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Gelsolin Activates DNase I *in Vitro* and in Cystic Fibrosis Sputum†

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ABSTRACT: Because actin can form a complex *in vitro* containing both gelsolin and DNase I, gelsolin and DNase I have been assumed to bind independently to actin. Although this assumption is consistent with the known crystalline structures of gelsolin with one actin and of actin with DNase I, which suggest that the binding sites on actin for both gelsolin and DNase I are distinct and separate, we propose that a second actin binding site on gelsolin competes with DNase I for actin. Since actin is an inhibitor of DNase I, competition at the second binding site results in activation of DNase I by gelsolin. Covalent cross-linking experiments confirm that DNase I prevents dimerization of actin by gelsolin, consistent with displacement of one actin from gelsolin by DNase I. Activation of DNase I by gelsolin is a novel function for a cytoskeletal protein and could have broad implications for biology, such as a role in initiating apoptosis. These results also may explain why both gelsolin and DNase I decrease sputum viscosity in cystic fibrosis (CF). While the activity of DNase I had originally been attributed to fragmentation of DNA, subsequent data suggested that both gelsolin and DNase I may affect viscosity by depolymerizing filamentous actin. The current results alternatively suggest that dissociation of the actin−DNase I complex by gelsolin in CF sputum results in activation of the nuclease activity of constitutive DNase I. The nuclease activity of DNase I alone is therefore sufficient to explain the effects of both gelsolin and DNase I on CF sputum.

The actin-filament severing protein gelsolin has repeatedly been identified as a tumor suppressor (*1*). Curiously, a mutant gelsolin that suppresses the tumorigenicity of c-Ha-*ras* oncogene-transformed NIH/3T3 cells has less severing activity than wild-type gelsolin, strongly suggesting that some activity other than actin-filament severing that accounts for tumor suppression (*2*). These results have provoked a search for alternative *in vivo* functions for gelsolin. Here we present data suggesting that one such potential function is the activation of DNase I. A model is described in which competition at one of the two actin binding sites on gelsolin results in dissociation of the DNase I−actin complex. Since DNase I is specifically inhibited by actin (*3*), DNase I is thereby activated. Our model is consistent with the isolation of gelsolin as a ternary complex with actin and DNase I (*4*, *5*), which has been previously interpreted to suggest that the interaction is noncompetitive (*6*). If, as we propose, competition occurs only at one of the two actin binding sites on gelsolin (*7*, *8*), then a ternary complex could result from DNase I that binds to the actin bound at the other site on gelsolin.

Our results have surprising implications for the biochemistry of CF[1] sputum. Aerosolized DNase I is used to reduce sputum viscosity in CF (*9*, *10*). The knowledge that DNase I has high affinity for monomeric actin and can depolymerize filamentous actin led to speculation that filamentous actin

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[1] Abbreviations: CF, cystic fibrosis; PBM, phenylene bismaleimide.

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contributes to sputum viscosity and that some or all of the clinical effects of DNase I could be explained by its actin-depolymerizing activity (*6*). Supporting this idea, the actin-filament severing protein gelsolin was also found to decrease CF sputum viscosity. However, contradicting this explanation, there was no correlation between the concentration of actin in sputum and the viscosity of CF sputum (*6*). More importantly, mutated DNase I with nuclease activity, but no actin binding activity, had greater ability than wild-type DNase I to decrease sputum viscosity (*11*). No satisfactory explanation for these conflicting observations has been previously provided. However, if gelsolin competes with DNase I for actin, gelsolin would augment the ability of DNase I to decrease CF sputum viscosity completely independent of gelsolin's effects on actin filament length.

EXPERIMENTAL PROCEDURES

In Vitro Measurement of DNase I Activity. The concentration of recombinant human plasma gelsolin (*12*) was determined by amino acid analysis. Beef pancreatic DNase I (molecular biology grade from Worthington Enzymes) was incubated with rabbit skeletal muscle actin and/or gelsolin for 8 min at room temperature before adding 100 μg/mL DNA (for 0.7% agarose gels) or 22 μg/mL DNA with ethidium bromide in a molar ratio of 1:34, ethidium bromide to base pairs (for fluorescence assay). The reaction mixture was in a buffer containing 21 mM NaCl, 0.1 mM $CaCl_2$, 2.0 mM $MgCl_2$, 0.1 mM ATP, 0.1 mM DTT, 0.1% sodium azide, and 5 mM Tris, pH 7.9. The rate of change in fluorescence intensity was measured in a photon-counting fluorimeter with excitation, 519 nm, and emission, 587 nm. A standard curve with fixed DNase I and variable actin concentration showed a linear relationship between dF/dt and free DNase I when the K_a between actin and DNase I was assumed to be 1×10^{10} M^{-1}, consistent with previous results (*13*). For DNase I and no actin, dF/dt was 21.0 arbitrary units/s. The theoretical curve was calculated by arbitrary variation of equilibrium constants using Chemical Kinetics Simulator software (IBM Almaden Research Ctr.).

Covalent Cross-Linking. Prior to cross-linking, actin and all other protein components were extensively dialyzed against 0.1 mM ATP and 2.5 mM imidazole, pH 7.4. Actin, gelsolin, and DNase I were mixed as indicated and, after the addition of 2 mM $MgCl_2$, were cross-linked with an equal volume of PBM[1] diluted in 20 mM sodium borate. The molar concentration of PBM equaled one-half that of actin. After 10 min at room temperature, the reaction was quenched. The efficiency of cross-linking has been previously suggested to be low, so that while the amount of cross-linked dimer seen on electrophoretic gels is proportional to the amount of dimer present in solution, the absolute amount of cross-linked dimer relative to monomer observed on the gels is not a meaningful quantity (*14*).

DNase I Activity in CF Sputum. Sputum obtained from a patient with CF, at routine follow-up and not receiving aerosolized DNase I, was collected on ice in a 1:1 volume of 10 mM $CaCl_2$, 0.5 mM PMSF, and 50 mM Imidazole, pH 6.9. After adding 0.3 mM diisopropyl fluorophosphate, the sample was divided with a razor blade into equal aliquots of 100 ± 10 μL. Zymograms were performed as previously described (*15*). Alternatively, gelsolin or DNase I was added as indicated along with 5 mM $MgCl_2$. The samples were gently agitated for 16 min at room temperature prior to quenching with 2.5 mM EDTA. Samples were either run on an agarose gel or DNA from the sample was extracted with phenol/chloroform/isoamyl alcohol, treated with calf intestinal phosphatase, and labeled at the 5′ ends using ATPγ-^{32}P and bacteriophage T4 polynucleotide kinase (*16*). After two cycles of ethanol precipitation, the number of labeled ends were quantified with a liquid scintillation counter.

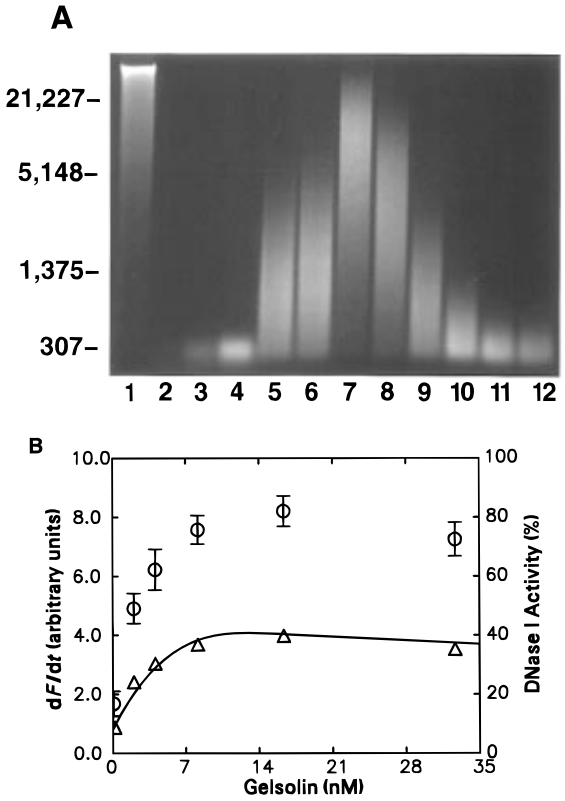

FIGURE 1: Activation of DNase I−actin complex *in vitro* by gelsolin. (A) Agarose gel of bovine double-stranded DNA stained with ethidium bromide (lane 1). DNA treated with 30 nM DNase I and actin (0, 7, 13, 18, 23, and 28 nM respectively) (lanes 2−7) or with 30 nM DNase I, 28 nM actin and gelsolin (5, 10, 19, 38, and 76 nM, respectively) (lanes 8−12). Saturating amounts of gelsolin are equivalent to 13−18 nM actin (compare lane 4 with lanes 11 and 12). Molecular size is indicated on the left in base pairs. (B) Rate of change in fluorescence of ethidium bromide-labeled DNA (dF/dt) in the presence of 12.5 nM DNase I, 12.5 nM actin, and variable amounts of gelsolin (circles). The error bars represent $\pm 2\sigma$. On the right axis, the fraction of total DNase I that is active is plotted as a function of gelsolin concentration (triangles). The solid line is the theoretical fraction of activated DNase I assuming the model and equilibrium constants described in the text.

RESULTS

Effect of Gelsolin on DNase I Activity as Assessed by Electrophoretic and Fluorescence Techniques. DNase I was added to samples of bovine double-stranded DNA in the presence of actin and/or gelsolin, and the samples were electrophoresed on an agarose gel. The gel was stained with ethidium bromide, thus showing the relative size of the remaining fragments of DNA (Figure 1A). DNase I was inhibited when bound to actin as previously reported (*3*), and this inhibition was partially reversed when gelsolin was added to the reaction mixture. At the highest concentrations of gelsolin used, actin inhibition of DNase I activity was reduced by about 50%. Four repetitions of this experiment

gave similar qualitative results. The nuclease activity of DNase I was quantitatively evaluated by measuring the rate of change in fluorescence of ethidium bromide bound to DNA. Fragmentation of DNA by DNase I releases ethidium bromide, with a severalfold decrease in fluorescence intensity. The rate of decrease in fluorescence intensity has been previously shown to be proportional to the concentration of DNase I (*17*). In a series of experiments in which DNase I and actin concentrations were held constant, addition of gelsolin increased the rate of change in fluorescence, consistent with activation of DNase I (Figure 1B). Human recombinant DNase I (Genentech) gave identical results (data not shown). In higher concentrations of NaCl (150 mM), DNase I activity was decreased, but the effect of gelsolin was qualitatively similar (data not shown). The actin concentration was well below the critical concentration for actin polymerization in all experiments in Figure 1, ensuring that none of the differences in inhibition could be explained by differences in amounts of unpolymerized, or G-actin.

The data in Figure 1B fits a simple model of competitive binding of gelsolin and DNase I to actin. If gelsolin binds two actin molecules sequentially and cooperatively and DNase I binds actin to competition with gelsolin at the second site, but not the first, then with equimolar DNase I and actin, at most 50% of DNase I could be activated by optimal concentrations of gelsolin, with the actual maximal percentage dependent on the equilibrium association constants. This is consistent with the data in Figure 1B, right axis. Thus, in the plateau region of the curves shown in Figure 1B, the model predicts that most of the actin and nearly 50% of the DNase I is arranged in a complex of one gelsolin, two actin, and one DNase I molecules. Very high concentrations of gelsolin would be expected to provide an excess of noncompetitive binding sites and the activity of DNase I should drop off, depending on the extent of cooperativity of the interaction between gelsolin and actin. This drop off at high concentrations of gelsolin is seen to a modest degree in Figure 1B. The theoretical curve in Figure 1B is derived assuming this model with a K_a of 1×10^{10} M^{-1} for gelsolin and one actin (*18*, *19*) and 800 times higher affinity at the second binding site (*7*, *19*, *20*), and DNase I binds actin with K_a of 1×10^{10} M^{-1} (*13*). These equilibrium constants are within the range of previous reports. The model is consistent with the known crystalline structure of the N-terminus of gelsolin bound to a single actin (*21*), in which there is no reason to expect that DNase I would compete at this binding site based on the crystalline structure of DNase I−actin (*22*). The model is also consistent with information suggesting that the two actin binding sites on gelsolin are structurally dissimilar (*23*, *24*).

While the kinetics of interaction between gelsolin and two actin subunits may be biphasic, with a slow, calcium binding step in which calcium is intercalated between actin and gelsolin (*7*, *25*), we note that the results of the fluorescence experiments were independent of the time of incubation of actin with gelsolin and independent of the order of addition of DNase I and gelsolin. The ability of gelsolin to bind an additional divalent cation upon binding to actin could possibly affect DNase I activity. However, the effect would be expected to be inhibitory, rather than stimulatory. Also, the fluorescence experiments gave identical results in 1 mM $CaCl_2$ and 0.1 mM $CaCl_2$ (data not shown), making this an improbable explanation for the observed effect of gelsolin.

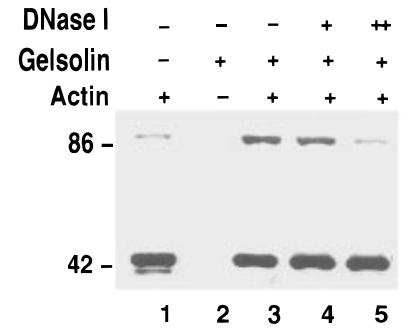

FIGURE 2: Immunoblot of cross-linked actin dimers: stimulation by gelsolin and inhibition by DNase I. Cross-linked actin (3.0 μM subunit concentration) yields a dimeric species with apparent molecular mass of 86 kDa, versus 42 kDa for the actin monomer (lane 1). Gelsolin (1.5 μM) treated with PBM in the absence of actin and DNase I doesn't react with the anti-actin antibody (lane 2), but gelsolin plus actin, at the same concentrations as in the previous lanes, significantly enhances the generation of cross-linked actin dimer under identical reaction conditions (lane 3). Adding DNase I (3.0 or 14 μM) to gelsolin and actin results in a lower yield of actin dimer (lanes 4 and 5, respectively).

Covalent Cross-linking Suggests That DNase I Disrupts Actin−Actin Interactions in the Complex of Two Actin Subunits and Gelsolin. The only structural information regarding the second gelsolin site that binds to actin comes from covalent cross-linking experiments using PBM, in which gelsolin has been shown to stimulate the formation of a cross-linked actin dimer (*26*). Other evidence suggests this dimer is formed by cross-linking two adjacent subunits in an antiparallel arrangement very close to their C-termini at cysteine-374 (*14*). Because gelsolin has nearly identical electrophoretic mobility to the actin dimer, the cross-linked actin dimer must be detected by immunoblot with an anti-actin antibody. We confirm that the complex of two actin and gelsolin is cross-linked by PBM only between the two actin subunits, resulting in a covalent dimer of apparent molecular mass of 86 kDa on SDS-Laemlli gels, but find that if DNase I is included in the reaction mixture, the amount of cross-linked actin dimer decreases (Figure 2). Because the interaction between gelsolin and actin is cooperative, relatively large amounts of DNase I are required to disrupt actin−actin interactions in the complex of gelsolin with two actin. In a control experiment not shown, large excesses of DNase I did not affect the extent of cross-linking of filamentous actin by PBM, confirming that the loss of cross-linking in Figure 2 is not simply related to depletion of PBM by DNase I. Gelsolin treated with PBM in the absence of actin and DNase I was employed as a control to verify that the antibody did not react with a maleimide derivative of gelsolin (Figure 2, lane 2).

DNase I could prevent cross-linking by preventing actin from binding to both sites on gelsolin, which is clearly not the case, since a ternary complex of gelsolin, actin, and DNase I is formed on DNase I-affinity matrix (*4*). DNase I could prevent cross-linking by steric interference with the cross-link site, but this is unlikely since DNase I binds to the opposite face of the molecule as that containing cysteine-374 (*22*). Alternatively, DNase I could interfere with the formation of a dimeric complex of actin by distorting potential actin−actin interactions to the extent that cross-linking is impossible. Competition at a single gelsolin actin binding site would be an example consistent with this final interpretation.

Activation of DNase I in CF Sputum. While no nuclease activity was present in untreated sputum, the zymogram technique detected actin-inhibitable DNase I activity in SDS-treated sputum from patients with CF. Zymograms (SDS-Laemmli-PAGE gels that contained DNA in the separating gel, incubated at room temperature overnight after rinsing to remove SDS, and stained with ethidium bromide) show nuclease activity only when incubated in the absence of actin (data not shown), consistent with the definition of DNase I (*27*). The technique is effective because actin, but not DNase I, is irreversibly denatured after SDS treatment (*15*). These results are as expected since actin is present in excess to DNase I in CF sputum (*6*).

When gelsolin was added to samples of CF sputum, the results were qualitatively similar to those seen in Figure 1A. Without added gelsolin, DNA molecules remained at the top of an agarose gel, consistent with a very large average size. With progressively more gelsolin, the DNA size shifted consistent with severing by DNase I. Typical results to those obtained with five different sputum samples are shown in Figure 3A. Once, in addition to the shift of DNA away from the top of the gel, we observed an ~200 base pair ladder pattern suggestive of internucleosomal fragmentation of DNA as seen in apoptotic cell death (*28*). Given the intrinsic heterogeneity of the collected samples, we speculate that this one sample may simply reflect better preservation of DNA chromatin structure at the time of sputum collection.

There are alternative explanations for the results in Figure 3A. A scaffold of F-actin and DNA could hinder migration in an agarose gel in the absence of the actin-severing activity of gelsolin. Treatment of CF sputum with sodium dodecyl sulfate, however, did not alter the electrophoretic mobility of DNA (data not shown). Further evidence of DNase I activity was provided by purifying DNA from gelsolin-treated sputum and labeling the 5′ ends that result from either single-stranded or double-stranded cuts by DNase I with bacteriophage T4 polynucleotide kinase and ^{32}P. Incorporation of radiolabeled phosphate was enhanced when samples were treated with either gelsolin or DNase I, consistent with the proposed similar molecular effects of the two proteins (Figure 3B).

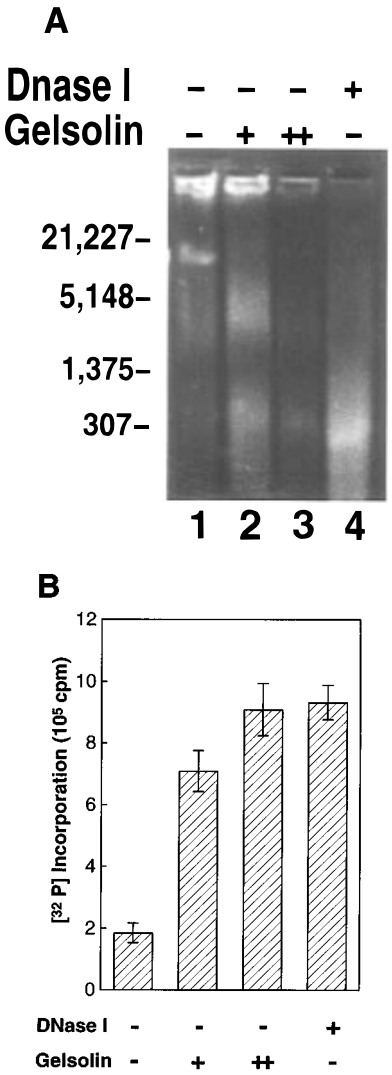

FIGURE 3: Gelsolin activates DNase I in CF sputum. (A) Sputum samples after incubation with gelsolin and/or DNase I were run on an agarose gel as in Figure 1A. Samples in lanes 1−3 have 0, 15, or 80 nM gelsolin, respectively, and in lane 4, 15 nM DNase I. (B) DNA was purified from samples prepared as in panel A and then labeled at the 5′ ends with ATPγ-^{32}P. The four bars in the diagram correspond to the samples in panel A, lanes 1−4, respectively (error bars represent $\pm\sigma$).

DISCUSSION

In retrospect, the results reported here might have been anticipated from the facts that DNase I causes a rapid depolymerization of filamentous actin from the pointed end (*29*), and the complex of gelsolin and two actins promotes actin filament polymerization at a rate consistent with pointed-end growth (*30*). As the gelsolin−actin complex presumably resembles the pointed end of an actin filament, it is not surprising that DNase I can cause dissociation of an actin monomer from the gelsolin−two actin complex, i.e., DNase I and at least one gelsolin site must bind actin competitively. As at the pointed end of the actin filament, the unavailable DNase I binding site on actin could be at the gelsolin−actin interface, the actin−actin interface, or sterically blocked by the other, bound DNase I molecule.

Our results explain why the effects of gelsolin and DNase I on CF sputum viscosity are additive (*6*). If binding of DNase I to actin was not hindered by gelsolin, then gelsolin−actin complexes would inhibit the nuclease activity of DNase I, just as does actin alone. Our results also explain why gc-globulin, an actin-monomer binding protein, did not lower viscosity when added by itself to sputum (*6*). Gc-globulin forms a ternary, noncompetitive complex with DNase I and actin and therefore would not be expected to dissociate the DNase I−actin complex (*31*). Finally, our results could be of clinical significance. Vasconcellos et al. (*6*) noted that gelsolin was more effective than DNase I in decreasing sputum viscosity. Free DNase I is a labile molecule (*32*), and it is likely stabilized, although inactive, when in a complex with actin (*3*). Since DNase I must diffuse into sputum (a very slow process) without degradation in order to be therapeutic, it may be more beneficial to use gelsolin to activate DNase I−actin complex (either constitutive or supplied exogenously) than to directly add active, free DNase I.

This is the first report of competition between DNase I and any other protein for actin. Activation of DNase I by gelsolin may be important *in vivo*. Given the cytoplasmic localization of DNase I (*33*), under appropriate circumstances, DNase I may interact directly with cytoplasmic gelsolin; alternatively, the previously unexplainable nuclear

localization of gelsolin-related proteins (*34*) may be responsible for *in vivo* activation of DNase I. Other data support this hypothesis. For example, DNase I is inactive in an *in vivo* assay for apoptosis unless preincubated with serum or cell extracts (*33*). Gelsolin may be responsible for activation of DNase I under these circumstances. Although an abundance of theories have been posulated regarding the mechanism of activation of DNase I in apoptosis, including the cleavage of actin by interleukin 1β-converting enzyme (*35*), an increase in the concentration of free gelsolin is an appealing alternative because it effectively links the observed cell-shape changes of apoptosis (via actin-filament severing) to DNase I activation. Additionally, speculation that gelsolin could lower the threshold for apoptosis is consistent with the abundant evidence that gelsolin functions as a tumor suppressor (*1*, *36*).

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