Cell Binding Fragments from a Sponge Proteoglycan-like Aggregation Factor*

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The marine sponge Microciona prolifera aggregation factor (MAF) is a 2 × 10^6 dalton proteoglycan. MAF mediates species-specific cell-cell recognition through two functionally different sites: a Ca^2+-independent species-specific cell binding site and a Ca^2+-dependent MAF-MAF binding site. Dissociation procedures combined with protease treatment were used to produce cell-binding pieces from the large complex. The seven different sized fragments produced were all uronic acid-rich glycoproteins of the apparent molecular weights: 15 × 10^3, 2.5 × 10^4, 1.2 × 10^4, 7 × 10^3, 2.7 × 10^3, 5 × 10^3, and 3.6 × 10^3. Each of the fragments retained species-specific binding to Microciona cells and was also capable of inhibiting MAF-promoted cell aggregation. However, the fragments were unable to bind to MAF-conjugated agarose beads in the presence or absence of Ca^2+ ions. These three properties are those expected for the cell binding site of MAF. Since the binding affinity decreased linearly with decreasing molecular weight of the fragments, we believe that the cell binding sites in MAF may be highly polyvalent, although to fully support such a concept, a detailed chemical characterization of each of the fragments is needed. A high valency of cell binding sites would overcome a relatively low K_d for the single site and would thereby not only guarantee specificity but also explain the need for the large size of the proteoglycan complex found to mediate species-specific sponge aggregation.

Studies with marine sponges have shown that dissociated cells are capable of species-specific aggregation (1–3). This phenomenon provides a very useful model for studying molecular mechanisms of selective cell-cell adhesion and intercellular recognition processes believed to play important roles in embryonic tissue formation (4–6). For such species-specific aggregation of sponge cells a minimum of three components are necessary: 1) a proteoglycan-like component called aggregation factor which can be removed from the cell surface by washing with Ca^2+-Mg^2+-free sea water (7, 8), 2) a cell surface receptor for aggregation factor, termed baseplate (9), and 3) Ca^2+ ions.

Microciona prolifera aggregation factor has been characterized as a proteoglycan-like component (M_w = 2.1 × 10^6) containing 50–60% protein and 40–50% polysaccharide by weight (11–13). MAF is a fibrous complex molecule appearing on electron micrographs as a sunburst with 15–16 arms extended like rays from a circular backbone (12). Functional analysis revealed that MAF has two types of sites: 1) a Ca^2+-independent species-specific cell binding site and 2) a Ca^2+-dependent MAF-MAF interaction site. The latter can be irreversibly inactivated using EDTA or periodate treatment without altering the species-specific cell binding site (14). If EDTA treatment is prolonged for several days the result is dissociation of MAF into the circular backbone, the arms, or arm fragments (12).

To understand the mechanism of MAF-cell surface recognition, the smallest functionally intact fragment will have to be isolated, a task that has resisted many efforts over the past decade, primarily due to the size and complexity of the starting material. We report here on the isolation of MAF fragments after dissociation of the complex by urea, EDTA, and heat, followed by protease treatment. The fragments obtained were chemically characterized and functionally tested for the presence of the specific cell binding site.

MATERIALS AND METHODS

Sponges—Live specimens of M. prolifera, Cliona celata, and Mycale fusca were collected by the Supply Department of the Marine Biological Laboratory and by the authors in the Woods Hole, MA area.

Buffers—Bicarbonate-buffered artificial seawater and Ca^2+-Mg^2+-free seawater were made according to Humphreys (7). 20 mM Tris, pH 7.4, was also used as buffer for artificial seawater and for Ca^2+-Mg^2+-free seawater. Ca^2+-Mg^2+-free seawater was sometimes supplemented with 2 mM CaCl_2. MBLT was used for aggregation assays with glutaraldehyde-fixed cells (15). Fixed cells were stored in CMFT-SW containing 0.02% NaN_3. MAF was stored in Ca CMFT-SW with 0.02% NaN_3.

Dissociation of Sponge Cells—Sponges were cut into small cubes (5 × 5 mm or 10 × 3 mm) and squeezed through 100-μm mesh nylon cloth (Nitex) either in bicarbonate-buffered artificial seawater (mechanical dissociation) or in Ca^2+-Mg^2+-free seawater (chemical dissociation) (7, 14). For MAF depletion, chemically dissociated sponge cells (10^7/ml) were incubated on a rotary shaker at +4 °C for 4 h. They were washed once in bicarbonate-buffered CMFSW and incubated for another hour under the same conditions. At the end of incubation, the cells were washed again twice in bicarbonate CMFSW. Such cells (CMF cells) were either fixed with glutaraldehyde (15) or used live immediately.

The abbreviations used are: MAF, Microciona prolifera aggregation factor; MBLT, bicarbonate-buffered artificial seawater; CMFSW, Ca^2+-Mg^2+-free seawater; MBLT, Tris-buffered artificial seawater; CMFT-SW, Tris-buffered Ca^2+-Mg^2+-free seawater; Ca CMFT-SW, Tris-buffered Ca^2+-Mg^2+-free seawater supplemented with 2 mM CaCl_2; SDS, sodium dodecyl sulfate; UEP1, UEP2, and UEP3, urea-EDTA-heat-generated peaks 1, 2, and 3; TP1, TP2, TP3, TP4, and TP5, trypsin-generated peaks 1, 2, 3, 4, and 5; B_{max}, amount bound at saturation.

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Purification and Radioiodination of Aggregation Factor—MAF was purified following largely Humphrey's procedure (11-13), with slight modification as described (14), except for gel filtration which was performed on a 3000 Å bead (Electro-Nucleonics Inc., Fairfield, NJ) column (90 × 1.5 cm) instead of Sepharose 2B (13). Chloramine-T iodination of MAF was carried out as described previously (14).

Dissociation of Microciona Aggregation Factor—Of purified radioiodinated MAF (1.63 × 10⁶ cpm/mg), 0.203 mg/ml was incubated in 40 mM EDTA (pH 7.0) and 5 M urea at 80 °C for 4 h in CMFT-SW. The dissociated material was either dialyzed against CMFT-SW or immediately applied to a Sepharose 4B column (0.8 cm × 75 cm) replacing dialysis. The 1-ml fractions were collected by eluting the column with CMFT-SW. Radioactive peaks were pooled and specific radioactivity was determined in a Packard Gamma spectrometer (Model 5385). The protein (16), neutral hexose (17), and uronic acid (18) content of the fractions were also assessed.

Protease Treatment of Urea, EDTA, and Heat-dissociated MAF—Of dissociated ¹²⁵I MAF (2.2 × 10⁶ cpm/mg) dialyzed against CMFT-SW, 0.05 mg/ml was mixed with trypsin (0.2 or 2 mg/ml final concentration; Sigma Type III from bovine pancreas) or of Staphylococcus aureus V8 protease: 2 mg/ml final concentration (Miles Laboratory), and incubated for 2 or 24 h at 37 °C. To terminate the reaction, phenylmethylsulfonyl fluoride was added (2 mM final concentration), and samples were immediately applied to a Sephacryl S-200 column (90 × 1.6 cm). Elution was carried out with 0.25 M NH₄HCO₃ at 4 °C. The 1-ml fractions were collected and their specific radioactivity was determined by measuring trichloroacetic acid-precipitable radioactivity in a Packard Gamma spectrometer (Model 5385). The protein (16), neutral hexose (17), and uronic acid (18) content of the fractions were also assessed.

RESULTS

Dissociation of the Aggregation Factor Complex with Urea and EDTA at Elevated Temperature—In an effort to obtain fragments of the M. prolifera aggregation factor that still bind species specifically to cells, several dissociation procedures known to inactivate MAF were tested individually and in various combinations. Only the simultaneous application of urea, EDTA, and heat provided dissociation into subcomponents within a reasonably short time and with reasonable yields.

The following conditions were eventually found to be optimal: 50–200 µg/ml of the radioiodinated MAF proteoglycan complex were treated at 80 °C for 4 h in 40 mM EDTA, 5 M urea (21, 22). Three major fractions, all of which contained carbohydrates, were separated on a Sepharose 4B column (Fig. 1). The first peak, representing the void volume, contained undisassociated factor as well as large subunits having an apparent molecular weight in excess of 7 × 10⁵. The second peak had a Vᵣ/V₀ of 0.63 and apparent M₀ = 100–200 × 10⁵ (based on molecular weight standards). The third peak coincided with the included volume of the column and could be dialyzed through a membrane with a cutoff at M₀ = 3,000. The first two fractions contained material with a ratio of protein:hexose:uronic acid of 4:3:1, which was the same as the untreated MAF. More than 95% of the total protein and carbohydrates were recovered in fractions UEP1 and UEP2. The third peak, consisting of very low molecular weight fragments as well as free iodine 125, was not investigated further.

Polyacrylamide-SDS-gel electrophoresis was performed to monitor the dissociation of MAF by the urea-EDTA-heat treatment (Fig. 2). About 60–80% of the radioactivity present in untreated MAF entering the separating gel and could be resolved into seven major bands. The remainder did not enter the stacking gel (Fig. 2A). The banding pattern of this autoradiogram was similar to the pattern seen with Coomassie blue staining (not shown). After the urea-EDTA-heat treatment (Fig. 2B) an apparent increase in a 90 × 10⁵ dalton fraction was observed along with the partial disappearance of higher molecular weight radioactive species (150–250 × 10⁵), as well
as a relative decrease in material that did not enter the stacking gel. Polyacrylamide-SDS-gel electrophoresis of denatured MAF fractionated on Sepharose 4B revealed that UEP1 contained only material that failed to enter the separating gel (Fig. 2C), a property typical of large proteoglycan complexes. A large portion of radioactivity in the UEP2 peak was present in the 90 X 10^3 dalton fragment (Fig. 2D). UEP3 contained only low molecular weight material which co-migrated with the dye marker (Fig. 2E), even in autoradiographs of gels which were overloaded with that fraction (data not shown).

To investigate the cell binding activity of urea-EDTA-heat-generated MAF fragments, binding assays to homotypic CMF cells were performed (Table I). For this analysis the UEP1 material was assumed to have a Mw = 15 X 10^6 based on the exclusion limits of the column (7 X 10^6) on one hand and the fact that the starting material had a Mw of 21 X 10^6 on the other hand. The UEP2 fraction was estimated to have a Mw = 1.5 X 10^6 based on molecular weight markers. The equilibrium association constant of the UEP1 fraction was approximately five times lower than that of the untreated aggregation factor, suggesting a direct relationship between size of the MAF complex subunits and the affinity of binding to cellular sites. The further decrease in the Kd for UEP2 binding was almost proportional to the decrease in molecular weight. The UEP3 peak material failed to bind to the homotypic CMF cells and was not studied further since it was mostly 125I. An increase in the maximal number of specific binding sites/cell was also evident with decreasing fragment size (Table I). To determine whether these "additional" MAF-fragment-binding sites were related to the binding sites of intact MAF, we investigated whether the binding of the fragments to cells would inhibit MAF-promoted cell aggregation (MAF-cell interaction) or would possibly interfere with MAF-MAF interaction. Both UEP1 and UEP2 (but not UEP3) proved to inhibit strongly cell aggregation (Table I). None of the dissociated MAF fragments bound to MAF-substituted agarose beads either in the presence or absence of Ca^{2+} ions, nor were the fragments capable of inhibiting Ca^{2+}-dependent binding of native MAF to MAF beads. These results indicate that degradative effects by EDEA-heat-treatment of MAF is restricted to sites mediating MAF-MAF interactions and not to cellular binding sites on the MAF molecule. Furthermore, the inhibition of cell aggregation by the fragments can be attributed to blocking of functional MAF binding sites on the cell surface rather than secondary inhibition of the Ca^{2+}-dependent linkage between factor molecules.

Isolation and Characterization of Small Cell Binding Fragments—In an effort to isolate the smallest fragment capable of species-specific binding to cells, the urea-EDTA-heat-treated MAF fragments were digested with different proteases. Of the proteases tested, discrete cleavage products were obtained with trypsin and S. aureus V8 protease, whereas others produced much more random digestion. The proteolytic fragments were fractionated by gel filtration on a Sephacryl S-200 column (Fig. 3, A and B). The tryptic fragments of MAF could be resolved into 4 peaks (Fig. 3A) with apparent molecular weight of 124 X 10^3, 70 X 10^3, 27 X 10^3, and 5 X 10^3 (based on molecular weight standards). Occasionally, as in Fig. 3A, peak 4 could be partially resolved into two subfractions, in which the subfractions were pooled. Each fraction from the Sephacryl S-200 column was analyzed for protein, neutral hexose, and uronic acid, all of which appeared to correspond exactly with the radioactive profile

\[ \text{TABLE I} \]

| Fragments | Mw (X 10^3) | Kd (X 10^-4) | B_{max} (X 10^3) |
|-----------|-------------|--------------|------------------|
| MAF       | 21,000      | 41,000 ± 510 | 0.24 ± 0.003     |
| UEP1      | 15,000      | 8,400 ± 282  | 0.35 ± 0.012     |
| UEP2      | 250         | 88 ± 28     | 24 ± 0.770       |
| UEP3      | 1           | ND          | ND               |

* ND, not determined.

Yields were proportional to the duration of the incubation time. After 24 h of incubation with trypsin, 60% of the total urea-EDTA-heat-treated material, based on total weight, was present in the four included peaks, whereas only 20% was contained in these fractions after a 2-h incubation. Retryptsinization under the same conditions of the void volume fractions gave the same four fragments in similar proportions. A 24-h incubation with SV8 protease also yielded more of the three fragments (20%) than a 2-h incubation (8%). In control samples incubated under the same conditions without proteases, no fragmentation could be detected and all the material was eluted in the excluded volume (data not shown). Lower protease to substrate ratios failed to produce fragments below 200 X 10^3 daltons within 24 h.

Polyacrylamide-SDS-gel electrophoretic analysis of the four fractions of the trypsin digestion products demonstrated that fractions of decreasing molecular weight on Sephacryl S-200, each contained a single labeled polypeptide of correspondingly decreasing size (data not shown). Each of the fractions was chemically characterized and shown to consist of a uronic acid-rich glycoprotein, each having a similar ratio of protein/neutral hexose/uronic acid (Table II). An approximately linear decrease of $K_d$ and increase in $B_{max}$ with decreasing molecular weight was observed for TP1, TP2, TP3, and TP4 similar to the relationship obtained for the large fragments UEP1 and UEP2 (Table II). The first three trypsin fragments were able to inhibit MAF-promoted CMF cell
FIG. 3. Gel filtration on Sephacryl S-200 of trypsin (A) and S. aureus V8 protease (B)-treated dissociated I25I-MAF. A, 0.05 mg/ml of I25I-MAF was treated with trypsin (final concentration 2 mg/ml) for a period of 2 h (---) and 24 h (---). At the end of the incubation time 0.5 mg of MAF (for details see "Materials and Methods") were applied on a Sephacryl S-200 column. The column was eluted with 0.25 M NH4HCO3 and 1-ml fractions were collected, counted in a Packard gamma spectrometer, and analyzed for protein.

TABLE II
Characteristics of I25I-MAF tryptic fragments: binding to homotypic cells, effect on MAF-promoted aggregation, and chemical composition

$K_a$, values, $B_{max}$, values + S.E. (from four different experiments), and the inhibitory effect of the fragments upon MAF-promoted aggregation were determined in the same way as described in the legend of Table I. Starting amounts/assay volume were: TP1, 1.89 $\times 10^{-7}$ mol, TP2, 2.86 $\times 10^{-7}$ mol, TP3, 2.67 $\times 10^{-7}$ mol, TP4, 3.3 $\times 10^{-7}$ mol.

| Peak fractions after trypsin | $M_a$ | $K_a$ ($\times 10^{-4}$) | $B_{max}$ | Inhibition of MAF-promoted aggregation | Composition as per cent of total |
|-----------------------------|------|------------------------|----------|--------------------------------------|--------------------------------|
|                             |      |                        |          |                                      | Protein | Neutral hexose | Uronic acid |
| TP1                         | $\times 10^{-3}$ | 124                   | 30 ± 4.0 | ++                                   | 41.1    | 48.3           | 10.6        |
| TP2                         | 70   | 21 ± 2.1               | 150 ± 15.6| ++                                   | 40.7    | 48.3           | 11.0        |
| TP3                         | 27   | 7.1 ± 1.8             | 270 ± 68.4| ++                                   | 46.9    | 42.6           | 10.5        |
| TP4                         | 5    | 1.4 ± 1.1             | 1500 ± 1175.5| +                                   | 39.5    | 46.5           | 14.5        |

aggregation completely, while the TP4 fragment was only partially effective (Table II). As observed previously with the fragments obtained by urea-EDTA-heat treatment, none of the tryptic fragments was able to bind to MAF-substituted agarose beads, regardless of the presence of Ca$^{2+}$ ions. Thus the inhibition of the aggregation by the tryptic fragments is likely to be due to direct inhibition of the MAF-receptor interaction.

Specificity and Activity of the Aggregation Factor Fragments—Each of the fragments showed species-specific binding to its homotypic cell, although the binding specificity of the trypsin fragments was somewhat reduced (Table III). The potency for inhibition of MAF-promoted aggregation was also reduced with decreasing fragment size, as expected from the corresponding decrease in the affinity of binding to cellular sites. These results suggest that polyvalency of MAF may be an important determinant of both the affinity and the specificity of MAF-cell interactions. A detailed chemical charac-

TABLE III
Binding specificity of I25I-MAF and the MAF fragments
Binding of radioiodinated MAF or fragments to CMF M. prolifera, M. fusca, and C. celata cells was carried out in CMFT-SW as described in legend to Table I. The number of bound molecules/cell was calculated from the specific radioactivity and Avogadro's number.

$\times 10^{-12}$

| MAF fragments | Number of molecules applied/assay | M. prolifera | C. celata | M. fusca |
|---------------|----------------------------------|-------------|----------|---------|
| MAF           | 0.1                              | 33          | 2.2      | 2.1     |
| UEP1          | 0.3                              | 29          | 2.7      | 2.3     |
| UEP2          | 12.7                             | 54          | 0.6      | 1.6     |
| UEP3          | <0.1                             | <0.1        | <0.1     |         |
| TP1           | 11.4                             | 31          | 8.1      | 7.9     |
| TP2           | 17.8                             | 29.4        | 7.3      | 6.8     |
| TP3           | 16.1                             | 31          | 7.8      | 6.7     |
| TP4           | 20.0                             | 45          | 12       | 11.1    |

* 1.1 $\times 10^6$ cpm applied.
terization of each individual fragment is still needed, however, to fully support such a concept.

**DISCUSSION**

Previous studies have shown that *Microciona* aggregation factor-promoted reaggregation of cells from the marine sponge *M. prolifera* is a two-step process involving: 1) a Ca\(^{2+}\)-independent, species-specific binding of MAF to surface receptors and 2) the formation of Ca\(^{2+}\)-dependent linkages between factor molecules (MAF-MAF) on adjacent cells (14). Other studies have demonstrated, in addition, that the MAF-MAF interaction site can be selectively inactivated by EDTA, periodate, or heat treatment without any effect on the cell binding site (14). In the absence of MAF-MAF interaction in treated factor preparations, it is possible to dissect out, characterize, and functionally test the cell binding sites. Such an approach may provide insights into the molecular mechanism of species-specific aggregation in sponges and may lead eventually to the identification of the active carbohydrate or protein moiety of the MAF.

When EDTA treatment, known to inactivate the MAF-MAF sites of interaction, is prolonged to several weeks, the MAF molecule has been shown to dissociate into a “sunburst” structure into a circular backbone and into arms and arm fragments (13). Assuming that enzymatic or heat degradation of covalent bonds does not largely contribute to this breakdown, Ca\(^{2+}\) ions must play an essential role in maintaining the structural as well as the functional integrity of MAF. With this in mind, we have combined an EDTA treatment together with urea as well as heat to provide a dissociation procedure that is more efficient and rapid than EDTA treatment alone (2–4 h instead of 4 weeks). Although preliminary electron microscopy of our Sepharose 4B fractions did reveal some ring and some smaller linear structures, we cannot yet determine unequivocally from which portions of the MAF molecule the cell binding fragments derive.

Polyacrylamide-SDS-gel electrophoresis revealed an increase in lower molecular weight bands after the urea-EDTA-heat treatment of MAF and a decrease in those of higher molecular weight. This could be explained in two different ways: 1) the sample buffer for SDS electrophoresis might not have broken all the covalent bonds but pretreatment with urea, EDTA, and heat would have achieved a better dissociation; or 2) if the sample buffer is indeed able to break all covalent bonds, then one must still consider that the urea-EDTA-heat pretreatment may have cleaved some of the covalent bonds (Fig. 2, A and B). It is interesting to point out that no new bands were detected after the urea-EDTA-heat treatment.

For optimal yields of proteolytic fragments with molecular weights below 200 × 10\(^3\), high ratios of protease to dissociated MAF were needed (Fig. 3). That the fragments obtained after protease treatment were generated by proteolytic action is confirmed by the following observations: 1) trypsin produced five fragments below 200 × 10\(^3\) daltons, while SV8 could generate only three (Fig. 3, A and B); 2) changes in the protease to substrate ratio affected the fragmentation, as did the duration of the incubation; 3) control incubations where protease was omitted did not produce any fragments below 200 × 10\(^3\) daltons. The apparent resistance to proteolytic cleavage may reflect the presence of 40–50% carbohydrate in MAF. Carbohydrate is known to confer such protection to other glycoproteins, and may also act as a protease inhibitor.

A Scatchard analysis of the binding data revealed a direct relationship between fragment size and binding affinity as well as an inverse relationship to the number of binding sites. The ability of the fragments to inhibit MAF-promoted cell aggregation also decreased with molecular weight (Tables I and II). Each of these results supports previous predictions of a highly polyvalent interaction between MAF and cell surface (23, 24). To what degree cooperativity between individual binding sites influence the affinity or specificity of MAF binding has yet to be assessed. Nor can it be assumed that all of the individual binding interactions are identical. In the simplest case—i.e., multiple, equivalent active sites on the MAF molecule and a single class of noninteracting cellular receptors— the influence of valency on the overall affinity of MAF binding can be easily interpreted in terms of the product of the individual affinity constants of the multiple binding interactions. An alternative explanation would be that the large fragments have a rare high affinity site that is lost during the preparation of the small fragments with low affinity sites. Preliminary experiments indicated however that not only MAF but also the large fragments (e.g. UEP1, UEP2, and TP1) could give rise to the same small fragments (e.g. TP3 and TP4). This is in agreement with the fact that the affinity of the fragments toward the homotypic cell will decrease proportionately with the size of each fragment. We believe therefore that MAF may be built up of many similar or identical small cell binding fragments. Support for such a concept will however require a detailed characterization at the chemical level of the small and large MAF fragments; this is now in progress.

Denaturation, dissociation, and enzymatic cleavage of the MAF molecule may also generate new binding sites on MAF, or possibly render the residual MAF binding sites capable of interaction with cell surface components which are normally inaccessible to the native factor molecule. This raises the additional question as to the extent to which not only qualitative but also quantitative differences in receptor number, density, or topographical distribution on the cell surface determine the biological specificity of MAF-induced cell aggregation. To settle such questions will require a detailed understanding of the chemical nature of MAF-receptor interactions.

We showed earlier that aggregation can be specifically inhibited when high concentrations of glucuronic acid are preincubated with the cells (23). Moreover, β-glucuronidase treatment of MAF decreased its aggregation efficiency, suggesting that the surface receptor (which we termed “baseplate”) may recognize MAF by a lectin-like interaction (25). The importance of the factor-promoted reaggregation of cells from the marine sponge has also been shown recently by Müller et al. (26, 27). We have found however that glucuronic acid failed to inhibit the direct binding of MAF (14). In view of our present results and the high K\(_a\) of MAF binding we would expect that glucuronic acid, which represents at best the outermost carbohydrate of the oligosaccharide chains of MAF, would have a relatively weak affinity for the cellular site and would require very high concentrations to block intact MAF binding. Such an approach to investigating the chemical basis of the minimal site of interaction between MAF and the cell surface, however, should become feasible following further purification, analysis, and modification of the smallest protease fragments described here.

Beside the species-specific MAF-cell receptor interaction, Ca\(^{2+}\)-dependent MAF-MAF polymerization is necessary for the species-specific aggregation of sponge cells. We do not yet know whether this second type of interaction is also species-specific. Recent results with polycations having different length and different distances between charge, indicate that MAF-MAF interaction may also contribute some specificity in the sponge aggregation process (28). If there are indeed two different systems providing specificity, which has not been
shown rigorously so far, the relative biological relevance of each of these will need to be evaluated.

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