DRUG DISCOVERY
AND GENOMIC TECHNOLOGIES

Research Report

Protein Chips Based on Recombinant Antibody Fragments: A Highly Sensitive Approach as Detected by Mass Spectrometry

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ABSTRACT

With the human genome in a first sequence draft and several other genomes being finished this year, the existing information gap between genomics and proteomics is becoming increasingly evident. The analysis of the proteome is, however, much more complicated because the synthesis and structural requirements of functional proteins are different from the easily handled oligonucleotides, for which a first analytical breakthrough already has come in the use of DNA chips. In comparison with the DNA microarrays, the protein arrays, or protein chips, offer the distinct possibility of developing a rapid global analysis of the entire proteome. Thus, the concept of comparing proteomic maps of healthy and diseased cells may allow us to understand cell signaling and metabolic pathways and will form a novel base for pharmaceutical companies to develop future therapeutics much more rapidly. This report demonstrates the possibilities of designing protein chips based on specially constructed, small recombinant antibody fragments using nanostructure surfaces with biocompatible characteristics, resulting in sensitive detection in the 600-amol range. The assay readout allows the determination of single or multiple antigen-antibody interactions. Mass identity of the antigens, currently with a resolution of 8000, enables the detection of structural modifications of single proteins.

INTRODUCTION

Genome-wide screens for protein function have been performed by expression of random cDNA libraries but with limited success because most clones in a library do not encode proteins in the right reading frame and most encoded proteins are not full length. A possible solution to this is the advent of miniaturized arrays of spotted proteins (3,8) compared to the widely used DNA chips (11). To perform global analysis of protein expression, we need to spot a catcher protein onto the surface of a microchip to be able to analyze its ligand interactions. This is complicated by the fact that globular proteins have a tendency to unfold and lose activity and to adsorb with different efficiency when spotted onto a solid support. Ideally, the spotted catcher molecules should be as identical as possible to minimize assay variations due to individual molecular properties, which is particularly important in global proteome analysis (1). In the present study, we demonstrate for the first time the performance of recombinant scFv antibody fragments, all based on the same single framework scaffold (12), resulting in almost identical molecules, in combination with rapid nanospotting onto micro-engineered silicon chips to yield functionalized protein chips (5). The antibody-antigen interactions were analyzed with mass spectroscopy, for which the absence of these single framework scaffold scFvs in the spectrograms was unexpected but further facilitated the interpretation. The ability to make such protein expression analysis on precoated microstructure formats is currently a major objective in proteomics and engages a number of investigators and biotech companies.

MATERIALS AND METHODS

Materials

αcyano-4-cinnamic acid (CHCA) was obtained from Aldrich (Steinheim, Germany). Trifluoroacetic acid (TFA) was obtained from Fluka Chemie AG (Buchs, Switzerland). The peptide mixture for the internal mass calibration of the matrix-assisted laser desorption ionization (MALDI) analysis came from PerSeptive Biosystems (Framingham, MA, USA). All other chemicals were purchased from Merck (Darmstadt, Germany).

Surface Coating

Five different surfaces were evaluated for adsorption of four different scFv antibody fragments. The surfaces were Costar 3590 High-Capacity Polystyrene 96-well assay plate (Corning Costar, Corning, NY, USA), Nunc-Immuno™ Plate, MaxiSorp™96 well (Nunc A/S, Roskilde, Denmark), black polystyrene 96-well fluorometer assay plate (BMG LabTechnologies, Offenburg, Germany), silicon chips (300 ×300 µm), and silicon chips coated with nitrocellulose. The scFv antibody fragments were s-MUC159 with specificity for MUC-1

(mucin 1, a carcinoma-associated antigen), FITC-8 with specificity for FITC-BSA, CT-17 with specificity for choleratoxin B, and anti-βgal with specificity for βgalactosidase. These antibodies originate from the n-CoDeR phage display antibody library (12) and were kindly supplied by Bo Jansson (BioInvent Therapeutics AB, Lund, Sweden). The antibodies were coated to the different surfaces overnight at room temperature, using 1.2 µg antibody/surface in 100 µL 0.026 M Na_2CO_3, 0.074 M $NaHCO_3$, pH 9.6, per well. The silicon chips were manipulated using the BMG plates. To evaluate the activity of the scFv antibodies after adsorption, each surface was washed with 1.5 M NaCl, 0.05% Tween® 20 (five times), and incubated with fluoroscein isothiocyanate (FITC)-labeled antigens at 4 ng to 2 µg in 100 µL buffer (10 mM sodium phosphate buffer, pH 8.0, containing 0.5 M NaCl and 0.1% Tween 20) for 2 h at 37°C. After antigen incubation, the surfaces were washed five times and analyzed using a FlouStar Galaxy® (BMG LabTechnologies).

Silicon chips with or without a thin coating of nitrocellulose were evaluated using a direct adsorption. Direct adsorption utilized 1.2 µg scFv antibody per 10 µL solution in a droplet put directly on the silicon chip and allowed to incubate in a humidified chamber at room temperature overnight. A small droplet (2 µL) of antigen of (0.8 ng to 0.1 µg) was also used in this setup and allowed to incubate for 2 h at room temperature.

Silicon Nanovial Protein Chip and Microdispensing

Silicon surfaces, square dice of 3 ×3 mm, were prepared from 3" <100> oriented wafers. The silicon dice were covered by a native silicon dioxide layer and were washed in deionized water before the antibody fragment adsorption experiments. Silicon chip nanovial MALDI target plates were fabricated by anisotropic etching of <100> silicon. Array chips holding 10 ×10 nanovials, each 300 ×300 µm in size, were used. To maintain the high mass resolution obtained when operating the mass spectrometry instrument in delayed extraction mode, the depth of the nanovials were limited to 20 µm (4). The accuracy in the microdispensing is currently set by the resolution of the x/y stage (10-µm increment) that translates the protein chip, where the droplet deposition is typically within 10 µm. The space between the nanovials is typically 300 µm. Thus, the risk of cross contamination of antibodies is extremely low.

A silicon micromachined dispenser developed in-house (6), having an internal volume from inlet to nozzle of 250 nL, was used to spot the desired number of antigen droplets. The droplet size was about 100 pL, and in each nanovial, 400 droplets (40 nL) of antibody solution (0.8 µM) were deposited and allowed to dry. The dispense rates were 50 Hz. After the scFv activation of the protein chip, the choleratoxin was spotted into each position. An important feature of nanospotting the antigen solution is the possibility of performing on-spot enrichment of the antigen. Enrichment factors of more than 50 times can been obtained using this technique (4). Furthermore, the low consumption of scFv antibody (≤ µL/antibody) is important. The total internal volume of the microdispenser system currently sets this limit.

Mass Spectrometry and Nanovial Target Plate Preparation

The MALDI-TOF mass spectrometry instrument was a Voyager DE-PRO® (PerSeptive Biosystems) with built-in delayed extraction and a linear path of 1.1 m. It is equipped with a video camera system to ensure precise focusing of the 337-nm nitrogen laser, having a focused laser spot diameter of approximately 100 µm. The sample plate can be moved with high resolution and precision using an x/y stage with a minimal increment of 3.2 µm. The mass spectra were acquired in the reflector mode. An accelerating voltage of 20 kV and a delay time of 150 ns were used. The nanovial protein chips were fixed onto standard MALDI target plates using double-sided adhesive and were mounted in a holder from PerSeptive Biosystems. The nitrocellulose thin film consisted of 5 mg/mL nitrocellulose dissolved in acetone/2-propanol (80%/20%, v/v). The entire target was homogeneously covered with the matrix solution using an airbrush (model 200; Badger Air-Brush, Franklin Park, IL, USA). The chip surface was subsequently scraped to ensure that only the nanovials contained the thin film.

Assay Procedure for MALDI Analysis

The following procedure was applied to run the assay: (*i*) form a thin polymer layer on the microchip; (*ii*) microdispense the scFv antibody fragment into the vial; (*iii*) flush 1 mL deionized water over the chip surface and repeat four times; (*iv*) microdispense the antigens into the vial; (*v*) flush 1 mL deionized water over the chip surface and repeat four times; (*vi*) apply the MALDI matrix by microdispensing; and (*vii*) analyze with MALDI-TOF mass spectrometry.

RESULTS AND DISCUSSION

To evaluate the dependence of solid supports on antigen binding capacity to adsorbed antibody fragments, five different surfaces were initially evaluated for their capacity to bind an scFv fragment with retained biological function. Three different plastic surfaces, a silicon surface, and a silicon surface coated with a nitrocellulose polymer were compared using a recombinant scFv antibody specific for choleratoxin. The results are shown in Figure 1, and scFv adsorbed to the nitrocellulose-coated silicon surface yielded by far the highest signal, using 100 ng fluorescently labeled antigen per chip with detectable signals down to 1 ng antigen, which indicated the presence of highly functional antibody fragments after absorption to this surface. The untreated silicon surface exhibited better performance compared to the different plastic surfaces, which behaved more similarly. However, antibody fragments adsorbed to the plastic surfaces needed several orders of magnitude more antigen to display a similar fluorescence signal, as compared to scFv fragments adsorbed to nitrocellulose-coated silicon. Furthermore, the specificity of different antibody fragments adsorbed onto silicon chips was evaluated using four frag-

ments with different specificities (see Materials and Methods), and the consecutive analysis showed that all scFvs retained their specificity after solid-phase adsorption (data not shown).

In the present study, the antibody fragments and the antigens were both deposited onto the nanovial array chip using piezoelectric flow-through dispensing technology developed in-house in array format of 300 × 300 µm nanovials with a density of 270 spots/cm^2. A shallow nanovial geometry configuration with 20 µm vial depth was used in these experiments. The microchip configuration and nanospotting principle are outlined in Figure 2. For comparison, the nanovial chip is depicted on top of a conventional target plate for MALDI-TOF mass spectrometry. We then chose to use mass spectrometry (2) to analyze specific antigen binding to the scFv antibody fragment spotted onto the chips. An organic solvent-based nitrocellulose solution was used to generate well-defined thin-layer coatings on the nanovial chip. In a recent study (9), we demonstrated the air-brush-based thin-film deposition technique on standard MALDI target plates to obtain well-defined nitrocellulose polymer coatings. Detailed investigation on the optimal thin-film properties are presented elsewhere (10). Liang et al. (7) have also demonstrated the benefits of using nitrocellulose films as solid supports for biomolecules in MALDI-TOF mass spectrometry. After the polymer film was deposited, the chip surface surrounding the nanovials was cleared

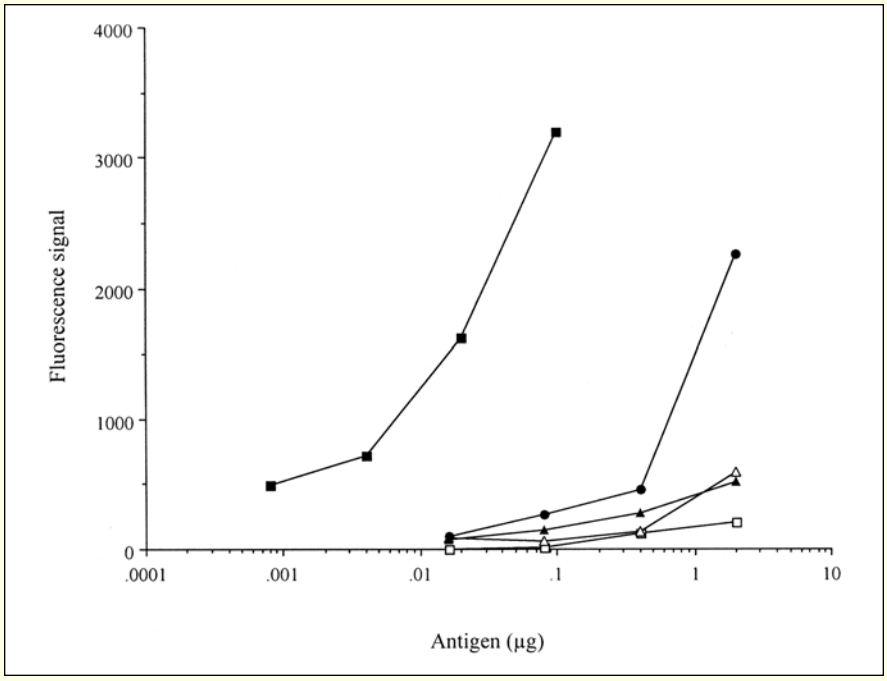

Figure 1. Adsorption of scFv antibody fragments onto different solid supports. The adsorption was detected using a fluorescence-based analysis. The different solid supports are denoted: BMG plastic (-□-), Nunc polystyrene (-Δ), Costar polystyrene (-▲-), Si (-●-), nitrocellulose-coated Si (-■-).

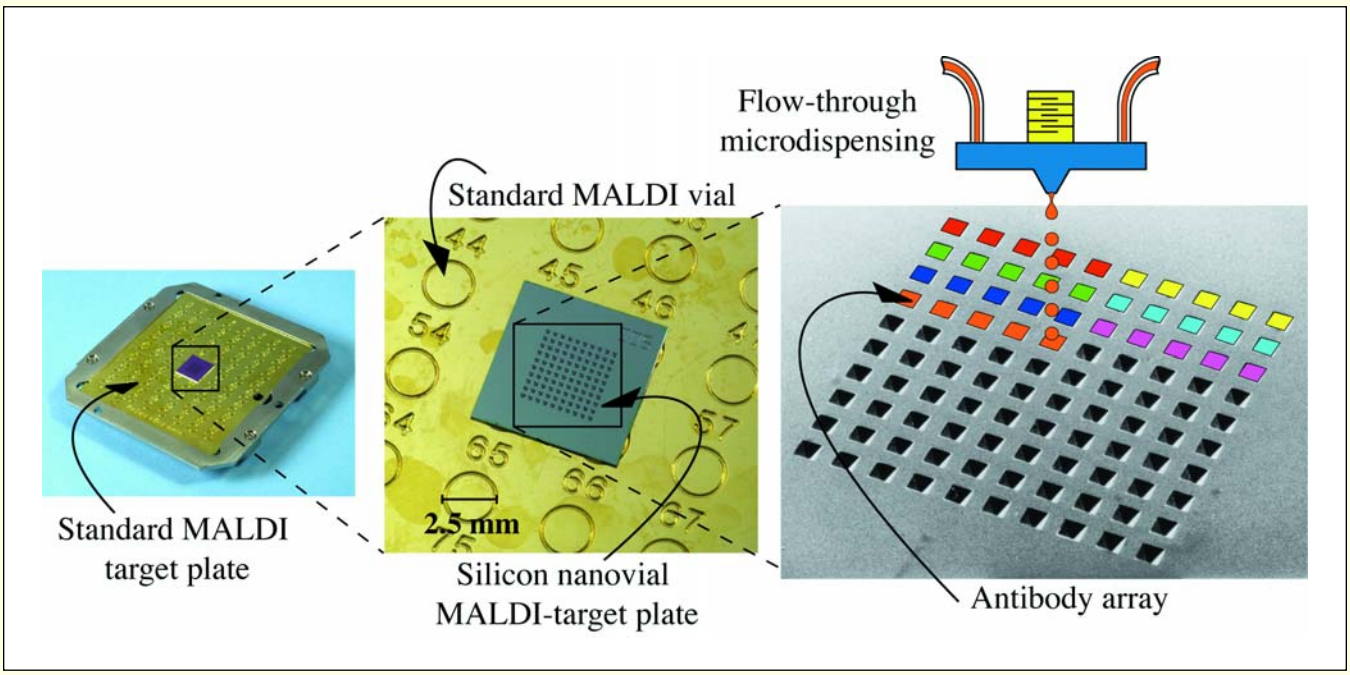

Figure 2. Silicon microchip arrays demonstrating the vial geometry of the chips used in this study. The nanovial arrays were mounted on a conventional MALDI target plate (Panel A). The miniaturization aspects are illustrated in Panel B showing a 100-position array compared to the surrounding circular standard MALDI spots. Panel C illustrates the principle of piezoelectric flow-through microdispensing of antibody arrays.

of nitrocellulose to ensure that the coating was only deposited in the chip vials, leaving the silicon surface naked in between the vials. This is important when dispensing the samples into the nanovials because the surface tension of the vial and the bare surrounding the silicon surface confines the protein sample to inside the nanovial. Thereby, a very beneficial concentrating effect is obtained within the vial (4).

The scFv fragment (CT-17), specific for choleratoxin, was spotted onto these chips and allowed to interact with a mixture of different antigens, as described in Materials and Methods. The antigen solution contained the choleratoxin subunit (11 kDa) at 50 nM and three different background proteins (ribonuclease, cytochrome C, and myoglobin) at a level of 500 nM. After microdispensing this protein mixture into the antibody-activated nanovials (20 fmol antigen/spot), the mass spectroscopy analysis was performed, and the resulting MALDI spectra are shown in Figure 3. The antigen mass identity makes it possible to discriminate protein structure modifications such as glycosylation or amino acid sequence variations. The combination of a highly selective affinity interaction and the high mass resolution obtained by MALDI mass spectrometry provides unique data features, in which both biorecognition and structural information are obtained simultaneously in one assay cycle. The data are typically obtained in less than 5 s for each array spot enabling a high-throughput format. Before and after washing the chip, the spectra clearly demonstrate the potential of this protein chip technology. The MALDI spectrum obtained before washing the chips (Figure 3A) revealed the presence of myoglobin, ribonuclease A, and cytochrome C in high yields, whereas the specific choleratoxin antigen signal was weak. However, after the washing step, a significant enhancement (40–50 times) in mass spectrometry signal ratio between choleratoxin and nonspecifically adsorbed proteins could be observed, and the unrelated proteins were now present at very low levels.

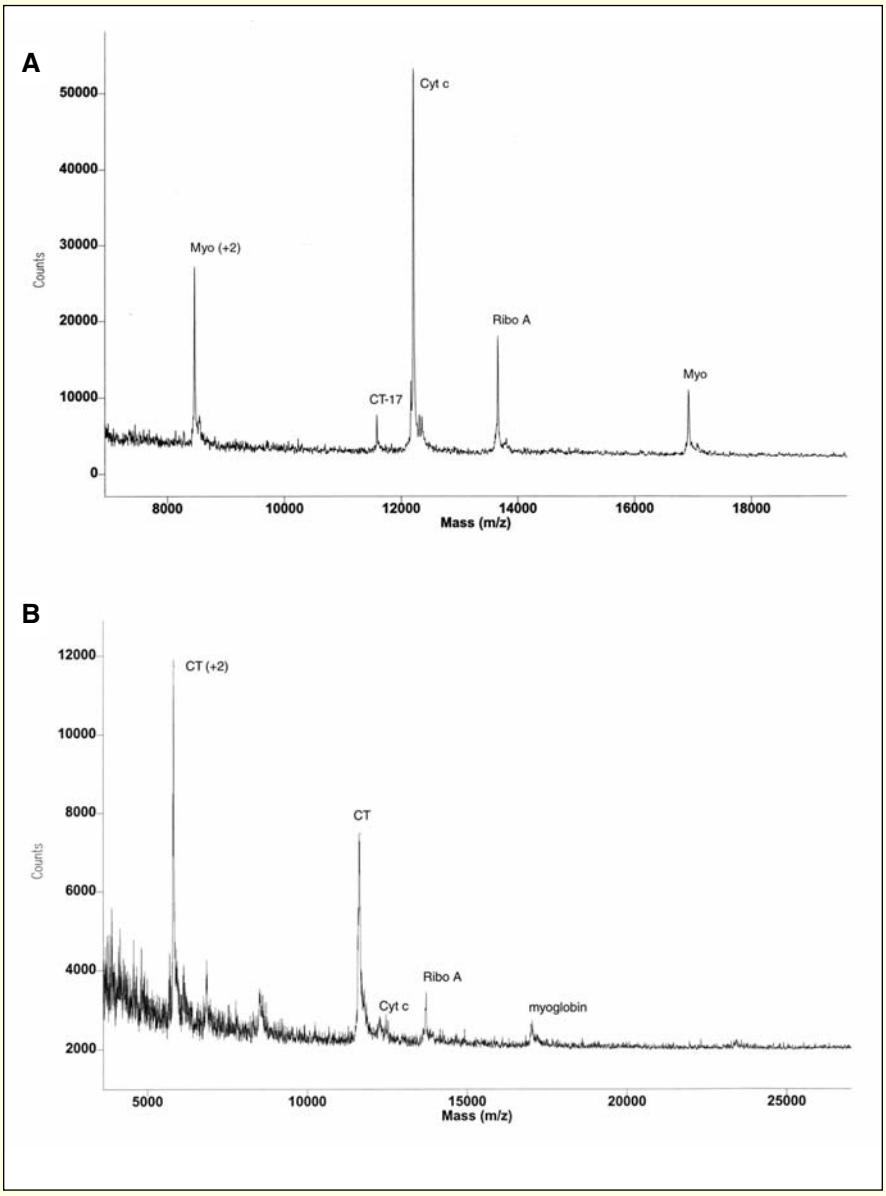

Figure 3. Mass spectrometry spectrum generated using a microchip coated with an scFv antibody fragment (CT-17) specific for choleratoxin. The chip was first allowed to react with a protein mixture before being washed. Mass spectroscopy spectra were performed before (A) and after (B) washing.

These advantages, on both molecular and mass spectroscopic levels, allowed a specific detection of choleratoxin at 600-amol levels (5 nM), as is demonstrated in Figure 4. A high degree of purity of the scFv antibody (≥95%) is desired to ensure a high surface density of the nanovials. The high purity of the antibody fragment is also a prerequisite to enhance the selectivity of the mass spectrometric assay. Detection of the specific antigen at these trace levels is believed to be the result of a dedicated surface binding of the antibody to the modified silicon chip surface because control experiments with unrelated scFv fragments (s-MUC159 and anti-βgal) did not exhibit any binding to choleratoxin. Furthermore, we were not able to detect a MALDI signal derived from the CT-17 scFv fragment, which should fall within the mass window of 24–26 kDa. Specific experiments were performed for detecting high molecular weight proteins, scanning the molecular weight area up to 80 kDa. The absence of this signal favors the explanation of a strong antibody binding to the chip surface, even while exposing the nanovials to the laser pulses, which might be a consequence of a favorable scFv molecular

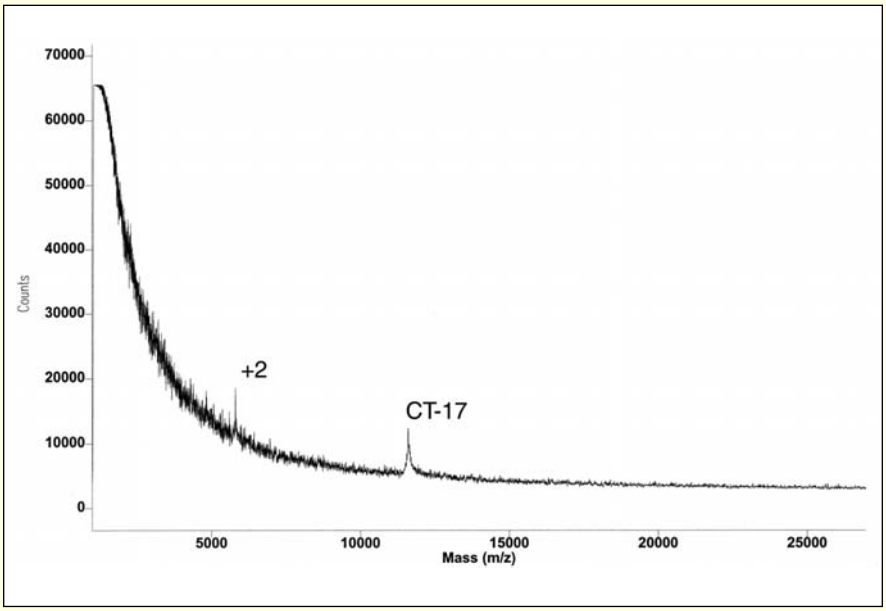

Figure 4. Mass spectrometry spectrum generated from the choleratoxin chip assay, showing an assay sensitivity of 600 amol. The MALDI spectrum shows both the singly charged and doubly charged choleratoxin antigens.

design based on the single framework scaffold. With this at hand, we were able to circumvent the ion suppression effects commonly appearing in MALDI ionization, which limits the detection of proteins at very low levels.

In summary, we have shown the application of recombinant scFv antibody fragments, based on a single framework scaffold, as a protein chip probe. In combination with piezoelectric microdispensing and on-chip enrichment, this probe yielded an assay sensitivity sufficient for the measurement of most clinically important proteins. Our results suggest directions for optimal utilization of small recombinant probes in a protein array format. In particular, the use of a flow-through format of the microdispenser enables on-spot enrichment, rapid and automated online switching between different antibodies, and, thus, speedy preparation of protein chips.

ACKNOWLEDGMENTS

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