Recombinant mussel adhesive protein Mgfp-5 as cell adhesion biomaterial

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Abstract

\textit{Mytilus galloprovincialis} foot protein type-5 (Mgfp-5) is one of the mussel adhesive proteins that participate in adhesion with the substratum. We previously reported the production of recombinant Mgfp-5 in \textit{Escherichia coli} and showed that the recombinant protein had superior adhesion abilities versus those of Cell-Tak, a commercially available mussel adhesive protein mixture. In the present work, we investigated the feasibility of using recombinant Mgfp-5 as a cell adhesion agent. Purified and tyrosinase-modified recombinant Mgfp-5 was used to adhere living anchorage-independent cells such as insect \textit{Drosophila} S2 cells and human MOLT-4 cells onto glass slides. Our results revealed that these cell lines efficiently attached to recombinant Mgfp-5-coated glass surfaces, and that surface-immobilized S2 cells were viable and able to undergo cell division for up to 1 week. Cytological studies with 4',6-diamidino-2-phenylindole (DAPI) staining of nuclei and immunofluorescence for secreted foreign human erythropoietin (hEPO) from recombinant S2 cells and quantitative comparative analyses of S2 cell binding ability with Cell-Tak and poly-l-lysine, the main cell adhesion agent, were performed to demonstrate successful usage of recombinant Mgfp-5 for cell biological applications. Collectively, these results indicate that recombinant Mgfp-5 may be a useful new cell adhesion biomaterial for anchorage-independent cells.

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1. Introduction

A number of marine invertebrates are equipped with a specialized adhesion aid, the byssus, which is a thread-like anchor that attaches the organism to the
substratum and fights buoyancy and tidal currents in the aqueous saline environment (Kamino et al., 2000; Waite, 1983a). The mussel, which thrives in coastal areas, boasts a byssus comprising of the byssal thread and an adhesion plaque that attaches to the substratum via specialized adhesive proteins (Waite, 1983b). Five distinctive types of foot protein (fp-1–fp-5) have been isolated from the adhesion plaque of the common mussel, Mytilus edulis (Inoue et al., 1995; Papov et al., 1995; Rzepecki, 1992; Waite, 1983b; Waite and Qin, 2001). Among the plaque proteins, fp-1, fp-3 and fp-5 appear to play important roles in adhesion; fp-1 is related with coating, while fp-3 and fp-5 were isolated from the interface between the adhesion plaque and the substratum, and appear to be primarily associated with adhesive functions (Waite et al., 2005). The foot proteins all have high levels (3–30 mole%) of the modified amino acid 3,4-dihydroxyphenyl-L-alanine (DOPA), which is derived from hydroxylation of the aromatic ring of tyrosine residues (Deming, 1999; Yu et al., 1999). Interestingly, the closer the protein is found to the adhesion interface, the higher the DOPA content (Waite, 2002). Indeed, studies have shown that the DOPA residues allow mussel adhesive protein molecules to not only adhere to substrates, but also to cross-link with each other via oxidative conversion to o-quinone (Waite, 2002). Furthermore, the absence of DOPA in mussel adhesive protein leads to a loss of adhesion ability (Yu and Deming, 1998; Yu et al., 1999), indicating that the adhesion and cross-linking of mussel adhesive proteins is associated with the presence of DOPA.

Mussel adhesive proteins are able to form strong bonds with various kinds of substrates including glass, plastics and metals. Moreover, their biodegradability and biocompatibility make them attractive candidates as environmentally friendly industrial or medical adhesives (Dove and Sheridan, 1986; Grande and Pitman, 1988). For commercialization of mussel adhesive proteins for biotechnological applications, researchers first attempted to refine natural extraction processes. However, this process is labor-intensive and inefficient, requiring around 10,000 mussels for 1 g protein. Cell-Tak is a unique commercialized naturally extracted mussel adhesive protein (primarily a mixture of fp-1 and fp-2). Over the past two decades, a number of groups have sought to develop recombinant systems for mussel adhesive protein production. The first cloned foot protein, fp-1, was intensively studied in various expression systems such as Escherichia coli, yeast, and plants. However, fp-1 could not be successfully mass produced because it contains an ~80-mer repeat of the decapetide, AKPSYPTYK, which causes plasmid instability, codon usage problems, and low productivity in recombinant systems (Filpula et al., 1990; Kitamura et al., 1999; Salerno and Goldberg, 1993). The subsequent identification of fp-3 and fp-5, which are located at the interface between the mussel adhesion plaque and the substratum (Waite, 2002), opened up new avenues for mussel adhesive protein research. We previously showed that recombinant fp-5 and fp-3A from Mytilus galloprovincialis could be successfully expressed in E. coli systems (Hwang et al., 2005, 2004). In addition, these proteins showed comparable or superior adhesion abilities versus Cell-Tak.

The development of biocompatible tissue and cell adhesive agents has become vital to the fields of tissue and medical engineering. Poly-L-lysine and (to a lesser degree) Cell-Tak are currently used as cell immobilizing agents or adhesives (Benthien et al., 2004; Mazia et al., 1975), but these materials have some limitations. For example, some cells and microorganisms do not readily settle out of suspension onto Cell-Tak coated surfaces (Cell-Tak manual, BD bioscience, Belford, MA, USA) while soluble poly-L-lysine can induce necrosis in some cell lines, and induces tumor necrosis factor production in monocytes (King et al., 2003; Strand et al., 2001). Thus, researchers continue to seek additional biocompatible adhesives. Here, we investigated the value of recombinant M. galloprovincialis foot protein type-5 (Mgfp-5) adhesive protein as a cell adhesive biomaterial. Insect Drosophila S2 cells and human MOLT-4 cells were used as models for cell adhesion analysis, because both cell types show anchorage-independent growth. S2 cells established from primary Drosophila embryo cultures (Schneider, 1972) were developed as an expression system for production of functional eukaryotic proteins, because they grow better than mammalian cells at 22–27 °C, do not require a CO2 incubator, and can grow in suspension cultures (Hill et al., 2001). MOLT-4 cells are a human T cell leukemia line established from the blood of a 19 year-old man with acute lymphoblastic leukemia in relapse (Minowada et al., 1972).
2. Materials and methods

2.1. Cell culture

The insect Drosophila melanogaster Schneider line-2 (S2) cells used in this study contained a plasmid encoding the human erythropoietin (hEPO) gene fused with a hexahistidine tag and a BIP signal sequence for secretion under the control of Drosophila metallothionein promoter, as previously described (Shin and Cha, 2002). Recombinant S2 cells were grown at 27 °C in M3 medium (Sigma, St. Louis, MO, USA) containing 10% IMS (insect medium supplement; Sigma), 1% antibiotic–antimycotic (Invitrogen, Carlsbad, CA, USA), and 3 μl ml⁻¹ hygromycin. Wild type human MOLT-4 cells (# CRL-1582; ATCC, Manassas, VA, USA) were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air.

2.2. Modification of tyrosine residues

Recombinant Mgfp-5 was expressed and purified as previously described (Hwang et al., 2004). To provide adhesion, the tyrosine residues of the purified recombinant Mgfp-5 (1.44 mg ml⁻¹) were converted to DOPA by modification with 50 μg ml⁻¹ tyrosinase (Sigma) in 0.1 M phosphate buffer, pH 7.0, containing 25 mM ascorbic acid at 25 °C for 1 h (Marumo and Waite, 1986). After modification, the samples were dialyzed, freeze-dried, and dissolved in 5% acetic acid.

2.3. UV spectroscopic analysis of DOPA residues

To measure the proportion of tyrosine residues modified to DOPA, we measured differences in UV spectroscopy (Waite, 1984). The UV absorbance spectrum of DOPA-containing Mgfp-5 can be changed by complexing DOPA with borate at high pH. The absorbencies of a 1 mM DOPA standard and those of modified Mgfp-5 in 0.2N HCl and 0.2 M sodium borate (pH 8.5) were scanned at 250–350 nm, using a UV–visible spectrophotometer (UV-1601PC; Shimadzu, Kyoto, Japan). The spectrum differences were measured by subtracting the spectra of the DOPA standard or modified Mgfp-5 in 0.2N HCl from that obtained from the modified sample in 0.2 M sodium borate. The 1 mM DOPA standard showed subtraction difference λ_max of 292 nm with a ΔC value of 3200 M⁻¹ cm⁻¹ (Waite, 1984). Using the λ_max and ΔC of the 1 mM DOPA standard, the percent of DOPA residues in the modified Mgfp-5 was calculated according to Beer’s law (Waite, 1984).

2.4. MALDI-TOF mass spectroscopy analysis

The amount of DOPA modification was also assessed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry analysis with time-of-flight (TOF), performed on a 4700 Proteomics Analyzer (Applied Biosystems, Foster, CA, USA) in the positive ion linear mode. Sinapinic acid in 30% acetonitrile and 0.1% trifluoroacetic acid was used as the matrix solution. Samples were diluted 1:25 with matrix solution, and 1 μl of the mixture was spotted onto the MALDI sample target plates and evaporated using a vacuum pump. Spectra were obtained in the mass range between 10,000 and 17,000 Da with ∼1500 laser shots. Internal calibration was performed using apomyoglobin with [M + H]+ at 16953.33 and apomyoglobin with [M + 2H]²⁺ at 8484.45.

2.5. Analysis of cell adhesion on adhesive coated surface

Cell adhesion assays were performed as previously described (Yuan et al., 2004) using hEPO-secreting recombinant S2 cells and wild type MOLT-4 cells. The cell adhesion ability of recombinant Mgfp-5 was compared to that of BSA in 5% acetic acid buffer (Sigma) as the non-adhesive negative control (Hwang et al., 2004) and Cell-Tak (BD Bioscience, San Jose, CA, USA) as the adhesive positive control. Each 2 μl drop of 1.44 mg ml⁻¹ protein solutions were dropped onto sterilized slide glasses (20 mm × 20 mm), incubated at room temperature for 10 min under laminar flow hood, and then washed twice with phosphate buffered saline (PBS; Invitrogen). The coated slide glasses were then submerged in 100 mm cell culture dishes containing >95% viable S2 (4 × 10⁶ cells ml⁻¹) or MOLT-4 (2 × 10⁶ cells ml⁻¹) cells. After incubation at 27 °C (for S2 cells) or 37 °C (for MOLT-4 cells) for 1 h to 7 days, unattached cells were rinsed off with PBS, and cell viability and the location of adhered cells with respect to the protein spots was checked by 0.4% (w/v) trypan blue (Sigma) staining.
2.6. Immunofluorescence analysis of recombinant proteins from attached S2 cells

S2 cells were attached to Mgfp-5 coated cover slips, which were then immersed in M3 medium in 30 mm cell culture dishes, and incubated at 27 °C for 6 h with 0.5 mM copper sulfate to induce production and secretion of hEPO. Each cover slip was then washed once with PBS and immersed in 4% formaldehyde in PBS for 20 min to fix the attached S2 cells. The fixed cells were washed with PBS and immersed in 1% Triton X-100 in PBS for 10 min. After washing with PBS, each cover slip was blocked with 3% BSA in PBS for 10 min and then incubated for 1 h with a 1:50 dilution of polyclonal anti-hEPO antibody (R&D Systems, Minneapolis, MN, USA) with 1% BSA and 0.02% Tween 20 in PBS. Each cover slip was then washed three times with 1% BSA and 0.02% Tween 20 in PBS for 5 min and incubated for 1 h with a 1:50 dilution of a Texas Red-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The cells were then examined by fluorescence microscopy (Olympus, Tokyo, Japan).

2.7. Nuclear staining

S2 or MOLT-4 cells were attached to Mgfp-5 coated cover slips, washed once with PBS, and immersed in 4% formaldehyde in PBS for 20 min. The fixed cells were then washed with PBS and immersed in 0.001% (w/v) of 4 ′,6-diamidino-2-phenylindole (DAPI) in PBS for 10 min. Finally, the cells were fixed with Lisbeth’s embedding medium (30 mM Tris–Cl, pH 9.5, 70% glycerol, 50 mg ml⁻¹ N-propyl gallate) and examined by fluorescence microscopy.

2.8. Quantitative and comparative analysis of S2 cell binding

S2 cell binding was quantitatively measured by WST-1 assay (Ishiyama et al., 1995). Cell-Tak, poly-L-lysine (Sigma), and unmodified Mgfp-5 were used as controls. Fifteen micrograms of each Cell-Tak, poly-L-lysine, unmodified Mgfp-5 or modified Mgfp-5 was coated on triplicate wells in 24-well polystyrene plate (SPL, Gyeongki, Korea). Cell-Tak and poly-L-lysine coated wells were prepared according to the each manufacture’s instruction. In the case of unmodified and modified Mgfp-5 proteins, coated wells were prepared based on the Cell-Tak manufacture’s instruction. After coating, 300 μl of 3 × 10⁵ cells ml⁻¹ (over 95% viable) was placed in each coated well to investigate cell binding for 2 h. After aspirating of unattached cells, 24-well plates were washed two times with PBS. Thirty microlitres of WST-1 (Roche, Mannheim, Germany) was placed into the wells and formazan dye was allowed to form for 4 h, and then absorbance was measured at 490 nm using a microplate reader (Wallac 1420 Victor 3; Perkin-Elmer, Wellesley, MA, USA). Absorbance of culture medium with WST-1 was used as a blank position.

3. Results

3.1. Modification of recombinant Mgfp-5 by tyrosinase

We previously showed that recombinant mussel adhesive Mgfp-5 proteins expressed in E. coli gain adhesive properties when their tyrosine residues are modified to DOPA residues (Hwang et al., 2004). Thus, we used tyrosinase treatment to modify our recombinant Mgfp-5. MALDI-TOF mass spectrometry and quantitative UV difference spectroscopy of DOPA–borate complexes (Waite, 1984) were used to examine the efficiency of tyrosinase modification. The MALDI-TOF results revealed that out of the 20 tyrosine residues in the modified recombinant Mgfp-5, 3–4 of them had been converted to DOPA (Fig. 1). Consistent with this, the UV spectroscopic analysis indicated that the average number of DOPA residues was 3.55 per Mgfp-5 molecule. Thus, it appears as though our tyrosinase modification yield for recombinant Mgfp-5 was about 15–20%.

3.2. Cell adhesion by recombinant Mgfp-5

Insect Drosophila S2 cells and human MOLT-4 cells were used as models for our cell adhesion analysis, because both cell types show anchorage-independent growth. We used these cells to compare cellular adhesion to glass slides coated with modified recombinant Mgfp-5, BSA subjected to the same modification conditions as the Mgfp-5 (negative control), and Cell-Tak, a commercialized cell adhesive extracted from mus-
Fig. 1. MALDI-TOF mass spectrometry analysis of recombinant Mgfp-5 modified by mushroom tyrosinase. Symbols: (···) unmodified recombinant Mgfp-5; (—) recombinant Mgfp-5 modified by mushroom tyrosinase.

sels (positive control). These proteins were spotted to glass slides and then washed; the Cell-Tak and BSA appeared to wash away, whereas most of the recombinant Mgfp-5 remained on the slide glass, despite our harsh washing conditions (Fig. 2A). Trypan blue staining was able to visualize the recombinant Mgfp-5 spots. We observed numerous S2 cells adhered to the Mgfp-5-coated areas (Fig. 2B). Light microscopy (Fig. 2C) revealed that most of the adhered S2 cells showed normal morphologies and high viability (as assessed by exclusion of trypan blue, a hallmark of living cells). The S2 cells adhered to recombinant Mgfp-5 within 20 min under our experimental conditions and showed good viability for up to 1 week (experimental microscopic observation). During our observation period, we found that some cells remained bound, while others disappeared and were replaced with daughter cells from dividing attached cells. We also observed successful adherence of human MOLT-4 cells to recombinant Mgfp-5-coated slides (Fig. 3A), but the adhesion of these cells was weaker and the viability was lower than that seen in S2 cells (experimental microscopic observation).

3.3. Cytochemical analysis of anchorage-independent cells adhered with recombinant Mgfp-5

To assess the feasibility of using recombinant Mgfp-5 as immobilizing biomaterials for cytochemical analysis of anchorage-independent cells, we performed DAPI staining of nuclei and immunofluorescence of the expression and localization of a recombinant protein (hEPO) in immobilized recombinant S2 cells. S2 cells were immobilized on glass slides using Mgfp-5, and copper sulfate was added to induce recombinant hEPO production for 6 h. We were successfully able to visualize DAPI-stained nuclei (Fig. 4A) and hEPO expression (Fig. 4B) in immobilized S2 cells. Higher resolution microscopy clearly showed the DAPI-stained nuclei of immobilized S2 cells (Fig. 4A, bottom), and revealed that recombinant hEPO was distributed throughout the cytoplasm prior to secretion (Fig. 4B, bottom). Moreover, DAPI staining revealed that some of the S2 cells contained two nuclei (Fig. 4C),
suggesting the presence of pre-division mitotic cells. DAPI staining of MOLT-4 cells also showed good co-localization of the cells with the recombinant Mgfp-5 coated areas (Fig. 3B), indicating that cytochemical analysis is possible in a second cell type adhered with Mgfp-5.

3.4. Quantitative and comparative analysis of S2 cell binding

Cell binding on Mgfp-5 coated polystyrene surface was quantitatively investigated using the WST-1 assay that is an index of cell number based on measure-
Fig. 5. Quantitative comparison of S2 cell binding ability on poly-l-lysine, Cell-Tak, unmodified Mgfp-5, and modified Mgfp-5 coated polystyrene surface using WST-1 assay. Abbreviations: US, uncoated surface; PLL, poly-l-lysine; Mgfp-5, unmodified Mgfp-5; Mgfp-5(T), modified Mgfp-5. Each value and error bar represents the mean of triplicate samples and its standard deviation.

We herein showed that modified recombinant Mgfp-5 may be a viable candidate for development as a new bioadhesive for cell biological applications. As a first step towards investigating bioadhesion by Mgfp-5, we expressed the protein in *E. coli* and used tyrosinase modification to convert the tyrosines of recombinant Mgfp-5 to the DOPA residues required for adhesive ability. Our protocol generated a modification yield of about 15–20% (Fig. 1). This relatively low conversion yield might be due to steric hindrance of the tyrosinase catalytic reaction by the structure of Mgfp-5, as tyrosinase can effectively convert free tyrosines but not those located inside the protein (Jee et al., 2000).

We then examined the feasibility of using recombinant Mgfp-5 as a cell adhesion agent for cytchemical studies, which requires effective adhesion to both the substrate and target cells. We previously showed that recombinant Mgfp-5 adhered to various substrates better than Cell-Tak (Hwang et al., 2004). Consistent with the prior findings, we observed that recombinant Mgfp-5 was able to coat slide glasses and remained in place after harsh washes, whereas Cell-Tak and BSA washed off under the same conditions (Fig. 2A). We then examined cell adhesion to the coated slides, and found that insect S2 cells (Fig. 2B) and human MOLT-4 cells (Fig. 3A) adhered to the Mgfp-5-coated areas, but not the uncoated glass. The S2 cells in particular showed tight adherence, maintained good viability for up to a week (experimental microscopic observation), and appeared to undergo cell division (Fig. 4C). Some of the daughter cells remained bound on the Mgfp-5-coated surface, while others were released into the medium. This is likely due to differences in the location of cytokinesis, i.e. whether the daughter cell was produced on the bound or unbound plane of the cell.

We further showed that Mgfp-5-immobilized S2 cells could successfully produce recombinant hEPO upon induction, indicating that it should be possible to perform expression studies in Mgfp-5-immobilized cells (Fig. 4B). The number of attached S2 cells gradually decreased to below 50% of the initially attached cells after 1 week, by which point the general cellular morphologies had deteriorated (experimental microscopic observation). This may indicate that S2 cells, which have been adapted to grow in the suspension state, are stressed by long-term surface adhesion.

To investigate the possibility of recombinant Mgfp-5 as an alternative for Cell-Tak and poly-l-lysine, comparative cell binding studies were performed. We also used unmodified Mgfp-5 as a reference because it has 16 lysine residues among total 76 amino acids and high lysine residue content may be good for cell adsorption like poly-l-lysine (Hwang et al., 2004; Waite and Qin, 2001). As results, modified Mgfp-5 showed the highest cell binding ability (Fig. 5). Interestingly, even though unmodified Mgfp-5 showed lower cell binding ability than modified Mgfp-5, it had comparable ability to...
Cell-Tak. This is similar pattern with the adsorption tests using quartz crystal microbalance in our previous study (Hwang et al., 2004). Comparable adsorption ability of unmodified Mgfp-5 might help cell binding. However, poly-L-lysine showed much lower cell binding ability than the others. One reason for lower cell binding of poly-L-lysine could be its lower binding strength. Actually, it was observed that almost equal amount of cells were attached on both poly-L-lysine and Mgfp-5 coated surfaces before PBS washing with shaking, but S2 cells on poly-L-lysine coated surface were easily detached according to washing times (experimental observation). We surmise that poly-L-lysine uses simply cationic strength of lysine for cell binding but Mgfp-5 uses interaction of DOPA or other amino acids as well as cationic strength of lysine.

Surface immobilization is critical for immune assays of T cells, such as human MOLT-4 leukemic T cells, which do not normally attach to glass slides or tissue culture plasticware (Benedict and Picciano, 1989). The ability to quantify the relative proportions of T cell subpopulations through cell adhesion on a surface could enable the rapid assessment of immunological competence in patients undergoing cancer therapy or suffering from specific immune system diseases. Although MOLT-4 cells also showed successful attachment to Mgfp-5-coated glass slides (Fig. 3), their adhesion was weaker than that of S2 cells, and MOLT-4 cell viability after immobilization was lower than that of S2 cells (experimental microscopic observation). Thus, our data collectively suggest that modified recombinant Mgfp-5 can be successfully used as an adhesive biomaterial for cells such as S2 and MOLT-4 cells.

Currently, Cell-Tak is the only commercially available mussel adhesive protein that can be used as a cell or tissue adhesive. However, our findings that modified recombinant Mgfp-5 outperforms Cell-Tak in terms of surface adhesion and cell immobilization suggest that recombinant Mgfp-5 is a good candidate for development as a less expensive and easily mass produced successor to Cell-Tak. Furthermore, recombinant Mgfp-5 may prove useful as a novel immobilizing agent for biochip systems or as a mediator for drug delivery. Poly-L-lysine, the main adhesion agent of anchorage-independent cells, has been widely studied as a mediator for drug delivery and as an immobilizing agent for microscopic or biochip work (Curiel et al., 1991; DeRisi et al., 1997; Mazia et al., 1975). In contrast to the adhesion mechanism of mussel adhesive protein (Mazia et al., 1975), poly-L-lysine acts by adherence of its polycationic sites to anionic sites on the cell surface. However, poly-L-lysine showed lower S2 cell binding ability than recombinant Mgfp-5 in the present work, and its use is limited by tendency to induce necrosis and/or provoke direct and indirect inflammatory responses (King et al., 2003; Strand et al., 2001). Therefore, recombinant mussel adhesive Mgfp-5 could also be an attractive alternative for poly-L-lysine as an adhesive in a variety of applications.

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