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Use of Penetrating Peptides Interacting with PP1/PP2A Proteins As a General Approach for a Drug Phosphatase Technology

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ABSTRACT

Protein phosphatase types 1 (PP1) and 2A (PP2A) represent two major families of serine/threonine protein phosphatases that have been implicated in the regulation of many cellular processes, including cell growth and apoptosis in mammalian cells. PP1 and PP2A proteins are composed of oligomeric complexes comprising a catalytic structure (PP1c or PP2AC) containing the enzymatic activity and at least one more interacting subunit. The binding of different subunits to a catalytic structure generates a broad variety of holoenzymes. We showed here that casein kinase 2α (Ck2α) and simian virus 40 small t antigen share a putative common β-strand structure required for PP2A1 trimeric holoenzyme binding. We have also characterized DPT-sh1, a short basic peptide from Ck2α that interacted only in vitro with the PP2A-A subunit and behaves as a nontoxic penetrating shuttle in several cultivated human cell lines and chick embryos. In addition, DPT-sh1 specifically accumulated in human red cells infected with *Plasmodium falciparum* malaria parasites. We therefore designed bipartite peptides containing DPT-sh1 and PP1- or PP2A-interacting sequences. We found that DPT-5, a DPT-sh1-derived peptide containing a short sequence identified in CD28 antigen, interacts with PP2A-Bα, and DPT-7, another DPT-sh1-derived peptide containing a short sequence identified in Bad as a PP1 catalytic consensus docking motif, induce apoptosis in cultivated cell lines. These results clearly indicate that the rational design of PP1/PP2A interacting peptides is a pertinent strategy to deregulate intracellular survival pathways.

In eukaryotic cells, biological activity of nearly 30% of proteins is modulated by phosphorylation. Reversible protein phosphorylation regulates multiple cellular processes, including metabolism, signal transduction pathways, cell cycle progression, oncogenic transformation, cell differentiation, and apoptosis (Garcia et al., 2000, 2003). Protein phosphorylation regulates the activities of protein kinases and protein phosphatases themselves (Hunter, 2000). Type 1 (PP1) and type 2A (PP2A) Ser/Thr protein phosphatases are major regulators of cell dephosphorylation. Binding of PP1 catalytic subunit (PP1c) to specific regulatory subunits generates a large family of PP1 holoenzymes (Cohen, 2002). For PP2A

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ABBREVIATIONS: PP, protein phosphatase; PP1c, PP1 catalytic subunit; PP2Ac, PP2A catalytic subunit; PP2A1, trimeric ABαC PP2A holoenzyme; PTD, protein transduction domain; DPT, phosphatase-derived drug technology; CK2α, casein kinase 2α; SV40, simian virus 40; SRP, streptavidin-conjugated peroxidase; DAB, 3,3′-diaminobenzidine tetrahydrochloride; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; OPD, orthophenyldiamine; CPP, cell-penetrating peptide; Hsp90, 90-kDa heat shock protein; PKA, protein kinase A.

protein family, the catalytic PP2Ac subunit associates with a structural PP2A-A subunit to form a dimeric core enzyme that interacts with variable regulatory (B) subunit(s) (McCright et al., 1996) and confers substrate specificity to the dephosphorylating activity. The multiple PP1/PP2A holoenzymes are differentially expressed and targeted to distinct subcellular compartments.

Recent studies have identified small protein domains, termed protein transduction domains (PTDs), that promote the delivery of peptides and proteins into cells. The PTDs identified in proteins such as HIV-1 Tat, the *Drosophila melanogaster* antennapedia homeoprotein (Antp), or HSV-1 VP22, can cross over the cell membrane by a process called protein transduction (which remains poorly defined) and can reach the nucleus, where they display their biological activity (Prochiantz, 2000). All known short cell-penetrating peptides derived from PTDs have a stretch of lysine or arginine-rich sequence required for intracellular delivery and peptide solubility. Because cellular delivery of conjugated (or fused) bio-molecules represent an interesting potential for drug design and molecular therapy (Ford et al., 2001), we have capitalized on these features to design peptides for intracellular delivery of phosphatase-targeting peptides.

We describe here a new phosphatase-derived drug technology (DPT), based on the concept of blocking the interaction of the PP1 or PP2A core catalytic subunit with their local regulatory partners using intracellular delivery of competing peptides to prevent specific substrate dephosphorylation. Several model systems have been investigated here. Based on structural analysis of PP2A interactions with casein kinase 2α (CK2α) from various species and the SV40 virus encoded small t antigen, a common β-strand structure interacting with the structural PP2A-A subunit was identified. A new cell-penetrating sequence was identified in a *Theileria parva* CK2α and was used as a new nontoxic shuttle for ex vivo and in vivo intracellular delivery of sequences interfering with intramolecular PP2A or PP1 holoenzymes. We show here successful intracellular peptide delivery in several ex vivo systems, including *Plasmodium falciparum* malaria parasites and in vivo chick embryos. In addition, and consistent with recent data concerning the role of PP1/PP2A proteins in apoptosis, we also found that intracellular delivery of sequences interacting with PP1c or PP2A Bα subunits by DPT-sh1-derived penetrating peptides induced cell death in several cell lines.

Materials and Methods

Cell Culture and Reagents. Adherent HeLa cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) as exponentially growing subconfluent monolayer on microplates or in 12-, 24-, or 96-well plates. Jurkat lymphoid T cells (clone E6; American Type Culture Collection) were cultured in RPMI 1640 GlutaMAX medium (Gibco; Invitrogen). Both cell lines were cultured in medium supplemented with 10% (v/v) fetal calf serum and antibiotics (100 U/ml streptomycin-penicillin). *P. falciparum* parasites (FUP/CB strain) were cultivated at 5% hematocrit in human A+ erythrocytes as described by Trager and Jensen (1976), in RPMI 1640 medium supplemented with 35 mM HEPES, 24 mM $NaHCO_3$, 5% human AB+ serum, 0.25% AlbuMAX, 1 mg/ml hypoxanthine, and 5 mg/ ml gentamycin at 37°C.

Streptavidin-conjugated peroxidase (SRP), 3,3′-diaminobenzidine tetrahydrochloride (DAB), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma. The annexin-V-fluorescent binding assay kit was from Roche.

Peptides. High-performance liquid chromatography-purified NH_2-biotinylated or FITC-conjugated peptides (purchased from Neosystem) were synthesized by solid-phase peptide synthesis. Peptides were dissolved in 150 mM NaCl and stored frozen at −20°C until use.

PP2A Proteins and Antibodies. Protocol for purification of PP2A proteins (trimeric PP2A1 and PP2A-A or Bα subunits) from pig brain was adapted from Waelkens et al. (1987). Characterization of PP2A antibodies (polyclonal guinea pig anti-A, or anti-B and anti-C subunits) has been described previously (Bosch et et al., 1995).

PP2A-Binding Assays on Cellulose-Bound CK2α and CD28 Peptides. Overlapping dodecapeptides scanning the whole *Theileria parva* CK2α sequence or the CD28 intracytoplasmic domain were prepared by automated spot synthesis (Abimed, Langerfeld, Germany) onto an amino-derived cellulose membrane, as described previously (Frank and Overwin, 1996; Valle et al., 1999). Membrane was blocked using SuperBlock (Pierce), incubated with purified PP2A-A subunit or PP2A1 holoenzyme and after several washing steps, incubated with anti-PP2A antibody, followed by peroxidase-conjugated secondary antibody. Positive spots were visualized using the ECL system.

Cellular Localization of Tagged Peptides. Exponentially proliferating cells were rinsed twice in phosphate-buffered saline (PBS). A total of 6×10^4 cells per well were seeded in 24 well plates and incubated in DMEM at 37°C. Twenty-four hours later, biotinylated peptides preincubated with streptavidin (ratio 1:4; 30 min at room temperature) were added to the cells and incubated for different periods of time at 37°C. Cells were rinsed twice in PBS, incubated with DAB for 5 min, washed in PBS, and analyzed by microscopy. For FITC-conjugated peptides, cells were incubated with the peptides and washed twice in PBS as above and then directly analyzed by fluorescence microscopy.

DPT-4 and DPT-5 biotinylated peptides were also directly incubated with cells for intracellular localization. Cells were then rinsed twice in PBS and fixed with 100% ethanol for 10 min before adding 10 μg/ml streptavidin peroxidase. After a 30-min incubation at 37°C, cells were rinsed twice in PBS, incubated with DAB for 5 min, washed in PBS, and analyzed by microscopy. For FITC-conjugated peptides, cells were incubated with the peptides and washed twice in PBS as above, and then directly analyzed by fluorescence microscopy.

Quantification of Peptide Internalization. Before incubation, peptides were preincubated with SRP conjugated in a 4:1 ratio. Cells at 50% of confluence were incubated with 10 or 100 μM peptide in 24-well plates. After 24 h, cells were washed twice in PBS and lysed in 300 μl of 0.1 M Tris, pH 8, and 0.5% Nonidet P-40 buffer for 10 min on ice. Cellular debris were removed by centrifugation at 13,000*g* for 10 min at 4°C. A total of 50 μl of cell lysate was mixed with 50 μl of OPD buffer (51.4 mM Na_2HPO_4 and 24.3 mM citric acid) then mixed with 100 μl of OPD solution [one OPD tablet from Sigma (St. Louis, MO) in 100 ml of OPD buffer in which 40 μl of 30% hydrogen peroxide was added just before use]. After 10 to 20 min, reaction was stopped by addition of 100 μl of 1 M HCl, and optical density was read at 490 nm.

Determination of Peptide Interaction with PP1 or PP2A Targets. 1×10^6 cells were washed twice then lysed 10 min on ice in 300 μl of lysis buffer (50 mM Tris, pH 7.4, 200 mM NaCl, 20% glycerol, 1% Nonidet P-40, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1 mM orthovanadate, and "complete, EDTA-free" protease inhibitor cocktail from Roche). Lysates were clarified at 13,000*g* for 10 min at 4°C and were preincubated with biotinylated peptides at 100 μM for 1 h at room temperature. After this preincubation, 30 μl of streptavidin-coated immunomagnetic beads (Calbiochem, San Diego, CA) were added for 2 h at 4°C. Biotinylated peptides were pulled down with streptavidin beads and

washed four times in 750 μl of lysis buffer. Bound proteins and clarified lysates were then analyzed by Western blotting using PP1 or PP2A antibodies.

Cytotoxicity and Annexin-V assays. The cytotoxicity of peptides in HeLa or Jurkat cell lines was analyzed by a colorimetric assay using MTT for adherent cells (Mosmann, 1983) or 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium for lymphocytic cells (Hansen et al., 1989), as described by the manufacturer (Sigma).

For detection of apoptotic cells, we used an Annexin-V-APC–conjugated kit (Roche) for the assessment of outer leaflet exposure of phosphatidylserine (PS) in the plasma membrane of apoptotic cells. Staining was performed according to the manufacturer's instructions. A total of 20,000 cells were analyzed by flow cytometry in a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Necrotic cells were excluded by propidium iodide staining, and single annexin V-positive cells were considered apoptotic.

Use of Penetrating Peptides in the Chick Embryo. Isa Brown chick eggs were incubated at 38°C and windowed on day 2 of incubation. Embryos were injected in the neural tube between the 10th and 15th somite under a dissecting microscope with 0.1 to 0.5 μl of 2 mM NH_2-biotinylated peptides in Hank's solution. After 24 h, embryos were collected and directly frozen at −70°C. Detection of NH_2-biotinylated peptides on 30-μm cryosections was analyzed as described above for cellular assays.

Analysis of Intracellular Peptide Delivery in Human Erythrocytes Infected with *P. falciparum*. Fluorescein-labeled DPT-sh1 or DPT-sh2 were added at a final concentration of 50 μM to uninfected erythrocyte control or a *P. falciparum* culture of plasma-gel-purified parasites (Lambros and Vanderberg, 1979) at 1% hematocrit in 24-well plates for 6 h under 5% CO_2 atmosphere. Erythrocytes were washed twice in PBS. Parasite nucleus was stained using 5 μg/ml Hoechst 33342 (Molecular Probes, Eugene, OR). DPT-sh1 and DPT-sh2 penetration was analyzed by fluorescence microscopy (Leica, Wetzlar, Germany).

Bio-Informatics and Structure Analysis. A homology model of bovine CK2α was constructed based on the X-ray crystal structure of 1lr4 from the Brookhaven Protein Data Bank (PDB) (Berman et al., 2000). The sequences of the target and template protein were aligned using clustalX (Thompson et al., 1997), and Modeler 4 (Fiser and Sali, 2003),was used to build a CK2α model. The model was refined by 100 steps of descent, followed by 200 to 300 steps of conjugate gradient energy minimization using Insight II (http://www.accelrys.com/insight/). The molecular assemblies between CK2α model (PDB 1b3u) and the X-ray crystal structure of PP2A-A subunit were then constructed by docking. The automated docking software Hex (http://www.biochem.abdn.ac.uk/hex/) with the macro molecular graphics package that uses a spherical polar Fourier correlation was used to accelerate docking calculations. After docking, energy minimization was applied on the whole complex with Insight II software. The figures were produced with GRASP (Nicholls et al., 1991).

Results

The DPT concept, illustrated in Fig. 1, is based on intracellular delivery of specific peptides interacting with Ser/Thr phosphatases PP1 or PP2A. We were first interested in the characterization of PP2A interacting sequences in CK2α proteins and we identified a new cell penetrating peptide. We took advantage of this result to generate hybrid CPPs containing a penetrating sequence derived from CK2α proteins fused to sequences interacting with PP1 or PP2A to assess their biological properties.

Structural Analysis of PP2A-Interacting Peptides in CK2α Proteins. To identify CK2α peptides able to bind in vitro to PP2A, a series of 205 overlapping dodecapeptides from the *T. parva* CK2α protein was produced onto a cellulose membrane and incubated with purified PP2A holoenzyme or with PP2A subunits. Three overlapping peptides corresponding to a major docking site (named site 1) that binds to trimeric PP2A1 were detected (Fig. 2A, top). This region is remarkably conserved among distinct CK2α proteins, such as mammalian (human, bovine) or *P. falciparum*, a parasite (Fig. 2B), and represents a promiscuous CK2α PP2A interaction sequence. It is noteworthy that the KHENRKLYRKD sequence from the SV40 virus small t antigen that contains a PP2A docking site (Mateer et al., 1998) presents significant similarity with the sequence immediately preceding the CK2α site1 (Fig. 2B). The peptide corresponding to residues 105 to 131 of SV40 small t antigen adopts a β-strand structure that is essential for high-affinity interaction with the PP2A dimer and inhibits phosphatase activity (Mateer et al., 1998). Bio-informatics and structural analysis of CK2α indicated that site 1 is also an antiparallel β-strand (Fig. 2, C and D). When the CK2α peptide array was incubated with a purified PP2A-A subunit, we were surprised to detect two additional sites (sites 2 and 3; Fig. 2A, bottom). These new potential docking sites are located in the region of the ATP binding site.

Identification of New PP2A-Interacting Cell-Permeable Peptides. All known short cell-permeable peptides (CPPs) that translocate into eukaryotic cells are polycationic peptides containing six to eight Arg and/or Lys residues. Because the CK2α sites 1 to 3 have a high Arg+Lys content, we hypothesized that they may represent a new source of CPP. To test this hypothesis, we chose to analyze the cell penetration potential of dodecamer 81 (VKKKKIKREIKI), which is part of site 3, called DPT-sh1 (Table 1).

At this time, we focused our interest on DPT-sh1 for its putative dual ability to interact with PP2A and to penetrate into cells as a result of its poly-basic sequence, similar to many known CPPs (Table 2). Biotinylated or FITC-labeled DPT-sh1 was used to investigate its ability to penetrate and to deliver a protein marker (streptavidin peroxidase) into lymphoid Jurkat or adherent HeLa cells. The amount of

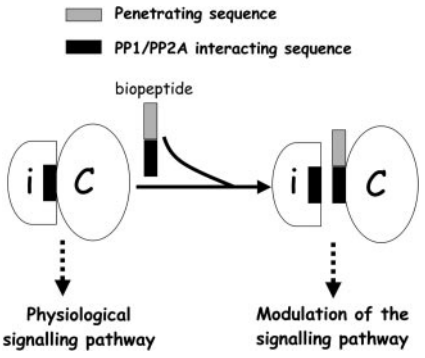

Fig. 1. Schematic illustration of the DPT concept. DPT consists of intracellular delivery of bipartite peptides, including a short cell-penetrating peptide fused to a peptide binding to the catalytic subunit of PP1, the core of PP2A, or to holoenzymes. PP1-/PP2A-interacting proteins are represented as i, and the phosphatase catalytic structures (PP1c or core PP2A) are schematically represented as C. This strategy derives from two lines of results: 1) identification of nontoxic shuttles derived from CK2α. Depending on the sequence, this shuttle (gray rectangle) localizes in the cytoplasm (DPT-sh1) or similarly to other CPPs both in nucleus and cytoplasm (DPT-sh2); 2) identification of docking sites (black rectangles) in PP1- or PP2A-binding proteins. A predictive signature for PP1 binding motifs has been published (Garcia et al., 2004). Signatures for PP1 are available at http://pp1signature.pasteur.fr.

DPT-sh1 peptide internalized in Jurkat cells at 37°C was time- and concentration-dependent (Fig. 3A). No uptake was detected at 4°C. In HeLa cells, FITC-tagged DPT-sh1 localized exclusively in the cytoplasm (Fig. 3A, bottom) in contrast to most known CPPs, which display nuclear or both nuclear and cytoplasmic localization. Thus, DPT-sh1 is of great interest to study regulation of cytoplasmic protein by PP2A or PP1. Furthermore, as a control of polycationic CPP with classic nuclear localization, we designed a new polycationic peptide named DPT-sh2 with an arbitrary polycationic penetrating sequence based on a trimeric repeat of RQKLRI. This sequence is closer to CK2α from *P. falciparum* than those from *T. parva* parasite (Table 1). It is noteworthy that DPT-sh2 (Fig. 3B) delivered a protein marker (human peroxidase) linked through a biotin-streptavidin bridge into the cytoplasm of HeLa cells as efficiently as DPT-sh1. Moreover,

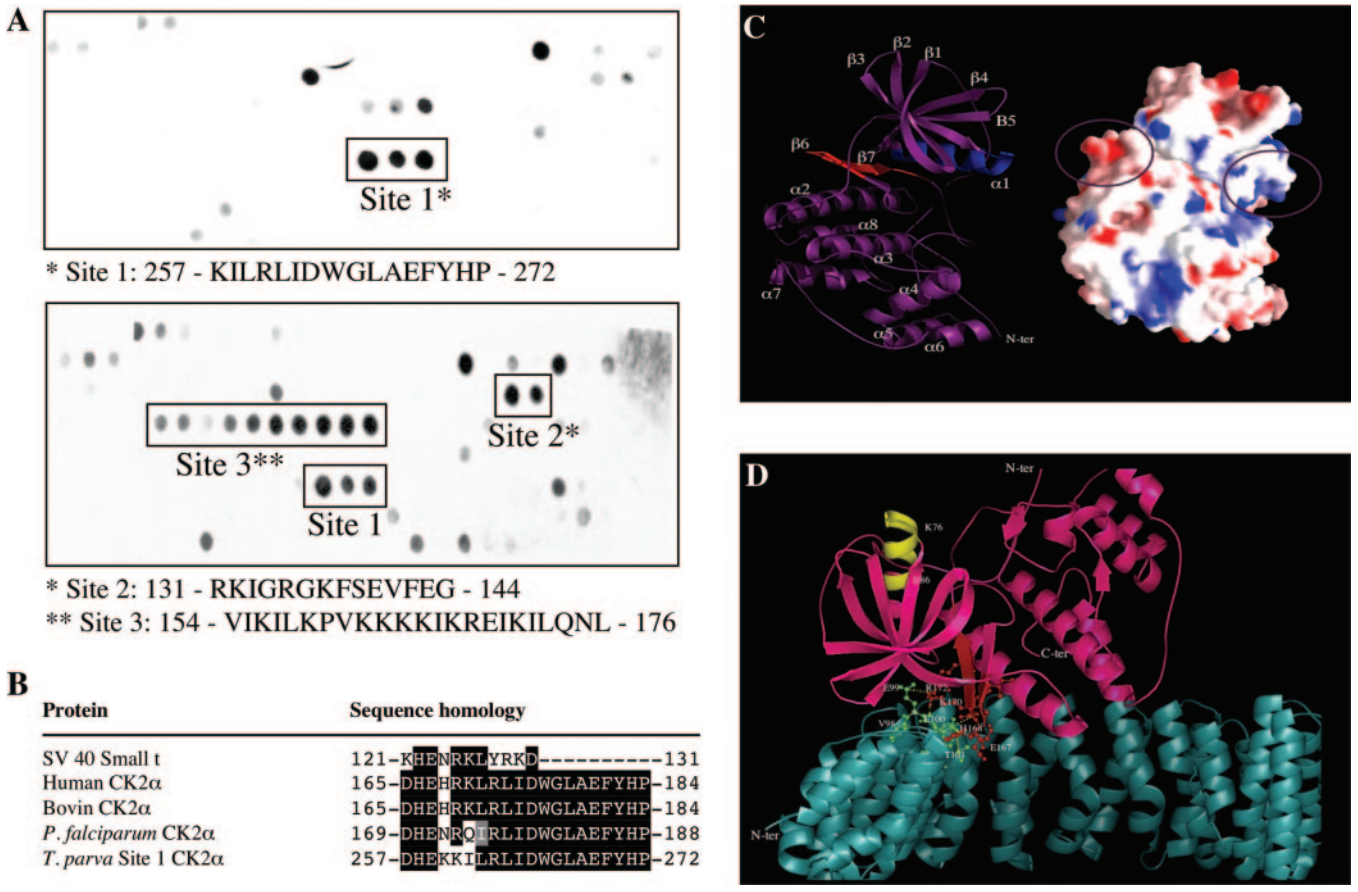

Fig. 2. PP2A binding assay on cellulose-bound CK2α peptides and structural analysis of PP2A interacting peptides. A, autoradiogram of the PP2A binding assay on cellulose-bound CK2α peptides. Two hundred five overlapping dodecapeptides with a two-amino acid shift scanning the entire *T. parva* CK2α sequence were synthesized on a cellulose membrane. The membrane was incubated with PP2A1 holoenzyme (top) or PP2A-A subunit (bottom) and subsequently with anti-PP2A antibodies followed by peroxidase-labeled anti-mouse antibodies. The interacting CK2α-derived peptides are boxed, and the corresponding protein sequence is indicated. B, sequence homology of the PP2A binding sequence of SV40 virus small t antigen and site 1 PP2A binding sequence from *T. parva* CK2α and homologous CK2α regions from various species. C, localization of the PP2A binding site and DPT-sh1 peptide on a ribbon representation deduced from the CK2α crystal structure (left) and on the charge density on the surface of electrostatic potential (right). Both figures are shown in the same orientation. The PP2A binding site (red) is located in the β7 antiparallel β-strand corresponding to residues 165 and 184. The DPT-sh1 sequence (blue), which encompasses residues 257 to 272, is located in the a1 α-helix. The electrostatic potential (red, −12 kiloTeslas; blue, +12 kiloTeslas) on the accessible surface of CK2α. The PP2A binding site and DPT-sh1 are on opposite sides of the molecule (circles) and electrostatic potentials. D, model (PDB 1b3u) of CK2α (blue) and PP2A-A (red) binding. The residues predicted to take part to binding are represented as surfaces. The DPT-sh1 peptide is yellow. The figure was created using the Insight II software.

TABLE 1

Conventional and new cell penetrating peptides containing PP1/PP2A-interacting motifs: origin and sequence of DPT peptides

Single-letter code for amino acids is used for all peptides. Residues corresponding to invariable amino acids in PP1c site are bold; mutations are underlined and bold.

Protein Origin	Peptide Sequence	Acronym
CKα (T. parva)	VKKKKIKREIKI	DPT-sh1
Synthetic sequence	RQKRLIRQKRLIRQKRLI	DPT-sh2
Protamin	RRRRRRRSRGRRRRTY	DPT-3
CD28 antigen	PRRPGPTRKHYQPYA[a]	DPT-4
CKa (T. parva) + CD28 antigen	VKKKKIKREIKI-PRRPGPTRKHYQPYA	DPT-5
Bad	**F**RG**R**S**R**-**F**RG**R**S**R**	DPT-6
CKa (T. parva) + Bad	VKKKKIKREIKI-**F**RG**R**S**R** **F**RG**R**S**R**	DPT-7
CKa (T. parva) + Bad mutant-1	VKKKKIKREIKI-**A**RG**R**S**R** **F**RG**R**S**R**	DPT-8
CKa (T. parva) + Bad mutant-2	VKKKKIKREIKI-**A**RG**R**S**RA**RG**R**S**R**	DPT-9

[a] This peptide binds in vitro to Bα but not to PP2A-A subunits.

DPT-sh2 displayed cytoplasmic and nuclear/nucleolar localization similar to Tat, a penetrating peptide derived from the viral transcription factor tat (Table 2). Cell viability 48 h after peptide penetration in HeLa or Jurkat cells was analyzed by the MTT or 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium colorimetric assay. No toxicity was observed in presence of DPT-sh1 even at concentrations up to 500 μM (Fig. 3C). In addition, as shown in Fig. 3D, internalization of FITC-conjugated DPT-sh1 and DPT-sh2 peptides in human red blood cells was undetectable. We were surprised to find that red blood cells infected with the human malaria parasite *Plasmodium falciparum* internalized the DPT-sh1 peptide. Further analysis indicated that fluorescence was restricted to parasites, as confirmed by Hoechst staining of the *P. falciparum* nucleus (data not shown). The FITC-labeled DPT-sh1 mostly localized to the cytoplasm of pigmented parasites but also appeared in merozoites before erythrocyte disruption. Similar uptake and localization were found with DPT-sh2 (data not shown).

Furthermore, as shown in Fig. 3E, based on internalization of SRP conjugated molecules by biotinylated peptides, we quantified in HeLa cells the cargo effect of DPT-sh1 and DPT-derived peptides compared with an HIV Tat-derived penetrating peptide. We observed that in contrast to DPT-sh1 alone, DPT-derived peptides displayed approximately 50% at 10 μM and equivalent efficiency of penetration as Tat alone at 100 μM.

In Vivo Analysis: Use of DPT-sh1 Shuttle in Chick Embryos. To test the in vivo effect of the penetrating peptides, we injected biotinylated DPT-sh1 into the chick embryo. The peptide was injected at a 2 mM concentration into the neural tubes of chick embryos (embryonic day 2), and 54 control embryos were injected with Hanks' balanced saline solution. Even at such a high concentration, the peptide showed limited toxicity, as the survival rate was similar (approximately 63%) in both groups. Peptide penetration was rapid, and after 24 h, the localization was restricted caudally to the 30 somite. Cryosections analyzed with streptavidin peroxidase showed that peptides were present predominantly in the notochord but also in the neural tube, and in a dorsolateral region of the somitic mesoderm underlying the ectoderm (Fig. 4). Taken together, these data indicate that DPT-sh1 and DPT-sh2 are new, nontoxic CPPs.

Effect of Intracellular Delivery of a Peptide That Binds in Vitro to the PP2A-Bα Subunit. The intracytoplasmic tail of the T lymphocyte surface receptor CD28 contains 41 residues that can associate with several members of the PP2A family (Chuang et al., 2000). To identify CD28 sequences able to bind the PP2A-Bα subunit, overlapping dodecapeptides scanning the cytoplasmic tail were produced onto a cellulose membrane and incubated with purified PP2A proteins. Two PP2A-Bα interacting peptide spots with an overlapping sequence (residues 196–210) were identified (Fig. 5A). The corresponding soluble peptide, called DPT-4, was produced, as well as DPT-5, which combines DPT-sh1 and DPT-4 sequences (Table 1). Using FITC-tagged peptides, we showed that DPT-5, but not DPT-4, was able to penetrate within HeLa cells and localize in the cytoplasm and the nucleus (Fig. 5A). Furthermore, as expected, pull-down experiments confirmed that DPT-4 and DPT-5 interacted with PP2A-Bα subunit (Fig. 5B). In addition, DPT-5, but not DPT-4, induced loss of cell viability (Fig. 5C). In Jurkat cells, DPT-5 penetrated into the cell and provoked apoptosis, whereas DPT-4 did not penetrate and was unable to induce apoptosis (Fig. 5D). These data suggest that intracellular delivery of a PP2A-Bα interacting motif is sufficient to affect cell viability.

Effect of Intracellular Delivery of a PP1c Binding Consensus Motif. We recently identified a new PP1c docking consensus (F-xx-R/K-x-R/K) motif in antiapoptotic Bcl-x_L (Ayllon et al., 2002) and in proapoptotic Bad (Garcia et al., 2004), two members of the Bcl-2 family. Because apoptosis induced by interleukin-2-deprivation in murine TS1$\alpha\beta$ cells requires activation of a PP1 Bad-associated phosphatase activity (Ayllon et al., 2000), we reasoned that intracytoplasmic delivery of a peptide containing the PP1c docking motif from Bad should interfere with cell death induction. We designed DPT-6, a peptide sequence corresponding to the dual repeat of PP1c docking motif from Bad (Table 1). Despite containing five Arg residues, DPT-6 was unable to serve as a CPP and penetrate into HeLa cells (Fig. 6A, left). However, DPT-7, which combines DPT-sh1 and the DPT-6 sequences (Table 2), penetrated and localized in the cytoplasm of HeLa cells (Fig. 6A, right). Consistent with this, DPT-7, but not DPT-6, provoked cell death in HeLa and Jurkat cells (Fig. 6, B and C). The invariant position of the amino acid F in the PP1c consensus docking motif was shown to be critical (Ayllon et al., 2000; Garcia et al., 2004). It is noteworthy that DPT-9, which carries a Phe-to-Ala mutation in both PP1c consensus docking motifs, no longer exhibited any toxic effect (while still penetrating into the cell). Peptide DPT-8 with a single mu-

TABLE 2

Conventional and new cell penetrating peptides containing PP1/PP2A-interacting motifs: origin, sequence, and biological properties of some known cell-penetrating peptides

Single-letter code for amino acids is used for all peptides. The minimum sequence is underlined.

Acronym	Sequence	Cell Penetration		Cytoxicity	Protein Carrier	
		Cytoplasm	Nucleus		Cytoplasm	Nucleus
TAT	YGRKKRRQRRR	+/–	+	+	+/–	+
ANTp	RQIKIWFQNRRMKWKK	+/–	+	+/–	+/–	+
Transportan[a]	GWTNLSAGYLLGKINLKALAALAKKIL	+/–	+	N.D.	+/–	+
MGP[a]	GALFLGFLGGAAGSTMGAWSQPKSKRKV	+/–	+	N.D.	+/–	+
VPR	HFRIGCRHSRIG	+/–	+	+++	N.D.	N.D.
VPR	HFRIGCRHSRIGVTRQRRARNGASRS	+/–	+	+++	N.D.	N.D.[b]

N.D., not determined; +, positive effect; –, negative effect.

[a] Non-naturally occurring CPPs.

[b] We have recently identified a new CPP binding PP2A-A and PP2A1 holoenzyme that contains part of this sequence and can carry 100-kDa proteins (A. Godet, V. Maire, J. Guergnon, X. Cayla, A. Rebollo, and A. Garcia, manuscript in preparation).

tation abrogating the first PP1c consensus docking motif still inhibited cell viability (Fig. 6B). Furthermore, pull-down experiments confirmed that DPT-6 and DPT-7 but not DPT-9 interacted with PP1c subunit (Fig. 6D). These data suggest that the presence of at least one PP1c docking motif is sufficient to affect cell viability.

Discussion

We described here new experimental evidences providing proof of principle for the drug phosphatase technology. This novel concept for rational drug design and potential therapy aims at targeting the families of PP1/PP2A holoenzymes,

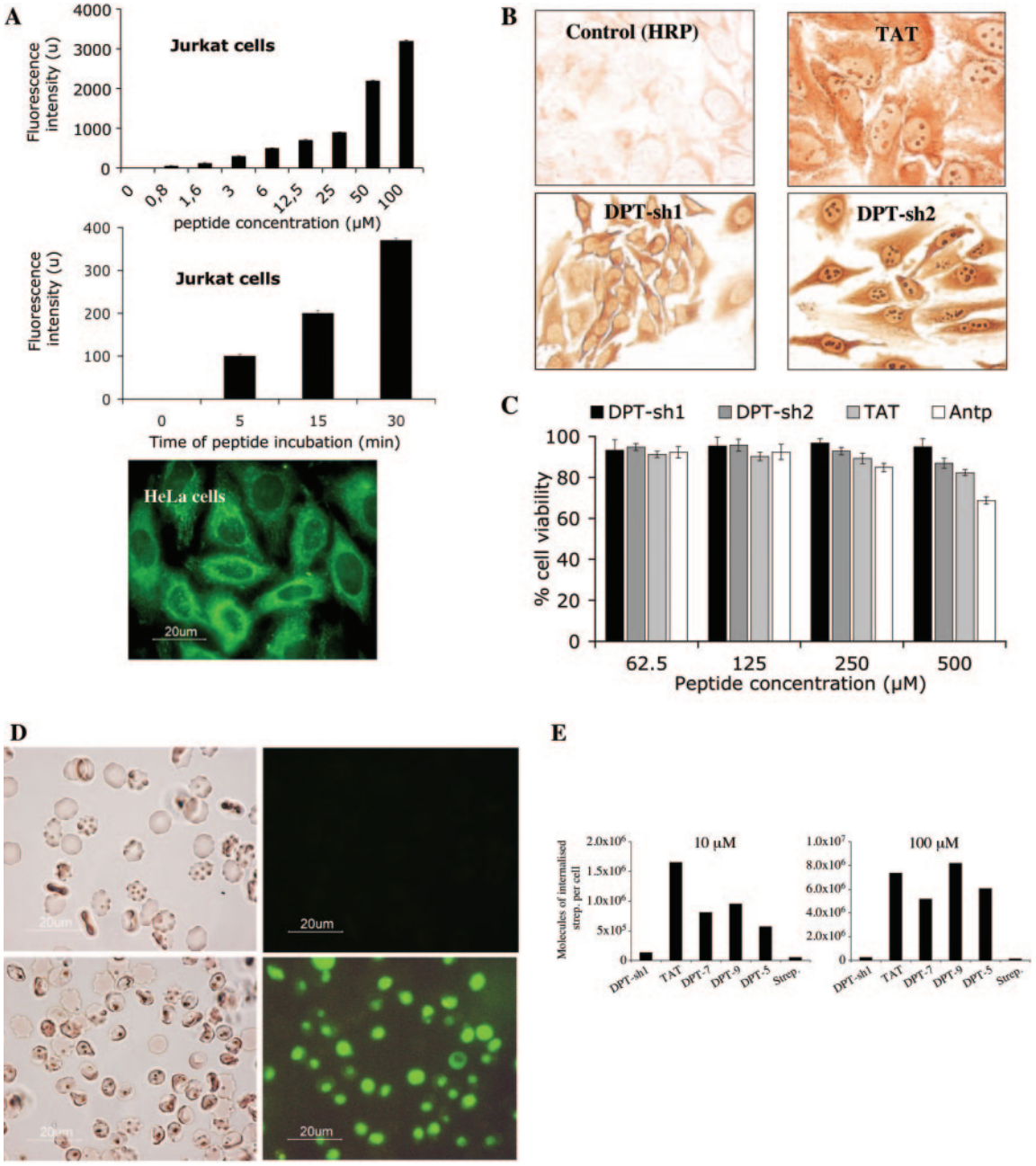

Fig. 3. Effect of peptides derived from PP2A-interacting proteins on cell penetration, intracellular delivery of streptavidin peroxidase, and cell viability. A, internalization of DPT-sh1 in HeLa and Jurkat cells. Top, dose dependence internalization was measured after a 60-min incubation at 37°C. Middle, kinetics of 10 μM DPT-sh1 cellular uptake by Jurkat cells at 37°C. Bottom, visualization of FITC-tagged DPT-sh1 peptide uptake by HeLa cells using epifluorescence. HeLa cells were incubated with 10 μM peptide for 1 h at 37°C and rinsed twice with PBS before fluorescence microscopy analysis. B, intracellular delivery of streptavidin peroxidase by biotinylated-DPT-sh1, DPT-sh2, and TAT in HeLa cells. Streptavidin peroxidase (HRP on figure) coupled with biotinylated peptides were incubated for 6 h at 37°C, and internalized complexes were visualized as indicated under *Materials and Methods*. C, cell viability assay. HeLa cells were incubated for 48 h with previous peptides before being processed for MTT staining. D, selective penetration of fluorescein-labeled DPT-sh1 peptide in *P. falciparum* FUP/CB-infected erythrocytes. Noninfected human erythrocytes (top) or *P. falciparum*-infected human erythrocytes (bottom) were analyzed by light transmission (left) and fluorescent staining (right). E, quantification of internalized peptides in HeLa cells. DPT-sh1, DPT-5, DPT-7, and DPT-9 were incubated 24 h at 10 or 100 μM and compared with the well characterized penetrating peptide derived from HIV-1 TAT protein. This figure corresponds to a representative experiment repeated three times.

which control a major portion of serine/threonine phosphatase activity in cell extracts. As illustrated in Fig. 1, phosphatase-derived drug technology, DPT, is based on cell delivery of PP1/PP2A interacting peptides.

DPT-Derived Peptides: Tools to Deregulate Intracellular Pathways. Our results suggest that PP2A is a source for new biologically active or inactive cell penetrating peptides (Table 3). Small cationic cell-penetrating peptides derived from the human immunodeficient virus (HIV)-1 Tat and *D. melanogaster* antennapedia homeoprotein (Antp) have been used to introduce multiple molecules, including synthetic small molecules, peptides, and proteins into cells and also animal models (Schwarze et al., 1999). In addition, non-naturally occurring CPP corresponding to chimeras containing charged residues such as MGP (Morris et al., 1999), a chimera between SV40 nuclear localization signal and GP41, have also been characterized (Table 2). We have clearly identified polycationic peptides located within PP2A-binding sequences of CK2α proteins. Bio-informatics and structural analysis suggested that both SV40 small t and CK2α proteins, two previously characterized PP2A interacting proteins, share a putative common β-strand structure interacting with the structural PP2A-A subunit. This β-strand structure favors heteromeric interaction within the trimeric PP2A1 holoenzyme. In contrast, the sequence corresponding to DPT-sh-1 permeable peptide is localized in a poorly accessible α-helix within CK2α (Fig. 2, C and D). Within the cell, the PP2A-A protein is recruited into two distinct dimeric and trimeric pools of PP2A holoenzyme and never has been found as a free subunit (Ruediger et al., 1997). These observations suggest that interaction between PP2A-A subunit and DPT-sh1 revealed by in vitro spot assay is not favored in situ and may explain the absence of DPT-sh1 toxicity illustrated in Fig. 3. The cytoplasmic localization of DPT-sh1 itself is an unusual feature in CPP designed from naturally occurring sequences. Indeed Tat, Antp, and various arginine-rich peptides mainly localize within the nucleus (Table 2). We proposed the use of DPT-sh1 as a new, nontoxic peptide shuttle to interfere with PP1/PP2A-dependent apoptotic pathways. The lack of toxicity and the rapid delivery of protein-bound DPT-sh1 and DPT-sh2, respectively, into the cytoplasm and into the nucleus represent two attractive features for their potential use in basic research or/and in therapeutic approach. In this context, our experiments in chick embryos indicate that DPT-sh1 microinjection in neural tubes will be

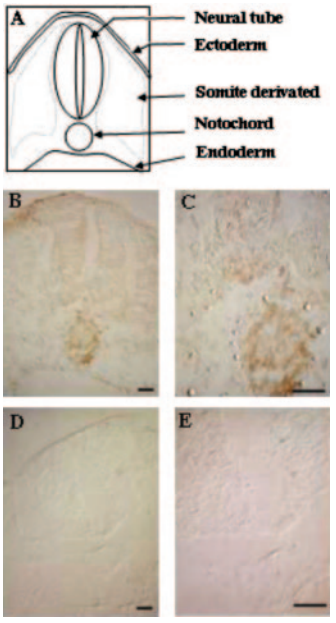

Fig. 4. Localization of biotinylated DPT-sh1 injected in the neural tube of embryonic day 2 chick embryos. A, schematic representation of the section of 3-day-old chick embryo presented in B and D. B, 24 h after injection in the neural tube (see *Materials and Methods*), the biotinylated DPT-sh1 peptide was detected on cryosections in the neural tube, in a dorsolateral region of the somitic mesoderm underlying the ectoderm, and in the notochord. C, enlargement of B shows the predominant signal in the notochord. D and E, absence of labeling on sections of control embryo injected with the Hanks' balanced saline solution alone. Scale bar, 30 mm.

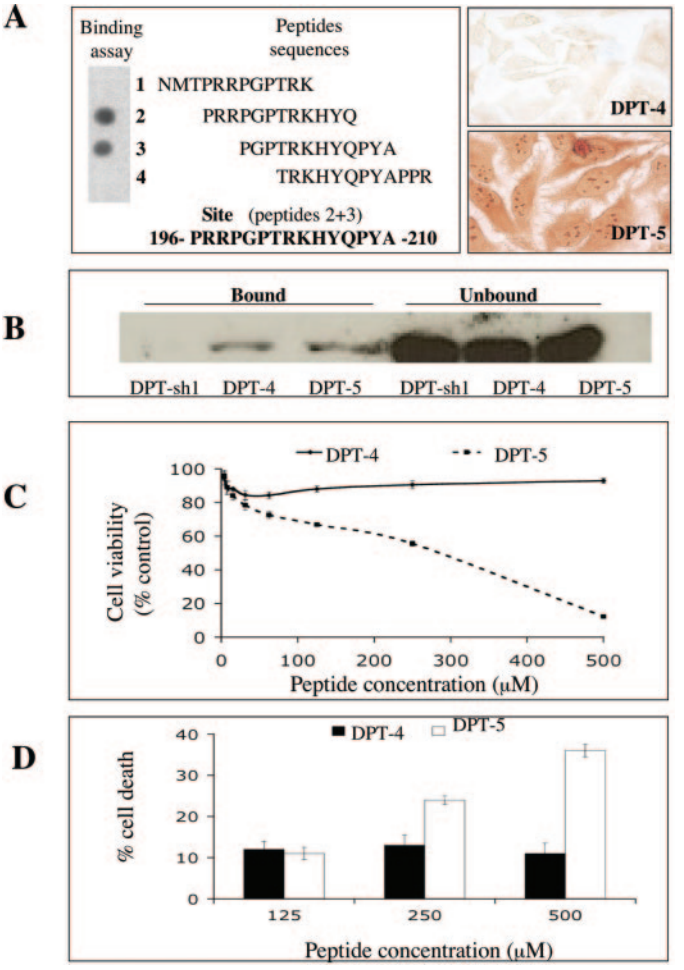

Fig. 5. Effect of intracellular delivery of a peptide mimicking a PP2A docking sequence in CD28 antigen. A, membrane with overlapping dodecapeptides deduced from the short 41-residue intracytoplasmic CD28 sequence was incubated with purified Bα, A subunits, or with PP2A1 holoenzyme and revealed as in Fig. 1A. Left, spots of interaction of CD28 with purified Bα subunit. Similar results were obtained with PP2A1 holoenzyme, which contains the Bα subunit (data not shown). Right, HeLa cells were incubated with 10 μM FITC-tagged DPT-4 or DPT-5 peptides for 1 h, rinsed, and fixed with mowiol before fluorescence microscopy analysis. B, identification of lysates (10%) or bound proteins (50%) in pull-down experiments was realized by immunoblotting using antibodies against PP2A-Bα. C, HeLa cells were incubated for 48 h with peptide before being processed for detection using MTT staining. DPT-sh1 peptide was used as control (data not shown). D, Jurkat cells were treated with FITC-tagged DPT-4 or DPT-5 peptides for 24 h in microplates. After treatment, cells were washed twice with PBS and stained with Annexin-V-APC. Cell death was measured by flow cytometry.

useful to interfere with regulatory pathways in vertebrate development.

PP2A, previously named polycation-stimulated phosphatase, is known to be activated by polycationic proteins such as protamines (Waelkens et al., 1987), suggesting that polycationic peptides derived from PP2A-interacting proteins, such as CK2α and protamin, may be used as new CPPs in DPT-technology. Consistent with this notion, previous reports indicate that Vpr, a 96-amino acid protein encoded by HIV-1, can penetrate and induce apoptosis in different types of human cell lines. It is noteworthy that this effect requires the presence of a short C-terminal domain containing the conserved sequence HFRIGCRHSRIG. Several CPPs containing this sequence penetrate and provoke apoptosis in human cells (Arunagiri et al., 1997). We have recently identified by in vitro experiments a PP2A binding region within this domain (A. Godet, V. Maire, J. Guergnon, X. Cayla, A. Rebollo, and A. Garcia, manuscript in preparation).

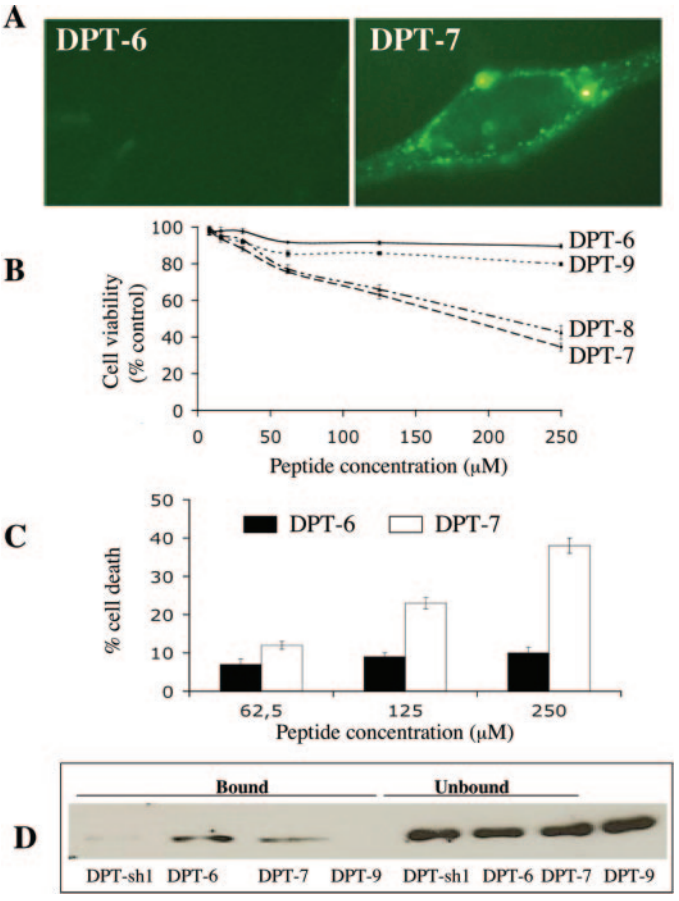

Fig. 6. Effect of intracellular delivery of a peptide mimicking the PP1 docking sequence of the pro-apoptotic protein Bad. A, HeLa cells were incubated with 10 μM FITC-tagged DPT-6 or DPT-7 peptides for 1 h before being rinsed twice with PBS and analyzed by fluorescence microscopy. B, HeLa cells were incubated 48 h with peptide before being processed for MTT staining detection. C, Jurkat cells were treated with FITC-tagged DPT-6 or DPT-7 peptides for 24. Cells were then rinsed twice with PBS and stained with Annexin-V-APC. Cell death was assessed by flow cytometry. D, identification of lysates (10%) or bound proteins (50%) in pull-down experiments was realized by immunoblotting using antibodies against PP1c.

Our recent results suggested that PP1 is a key phosphatase player in apoptosis (for review, see Garcia et al., 2003). The simultaneous presence in most PP1 interacting proteins of two distinct PP1c consensus docking motifs, R/K-x$_{(0,1)}$-V-x-F and F-xx-R/K-x-R/K, also identified in some antiapoptotic members of the Bcl-2 family, led us to propose a new concept of PP1 predictive signature (for details, see Garcia et al., 2004 and http://pp1signature.pasteur.fr). We hypothesized that the bioinformatics identification of PP1c docking sites in putative medically important PP1c-interacting proteins, combined with the use of DPT shuttles and/or nontoxic CPPs, may be relevant to develop DPT. We have previously shown that growth factor deprivation-induced apoptosis operates by regulating Bad-phosphatase-PP1 activity in murine interleukin-4-dependent T lymphocyte (Ayllon et al., 2002), and we also characterized in vitro a PP1c docking motif in Bad (Garcia et al., 2004). This system was used to carry out proof of principle experiments for this strategy. Indeed as reported in Fig. 6, introduction of PP1c-interacting motif, deduced from Bad interacting sequence, triggered cell death in HeLa and Jurkat cells.

The Dpt Concept Is a New Approach for Drug Discovery against Tumors and Infections. With regard to cancer, within the last 3 decades, the major technical progress in molecular biology, combined with the discovery of oncogenes, and more recently the identification of apoptotic factors, opens a way to rational drug design. The better understanding of signaling pathways driven by Ras oncogen and by protein kinases or phosphatases allowed the development and use of small inhibitory molecules. Consequently, distinct farnesyl transferase inhibitors, initially designed as a potential anti-ras therapy or different specific pharmacological inhibitors of tyrosine kinases or antisense therapy against protein kinase C or PKA have been already used in clinical trials (Liu et al., 1998; Yuen et al., 1999; Zhang et al., 1999). However, appropriate utilization of these new mole-

TABLE 3
Conventional and new cell penetrating peptides containing PP1/PP2A-interacting motifs: biological properties of DPT peptides

Peptide	In Vitro Binding (Spot Assay)			Cell Binding (Pull-Down Assay)			Protein Carrier (SRP Assay)	Apoptosis
	PP1c	PP2A-A	PP2A Bα	PP1c	PP2A-A	PP2A Bα		
DPT-sh1	N.D.	+	−	−	−	−	+	−
DPT-sh2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	+	−
DPT-3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	+	+
DPT-4	−	−	+	N.D.	N.D.	+	−	−
DPT-5	N.D.	N.D.	N.D.	N.D.	N.D.	+	+	+
DPT-6	+[a]	N.D.	N.D.	+	N.D.	N.D.	−	−
DPT-7	N.D.	N.D.	N.D.	+	N.D.	N.D.	+	+
DPT-8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	+	+
DPT-9	−[a]	N.D.	N.D.	−	N.D.	N.D.	+	−

N.D., not determined; +, positive effect; −, negative effect.
[a] The spot assays involving PP1c consensus docking sequences, used in wild-type (DPT6) or mutated (DPT9) peptides, have been published previously (Garcia et al., 2004).

cules alone or in combination with chemotherapy is still a matter of debate.

In this context, how might PP1/PP2A protein phosphatases and DPT help us to design more specific and less toxic new cancer drugs? Viruses have developed specific pro and anti-transforming strategies to deregulate cells via PP2A. Interaction of the PP2A-A subunit with small t antigen encoded by transforming papovaviruses activates MEK/ERK, two major components of the ras transforming pathway (Sontag et al., 1997). In contrast, interaction between PP2A-Bα subunit and adenoviral protein E4orf4 induces cancer-cell specific apoptotic pathways that, depending on the cell type, operate through caspase-independent or -dependent apoptosis (Shtrichman et al., 1999). Given that interaction of E4orf4 with PP2A-Bα regulatory subunit induces apoptosis of transformed cells in a p53-independent manner (Livne et al., 2001), we speculated that peptides interacting with PP2A-Bα may also induce apoptosis. Consistent with this hypothesis, intracellular delivery of PP2A-Bα interacting sequences deduced from CD28 antigen in the PP2A-Bα-interacting DPT-5 peptide, clearly provoked cell death. This result suggest that DPT-derived peptides mimicking signaling generated by E4orf4-PP2A interaction could represent a new creative approach to counteract resistance of cancer cells against drug-induced apoptosis. Contrary to PP1 interacting subunit, the motifs required for PP2A interaction are not well characterized, because no consensus is clearly established. Bio-informatics studies are under progression to elucidate this mystery. Data obtained from bio-informatics will give us some clues to design PP2A-interacting peptide mutants to validate the specificity of such peptides as DPT-5. This work illustrates the relevance of targeting regulatory sites, not only catalytic motifs of PP1 and PP2A, to specifically induce apoptosis of tumor or infected cells. We now are pursuing this work by crystallography studies to understand how DPT peptides interact with PP1 and PP2A compared with the full-length proteins. In this way, the use of CPPs, especially when targeting PP1 and PP2A, is much more relevant than the use of known pharmacological small molecules that directly inhibit the catalytic function in a toxic way for all nontransformed and transformed cells.

Two recent breakthrough studies upgrade the interest in both small molecules and penetrating peptides for rationale drug design. First, a recent work by Plescia et al. (2005) describes a penetrating peptide based on tat penetrating sequence fused to nine amino acids deduced from survivin sequence. This peptide, named Shepherdin, is the first non–small-molecule inhibitor of Hsp90 and is shown to inhibit tumor growth in mice and to be very well tolerated in vivo, suggesting that peptidomimetic inhibitors of Hsp90 may circumvent some of the toxicity issues currently limiting the clinical utility of small molecule Hsp90 inhibitors. The second concerns small molecules, based on chemical biology approach. Boyce et al. (2005) identified salubrinal as a small molecule that protects against endoplasmic reticulum-stress-induced cell death, through its ability to prevent dephosphorylation of eIF2α by PP1. Salubrinal also blocks eIF2α dephosphorylation mediated by a herpes simplex virus protein and inhibits viral replication. This study showed that it is possible to target dephosphorylation of selected protein phosphatase substrates for future therapeutic development.

Concerning infection, we focused on malaria because the absence of vaccine and the emergence of drug-resistant malaria parasites represents an important public health problem in sensitive countries. Because there is no evidence for endocytosis in noninfected mature erythrocytes (Kirk, 2001), the selective penetration of DPT shuttles in red cells infected by *P. falciparum* opens the way to a new drug therapy. In this context, pharmacological inhibition of PKA, as well as abrogation of PP1 expression by short interfering RNA, led to inhibition of the parasite intraerythrocytic development (Syin et al., 2001; Kumar et al., 2002). It is noteworthy that our data (J. Guergnon, F. Dessauge, F. Fraincard, X. Cayla, A. Rebolla, P.-E. Bost, G. Langsley, and A. Garcia, submitted) demonstrated that DPT-PKI, a new specific PKA inhibitor that combines DPT-sh1 and the PKI sequences defined by Scott et al. (1985), inhibits *Theileria annulata*-directed PKA survival pathway in parasitized bovine lymphocytes, suggesting that it is useful for cell culture experiments and to develop novel therapies based on PKA modulation. This tool is under evaluation in both cancer and *P. falciparum* parasites.

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References

Arunagiri C, Macreadie I, Hewish D, and Azad A (1997) A C-terminal domain of HIV-1 accessory protein Vpr is involved in penetration, mitochondrial dysfunction and apoptosis of human CD4+ lymphocytes. *Apoptosis* **2:**69–76.

Ayllon V, Cayla X, Garcia A, Fleischer A, and Rebollo A (2002) The anti-apoptotic molecules Bcl-xL and Bcl-w target protein phosphatase 1α to Bad. *Eur J Immunol* **32:**1847–1855.

Ayllon V, Martinez AC, Garcia A, Cayla X, and Rebollo A (2000) Protein phosphatase 1alpha is a Ras-activated Bad phosphatase that regulates interleukin-2 deprivation-induced apoptosis. *EMBO (Eur Mol Biol Organ) J* **19:**2237–2246.

Berman HM, Bhat TN, Bourne PE, Feng Z, Gilliland G, Weissig H, and Westbrook J (2000) The Protein Data Bank and the challenge of structural genomics *Nat Struct Biol* **7(Suppl):**957–959.

Bosch M, Cayla X, Van Hoof C, Hemmings B, Ozon R, Merlevede W, and Goris J (1995) The PR55 and PR65 subunits of protein phosphatase 2A from *Xenopus laevis*: molecular cloning and developmental regulation of expression. *Eur J Biochem* **230:**1037–1045.

Boyce M, Bryant KF, Jousse C, Long K, Harding HP, Scheuner D, Kaufman RJ, Ma D, Coen DM, Ron D, et al. (2005) A selective inhibitor of eIF2alpha dephosphorylation protects cells from ER stress. *Science (Wash DC)* **307:**935–939.

Chuang E, Fisher TS, Morgan RW, Robbins MD, Duerr JM, Vander Heiden MG, Gardner JP, Hambor JE, Neveu MJ, and Thompson CB (2000) The CD28 and CTLA-4 receptors associate with the serine/threonine phosphatase PP2A. *Immunity* **13:**313–322.

Cohen PT (2002) Protein phosphatase 1-targeted in many directions. *J Cell Sci* **115:**241–256.

Fiser A and Sali A (2003) Modeller: generation and refinement of homology-based protein structure models. *Methods Enzymol* **374:**461–491.

Ford KG, Souberbielle BE, Darling D, and Farzaneh F (2001) Protein transduction: an alternative to genetic intervention? *Gene Ther* **8:**1–4.

Frank R and Overwin H (1996) SPOT synthesis. *Methods Mol Biol* **66:**149–169.

Garcia A, Cayla X, Caudron B, Deveaud E, Roncal F, and Rebollo A (2004) New insights in protein phosphorylation: a signature for protein phosphatase 1 interacting proteins. C R *Biologies C R Biologies* **327:**93–97.

Garcia A, Cayla X, Guergnon J, Dessauge F, Hospital V, and Rebollo MP, Fleischer A, and Rebollo A (2003) Serine/threonine protein phosphatases PP1 and PP2A are key players in apoptosis. *Biochimie* **85:**721–726.

Garcia A, Cayla X, and Sontag E (2000) Protein phosphatase 2A: a definite player in viral and parasitic regulation. *Microbes Infect* **2:**401–407.

Hansen MB, Nielsen SE, and Berg K (1989) Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J Immunol Methods* **119:**203–210.

Hunter T (2000) Signaling-2000 and beyond. *Cell* **100:**113–127.

Kirk K (2001) Membrane transport in the malaria-infected erythrocyte. *Physiol Rev* **81:**495–537.

Kumar R, Adams B, Oldenburg A, Musiyenko A, and Barik S (2002) Characterisation and expression of a PP1 serine/threonine protein phosphatase (PfPP1) from the malaria parasite, *Plasmodium falciparum*: demonstration of its essential role using RNA interference. *Malar J* **2:**1–11.

Lambros C and Vanderberg JP (1979) Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J Parasitol* **65:**418–420.

Liu M, Bryant MS, Chen J, Lee S, Yaremko B, Lipari P, Malkowski M, Ferrari E, Nielsen L, Prioli N, et al. (1998) Antitumor activity of SCH 66336, an orally

bioavailable tricyclic inhibitor of farnesyl protein transferase, in human tumor xenograft models and wap-ras transgenic mice. *Cancer Res* **58:**4947–4956.
Livne A, Shtrichman R, and Kleinberger T (2001) Adenovirus type 5 E4 open reading frame 4 protein induces apoptosis in transformed cells. *J Virol* **75:**789–798.
Mateer SC, Fedorov SA, and Mumby MC (1998) Identification of structural elements involved in the interaction of simian virus 40 small tumor antigen with protein phosphatase 2A *J Biol Chem* **273:**35339–35346.
McCright B, Rivers AM, Audlin S, and Virshup DM (1996) The B56 family of protein phosphatase 2A (PP2A) regulatory subunits encodes differentiation-induced phosphoproteins that target PP2A to both nucleus and cytoplasm. *J Biol Chem* **271:** 22081–22089.
Morris MC, Chaloin L, Méry J, Heitz F, and Divita G (1999) A novel potent strategy for gene delivery using a single peptide vector as a carrier. *Nucleic Acids Res* **27:**3510–3517.
Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65:**55–63.
Nicholls A, Sharp KA, and Honig B (1991) Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. *Proteins* **11:**281–296.
Plescia J, Salz W, Xia F, Pennati M, Zaffaroni N, Daidone MG, Meli M, Dohi T, Fortugno P, Nefedova Y, et al. (2005) Rational design of shepherdin, a novel anticancer agent. *Cancer Cell* **5:**457–468.
Prochiantz A (2000) Messenger proteins: homeoproteins, TAT and others. *Curr Opin Cell Biol* **12:**400–406.
Ruediger R, Brewis N, Ohst K, and Walter G (1997) Increasing the ratio of PP2A core enzyme to holoenzyme inhibits Tat-stimulated HIV-1 transcription and virus production. *Virology* **238:**432–443.
Schwarze SR, Ho A, Vocero-Akbani A, and Dowdy SF (1999) In vivo protein transduction: delivery of a biologically active protein into the mouse. *Science (Wash DC)* **285:**1569–1572.
Scott JD, Fischer EH, Takio K, Demaille JG, and Krebs EG (1985) Amino acid sequence of the heat-stable inhibitor of the cAMP-dependent protein kinase from rabbit skeletal muscle. *Proc Natl Acad Sci USA* **82:**5732–5736.
Shtrichman R, Sharf R, Barr H, Dobner T, and Kleinberger T (1999) Induction of apoptosis by adenovirus E4orf4 protein is specific to transformed cells and requires an interaction with protein phosphatase 2A *Proc Natl Acad Sci USA* **96:**10080–10085.
Sontag E, Sontag JM, and Garcia A (1997) Protein Phosphatase 2A is a critical regulator of protein kinase C ζ signaling targeted by SV40 small t to promote cell growth and NF-κB activation. *EMBO (Eur Mol Biol Organ) J* **16:**5662–5671.
Syin C, Parzy D, Traincard F, Boccaccio I, Joshi MB, Lin DT, Yang XM, Karine Assemat K, Doerig C, and Langsley G (2001) The H89 cAMP-dependent protein kinase inhibitor blocks *Plasmodium falciparum* development in infected erythrocytes. *Eur J Biochem* **268:**4842–4849.
Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, and Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25:**4876–4882.
Trager W and Jensen J (1976) Human malaria parasite in continuous culture. *Science (Wash DC)* **193:**673–675.
Valle M, Munoz M, Kremer L, Valpuesta JM, Martinez AC, Carrascosa JL, and Albar J (1999) Selection of antibody probes to correlate protein sequence domains with their structural distribution. *Protein Sci* **8:**883–889.
Waelkens E, Goris J, and Merlevede W (1987) Purification and properties of polycation-stimulated phosphorylase phosphatases from rabbit skeletal muscle. *J Biol Chem* **262:**1049–1059.
Yuen AR, Halsey J, Fisher GA, Holmlundg JT, Geary RS, Kwoh TJ, Dorr A, and Sikic BI (1999) Phase I study of an antisense oligonucleotide to protein kinase C-alpha (ISIS 3521/CGP 64128A) in patients with cancer. *Clin Cancer Res* **5:**3357–3363.
Zhang L, Lau YK, Xia W, Hortobagyi GN, and Hung MC (1999) Tyrosine kinase inhibitor emodin suppresses growth of HER-2/neu-overexpressing breast cancer cells in athymic nude mice and sensitizes these cells to the growth inhibitory effect of paclitaxel. *Clin Cancer Res* **5:**343–353.

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