

A chimera of a gelatinase inhibitor peptide with streptavidin as a bifunctional tumor targeting reagent

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Abstract A chimeric protein, consisting of streptavidin fused to a cyclic decapeptide with potent inhibitory activity for matrix metalloproteinases (MMP), has been produced in *Escherichia coli* and purified. The purified chimera formed a tetramer and showed full biotin-binding ability. The chimera was also capable of both binding to MMP-2 and inhibiting its activity. Thus, both the streptavidin moiety and the decapeptide of the chimera are fully functional. This bifunctional nature of the chimera should facilitate the application of the decapeptide since the streptavidin moiety can be used as a specific conjugation site for almost any materials upon biotinylation. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Streptavidin; Biotin; Cyclic peptide; Matrix metalloproteinase

1. Introduction

A cyclic decapeptide with the sequence CTTHWGFTLC was recently isolated as a selective inhibitor of matrix metalloproteinase (MMP)-2 and MMP-9 [1], which play key roles in tumor migration and invasion [2–5]. This peptide also showed the potential ability to co-localize with tumor vasculature and suppress the growth of tumors. These characteristics suggest great potential for the CTTHWGFTLC peptide to serve as a useful reagent for tumor targeting [1,6]. To test and realize the application of the CTTHWGFTLC peptide (and other similar peptide-based targeting reagents) for tumor targeting, it is essential that the peptide can be conjugated to or labeled with other molecules without disturbing its structure and properties. However, an extensive effort is generally required for conjugation and labeling of such peptides with each partner molecule. Thus, the number of molecular species that can actually be tested as partner molecules would be limited. It would be enormously useful if such conjugation

or labeling of the peptide could be done in a simple, yet efficient and controlled manner.

In this work, we have fused, recombinantly, the CTTHWGFTLC peptide to streptavidin. Streptavidin, a tetrameric protein produced by *Streptomyces avidinii*, binds D-biotin with an extremely high affinity ($K_d \sim 10^{-14}$ M) [7,8]. The strength of the biotin binding by streptavidin and the fact that biotin can easily be incorporated into various biological materials have made the streptavidin-biotin system one of the most useful molecular tools in biological and medical sciences [9–11]. A chimeric protein consisting of the CTTHWGFTLC peptide and streptavidin, if successfully produced, should allow specific, tight conjugation of the CTTHWGFTLC peptide to almost any molecules upon biotinylation by using the streptavidin moiety as the conjugation site. This should facilitate the development of the application of the CTTHWGFTLC peptide as a tumor-targeting reagent. Here we describe the design and production of a chimera, which consists of the CTTHWGFTLC peptide with streptavidin. We show that the resulting chimera is bifunctional and possesses the abilities to bind to both MMP and biotin, derived from the CTTHWGFTLC peptide and the streptavidin moiety, respectively.

2. Materials and methods

2.1. Construction of an expression vector for an Stv-CTTHWGFTLC chimera

A double-stranded oligonucleotide (5'-CGTGC ACCAC CCACT GGGGT TTCAC CCTGT GCT-3' and 5'-GATCA GCACA GGGTG AAACC CCAGT GGGTG GTGCA CGAGC T-3'), encoding the sequence CTTHWGFTLC with a translation termination codon, was cloned between the *SacI* and *BamHI* sites of pTSA-18F, an expression vector for streptavidin-containing chimeras [12]. The resulting expression vector, pTSA-CTTHWGFTLC, encodes a chimeric protein (Stv-CTTHWGFTLC chimera) consisting of the CTTHWGFTLC peptide fused to the C-terminus of the core region of streptavidin with a four-residue linker (sequence, NSSS).

2.2. Expression and purification of the Stv-CTTHWGFTLC chimera

Expression of the Stv-CTTHWGFTLC chimera was carried out by using *Escherichia coli* strain BL21(DE3)(pLysE) [13] carrying pTSA-CTTHWGFTLC, as described [14,15]. Purification of the expressed chimera was carried out by the method described earlier [14–17] with several modifications. An inclusion body fraction was collected from cell lysates of BL21(DE3)(pLysE)(pTSA-CTTHWGFTLC), which had been incubated for 4 h after induction, and dissolved in 7 M guanidine hydrochloride (Gdn·HCl; pH 7.0), 0.5 mM EDTA, 10 mM dithiothreitol (DTT), 10 mM 2-mercaptoethanol (2-ME). The resulting solution was diluted slowly with 20 volumes of Tris-buffered saline (TBS) [150 mM NaCl, 20 mM Tris-Cl (pH 7.4)] containing 0.5 mM EDTA and 10 mM 2-ME. After the addition of Na₂CO₃ to 100

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Abbreviations: APMA, *p*-aminophenylmercuric acetate; DTT, dithiothreitol; Gdn·HCl, guanidine hydrochloride; 2-ME, 2-mercaptoethanol; MMP, matrix metalloproteinase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline

mM, the suspension was filtered through a 5- μ m Durapore membrane (Millipore). The filtrate was adjusted to pH 10.5 by the addition of NaOH and applied to a 2-aminobiotin-agarose column (Sigma) [18], equilibrated with 150 mM NaCl, 100 mM Na₂CO₃ (pH 11.0), 0.5 mM EDTA, 10 mM 2-ME. Unbound proteins were removed, and bound proteins were eluted with 2 M urea, 50 mM sodium acetate (pH 4.0), 0.5 mM EDTA, 10 mM 2-ME. The eluted protein was dialyzed against phosphate-buffered saline (PBS) [137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄ (pH 7.2)] containing 0.5 mM EDTA and 10 mM 2-ME, followed by filtration through a 5- μ m Durapore membrane. The filtrate, containing purified Stv-CTTHWGFTLC chimeras, was stored at -75°C until used. Under these reducing conditions, the CTTHWGFTLC peptide in the chimera should be in a reduced, linear form.

Mild oxidizing conditions were used to form an intra-molecular disulfide bond of the CTTHWGFTLC peptide in the chimera (circularization of the CTTHWGFTLC peptide). Purified chimeras were dialyzed against PBS containing 0.02% Tween 20 and 0.1 μM ZnCl₂. The concentration of the chimera during dialysis was adjusted to 10 $\mu\text{g}/\text{ml}$ or lower to minimize the formation of inter-molecular disulfide bonds. The dialyzed sample was centrifuged at $10000\times g$ for 30 min at 4°C , and the supernatant was used in subsequent experiments.

2.3. Biotin-binding assays

The biotin-binding ability of the Stv-CTTHWGFTLC chimera was determined by a gel filtration method [19] using PD-10 columns (Amersham Pharmacia) or an ultrafiltration method [20] using Ultra-free-MC centrifugal filtration units (Millipore).

2.4. MMP inhibition assays

The ability of the Stv-CTTHWGFTLC chimera to inhibit the activity of MMP-2 was analyzed by using β -casein as the substrate. Pro-MMP-2 (1 μg ; Oncogene Research Products) in 50 mM borate (pH 7.5), 5 mM CaCl₂, 10 μM ZnCl₂, 20% glycerol, 0.005% Brij 35 was incubated at 37°C for 2 h in the presence of 0.5 mM *p*-aminophenyl-mercuric acetate (APMA) [21]. The resulting activated MMP-2 (5 ng) was incubated with the chimera (0–12 ng) at 24°C for 1 h in 50 mM NaCl, 50 mM Tris-Cl (pH 7.5), 5 mM CaCl₂. Then, β -casein (500 ng; Sigma) was added to each mixture and incubated at 24°C for 1 h. The reaction was terminated by the addition of sodium dodecyl sulfate (SDS) to 0.2%. Each reaction mixture was analyzed by SDS-PAGE (polyacrylamide gel electrophoresis) [22], and the degradation of β -casein was used as a measure of MMP activity.

2.5. MMP-binding assays

The ability of the Stv-CTTHWGFTLC chimera to bind to MMP-2 was analyzed by using the chimera immobilized on biotin-coated microwell plates. The chimera (0–11 ng) was allowed to bind to biotin-coated microwells (Pierce Chemical), followed by washing the microwells with PBS. APMA-activated MMP-2 (20 ng) in PBS was added to each microwell and incubated at 4°C for 3 h. Unbound MMP-2 was collected by washing the microwells with PBS. Bound MMP-2 was released from the microwells by incubation at 50°C for 20 min after the addition of Gdn-HCl to 2 M. Each of the bound and unbound fractions was blotted onto polyvinylidene fluoride membrane

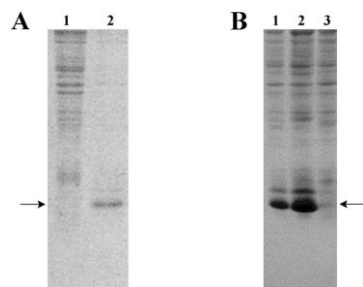


Fig. 1. Expression of the Stv-CTTHWGFTLC chimera in *E. coli*. Proteins were analyzed by SDS-PAGE and stained with Coomassie brilliant blue R. The position where the chimera migrates is shown by arrows. A: Lanes 1, total protein of uninduced cells; 2, total protein of cells at 4 h after induction. B: Lanes 1, total cell protein at 4 h after induction; 2, insoluble fraction of cell lysate; 3, soluble fraction of cell lysate.

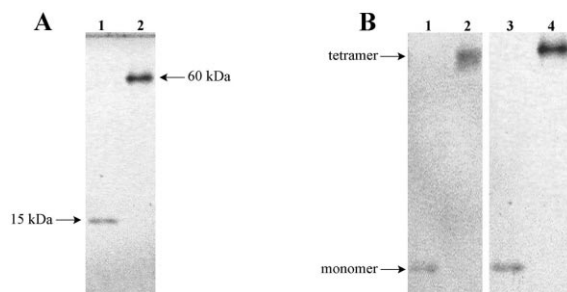


Fig. 2. SDS-PAGE of the purified Stv-CTTHWGFTLC chimera. A: Purified chimeras under reducing conditions. Lanes 1, heated for 3 min in boiling water in the presence of SDS, DTT, and 2-ME; 2, in the presence of SDS, DTT, and 2-ME without heat treatment. B: Purified chimera after oxidation. Lanes 1, with heat treatment in the presence of SDS; 2, in the presence of SDS without heat treatment; 3, with heat treatment in the presence of SDS and DTT; 4, in the presence of SDS and DTT without heat treatment.

(Millipore), and MMP-2 on the blots was analyzed by immuno-detection using a rabbit anti-MMP-2 polyclonal antibody (Sigma) with an ECL Plus Western Blotting Detection System (Amersham Pharmacia).

3. Results and discussion

3.1. Design of an Stv-CTTHWGFTLC chimera

The plasmid pTSA-18F [12] was used as a platform for the construction of an Stv-CTTHWGFTLC chimera. pTSA-18F carries a truncated streptavidin gene, encoding a core region of streptavidin [23], under a bacteriophage T7 promoter, $\Phi 10$ [13]. The coding sequence is followed by a polylinker region, which facilitates the construction of gene fusions with streptavidin. A double-stranded oligonucleotide encoding the sequence CTTHWGFTLC was inserted into the polylinker region of pTSA-18F. The resulting expression vector pTSA-CTTHWGFTLC encodes a chimeric protein, which consists of the CTTHWGFTLC peptide fused to the C-terminus of streptavidin (Stv-CTTHWGFTLC chimera; 14.1 kDa) with a four-residue linker sequence (NSSS).

Both the N- and C-termini of streptavidin are located on the surface of the molecule [24,25]. Thus, the CTTHWGFTLC peptide, fused to the C-terminus of streptavidin, should be located on the surface of the streptavidin molecule, maximizing the accessibility to MMPs. The highly hydrophilic, flexible four-residue linker, NSSS, between the CTTHWGFTLC peptide and streptavidin might also help to expose the peptide to solvent. The chimera is likely to form a tetramer due to the natural tetrameric structure of streptavidin [7,8]. Thus, each chimera molecule, consisting of four subunits, would have four CTTHWGFTLC sequences present on the surface.

Two structural characteristics of streptavidin may facilitate the circularization of the CTTHWGFTLC peptide (intra-subunit disulfide bonds) in the chimera, which is essential for its potent inhibitory activities for MMPs [1]. First, natural streptavidin has no cysteine. Thus, the streptavidin moiety should not interfere with the circularization of the CTTHWGFTLC peptide via disulfide formation. Second, streptavidin has D_2 symmetry with a pseudo-tetrahedral structure [24,25]. The formation of a disulfide bond between any two CTTHWGFTLC peptides in a single chimera molecule (inter-subunit

disulfide bonds) should not be favored due to the relatively long distance between them.

3.2. Production and purification of the Stv-CTTHWGFTLC chimera

The bacteriophage T7 expression system [13] was used to produce the Stv-CTTHWGFTLC chimera in *E. coli*. SDS-PAGE of total cell protein (Fig. 1A) shows that the Stv-CTTHWGFTLC chimera was expressed efficiently. Expressed chimeras formed insoluble inclusion bodies in host cells (Fig. 1B). Insoluble fractions were isolated from cell lysates and dissolved in Gdn-HCl under the conditions where any disulfide bonds are reduced to sulfhydryl groups. The resulting solution was diluted with TBS under reducing conditions to allow the expressed chimera to refold. Refolded chimeras were purified by 2-iminobiotin affinity chromatography [18] under reducing conditions, where the CTTHWGFTLC peptide should be in a reduced, linear form. SDS-PAGE of the purified chimera showed a single band at 15 kDa (lane 1, Fig. 2A) when the chimera was heated in boiling water in the presence of SDS, under which conditions streptavidin dissociates into subunits [26]. On SDS-PAGE of the purified chimera without heat treatment, under which conditions streptavidin maintains its tetrameric structure [26], the chimera showed a single band at 60 kDa (lane 2), indicating that the chimera forms a subunit tetramer, as predicted.

The purified Stv-CTTHWGFTLC chimera was subjected to mild oxidizing conditions to circularize the CTTHWGFTLC peptide. To minimize the formation of inter-molecular disulfide bonds, low protein concentrations (10 µg/ml or lower) were used. When higher protein concentrations were used, significant amounts of insoluble aggregates were seen, indicating the formation of extensive inter-molecular disulfide bonds. SDS-PAGE of the chimera after mild oxidation shows that the chimera subunit migrated at 15 kDa, and no subunit dimer is seen in the absence of reducing agents (lane 1, Fig. 2B). Under the conditions where the tetrameric structure of streptavidin is maintained, the chimera migrated at 60 kDa, indicating virtually no multimer formation (lane 2). These data suggest that the conditions used for the circularization of the CTTHWGFTLC peptide effectively prevented the formation of inter-molecular disulfide bonds. The migration of the oxidized chimera subunit (lane 1) is almost the same as that under reducing conditions. Thus, the addition of DTT to

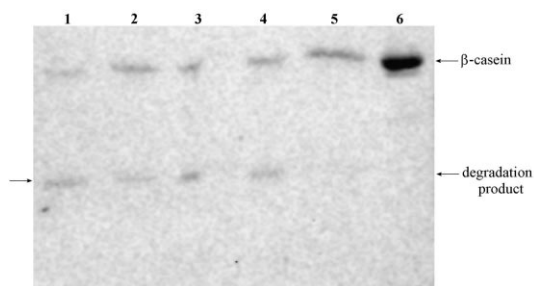


Fig. 3. Inhibition of MMP-2 activity by the Stv-CTTHWGFTLC chimera. APMA-activated MMP-2 was pre-incubated with the oxidized chimera. β -Casein was added as the substrate to each mixture, which was analyzed by SDS-PAGE for the degradation of β -casein. The amounts of the chimera used in lanes 1–5 are: 0, 0.1, 0.5, 2.4, and 12 ng, respectively. Lane 6 shows a control sample, in which no MMP-2 was included in the mixture. The positions where β -casein and its degradation product migrate are shown by arrows.

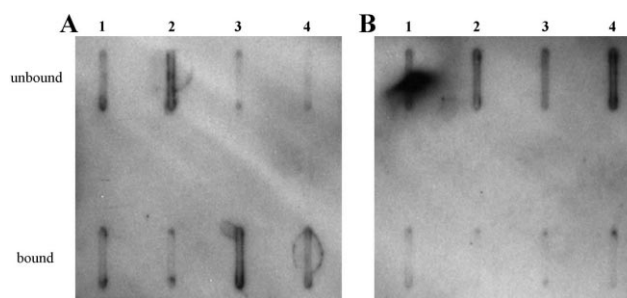


Fig. 4. Binding of MMP-2 to the Stv-CTTHWGFTLC chimera. APMA-activated MMP-2 was allowed to bind to the oxidized chimera, which had been immobilized on biotin-coated microwells. Unbound and bound MMP-2 fractions were blotted on polyvinylidene fluoride membrane and analyzed by immuno-detection using an anti-MMP-2 antibody. A: Stv-CTTHWGFTLC chimera. The amounts of the chimera used in each reaction are: wells 1, 0; 2, 0.11 ng; 3, 1.1 ng; 4, 11 ng. B: Natural streptavidin. The amounts of natural streptavidin used in each reaction are: wells 1, 0; 2, 0.25 ng; 3, 2.5 ng; 4, 25 ng.

the oxidized chimera had little effect on the migration of the chimera subunit (lane 3). In contrast, the migration of the tetramer became slightly faster after oxidization (lane 2). The addition of DTT to the oxidized chimera (lane 4) made the migration of the tetramer slower than the level seen with the chimera before oxidation. These results reveal that the overall structure of the chimera in the presence of SDS became more compact upon oxidation, suggesting that intra-subunit disulfide bonds of the CTTHWGFTLC peptide were formed efficiently under the conditions used.

3.3. Characterization of the Stv-CTTHWGFTLC chimera

The purified Stv-CTTHWGFTLC chimera was characterized for the functionality of each of the two moieties. The chimera showed a full biotin-binding ability, both before and after the circularization of the CTTHWGFTLC peptide, indicating that the fusion of the CTTHWGFTLC peptide has little, if any, effect on the biotin-binding ability of the streptavidin moiety.

The ability of the Stv-CTTHWGFTLC chimera to inhibit the activity of MMPs was investigated by using MMP-2. The chimera with circularized CTTHWGFTLC peptides was mixed with APMA-activated MMP-2 at various ratios. Then, β -casein was added to the mixtures, and the degradation of β -casein was monitored by SDS-PAGE (Fig. 3). When the chimera was mixed with activated MMP-2 at approximately 10-fold molar excess, caseinolysis was completely inhibited (lane 5). However, little or no inhibition of caseinolysis was seen when the chimera was not included in reaction mixtures or lower molar ratios of the chimera to MMP-2 were used (lanes 1–4). Natural streptavidin, used in place of the Stv-CTTHWGFTLC chimera, showed no effect on caseinolysis by MMP-2 (data not shown). These results suggest that the CTTHWGFTLC peptide in the chimera retains its inhibitory activity for MMPs. However, relatively high concentrations of the chimera were required for complete inhibition of MMP activity. This is in good agreement with a previous study on the CTTHWGFTLC peptide [1].

The Stv-CTTHWGFTLC chimera was then examined for its ability to bind to MMP-2. The chimera with circularized CTTHWGFTLC peptides was immobilized on biotin-coated microwells. Then, APMA-activated MMP-2 was allowed to

bind to immobilized chimeras, and MMP-2 in both the bound and unbound fractions was analyzed. The immobilized chimera was able to capture MMP-2, when 1.1 or 11 ng of the chimera was used for binding reactions (Fig. 4A). Almost no binding of MMP-2 was seen when the chimera was not included or only 0.11 ng of the chimera was used. The binding of MMP-2 was not seen when natural streptavidin, in place of the chimera, was immobilized, independent of the amount used (Fig. 4B). This suggests that the binding of MMP-2 to the immobilized chimera is specific and mediated by the CTTHWGFTLC peptide of the chimera. These results demonstrate that the CTTHWGFTLC peptide of the chimera retains the binding ability for MMP-2. As predicted from the MMP-2 inhibition assays above, the binding affinity of the chimera for MMP-2 appears to be relatively low, and high concentrations of the chimera may be required for efficient binding to MMP-2.

3.4. Conclusion

We have successfully produced a chimeric protein, consisting of the CTTHWGFTLC peptide with streptavidin, in which both the CTTHWGFTLC peptide and the streptavidin moiety are fully functional. The bifunctional nature of the chimera should facilitate the development of the application of the CTTHWGFTLC peptide since the streptavidin moiety allows specific, indirect conjugation of the peptide to almost any materials upon biotinylation. Additionally, recombinantly fusing to streptavidin, which serves as a specific, versatile conjugation site, could be useful as a general strategy for the characterization and application of many other peptide-based targeting reagents. The tetrameric structure of such chimeras might also enhance the binding affinity of the peptides for targets expressed in high densities on the cell surface.

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