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PROTEOLYTIC ENHANCEMENT OF GELATION OF ASCITES TUMOR CELL EXTRACTS. RELATIONSHIP TO ACTIN BINDING PROTEIN

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Summary: Proteolysis of cytoplasmic extracts of sarcoma 180 and MAT-C1 adenocarcinoma ascites cells enhances the rate of gelation. Only high molecular weight polypeptides, including actin binding protein and myosin, are cleaved during the process; actin is not cleaved. In MAT-B1 adenocarcinoma extracts the gelation rate was not enhanced by proteolysis and actin binding protein was not readily cleaved. Electrophoretic comparisons of trypsin-treated and untreated extracts of MAT-B1 and MAT-C1 cells show that actin binding protein is the only readily discernible polypeptide which is cleaved in the C1 cells but not in the B1 cells. These results suggest that actin binding protein may act as an inhibitor of gelation.

Cell motility is widely believed to involve contractile systems similar to those found in muscle (1). In lower organisms there is good evidence that a sol-gel transformation plays an important role in directed movement (2). This transition has been studied in cytoplasmic extracts of amoebae and appears to be calcium-regulated as well as temperature-dependent (3). Although motility of mammalian cells is less well understood, cytoplasmic extracts of these will also gel upon warming (4, 5). The processes in the two systems show some interesting similarities, including the presence of a high molecular weight polypeptide (6, 7). This protein was isolated from macrophages and called actin binding protein by Hartwig and Stossel (6) and was shown to cause formation of gels when added to actin (8). However, experiments in other systems suggest that the high molecular weight component is not absolutely necessary for gelation (9). Maruta and Korn (10) have purified four smaller proteins which cause purified F-actin to gel. Moreover, Tilney (11) has described high molecular weight polypeptides in Thyone sperm which appear to form a complex with actin and inhibit its polymerization. Thus the role of high molecular weight factors such as actin binding protein is unclear.

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We report here studies of the proteolysis of extracts of three ascites tumor lines that suggest a correlation between the enhancement of gelation and the cleavage of actin binding protein and indicate that the actin binding protein is more likely an inhibitor than a promoter of actin polymerization.

Methods: Sarcoma 180 ascites cells were grown in and isolated from mouse peritoneal cavities as previously described (12). MAT-B1 and MAT-C1 ascites cells were grown in the peritoneal cavities of rats and isolated by similar methods (13). After the cells were washed in isotonic buffer, they were swollen in 40 mM Tris-HCl, pH 7.4, for 4 min and collected by low speed centrifugation. The packed cell volume was measured and an equivalent amount of 40 mM Tris-HCl (pH 7.4) containing ATP, EDTA, and β-mercaptoethanol was added to give final concentrations of 5 mM, 1 mM, and 10 mM respectively. The cells were homogenized in a Dounce homogenizer fitted with a B pestle until essentially all cells were disrupted. The cell homogenates were centrifuged at 26,000 rpm in an SW 27 rotor on a Beckman Model L-5 ultracentrifuge at 4° for 1 hr. The floating layer was removed and the supernatant decanted and kept on ice. Aliquots of 0.5 ml were removed to 10 x 75 mm glass tubes for assay. Trypsin, papain (5-20 µg per mg homogenate protein) or other effector was mixed with the extract (12-20 mg protein per ml) and warmed to room temperature. Gelation was assayed by inversion of the sample tubes at invervals. A positive gel(+) was scored only if the formed gel remained in the tube bottom upon inversion.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed as previously described (14). Gels were scanned on a Helena Quik Scan equipped with an automatic integrator.

Results: As previously demonstrated with amoebae (7), macrophages (4) and HeLa cells (5), cytoplasmic extracts of sarcoma 180 ascites cells undergo gelation when allowed to warm to room temperature (Table I), requiring about 30 min to form a solid gel. Gelation was inhibited by cytochalasin B added in dimethylsulfoxide (5); dimethylsulfoxide alone had essentially no effect. Gelation was significantly promoted by addition of either trypsin or papain. With 5 µg trypsin per mg of extract protein a stable gel was formed in about 3 min and remained stable for at least an hour. At higher concentrations of trypsin or papain gels formed but subsequently appeared to destabilize, giving a semisolid mass (scored as negative in Table I). Although the assay for gelation is somewhat subjective, the trend of gelation followed by gel dissolution was very clear and was reproducible with extracts from different batches of cells.

Proteolysis also eliminated the temperature dependence of gelation of sarcoma cytoplasmic extracts. When extracts were treated with 7 µg trypsin per

TABLE I

Proteolytic Enhancement of Gelation of Sarcoma 180 Cytoplasmic Extracts

Time (min)	Control	Trypsin 5 μg/mg	Trypsin 20 μg/mg	Papain 10 μg/mg	Cytochalasin B 20 μg/mg
5	-	+	+	+	-
10	-	+	+	+	-
15	-	+	-	-	-
20	-	+	-	-	-
25	-	+	-	-	-
30	-	+	-	-	-
35	+	+	-	-	-
40	+	+	-	-	-
50	+	+	-	-	-
60	+	+	-	-	-

mg protein and held at 4°, gelation occurred in 5 min. Treated extracts warmed to 25° gelled in less than 3 min. However, the untreated extracts held at 4° did not gel over a 6.5 hr observation period.

To determine what proteins were being cleaved by protease during the enhancement of gelation, the gels and extracts were solubilized with hot sodium dodecyl sulfate and subjected to polyacrylamide gel electrophoresis (Fig. 1). Under the minimal conditions for gel formation only the high molecular weight polypeptides (>100,000) were substantially cleaved. These include myosin, actin binding protein and an unidentified slowly migrating species near the origin (band E, ref. 14). The first two of these have been identified by isolation from extracts or membranes and by their amino acid analyses, electrophoretic behavior and enzyme activities (14). There was no significant

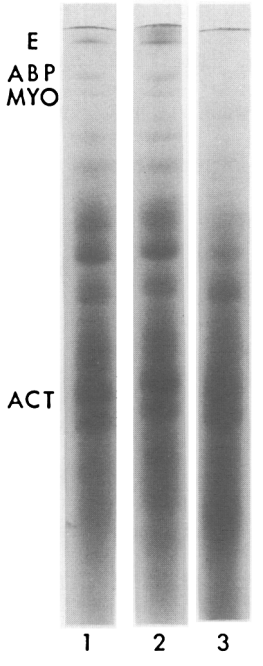

Fig. 1 - Polypeptide patterns of sarcoma 180 cytoplasmic extracts from gelation experiments. Sample 1 is a control aliquot of the cold high speed supernatant which was solubilized prior to the initiation of the gelation experiment. Sample 2 was incubated under gelation conditions (allowed to warm to room-temperature) and formed a gel in about 30 min. It was solubilized after 50 min. Sample 3 was incubated with 5 µg trypsin per mg protein under gelation conditions and solubilized after 50 min. It formed a gel in less than 5 min. There are no significant polypeptide differences between 1 and 2, but the higher molecular weight polypeptides have all been cleaved in 3. Cleavage of the proteins is rapid, occurring in less than 3 min. Polypeptide patterns are essentially the same over a 3-50 min incubation with trypsin. ABP, actin binding protein; MYO, myosin; ACT, actin.

cleavage of actin even when the gels were disintegrating (Fig. 1). The cleavage of the high molecular weight polypeptides is rapid, occuring in less than 3 min in cytoplasmic extracts being warmed to room temperature with 5 µg trypsin per mg extract protein. There are essentially no significant changes in the cleavage patterns in the period 3-50 min.

The results are particularly interesting in view of the question of the role of actin binding protein in gelation. In the sarcoma 180 system enhancement of gelation occurs in concert with cleavage of actin binding protein, suggesting that this protein may be an inhibitor rather than an activator of

gelation. To pursue this question further we examined gelation in extracts of two other types of ascites cells. The 13762 MAT-B1 and MAT-C1 rat mammary adenocarcinoma ascites sublines were both derived from the solid 13762 mammary adenocarcinoma. However, they differ markedly in their cell surface characteristics and cytoskeletal organization (15).

These two sublines were tested for gelation and its enhancement by proteolysis. MAT-C1 extracts behaved essentially as did the sarcoma 180 (Table II); however, those of the MAT-B1 line failed to show gelation with trypsin treatment over the time invervals tested. Trypsinized and control samples from these lines and the sarcoma 180 were analyzed by gel electrophoresis. The gels were scanned and peaks for actin binding protein and myosin quantified from the areas under the curves. Extracts which gelled have no actin binding protein. In the case of the MAT-B1 extracts, which did not gel, the actin binding protein had not been substantially cleaved. At longer times some cleavage of actin binding protein was noted and a loose gel was formed. However, in no case was a solid gel formed, and some of the actin binding protein was always resistant to cleavage. Comparisons of electrophoretic gels of B1 and C1 extracts (treated and untreated) indicate that actin binding protein is the only readily discernible polypeptide which is cleaved in the C1 samples but not in the B1.

When equal volumes of MAT-B1 and MAT-C1 homogenates are mixed, gelation still occurs. This indicates that there is not a free inhibitor of gelation in the MAT-B1 extracts, and further suggests that any inhibitor present must already be bound to the site of inhibition in the MAT-B1 extracts.

Discussion: The results presented above clearly indicate that proteolysis can promote gelation of cytoplasmic extracts containing soluble proteins. They also shed light on the possible role of the high molecular weight actin binding protein in gelation. The correlation of gelation with cleavage of actin binding protein by proteolysis indicates that it is probably an inhibitor of gelation. This finding is in accord with recent results indicating that actin binding protein is not a necessary constituent of actin-containing gels (9),

TABLE II

Relationship of Proteolytic Cleavage of Actin Binding Protein and Myosin to Enhancement of Gelation of Cytoplasmic Extract of Sarcoma 180, MAT-B1 and MAT-C1 Ascites Cells

Cell	Time (min)	5 µg/ml Trypsin added	Gelation	Myosin	Actin Binding Protein
MAT-C1	6	-	-	4	10
	6	+	+	0	0
SA-180	4	-	-	3	2
	4	+	+	0	0
MAT-B1	2	-	-	6	8
	2	+	-	0	7
MAT-B1	4	-	-	5	9
	4	+	-	0	8
MAT-B1	10	-	-	5	6
	10	+	-	0	3

The values for the amounts of proteins are given as a percentage of the amount of the actin band stained on the acrylamide gels.

even though there is ample evidence of an association between actin and actin binding protein (4-6, 8, 9, 16). In addition the ability of proteolysis to overcome the temperature dependence of gelation is consistent with a role for actin binding protein, since its physical properties and its association with actin are temperature dependent (9, 16). Tilney (11) has suggested that high molecular weight proteins can inhibit actin polymerization in Thyone sperm and erythrocytes; however, it is uncertain whether he is dealing with proteins that are functionally similar to actin binding protein.

In view of the results presented above, we propose a model for the role of actin binding protein based on two possible modes of binding to actin. 1) Actin binding protein binds to free ends of the short actin filaments, which

act as nucleation sites for polymerization, to prevent addition of actin monomer. This interaction is equilibrium-controlled and is thus dependent on the concentrations of actin, actin binding protein and any substances which might alter their association (e.g., calcium, see ref. 9). Proteolytic cleavage of actin binding protein decreases this affinity for actin. An alternative possibility is that cleavage of actin binding protein might produce gelation activation factors such as those isolated by Maruta and Korn (10). It should be possible to distinguish between these alternative mechanisms using purified actin binding protein and its proteolysis products. In either case proteolysis could serve as a valuable control mechanism over actin polymerization.

2) Actin binding protein may also bind to central regions of actin filaments that are already formed to crosslink the filaments into a network forming a gel. The requirements for the two types of association of actin and actin binding protein would need to be different in order to exert a controlling effect on actin polymerization. In our model the initial proteolytic cleavage of actin binding protein would not decrease this second type of binding, but further cleavage would break linkages between the actin filaments. These requirements are based on the observations reported above in which more severe proteolytic treatments cause gel loosening without apparent cleavage of actin. This second type of binding might also explain results in which actin binding protein or filamin (17), an apparently similar protein, promotes gelation (4, 8) or filament bundle formation (18). It is not yet clear whether either of these two postulated binding mechanisms is important in the cell or how they might operate and be controlled. Further experimentation is needed with both purified actin and actin binding protein and with appropriate native systems to answer some of these intriguing questions.

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