Efficient Internalization of the Polo-Box of Polo-like Kinase 1 Fused to an Antennapedia Peptide Results in Inhibition of Cancer Cell Proliferation

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Abstract

Polo-like kinases (Plk) regulate multiple stages in mitosis. Plk1 is overexpressed in tumors. The COOH-terminal regions of Plks contain a conserved domain, termed polo-box, which is required for subcellular localization and for physical interaction with substrates. We linked the polo-box (amino acids 410–429) of Plk1 to an Antennapedia peptide and studied its impact on tumor cells. Whereas the wild-type polo-box inhibited the proliferation of tumor cells associated with induction of apoptosis, a mutated derivative was much less effective. The treatment caused mitotic arrest, misaligned chromosomes, and multiple centrosomes. Taken together, membrane-permeable polo-box peptides inhibit cancer cell proliferation efficiently.

Introduction

Plks are serine-threonine kinases that are highly conserved during evolution. This family includes mammalian Plk1, Plk2 (Snk), and Plk3 (Fnk/Pfk), Xenopus laevis Plk1, Drosohphila melanogaster polo, Schizosaccharomyces pombe Pob1, and Saccharomyces cerevisiae Cdc5. Increasing evidence supports the concept that Plks regulate pivotal stages throughout mitosis including its initiation by activating Cdc2 through Cdc25 and direct phosphorylation of cyclin B1 targeting Cdc2/cyclin B1 to the nucleus. Furthermore, Plk1 contributes to centrosome maturation, bipolar spindle formation, DNA damage checkpoint adaptation, and activation of Cdc16, Cdc27 as components of the anaphase-promoting complex for mitotic exit. Finally, Plks are key regulators of cytokinesis (reviewed in Refs. 1, 2).

Mammalian Plk1 is overexpressed in rapidly proliferating cells and various human tumors (3). An increasing body of evidence suggests that the frequency of Plk1 expression is of prognostic value for patients suffering from different types of tumors like non-small cell lung cancer, squamous cell carcinomas of head and neck, melanomas, oropharyngeal carcinomas, and ovarian and endometrial carcinomas (4). Many data implicate that Plk1 participates in pathways, which override checkpoint arrests. Plk1 is needed for the Ca2+-induced release of Xenopus egg extracts from the meiotic M phase arrest (5). Cdc5p is clearly required for adaptation to a DNA damage checkpoint in Saccharomyces cerevisiae (6). Moreover, expression of active Plk1 can override the G2 arrest induced by DNA damage in mammalian cells (7). Thus, it is tempting to speculate that Plks may play a role in overriding spindle and/or DNA damage checkpoints. Overexpression of Plk1 might be involved in malignant proliferation. Furthermore, constitutive expression of Plk1 in NIH-3T3 cells causes oncocgenic focus formation and induces tumor growth in nude mice suggesting that Plk1 may contribute to cancer progression (8). Disrupting the function of Plks could be an important application for cancer therapy.

From the view of the primary structure, Plks contain a strikingly conserved sequence within their COOH-terminal domain, termed the polo-box, which is 30 amino acids in length. Without impairing kinase activity, three mutations in the polo-box of Plk1 abolish its ability to functionally complement the defect associated with a Cdc5–1 temperature-sensitive mutation (9). Recent studies revealed that fission yeast Plol interacts with the anaphase-promoting complex through the polo-box and the tetratricopeptide repeat domain of the subunit, Cut23 (10). A mutation in Cut23, which specifically disrupts the interaction with the polo-box, results in metaphase arrest. Taken together, the data suggest that the polo-box of Plks plays a critical role for the function of Plk1, in particular for its spatial distribution and for the physical interaction with substrates.

Because of the rapid development of technical protein synthesis and the advantage of nongene interference, the treatment with peptides is becoming a powerful new approach for tumor therapy (11). A 16-mer peptide, derived from the homeodomain of Antennapedia, has been reported to enter cells readily via a nonendocytotic, and receptor- and transporter-independent pathway (12). In this communication we have linked this Antennapedia peptide to the wild-type polo-box or to a mutated polo-box and analyzed its impact on the proliferation of cancer cells.

Materials and Methods

Synthesis of Peptides. Peptides were synthesized according to N-(9-fluorenyl)methoxycarbonyl synthesis protocols with double or triple coupling reactions using TBTU as activator on a Symphony synthesizer (Rainin Instrument Co., Woburn, MA). Purifications were performed by reverse-phase-high-performance liquid chromatography on a Waters (Milford, MA) Delta-Pak C18 column with a Waters liquid chromatography system. Quality control was performed by analytical RP-high-performance liquid chromatography using a Waters Alliance 2690 separation module equipped with a Waters 996 photodiode array detector and by MALDI-TOF mass spectrometry. Peptide sequences: P1 [polo-box (aa 410–429)] linked to a 16-mer carrier from Antennapedia, H2N-WVSKYWDYSDKYGLG-YQLCDRQIKIWIFQQNRRMKWKK-COOH. P2 [mutated polo-box (aa 410–429)] linked to a 16-mer carrier from Antennapedia: H2N-WVSKYTDDYSDKYGLG-YQLCDRQIKIWIFQQNRRMKWKK-COOH. P3 (16-mer carrier from Antennapedia as a control): H2N-RQIKIWFQNRRMKWKK-COOH.

Cell Culture and Growth Inhibition Assays. Cancer cell lines HeLa S3 (cervix), MCF-7 (breast), and Saos-2 (osteosarcoma) were grown at 37°C in 5% CO2 in Ham’s F12, RPMI 1640, and McCoy’s 5a medium, respectively, containing 10% fetal bovine serum and 2 mM glutamine. To assay for growth inhibition, exponentially growing cells (0.3–2.5 × 105) were seeded into 24-well plates. On the following day cells were incubated with peptides at various concentrations without serum for 3 h followed by addition of complete medium. On days 3, 6, and 8 cells were treated again and harvested on days 3, 6, 8, and 10 to determine cell numbers using a hemacytometer. Cell viability was assessed by trypan blue staining. Each experiment was repeated at least three times.
Flow Cytometry and Indirect Immunofluorescence Staining. Cell cycle analysis was performed using a Cycle TEST PLUS DNA reagent kit (Becton Dickinson) according to the manufacturer’s instructions. Briefly, cells were washed with PBS, treated with RNase A, and stained with PI. The analysis was performed using a Becton Dickinson FACScan flow cytometer. Using the MODFIT LT 2.0 software (Verity Software House, Topsham, ME) for each individual sample 30,000 cells were analyzed.

For staining cells were grown in slide flasks and treated with peptides for 24 h. Then, cells were fixed in 4% paraformaldehyde for 30 min, permeabilized in 0.2% Triton X-100 for 20 min, and stained with α-tubulin (Cedarlane, Ontario, Canada) 1:100, polyclonal rabbit PLK1 1:100, or monoclonal β-tubulin (Sigma) 1:100. Stained cells were analyzed with a confocal laser scan microscope or a fluorescence microscope.

Annexin V Apoptosis Assay. Cells were seeded into six-well plates, allowed to attach overnight, and then treated with different peptides at a concentration of 10 μM. Cells were trypsinized after 16 h and incubated with Annexin V according to the manufacturer’s recommendations (Mo Bi Tech).

Results

The Polo-Box Fused to an Antennapedia Carrier Translocates to the Cytoplasm and Nucleus of Cancer Cells. PLK1 was shown to be of importance for the G2/M transition (1, 2). The polo-box, a highly conserved domain of PLK1 (aa 410–439), contributes to binding of substrates as well as for its correct subcellular localization (9, 10). We assumed that peptides representing the polo-box might be able to compete with PLK1 for endogenous substrates and docking proteins. Thus, we tested polo-box-specific peptides for their ability to suppress the function of endogenous PLK1. In previous experiments an Antennapedia homeodomain sequence was linked to peptides and allowed the resulting chimerical peptides to be transported across the cell membrane directly from the cell culture medium to both the cytoplasm and nuclear compartment (12). In our study we linked the Antennapedia homeodomain (16 aa) to the core region of the polo-box derived from PLK1 (aa 410–429; Fig. 1A) or to a mutated version (9) to find out whether fusion peptides could enter tumor cells from the culture medium. The transport kinetics revealed that FITC-labeled peptides reached the cytoplasm within 15 min and then emerged immediately to the nucleus. After 30 min peptides entered 98–100% of cells in culture. Both fusion peptides (wild-type P1 and mutant P2) were efficiently delivered into the cytoplasm and nucleus of MCF-7 (Fig. 1B, panels e and f), HeLa S3 cells (Fig. 1B, panels g and h), and Saos-2 cells (data not shown). The median values of intracellular FITC intensity of wild-type P1 and its mutant form P2 were 155 and 164, respectively, at 30 min in HeLa S3, as determined by a flow cytometric analysis. The intracellular FITC intensity of wild-type polo-box P1 and its mutant form P2 was additionally examined at 2, 4, and 24 h. No substantial difference of intracellular FITC intensity was observed (data not shown). Peptides were distributed diffusely in the cytoplasm and accumulated at the nuclear membrane. Elevated concentrations of peptides were detected at nucleoli. The overall distribution and uptake of both peptides (wild-type P1 and mutant P2) in HeLa S3 and MCF-7 cells was similar. Four h after peptide treatment, morphological changes in MCF-7 and HeLa S3 cells attributable to toxic effects were not observed (Fig. 1B, panels a–d). FITC-labeled peptides were still detectable after 24 h (data not shown).

Wild-type Polo-Box P1 Inhibits the Proliferation of Human MCF-7, Saos-2, and HeLa S3 Cells. At first the human breast cancer cell line MCF-7 was tested to assay effects exerted by polo-box-specific peptides on its proliferation. A dose kinetics was established to choose an appropriate working concentration. Whereas the wild-type peptide P1 at concentrations between 0.01 and 0.5 μM did not affect the growth behavior, concentrations >1 μM started to inhibit the proliferation of MCF-7 cells (Fig. 1C). Inhibition of proliferation occurred in a dose-dependent manner in the range between 1 and 10 μM of P1. A general toxic effect was observed above 20 μM of peptide P1 (data not shown).

To investigate whether antiproliferative effects exerted by polo-box peptides are restricted to certain cell types, we tested different human cancer lines: MCF-7 (breast), Saos-2 (osteosarcoma), and HeLa S3 (cervix). As shown in Fig. 2, A–C, the wild-type polo-box (P1) exerted an inhibitory effect on cell proliferation, which was most prominent in MCF-7 cells. Interestingly, the mutated polo-box (P2), which differed from P1 at three positions, reduced the antiproliferative effects on MCF-7 cells in a dose-dependent manner in the range between 0.01 and 0.1 μM of P2. Consequently, MCF-7 started to inhibit cell proliferation of NCI-H11022 and NCI-H9253 at a concentration of 10 μM of P1 and P2.
potency of P1 significantly. Whereas an intermediate effect was seen for Saos-2 cells, little inhibition occurred in HeLa S3 cells. The control 16-mer carrier P3 alone had eventually no effect at concentrations between 5 and 10 μM. Thus, the results suggested that the inhibitory effect exerted by polo-box peptides is sequence-specific. In addition, testing of human primary mammary epithelial cells (HMEC) revealed that the polo-box P1 inhibited proliferation of HMEC but to a much lower extent than MCF-7 cells (data not shown).

**Treatment of Cancer Cells with Polo-Box-specific Peptides Induces G2/M Cell Cycle Arrest.** Because the functional down-regulation of Plk1 by microinjection of Plk1-specific antibodies or overexpression of a dominant-negative Plk1 induced G2/M arrest (17, 18), we investigated the impact of polo-box-specific peptides on the cell cycle of tumor cells. Fig. 2D depicts a representative analysis of HeLa S3 cells at day 6. Incubation with the wild-type polo-box P1 induced an increase of cells in G2/M by 25–35% compared with control cells. We decided to additionally determine the subpopulation of mitotic cells by microscopical means. The percentage of mitotic cells increased 3–4-fold after wild-type polo-box (P1) treatment compared with control cells. HeLa S3 cells were treated 24 h with different peptides at 5 μM, and stained for DNA and α-tubulin for subsequent fluorescence analysis. To determine the percentage of the mitotic population 500 cells were inspected. Each experiment was repeated three times independently; bars, ±SE.

Wild-type Polo-Box Induces Abnormal Mitotic Phenotypes with Misaligned Chromosomes and Multiple Spindle Poles. Polo-box-treated cells were additionally analyzed using DNA and α-tubulin staining to monitor spindle apparatus and chromosomal figures. Many treated cells showed multiple (Fig. 3D, panels d and f) or monoastral spindle poles (Fig. 3D, panel e). Furthermore, chromosomes in P1-treated HeLa S3 cells were misaligned, not properly segregated, and partially condensed (Fig. 3D, panels a–c).

**Discussion**

The Antennapedia homeodomain corresponding to the third helix of the DNA binding domain of a *Drosophila* transcription factor is
internalized into eukaryotic cells by a receptor-independent process (12). The internalization peptide has been used as a vector for small peptides derived from c-myc, p21, and p16 to traverse the cell membrane (13–15). We linked this carrier to a peptide (P1) representing the polo-box of Plk1 or to the mutated polo-box (P2). Fused peptides entered the cells quickly and efficiently. Both polo-box peptides P1 and P2 were found in the cytoplasm and nucleus of cancer cells. No difference in import efficiency between the wild-type polo-box P1 and its mutated form P2 could be detected in HeLa S3, MCF-7, and Saos-2 cells. Toxic side effects were not observed in cell lines at concentrations between 0.01 and 10 \( \mu M \).

Plk1 plays various critical roles in the passage of cells through M phase. It is overexpressed in rapidly proliferating cells and tumors (3, 4). Data implicate that Plk1 contributes to override spindle and DNA damage checkpoints (5–7), which makes Plk1 an attractive target for cancer therapy. Recently, it was reported that overexpression of the COOH-terminal domain of Plk1 is more efficient in causing mitotic delay or arrest than wild-type or kinase-defective Plk1 (16). This observation is attributable to the binding of the COOH terminus to full-length Plk1 or to the catalytic domain of Plk1, which causes the inhibition of its kinase activity (16). The region within the COOH-terminal domain mediating this effect is unknown. The polo-box represents a highly conserved sequence within the COOH-terminal noncatalytic region of the Plk family and has not been observed in proteins other than Plks yet. The exchange of three amino acids within the polo-box abolished the proper localization of Plk1 and disrupted its kinase function (9). In this communication we demonstrated for the first time that a fusion protein containing the polo-box of Plk1 and a
transmembrane carrier from Antennapedia used for the treatment of cancer cells is a novel strategy to inhibit the function of Plk1. We revealed that the polo-box inhibits the proliferation of various cancer cell lines by inducing apoptosis. Strong effects were observed in MCF-7 cells, which is possibly connected to the functional integrity of wild-type p53 and Rb, two tumor suppressor proteins leading to better apoptotic reaction. The inhibitory effect began after 10 h of treatment and reached its apoptotic peak at 24 h. The typical morphology of apoptotic cells was observed in all three of the cancer cell lines. As reported for microinjecting of Plk1 antibodies or for expression of a dominant-negative form of Plk1 (17, 18), polo-box peptide induced also mitotic arrest. The fluorescence-activated cell sorter analysis documented an increase of the G2/M population and in particular a 3–4-fold increase of mitotic cells in polo-box-treated cells.

In many treated cells chromosomes appeared to be randomly distributed and improperly condensed. Multiple or monoastral spindle poles were observed, which is in line with observations in cells transfected with COOH-terminal domain of Plk1 (16). In addition, P1-treated cells displayed daughter cells still connected by strings of cytoplasm in contrast to P3 treatment (data not shown). So we suggest that polo-box-mediated functions seemed also to be involved in cytokinesis.

Major abnormalities in cancer cells including the inhibitory effect on proliferation were induced only by the wild-type polo-box but not by the mutated form P2. Different mechanisms could contribute to the apoptotic impact exerted by polo-box peptides. At first, kinase assays revealed an inhibitory effect on substrate phosphorylation by Plk1 (data not shown): The polo-box peptide could prevent the binding of Plk1 to its substrate thereby acting in a competitive manner. Secondly, recent evidence documents that the COOH-terminal domain of Plk1 can bind to full-length or the catalytic domain of Plk1 (16). This interaction is interrupted when Thr-210 is substituted with an aspartic residue. In addition, the function of Plk3 was also shown to depend on its COOH-terminal domain (19). Still, the region within the COOH-terminal domains of Plk1 and Plk3 responsible for regulating the kinase activity remains to be elucidated. It is intriguing to consider the polo-box, a domain very well conserved during evolution, as a candidate for this regulatory function possibly by binding to a region surrounding Thr-210 in Plk1. This hypothesis gains additional support from previous observations, which demonstrated that mutations in the polo-box reduce the kinase activity of Plk1 (9). Future investigations are required to study the polo-box as a structural component for an intramolecular modulation of the activity of Plks.

Whereas in yeasts and Drosophila only a single Plk has been identified to date, the genome of higher vertebrates encompasses at least three Plks. The remaining two family members, Plk2 (Snk) and Plk3 (Fnk/Pkr), belong to proteins of immediate early response genes (1, 2). Functional assays imply that Plk1 and Plk3 are likely to have both overlapping and unique functions within the cell cycle (1). Plk3 links DNA damage functionally to cell cycle arrest and apoptosis partially via the p53 pathway (20). Overexpression of Plk3 induces incomplete cytokinesis and apoptosis (19). Considering the high homology (74%) of the polo-boxes belonging to Plk1–3, we could not exclude that the polo-box-specific peptide (P1) derived from Plk1 may also inhibit at least partially the function of Plk2 and Plk3, which might contribute to the effects observed in our study. Inhibition of Plk3 might be especially involved in inducing apoptosis and incomplete cytokinesis. Taken together, polo-box-specific peptides inhibit proliferation of tumor cell lines by inducing mitotic arrest and apoptosis. In line with the rapid development of peptide synthesis, polo-box could be a powerful inhibitor for proliferation. Up to date Plk1 function was inhibited by expression of dominant-negative forms or by application of antibodies (17, 18). Beyond perspectives offered by these techniques the use of fusion peptides such as P1 could open new ways for the systemic treatment of animals with localized tumors or even with disseminated disease. Thus, additional studies in tumor-bearing animals will shed light on the potential of polo-box-specific peptides as candidates for tumor therapy.

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References

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