Constrained Cell Recognition Peptides Engineered into Streptavidin

ARTICLE *in* BIOTECHNOLOGY PROGRESS · DECEMBER 1998

Impact Factor: 2.15 · DOI: 10.1021/bp990043n · Source: PubMed

3 AUTHORS, INCLUDING:

Todd C. McDevitt
Gladstone Institutes
82 PUBLICATIONS **2,023** CITATIONS
SEE PROFILE

Kjell Nelson
University of Maryland, College Park
16 PUBLICATIONS **1,550** CITATIONS
SEE PROFILE

Constrained Cell Recognition Peptides Engineered into Streptavidin

Todd C. McDevitt,† Kjell E. Nelson,† and Patrick S. Stayton*

Department of Bioengineering, University of Washington, Seattle, Washington 98195

Streptavidin is widely used as an adaptor molecule in diagnostics, separations, and laboratory assay applications. We have here engineered cell adhesive peptides into the three-dimensional scaffolding of streptavidin to convert streptavidin into a functional protein. The mutations did not alter refolding or tetramer assembly and the slow biotin dissociation rate of wild-type streptavidin was retained. The peptide targets were hexapeptide sequences derived from osteopontin and fibronectin that contain the RGD cell adhesion sequence. Cell binding assays directly demonstrated that rat aortic endothelial cells and human melanoma cells adhered to surfaces coated with either of the two RGD streptavidin mutants in a dose-dependent fashion. Wild-type streptavidin displayed no significant cell binding activity. Inhibition studies with soluble RGD peptides confirmed that the cell adhesion was RGD mediated. Further inhibition studies with antibodies directed against $\alpha_v\beta_3$ demonstrated that the RGD−streptavidin interaction was primarily mediated by this integrin with melanoma cells. These results demonstrate that peptide recognition sequences can be engineered into accessible surface regions of streptavidin without disrupting biotin binding properties. This approach to introducing secondary functional activities into streptavidin may improve streptavidin's utility in existing applications or provide new technology opportunities.

Introduction

Streptavidin is a commonly used molecular adaptor in many diagnostic, affinity separations, and drug targeting applications. Its utility arises from the unique biotin binding properties of the four binding sites ($K_a = 10^{13-15}$ M^{-1}) and their dyad symmetry that creates oppositely aligned surfaces for binding biotinylated molecules or coatings. The genetic engineering of streptavidin has provided new opportunities to tailor the protein for new or improved technologies. Streptavidin fusion proteins have been created with a variety of proteins such as protein A, metallothionein, single-chain antibodies, luciferase, and green fluorescent protein (*1*−*5*). These fusion proteins are intended to improve diagnostic and therapeutic applications which require conjugation of multiple proteins or moieties through dual ligand recognition capabilities.

We have here investigated a complementary approach toward re-engineering streptavidin to contain second functional domains. Specifically, our goal was to engineer functional peptide sequences into the streptavidin three-dimensional scaffolding at defined surface locations. We have utilized the Arg-Gly-Asp (RGD) cell adhesive sequence as an initial target, but the peptide sequences could represent a large number of functional activities. This particular motif is a well-characterized peptide sequence responsible for integrin-mediated cell adhesion that is found in fibronectin and many other extracellular matrix and matricellular proteins. Proteins without a native RGD domain have previously been genetically engineered to incorporate an RGD site that confers cell adhesive properties (*6*−*11*). Here we report the design of two streptavidin mutants that incorporate the RGD sequence and flanking residues from fibronectin (FN-SA) and osteopontin (OSN-SA), respectively (Figure 1). These RGD streptavidin mutants are bifunctional proteins that retain wild-type biotin dissociation rates, yet also mediate cell adhesion in an RGD-dependent manner. Streptavidin itself may thus be used as both an adaptor and a biological effector, which may provide opportunities for improving diagnostics, separations, and drug targeting applications.

Materials and Methods

Construction and Production of Mutants. 60mer oligonucleotides were initially purchased from Integrated DNA Technologies (IDT) and 5′ phosphorylated with T4 kinase (Gibco BRL). The FN-SA cassette was obtained by annealing the following complementary strands: 5′CTAGGTACGTTCTGACCGGTCGTTACGACTCCGCTCCGGGTCGTGGTGACTCCCCGGGTT3′ and 5′CCGGAACCCGGGGAGTCACCACGACCCGGAGCGGAGTCGTAACGACCGGTCAGAACGTAC3′. The OSN-SA cassette was constructed with the following strands: 5′CTAGGTACGTTCTGACCGGTCGTTACGACTCCGCTCCGGGTCGTGGTGACTCCGTTGGTT3′ and 5′CCGGAACCAACGGAGTCACCACGACCCGGAGCGGAGTCGTAACGACCGGTCAGAACGTAC3′. The previously described streptavidin construct in pUC18 was digested with restriction enzymes XbaI and BspEI (New England

† Co-first authors.

* Corresponding author. Address: Department of Bioengineering, Box 352125, University of Washington, Seattle, WA 98195. Telephone: 206-685-8148. Fax: 206-685-8256. E-mail: stayton@bioeng.washington.edu.

10.1021/bp990043n CCC: $18.00
Published on Web 05/07/1999

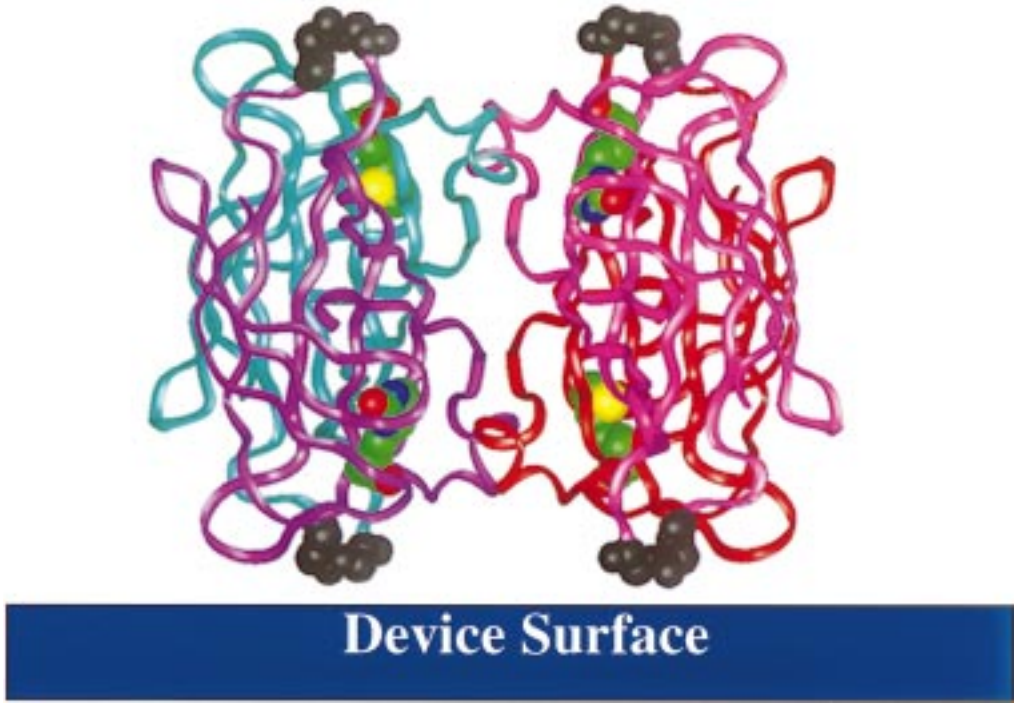


Figure 1. Schematic model of the streptavidin tetramer structure and its orientation on a biotinylated surface. Black atoms represent the RGD residues, and biotin is represented as the gray CPK surfaces. The model does not attempt to predict the precise structure of the loop, but simply to depict the spatial relationships of biotin and the RGD mutations.

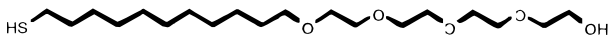
Biolabs) to create complementary ends for the annealed cassettes, which were subsequently ligated into the plasmid DNA (*12*). The ligation products were transformed into NovaBlue competent cells (Novagen). The 60mer oligos contained a single nucleotide mutation which abolished the XbaI site from the streptavidin gene in order to screen for the mutants. Successful production of the mutant sequences was confirmed by fluorescent dye terminator cycle PCR sequencing. The FN-SA and OSN-SA gene constructs were subsequently subcloned into the pET21a expression plasmid and transformed into BL21(DE3) (Novagen) competent cells in preparation for large-scale streptavidin expression. The FN-SA and OSN-SA constructs in pET21a were expressed and purified as previously described (*13*).

Protein Analysis. 10−20% Tris-Gly SDS-PAGE gels (Novex) were run with boiled and unboiled samples of the mutants, wild-type streptavidin, and a Kaleidoscope molecular weight marker to characterize the oligomeric state of the proteins. In addition, a native PAGE gel analysis was performed by omitting SDS from the running buffer. Mass spectrometry was performed on samples of the two streptavidin mutants by electron spray ionization mass spectrometry (Micromass Quattro II tandem quadrupole mass spectrometer). The protein samples were prepared by dialyzing overnight versus distilled water and then boiled for 30 min in 25% methanol and 1% formic acid immediately prior to being run on the mass spectrometer. Off-rate measurements were performed at 25 °C as previously described to quantitatively compare the biotin binding properties of the mutants to wild-type streptavidin (*13*).

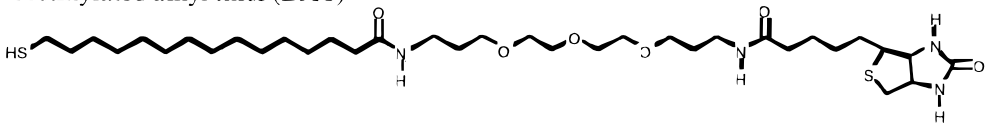
Cell Adhesion. Cell adhesion assays were performed as previously described (*14*). Briefly, rat aortic endothelial cells were cultured in MDCB131 media (Gibco BRL) with 10% fetal bovine serum and used between passages 20−25. Wild-type and RGD streptavidin mutants were incubated in 96-well polystyrene plates overnight at 4 °C at various concentrations and then blocked with 1% BSA in PBS for 1 h at 37 °C prior to the plating of cells ($n = 3$). Alternatively, biotinylated BSA was incubated in the 96 wells overnight at 4 °C, blocked with 1% BSA in PBS for 1 h at 37 °C, and then streptavidin (wild-type or RGD) was incubated in the wells for 1 h at 37 °C. The protein solution was aspirated and the wells were rinsed with sterile PBS. A total of 50 000 cells in serum-free media containing 0.1% BSA were plated onto each well and placed in a humidified incubator for 1 h at 37 °C with 5% CO_2. The media was then removed, and the wells were rinsed gently twice with warm PBS (37 °C) containing Ca^{2+} and Mg^{2+}. The adherent cells were fixed with 4% paraformaldehyde (PFA) for 5 min and stained with 0.5% toluidine blue in 4% PFA for another 5 min at room temperature before the wells were rinsed by immersing the plate into a large bowl of tap water. The plate was dried by blotting the inverted plate onto paper towels, and adherent cells were lysed with 1% SDS to release the dye prior to reading the absorbance of the wells with a plate reader at 595 nm. The inhibition assay was performed with the same protocol as for the cell adhesion assay except that the cells were incubated with the peptides for 15 min at room temperature prior to being plated onto the protein-coated wells. Synthetic hexapeptides GRGDSP, GRGESP, and GRADSP were purchased from Gibco BRL.

Melanoma cell adhesion assays on streptavidin coated polystyrene dishes were performed as described above for endothelial cell adhesion assays, except that DMEM with 0.1% BSA and 10 mM HEPES was used as the culture medium and 2×10^5 cells were added to each well. Moα_v cells that express high levels of $\alpha_v\beta_3$ integrin were derived from M21 melanoma cells. These cells were generously provided by Dr. Cecilia Giachelli (University of Washington). Adhesion assays were also performed on self-assembled monolayers constructed on 24-well tissue-culture polystyrene dishes. The dishes were evaporated

(1-Mercaptoundec-11-yl)tetra(ethylene glycol) (PEG)



Biotinylated alkyl thiol (BAT)



with ~400 Å gold, then incubated with a 0.1 mM ethanolic thiol solution of 20% biotinylated alkanethiol (BAT) and 80% (1-mercaptoundec-11-yl)tetrakis(ethylene glycol) (PEG) overnight. The wells were rinsed three times with 100% ethanol, blown dry with nitrogen, then incubated with 0.05 mg/mL of streptavidin in PBS (wild-type or mutant) at 37 °C for 1 h with side−side rocking. The protein solution was then removed, and the wells were rinsed three times with PBS before the addition of cells. Antibody inhibition assays were performed essentially identically as for the peptide inhibition assays. LM609 and control IgG antibodies were obtained from Chemicon International, Inc. (CA), and were used at 1:1000 dilution (10 μg/mL).

Results

Characterization of Streptavidin Mutants. Wild-type streptavidin runs as a tetramer on denaturing SDS-PAGE gels if the samples are not boiled prior to electrophoresis. Both mutants exhibited faint bands at the tetramer molecular weight under these conditions, with the primary band running at the monomer mass. Native PAGE analysis indicated that the mutant proteins do migrate as tetramers, demonstrating that the proteins exist as tetramers in the absence of high SDS concentrations. Mass spectrometry provided accurate mass measurements for both of the streptavidin mutants which have calculated molecular weights of 13 554 for FN-SA and 13 556 for OSN-SA. The measured molecular weights of 13 551 for FN-SA and 13 553 for OSN-SA are within experimental error of the predicted masses. To determine whether the altered sequences affect the biotin binding site, we have measured the biotin off-rates, which are very sensitive to structural perturbations. At 25 °C, the k_{off} for wild-type is 3.3×10^{-6}/s compared to 3.28×10^{-6}/s for FN-SA and 3.13×10^{-6}/s for OSN-SA.

Cell Adhesion. Adhesion assays with rat aortic endothelial cells and human melanoma cells were performed with both of the RGD mutants and wild-type streptavidin adsorbed to 96-well polystyrene wells. Wild-type streptavidin did not support cell adhesion, whereas both of the RGD streptavidin mutants displayed high cell binding activities. Endothelial cell adhesion was also supported when the RGD mutants were immobilized via biotinylated BSA that had been preadsorbed onto the polystyrene wells. To assess cell adhesion, the concentration dependence was determined in titration assays where the RGD streptavidin concentration was varied (Figure 2). For both the FN-SA and OSN-SA mutants, maximum endothelial cell adhesion was observed at a solution protein concentration of ca. 100 nM (5 μg/mL). Statistical analyses comparing the two mutants suggested that any differences in cell binding activity were at the limit of significance. Wild-type streptavidin does not display any cell adhesion above control levels. This was also confirmed by visually inspecting the wells with a phase-contrast microscope prior to introducing the fixative and cell stain. A high number of cells remained adherent to the wells with the RGD streptavidin mutants, whereas very few cells remained in the wells coated with wild-type streptavidin. To confirm that the observed cell adhesion was directly RGD-dependent, inhibition studies were conducted with GRGDSP, GRGESP, and GRADSP peptides. The RGE and RAD control peptides had no noticeable inhibitory effect on cell adhesion, but the RGD peptide inhibited cell adhesion in a dose-dependent manner (Figure 3).

Comparable cell adhesion and inhibition results were obtained using human melanoma cells in addition to the endothelial cells. Figure 4 demonstrates that the RGD mutants show strong cell adhesive activity and that this activity can be inhibited by the soluble RGD peptide. The cell adhesive activity can be inhibited by an anti-$\alpha_v\beta_3$ integrin monoclonal antibody but not by a control non-specific antibody (Figure 5). These results thus demonstrate that the RGD sequence mediates cell adhesion, and that these sequences interact specifically with the $\alpha_v\beta_3$ integrin present on the melanoma cells.

Discussion

We have here demonstrated that cell adhesive peptides can be incorporated directly into the streptavidin structural scaffolding. RGD sequences are commonly found at the apex of a β-turn loop, such as the type III β-turn for fibronectin (*15*). The antiparallel β strands provide a constrained framework for the RGD loop. Cyclical peptides, which are comparable to loop structures, can demonstrate an enhanced activity over linear peptides of the same amino acid sequence (*16*). An exposed loop of the streptavidin molecule between opposing β strands was thus chosen for the site of the RGD mutations (Figure 1). This loop lies near the binding site, but does not involve any direct contact residues, and is on the symmetry-related surface which is maximally exposed to solution when streptavidin is bound to biotinylated surfaces. Our initial design for introducing the RGD sequence into streptavidin substituted an Arg and Gly for residues Ala and Thr (65 and 66) before a naturally occurring Asp residue (67), but this mutant did not promote cell adhesion. Two subsequent mutants included additional flanking residues mimicking those found in fibronectin and osteopontin. A Gly was inserted between residues 64 and 65 of streptavidin, and a Ser and either Val or Pro (corresponding to fibronectin and osteopontin, respectively) were placed between amino acids 67 and 68.

The additional flanking residues may increase the exposure of the loop and/or sterically optimize the adhesive sequences in a more favorable orientation. The flanking residues surrounding the RGD sequence also play a role in determining the adhesive activity of the peptide, as well as the specificity for individual integrins (*17*). Nearly half of the known integrins display an RGD ligand binding dependence, and certain integrins require

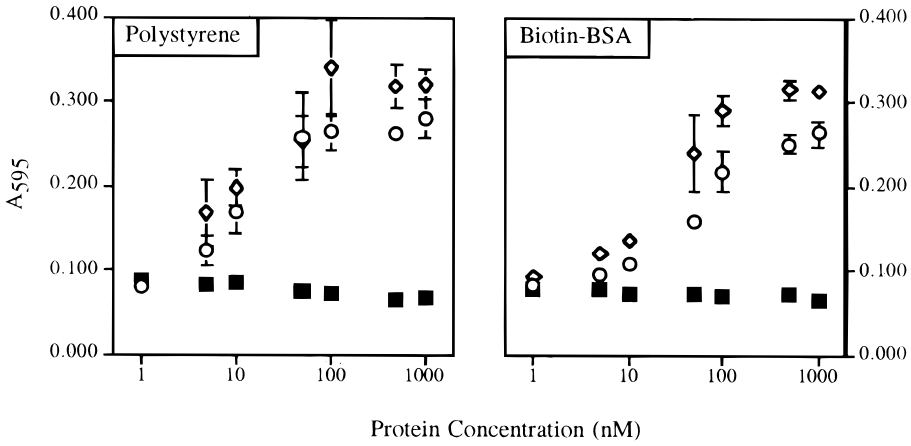


Figure 2. Concentration dependence of FN-SA- and OSN-SA-mediated endothelial cell adhesion. FN-SA (diamonds) and OSN-SA (circles) were coated on either polystyrene or biotin-BSA adsorbed onto polystyrene and compared to wild-type streptavidin (black squares). Some standard deviation values were too small to be depicted.

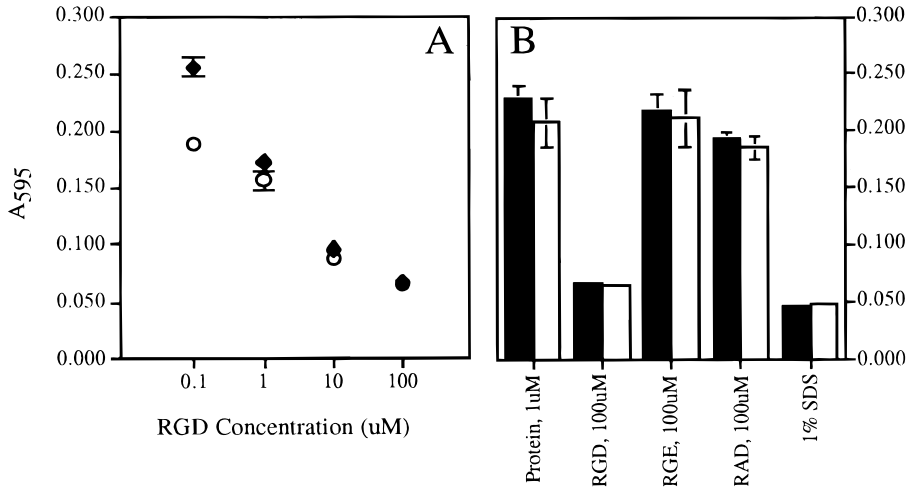


Figure 3. RGD peptide inhibition of FN-SA- and OSN-SA-mediated endothelial cell adhesion: (A) inhibition of FN-SA (black diamonds) or OSN-SA (white circles) with soluble GRGDSP peptide; (B) comparison of GRGDSP, GRGESP, and GRADSP soluble peptide inhibition of FN-SA (black bars) or OSN-SA (white bars). Some standard deviation values were too small to be depicted.

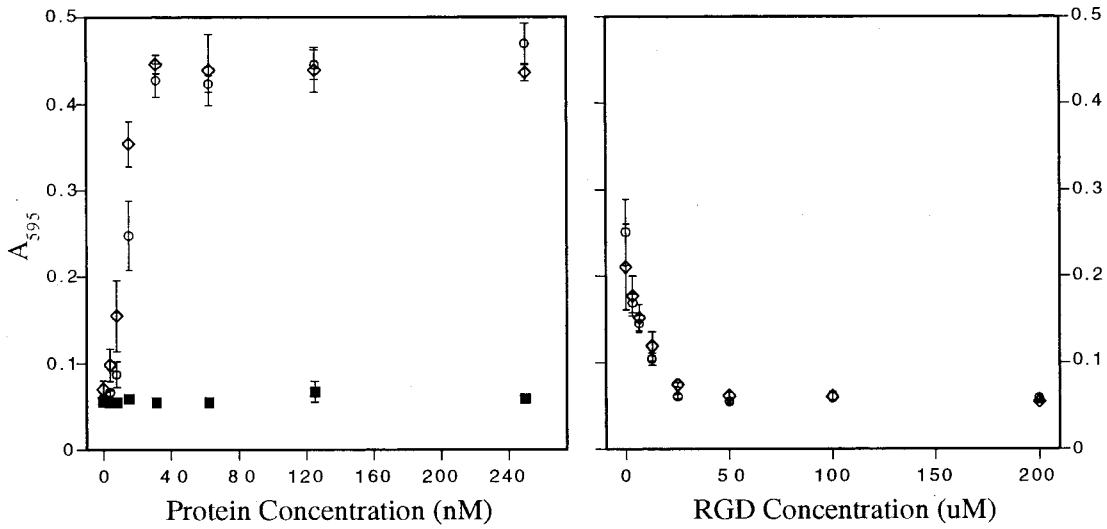


Figure 4. Melanoma cell adhesion to polystyrene dishes coated with FN-SA (diamonds), OSN-SA (circles), and wild-type streptavidin (black squares). The left-hand plot displays dose-dependent cell adhesion at various coating concentrations, while the right-hand plot displays dose-dependent inhibition of adhesion in the presence of various concentrations of soluble RGD peptides. The streptavidin coating concentration in the experiment shown on the right was kept constant at 200 μM (see the Materials and Methods). Error bars indicate $\pm$ standard deviation ($n = 3$).

specific flanking sequences that differ among the various extracellular and matricellular proteins (*18*). The different flanking residues are believed to alter the conformation of the RGD domain, thus providing a structural basis for integrin specificity. Previous reports have suggested that an RYD site in streptavidin can direct association

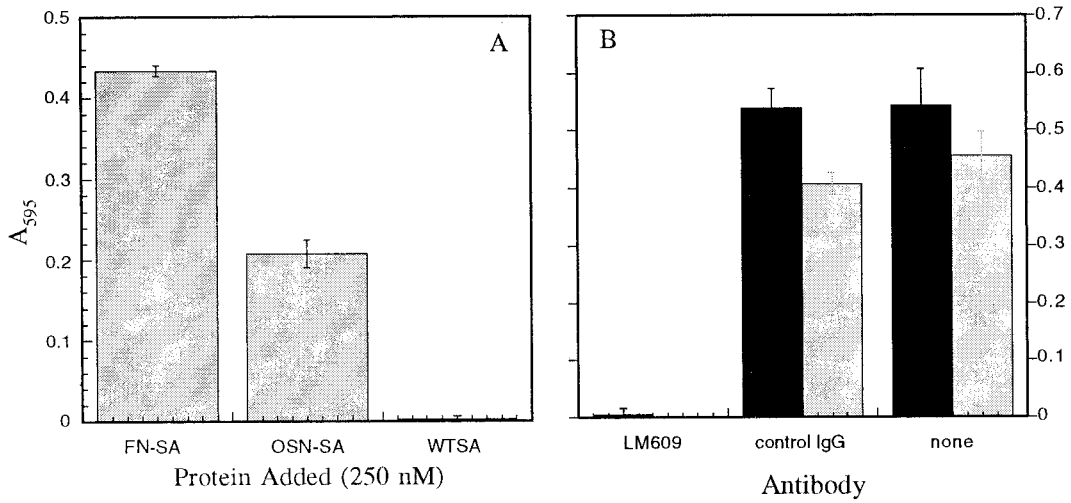


Figure 5. Melanoma cell adhesion to mixed self-assembled monolayers (SAMs) containing biotin and poly(ethylene glycol) thiols. Part a compares cell adhesion to SAMs coated with FN-SA, OSN-SA, and wild-type streptavidin. Part b demonstrates integrin specificity of melanoma cell adhesion by preincubating the cell suspension in the presence of 10 μg/mL anti-$\alpha_v\beta_3$ integrin complex (LM609) or isotype-matched nonimmune control antibody, FN-SA (black bars) or OSN-SA (grey bars). Error bars indicate ± standard deviation ($n = 3$). Results are representative of at least two independent experiments.

with Chinese hamster ovary cells, M4 murine melanoma cells, ADP-activated platelets, and $CD4^+$ T lymphocytes cells and that soluble RGD peptides will compete with the native protein for integrin engagement (*19*−*21*). We did not observe this activity with the endothelial and melanoma cells used in this study, or in other studies with a variety of cell types (unpublished results).

Streptavidin is used as an adapter in a wide variety of diagnostic, separation, and drug targeting applications. It is thus interesting that streptavidin itself could be used in some applications as both an adapter and as an effecter, which could provide opportunities to simplify and improve many of these applications. The cell adhesive streptavidin derivatives described here are directly useful as tissue culture coatings to immobilize cells. We have also demonstrated that the mutants can be used in self-assembled monolayer systems for controlled immobilization on a non-fouling poly(ethylene glycol) background surface. Integrin-mediated cell adhesion is closely tied to important signaling pathways that control cell behavior, and these streptavidin derivatives might also be used to stabilize cell phenotype through the incorporation of RGD or related sequences that mediate specific receptor-mediated engagement and biological responses. It is likely that a number of different peptide sequences with a variety of receptor specificities can be incorporated into this loop or other surface locations, which could provide additional opportunities for cell separations and diagnostics applications. The incorporation of receptor specific peptide sequences could also provide opportunities to evaluate streptavidin as both the targeting and capture agent in pretargeting drug delivery applications utilizing biotinylated therapeutics.

Acknowledgment

We thank Dr. Cecilia Giachelli and Dr. Marta Scatena for the generous donation of rat aortic endothelial cells and melanoma cells and Drs. Laura Smith and Owen Lawrence for technical assistance. This work was supported by the NIH (DK49655), by the NSF through the University of Washington Engineered Biomaterials Engineering Research Center, and by a NSF Molecular Bioengineering training grant fellowship to K.E.N.

References and Notes

(1) Sano, T.; Cantor, C. R. A streptavidin-protein A chimera that allows one-step production of a variety of specific antibody conjugates. *Bio/Technology* **1991**, *9*, 1378−1381.

(2) Sano, T.; Glazer, A. N.; Cantor, C. R. A streptavidin-metallothionein chimera that allows specific labeling of biological materials with many different heavy metal ions. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 1534−1538.

(3) Dubel, S.; Breitling, F.; Kontermann, R.; Schmidt, T.; Skerra, A.; Little, M. Bifunctional and multimeric complexes of streptavidin fused to single chain antibodies (scFv). *J. Immunol. Methods* **1995**, *178*, 201−209.

(4) Karp, M.; Lindqvist, C.; Nissinen, R.; Wahlbeck, S.; Akerman, K.; Oker-Blom, C. Identification of biotinylated molecules using a baculovirus-expressed luciferase-streptavidin fusion protein. *Biotechniques* **1996**, *20*, 452−459.

(5) Oker-Blom, C.; Orellana, A.; Keinanen, K. Highly efficient production of GFP and its derivatives in insect cells for visual in vitro applications. *FEBS Lett.* **1996**, *389*, 238−243.

(6) Maeda, T.; Oyama, R.; Ichihara-Tanaka, K.; Kimizuka, F.; Kato, I.; Titani, K.; Sekiguchi, K. A novel cell adhesive protein engineered by insertion of the Arg-Gly-Asp-Ser tetrapeptide. *J. Biol. Chem.* **1989**, *264*, 15165−15168.

(7) Hashino, K.; Shimojo, T.; Kimizuka, F.; Kato, I.; Maeda, T.; Sekiguchi, K.; Titani, K. Engineering of artificial cell adhesion proteins by grafting the Arg-Gly-Asp cell adhesive signal to a calpastatin segment. *J. Biochem.* **1992**, *112*, 547−551.

(8) Barbas, C. F., III; Languino, L. R.; Smith, J. W. High-affinity self-reactive human antibodies by design and selection: Targeting the integrin ligand binding site. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 10003−10007.

(9) Rossi, F.; Billeta, R.; Ruggeri, Z.; Zanetti, M. Engineered idiotypes. Immunochemical analysis of antigenized antibodies expressing a conformationally constrained Arg-Gly-Asp motif. *Mol. Immunol.* **1995**, *32*, 341−346.

(10) Smith, J. W.; Tachias, K.; Madison, E. L. Protein loop grafting to construct a variant of tissue-type plasminogen activator that binds to platelet integrin $\alpha_{IIb}\beta_3$. *J. Biol. Chem.* **1995**, *270*, 30486-30490.

(11) Yamada, T.; Song, H.; Inaka, K.; Shimada, Y.; Kikuchi, M.; Matsushima, M. Structure of a conformationally constrained Arg-Gly-Asp sequence inserted into human lysozyme. *J. Biol. Chem.* **1995**, *270*, 5687−5690.

(12) Chilkoti, A.; Tan P. H.; Stayton, P. S. Site-directed mutagenesis studies of the high-affinity streptavidin−biotin complex: Contributions of tryptophan residues 79, 108, and 120. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 1754−1758.

(13) Klumb, L. A.; Chu, V.; Stayton, P. S. Energetic roles of hydrogen bonds at the ureido oxygen binding pocket in the streptavidin−biotin complex. *Biochemistry* **1998**, *37*, 657−7663.
(14) Liaw, L.; Almeida M.; Hart, C. E.; Schwartz, S. M.; Giachelli, C. M. Osteopontin promotes vascular cell adhesion and spreading and is chemotactic for smooth muscle cells in vitro. *Circ. Res*. **1994**, *74*, 214−224.
(15) Main, A. L.; Harvey, T. S.; Baron, M.; Boyd, J.; Campbell, I. D. The three-dimensional structure of the tenth type III module of fibronectin: An insight into RGD-mediated interactions. *Cell* **1992**, *71*, 671−678.
(16) Pierschbacher, M. D.; Ruoslahti, E. Influence of stereochemistry of the sequence Arg-Gly-Asp-Xaa on binding specificity in cell adhesion. *J. Biol Chem*. **1987**, *262*, 17294−17298.
(17) Maeda, T.; Hashino, K.; Oyama, R.; Titani, K.; Sekiguchi, K. Artificial cell adhesive proteins engineered by grafting the Arg-Gly-Asp cell recognition signal: Factors modulating the cell adhesive activity of the grafted signal. *J. Biochem*. **1991**, *110*, 381−387.
(18) D'Souza, S. E.; Ginsberg, M. H.; Plow, E. F. Arginyl-glycyl-aspartic acid (RGD): a cell adhesion motif. *Trends Biochem. Sci*. **1991**, *16*, 246−250.
(19) Alon, R.; Bayer, E. A.; Wilchek, M. Streptavidin contains an RYD sequence which mimics the RGD receptor domain of fibronectin. *Biochem. Biophys. Res. Commun*. **1990**, *170*, 1236−1241.
(20) Alon, R.; Bayer, E. A.; Wilchek, M. Cell-adhesive properties of streptavidin are mediated by the exposure of an RGD-like RYD site. *Eur. J. Cell Biol*. **1992**, *58*, 271−279.
(21) Alon, R.; Hershkoviz, R.; Bayer, E. A.; Wilchek, M.; Lider, O. Streptavidin blocks immune reactions mediated by fibronectin-VLA-5 recognition through an Arg-Gly-Asp mimicking site. *Eur. J. Immunol*. **1993**, *23*, 893−898.

Accepted March 19, 1999.

BP990043N