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Conformational Change Precedes the Formation of Multimeric Fibronectin

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Plasma fibronectin incubated with a low concentration of SH reagent under physiological conditions without cells formed a multimer which retained the ability of heparin-binding and cell-binding but lost gelatin affinity [Sakai, K., Fujii, T., and Hayashi, T. (1994) *J. Biochem.* 115, 415-421]. The conformation of the multimeric fibronectin, as observed by ultraviolet circular dichroism and fluorescence spectroscopy was different from that of dimeric plasma fibronectin. Monitoring the change in ellipticity indicated that conformational change was mostly accomplished within the first 3 h of incubation with 0.5 mM dithiothreitol at 37°C. In contrast, multimers became detectable after 4 h of incubation. The results indicate that the overall reaction of multimerization of plasma fibronectin consists of two steps: the initial step of conformational change of dimeric fibronectin, and the later polymerization step of the polypeptide in an altered conformation. The initial step, involving the conformational change of fibronectin, depended on temperature: it proceeded at 37°C but not at 25°C. In contrast the second step took place at 25°C at a low, yet significant rate. Proteolytic susceptibility of the fibronectin to thermolysin or *m*-calpain changed within 3 h of incubation with dithiothreitol at 37°C in accordance with the conformational change detected by circular dichroism. Namely, the fibronectin in an altered conformation appeared to be less susceptible to thermolysin, but more susceptible to *m*-calpain. The changes in enzymatic susceptibilities tended to be localized in the amino- and carboxyl-terminal regions, which are consistent with the implications from the spectroscopic analysis.

Key words: conformation, disulfide-bond, fibronectin, multimerization.

Two forms of fibronectin (FN) are found in organisms: plasma FN in a soluble dimeric form is found in blood and body fluids, and cellular FN in an insoluble multimeric form with disulfide bonds is found in connective tissues (*2-4*). Plasma FN diffuses from blood or body fluids into connective tissues, where it is incorporated into fibrillar aggregates as multimeric FN (*5*). It is generally accepted that the multimer formation resulting in exchange of interchain disulfide bonds requires the presence of cells under physiological conditions at 37°C (*6-8*). The cell-mediated aggregation of FN appeared to involve intermolecular interactions of either homotypic domains or heterotypic domains (*9, 10*). The heterotypic interactions involved the binding of the amino-terminal region of one FN molecule to the carboxyl-terminal heparin-binding domain of an adjacent FN molecule (*11-13*). On the other hand, FN multimerization without living cells was induced with a high salt concentration by weakening the electrostatic interactions that maintain the compact structure of FN, and thus converting the structure to an extended form. The change of the overall structure of FN was reflected in the conformational change detected by spectroscopic methods such as circular dichroism (CD), fluorescence (*14-17*), and magnetic resonance (*18, 19*).

In connective tissue, FN expresses functions such cell adherence, cell migration, or cell differentiation in a multimeric form. Until now, it has been thought that a physiologically functional multimeric FN was formed only by mediation of cells. Since FN is a multifunctional protein, it is important to study the relation of structure to the regulation of the function expressed by multimeric FN. We have recently reported (*1*) that purified plasma FN formed a disulfide-bonded multimer without cells under simple but physiologically possible conditions in the presence of a low concentration of SH reagent such as glutathione. The concentration of the reduced form of glutathione could increase to the mM level upon lysis of red cells (*20*). The multimeric FN formed by the SH reagent retains the heparin-binding ability and the cell-attachment activity but did not show gelatin binding (*1*). The experimental system we reported previously for the FN multimerization was simple, without requiring living cells, consisting of the incubation of plasma FN with dithiothreitol (DTT) in Tris-buffered saline (TBS) at 37°C. Thus allowed us to examine the timing of the conformational change of FN spectroscopically during the process of multimerization.

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Abbreviations: CD, circular dichroism; DTT, dithiothreitol; FN, fibronectin; TBS, Tris-buffered saline.

MATERIALS AND METHODS

Materials—Plasma FN was purified from bovine serum as described previously (*1*). DTT was from Nacalai Tesque (Kyoto). TPCK-treated trypsin and thermolysin were from Sigma (St. Louis, USA). *m*-Calpain was a kind gift from Dr. Kawashima (The Tokyo Metropolitan Institute of Medical Science). Multimeric FN was prepared by incubation with DTT from bovine plasma FN as described before (*1*).

Protease Treatments—Protease treatment was performed at 37°C at an FN concentration of 0.3 mg/ml in TBS containing 10 mM $CaCl_2$, with trypsin (2 μg/ml), thermolysin (0.2 μg/ml), or *m*-calpain (2.4 U/ml). The enzymatic reactions were terminated by adding SDS-buffer supplemented with 20 mM EDTA, followed by the analysis on SDS-PAGE.

SDS-PAGE—SDS-PAGE was performed on 5 or 10% polyacrylamide gel with a stacking gel of 2.5% in a discontinuous buffer system (*21*). Proteins were stained with Coomassie Brilliant Blue R-250.

Fluorescence Measurements—Fluorescence intensities and spectra were obtained with a Jasco FP770 Spectrofluorometer. The excitation wavelength was 280 nm. The emission spectrum was recorded from 287 to 500 nm at 37°C.

CD Measurements—A CD spectrum of the FN sample was obtained at 37 or 25°C with a Jasco J600 Spectropolarimeter in a jacketed-cylindrical cell with 5 or 10 mm light path length.

RESULTS

CD Spectra of Dimeric, Multimeric, and Heat-Treated FNs—The CD spectrum of multimeric FN was distinguishable from that of dimeric FN (Fig. 1): the peak at 227 nm disappeared and the trough at 215 nm became deeper upon multimerization. The CD spectrum of FN that had been heat-treated at 70°C for 15 min did not show the positive maximum at 227 nm. However, the multimer showed a distinct difference: ellipticity at 227 nm was higher in the multimer, while the trough at 215 nm was deeper with the minimum shifted to a slightly shorter wavelength in heat-treated FN. The CD spectrum of the dimeric FN incubated at 37°C without DTT was the same as that of the dimeric FN without incubation, suggesting that reduction of S-S cross-links is essential for the conformational change.

Effects of Temperature and DTT Concentration on Multimer Formation—Multimer formation from plasma FN depended on the DTT concentration at 37 or 25°C as shown in Fig. 2. Incubation at 37°C for 18 h in the presence of 0.1 mM DTT converted dimeric FN to the multimers that were detected at the top of the running gel and bottom of the stacking well. At 0.5 mM DTT, the dimeric band disappeared completely. On the other hand, on incubation at 25°C at 0.5 mM DTT, more than half of the dimeric FN remained. At 5.0 mM DTT, the dimeric FN band decreased to less than 20% of the total protein. The results indicated that the multimer formation was accelerated synergistically by increases of temperature and DTT concentration. A 10-fold concentration of DTT was required at 25°C for the multimerization of FN.

Change in the CD Spectra of FN during DTT Treatment—CD spectra of plasma FN in the ultraviolet region of 200-250 nm are shown for different incubation times with DTT in Fig. 3. The initial CD spectrum of plasma FN showed a maximum at 227 nm and a minimum at 215 nm. The positive peak at 227 nm gradually decreased with the incubation time from 0.5 to 3 h at 37°C in the presence of 0.5 mM DTT (Fig. 3). By 5 h the change had leveled off and the CD spectrum became essentially the same as that of multimers (Fig. 1). The trough at 213 nm also became deeper with incubation time from 0.5 h and the change leveled off by 5 h. On the other hand, the multimer became detectable on SDS-PAGE only after 4 h of incubation (*1*). The results suggest that the multimerization was preceded by a conformational change of FN. The decrease in ellipticity at 227 nm was slow at 25°C (Fig. 3, insert), where multimer formation took place more slowly.

Change in Fluorescence of FN by Treatment with DTT—The emission peak of plasma FN was at 325 nm when excitation was at 280 nm. The fluorescence intensity increased during the incubation at 37°C in the presence of 0.5 mM DTT from 0 to 4 h. The increase of intensity is presumably due to a conformational change in the region of FN that is responsible for the decrease in the ellipticity at

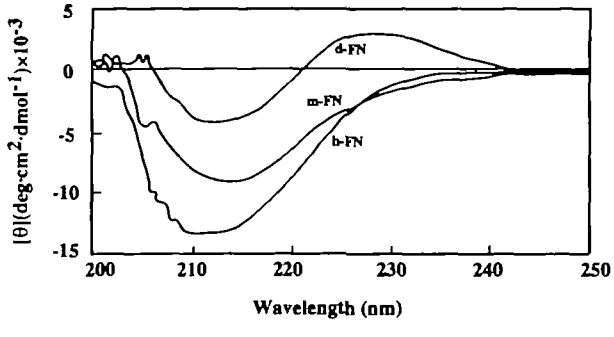

Fig. 1. **Far-ultraviolet CD spectra of dimeric FN (d-FN), multimeric FN (m-FN), and heat treated FN (h-FN).** FN treated with 1 mM DTT at 37°C for 18 h was dialyzed against TBS and used as m-FN. The m-FN did not contain d-FN detected by SDS-PAGE under non-reduced conditions. All FNs used the experiment were incubated at 37°C before measuring the CD spectra.

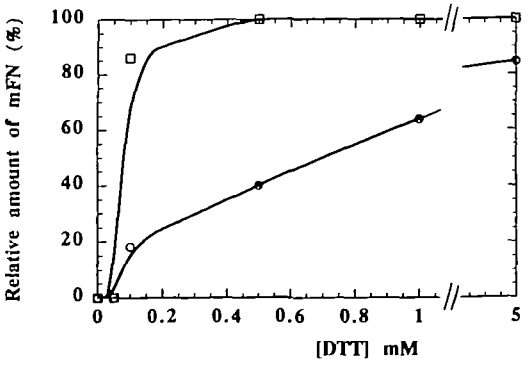

Fig. 2. **The effect of temperature on multimerization of plasma FN induced with DTT.** Plasma FN was incubated at 37 or 25°C in TBS containing DTT at the concentrations indicated for 18 h. Reaction products after dialyzed were analyzed by SDS-PAGE without reduction. The relative amount of m-FN (the band at sample well bottom and at the running gel top) found at 37°C (□) or 25°C (○) was obtained by densitometric quantification.

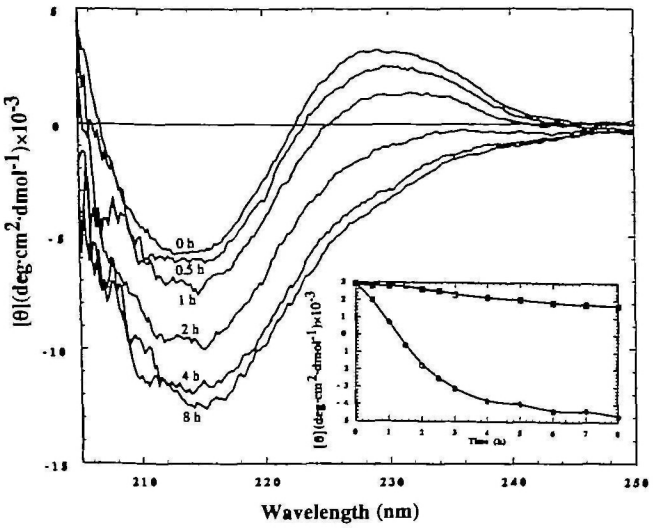

Fig. 3 **Far-ultraviolet CD spectra of plasma FN during incubation with 0.5 mM DTT in TBS.** Indicated numbers are the incubation time in hours after adding DTT. Inserts show the change of ellipticity at 227 nm during incubation at 37°C (○) or 25°C (□)

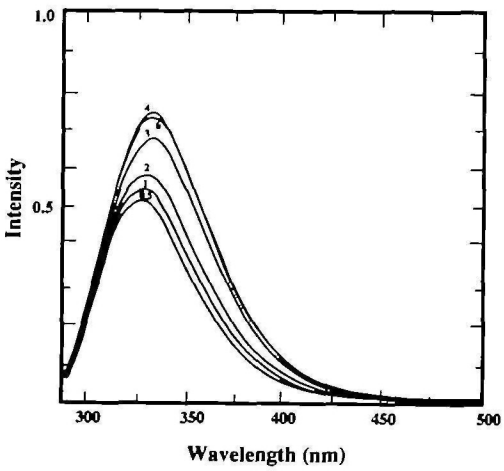

Fig. 4. **Intrinsic tryptophan fluorescence derived from plasma FNs incubated with 1 mM DTT.** Indicated numbers are the incubation time in hours at 37°C after adding DTT.

227 nm (Fig. 4). The emission maximum wavelength shifted from 325 to 335 nm between 1 and 6 h of incubation suggesting that the tryptophan residue became partially exposed to the polar environment (*22*).

Susceptibility of FN Incubated with DTT to Proteolytic Enzymes—FN that had been incubated with DTT for 3 h at 37°C was treated with trypsin, thermolysin, or *m*-calpain for 10 min, followed by analysis on SDS-PAGE (Fig. 5). Trypsin and thermolysin have been used for fragmentation of human plasma FN into functional domains (*23-25*), while *m*-calpain can be used as a probe for the conformational analysis of protein (*26*). *m*-Calpain fragmented the DTT-treated FN into smaller sizes (180 and 150 kDa) than it did the FN untreated (200 kDa), suggesting that new cleavage sites may be exposed and become susceptible to *m*-calpain through conformational change (Fig. 5). On the contrary, DTT-treated FN was cleaved by thermolysin into larger fragments (190 and 200 kDa) than the untreated FN (145 and 155 kDa). The proteolytic fragments of 145 and 155 kDa obtained with thermolysin seemed to be derived from the region containing the cell-binding domain of FN chains (*28*). Banding patterns of thermolysin fragments from DTT-treated FN could be interpreted as indicating that either the gelatin-binding region or the heparin-bind-

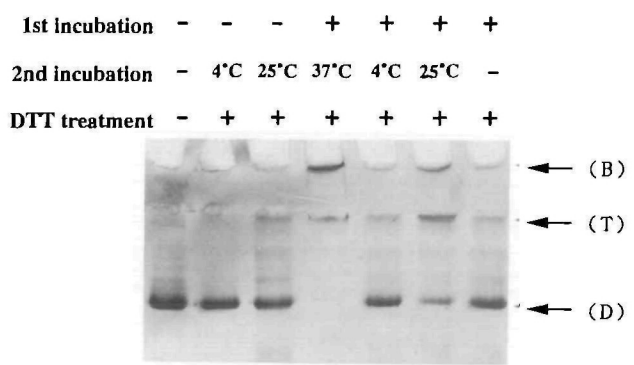

Fig 6. **Effect of changes of temperature on multimerization.** Plasma FN was incubated in TBS with 0.1 mM DTT at 37°C for 4 h (first incubation), then at 37, 25, or 4°C for 18 h (second incubation). The second incubation temperature is indicated above each lane. Arrows show the positions of sample well bottom (B), gel top (T), and dimeric FN band (D). The left end lane is untreated plasma FN for reference.

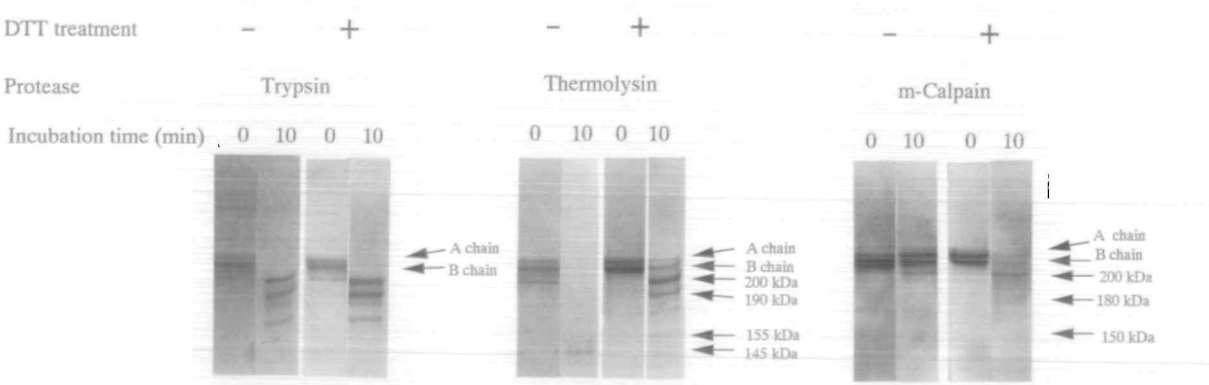

Fig 5. **The effect of protease treatment of plasma FN after incubation with 1 mM DTT for 4 h at 37°C.** DTT-treated plasma FN was incubated with trypsin, thermolysin, or *m*-calpain for 10 min at 37°C. Proteolytic fragments were analyzed by SDS-PAGE with reduction.

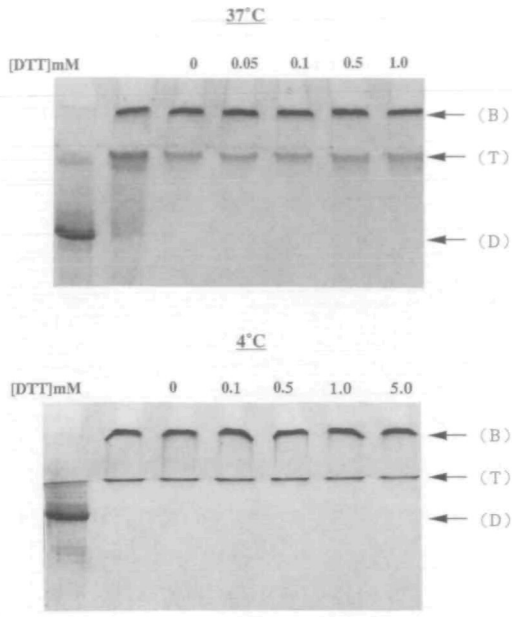

Fig. 7. **Retreatment of multimeric FN with DTT.** Multimer formed from plasma FN by DTT treatment was reincubated with various concentrations of DTT at 37 or 4°C for 18 h. DTT concentrations in mM are indicated above each lane. Arrows show the position of sample well bottom (B), gel top (T), and dimeric FN band (D). The left end lane is untreated plasma FN as reference and second lane from left is multimer formed from plasma FN.

ing domain II in the carboxyl-terminal region was involved in the major conformational change. The banding pattern of the fragments (200, 180, and 160 kDa) produced by trypsin treatment was essentially the same for DTT-treated FN and untreated FN. These fragments may have been generated by cleavage in the heparin-binding domain of the amino-terminal region and/or fibrin-binding domain II in the carboxyl-terminal region, the conformation of which may not have been changed greatly with DTT.

Effect of Changes of Incubation Temperature on Multimerization—At a DTT concentration of 0.5 mM, multimer formation occurred at 37°C but not at 25 or 4°C (Fig. 6). Plasma FN that had been incubated at 37°C for 4 h, at which time conformational change became observable by CD and fluorescence but the multimer was not yet detectable by SDS-PAGE, was transferred to 25°C and the incubation was continued up to 18 h. By this procedure, about a half of the FN in an altered conformation was converted to multimeric form. However, no multimer formation was observed when the temperature was changed from 37 to 4°C. The results suggested that plasma FN in an altered conformation induced by the initial incubation at 37°C was adequate for the multimerization to take place slowly but significantly at 25°C. Alternatively, any multimer formed by the incubation at 37°C for 4 h might have had high stability at 25°C but low stability at 4°C. However, since the multimer detectable on SDS-PAGE was found to be highly stable at 4°C as described below, this possibility is unlikely. A third possibility is that the FN conformation induced by incubation at 37°C with DTT but without multimerization might have reverted to its original state at 4°C.

Retreatment of Multimeric FN with DTT—The multimeric FN was retreated with DTT at 37 or 4°C for 18 h to see whether disulfide bonds could be reversibly reduced to yield dimeric FN. At a concentration of DTT from 0.05 to 5 mM, the multimeric FN incubated both at 37 and 4°C remained at the bottom of the stacking well, and no staining was detectable at the site of the dimeric or monomeric FN band (Fig. 7). Intermolecular disulfide bonds in FN multimer were irreversibly stabilized at 37°C.

DISCUSSION

We reported previously that plasma FN multimerized was promoted under conditions of physiological pH, ionic strength, and temperature by adding an SH reagent (*1*). This may provide us with an experimental system in which changes can be followed spectroscopically, since it contains only purified plasma FN, TBS, and DTT.

The conformation of multimeric FN was distinguishable from that of dimeric plasma FN in CD spectra. The lower ellipticity at 227 nm in the multimer might reflect the change in the environment of aromatic residues, especially a tyrosine residue, while the deeper trough at 215 nm might reflect an increase in β-sheet conformation localized in type III modules in the central region of FN (*15*). The requirement for a 10-fold increase in concentration SH reagent in the multimerization at 25°C suggests a cooperative action of high temperature and reducing reagent on the conformational change with concomitant reduction of intrachain disulfide bonds toward an extended structure of the protein.

Concurrent changes in CD spectrum and fluorescence emission spectrum of FN were observed with the progress of incubation with DTT at 37°C. Baron *et al.* (*18*) reported that type I modules of plasma FN have a compact structure containing two antiparallel β-sheets with chromophores such as tyrosine, tryptophan, and two disulfides (*27*). The CD spectrum of a proteolytic fragment from the heparin-binding domain I and the gelatin-binding domain, where type I modules are aligned, showed an unusual peak at 227 nm (*15*). The decreased ellipticity at 227 nm resulting from incubation of plasma FN with DTT at 37°C might reflect a change in conformation of the amino-terminal region. The polypeptide chain of FN contains 28 intrachain disulfide bonds altogether. They are localized in the type I and type II modules, which are in turn concentrated in the amino and carboxyl termini of FN. By incubation with DTT, disulfide bonds in the modules might have been reduced, resulting in a loosened structure of the modules, which changed the microscopic environments for tryptophan and tyrosine residues. Exposure of the tryptophan residue could be the cause of the red shift of the emission maximum, while a change in the surrounding of the tyrosine residue could be responsible for the decrease in ellipticity at 227 nm. Alternatively, but not excluding the above possibility, the change in CD spectra induced by the incubation with DTT at 37°C might have been due to changes in secondary structures in the amino- and carboxyl-terminal regions.

Protease susceptibilities of the DTT-treated FN implied that the conformational changes tended to be localized in particular regions of FN. The carboxyl-terminal side of the gelatin-binding domain or the amino-terminal side of heparin-binding domain II, where the thermolysin cleavage sites for the DTT-untreated FN reside, became resistant to thermolysin in the DTT-treated FN, leaving the cleavage site only at the carboxyl-terminal side of heparin-binding

domain I. On the other hand, the fragments generated by trypsin did not differ between DTT-treated and untreated FNs (200, 180, and 160 kDa) (*28*), suggesting that the conformation of the carboxyl-terminal side of heparin-binding domain I and/or the amino-terminal side of fibrin-binding domain II was little affected by DTT treatment. The change in CD spectrum at 227 nm might possibly correspond to conformational change of the gelatin-binding domain (*15*). Taken together, these findings suggest that the thermolysin-resistant region of the DTT-treated FN might be at the carboxyl-terminal side of the gelatin-binding domain. On the other hand, *m*-calpain cleavage sites of the FN chain cannot be assigned from the results obtained in the present study, since the cleavage sites preferential for *m*-calpain may not be dependent on primary structure. Above all, the finding that smaller fragments were obtained from DTT-treated FN with *m*-calpain suggested that the conformational change might have occurred at various regions of FN including the gelatin-binding domain.

The change in susceptibilities of FN to thermolysin and *m*-calpain was completed by 3 h of incubation with DTT, suggesting that conformational change of plasma FN was completed before the multimer formation started. Namely, the plasma FN in the altered conformation would be capable of assembling. The multimerization of FN with an SH reagent thus should be proceeded by conformational change of the protein to an extended structure, following by assembly of the extended structure of FN, with accompanying oxidation of SH groups to interchain disulfide bonds.

Since the incubation at 37°C followed by the incubation at 25°C yielded a significant amount of multimer, but the incubation at 25°C followed by the incubation at 37°C did not, the requirement of a high temperature for the overall reaction process may be ascribed to the first step, that involving conformational change of FN. A temperature-dependent conformational difference of FN in the presence of DTT was observed in differential reactions of conformation-discernible monoclonal antibody (*27*). A Perrin plot of the fluorescence anisotropy of FN (*29*) also suggested a temperature dependency of FN conformation. Disulfide-bonded multimer was not converted to dimeric or monomeric FN by retreatment with DTT (Fig. 7), implying that the conformation of FN in the multimer is more strongly stabilized than that in the dimer under reducing conditions.

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