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Tribody: Robust Self-Assembled Trimeric Targeting Ligands with High Stability and Significantly Improved Target-Binding Strength

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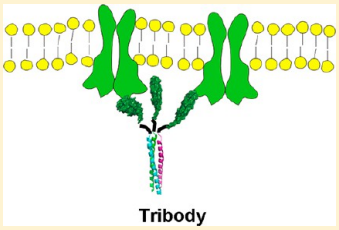
ABSTRACT: The C-terminal coiled-coil region of mouse and human cartilage matrix protein (CMP) self-assembles into a parallel trimeric complex. Here, we report a general strategy for the development of highly stable trimeric targeting ligands (tribodies), against epidermal growth factor receptor (EGFR) and prostate-specific membrane antigen (PSMA) as examples, by fusing a specific target-binding moiety with a trimerization domain derived from CMP. The resulting fusion proteins can efficiently self-assemble into a well-defined parallel homotrimer with high stability. Surface plasmon resonance (SPR) analysis of the trimeric targeting ligands demonstrated significantly enhanced target-binding strength compared with the corresponding monomers. Cellular-binding studies confirmed that the trimeric targeting ligands have superior binding strength toward their respective receptors. Significantly, the EGFR-binding tribody was considerably accumulated in the tumor of mice bearing xenografted EGFR-positive tumors, indicating its effective cancer-targeting feature under in vivo conditions. Our results demonstrate that CMP-based self-assembly of tribodies can be a general strategy for the facile and robust generation of trivalent targeting ligands for a wide variety of in vitro and in vivo applications.



Targeting ligands play a pivotal role in all in vitro and in vivo targeting applications.[1] Depending on the nature of the application and the molecules to be targeted, some basic properties of the target ligand, including molecular weight, surface charge, target-binding specificity, affinity, and valency, should be optimized. It is now clear that one of the most critical parameters for satisfactory in vivo targeting is the valency of the targeting ligand. Most monovalent targeting ligands, even those with very high binding affinities, have fast dissociation rates and provide only modest retention time on the target antigen under nonequilibrium physiological conditions.[1] Impressively, nature addresses this problem by extensively using multivalent interactions, as observed in almost all types of antibodies and numerous multimeric interactive proteins. Currently, there is an unmet need for a technology platform that allows for the facile and robust development of desired panels of multivalent targeting ligands that possess significantly increased target-binding strength and decreased dissociation from the target and thus longer and more accumulation in diseased tissues.

Monoclonal antibody is the major class of targeting ligands that has been widely used in many biomedical fields.[2] More than 20 monoclonal antibodies are being clinically used as therapeutic agents and many more are under preclinical development.[3] However, immunoglobulin-scaffold-based antibodies have intrinsic limitations, including large size (~150 kDa), the presence of disulfide bonds, complex tetrameric structure, and high cost of production, that complicate their many applications.[3] Substantial efforts have been made to develop targeting ligands that can be quickly tuned to mimic antibodies with multivalent features.[2−5] The protein domains capable of forming a multimeric complex have been extensively investigated to generate recombinant proteins to achieve an avidity effect through multivalency.[1,6] To develop a robust system that allows for the facile generation of targeting ligands with multivalent features, the multimerization domains should be of small sizes and possess favorable biophysical properties, including thermal stability, resistance to proteases, and cost-effective production, while still being able to generate a highly stable multimeric complex that can display multiple target-binding moieties in parallel. Different scaffolds that allow for enhanced avidity have been reported.[1,2,5−7] These scaffolds include the bacteriophage T4 foldon domain, collagen-like peptide $(Gly\text{-}Pro\text{-}Pro)_{10}$, and NC1 domain of the collagen XV and XVIII domains as well as the GCN leucine-zipper domain for trimers,[1,2,8−10] streptavidin and transcription factor p53 for tetramers,[11] the B-subunit of bacterial verotoxin and cartilage oligomeric matrix protein (COMP) for pentamers,[12,13] and, recently, the hyperthermophilic Sm protein for heptamers.[6]

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However, most of these scaffolds are derived from nonhuman proteins and have limited clinical application because of immunogenicity. Ideally, the multimerization domain should be a highly conserved extracellular protein that is abundant in mouse and human proteomes, which could result in less immunogenicity and allow for a smooth transition from animal studies to translational and clinical investigations. Although protein domains forming trimeric structures are widely used in nature, few possess the desired features that allow for the effective development of trivalent ligands that are clinically amenable. To address this unmet need, we chose the C-terminal domain from mouse CMP-1 (matrilin), which is highly homologous to human CMP-1, for the development of self-assembly trivalent targeting ligands on the basis of its remarkable property of forming stable trimeric structures.[14−16] As a major component of various cartilages, CMP-1, a 148 kDa extracellular matrix glycoprotein, serves as an adaptor in the assembly of the extracellular matrix structure. The oligomerization characteristic of CMP or matrilin has been extensively studied.[14−16] There are four members of matrilins, namely, matrilin-1, -2, -3, and -4, whose domain structures are highly conserved among species from chicken to human. It has been reported that matrilin-1 and -4 form trimeric complexes, whereas matrilin-2 and -3 form tetrameric complexes.[16] The last 40 amino acids at the C-terminus of CMP-1 play an important role in the formation of the trimeric structure.[14,16] This C-terminal coiled-coil domain has a heptad repeat in which hydrophobic residues reside in the first and the fourth positions, whereas polar residues are common in the fifth and the last positions.[14] The strong hydrophobic and ionic interactions within this α-helical coiled-coil result in a parallel, disulfide-linked three-stranded domain. The resulting rod-shaped trimeric structure, with a length of about 5.1 nm and a diameter of 3 nm, has a high degree of co-operativity and a high stability, with a T_m higher than 60 °C at neutral pH, which can be attributed to the hydrophobic packing and a salt bridge between the R27 and E32 residues.[14] It appears that the trimeric structure was still well maintained when the disulfide bonds that flank the N-terminus of the coiled-coil were abolished.[14]

Overexpression of epidermal growth factor receptor (EGFR) and prostate-specific membrane antigen (PSMA) receptor is frequently associated with various cancers including lung, breast, prostate, and glioblastoma.[3,17] Selective targeting toward EGFR or PSMA holds the promise of early diagnosis and improved treatment.[18,19] In this report, we describe the use of the trimerization domain derived from the cartilage matrix protein that is abundant and highly homologous in mouse and human to develop trivalent targeting molecules (tribodies) with high stability and significantly enhanced avidity against EGFR and PSMA. The resulting tribodies are extensively characterized and used for in vivo tumor targeting in xenograft mouse models.

MATERIALS AND METHODS

Plasmid Construction of Tribodies. A mouse cDNA library was used for the amplification of the gene encoding the mouse CMP-1 trimerization domain. Sequences encoding the EGFR-binding Z domain (Z1907) or PSMA-binding homing peptide were constructed as we reported previously.[6] Each PCR fragment was ligated into pET28b between the NcoI and XhoI sites followed by sequencing to ensure that each affinity molecule was generated correctly.

Expression and Purification of Tribodies. The plasmid containing a gene of interest was transformed into *Escherichia coli* BL21 (DE3) Rosseta cells, and the positive clones were selected on LB plates containing kanamycin (50 μg/mL) and chloramphenicol (34 μg/mL). A single colony was picked and grown in 5 mL of LB overnight at 37 °C. The overnight culture was added to 500 mL of LB containing kanamycin and chloramphenicol. The culture was grown at 37 °C until the OD600 reached between 0.5 and 1.0, at which time 1 mM of IPTG was added to induce protein expression. After induction at 22 °C for 16 h, the cells were spun down at 3000*g* for 10 min at 4 °C, and the pellet was stored at −20 °C prior to purification. To purify each monomeric and trimeric molecule, each cell pellet was resuspended in buffer A (25 mM HEPES pH 7.4 and 50 mM NaCl) and sonicated for 1 min for a total of three to four times. After cell lysis, the soluble fraction was recovered by centrifugation at 12 000*g* for 10 min at 4 °C. The resulting soluble fraction was loaded into a TALON metal-affinity column (Clontech, Mountainview, CA) pre-equilibrated with buffer B (25 mM HEPES pH 7.4 and 300 mM NaCl). The column was washed with 20 column volumes of buffer B followed by extensive washing with buffer C (buffer B containing 20 mM imidazole). The targeting molecules were eluted with buffer D (buffer B containing 200 mM imidazole). The quality of the purified proteins was examined by SDS-PAGE.

Native-Gel Electrophoresis. An 8% discontinuous native gel was prepared in the absence of SDS and reducing agent using the standard Laemmli SDS-PAGE protocol. About 5 μg of highly purified monomer or tribody was loaded for separation. Proteins were stained with Coomassie brilliant blue R-250.

Circular Dichroism Spectroscopy. Highly purified monomeric and trimeric proteins were prepared in 10 mM phosphate buffer (pH 7.5) and used for circular dichroism (CD) scanning with an AVIV model 202-01 Spectropolarimeter at the UNC Macromolecular Interaction facility. To determine thermal stability, the CD spectra at 220 nm were measured from 25 to 94 °C, and the ramp was cooled to 25 °C followed by measuring the CD spectra at 220 nm again from 25 to 94 °C. The melting temperature (T_m) was calculated as the temperature that has the midpoint CD spectra between the lowest and the highest CD spectra with a reversible melting curve.

Protease Resistance. Thermolysin, papain, and MMP-9 were used for the protease-resistance assays. Thermolysin and papain were purchased from Sigma-Aldrich (St. Louis, MO). MMP-9 was purchased from EMD Millipore (Billerica, MA). Approximately 2 μg of monomeric or trimeric targeting ligands were incubated with thermolysin (10 ng of thermolysin per microgram of protein). The protease digestion was performed in an HBS buffer (10 mM HEPES pH 7.4 and 150 mM NaCl) for 30 min at 37 °C. The reaction mixtures were applied to SDS-PAGE to examine protein degradation. Approximately 2 μg of monomeric or trimeric targeting ligands were incubated with 20 ng of papain for 30 min at 37 °C. The reaction was performed in a papain digestion buffer (50 mM phosphate buffer pH 7.0 and 0.5 mM EDTA). Papain was activated using 5 mM L-cysteine followed by removing L-cysteine using a MWCO 5 kDa spin column through centrifugation. The reaction mixtures were applied to SDS-PAGE to examine protein degradation. Approximately 2 μg of monomeric or trimeric targeting ligands were incubated with 100 ng of MMP-

9 for 4 h at 37 °C. The reaction was performed in a MMP-9 digestion buffer (50 mM HEPES pH 7.5, 10 mM $CaCl_2$, and 0.02% BRIJ-35). The reaction mixtures were applied to SDS-PAGE to examine protein degradation.

Target-Binding Analysis using Biacore. BIAcore 2000 (BIAcore AB, Uppsala, Sweden) was used for surface plasmon resonance analysis. The extracellular domain of recombinant human EGFR or PSMA (R&D Systems, Minneapolis, MN) was diluted in 10 mM sodium acetate pH 5.0 and immobilized on a CM5 sensor chip (GE healthcare, Piscataway, NJ) to achieve ~2500 resonance units by amine coupling according to the manufacturer's instruction. Various concentrations of monomeric and trimeric ligands were injected into the flow cell in a HBS-P buffer (10 mM HEPES pH 7.4, 150 mM NaCl, and 0.005% surfactant P20) at a flow rate of 20 μL/min. The association and dissociation constants were calculated using BIA evaluation software by fitting the data to a one-to-one Langmuir binding model.

Cell Culture. All cell lines, including EGFR-positive A431, EGFR-negative Jurkat, PSMA-positive LNCaP, and PSMA-negative PC3 cancer cells, were obtained from the Tissue Culture Facility at UNC-Chapel Hill. All cell lines were maintained by serial passage in the appropriate media containing 10% fetal bovine serum in a 5% CO_2 incubator at 37 °C.

Confocal Microscopy. Each cell type (about 2×10^4) was seeded onto coverslips and allowed to grow in an appropriate media for 16 h. The resulting coverslips were washed twice with 1× PBS followed by incubation with different concentrations of FITC-labeled monomeric and trimeric ligands for 30 min at room temperature. After incubation, the coverslips were washed three times with 1× PBS. The resulting samples were examined with a Zeiss LSM 510 confocal microscope at the UNC microscopy core facility.

Flow Cytometry. Cell binding of the monomeric and trimeric ligands was evaluated using flow cytometry. Approximately 2×10^4 cells were grown on a 24-well plate for 16 h. After washing with 1× PBS, the cells were incubated with an appropriate amount of FITC-labeled monomeric or trimeric ligand for 30 min at room temperature followed by washing twice with 1× PBS. The resulting samples were analyzed by flow cytometry (BD FACS Canto flow cytometer), and the data was analyzed by the Flow Jo System (Tree star, Inc., Ashland, OR). FITC-labeled anti-PSMA antibody (MBL, Nagoya, Japan) was used as a positive control.

Serum Stability. Purified tribody (5 μg) was incubated at 37 °C with 100 μL of mouse serum (50 mg/mL, Sigma-Aldrich) for 6 and 24 h. After incubation, 10 volumes of buffer A were added, and the protein was recovered using a Co^{2+}-NTA column as described above.

Cell Proliferation MTS Assay. The CellTiter96 Aqueous Non-Radioactive Cell Proliferation Assay kit from Promega (Madision, WI) was used for the MTS cell proliferation assays. Approximately 1×10^4 cells were seeded in each well of a 96-well plate and grown for 16 h at 37 °C. Appropriate amount of each targeting ligand was added and incubated with the cells for 36 h. Ten micromolar Cis-platinum(II) diamine dichloride (Sigma-Aldrich) was used as a positive control. After incubation, approximately 20 μL of the MTS/PMS solution was added to each well followed by incubation for 4 h at 37 °C. The absorbance at 490 nm was recorded using an ELISA plate reader.

PEGylation and Fluorescent Labeling with Alexa 750. Highly purified monomeric or trimeric ligand was incubated with 30 kDa mPEG-NHS ester (Creative PEGWorks, Winston Salem, NC) in 1× PBS (1:15 molar ratio) at room temperature for 4 h. The reaction mixture was purified by Co^{2+}-NTA column chromatography to remove unreacted PEG. After PEGylation, Alexa 750 succinimidyl ester (Life Technology, Grand Island, NY) was added (10:1 molar ratio) and incubation for 2 h at room temperature. Unreacted Alexa 750 was removed through extensive dialysis against 1× PBS using a dialysis membrane with a 5 kDa MWCO.

Animal Study. Female athymic nude mice (6−8 weeks of age, ~20 g in weight) were purchased from Charles River Laboratories (Wilmington, MA). All work performed on animals was in accordance with and approved by the UNC Institutional Animal Care and Use Committee. Specifically, A431 cells (5×10^6) were subcutaneously injected into the lower back of female nude mice and were allowed to grow until the tumor reached a size of about 200 mm^3. Approximately 0.5 nmol of PEGylated, Alexa750-labeled Z^{EGFR} monomer or trimer in DPBS was applied to the mice by intravenous injection. Six hours postinjection, mice were sacrificed by followed by cervical dislocation, and the organs were isolated. The images of the tumor and each organ were taken with a Kodak In Vivo Imaging System FX-PRO (Carestream Health, Inc.). Densitometric analysis of the tumor signal was performed using Image J (NIH, Bethesda, MD).

RESULTS

Design and Generation of Tribodies. To generate trimeric targeting ligands, we started with the 43-residue trimerization domain from mouse CMP-1: EEDPCACESIL-KFEAKVEGLLQALTRKLEAVSGRLAVLENRII. This sequence is highly homologous to the same trimerization domain of human CMP-1: EEDPCACESLVKFQAKVEGLLQAL-TRKLEAVSKRLAILENTVV, making it easy to switch to the human version if a translational application is desired. In principle, many target-binding moieties can be fused with this trimerization domain for the facile generation of and conversion into the trimeric form. These targeting moieties include the V_H or V_L domains from the natural antibodies, nonimmunoglobulin protein domains that mimic natural antibodies, and various short homing peptides isolated from phage display or other selection strategies.[1] To demonstrate that this tribody approach can be generally applied to fuse with targeting moieties with different 3D structures from non-structural homing peptides to well-folded protein domains and with target-binding affinities from low to high, we chose a PSMA-binding short homing peptide HP^{PSMA} (WQPDTAHH-WATL) as an example for a low-affinity target binder[17] and an EGFR-binding Z domain as an example for a high-affinity target binder (Figure 1A).[4] To compare the tribody with its monomeric form, the monomeric Z^{EGFR} targeting molecule was constructed without the trimerization domain. We also truncated the trimerization domain by deleting the last 14 residues that are critical for intermolecular interaction and mutated Cys to Ala to disrupt the disulfide bond (Table 1). The resulting domain cannot trimerize and was used for the generation of monomeric PSMA-binding protein. The coding sequence for each recombinant fusion protein was synthesized and cloned into expression vector pET-28b for overexpression in *E. coli* (Table 1). A C-terminal His-tag was introduced to facilitate affinity purification. The expression of each tribody

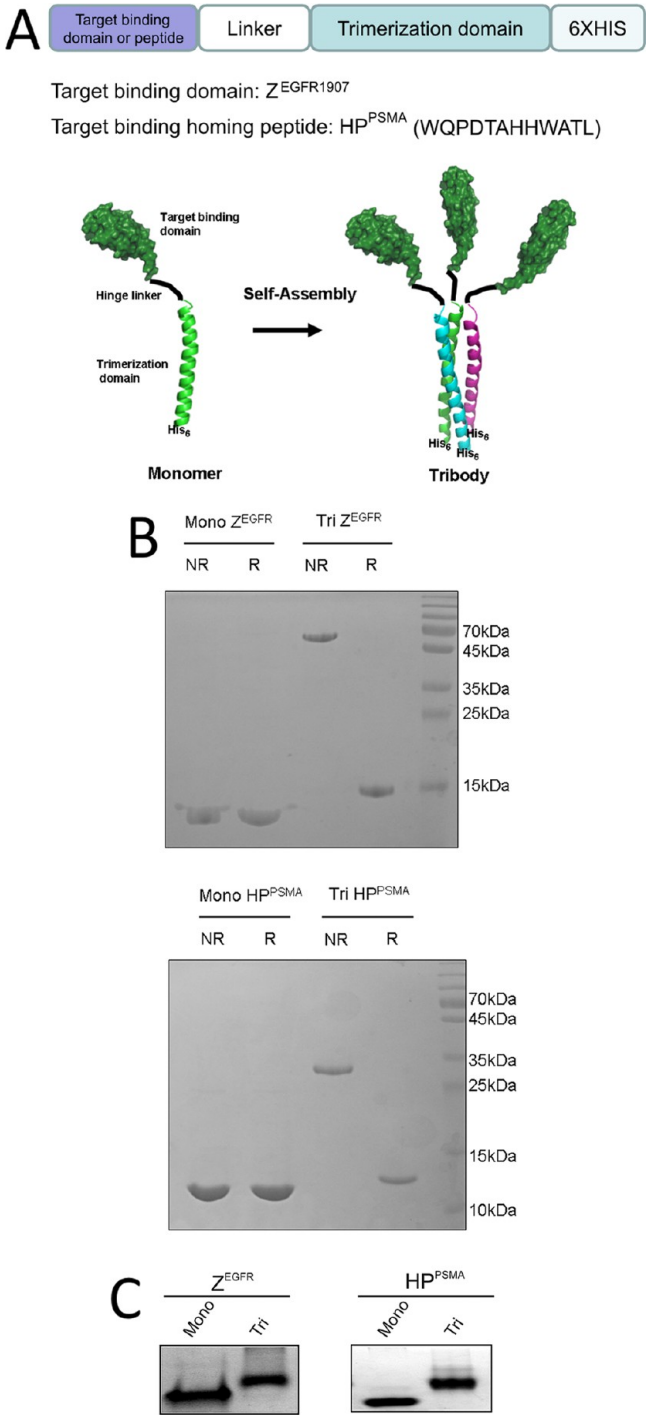

Figure 1. Schematic diagram and purication of the tribody. (A) Self-assembled trimeric targeting ligand consists of a target-binding domain (Z^{EGFR}) or homing peptide (HP^{PSMA}), a flexible hinge linker, and a trimerization domain derived from the C-terminus of mouse CMP-1. A 6× His tag was engineered at the C-terminus of each ligand to facilitate affinity purification. (B) SDS-PAGE of the purified monomeric and trimeric targeting ligands. The Z^{EGFR} monomer, Z^{EGFR} trimer, HP^{PSMA} monomer, and HP^{PSMA} trimer were purified by metal-affinity column chromatography and loaded on a 12% SDS-PAGE gel for analysis. NR, nonreducing conditions; and R, reducing conditions with 10 mM DTT. (C) Native-gel electrophoresis. Purified monomeric and trimeric proteins (5 μg) were loading on an 8% native gel to analyze the oligomeric status. Proteins were stained by Coomassie brilliant blue.

and its corresponding monomer was induced by the addition of 1 mM IPTG. One of the key advantages of using the trimerization domain of CMP-1 is that its self-assembly considerably favors a trimeric form compared to other trimeric and pentameric scaffolds.[20,21] The CMP-based recombinant proteins were highly expressed and purified to near homogeneity through Co^{2+}-NTA purification. Indeed, more than 99% of the molecules exist in the trimeric form following purification with only a trace amount of monomer and dimer (Figure 1B). To verify the prominence of the trimer, native-gel electrophoresis was performed. As shown in Figure 1C, both EGFR-targeting and PSMA-targeting tribodies existed only as a trimer under native and nondenaturing conditions. These results indicated that the tribodies are self-assembled highly efficiently and can be purified as homogeneous trimeric complexes by a one-step Co^{2+}-NTA chromatography procedure.

Thermal Stability and Protease Resistance of Tribodies. To use the tribodies under in vivo conditions, the biophysical properties, such as solubility, thermal stability, and resistance against protease degradation, should be investigated. The CMP-based tribodies were highly expressed in *E. coli* as soluble recombinant proteins. To determine the thermal stability of the tribodies, we first performed circular dichroism (CD) analysis using highly purified monomeric and trimeric molecules by gradually increasing the temperature from 25 to 94 °C to examine the secondary-structure changes (Figure 2A–F). It was found that the melting temperature (T_m) of the monomeric HP^{PSMA} was about 65 ± 1 °C (Figure 2B). The HP^{PSMA} trimer showed remarkable stability when the temperature was increased from 25 to 94 °C, with a reversible melting curve (Figure 2B). Significantly, trimeric HP^{PSMA} appears to be more stable than the monomeric HP^{PSMA} at higher temperatures (Figure 2C). Because both the targeting moiety and the flexible linker in trimeric HP^{PSMA} are short peptides that are presumably nonstructural, the observed remarkable thermal stability of the HP^{PSMA} tribody can be attributed to the highly stable trimerization domain. The T_m of the Z^{EGFR} monomer was approximately 60 ± 2 °C (Figure 2E). The Z^{EGFR} trimer also showed high stability when the temperature was increased from 25 to 94 °C (Figure 2E). Although the melting curve of the Z^{EGFR} trimer was reversible only in the range from 25 to 60 °C (Figure 2F), the second and third melting curves showed similar patterns as their corresponding monomer (Figure 2E), suggesting a T_m of around 61 ± 1 °C for the Z^{EGFR} trimer. Because the Z^{EGFR} tribody was very stable when the temperature was increased to 60 °C, it is likely that the T_m of the Z^{EGFR} tribody is much higher than 61 °C and that the first heating to 94 °C causes irreversible changes that result in a reduction of the T_m of subsequent measurements. It appears that another transition above 80 °C in the Z^{EGFR} monomer and trimer melting curves was evident. Although the results were reproducible, the nature of this transition is not clear.

Protein degradation by serum proteases is another obstacle that limits the in vivo applications of many targeting ligands. To investigate this issue, we performed protease-mediated digestion of the targeting ligands by using the thermostable metallopeptidase thermolysin, cysteine protease papain, and matrix metallopeptidase-9 (MMP-9). Significantly, all of the monomeric and trimeric molecules were resistant to thermolysin digestion (Figure 3). Figure 4A shows that both the monomeric and trimeric molecules were resistant to papain. MMP-9 is one of the best characterized extracellular proteases

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Table 1. Amino Acid Sequences of Tribody Components

	amino acids
Z^{EGFR}	MVDNKFNKEMWAAWEEIRNLPNLNGWQMTAFIASLVDDPSQSANLLAEAKKLNDAQAPK
TP^{PSMA}	WQPDTAHHWATL
hinge linker	GPQPQPKPQPKPEPEPQPQGG
trimerization domain	EEDPCACESILKFEAKVEGLLQALTRKLEAVSGRLAVLENRII
truncated trimerization domain	EEDPAAAESILKFEAKVEGLLQALTRKLE

that are overexpressed in numerous tumors. Figure 4B shows that both the monomeric and trimeric molecules were resistant to MMP-9 digestion (Figure 4B), whereas the control protein that contains an MMP-9 cleavage site was readily cleaved. These results suggest that these tribodies can be used under in vivo conditions when biologically active proteases are highly abundant.

In Vitro Target-Binding toward Immobilized and Cell-Surface Targets. To examine whether these tribodies have the desired targeting feature, we first used surface plasmon resonance (SPR) to compare the target-binding properties of the monomeric and the corresponding trimeric targeting ligands. Each purified protein target (the extracellular domain of EGFR or PSMA) was immobilized on a CM5 sensor chip through amine coupling. Various concentrations of monomeric or trimeric targeting ligands were injected into the flow cell for binding-strength analysis. To examine the multivalent effect, relatively low concentrations of tribodies were used in the flow phase. As shown in Figure 5 and Table 2, the monomeric Z^{EGFR} rapidly dissociated from EGFR immobilized on chip surface (k_d $\sim 6.78 \times 10^{-4}$ s^{-1}) with a binding affinity of around 2.06 ± 0.51 nM. Significantly, the Z^{EGFR} tribody dissociated very slowly compared to the monomer. The multiple target-binding sites involved in the interaction between tribody and EGFR significantly increase the local concentration of the affinity unit, effectively providing a decreased overall off-rate. This multivalent avidity or functional affinity is distinct from the intrinsic affinity used to describe the single antibody−antigen interaction and can be orders of magnitude higher than the conventional affinity of a monovalent interaction. As shown in Figure 5B, the dissociation rate of the tribody observed from the SPR experiments was indeed significantly decreased. However, it is difficult to fit the data on any BIAevaluation model for multivalent kinetic analysis, presumably due to the complexity of the multivalent interaction between the tribody and the dimeric EGFR receptor. Therefore, the kinetic parameters of this EGFR-binding tribody were not calculated. In the case of the PSMA tribody, we found that PSMA immobilized on a CM5 sensor chip was not functional when examined with a widely used anti-PSMA antibody, making it impossible to measure the PSMA binding of the HP^{PSMA} tribody. To address this issue, flow cytometry was adopted to estimate the binding of FITC-labeled HP^{PSMA} monomer and tribody with native PSMA present on the surface of cancer cells (Figure 6), and a FITC-labeled anti-PSMA antibody was used as a positive control. As shown in Figure 6A, the anti-PSMA antibody bound to LNCaP cells but not PC3 cells when used at 100 nM. The HP^{PSMA} monomer showed no obvious binding with both PSMA-positive LNCaP cells and PSMA-negative PC3 cells (Figure 6B). However, as shown in Figure 6B, the HP^{PSMA} tribody showed selective binding toward LNCaP but not PC3 cells, with a K_d of 455 ± 24 nM.

We further confirmed the significantly enhanced target binding of the tribodies by using biomarker-expressing cell lines. A431 cells have a high expression level of EGFR and were used as positive cells, whereas Jurkat cells were used as negative cells. As shown in Figure 7A, the trimeric targeting ligands demonstrated much stronger cell binding compared to the monomeric forms. In the negative-control experiments, both the FITC-labeled Z^{EGFR} monomer and trimer failed to bind to Jurkat cells even at high concentration (100 nM). In contrast, the Z^{EGFR} tribody can recognize A431 cells with a concentration as low as 1 nM, whereas a more than 10-fold higher concentration of the Z^{EGFR} monomer was required to observe a similar cell-binding extent. In the case of HP^{PSMA}, a FITC-labeled HP^{PSMA} tribody can bind to PSMA-positive LNCaP cells with a concentration as low as 100 nM (Figure 7B). We found that the monomeric PSMA homing peptide did not result in specific cell-binding signal even when used at high micromolar concentrations (Figure 7B and unpublished data). Because the target-binding affinity of monomeric HP^{PSMA} was too weak to measure, we were not able to determine the exact fold increase in the target-binding strength for HP^{PSMA}. Nevertheless, the results clearly indicate that the CMP-based trimerization approach allows for a quick conversion of a short homing peptide with an undetectable binding affinity to a trivalent form with a target-binding strength close to many monoclonal antibodies. Taken together, both the Z^{EGFR} and HP^{PSMA} tribodies recognize their target receptors with greatly enhanced binding strength without sacrificing specificity compared with their corresponding monomers.

Serum Stability and Toxicity. The successful in vivo application of tribodies, including imaging or therapy, requires excellent serum stability and nontoxicity. To address the stability of the tribody in serum, trimeric Z^{EGFR} was incubated with mouse serum at 37 °C for up to 24 h. After incubation, the protein was recovered by Co^{2+}-NTA chromatography. As shown in Figure 8A, the Z^{EGFR} tribody displayed unusually high stability in mouse serum without detectable degradation even after 24 h at 37 °C. The toxicity of the Z^{EGFR} tribody was also examined using the cell proliferation assay. As illustrated in Figure 8B, no cell growth inhibition was observed when the Z^{EGFR} tribody was used at 500 nM, a concentration that results in very strong cell-binding signals. Taken together, these results demonstrate that the Z^{EGFR} tribody showed high serum stability and nontoxicity and holds great potential for various in vivo and translational applications.

In Vivo Tumor Targeting. To examine the in vivo application of the trimeric targeting ligands, we used the EGFR-binding tribody to target the tumor in a xenograft mouse model. The molecular weight of the tribody is in the range of 30 kDa (for HP^{PSMA}) to 58 kDa (for Z^{EGFR}). To increase the circulation time, both the monomeric and trimeric Z^{EGFR} were PEGylated using 30 kDa PEG-NHS ester. To examine whether the Z^{EGFR} tribody can efficiently target a tumor in which EGFR is highly expressed, Alexa 750-labeled, PEGylated Z^{EGFR} monomer and tribody were intravenously injected into A431-xenografted nude mice. Six hours after injection the mice were

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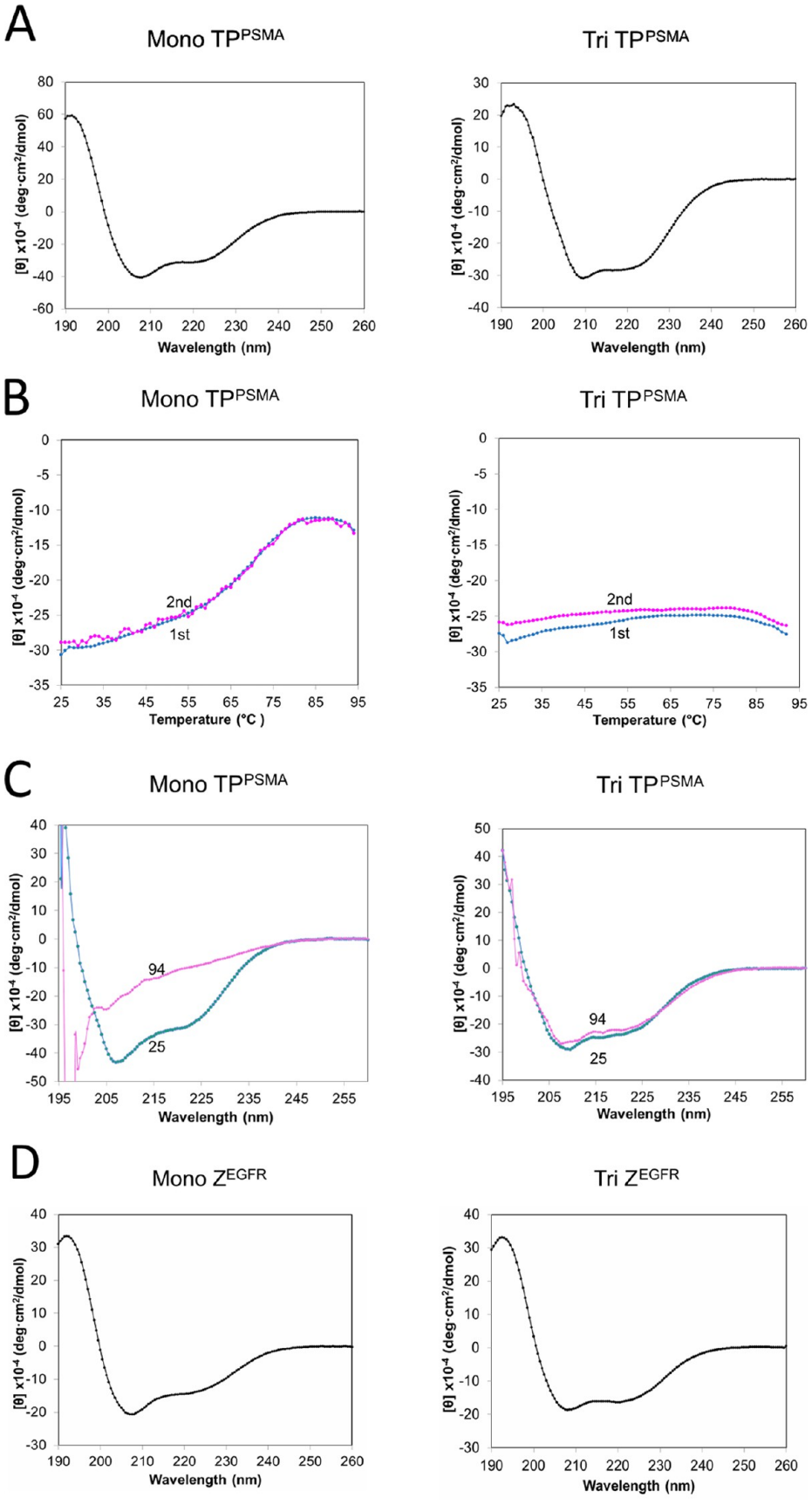

Figure 2. continued

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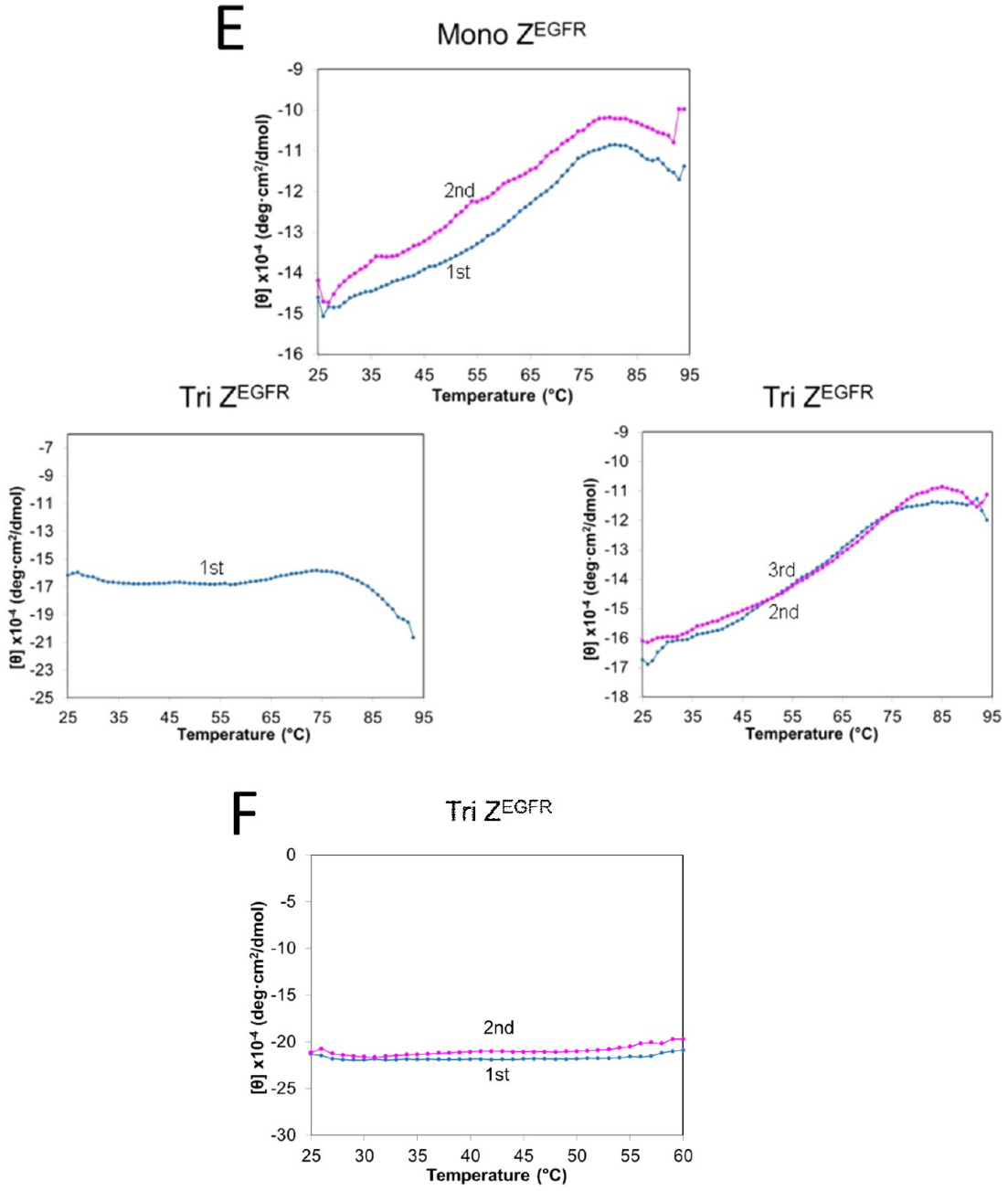

Figure 2. Analysis of heat-induced denaturation using circular dichroism. (A) CD spectra of the HP^{PSMA} monomer and HP^{PSMA} trimer recorded from 190 to 260 nm at 25 °C, respectively. (B) CD spectra were recorded at various temperatures from 25 to 94 °C. The ellipticity at 220 nm was used for analysis. (C) CD spectra of the HP^{PSMA} monomer and HP^{PSMA} trimer recorded from 190 to 260 nm at 25 and 94 °C, respectively. (D) CD spectra of the Z^{EGFR} monomer and Z^{EGFR} trimer recorded from 190 to 260 nm at 25 °C, respectively. (E) CD spectra of the Z^{EGFR} monomer and Z^{EGFR} trimer recorded at various temperatures from 25 to 94 °C. The ellipticity at 220 nm was used for analysis. (F) CD spectra of the Z^{EGFR} trimer recorded at various temperatures from 25 to 60 °C. The ellipticity at 220 nm was used for analysis.

sacrificed, and the organs were isolated. The images of each organ and tumor were taken with a Kodak In Vivo Imaging System FX-PRO. As shown in Figure 9, when the PEGylated tribody was used, the strongest accumulation was found in the tumor with no or minimal accumulation in the heart, lungs, spleen, and liver. There was modest accumulation in the kidney, presumably due to the well-known renal elimination of the animal. Approximately 54% of the fluorescent signal from Z^{EGFR} tribody was accumulated in the tumor compared to that of 7.7% from the monomer. These results clearly indicate that the tribody holds great promise for in vivo tumor-targeting applications.

DISCUSSION

In this study, we report the successful development and application of a novel class of trimeric targeting ligands based on the highly conserved trimerization domain of mammalian CMP-1. Compared with other trimeric scaffolds that have been reported,[8−10,21,22] this tribody approach possesses several key advantages. First, this trimerization domain is derived from a highly conserved extracellular protein that is abundant in both

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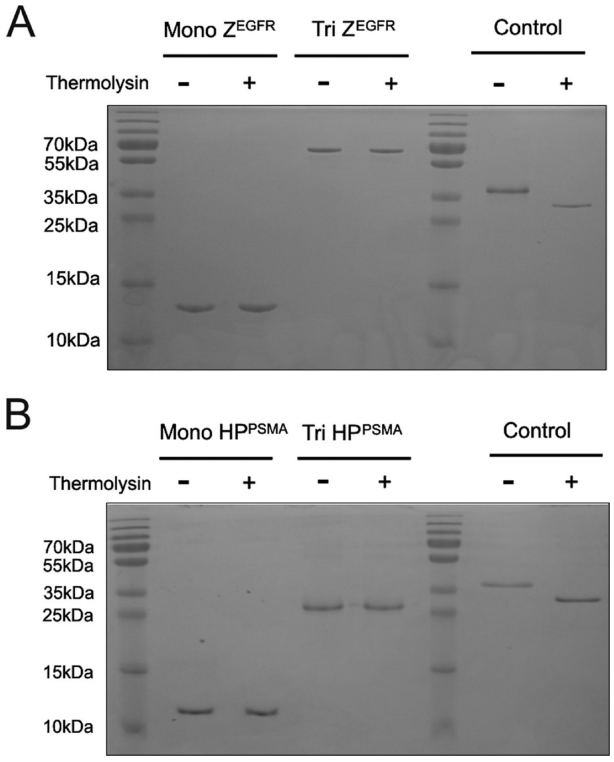

Figure 3. Analysis of protease resistance using thermolysin. Each purified protein was incubated with thermolysin at 37 °C for 30 min. (A) Z^{EGFR} monomer and trimer. (B) HP^{PSMA} monomer and trimer. The reaction mixtures were loaded on a 12% SDS-PAGE gel for analysis.

mouse and human. Presumably, the tribodies that are used for animal studies and translational applications can be readily generated with similar targeting features and low immunogenicity. This will greatly facilitate a smooth transition from in vitro investigation to in vivo application. Second, the tribody has remarkable biophysical features with regard to thermal stability and protease resistance (Figures 2−4). They were very stable when the temperature was 90 °C (Figure 2). Also, the tribodies were resistant to degradation mediated by different proteases, including physiologically relevant papain and MMP-9 (Figures 3 and 4). Although these tribodies existed as a trimer predominantly under denaturation conditions, such as boiling and in the presence of SDS (Figure 2), the bacteriophage T4 foldon-derived trimer dissociated into a monomeric form after boiling.[8] The human collagen XVIII NC1 domain, another trimerization domain that has been used for the generation of multimeric antibody, was sensitive to heat-induced denaturation and protease degradation.[9] Third, the trimerization of tribody is a convenient and highly efficient self-assembling process (Figure 1). Compared to most other multimerization domains such as mucin 1-based trimer and COMP-based pentamer in which intermolecular disulfide bond linkages are indispensable for the formation of multimeric complexes,[20,21] it was found that the tribody efficiently self-assembled into a stable trimeric form in the absence of any disulfide bonds, presumably due to the strong and extensive intermolecular hydrophobic and ionic interactions among the trimeric complex. This feature makes the tribody very useful in many in vitro and in vivo applications under reducing conditions. The trimer predominance of this system also provides an advantage in the cost effectiveness of this approach over other disulfide-

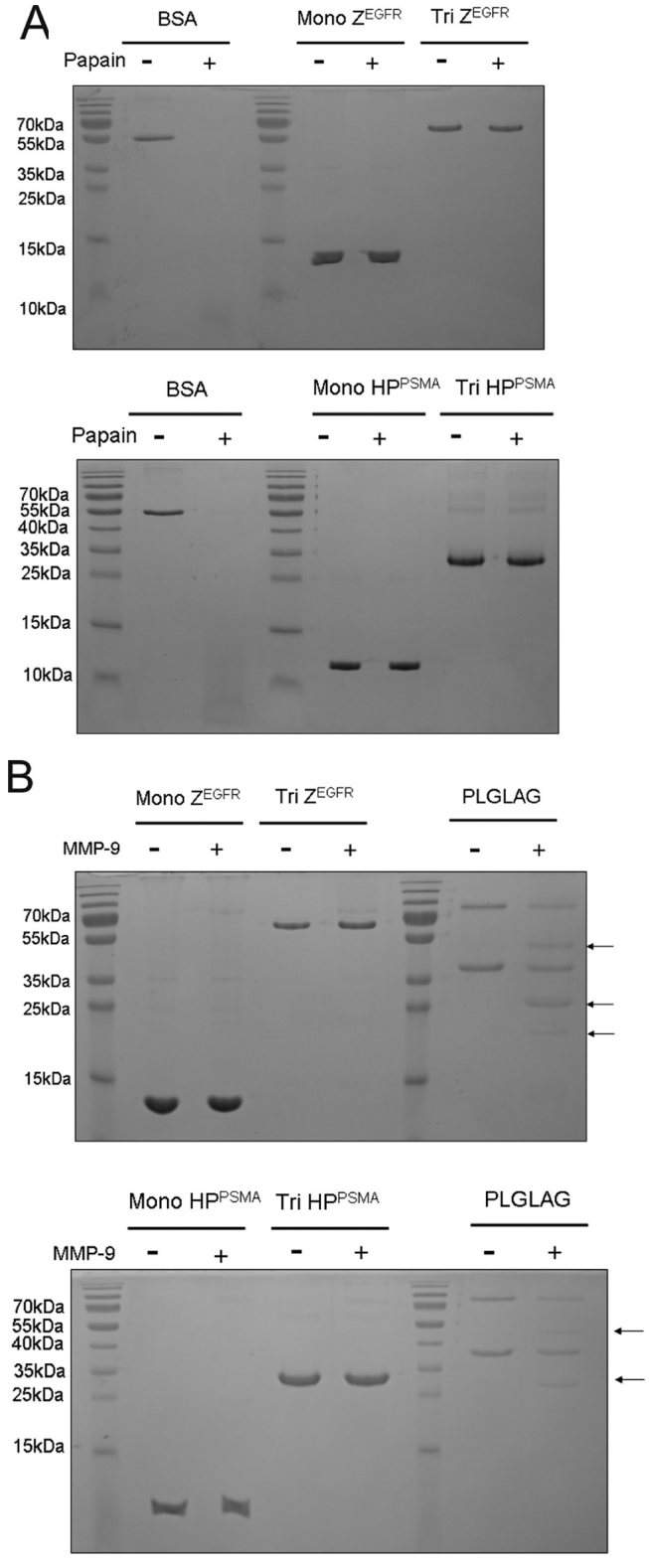

Figure 4. Protease resistance assay using papain and MMP-9. (A) Each purified protein (Z^{EGFR} monomer and trimer, HP^{PSMA} monomer and trimer) was incubated with papain at 37 °C for 30 min with BSA as a positive control. The reaction mixtures were loaded on 12% SDS-PAGE. (B) MMP-9 was incubated with each purified protein (Z^{EGFR} monomer and trimer, HP^{PSMA} monomer and trimer) as well as an engineered protein which contains MMP-9 clevage site (PLGLAG). The reactions were performed at 37 °C for 2 h. The reaction mixtures were loaded on 12% SDS-PAGE.

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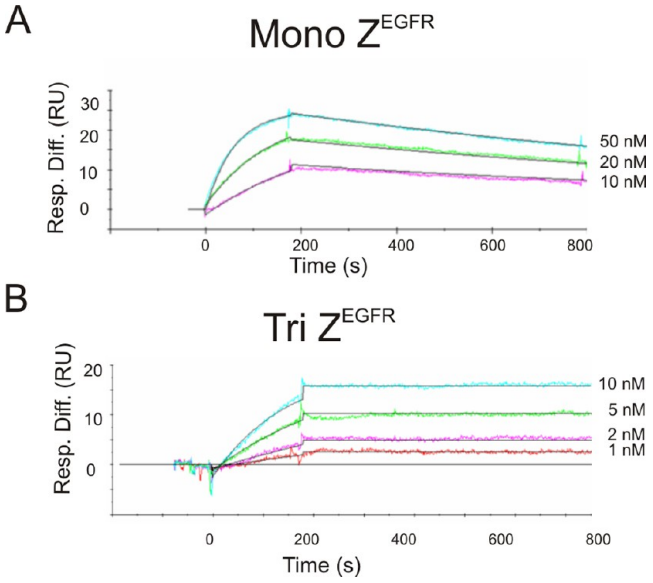

Figure 5. Biacore analysis of target binding properties. Recombinant human EGFR-Fc was immobilized on a CM5 biosensor chip via amine coupling. Different concentrations of targeting ligands were injected into the biosensor channels, and the analysis was performed at a flow rate at 20 μL/min at room temperature. (A) Z^{EGFR} monomer and (B) Z^{EGFR} trimer.

bond-mediated trimers and pentamers, which are often generated with a significant amount of intermediate forms that are extremely difficult and time-consuming to purify.[13,23,24] For example, the GCN4 isoleucine-zipper-based trimer as well as the chicken tenascin C-mediated trimer often coexist with the corresponding monomer and dimer,[23,24] making it critical to remove the intermediate forms through often inefficient gel-filtration chromatography. Fourth, the self-assembly multimerization process results in a significant increase in the molecular weight for short homing peptides and small target-binding domains, which otherwise display a much shorter half-life in vivo because of rapid proteolytic cleavage and renal clearance.

The enhancement of the binding strength of a tribody against a cell-surface receptor was demonstrated by SPR experiments (Figure 5), supporting the avidity effect of the tribody and further making it an attractive reagent for in vivo tumor detection and targeting. Cellular-binding analysis using biomarker-expressing cell lines demonstrated that the tribodies recognized their corresponding receptors much more tightly than their monomer counterparts (Figure 7). It is worth mentioning that the length of the flexible linker could be critical to achieve the desired multivalency and should be tuned depending on the nature of the targeting moiety and the receptor. The experimental conditions for SPR are artificial. The density of the receptor used in SPR is different from the native biomarker on the cell surface. It should be noted that unlike the surface of cancer cells where a biomarker of interest

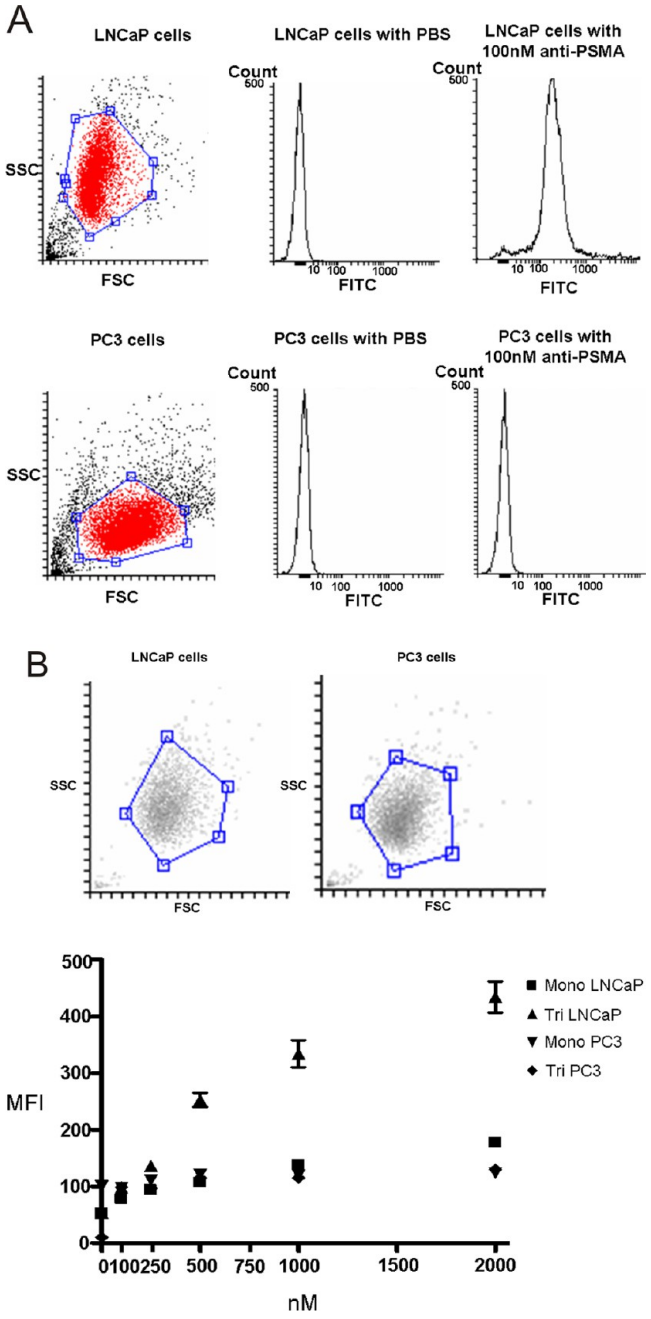

Figure 6. Flow cytometry for cell binding. (A) PSMA-positive LNCaP cells and PSMA-negative PC3 cells were grown on a 6-well plate overnight. FITC-labeled anti-PSMA antibody (100 nM) was incubated with LNCaP or PC3 cells at 25 °C for 30 min. The cell-binding signals were analyzed by flow cytometry. (B) PSMA-positive LNCaP cells and PSMA-negative PC3 cells were grown on a 24-well plate overnight. FITC-labeled HP^{PSMA} monomer and HP^{PSMA} trimer at various concentrations were incubated with LNCaP or PC3 cells at 25 °C for 30 min. The cell-binding signals were analyzed by flow cytometry. MFI, mean fluorescent intensity.

Table 2. Binding Constants of Z^{EGFR} Monomer[a]

	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (M)
Z^{EGFR} monomer	$3.29 \pm 0.82 \times 10^5$	$6.78 \pm 1.69 \times 10^{-4}$	$2.06 \pm 0.51 \times 10^{-9}$

[a]The association and dissociation constants were calculated using BIAevaluation software by the fitting data to a one-to-one Langmuir binding model.

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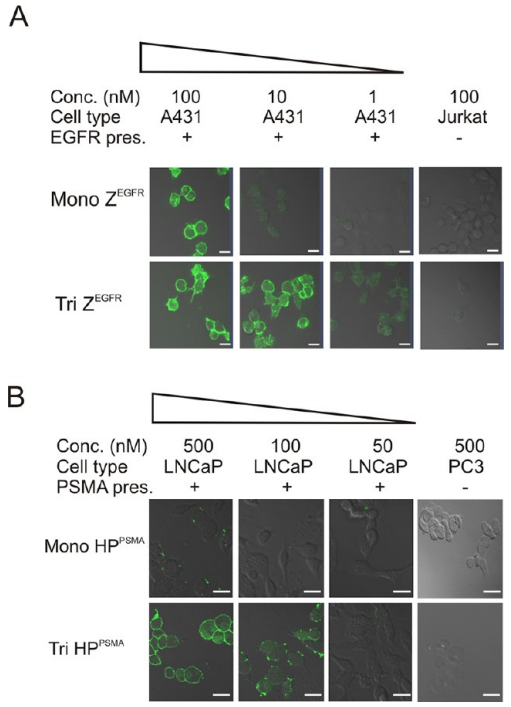

Figure 7. Comparison of the binding of monomeric and trimeric targeting ligands with targets on cell surface. (A) EGFR-positive A431 cells and EGFR-negative Jurkat cells were grown on cover slides. Different concentrations of FITC-labeled Z^{EGFR} monomer and Z^{EGFR} trimer were incubated with the cells for 30 min at 25 °C. (B) PSMA-positive LNCaP cells and PSMA-negative PC3 cells were grown on cover slides. Different concentrations of FITC-labeled HP^{PSMA} monomer and HP^{PSMA} trimer were incubated with the cells for 30 min at 25 °C. The cell-binding signals were visualized by confocal microscopy. The scale bars indicate 20 μm.

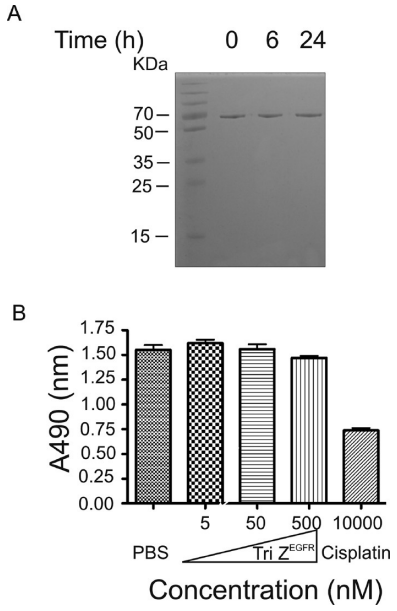

Figure 8. Serum stability and toxicity. (A) Serum stability. Purified Z^{EGFR} tribody (5 μg) was incubated with mouse serum (5 mg) for 0, 6, and 24 h as indicated. After incubation, the proteins in the reaction mixture were recovered with Co^{2+}-NTA resin. The recovered tribodies were loaded on a 12% SDS-PAGE gel to examine stability. (B) Toxicity. A431 cells were incubated with various concentrations of the Z^{EGFR} tribody for 36 h at 37 °C. Cisplatin (10 μM) was used as a positive control. Cell proliferation was determined using the absorbance at 490 nm (A490). Three repeated assays were performed.

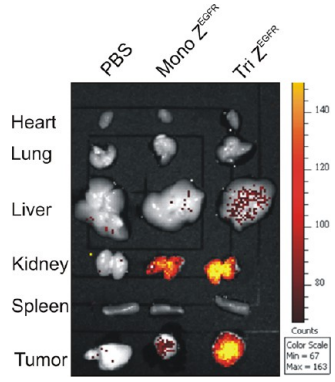

Figure 9. In vivo tumor targeting using PEGylated monomeric and trimeric Z^{EGFR}. PBS (column 1), 0.5 nmol of PEGylated Alexa750-labeled monomeric Z^{EGFR} (column 2), and 0.5 nmol of PEGylated Alexa750-labeled trimeric Z^{EGFR} (column 3) in PBS were injected into A431-xenografted nude mice. Six hours after injection the mice were sacrificed, and the images of the tumor and each tissue were taken with a Kodak In Vivo Imaging System FX-PRO.

is present with thousands of other receptors and membrane-bound proteins, only purified biomarker was present on the surface of SPR biosensor. It is expected that this absence of competition during the in vitro assays facilitates the binding of the tribody through multivalent interactions with more than one receptor. Although the results from such in vitro analysis do not necessarily correlate well with those observed using living cells, they reveal more precisely the biochemical and biophysical interactions (i.e., k_{on} and k_{off}) between a trimeric targeting ligand and a purified receptor of interest, which facilitates the rational design of targeting ligands with desired features.

Tribodies showed remarkable stability against protease degradation and serum proteases (Figures 3, 4, and 8A). In addition, they did not show detectable cell toxicity when applied up to 500 nM (Figure 8B), suggesting that they have the potential of being utilized for in vivo tumor targeting. Compared to full-length monoclonal antibodies and small protein-domain-based antibody mimics, the modest size and slower off-rate of tribody (i.e., 58 kDa for Z^{EGFR} tribody) could presumably provide rapid tissue penetration and longer retention time for a target receptor in diseased tissues.[9,19] To demonstrate the in vivo application of the tribody, we performed in vivo tumor targeting using a PEGylated tribody in mice bearing an EGFR-positive tumor. It is well-known that PEGylation of a targeting ligand aids in the reduction of nonspecific binding and increases the circulatory half-life.[25] Indeed, effective targeting to the EGFR-positive A431 tumor was demonstrated 6 h postinjection (Figure 9). The tribody was accumulated most in the tumor, with no or minimal accumulation in the heart, lungs, spleen, and liver. It was not surprising that a modest accumulation was found in kidney because of its well-known function in the clearance of macromolecules. It has been reported that the Z^{EGFR} monomer also bound murine EGFR.[26] This probably explained why there was some accumulation in mouse liver, which is known to have more expression of EGFR compared to other organs.

Despite the advantages we have demonstrated in this work, the CMP-based tribody system does have its limitations. First, it

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is unrealistic to fuse it with a very large protein domain, particularly those with complex structures or multiple cysteine residues, while still expecting it to achieve the dramatic multivalent effect as we demonstrated for short homing peptides and target-binding small protein domains. Presumably, the close proximity of the three large domains in the tribody will greatly complicate the intermolecular interaction and disulfide-bond exchange, resulting in incorrect disulfide-bond formation and misfolding of the protein, despite the use of a long flexible linker. This will greatly reduce the multivalent effects, a slightly less than 10-fold increase was recently reported by Saha and co-workers,[27] even under in vitro conditions such as in SPR experiments. Second, one critical question to ask is which valency is enough and better to achieve the desired avidity effect? We have reported previously pentameric and heptameric targeting ligands using distinctive self-assembly approaches.[6,19] On the basis of the in vitro biochemical and biophysical characterization that can be quantified, it appears that the trimeric targeting ligands perform as well as, and sometimes even better than, the pentameric and heptameric targeting ligands.[6,19] However, caution should be taken in drawing a general conclusion on this point because the interaction between a multivalent ligand with a cell-surface receptor is highly dependent on the molecular nature, oligomeric status, surface density, correct conformation, post-translational modification, and other biochemical and biophysical properties of the receptor. Our studies shed light on the further development of multivalent targeting ligands against numerous cell-surface receptors that are implicated in different human diseases.

We have developed trimeric targeting ligands based on the highly conserved CMP trimerization domain, found in mouse and human, with a remarkable stability against heat and proteolysis as well as a significantly improved target-binding affinity and an effective in vivo tumor-targeting feature in animals. The facile and tunable production of tribody suggests that it has great potential for use in specific tumor imaging and targeted delivery of numerous therapeutic agents.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

CMP, cartilage matrix protein; Tribody, trimeric targeting ligand; EGFR, epidermal growth factor receptor; PSMA, prostate-specific membrane antigen; SPR, surface plasmon resonance; Z^{EGFR}, EGFR-binding affibody; HP^{PSMA}, PSMA-binding homing peptide

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