Engineering a Thermostable Human Prolyl Endopeptidase for Antibody-Directed Enzyme Prodrug Therapy

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ABSTRACT: We present a new antibody-directed enzyme prodrug therapy strategy (ADEPT) based on a post-proline cleaving endopeptidase and prodrugs, in which cytotoxic moieties are linked to a proline-containing peptide. Human prolyl endopeptidase was expressed in *Escherichia coli* and purified to homogeneity. The enzyme was active in buffer and in human serum but was rapidly thermally inactivated by incubation at 37 °C, thus preventing applications in vivo. While prolyl endopeptidase display on filamentous phage abolished viral infectivity and prevented directed evolution strategies based on phage display, we robotically screened 10752 individual colonies of mutant enzymes using a fluorogenic assay to improve enzyme stability. A single amino acid mutation (Glu289 → Gly) improved protein stability, resulting in a half-life of 16 h at 37 °C in phosphate buffer. Two prodrugs were synthesized, in which an N-protected glycine-proline dipeptide was covalently coupled to doxorubicin and melphalan. (Benzyloxycarbonyl)glycylprolylmelphalan, but not the more sterically hindered doxorubicin prodrug, could be efficiently activated by prolyl endopeptidase [specific activity = 813.3 nmol min^{-1} (mg of enzyme)$^{-1}$ at 25 °C]. The melphalan prodrug was essentially nontoxic to CHO, F9 teratocarcinoma, MCF7 breast adenocarcinoma, and p3U1 mouse myeloma cells up to millimolar concentrations, while prodrug incubation with the engineered prolyl endopeptidase mutant led to a cell killing profile superimposable to the one of melphalan. The prolyl endopeptidase mutant was then chemically coupled to the human antibody L19, specific to the EDB domain of fibronectin, a marker of angiogenesis. The resulting immunoconjugate retains antigen binding and enzymatic activity, thus opening the way to anticancer ADEPT applications.

Cancer chemotherapy relies on the expectation that anticancer drugs will preferentially kill rapidly dividing tumor cells, rather than normal cells. Since a large portion of the tumor cells has to be killed in order to obtain and maintain a complete remission, large doses of drugs are typically used, with significant toxicity toward proliferating nonmalignant cells. Indeed, the majority of pharmacological approaches for the treatment of solid tumors suffers from poor selectivity, thus limiting dose escalation (i.e., the doses of drug which are required to kill tumor cells cause unacceptable toxicities to normal tissues) (*1*). The development of more selective anticancer drugs, with better discrimination between tumor cells and normal cells, is possibly the most important goal of modern anticancer research.

A number of monoclonal antibodies, which specifically bind to tumor-associated antigens, have been shown to display a selective localization on solid tumors, following intravenous administration (*2*). However, it is not obvious how to turn a tumor-targeting antibody into a therapeutic agent, and many research efforts are being devoted to the engineering of therapeutic antibody derivatives (*3*). In contrast to many antibody derivatives which are toxic immediately after intravenous administration (e.g., radiolabeled antibodies), ideal therapeutic strategies would display a "delayed" biocidal activity a few hours after injection, when the dose of antibody in tumor is significantly higher than in normal tissues and blood.

Antibody-directed enzyme prodrug therapy (ADEPT)[1] is a two-step targeting approach designed to improve the selectivity of antitumor agents. In the first step, an antibody−enzyme conjugate is administered, which is capable of selective localization at the tumor site. In the second step, a nontoxic prodrug is administered, when most antibody−enzyme conjugate has cleared from circulation. The prodrug is then preferentially converted into a toxic drug at the tumor site, thus sparing normal tissues (*4*, *5*).

A number of enzymes have been used for prodrug activation in ADEPT, including alkaline phosphatase (*6*−*8*), carboxypeptidase G2 (*9*−*13*), carboxypeptidase A (*14*, *15*), carboxypeptidase A1 (*16*, *17*), cytosine diaminase (*18*, *19*), β-glucuronidase (*20*−*22*), β-lactamase (*23*−*25*), nitroreductase (*26*, *27*), penicillin-V amidase (*28*), and penicillin-G amidase (*29*, *30*). Several limitations such as inefficient

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[1] Abbreviations: PEP, prolyl endopeptidase; ADEPT, antibody-directed enzyme prodrug therapy; AMC, 7-amino-4-methylcoumarin; Z, benzyloxycarbonyl; CHO, Chinese hamster ovary; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PEG, poly(ethylene glycol); MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; HRP, horseradish peroxidase; tu, transforming units.

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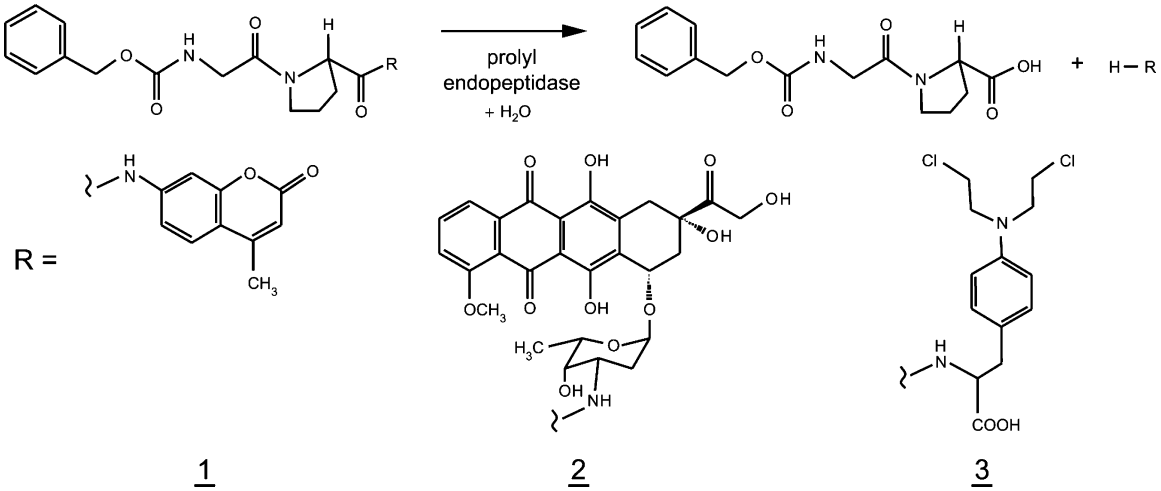

FIGURE 1: Enzymatic cleavage of prodrugs by PEP to yield the cytotoxic drugs: fluorogenic substrate of PEP (benzyloxycarbonyl)-glycylprolyl(aminomethyl)coumarin (**1**), (benzyloxycarbonyl)glycylprolyldoxorubicin (**2**), and (benzyloxycarbonyl)glycylprolylmelphalan (**3**).

tumor targeting, undesired prodrug activation by host enzymes, enzyme inhibition by endogenous inhibitors, and inefficient conversion of prodrug to drug have so far prevented most ADEPT strategies from entering clinical trials. However, an ADEPT strategy based on the bacterial carboxypeptidase G2 fused to an antibody fragment specific for the carcinoembryonic antigen has exhibited an impressive performance in animal models (*11*−*13*, *31*) and is being tested in the clinic (*9*, *10*, *32*). The carboxypeptidase activates a benzoic acid mustard−glutamate prodrug. Unfortunately, the bacterial enzyme may be recognized by the immune system of the host as foreign protein. Administration of cyclosporin delayed immune reactions, but antibodies specific to the enzyme were found in patients after 2 weeks and prevented repeated administrations (*9*, *32*).

Attempts have been made to overcome the immunogenicity of ADEPT by using human enzymes, such as β-glucuronidase or a mutant of carboxypeptidase A1. The enzymes turned out to be not sufficiently active in vivo, as the activity of human β-glucuronidase is low at physiological pH (*22*) and carboxypeptidase A1 is not sufficiently stable (*16*).

Bearing in mind the limitations of existing ADEPT strategies, we have selected the human prolyl endopeptidase (PEP; EC 3.4.21.26) as a candidate for use in ADEPT. The highly active cytosolic serine endopeptidase cleaves peptide bonds on the carboxyl side of proline in peptides (*33*, *34*). The low activity of this enzyme in human blood should prevent nonspecific activation of the prodrug (*35*). Inhibition of PEP in human blood has not been reported, and the selectivity of PEP for polypeptides smaller than 30 amino acids prevents the degradation of serum proteins (*36*). The human origin of the peptidase is likely to lower the risk of immunogenic reactions in humans and may allow the administration of multiple doses of antibody−enzyme conjugate.

We have expressed PEP in *Escherichia coli* and tested its activity in human serum. Because of the moderate thermostability of PEP in human serum, we applied directed evolution approaches based on phage display or robotized screening technologies to improve the stability of the enzyme. Furthermore, we designed two prodrugs based on the widely used chemotherapeutic drugs, doxorubicin and melphalan, and we tested their cytotoxicity and their enzyme-catalyzed activation (Figure 1).

EXPERIMENTAL PROCEDURES

Chemicals and Reagents. Ampicillin and IPTG were purchased from Applichem (Darmstadt, Germany). All of the restriction enzymes, polymerases, and deoxynucleoside triphosphates were purchased from Qbiogene (Basel, Switzerland) and Roche (Mannheim, Germany). T4 DNA ligase was obtained from Qbiogene (Basel, Switzerland). DNA primers were purchased from Microsynth (Balgach, Switzerland).

Measuring the Activity of Proline Endopeptidase in Human Serum. Human serum (Sigma, Buchs, Switzerland) was heated to 37 °C, and the proline-specific peptidase activity was immediately measured using the fluorogenic substrate *N*-(benzyloxycarbonyl)glycylprolyl-7-amino-4-methylcoumarin (Z-Gly-Pro-AMC). Z-Gly-Pro-AMC (2 mM in DMSO) (5 μL) was added to 95 μL of serum (or PBS), and the fluorescence intensity was measured at 381/455 nm.

Bacterial Strains and Vector Construction. All expression work was performed in *E. coli* strain TG1 [K12, Δ(*lac-pro*), *sup*E, *thi*, *hsdD5*/F′*traD*36, *proA*$^+$B$^+$, *lacI*^{q}, *lacZ*Δ*M*15].

The plasmid vectors were constructed using standard techniques (*37*).

pCHH28 is a pQE12-based expression vector which contains a noncoding 1500 bp stuffer fragment between the restriction sites *Eco*RI and *Bgl*II. A 1500 bp PCR product was ligated into the *Eco*RI and *Bgl*II sites of pFV46.3 (*38*).

pCHH29 is a vector for cytoplasmic expression of hexahistidine-tagged human PEP. The human PEP gene was amplified from a human liver cDNA library (Invitrogen, Basel, Switzerland) with the primers hupeecoba and hupebglfo and inserted into the *Eco*RI and *Bgl*II sites of the pQE12-based expression vector pCHH28.

pEW1 is a phage plasmid for the display of hexahistidine-tagged human PEP on phage. The gene for human PEP was amplified from pCHH29 with the primers sfihispepba and pepnotfo and inserted into the *Sfi*I and *Not*I sites of the vector pCHH2 (*39*).

pEW3 is a phage plasmid based on the vector fd-tet-DOG1 (*40*) with the unique restriction sites *Sac*I and *Kpn*I between the two N-terminal domains and the C-terminal domain of the pIII gene. The gene of the two N-terminal domains of pIII was amplified with the primers bspbafd/sactetfo from pCHH2, and the gene of the C-terminal domain of pIII was amplified with the primers kpntetba/pactetfo from pCHH2. The PCR products were assembled in a PCR reaction using the primers bspbafd/pactetfo and ligated into the *Not*I and *Pac*I sites of pCHH2.

pEW4 is a phage vector for the display of human PEP on phage inserted between the two N-terminal domains and the C-terminal domain of pIII. The gene of the human PEP was amplified with the primers N2pepsacba/Cpepkpnfo from pCHH29 and ligated into the *Sac*I and *Kpn*I sites of pEW3.

DNA Primers. The following DNA primers were used: Bspbafd, 5′-CCGTTTAATGGAAACTTCCTCATG-3′; Cpepkpnfo, 5′-CAAAATCACCGGTACCGGAGCCGCCTGGAATCCAGTCGACATTCAGGCAC-3′; huepbglfo, 5′-ACTAGATCTCTATCAGTGATGGTGATGGTGATGGCCACCTGGAATCCAGTCGACATTCAGGCAC-3′; hupeecoba, 5′-ACTGAATTCATTAAAGAGGAGAAATTAACTATGCTGTCCTTCCAGTACCCCGACGTGTACCGCGAC-3′; Kpntetba, 5′-GGTGGAGGCTCTGGTGGAGGCGGTACCGGTGGCGGCTCCGGTTCCGGTGATTTTG-3′; N2pepsacba, 5′-CAATGCTGGCGGGAGCTCTGGTATGCTGTCCTTCCAGTACCCCG-3′; Pactetfo, 5′-ATATATGTGATGGAATAACCTTGC-3′; Pepnotfo, 5′-GCATGCGGCCGCACCTGGAATCCAGTCGACATTCAGGCAC-3′; Sactetfo, 5′-GCCTCCACCAGAGCCTCCACCAGAGCTCCCACCACCAGAGCCGCCGCCAGCATTG-3′; Sfihispepba, 5′-ATGCGGCCCAGCCGGCCATGGCCCATCACCATCACCATCACGGTGGCATGCTGTCCTTCCAGTACCCCG-3′.

Expression and Purification of Prolyl Endopeptidase. A 5 mL overnight culture of *E. coli* strain TG1 containing the expression vector pCHH29 was used to inoculate 400 mL of 2YT media containing 100 μg/mL ampicillin. Cells were grown at 37 °C with vigorous shaking until an OD_{600} of 0.6 was reached. Protein expression was induced by adding IPTG to a final concentration of 1 mM. Cells were incubated for an additional 4 h at 25 °C and then harvested. The cell pellet was lysed with a buffer containing 50 mM Tris-HCl, pH 8, 300 mM NaCl, 1 mM EDTA, 10 mM imidazole, and 1 mg/mL lysozyme. Cells were additionally sonicated. The native fraction of the expressed protein was purified by nickel affinity chromatography using 300 μL of Ni-NTA−agarose (Qiagen, Hilden, Germany). The lysate−Ni-NTA mixture was loaded into a column (Bio-Rad, Glattbrugg, Switzerland) and washed twice with 6 mL of a buffer containing 50 mM Tris-HCl, pH 8, 300 mM NaCl, and 15 mM imidazole by gravity flow. The enzyme was eluted in 600 μL of buffer containing 50 mM Tris-HCl, pH 8, 300 mM NaCl, and 250 mM imidazole. Expression yields for cytoplasmic expression were 1 mg/L of culture. The enzyme was analyzed by SDS−polyacrylamide gel electrophoresis using precast 10% Bis-Tris gels (Invitrogen, Basel, Switzerland) and Coomassie staining.

Enzyme Activity Assay. Fluorescence assays were performed by following the release of free (aminomethyl)-coumarin (AMC) from the substrate Z-Gly-Pro-AMC. Time-dependent fluorescence intensity was measured with a VersaMAX Gemini plate spectrofluorometer (Molecular Devices, Sunnyvale, CA). A typical assay consisted of a 100 μL volume of 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5% DMSO, 100 μM Z-Gly-Pro-AMC, and 10−100 nM PEP mutant from either purified protein or bacterial cell lysate at 25 °C for 30 min. Fluorescence measurements were carried out with an excitation wavelength of 381 nm and an emission wavelength of 455 nm. The specificity rate constant (k_{cat}/K_M) of PEP was measured at a substrate concentration of 6 μM.

Phage Preparation and Characterization. Phage were produced in *E. coli* TG1 cells at 20 or 30 °C for 16 h in 2YT medium supplied with tetracycline (10 μg/mL final concentration). Phage were PEG purified according to standard procedures (*41*).

The number of infective phage particles was determined by incubating various phage dilutions with an excess of exponentially growing *E. coli* TG1 cells for 30 min at 37 °C and plating them immediately on selective agar plates.

The display of protein on phage was analyzed by Western blot. A total of 5×10^8 infective phage were denatured by heating at 90 °C for 5 min in the presence of 20 μL of reducing SDS sample buffer. The samples were loaded on a 10% (w/v) polyacrylamide gel (Novex, Frankfurt, Germany), separated by electrophoresis, and transferred onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membrane was blocked with PBS buffer (phosphate-buffered saline: 50 mM phosphate (pH 7.4) and 100 mM NaCl) containing 1% (w/v) milk powder, and proteins were detected with an anti-pIII murine monoclonal antibody (Mo Bi Tec, Göttingen, Germany) followed by a secondary anti-mouse HRP-goat IgG antibody (Sigma, St. Louis, MO). The peroxidase activity was detected by electrochemiluminescence using an ECL kit from Amersham Biosciences (Dübendorf, Switzerland).

Error-Prone PCR for Random Mutagenesis. The error-prone PCR method described by Leung et al. (*42*) was used to create randomly mutated DNA of the PEP gene. As template for the error-prone PCR reaction a 3000 bp long DNA fragment containing the 2100 bp PEP gene was amplified in a PCR reaction with the primers pqe12seqba and pqetempfo from pCHH29. An error-prone PCR reaction mixture contained 10 mM Tris-HCl, pH 9, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mg/mL BSA, 0.2 mM each of four dNTPs, 0.2 μg/mL template PCR, 0.5 μM primers pqe12seqba and pqe12seqfo, 10−100 units/mL Taq DNA polymerase, and 60−240 μM $MnCl_2$. The PCR was carried out in a T3 Biometra thermocycler with a program of 94 °C for 30 s; 94 °C for 15 s, 55 °C for 30 s, 72 °C for 2 min 30 s (10 cycles); 94 °C for 15 s, 55 °C for 30 s, 72 °C for 3 min (10 cycles); 94 °C for 15 s, 55 °C for 30 s, 72 °C for 3 min 30 s (10 cycles); and finally 72 °C for 4 min. The 2100 bp PCR product was separated by agarose gel electrophoresis from the 3000 bp DNA template, *Eco*RI/*Bgl*II digested, and ligated into the pQE12-based expression vector pCHH28. A total of five PEP libraries were cloned with different $MnCl_2$ concentrations in the PCR reaction (pCHH61, no $MnCl_2$; pCHH62, 60 μM $MnCl_2$; pCHH63, 120 μM $MnCl_2$; pCHH64, 180 μM $MnCl_2$; pCHH65, 240 μM $MnCl_2$). Members of the PEP libraries were sequenced with the primers Hupepseq1ba, Hupepseq2ba, Hupepseq3ba, Hupepseq4ba, and Hupepseq5ba on a ABIprism sequencer.

DNA Primers. The following DNA primers were used: Pqe12seqba, 5′-CATTATTATCATGACATTAACC-3′; Pqe-12seqfo, 5′-GTCATTACTGGATCTATCAACAGG-3′; Pqe-tempfo, 5′-TTGATGCCTCAAGCTAGAGAG-3′; Hupepseq-1ba, 5′-GGTTTGCAGAACCAGCGAG-3′; Hupepseq2ba, 5′-CCATGTCTTGGGAACCGATC-3′; Hupepseq3ba, 5′-CCTGAGCATGAGAAAGATGTC-3′; Hupepseq4ba, 5′-GAAGATTCCAATGTTCATTGTGC-3′; Hupepseq5ba, 5′-GTTGTGTTATTGCCCAAGTTGG-3′.

Screening for Thermostable Mutant Clones. The PEP library pCHH63 was electroporated into electrocompetent TG1 *E. coli* cells and spread on a 20 × 20 cm agar plate containing ampicillin (100 μg/mL final concentration). The plate was incubated overnight at 37 °C. Colonies were picked with a QpixII colony picker (Genetix, New Milton, U.K.) and transferred to two Nunclon U-shaped 96-well plates (Nalge Nunc, Rochester, NY) containing 140 μL of 2YT/ampicillin (100 μg/mL final concentration). The plates were incubated in a shaker at 37 °C until cells were visible. IPTG (15 μL, 23.8 mg/mL) was added to each well of the first plate, and the plate was shaken at 200 rpm at 25 °C overnight. Then 50 μL of 40% glycerol was added to each well of the replica plate, and the plate was stored at −80 °C. The cells in the first plate were lysed by sonication for 3 min in a sonication bath. The plates were centrifuged at 4000 rpm for 15 min, and 80 μL of the supernatant was distributed equally to two different 96-well plates. The first plate was stored at 4 °C, and the second plate was incubated at 44 °C for 40 min and then chilled to 4 °C. The differently treated cell lysates were then assembled on a 384-well plate in two neighboring wells. The fluorogenic substrate Z-Gly-Pro-AMC (30 μL, 267 μM) was pipetted to each well, and the fluorescence intensity was measured at an excitation wavelength of 381 nm and an emission wavelength of 455 nm in 3 min intervals for 30 min. All pipetting steps in microtiter plates were carried out from a liquid-handling robot from Tecan (Männedorf, Switzerland).

Measuring Thermostability of Mutant Clones. Heat treatment of PEP in cell lysate or of the purified PEP was performed with a programmable temperature controller. The cell lysate or the purified PEP in TBS, pH 7.4, or in PBS, pH 7.4, was incubated for 30 min at different temperatures and immediately cooled to 4 °C. The PEP activity remaining after the heat treatment was measured as described above.

The stability of PEP wild type or PEP mutants in human serum (or PBS) at 37 °C was performed by incubating 5 μL of purified PEP in 90 μL of human serum (or PBS, pH 7.4) for 24 h, 6 h, 3 h, 90 min, 20 min, or 5 min and measuring the residual enzymatic activity. The activity was measured with the fluorogenic substrate Z-Gly-Pro-AMC (5 μL of 2 mM) as described above.

Chemical Synthesis of Prodrugs. The (benzyloxycarbonyl)-glycylprolyldoxorubicin was prepared by reacting doxorubicin (2 mg, 3.4 μmol) in DMSO (0.2 mL) with a 10-fold molar excess of (benzyloxycarbonyl)glycylprolylhydroxysuccinimide ester (13.7 mg, 34 μmol) in DMSO (0.25 mL) and 50 μL of phosphate-buffered saline (pH 8.5) for 1 h at room temperature. The reaction product was purified by HPLC on a C-18 reversed-phase column using a linear gradient at 1.5 mL/min consisting of 0−100% solvent B (acetonitrile) against solvent A (H_2O and 0.1% trifluoroacetic acid). A detector wavelength of 496 nm was used to monitor doxorubicin and the doxorubicin prodrug, respectively. The product was dried under vacuum.

The (benzyloxycarbonyl)glycylprolylmelphalan was prepared by reacting melphalan (30.4 mg, 0.1 mmol) in DMSO (0.5 mL) with a 3-fold molar excess of (benzyloxycarbonyl)-glycylprolylhydroxysuccinimide ester (120.9 mg, 0.3 mmol) in DMSO (0.4 mL) and 0.1 mL of phosphate-buffered saline (pH 8.5) for 1 h at room temperature. The reaction product was purified by reversed-phase HPLC on a C-18 reversed-phase column using a linear gradient at 1.5 mL/min consisting of 0−100% solvent B (100 mM triethylamine, 100 mM acetic acid, pH 8, and 80% acetonitrile) against solvent A (100 mM triethylamine and 100 mM acetic acid, pH 8). A detector wavelength of 257 nm was used to monitor melphalan and the melphalan prodrug, respectively. The solvent was removed by freeze-drying.

Prodrug Activation by Prolyl Endopeptidase. The prodrug (benzyloxycarbonyl)glycylprolyldoxorubicin or (benzyloxycarbonyl)glycylprolylmelphalan (100 μM) or the fluorogenic substrate (benzyloxycarbonyl)glycylprolyl(aminomethyl)-coumarin (100 μM) in TBS (50 mM Tris-HCl, pH 7.4, 100 mM NaCl) and 5% DMSO was treated with prolyl endopeptidase (25 μg/mL, 2.5 μg/mL, 0.25 ng/mL, and 25 ng/mL final concentration) in a total volume of 200 μL for 30 min at room temperature. DMSO (200 μL) was added to stop the reaction, and the enzyme was removed by centrifugation in a Microcon YM-10 (Millipore, Volketswil, Switzerland). The reaction (300 μL) was analyzed by reversed-phase HPLC using a C-18 column. A linear gradient at 1.5 mL/min consisting of 0−100% solvent B (100 mM triethylamine, 100 mM acetic acid, pH 8, and 80% acetonitrile) against solvent A (100 mM triethylamine and 100 mM acetic acid, pH 8) was used. Doxorubicin and melphalan derivatives were monitored using a detector wavelength of 496 and 257 nm, respectively.

Cytotoxicity Assays. CHO (100 μL, 3 × 10^4 cells/mL) or F9 murine teratocarcinoma cells (100 μL, 1.5 × 10^4 cells/mL) in DMEM medium (Invitrogen, Basel, Switzerland) containing 10% calf serum, 100 μL of MCF7 cells (3 × 10^4 cells/mL; ATCC, Rockville, MD) in MEM medium (Invitrogen, Basel, Switzerland) containing 2 mM L-glutamine, 0.01 mg/mL bovine insulin, and 1 mM sodium pyruvate, or 100 μL of p3U1 mouse myeloma cells (3 × 10^4 cells/mL) in RPMI medium (Invitrogen, Basel, Switzerland) containing 10% calf serum were incubated in 96-well plates for 24 h at 37 °C. Media (100 μL) containing melphalan or *N*-(benzyloxycarbonyl)glycylprolylmelphalan with or without prolyl endopeptidase (0.5 μg/mL final concentration) were added to the wells to reach a final concentration of drug or prodrug of 2 mM, 1 mM, 300 μM, 100 μM, 30 μM, 10 μM, 3 μM, or 1 μM. The plate was incubated for 24 h at 37 °C, and the fraction of surviving cells was measured with the tetrazolium salt (MTT) test.

Synthesis of the Immunoconjugate. Prolyl endopeptidase mutant (PEP) and SIP(L19) (*43*) were chemically coupled using the heterobifunctional cross-linker *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) (Bachem, Bubendorf, Switzerland). SIP(L19) was purified as described (*43*). The antibody, dissolved in 0.1 M sodium phosphate and 0.15 M NaCl, pH 7.2, at the concentration of 1 mg/mL, was reacted with a 50-fold molar excess of MBS for 1 h at room temperature. Excess MBS was removed using a disposable

gel filtration PD10 column (Amersham Pharmacia Biotech, Dübendorf, Switzerland). The modified SIP(L19), carrying reactive maleimido groups, was then mixed at a 1:4 molar ratio with purified PEP, previously treated with 1 mM DTT and desalted on PD-10. The mixture was allowed to react for 5 h on ice. Isolation of the conjugate from the reaction mixture required a two-step purification procedure consisting of two gel filtration chromatographies on a Superdex 200 column (Amersham Pharmacia Biotech, Dübendorf, Switzerland). The size and the purity of the conjugate were analyzed by SDS−PAGE. The yield of the purified SIP(L19)-PEP protein was 300 μg (30% of the originally employed L19). The presence of both SIP(L19) and PEP in the immunoconjugate was confirmed by ELISA on EDB-coated wells (*44*). The SIP(L19) moiety could be detected using both peroxidase-conjugated protein A and peroxidase-conjugated rabbit anti-human IgE (*43*). The chemical conjugate was also positive for a peroxidase-conjugated anti-His antibody, indicating the presence of His-tagged PEP.

RESULTS

Activity and Stability of Prolyl Endopeptidase. The activity of recombinant human PEP was measured in human serum at 37 °C to assess its catalytic performance at conditions that are similar to the in vivo situation. The gene of human PEP was amplified from a human liver cDNA library and ligated into a bacterial expression vector (*45*). The enzyme was expressed in *E. coli* cells and purified to homogeneity by nickel affinity chromatography. The recombinant human PEP hydrolyzed the fluorogenic substrate (benzyloxycarbonyl)glycylprolyl(aminomethyl)coumarin efficiently in human serum ($k_{cat}/K_M = 41000\ s^{-1}\ M^{-1}$).

The stability of recombinant PEP in PBS or human serum at body temperature was measured by incubating the enzyme for different time periods and measuring the residual activity with the fluorogenic substrate. The enzyme lost 15% and 69% of its activity after 20 min incubation at 37 °C in PBS or human serum, respectively. Gel electrophoretic analysis of the protein samples in PBS at different time points confirmed that activity loss was not due to proteolytic degradation (Figure 2A). Incubation of the enzyme at different temperatures and subsequent measurement of the residual activity revealed that the human PEP is thermally inactivated. The T_{50}, the temperature at which the enzyme loses 50% of its activity in 30 min, was 34 °C in PBS, pH 7.4.

An unexpected stabilization of PEP by serum albumin was found when the enzyme was incubated with different BSA concentrations ranging from 10 μg/mL to 100 mg/mL (Figure 2B). At the highest BSA concentration tested the T_{50} of PEP was shifted by 10 °C to a higher temperature. SDS−PAGE analysis of heat-treated PEP samples showed that inactivated PEP is not proteolyzed. These findings suggest that the stabilization effect of BSA does not rely on saturating traces of proteases by BSA (Figure 2C).

Improving the Thermostability of Prolyl Endopeptidase. Two different approaches were applied to isolate PEP mutants with improved thermal stability from a large repertoire of randomly mutated PEP clones. In the first approach protein stability was linked to phage infectivity (*46, 47*) by insertion of the PEP between the N-terminal and the

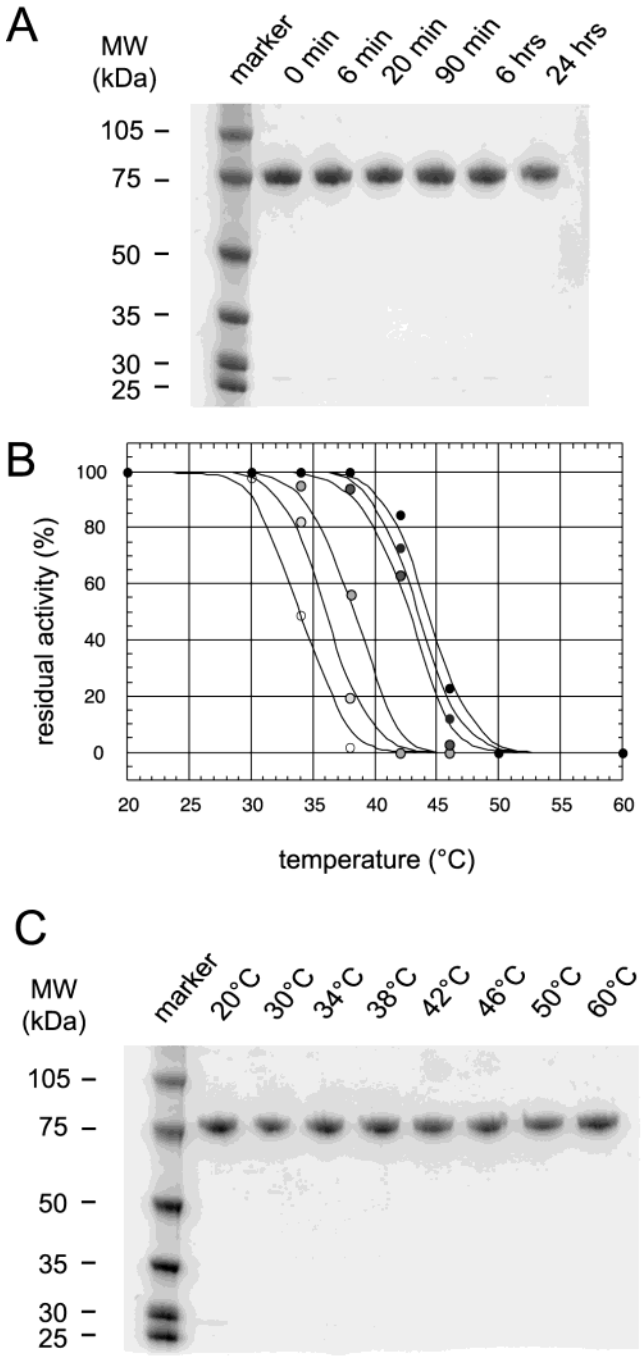

FIGURE 2: Stability of PEP. (A) Gel electrophoretic analysis of PEP samples incubated at 37 °C in PBS for different time points. (B) Thermal inactivation curves for wild-type PEP measured in the presence of increasing BSA concentrations (circles with gray scale colors from white to black: 0, 0.01, 0.1, 1, 10, and 100 mg/mL). The enzyme was incubated for 30 min at various temperatures, and the residual activity was measured. (C) SDS−PAGE analysis of PEP incubated at various temperatures for 30 min.

C-terminal domains of pIII of a filamentous phage. Thermally inactivated proteins are more susceptible to proteolysis, and phage with truncated pIII fusion proteins lose their ability to infect bacterial cells and consequently cannot propagate their DNA. The susceptibility of thermally inactivated PEP to proteolysis was tested by incubation with trypsin, followed by SDS−PAGE analysis. The heat-treated PEP was completely degraded at trypsin concentrations of 1 μM whereas 100-fold higher trypsin concentrations were needed to

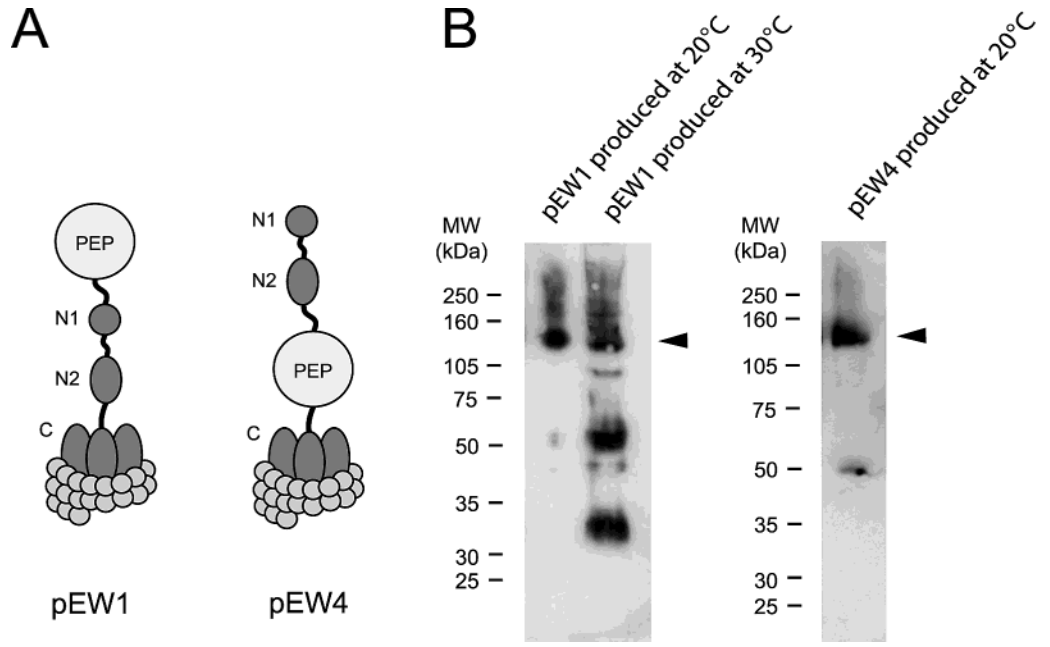

FIGURE 3: Display of PEP on phage. (A) Schematic representation of phage displaying human PEP at the N-terminal end of pIII (pEW1) or between the N2 and C domains of pIII (pEW4). (B) Western blot analysis of pIII-PEP fusion protein. Denatured proteins from phage were loaded on a 4−12% (w/v) polyacrylamide gel, separated by electrophoresis, and transferred onto nitrocellulose membrane. pIII fusion proteins were immunostained with mouse monoclonal antibodies against pIII and anti-mouse HRP. The expected molecular size of the pIII-PEP fusion proteins is indicated with an arrow.

completely degrade untreated PEP probes (data not shown). Phage displaying the human PEP at the N-terminal end of pIII (pEW1) were produced to test if the large 76 kDa enzyme can be displayed on phage. Phage displaying the human PEP between the N2 and C domains of pIII (pEW4) were produced to test if this fusion protein is incorporated into phage particles and if these phage particles are infective (Figure 3A). Western blot analysis using an anti-pIII monoclonal antibody indicated an efficient display of the large pIII-PEP fusion proteins on phage (Figure 3B). Infectivity measurements of the phage revealed that phage displaying PEP at the N-terminal end of pIII (pEW1) are highly infective (1.2×10^{11} tu/mL) whereas no infectivity could be detected in preparations of phage displaying PEP between the N2 and C domains of pIII (pEW4). Because of the unexpected loss of infectivity of these phage particles, we could not apply phage-based approaches to select for PEP mutants with improved stability.

In a second approach, we mutagenized PEP by error-prone PCR (*42*) and used a robotic microtiter plate-based activity assay in order to screen for improved thermostability. The error-prone PCR conditions were optimized in order to introduce a limited number of base substitutions (on average, one or two), thus preventing the generation of an excessive number of inactive PEP mutants. A total of five PEP libraries were cloned in which the $MnCl_2$ concentration was varied in the PCR reaction between 0 and 240 μM. The presence of the PEP gene in the expression vector of individual clones of the libraries was confirmed by PCR screening, using two primers flanking the cloning sites. Nearly 100% of the clones screened contained a PEP gene. The portion of active mutants in the libraries pCHH61−pCHH65 was determined by expressing individual enzyme mutants and measuring the PEP activity of unpurified enzyme. PEP library pCHH61 (in which $MnCl_2$ was omitted in the PCR reaction) and the PEP libraries pCHH62 (60 μM $MnCl_2$) and pCHH63 (120 μM $MnCl_2$) contained 40% of active clones. In the PEP libraries pCHH64 (180 μM $MnCl_2$) and pCHH65 (240 μM $MnCl_2$), the portion of clones expressing an active enzyme was reduced to 16% and 6%, respectively. 10752 clones of the library pCHH63 were expressed in individual wells of microtiter plates. The cell lysate was distributed to two different 96-well plates, which were incubated at 4 or 44 °C for 30 min. The differentially treated cell lysates were then reassembled in neighboring wells of a 384-well plate, and the activity was measured with the fluorogenic substrate (benzyloxycarbonyl)glycylprolyl(aminomethyl)coumarin (Figure 4A). PEP mutants that showed improved stability in the robotic screening assay were expressed and purified, and their T_{50} was determined by incubating the protein at various temperatures and measuring the residual activity of the enzyme. A PEP mutant (pCHH63/21-1F) with a single amino acid mutation (Glu289 → Gly) was identified that has a T_{50} of 43 °C, which is 4 °C higher than the T_{50} of wild-type PEP (Figure 4B). The mutant was incubated in PBS or human serum for various time periods at 37 °C, and the residual activity was measured. The pCHH63/21-1F PEP mutant showed a 4-fold longer half-life in human serum or buffer at 37 °C, compared to the wild-type enzyme (Figure 4C). The activity (specificity rate constant, k_{cat}/K_M) of the PEP mutant was not changed by the amino acid mutation.

Enzymatic Hydrolysis and Cytotoxic Activity of Prodrugs. The (benzyloxycarbonyl)glycylprolyl derivatives of doxorubicin and melphalan were prepared by reacting the primary amines of doxorubicin or melphalan with the activated ester (benzyloxycarbonyl)glycylprolylhydroxysuccinimide. The PEP-specific activation of the doxorubicin and melphalan prodrugs was monitored by reversed-phase HPLC and compared to the activtion of the fluorogenic PEP substrate

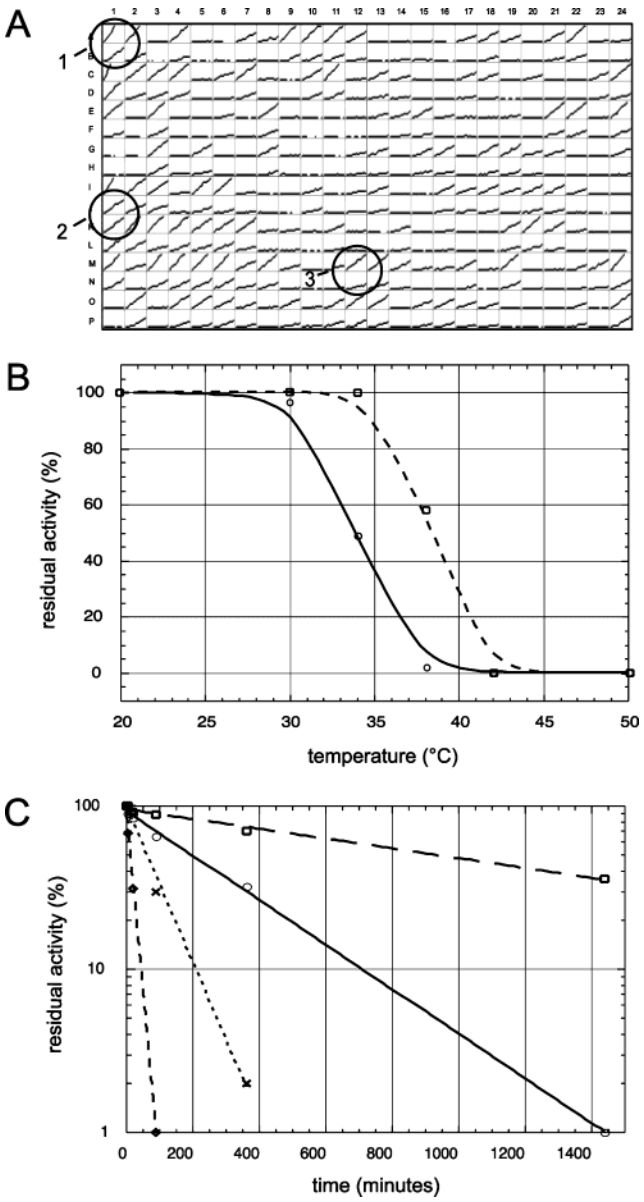

FIGURE 4: Themostability of PEP wild type and mutants. (A) Typical thermostability screen in a 384-well microtiter plate. PEP mutants that were incubated at 4 or 44 °C were assayed in neighboring wells. (1) Wild-type PEP, (2) mutant PEP with improved thermal stability, and (3) mutant PEP with lower stability. (B) Thermal inactivation curves for wild-type PEP (solid line, open circles) and the thermostable mutant pCHH63/21-1F (dashed line, open squares). (C) Thermal inactivation of wild-type PEP in PBS (solid line, open circles) or human serum (dashed line, open diamonds) and the thermostable mutant pCHH63/21-1F in PBS (dashed line, open squares) or human serum (dashed lines, crosses).

(benzyloxycarbonyl)glycylprolyl(aminomethyl)coumarin. The prodrugs or the fluorogenic substrate was incubated with PEP concentrations ranging from 12.5 ng/mL to 12.5 μg/mL for 30 min at room temperature, and the extent of hydrolysis was determined by HPLC. The fluorogenic substrate and the melphalan prodrug were efficiently activated while the hydrolysis of the doxorubicin prodrug was very slow (Table 1).

The cytotoxic activities of melphalan and (benzyloxycarbonyl)glycylprolylmelphalan on CHO, F9 (mouse teratocarcinoma), MCF7 (human breast adenocarcinoma), and p3U1 (mouse myeloma) cells were tested. The cells were exposed

Table 1: Enzymatic Hydrolysis of Prodrugs[a]

compound	specific activity [nmol min^{-1} (mg of PEP)$^{-1}$]
Z-glycylprolyl(aminomethyl)coumarin	4066.7
Z-glycylprolyldoxorubicin	96
Z-glycylprolylmelphalan	813.3

[a] The fluorogenic substrate and the prodrugs at a concentration of 100 μM in TBS, pH 7.4, were incubated with PEP at room temperature for 30 min. Substrate and product concentrations were quantified by HPLC, and the specific activity was calculated. Z = benzyloxycarbonyl.

to the drugs at concentrations ranging from 1 μM to 2 mM in the presence or absence of PEP (0.5 μg/mL) for 24 h in triplicate; cell survival was measured with the tetrazolium salt (MTT) test (Figure 5). The cytotoxicity of the melphalan prodrug on CHO and MCF7 cells was reduced at least 10-fold, compared to melphalan. In the case of p3U1, an 80-fold difference in toxicity was observed (Figure 5D). The highest concentration of melphalan prodrug tested (2 mM) was not toxic to F9 mouse teratocarcinoma cells. However, incubation of the prodrug with PEP restored a cytotoxicity equivalent to the one of melphalan, indicating that the prodrug is quantitatively cleaved.

Synthesis of the Immunoconjugate. The purified prolyl endopeptidase mutant was chemically coupled to SIP(L19) using the heterobifunctional cross-linker *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester. Figure 6A shows a SDS−PAGE analysis of the starting proteins and of the immunoconjugate. SIP(L19) is a recombinant human miniantibody specific to the EDB domain of fibronectin, a marker of angiogenesis (*43*). Recombinant antibody formats of the L19 antibody have shown an impressive ability to selectively localize to tumor neovasculature in animal models and in patients with cancer (*43*, *48*−*51*). Figure 6B confirms that the SIP(L19)-PEP immunoconjugate retains antigen-binding activity. The prolyl endopeptidase activity of SIP(L19)-PEP was 63% of the enzymatic activity of PEP (data not shown).

DISCUSSION

We have developed a new ADEPT strategy, based on the human enzyme prolyl endopeptidase and suitable prodrugs. The highly active cytosolic serine endopeptidase has several characteristics which make the human enzyme an interesting candidate for use in ADEPT: the enzyme is of human origin, the activity of endogenous PEP or of enzymes with the same substrate specificity is low in human blood (*35*), PEP is not inhibited in blood, and the selectivity of the peptidase for proteins smaller than 30 amino acids prevents degradation of serum proteins. We have demonstrated that the recombinantly produced PEP efficiently hydrolyzes the fluorogenic substrate (benzyloxycarbonyl)glycylprolyl(aminomethyl)-coumarin in human serum. Yet, the thermal inactivation of the PEP in human serum at 37 °C forced us to improve the thermostability of the enzyme.

A first protein engineering approach, in which the stability of PEP was linked to the infectivity of filamentous phage, failed. The 78 kDa enzyme was efficiently displayed on phage, but the resulting phage particles were not infective. It is possible that the large size of the enzyme (MW = 78 kDa) prevents an interdomain interaction within the minor coat protein pIII, which is essential for the infection of

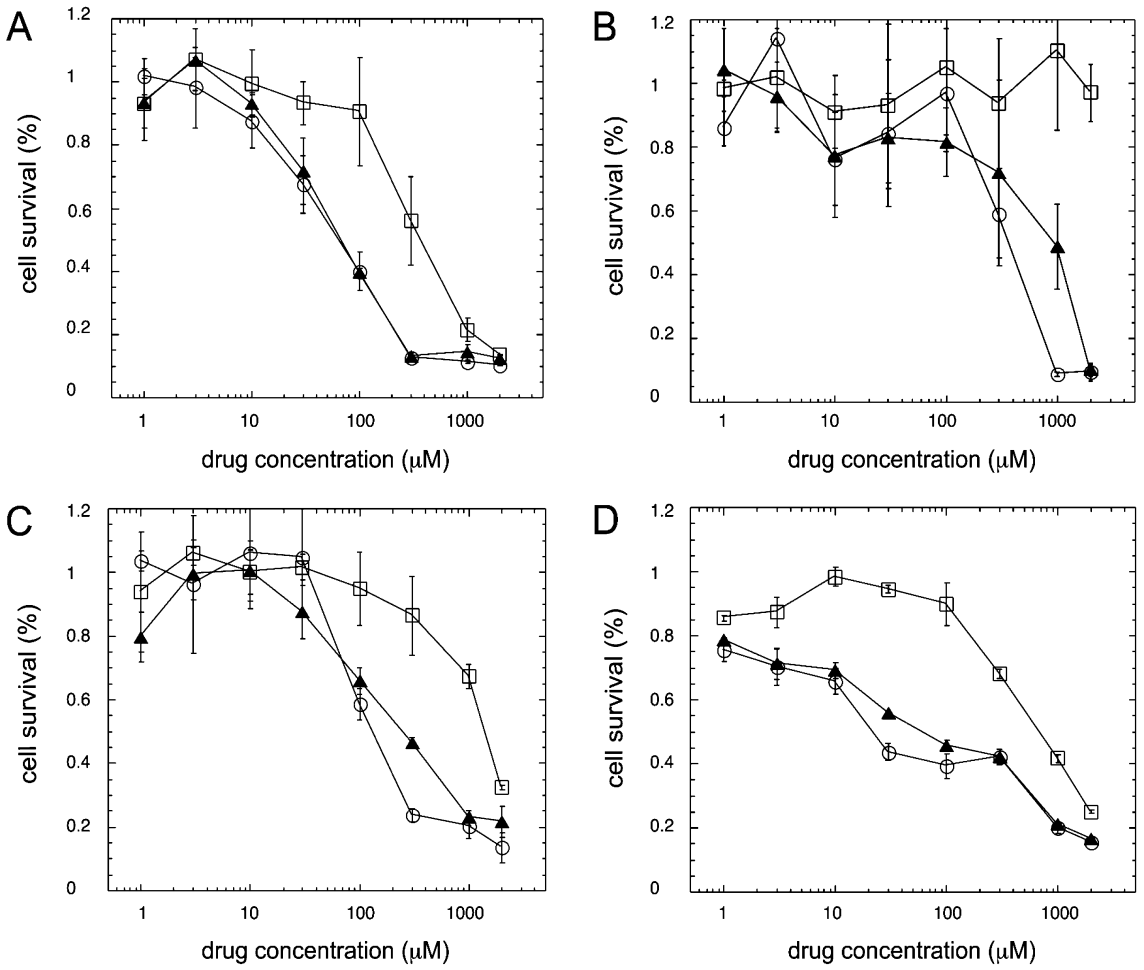

FIGURE 5: In vitro cytotoxicity of melphalan (open circle) and (benzyloxycarbonyl)glycylprolylmelphalan in the presence (closed triangle) or absence (open square) of PEP (0.5 μg/mL final concentration) on CHO (A), F9 mouse teratocarcinoma (B), MCF7 human breast adenocarcinoma (C), or p3U1 mouse myloma (D) cells. The cells were incubated with drug or prodrug concentrations ranging from 1 μM to 2 mM, and cell survival was measured with the tetrazolium blue (MTT) test.

bacterial cells (*52*). In a second approach, we screened a large library of 10752 randomly mutated enzymes in a robotized activity assay for improved thermostability. Screening of a large number of clones was possible because human PEP could be expressed in sufficient yields in bacterial cells and because the activity could be measured in the cell lysate as the level of endogenous proline-specific *E. coli* proteases is very low. A PEP mutant with a single amino acid mutation and a 4-fold longer half-life in human serum at 37 °C was identified. The enzyme may be further stabilized in a suitable environment (e.g., at the tumor site), in analogy to the observation that PEP is stabilized by incubation with BSA.

We have designed and synthesized suitable prodrugs for PEP which are composed of a proline-containing dipeptide linked to the primary amines of the chemothertherapeutic drugs doxorubicin and melphalan via an amide bond. The melphalan prodrug was efficiently hydrolyzed by PEP, and its activity should be sufficiently high to produce toxic levels of drug at the tumor site. The inefficient activaton of the doxorubicin prodrug can be explained by the bulky tetracycle of doxorubicin which does not fit into the S1′ site of PEP. Modification of the N-terminal benzyloxycarbonyl protection group and of the P2 residue of the dipeptide might even lower the cytotoxicity of the prodrug. Such modifications are unlikely to reduce the catalytic activity of PEP as the enzyme is very tolerant for many different amino acids in the S2−S4 subsite. The high tolerance of PEP for substituents at the S1′ subsite allows use also of other chemotherapeutic drugs containing primary amines, such as methotrexate and cephalosporine analogues. The main challenge for the future prolyl endopeptidase in ADEPT strategies may be represented by the availability of even more toxic drugs, bearing a primary amino group suitable for modification with a prolyl-containing peptide. Cytotoxic drugs with IC_{50} in the subnanomolar concentration range have been described. It should be possible to use prolyl derivatives of epothilones (*53*), tubulysins (*54*), and dolostatins (*55*) in ADEPT strategies featuring PEP as the prodrug converting enzyme.

In principle, a number of monoclonal antibody fragments could be considered for fusion to PEP and use in ADEPT. Our laboratory has developed the human antibody L19, specific to the EDB domain of fibronectin, a marker of angiogenesis (*56*). EDB is strongly overexpressed in the majority of solid tumors but is virtually undetectable in normal adult tissues, with the exception of the endometrium in the proliferative phase and some vessels in the ovaries (*57*, *58*). The selective targeting ability of the L19 antibody and of its derivatives has been demonstrated in animal models of angiogenesis-related diseases (*43*, *44*, *48*, *50*, *51*, *59*−*62*) and in patients with cancer (*49*). The long residence time

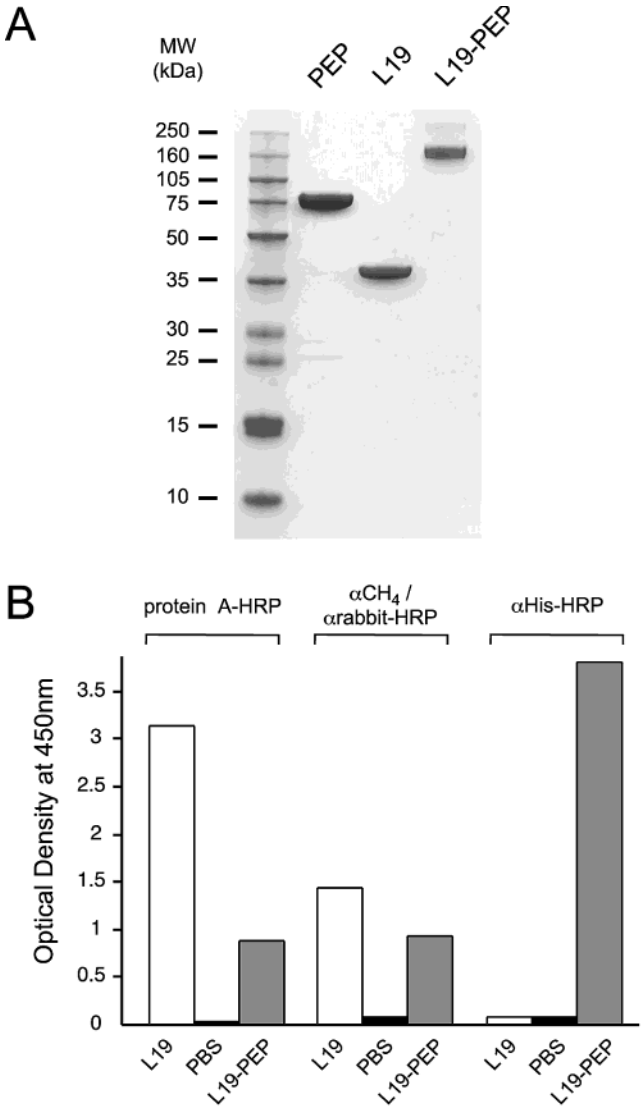

FIGURE 6: Immunoconjugate of PEP with SIP(L19), a recombinant miniantibody specific to the EDB domain of fibronectin. (A) SDS−PAGE analysis of purified PEP (lane 1), SIP(L19) (lane 2), and SIP(L19)-PEP (lane 3). (B) ELISA analysis of the SIP(L19)-PEP immunoconjugate on microtiter plates coated with purified recombinant ED-B using three different detection methods. The SIP(L19) moiety could be detected using both peroxidase-conjugated protein A and peroxidase-conjugated rabbit anti-human IgE. The chemical conjugate was also positive for a peroxidase-conjugated anti-His antibody, indicating the presence of His-tagged PEP.

of the L19 antibody in the tumor environment (*43*, *48*) makes it an ideal candidate for use in ADEPT. The retention of both antigen binding and enzymatic activity of SIP(L19)-PEP encourages the use of this immunoconjugate in tumor-targeting applications.

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