Antitumor Effect of a Novel Proapoptotic Peptide that Impairs the Phosphorylation by the Protein Kinase 2 (Casein Kinase 2)

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ABSTRACT

Protein Kinase (casein kinase 2, CK2) is a serine-threonine kinase that is frequently dysregulated in many human tumors. Therefore we hypothesized that peptides capable of binding to the CK2 acidic domain may exhibit potential anticancer properties. By screening a random cyclic peptide phage display library, we have identified a novel peptide, P15, that abrogated CK2 phosphorylation by blocking the substrate in vitro. To verify its potential antineoplastic effect, P15 was fused to the cell-penetrating peptide derived from the HIV-Tat protein. Interestingly, P15-Tat induced apoptosis as evidenced by rapid caspase activation and cellular cytotoxicity in a variety of tumor cell lines. Furthermore, direct injection of P15-Tat into C57BL/6 mice bearing day 7-established solid tumors, resulted in substantial regression of the tumor mass. Our findings describe a new proapoptotic cyclic peptide that blocks the CK2 phosphorylation and exhibits antitumor effect in vivo, indicating that the P15 peptide may potentially be used clinically to treat solid tumors or as an adjuvant for cancer therapy.

INTRODUCTION

Previous findings have suggested that phosphorylation by casein kinase 2 (CK2) is a biochemical event involved in human oncogenesis, and several groups have reported a marked elevation of the CK2 enzymatic activity in various solid tumors (1), as well as a determinant role in cell growth and proliferation (2), cell viability (3), and apoptosis (4, 5). Furthermore, direct inhibition of CK2 through an antisense approach (6) or by a specific inhibitor like the 4,5,6,7-tetramethoxy-2-nitrobenzofuran (TMB; ref. 7) has been shown to induce apoptosis in vitro. Therefore, CK2 phosphorylation constitutes a biochemical event that represents a suitable target for developing cancer therapeutics. Using a peptide-based approach exploiting the feasibility of cell permeable peptides as carriers, we describe the identified and characterized proapoptotic cyclic peptide that impairs the phosphorylation by CK2 in vitro and that leads to regression when administered directly to solid tumors. This peptide, termed P15, was originally identified by screening a 9-mer random cyclic peptide phage library and by using the human papillomavirus (HPV-16)E7 acidic domain as target. Overall, this work suggests that P15 cyclic peptide may be a potential agent in the treatment of solid tumors.

MATERIALS AND METHODS

Phage Selection. A random 9-mer cyclic peptide phage library (8) was used for the screening. Phage were selected by panning on streptavidin-coated beads (6.4 mm in diameter, Precision Plastic Ball Co., Ilkley, West Yorkshire, United Kingdom) containing a biotinylated synthetic peptide of the HPV-16 E7 onco-protein (28 LNDSSEEDEI 38) as target. After three rounds of selection, positive clones were identified by their specific binding to the E7 (amino acid 28–38)

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synthetic peptide in ELISA (phage ELISA). DNA of individual positive phage was sequenced, and the corresponding amino acid sequence was deduced from the nucleotide sequence. Finally, selected peptide-presenting phages were checked for their ability to block the phosphorylation of the HPV-16 E7 fusion protein by CK2.

Design and Synthesis of Peptide Chimeras. The target peptide used for screening the Phage Display Library corresponds to the acidic domain for CK2 on the HPV-16 E7 (28 LNDSSEEDEI 38). The two synthetic peptide chimeras used in this work contain the cell penetrating peptide Tat (48–68; GRKKRRQRRRPPR) in the NH2 terminus separated from the respective peptide cargoes at the COOH-terminus. One of these cargoes represents the cyclic peptide P15 (CWMSPRHLKCOEAPAS (19)GTC; chimera P15-Tat), and the other corresponds to the HPV-16 E7 acidic domain in which the two phosphorylatable residues have been substituted by alamine (chimera F202-Tat, negative control). All peptides were synthesized on solid phase and purified by reverse-phase high-performance liquid chromatography to >95% purity on an acetonitrile/H2O-trifluoroacetic acid gradient and confirmed by ion-spray mass spectrometry (Micromass, Manchester, United Kingdom). Lyophilized peptides were reconstituted in PBS for use in vitro and in vivo. O-phthalaldehyde (Merck) and 9-fluoromethyl-chlorofluoromate (Merck) for derivatization of amino acids was used to quantitate solubilized peptides by amino acid analysis. For the synthesis of P15-Tat, an additional cyclization step was done by oxidation of the peptide (0.1 mg/ml) in ammonia aqueous solution (pH 8.2) for 72 hours with agitation. Formation of disulfide bridges between the two cysteine residues available in P15 was verified by reverse-phase high-performance liquid chromatography and ion-spray mass spectrometry analysis.

In vitro Phosphorylation of HPV-16 E7-GST Fusion Protein. The recombinant HPV-16 E7-glutathione S-transferase (GST) fusion protein that served as a CK2 substrate for the in vitro phosphorylation in this work was purified by Glutathione Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden). For in vitro phosphorylation, about 30 μL of Glutathione Sepharose beads containing 10 μg of HPV-16 E7-GST in CK2 kinase buffer [20 mmol/L HEPES (pH 7.8), 20 mmol/L MgCl2] were used in each reaction. Before the phosphorylation reaction, each 10 μg transduction unit of peptide-presenting phages or 100 μmol/L, from synthetic peptides were preincubated with the E7-GST beads for 1 hour at 37°C with occasional shaking. Phosphorylation was done by adding 10 μCi of [γ32P]ATP (Amersham Biosciences) and 1 unit of recombinant CK2 (Promega), and further incubated for 30 minutes at 37°C. The kinase reactions were stopped by adding 2X sample buffer, boiling, and applying to a 10% denaturing polyacrylamide gel. The gel was blue stained and further dried in a gel dryer (Bio-Rad, Hercules, CA). The CK2 activity was expressed as phosphorylated HPV-16 E7 relative levels with respect to the blue-stained HPV-16 E7 levels.

Cell Viability Assay. H-125, H-82, TC-1, and Jurkat cell lines were maintained at 37°C and 5% CO2 in RPMI 1640 supplemented with 10% fetal serum (HyClone, Logan, UT), Ca/Ski, SiHa, and HeLa cells were cultured in DMEM at 37°C and 5% CO2 in RPMI 1640 supplemented with 10% fetal serum (HyClone, Logan, UT). Ca/Ski, SiHa, and HeLa cells were cultured in DMEM at 37°C and 5% CO2 in RPMI 1640 supplemented with 10% fetal serum (HyClone, Logan, UT). Ca/Ski, SiHa, and HeLa cells were cultured in DMEM at 37°C and 5% CO2 in RPMI 1640 supplemented with 10% fetal serum (HyClone, Logan, UT). Ca/Ski, SiHa, and HeLa cells were cultured in DMEM at 37°C and 5% CO2 in RPMI 1640 supplemented with 10% fetal serum (HyClone, Logan, UT). Ca/Ski, SiHa, and HeLa cells were cultured in DMEM at 37°C and 5% CO2 in RPMI 1640 supplemented with 10% fetal serum (HyClone, Logan, UT). Ca/Ski, SiHa, and HeLa cells were cultured in DMEM at 37°C and 5% CO2 in RPMI 1640 supplemented with 10% fetal serum (HyClone, Logan, UT). Ca/Ski, SiHa, and HeLa cells were cultured in DMEM at 37°C and 5% CO2 in RPMI 1640 supplemented with 10% fetal serum (HyClone, Logan, UT). Ca/Ski, SiHa, and HeLa cells were cultured in DMEM at 37°C and 5% CO2 in RPMI 1640 supplemented with 10% fetal serum (HyClone, Logan, UT). Ca/Ski, SiHa, and HeLa cells were cultured in DMEM at 37°C and 5% CO2 in RPMI 1640 supplemented with 10% fetal serum (HyClone, Logan, UT). Ca/Ski, SiHa, and HeLa cells were cultured in DMEM at 37°C and 5% CO2 in RPMI 1640 supplemented with 10% fetal serum (HyClone, Logan, UT). Ca/Ski, SiHa, and HeLa cells were cultured in DMEM at 37°C and 5% CO2 in RPMI 1640 supplemented with 10% fetal serum (HyClone, Logan, UT). Ca/Ski, SiHa, and HeLa cells were cultured in DMEM at 37°C and 5% CO2 in RPMI 1640 supplemented with 10% fetal serum (HyClone, Logan, UT). Ca/Ski, SiHa, and HeLa cells were cultured in DMEM at 37°C and 5% CO2 in RPMI 1640 supplemented with 10% fetal serum (HyClone, Logan, UT). Ca/Ski, SiHa, and HeLa cells were cultured in DMEM at 37°C and 5% CO2 in RPMI 1640 supplemented with 10% fetal serum (HyClone, Logan, UT). Ca/Ski, SiHa, and HeLa cells were cultured in DMEM at 37°C and 5% CO2 in RPMI 1640 supplemented with 10% fetal serum (HyClone, Logan, UT).

Detection of Caspase Activation. Caspase activation was monitored by CaspACE FITC-VAD-FMK in situ marker (Promega, Madison, WI). Briefly, 100,000 TC-1 cells were seeded on multichamber slides (Dako Corp., Carpinteria, CA) and 24 hours later, peptides were added to a final concentration of 100 μmol/L and incubated for 30 minutes at 37°C, 5% CO2. Subsequently, the fluorescent in situ caspase marker was added to 10 μmol/L, and incubation was
extended for 20 minutes. Cells were fixed in 10% buffered formalin for 30 minutes at room temperature; slides were rinse extensively, mounted with 10% glycerol, and observed under a fluorescence microscope (Olympus). For monitoring caspase 3 activation, samples were first fixed with 4% paraformaldehyde in PBS at 4°C for 12 hours and subsequently mounted on coated gelatin glass slides (Dako). After permeabilization with 0.2% Triton X-100 in PBS and blocking with 0.2% BSA, a rabbit polyclonal antibody anticaspase 3 (R&D Systems Inc., Minneapolis, MN) was added and further incubated overnight at 4°C. Finally, samples were incubated with a FITC-conjugated antirabbit IgG during 1 hour at room temperature, and fluorescence was observed under a Nikon microscope with attached laser confocal scanning system MRC 600 (Bio-Rad, Watford, Herts, United Kingdom). Ten to twelve fields were imaged from each sample. Four to fifteen serial optical z-sections (0.2–0.5 μm thick) were collected from each observed field.

In vivo Administration of P15-Tat. C57BL/6 mice were challenged with 500,000 TC-1 cells on the right flank. After 7 days, tumor-bearing animals received intratumor daily injections for 5 consecutive days with 200 μL of 0.24 mmol/L P15-Tat or F20.2-Tat in PBS (200 μg/dose), or received only the vehicle PBS as a control. Ten mice were used in each group and were sacrificed by cervical dislocation at the end of the trial. Animals were maintained in pathogen-free conditions, and procedures were done in accordance with recommendations for the proper use and care of laboratory animals. Tumors were measured with a caliper, and the respective volumes were calculated with the following formula: volume = width² (mm²) × length (mm)/2.

RESULTS AND DISCUSSION

In the present study, a peptide-based approach was used to interfere with CK2 phosphorylation by blocking substrate interaction. Tumor cell lines and a relevant tumor model were used to further investigate the potential antineoplastic effect of such a peptide inhibitor both in vitro and in vivo. To identify peptides that targeted the CK2 phosphorylation site, we screened a 9-mer random cyclic peptide phage library, using a synthetic peptide spanning from Leu-28 to the Iso-38 of the HPV-16 E7 oncoprotein. This region contains multiple acidic residues downstream of the phosphorylatable aminocids, Ser-31/Ser-32, which represent an excellent substrate for phosphorylation by CK2 (9). After consecutive rounds of selection, 11 different phage clones were identified, on the basis of the sequence analysis of the respective nanopeptides (Fig. 1A).

The inhibitory effect of the selected phages on the phosphorylation of recombinant E7-GST fusion protein by CK2 was next examined. Phages 1 and 15 exhibited the strongest inhibition of E7 phosphorylation when compared with that observed for the PC89 phage used as a negative control (Fig. 1A). The antineoplastic properties of the cyclic peptide corresponding to phage 15 were next examined, and initial experiments showed that P15 largely abrogated the phosphorylation of E7-GST when preincubated with the substrate (Fig. 1B).

As earlier data have shown that induction of apoptosis could be achieved by targeting the CK2 enzyme within tumor cells (6, 7), we hypothesized that P15-Tat could exert a similar effect by virtue of its potential antineoplastic effect of such a peptide inhibitor both in vitro and in vivo. To identify peptides that targeted the CK2 phosphorylation site, we screened a 9-mer random cyclic peptide phage library, using a synthetic peptide spanning from Leu-28 to the Iso-38 of the HPV-16 E7 oncoprotein. This region contains multiple acidic residues downstream of the phosphorylatable aminocids, Ser-31/Ser-32, which represent an excellent substrate for phosphorylation by CK2 (9). After consecutive rounds of selection, 11 different phage clones were identified, on the basis of the sequence analysis of the respective nanopeptides (Fig. 1A).

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As earlier data have shown that induction of apoptosis could be achieved by targeting the CK2 enzyme within tumor cells (6, 7), we hypothesized that P15-Tat could exert a similar effect by virtue of its
capacity to block CK2 substrates. To investigate this possibility, P15 was synthetically fused to the cell penetrating peptide Tat 48–68 that has been extensively used for delivering peptides cargoes within cells (10). After exposure of TC-1 cell to P15-Tat, in situ caspase detection experiments clearly indicated that P15-Tat treatment led to a rapid activation of caspase pathways within 30 minutes of exposure to peptide (Fig. 2A). Likewise, laser confocal microscopy (Fig. 2B) was used to observe activation of caspase-3 in the cytoplasm, whereas in negative controls no caspase activation was detected. Thus, P15 behaves as a proapoptotic peptide in tumor cells when introduced as a cargo together with the HIV-1 Tat peptide.

The biological effect of P15-Tat was then examined in various tumor cell lines from different origins; importantly, P15-Tat exhibited differential cytotoxic effects on various tumor cell lines as determined by the IC50 values (Table 1). Human lung cancer cells had the highest sensitivity to the cytotoxic effect of P15-Tat. Previous findings have shown that basic peptide (amino acid 46–60) on the HIV Tat protein interacts functionally with vascular endothelial growth factor receptors (11) and the cell surface heparan sulfate proteoglycans (12), and such a domain is responsible for the internalization of the Tat protein into the cell. Whether the differential cellular response toward P15-Tat in tumor cells is influenced by a differential membrane receptor density for vascular endothelial growth factor and/or Heparin merits additional investigation.

Finally, to explore whether P15-Tat could represent a therapeutic strategy to target exponential growing tumors, the efficacy of daily intratumoral administration of P15-Tat into the TC-1 lung epithelial tumor implanted in syngeneic C57BL/6 mice was investigated. Administration of P15-Tat led to a significant reduction or complete inhibition of tumor growth compared with the groups treated either with PBS or F20.2-Tat (Fig. 3A). By the sixth day, \( P = 0.0011 \) was achieved between the P15-Tat versus PBS group and \( P = 0.0140 \) between P15-Tat versus F20.2-Tat group as determined by the Mann-Whitney \( U \) test (confidence intervals 95%). Interestingly, the differences between P15-Tat and the control groups were maintained after cessation of treatment and until the end of the assay. A plot of individual tumor sizes (Fig. 3B) indicated that only 1 of 10 mice from the F20.2-Tat-treated group overlapped in tumor volume with the P15-Tat-treated group from day 6 to day 15. Thus, these results show a vigorous antitumor effect of P15-Tat when directly administered into the TC-1 solid tumor for 5 days. The proapoptotic effect of P15-Tat shown in vitro along with previous findings indicating a role for apoptosis in priming the immune response (13) suggest that the P15 peptide may enhance the cellular immune response against tumor antigens by triggering massive apoptosis in the tumor milieu.

Taken together, our data provides proof-of-concept that a peptide inhibitor that blocks CK2 phosphorylation by targeting the substrate can exhibit antitumor properties in both tumor cell lines and in an animal model. P15-Tat may serve as the prototype of a peptide-based drug with the potential to reduce tumor burden alone or when coadministered with conventional chemotherapy.

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