6×His Promotes Binding of a Recombinant Protein to Heparan Sulfate

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Commercially available systems provide a variety of strategies for the expression and purification of recombinant proteins. One popular method is the polyhistidine fusion tag, which usually consists of six consecutive histidines (6×His) added to the amino or carboxyl terminus of a protein (7). The high-affinity binding of 6×His to metal-chelate resins allows easy purification of 6×His-tagged proteins under both nondenaturing and denaturing conditions (6,8,15). Purified under nondenaturing conditions, 6×His-tagged proteins have been shown to maintain native conformations and biological activity (8), and the 6×His tag does not alter the activity of such recombinant proteins as endonuclease *EcoRV* (11) and human serum response factor (8). One producer of a 6×His-based expression system (Qiagen, Valencia, CA, USA) states in the technical manual, "the 6×His tag is very small and does not interfere with the structure or function of the recombinant protein". However, we find that the addition of a 6×His tag to a recombinant protein increases the protein's capacity for binding heparan sulfate.

Sp17, a 17.5-kDa protein expressed on acrosome-reacted sperm, promotes sperm-egg adhesion by interacting with carbohydrates in the oocyte zona pellucida (10). In vitro analysis indicates Sp17 binds dextran sulfate and perhaps other sulfated carbohydrates such as heparan sulfate (13). We recently discovered that malignant plasma cells (myeloma cells) express Sp17 on their surface (9). To enable us to investigate the role of Sp17 in heparan sulfate-mediated cell-cell adhesion of myeloma cells, we expressed two forms of recombinant Sp17 (rSp17) with different purification tags at the N-terminus: glutathione-S-transferase (GST)-rSp17 and 6×His-rSp17. The GST tag is removed via a thrombin cleavage site allowing for purification of rSp17 with no tag, while 6×His-rSp17 retains its

amino acid tag after purification. Preliminary experiments suggested that the 6×His tag interferes with the heparan sulfate-binding capacity of rSp17. Thus, to determine the extent of interference by the 6×His tag, we compared the ability of rSp17 and 6×His-rSp17 to interact with heparan sulfate.

To express GST-rSp17 for the purification of rSp17 alone, the 456-bp open reading frame of human Sp17 was amplified by RT-PCR from the total RNA of a human plasma cell leukemia line, ARH-77 (3) (ATCC, Manassas, VA, USA), then ligated in-frame into the pGEX-4T-2 vector (Amersham Biosciences, Piscataway, NJ, USA) 3' to the sequence encoding the GST gene and thrombin cleavage site. The Sp17 cDNA insert was sequenced for accuracy, and GST-rSp17 was expressed in *E. coli* strain BL21 (Amersham Biosciences). After purification under nondenaturing

conditions using glutathione-Sepharose® beads (Amersham Biosciences), rSp17 was released from the GST-rSp17-glutathione bead complex with thrombin. The thrombin was then removed from the released rSp17 using benzamidine-Sepharose (Amersham Biosciences). SDS-PAGE analysis of thrombin-released rSp17 showed one band at the appropriate size (not shown), and western analysis with Sp17 antiserum (generously provided by Dr. Michael O'Rand) confirmed the band to be rSp17 (9). To express and purify 6×His-rSp17, the cDNA of Sp17 was amplified by PCR and ligated in-frame into the pQE-30 vector (Qiagen) 3' to the sequence encoding the 6×His tag. Before expressing the 6×His-rSp17 in *E. coli* strain M15, the Sp17 cDNA insert was sequenced to confirm accuracy. The 6×His-rSp17 was purified under nondenaturing conditions by affinity chro-

Figure 1. A 6×His-tag promotes the binding of rSp17 to cell surface heparan sulfate. 6×His-rSp17 or rSp17 (50 µg/mL) was incubated with myeloma cell line ARP-1, and the level of cell surface Sp17 was determined by immunofluorescent staining and flow cytometric analysis. Data are expressed as a percent of control, where the control is the value of Sp17 staining in the absence of rSp17 or 6×His-rSp17. Before the addition of the recombinant proteins, some cells were treated with heparitinase (H'ase) to remove heparan sulfate chains. Approximately three times more 6×His-rSp17 than rSp17 binds to untreated cells. Removal of heparan sulfate decreases binding of both 6×His-rSp17 and rSp17, demonstrating that both are attaching to cells via heparan sulfate. Values represent the mean of three experiments ± standard error.

Benchmarks

matography using metal-chelating adsorbent nickel-NTA agarose (Qiagen). SDS-PAGE analysis showed one major band with a few minor bands, and western analysis confirmed the bands to be rSp17 (not shown).

To determine whether the 6×His tag alters the binding of rSp17 to cell surface heparan sulfate, rSp17 or 6×His-rSp17 (50 µg/mL) was incubated with the myeloma cell line ARP-1 (4) that expresses syndecan-1, a cell surface heparan sulfate-bearing proteoglycan. Some cells were pretreated with heparitinase (Seikagaku America, Falmouth, MA, USA), an enzyme that strips heparan sulfate from the cell surface. Sp17 levels were determined by flow cytometry after staining with a well-characterized rabbit Sp17 antiserum (13) (Figure 1). Following the addition of rSp17, the level of detectable Sp17 at the cell surface increases over 2-fold. In

contrast, addition of 6×His-rSp17 increases cell surface Sp17 over 7-fold. Removal of heparan sulfate dramatically reduces the amount of bound rSp17 and 6×His-rSp17, indicating that both are binding to cells via heparan sulfate. These data demonstrate that the 6×His tag dramatically increases the ability of 6×His-rSp17 to bind to cells via heparan sulfate.

Previous work from our laboratory demonstrates that cell-surface heparan sulfate mediates cell-cell adhesion of myeloma cells (14), and recently published work shows that rSp17 promotes heparan sulfate-mediated cell-cell adhesion (9). Thus, to further evaluate the interaction of 6×His with heparan sulfate, we compared the ability of rSp17 and 6×His-rSp17 to promote heparan sulfate-mediated cell-cell adhesion. ARP-1 cells were incubated with 100 µg/mL rSp17 or 6×His-rSp17. As con-

trols, cells were either untreated or incubated with 100 µg/mL recombinant GST (rGST) that had been expressed in, and purified from, *E. coli*. After 2 h on a gyratory shaker (100 revolutions/min) at 37°C, cells were evaluated microscopically for the number of cells in aggregates of three or more (Figure 2). Addition of rSp17 increases the extent of cell aggregation significantly ($P < 0.001$). In comparison, 6×His-rSp17 dramatically increases the extent of cell aggregation significantly more than rSp17 (rSp17 vs. 6×His-rSp17; $P < 0.002$). Treatment of cells with heparitinase before the addition of 6×His-rSp17 decreases cell aggregation to control levels, indicating that 6×His-rSp17 interacts with heparan sulfate to mediate cell-cell adhesion. Addition of control protein rGST has no effect on aggregation. These data demonstrate that the 6×His tag dramatically increases 6×His-rSp17 interaction with heparan sulfate and, in doing so, enhances the ability of 6×His-rSp17 to promote cell-cell adhesion.

In this study, two forms of recombinant Sp17 (rSp17 and 6×His-rSp17) were expressed and tested for their ability to (i) bind cells via heparan sulfate and (ii) promote heparan sulfate-mediated cell-cell adhesion. Three times more 6×His-rSp17 than rSp17 binds heparan sulfate on ARP-1 cells, and 6×His-rSp17 increases heparan sulfate-mediated cell-cell adhesion of myeloma cells two times more than rSp17. These data demonstrate that the 6×His tag increases the capacity of rSp17 to interact with heparan sulfate and, in doing so, alters rSp17 adhesive function.

Recent work by Bauman and Church (1) lends strong support to our report. When recombinant heparin cofactor II (rHCII) was expressed with a 6×His tag at the carboxyl terminus, the 6×His endowed the rHCII with increased heparin binding. Additionally, the 6×His tag augmented the ability of rHCII to inhibit thrombin in the presence of heparin. Furthermore, their data support the concept that the 6×His tag induced these functional changes in rHCII by altering the protein's conformation (1). In light of their report, it is plausible that the 6×His tag, when present on the rSp17, increases binding of heparan sulfate to Sp17 by altering the

Figure 2. A 6×His tag increases the ability of rSp17 to promote heparan sulfate-mediated cell-cell adhesion. ARP-1 cells were incubated at 37°C with 100 µg/mL rSp17, 6×His-rSp17, or control rGST. Before the addition of 6×His-rSp17, some cells were treated with heparitinase (H'ase) to remove heparan sulfate chains. After 2 h of incubation, cells were evaluated microscopically for the number of cells in aggregates of three or more. Although the addition of both rSp17 and 6×His-rSp17 increases the extent of aggregation of cells, 6×His-rSp17 increases aggregation significantly more than rSp17 ($P < 0.002$). Removal of heparan sulfate abolishes the ability of 6×His-rSp17 to promote aggregation. Values represent the mean of a minimum of three experiments $\pm$ standard error.

Benchmarks

protein's conformation to expose additional heparan sulfate binding sites.

Another possibility is that the 6×His itself binds heparan sulfate. Heparan sulfate-protein interactions are predominantly electrostatic in nature, with the negatively charged sulfate groups on the heparan sulfate chains interacting with clusters of basic amino acids within a protein. Some heparan sulfate-binding proteins contain linear clusters of basic amino acids (2,5). Since histidine is a basic amino acid, a linear arrangement of six consecutive histidines may well constitute a heparan sulfate-binding site.

The fact that 6×His can alter the heparan sulfate-binding capacity of a protein should be given serious consideration when choosing a strategy for the expression of recombinant proteins, especially if the protein is to be tested in vivo and in vitro with intact cells. Heparan sulfate is ubiquitously expressed in extracellular matrices and on cells. Secreted heparan sulfate proteoglycans, such as perlecan, are important constituents of basement membranes and

extracellular matrices. Most mammalian cells express at least one of the two major cell-surface heparan sulfate proteoglycan families, syndecans and glypicans. Furthermore, members of the syndecan family are shed from the cell surface into extracellular spaces where this soluble form of syndecan can bind and act as reservoirs for heparan sulfate-binding molecules such as growth factors (2,12). Thus, abundant heparan sulfate is present in both in vivo and in vitro systems where intact cells are present. Testing a 6×His-tagged protein in such heparan sulfate-rich systems may result in unintended and unwanted interactions. The tagged protein could bind via heparan sulfate to cells, extracellular matrices, or even soluble shed syndecan to form molecular complexes of 6×His-tagged protein, syndecan, and growth factors. Any of these interactions have the potential to produce erroneous experimental results.

In summary, we present evidence that the 6×His tag is not as inert as previously believed—it increases the heparan sulfate-binding capability of a recombinant protein and, in doing so, increases the protein's ability to promote cell-cell adhesion.

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