pers. The center wells have filter paper strips moistened with methylbenzethonium hydroxide to trap $^{14}CO_2$. Homogenate was added to start the incubation for 1 hour at 37°C, and the reaction was terminated by the addition of 40% trichloroacetic acid. After further incubation for 20 min at 37°C, the filter paper strips were transferred to scintillation vials and the radioactivity determined by liquid scintillation counting. Commercially available *Escherichia coli* enzyme (Sigma) was used to verify the assay conditions.

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Reduction in Viscosity of Cystic Fibrosis Sputum in Vitro by Gelsolin

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Obstruction of airways by viscous sputum causes lung damage in patients with cystic fibrosis (CF). Sputum samples from CF patients were shown to contain filamentous actin. Human plasma gelsolin, a protein that severs actin filaments, rapidly decreased the viscosity of CF sputum samples in vitro. Gc globulin and deoxyribonuclease I, proteins that sequester monomeric actin but do not sever actin filaments, were less efficient than gelsolin in diminishing sputum viscosity. These results suggest that gelsolin may have therapeutic potential as a mucolytic agent in CF patients.

Patients with CF accumulate thick secretions in their airways that cause progressive pulmonary destruction (*1*). CF sputum is a complex material, but a major cause of its thick consistency is pus, derived from masses of degenerating leukocytes (*2*). The ability of bovine pancreatic deoxyribonuclease I (DNase I) and, more recently, human recombinant DNase I to liquify CF sputum has been ascribed to enzymatic degradation of leukocyte-derived DNA (*3*). We considered the possibility that filamentous actin might also contribute to the thickness of CF sputum. Actin comprises 10% of total leukocyte protein (*4*) and forms long protease-resistant (*5*) filaments that are highly viscoelastic (*6*). Furthermore, DNase I is known to bind monomeric actin and to slowly depolymerize actin filaments (*7*), which suggests that some of its reported mucolytic effect might be due to actin disaggregation rather than to DNA hydrolysis.

We documented the presence of actin in CF sputum (*8*) by three independent methods. First, we did an immunoblot analysis of denatured CF sputum and identified a polypeptide that was similar in size to actin and that reacted with an actin-specific antibody (Fig. 1, inset) (*9*). Second, we showed that CF sputum eliminated the typical time delay in actin polymerization that has been attributed to cation-induced nucleation of monomeric actin (Fig. 1) (*10*). Nucleation of actin polymerization is a property of the ends of preformed actin filaments, and the increased polymerization rate may be due to the introduction of filaments from the sputum (*10*). Third, we showed that phalloidin, a fungal peptide that specifically ligates filamentous actin, bound to CF sputum.

Analysis of 10 CF sputum samples revealed that the concentration of polymeric actin (*11*) varied widely among samples (ranging from 0.1 to 5 mg/ml) and did not appear to correlate with sample viscosity (Fig. 1B). This is perhaps not surprising, however, as the viscosity of filamentous actin, like that of other linear polymers, depends on the shear rate, the polymer concentration, the orientation of the filaments, and, most importantly, the average filament length. The viscosity of purified filamentous actin, for example, varies with the fifth power of the average filament length (*6*). We reasoned that if filamentous actin contributes substantially to the viscosity of CF sputum, then reducing actin filament length should markedly diminish sputum viscosity.

To investigate the effect of actin filament length on viscosity, we added gelsolin, a protein that rapidly severs noncovalent bonds between monomers within a filament (*12*), to CF sputum samples and then monitored their rheological properties. Prior to gelsolin treatment, the viscosity of 32 CF sputum samples was 322 ± 199 Pa-s (mean ± SD) (*13*). At concentrations of 100 to 500 nM, gelsolin, purified from human plasma (*14*), rapidly diminished the viscosity of these sputum samples by an average of 62 ± 44% (mean ± SD), irrespective of the initial viscosity of the untreated sputum. By contrast, at a concentration of 250 nM, bovine pancreatic DNase I and another actin monomer–binding protein, Gc globulin (a vita-

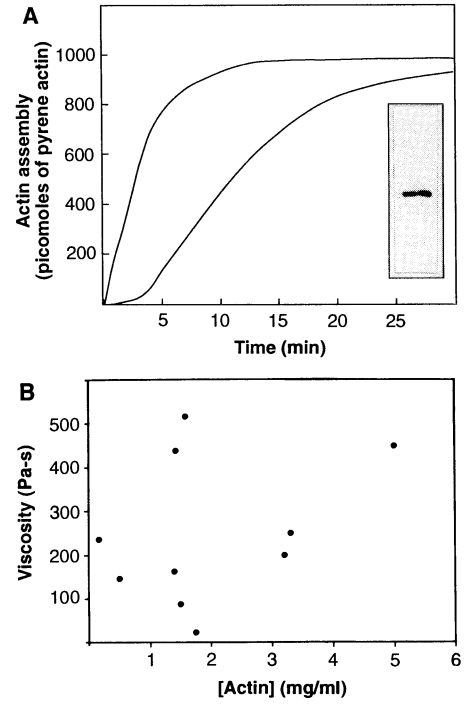

Fig. 1. (**A**) Identification of actin in CF sputum. The graph shows the increase in fluorescence of pyrene actin as the actin polymerized in the absence (lower curve) or presence (upper curve) of extracts of CF sputum. The inset shows an immunoblot of proteins detected in the sputum of two different CF patients by an antibody to actin. (**B**) Analysis of actin concentration and viscosity of 10 CF sputum samples. The concentration of polymeric actin was estimated by adding rhodamine-labeled phalloidin to the sputum samples and measuring specifically bound fluorescence (*11*). The viscosity of the samples was derived from their flow rate in response to a constant stress (*13*).

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min D–binding protein) (*15*), had no effect on the viscosity of CF sputum over the same time interval, although both of these proteins enhanced gelsolin's activity (Fig. 2A). A dose-response curve showed that gelsolin was effective in reducing sputum viscosity at much lower concentrations than DNase I (Fig. 2B).

CF sputum is a viscoelastic material capable of recovering its shape after deformation, and the rate of this recovery permits calculation of its elastic shear modulus (6). The shear modulus of sputum samples from five different patients was 21.0 ± 6.7 Pa (mean ± SD) (*16*). At a concentration of 200 nM, gelsolin reduced the shear modulus of the samples by 58 ± 10% (mean ± SD). The dependence of viscosity on filament length is especially manifest when the filaments are imbedded in other solids, as in CF sputum, where actin filaments would be mixed with mucopolysaccharides and other polymers. For example, actin filaments have been shown to strongly increase the shear modulus of fibrin clots, and low gelsolin concentrations diminish the viscosity of fibrin clots that contain interpenetrated actin filaments (*17*).

High-affinity actin monomer–binding proteins, such as DNase I and Gc globulin, can also shorten actin filaments by preventing monomers from adding back onto filaments after they dissociate. Compared to severing by gelsolin, however, this mechanism of filament shortening is kinetically and stoichiometrically inefficient, because it depends on the intrinsic rates at which monomers dissociate from actin filament ends (0.2 to 2 s^{-1}), and it requires a comparatively large amount of an actin monomer–binding protein to substantially reduce filament length. For example, a single gelsolin molecule can shear in half a 1-μm-long actin filament [containing 370 subunits (*18*)] in less than 1 s, whereas 185 DNase I molecules working for 90 s or longer would be required to achieve the same effect.

Although DNase I has been reported to degrade DNA in CF sputum (*3*), our findings raise the possibility that it may also reduce viscosity through its action as an actin-binding protein. Purified actin inhibits the DNA-hydrolyzing activity of DNase I (*7*); the actin in CF sputum may have the same effect. If so, then gelsolin and other actin-binding proteins could enhance DNase I activity by facilitating clearance of actin. We found that both DNase I and Gc globulin had an additive effect with gelsolin in lowering the viscosity of CF sputum. This synergy may arise in part because gelsolin produces more filament ends from which actin monomers can dissociate and then bind to the actin monomer–binding proteins. The slightly greater additive effect of Gc globulin may reflect its ability to protect gelsolin's actin-severing function from inactivation by binding actin monomers. Because actin can simultaneously bind gelsolin and DNase I, DNase I would not have this protective effect (*19*).

Plasma gelsolin is a normal constituent of extracellular fluids and in concert with Gc globulin is thought to scavenge actin released from cells during inflammation and injury. Because inflamed airways contain plasma proteins, it is likely that Gc globulin and plasma gelsolin are naturally present in the airways of CF patients, but perhaps in insufficient quantities to shorten actin filaments released from the prodigious accumulation of pus. Our findings suggest that gelsolin merits further investigation as a potential mucolytic agent in CF patients.

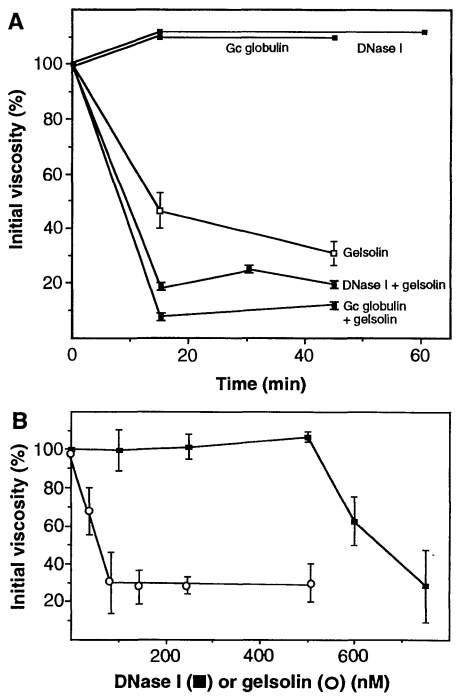

Fig. 2. (**A**) Effect of 250 nM gelsolin, purified from human plasma (*14*); of proteinase-free DNase I (Worthington Biochemical, Freehold, New Jersey); of Gc globulin (Calbiochem, La Jolla, California); and of combinations of these reagents on the viscosity of CF sputum as a function of time. (**B**) Effect of gelsolin and DNase I concentrations on the viscosity of CF sputum after a 60-min incubation. A series of measurements at each time point (A) or concentration of additive (B) was made in duplicate on sputum samples from a single CF patient. The experiments were repeated three to five times with samples from different CF patients. The volume of reagents added was less than 6% of the total sample volume. The values represent means ± SD.

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Kinetics of Molecular Chaperone Action

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Molecular chaperones of the Hsp70 type transiently sequester unfolded segments of proteins and promote their correct folding. Target peptides were labeled with an environmentally sensitive fluorophore so that their binding to the molecular chaperone DnaK of *Escherichia coli* could be followed in real time. The two-step process was characterized by relaxation times of 27 seconds and 200 seconds with 2 μM DnaK and 0.1 μM ligand at 25°C. In the presence of adenosine triphosphate, the formation of the complex was greatly accelerated and appeared to be a single-exponential process with a relaxation time of 0.4 second. The binding-release cycle of DnaK thus occurs in the time range of polypeptide chain elongation and folding and is too fast to be stoichiometrically coupled to the adenosine triphosphatase activity of the chaperone (turnover number, 0.13 per minute at 30°C).

The molecular chaperone DnaK of *Escherichia coli* belongs to the 70-kD heat shock protein (Hsp70) family (*1*, *2*). These proteins were first identified as part of a set of specific proteins that are induced during the cellular response to heat shock. Most of them are constitutively expressed and are essential for cell viability under normal growth conditions. Constitutive Hsp70s are thought to interact transiently with hydrophobic segments of newly synthesized proteins and thus to assist in folding and translocation of the proteins through membranes. DnaK, like its eukaryotic homologs, consists of an evolutionarily variable COOH-terminal peptide recognition domain and a conserved NH_2-terminal adenosine triphosphatase (ATPase) domain (*3*), which has been reported to trigger the release of a bound ligand upon adenosine triphosphate (ATP) hydrolysis (*4*, *5*). The rate of binding of a protein or peptide ligand has not been determined to date, because no kinetic assay for chaperone action has been available. We used peptide ligands labeled with an environmentally sensitive fluorophore, together with a stopped-flow spectrofluorimeter, to determine the kinetics of peptide binding to DnaK and the effects of ATP on peptide binding.

The targeting sequence of the precursor of mitochondrial aspartate aminotransferase is tightly bound by DnaK (*6*). Here, a synthetic 21-residue prepiece was used as a ligand for DnaK (Fig. 1). It was synthesized with an additional cysteine at the NH_2-terminus for the covalent attachment of the fluorescent probe acrylodan (a dimethylaminonaphthalene fluorophore with an acryl group) (*7*). The binding site of BiP, the Hsp70 in the endoplasmic reticulum, accommodates a peptide of seven amino acid residues. It selects for aliphatic residues and excludes charged residues at all positions with the exception of position 7, where positively charged residues are slightly preferred (*8*). If DnaK has a similar binding specificity, we expect that the prepiece contains two preferential sites for binding to DnaK: residues 1 to 7 and 8 to 14 (Fig. 1). On binding to DnaK, the fluorescence spectra of the acrylodan-labeled prepiece (a-pp) and the shorter acrylodan-labeled peptide a-p1 (corresponding to residues 1 to 7 of a-pp) underwent a blue shift; the emission maximum shifted from 525 to 505 nm. Binding to DnaK increased the fluorescence intensity at 500 nm to 185% (a-pp) and 300% (a-p1) of that of the unbound ligand (Fig. 1). The changes in fluorescence arise from an increased hydrophobicity of the microenvironment of the label (*9*). The dissociation constants (K_d), as determined from the hyperbolic binding curves (*10*) obtained by titration of a constant concentration of ligand (50 nM) with increasing concentrations of DnaK, were 63 nM for a-pp and 1.4 μM for a-p1. For the experiments below, a-pp, which bound more tightly, was used as the ligand if not stated otherwise.

Complex formation followed double-exponential kinetics. At 0.1 μM a-pp and 2 μM DnaK, the reaction equilibrated with τ_1 = 27 s and τ_2 = 200 s (Fig. 2). The pseudo–first-order rate constant (k_{obs}) of the first phase of the reaction was linearly proportional to DnaK concentration and corresponded to a bimolecular reaction with a second-order binding rate constant (k_{+1}) of 9400 M^{-1} s^{-1} and a dissociation rate constant (k_{-1}) of 0.004 s^{-1}. The k_{obs} value of the second, slower phase of complex formation increased on raising the concentration of DnaK, which suggested that the formation of an encounter complex might be followed by its isomerization.

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and the mixtures were incubated for 30 min on ice in the dark. Rhodamine-labeled phalloidin (10 μM; Sigma) was then added to all samples, and the mixtures were incubated for 1 hour on ice in the dark. The samples were washed three times with buffer A and extracted overnight at 4°C with 0.1 N NaOH. An excitation wavelength of 550 nm and an emission wavelength of 580 nm were used to measure the fluorescence of rhodamine-phalloidin. The fluorescence of the extract that had been treated first with unlabeled phalloidin was subtracted from the fluorescence of the sample not treated with unlabeled phalloidin, and the difference was ascribed to filamentous actin. The rhodamine-phalloidin concentrations in the extracts were calculated from a standard curve generated by exposing phalloidin at a range of concentrations to the same treatment as the sputum samples.

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