar proportions 3:1.5:2) led to disappearance of the most anodal peak and a further increase in the height of the intermediate peak (Fig. 1*d*), while equimolar concentrations of DNAase, Gc and G-actin (Gc:G-actin:DNAase molar proportions 1:1:1) gave only the intermediate peak (results not shown). The formation of ternary complexes was confirmed by autoradiography (Figs. 1*g* and 1*h*). In contrast, with addition of DNAase only to Gc, no alteration in the Gc immunoprecipitate was observed, indicating a lack of direct interaction between these two proteins. Identical profiles were obtained with normal human serum of Gc1 and Gc1,2 phenotypes.

The putative ternary complex was further investigated by two-dimensional electrophoresis of mixtures of these proteins in purified form. When complexes of Gc1 with G-actin (molar ratio 3:1) were examined, Gc focused in the first dimension to three locations with pI values of 4.95, 4.90 and 4.80 (Fig. 2*a*), corresponding to the slow native isotype, the fast native isotype and Gc–G-actin complexes respectively (Emerson *et al.*, 1984). An M_r 42000 species representing actin was detected at pH4.80, confirming the presence of Gc–G-actin complexes at this pI which were dissociated during the SDS/polyacrylamide-gel step in the second dimension. Parallel experiments with the further addition of purified DNAase (Gc:G-actin:DNAase molar proportions 3:1:1) showed identical pI values for the three Gc bands, but a third band (M_r 33000) corresponding to DNAase was also clearly seen at pH 4.80 after SDS/polyacrylamide-gel electrophoresis (Fig. 2*b*). Moreover, when DNAase or G-actin were run in the absence of Gc, neither was detectable at pH4.80.

Further evidence of the formation of a ternary complex between Gc, G-actin and DNAase was obtained by chromatography of equimolar mixtures of G-actin, Gc and DNAase in G-actin buffer on a calibrated column of Sephadex G-100. This showed a single peak containing Gc, G-actin and DNAase at a calculated M_r of approx. 131000, and confirmed the stoichiometry of interaction to be equimolar for Gc:G-actin:DNAase.

Further characterization of the ternary complex

By rocket immunoelectrophoresis, addition of G-actin to purified Gc or normal human serum gave an increase in rocket height as anticipated (Goldschmidt-Clermont *et al.*, 1985). DNAase was then added to such samples in amounts sufficient to saturate the G-actin present, which resulted in rockets smaller than those obtained with the same amounts of Gc–G-actin complex but still higher than those obtained with native Gc alone. Autoradiography performed with ^{125}I-DNAase and ^{125}I-G-actin showed that both G-actin and DNAase were bound and the entire rocket was radiolabelled. No effect upon Gc rocket height was observed after addition to Gc of DNAase only, and no overall differences were observed upon comparison of the different Gc phenotypes tested.

The profiles obtained for Gc after addition of G-actin and DNAase were further analysed by isoelectric focusing. As with crossed immunoelectrophoresis, the addition of DNAase to samples containing purified Gc had no discernible effect upon the position of the Gc bands. The Gc–G-actin bands were anodal to those of native Gc (Figs. 3*a* and 3*b*), as expected (Emerson *et al.*, 1984). However, no difference could be discerned upon the further addition of DNAase, and the profile obtained was identical with that of Gc–G-actin over a wide range of added DNAase, corresponding to G-actin–DNAase molar ratios of 10:1 to 1:10 (Figs. 3*c* and 3*d*). The use of tracer amounts of ^{125}I-G-actin or ^{125}I-DNAase and print immunofixation for Gc showed that the relevant anodal bands contained Gc and were radiolabelled and therefore contained all three proteins (Figs. 3*d*–3*g*). In addition, experiments in which 25-(OH)D_3 was added demonstrated that this metabolite appeared to bind to the ternary complex, resulting in a further anodal shift to a pI characteristic of Gc–G-actin–25-(OH)D_3 complexes (Figs. 3*e* and 3*g*). These results, which did not indicate any detectable modification in pI of Gc–G-actin or Gc–G-actin–25-(OH)D_3 complexes upon interaction with DNAase, further confirmed the observations made by two-dimensional electrophoresis (Fig. 2).

Equimolar mixtures of purified Gc, G-actin and DNAase were also analysed by gradient polyacrylamide-gel electrophoresis in the absence of SDS (Fig. 4). As compared with purified Gc only (Figs. 4*e* and 4*f*), the single Gc–G-actin bands obtained (M_r approx. 100000) showed reduced mobility (Figs. 4*c* and 4*d*). The addition of DNAase as well as G-actin led to a further decrease in electrophoretic mobility of the single band obtained (M_r approx. 130000) (Figs. 4*a* and 4*b*), and autoradiography with ^{125}I-G-actin, ^{125}I-DNAase and ^{125}I-Gc indicated that these bands contained all three components. In contrast, inclusion of 0.1% SDS in the polyacrylamide gels resulted in every case in the reappearance of individual protein bands of M_r values 56000, 42000 and 33000. These results

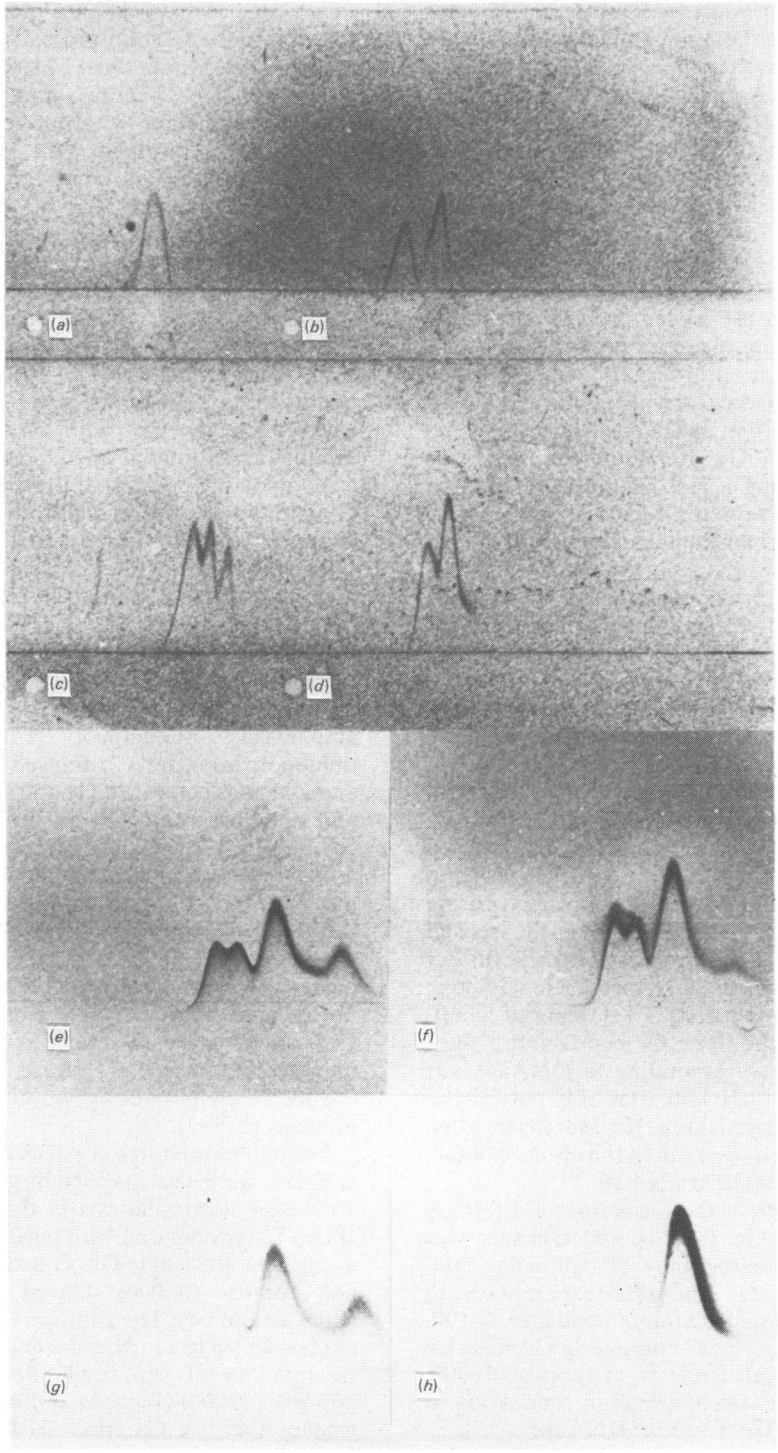

Fig. 1. *Crossed immunoelectrophoresis of mixtures of purified Gc, G-actin and DNAase against a monospecific antiserum to human Gc*

The cathode was at the left. (*a*)–(*f*), Coomassie Blue stained: (*a*), native Gc; (*b*), Gc–G-actin (molar ratio 3:1); (*c*), Gc–G-actin–DNAase (molar proportions 3:1.5:1); (*d*), Gc–G-actin–DNAase (3:1.5:2); (*e*) and (*f*), as (*c*) but

indicated that gradient polyacrylamide-gel electrophoresis performed without SDS reproducibly distinguished between native Gc, Gc–G-actin complexes and a ternary Gc–G-actin–DNAase complex in order of decreasing migration. Moreover, under these conditions, no evidence of dissociation of these complexes was observed, either by autoradiography or direct counting of gel slices. Further experiments were then performed to determine the K_a of ^{125}I-Gc binding to G-actin and, in parallel, to G-actin–DNAase. Scatchard analysis yielded calculated K_a values of $1.9 \times 10^8 M^{-1}$ and $1.5 \times 10^8 M^{-1}$ respectively.

Discussion

Previous studies have suggested formation *in vitro* of a ternary complex between DNAase, G-actin and Gc (Cooke *et al.*, 1979*a*; Van Baelen *et al.*, 1980; Haddad, 1982). The evidence obtained in the present study with a variety of methods collectively provides further confirmation. Thus there appeared to be no direct interaction between Gc and DNAase. However, both these components could bind to G-actin contemporaneously and independently, indicating that the binding sites for Gc and DNAase on the G-actin molecule are structurally or sterically distinct. Moreover, although the absolute K_a values obtained for Gc interaction with G-actin and G-actin–DNAase will require further confirmation, the lack of any major difference indicates that the relevant binding sites for Gc and for DNAase may be widely separated on the G-actin molecule. Studies with trypsin and chymotrypsin have shown that DNAase protects the major sites of proteolysis at, respectively, Arg-62 and Lys-68 for trypsin, and Leu-67 for chymotrypsin (Burtnick & Chan, 1980). This suggests that interaction with DNAase occurs at the *N*-terminal end of the G-actin molecule. However, comparable information is not yet available for Gc.

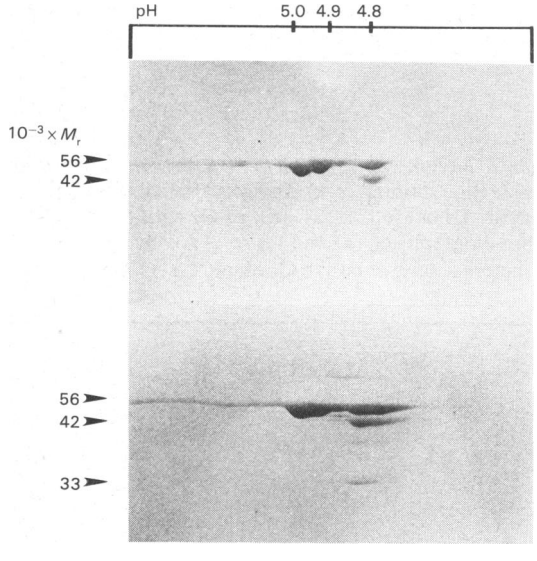

Fig. 2. *Two-dimensional electrophoresis of mixtures of Gc, G-actin and DNAase as described in the text*

Top, Gc–G-actin (molar ratio 3:1); bottom, Gc–G-actin–DNAase (3:1:1). Note in both cases the presence of single Gc spots (M_r 56000) at pH4.90 and 4.95 corresponding to the isotypes of native Gc1, with a third Gc spot at pH4.8 which had migrated in the first dimension together with G-actin (M_r 42000) or G-actin complexed with DNAase (M_r 33000).

The results obtained in the present study suggest certain additional points of interest. For example, interaction of Gc with G-actin has been found both previously (Emerson *et al.*, 1984; Goldschmidt-Clermont *et al.*, 1985) and in the present study to lead to anodal shifts on isoelectric focusing and increased electrophoretic mobility on crossed and rocket immunoelectrophoresis. However, the present results indicate that, although the superimposed binding of DNAase to Gc–G-actin complexes could alter electrophoretic mobility, the profiles as obtained by isoelectric focusing were not discernibly modified. It is important to note in contrast that binding of 25-(OH)D_3 to native Gc, to Gc–G-actin and to Gc–G-actin–DNAase complexes is associated in all three cases with obvious alterations in isoelectric point (Fig. 3) but almost imperceptible changes in electrophoretic mobility by crossed (Emerson *et al.*, 1984) and by rocket (Goldschmidt-Clermont *et al.*, 1985) immunoelectrophoresis.

The biological relevance of these findings is still hypothetical. Gc, G-actin and DNAase are known to be present within cells, and all three have also been found in the extracellular space (Cleve *et al.*, 1963; Carlsson *et al.*, 1977; Hitchcock, 1980; Cooke *et al.*, 1979*a*,*b*; Mannherz *et al.*, 1980; Chitrabamrung *et al.*, 1981; Korn, 1982; Weeds, 1982; Accini *et al.*, 1983). Consequently, interactions between these three molecules would

containing tracer amounts of ^{125}I-G-actin and ^{125}I-DNAase respectively and with electrophoresis for twice the time in the first dimension; (*g*) and (*h*), autoradiographs of (*e*) and (*f*). Note the formation of distinct Gc, Gc–G-actin–DNAase and Gc–G-actin peaks in order of increasing migration, and the bifid cathodal peak (native Gc) in (*e*) and (*f*) due to partial resolution of the Gc1 isotypes.

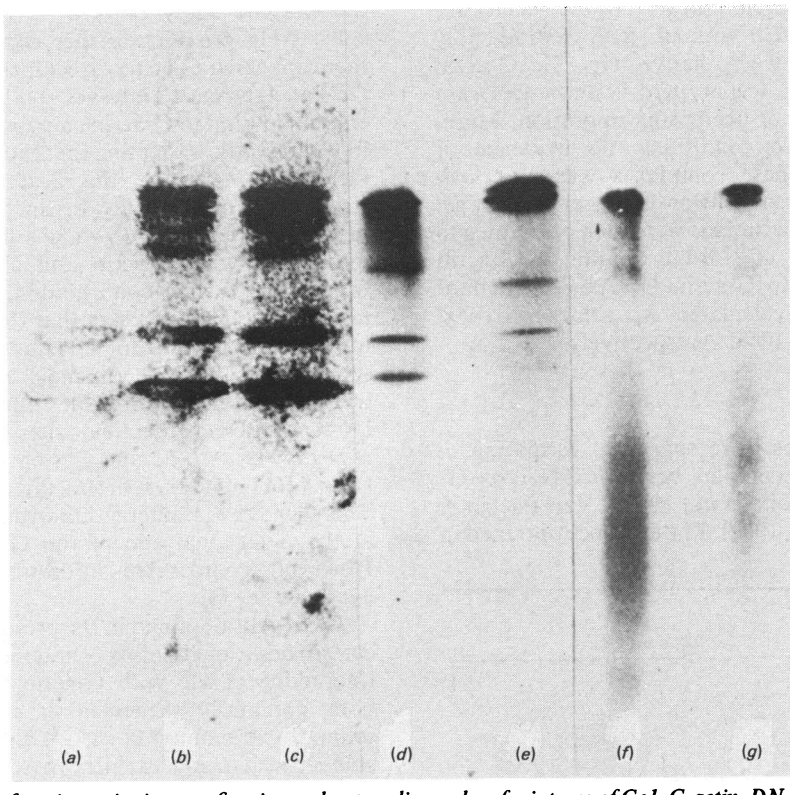

Fig. 3. *Isoelectric focusing, print immunofixation and autoradiography of mixtures of Gc1, G-actin, DNAase and 25-(OH)D_3* Results are shown for both cellulose acetate strips after Coomassie Blue staining (*a–c*), and gels after silver staining (*d–e*). (*a*), Gc; (*b*), Gc–G-actin (molar ratio 2:1); (*c*), Gc–G-actin–DNAase (2:1:2); (*d*), as (*c*) with tracer ^{125}I-DNAase; (*e*), as (*d*) with excess 25-(OH)D_3; (*f*) and (*g*), autoradiographs of (*d*) and (*e*) respectively. Note that addition of DNAase had no detectable effect on the pI or microheterogeneity of native Gc alone, Gc–G-actin or 25-(OH)D_3–Gc–G-actin.

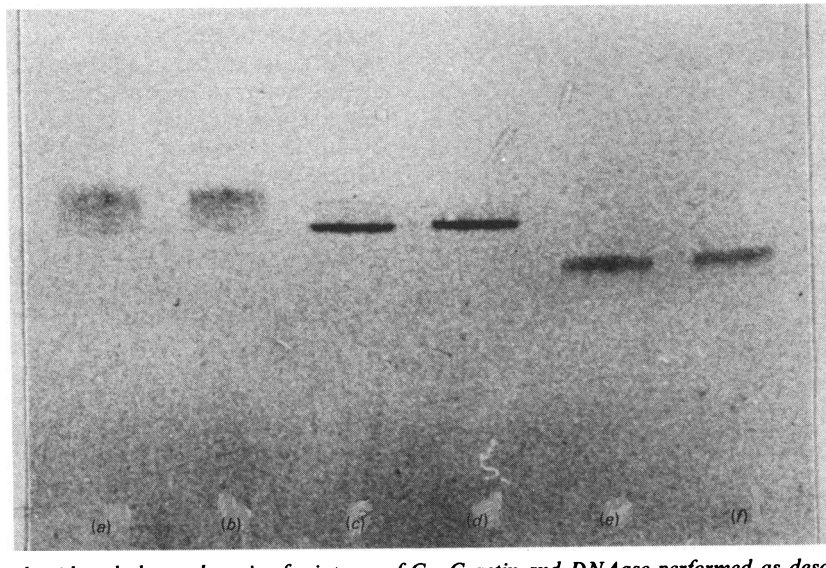

Fig. 4. *Polyacrylamide-gel electrophoresis of mixtures of Gc, G-actin and DNAase performed as described in the text* (*a*) and (*b*), Gc–G-actin–DNAase (molar proportions 1:1:1); (*c*) and (*d*), Gc–G-actin (1:1); (*e*) and (*f*), Gc alone. Note the presence of a sieving effect leading to decreasing mobility in the order of Gc > Gc–G-actin > Gc–G-actin–DNAase.

appear also to be possible in both intracellular and extracellular compartments. Moreover, ternary complexes continue to bind 25-(OH)D_3, even though Gc is complexed with both G-actin and DNAase, and our preliminary results suggest that the K_a of interaction between Gc and 25-(OH)D_3 is unaffected by ternary complex formation (B. Boutin, P. J. Goldschmidt-Clermont, R. M. Galbraith & P. Arnaud, unpublished work). The functional consequences of such interactions are unknown, but the occurrence of a ternary complex may hold certain implications for the respective roles of Gc and DNAase in the actin polymerization/depolymerization cycle.

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