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A chimera of green fluorescent protein with gelatinase binding and tumor targeting peptide

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ABSTRACT

Matrix metalloproteinases (MMPs) are enzymes that can hydrolyze almost all constituents of extracellular matrix. An MMP subgroup, the gelatinases, has been focused during last years, since over-expression of gelatinase A (MMP-2) and gelatinase B (MMP-9) has been linked with severe homeostasis disorders such as tumor growth, metastasis formation, and chronic inflammation. In this study, a phage display library-derived novel antigelatinolytic decapeptide, the CTT-peptide, was expressed as a carboxyl terminal, histidine-tagged fusion with the green fluorescent protein (CTT–GFP) in *Escherichia coli*. In addition, a biologically intact chimera, in which residues in the CTT-peptide critical for gelatinase binding were replaced with alanine (Ala–CTT–GFP), was constructed. The GFP-fusion proteins were purified to homogeneity with a simple one-step procedure utilizing nickel affinity chromatography. The purified chimeras were tested for their binding properties to 4β-phorbol-12,13-butyrate (PdBu) activated, MMP-9 expressing THP-1 cells, and it was demonstrated that the CTT–GFP strongly bound to the cells, whereas Ala–CTT–GFP lacked the binding ability. Furthermore, the adherence of the CTT–GFP to MMP-9 expressing cells was demonstrated to be mediated by the CTT-moiety, since the binding could be dose-relatedly inhibited with increasing concentrations of synthetic soluble CTT-peptide. In conclusion, this novel tool, combining the gelatinase binding ability of the CTT-peptide with the fluorescing property of the GFP, should clearly improve both experimental and clinical studies of the role and function of gelatinases.

Introduction

Matrix metalloproteinases (MMPs)[4] are a group of structurally related but genetically distinct zinc-dependent proteolytic enzymes capable of degrading almost all components of the extracellular matrix (ECM) and basement membranes [1,2]. MMPs can additionally modify multiple cellular and immune responses by processing various non-matrix bioactive substrates. Under normal physiological conditions MMPs have important functions in tissue remodeling, cell migration, wound healing, and embryogenesis [3]. According to their substrate specificity and structure, MMPs can be divided into several subgroups [4], one of which is represented by gelatinases, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) [5]. The gelatinases have been intensively studied because of their roles in various tissue destructive disorders, including cancer and chronic inflammatory diseases [6]. Furthermore, as gelatinases are often over-expressed in diseased tissues as compared to healthy tissues [7], and since they are mostly found on the surfaces of invading cells [8–10], the gelatinases have been considered as promising candidates for targeted drug delivery in various cancers and inflammatory diseases.

A phage display library-derived cyclic decapeptide with a sequence CTTHWGFTLC (CTT-peptide) has been shown to both bind to and inhibit the proteolytic activity of the gelatinases A and B. *In vitro*, the CTT-peptide inhibited migration of HT1080 fibrosarcoma and other tumor cell lines [11]. *In vivo*, the CTT-peptide inhibited the growth of tumors after intraperitoneal administration, and a bacteriophage expressing the CTT-peptide on its surface

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[4] *Abbreviations used:* MMP, matrix metalloproteinase; GFP, green fluorescent protein, *E. coli*, *Escherichia coli*; PdBu, 4β-phorbol 12,13-butyrate; ECM, extracellular matrix; IPTG, isopropyl-β-D-thiogalactopyranoside; RFU, relative fluorescence unit.

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efficiently targeted to tumors after intravenous injection, as studied in mice [11]. Also, liposomes harboring the CTT-peptide at their exterior showed increased binding properties into CHO, HT1080, and U937-cells, and they were also demonstrated to efficiently unload their cargo into the cells [12].

The aim of this study was to make available two novel protein chimeras for gelatinase studies. The first chimera developed is a fusion protein composed of an intact CTT-peptide fused to the green fluorescent protein (GFP), and the other a chimera of the GFP and Ala–CTT, a biologically inactive form of the CTT-peptide [13], in which the tryptophan and phenylalanine residues of the CTT-peptide have been changed into alanine moieties. In this study we demonstrate that the CTT–GFP chimera is able to bind to MMP-9 expressing cells in a CTT-peptide-dependent manner, whereas the Ala–CTT-chimera lacks this binding capability.

Materials and methods

Construction of expression vectors

A coding sequence for the CTT-peptide with a C-terminal His-tag followed by a translation termination codon was introduced into a *Sac*I/*Eco*RI restricted expression vector pGFPuv (Clontech) encoding for the brightly fluorescing "cycle 3" variant of GFP not to be mixed with the EGFP by the aid of 5́-CTGTACAA CTCATTGGGGTTTTACATTATGTAGGCCTCATCATCATCACCATCATTA AG-3́ and 5́-AATTCTTAATGATGGTGATGAT GATGAGGCCTACATAA TGTAAAACCCCAATGAGTTGTACAGAGCT-3́oligonucleotides, that after hybridization with each other formed a two-stranded coding region with single-stranded overhangs complementary to the overhangs formed by *Sac*I and *Eco*RI. The resulting plasmid pLEB633, encoding for the CTT–GFP chimera (GFPuv + CTTHWGFTLCRP at the C-terminus), was transformed into an *Escherichia coli* strain ABLE K (Stratagene). The new strain was given name ECO655. An expression vector for the negative control, the Ala–CTT–GFP chimera (GFPuv + CTTHAGATLCRP at the C-terminus), was constructed in a similar manner with 5́-CTGTACAAC TCATGCTGGTGCTACATTATGTAGGCCTCATCATCATCACCATCATTA AG-3́ and 5́-AATTCTTAATGATGGTGATGATGATGAGGCCTACATAA TGTAGCACCAGCATGAGTTGTACAGAGCT-3́ oligonucleotides. The resulting plasmid pLEB634 was electroporated into the *E. coli* strain TG1, and the new strain was designated as ECO656.

Expression and purification of CTT–GFP and Ala–CTT–GFP chimeras

Prior to protein production the plasmids pLEB633 and pLEB644 were transformed into the *E. coli* strain BL21Star(DE3)pLysS (Invitrogen), and the transformed cells were grown in 1000 ml to early logarithmic growth phase ($OD_{600\ nm}$ = 0.5) in prewarmed LB-medium supplemented with ampicillin (100 μg/ml) at 37 °C with shaking (220 rpm). The expression of chimeras was induced by adding IPTG to a final concentration of 0.3 mM, and the bacteria were cultured for 3 h. The cells were harvested by centrifugation at 7000*g* for 10 min. The bacterial pellets (wet weights 7.8 g and 9.8 g for CTT–GFP and Ala–CTT–GFP, respectively) were resuspended in 40 ml binding buffer (20 mM sodium phosphate, 500 mM sodium chloride, pH 7.8) containing 1 mg/ml of lysozyme, incubated 10 min at 37 °C with shaking followed by 15-min sonication in a waterbath sonicator. The bacterial debris was removed by centrifugation at 25,000*g* for 20 min, and the supernatants were filtered through a 0.45 μm filter (Sartorius). The CTT–GFP and Ala–CTT–GFP chimeras were recovered from the filtrates with HisBind® Quick 900 Cartridges (Novagen) according to manufactureŕs instructions. The elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9) was replaced with PBS by Biomax-5 filters (Amicon), and the purified chimeras were stored at 4 °C.

SDS–PAGE and Western blot analysis

The purity of eluates was analyzed with SDS–PAGE (polyacrylamide gel electrophoresis) [14] on 15% gels. Immunoreactivity of a 1:500 diluted rabbit polyclonal anti-CTT serum [13] against CTT–GFP and Ala–CTT–GFP was verified by WesternBreeze® Chromogenic Western Blot Immunodetection Kit (Invitrogen) on Immobilon-P PVDF-membrane (Millipore).

Binding to MMP-9 expressing cells

CTT–GFP, Ala–CTT–GFP, and glutathione S-transferase (GST; produced and purified as described elsewhere) [15] were coated on microtiter wells over night at 4 °C in a concentration of 40 μg/ml in PBS. Blocking was carried out with 3% BSA in PBS for 2 h at room temperature. After blocking the wells were washed five times with PBS.

MMP-9 expressing THP-1 cells were activated with 50 nM 4β-phorbol-12,13-butyrate (PDBu; Sigma) in RPMI-1640 cell medium (Cambrex Bio Science) containing 0.1% BSA and 1 mM $MgCl_2$ for 30 min at 37 °C, 5% CO_2. Activator medium was removed and the cells were further incubated in BSA/$MgCl_2$ supplemented medium with or without competing synthetic CTT-peptide for 20 min. Cell suspensions were then transferred into microtiter wells (100,000 cells/well) coated with the GFP-chimeras or GST for adhesion experiments. As a control, THP-1 binding to non-coated, BSA-blocked wells was done as well.

After a 30-min incubation with the chimeras at 37 °C, 5% CO_2, the wells were washed twice with PBS. Detection of bound cells was performed with cellular phosphatase assay [16]. Briefly, 100 μl of substrate buffer (3 mg/ml *p*-nitrophenyl phosphate salt in acetate buffer, pH 5.0 with 1% Triton X-100) was incubated with cells at 37 °C for 30 min. After addition of 50 μl of 1 M NaOH, the yellow color was read at 405 nm in a microtiter plate reader.

Table 1
Purification of His-tagged CTT–GFP and Ala–CTT–GFP from *E. coli*.

Purification step	Total protein (mg)[b]	Total RFU ($\times 10^3$)[c]	RFU/mg ($\times 10^3$)	Yield (%)	Purification factor
CTT–GFP					
Crude supernatant[a]	43	126	2.93	100	(1)
Affinity eluate	0.665	8.51	12.8	6.6	4.3
Ala–CTT–GFP					
Crude supernatant[a]	51	163	3.21	100	(1)
Affinity eluate	1.2	19.1	15.9	11.7	4.96

[a] The starting material was 40 ml crude *E. coli* supernatant containing 7.8 g and 9.8 g (wet weight) lysed CTT–GFP and Ala–CTT–GFP producing cells, respectively.
[b] The protein concentrations were measured using the Bradford assay using BSA as a standard.
[c] Relative fluorescence units (RFU) were measured with Fluoroscan Ascent 374 fluorometer (Labsystems) the excitation and emission filters being 374 and 520 nm, respectively.