Disrupting Skp2-cyclin A interaction with a blocking peptide induces selective cancer cell killing

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Abstract

Skp2 fulfills the definition of an oncoprotein with its frequent overexpression in cancer cells and oncogenic activity in various laboratory assays and therefore is a potential cancer therapy target. The best-known function of Skp2 is that of an F-box protein of the SCF^{Skp2}-Roc1 E3 ubiquitin ligase targeting the cyclin-dependent kinase inhibitor $p27^{Kip1}$. Knockdown of Skp2 generally leads to accumulation of p27 but its effects on cancer cells are less certain. Another function of Skp2 is its stable interaction with cyclin A, which directly protects cyclin A from inhibition by p27 in *in vitro* kinase assays. Here, we report that an 18-residue blocking peptide of Skp2-cyclin A interaction can indirectly inhibit cyclin A/Cdk2 kinase activity dependent on the presence of p27 in *in vitro* kinase assays. Transmembrane delivery of this blocking peptide can induce cell death in a panel of four cancer cell lines in which Skp2 knockdown only have mild inhibitory effects. This Skp2-cyclin A interaction blocking peptide can synergize with a previously identified E2F1-derived LDL peptide, which blocks its access to cyclin A, in killing cancer cells. IC_{50} of the Skp2-cyclin A blocking peptide correlated with abundance of Skp2, its intended target, in cancer cells. These results suggest that Skp2-cyclin A interaction plays an important role in cancer cell survival and is an attractive target for cancer drug discovery. [Mol Cancer Ther 2007;6(2):684–91]

Received 8/29/06; revised 11/16/06; accepted 12/21/06.

Grant support: NIH (National Cancer Institute, National Institutes of Diabetes, Digestive and Kidney Diseases), Albert Einstein Comprehensive Cancer Research Center and Liver Research Center (core facility support), and the Irma T. Hirschl Career Scientist Award (L. Zhu).

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doi:10.1158/1535-7163.MCT-06-0538

Introduction

Skp2 was identified and cloned as a cyclin A–binding protein (1) and an F-box protein (2). It is now well established that Skp2 is the substrate recruiting subunit of the SCF^{Skp2}-Roc1 E3 ubiquitin ligase targeting p27 for ubiquitylation (3). Because p27 is a negative regulator of cell proliferation (4), the p27 ubiquitylation activity of Skp2 and its overexpression in cancer cells suggested that it might be an oncoprotein. Indeed, Skp2 meets the criteria for an oncoprotein with frequent overexpression in a wide range of cancer specimens and oncogenic activity in various experimental assays (3).

Importantly, in experimental oncogenic studies, oncogenic activities of Skp2 overexpression were not mimicked by inactivation of p27, indicating that the oncogenic functions of Skp2 involve activities in addition to p27 ubiquitylation and degradation. This contrasts with the role of Skp2 in mouse development, in which phenotypes of Skp2 knockout can mostly be corrected by p27 knockout (5, 6). Thus, mechanisms of Skp2 functions in normal physiology and in oncogenesis must be different. This difference may provide an opportunity to develop anti-Skp2 therapeutics for cancer cell–selective therapy.

Molecular details of the activity of Skp2 in recruiting p27 into the SCF^{Skp2}-Cks1-Roc1 complex have been revealed in great details (7), which form the basis for inhibitor development for this aspect of Skp2 function (3). In the meantime, a number of studies have already used gene silencing to determine the effects of targeting Skp2 in cancer cells (8–11). Consistent with Skp2 as a substrate recruiting subunit of the SCF^{Skp2}-Roc1 ubiquitin ligase targeting p27, knockdown of Skp2 generally leads to increases in p27 protein levels. However, the biological effects of Skp2 knockdown in cancer cells vary among various degrees of proliferation inhibition, cell death, or lack of effect. These studies provided ''proof-of-principle'' that targeting Skp2 could be therapeutic for certain cancers but raised the need to more carefully determine its potential in this regard. We therefore initiated our study by determining the effects of Skp2 knockdown in four human cancer cell lines with various functional status of p53 and Rb.

We have recently determined that the functional significance of the stable Skp2-cyclin A interaction is for Skp2 to directly protect cyclin A/Cdk2 from inhibition by p27 (12). Because this interaction is specific for cyclin A in the cyclin family, specific inhibition of Skp2-cyclin A interaction could create a cellular condition that is distinct from knockdown of Skp2, which leads to inhibition of multiple cyclins together by increased levels of p27. We therefore determined whether specific inhibition of Skp2-cyclin A interaction by a blocking peptide could lead to inhibition of cyclin A/Cdk2 kinase activity and could have different effects on cancer cells than knockdown of Skp2.

Materials and Methods

Skp2 Knockdown and Overexpression

The target sequences for Skp2 knockdown are 5′-CCTTAGACCTCACAGGTAA-3′ and 5′-CAGTCGGTGCTATGATATA-3′. The negative control is an irrelevant sequence (5′-GTTACAAAGCAGAAGTTAA-3′, which is derived from the 3′ untranslated region of mouse p27 and does not match any human sequence in the databases). Construction of lentivirus-based multi–micro-RNA hairpin constructs, production of lentivirus, and infection with lentivirus were previously described (13). Overexpression of Skp2 was achieved by transduction with a lentivirus expressing a flag-tagged Skp2 expressed from a cytomegalovirus promoter. Cell lines U2OS, HeLa, C33A, and Saos2 were maintained in standard conditions.

Antibodies, Peptides, and Treatment of Cells with Cell-Permeable Peptides

Antibodies to actin (C-2), Cdk2 (M-2), and cyclin A (H-432) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–cyclin A (E23) was obtained from Lab Vision NeoMarkers (Fremont, CA). Anti-p27 (K25020) was from BD Transduction Laboratories (Franklin Lakes, NJ). Anti-Skp2 (51–1,900 and 32–3,300) was purchased from Invitrogen Zymed (Carlsbad, CA). The following peptides were custom-ordered from Genescript (Piscataway, NJ): 4060 (KTSELLSGMGVSALEKEE), 4060AAAAA (KTSEAASGMGVAAAEKEE), TAT-4060 (YGRKKRRQRRRGKTSELLSGMGVSALEKEE), TAT-4060AAAA (YGRKKRRQRRRGKTSEAASGMGVAAAEKEE), TAT-LDL (YGRKKRRQRRRGPVKRRLDL), and TAT-Umt (YGRKKRRQRRRGETDHQYLAESS).

Western Blotting, Immunoprecipitation, Kinase Assays, and Fluorescence-Activated Cell Sorting

These assays were all done as previously described (12).

Cell Proliferation and Survival Assays

For cell proliferation assay shown in Fig. 1D, 1×10^4 cells were plated in each well of 24-well plates and allowed to grow for a course of 4 days in the presence of 10% fetal bovine serum. Cell numbers were counted every day from triplicate wells. For cell survival assays in Figs. 4–6, 1×10^3 cells were plated in each well of 96-well plates and allowed to adhere overnight in the presence of 10% fetal bovine serum. The cells were washed once with Opti-MEM (Invitrogen Life Technologies, Carlsbad, CA) followed by incubation with different concentrations of peptides in Opti-MEM for 24 h in

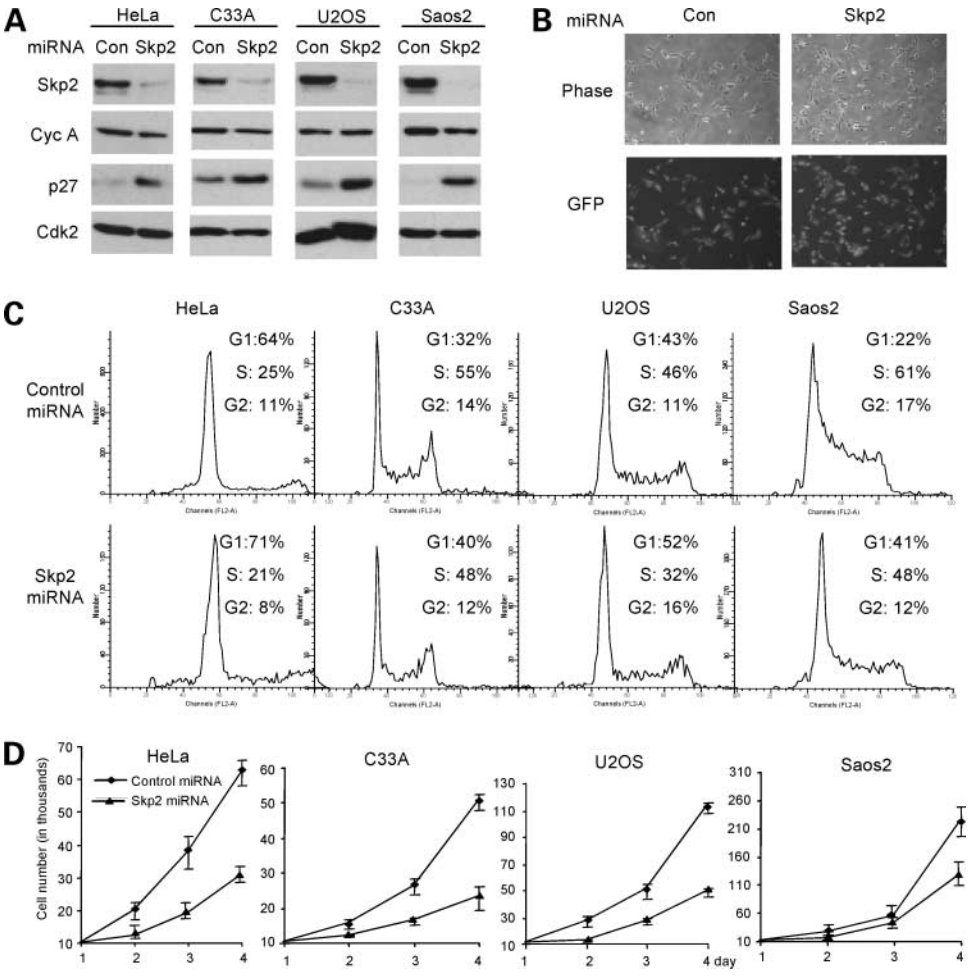

Figure 1. Skp2 knockdown in four human cancer cell lines. **A,** Western blot of indicated cells infected with lentivirus expressing micro-RNA (*miRNA*) hairpins targeting Skp2 or an irrelevant mouse sequence as control (*Con*) for 48 h. **B,** phase contrast and green fluorescent protein (*GFP*) pictures of U2OS cells infected with the indicated lentiviruses for 48 h. **C,** fluorescence-activated cell sorting analysis of the indicated cells 48 h after infection. **D,** cell proliferation assay of indicated cancer cells infected with the indicated lentiviruses. Cells in 24 wells, in triplicates, were counted everyday for 4 d.

triplicate. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (inner salt; MTS) assay was then done following the manufacturer's protocol (Promega, Madison, WI).

Results

Knockdown of Skp2 in a Panel of Four Human Cancer Cell Lines Results in Mild Proliferation Inhibition

We used a recently established multi–micro-RNA hairpin method to achieve effective knockdown of Skp2 in a panel of four human cancer cell lines, including cervical carcinoma cell lines HeLa and C33A and osteosarcoma cell lines U2OS and Saos-2. HeLa cells contain papilloma virus oncoprotein E6 and E7, which disrupt functions of *p53* and *Rb*, respectively, whereas C33A cells contain a nonfunctional *Rb*. Saos-2 cells are defective in *p53* and *Rb* functions due to mutations in both genes, whereas *p53* and *Rb* functions are intact in U2OS cells. Knockdown hairpins were expressed from a cytomegalovirus promoter in a single transcript with green fluorescent protein from a lentivirus vector. An irrelevant hairpin expressed from the same vector was used as control. As shown in Fig. 1A, Skp2 was efficiently knocked down in all four cancer cell lines at 48 h after lentiviral transduction of all the cells in culture as shown by green fluorescent protein expression (Fig. 1B and data not shown). Knockdown of Skp2 resulted in accumulation of p27 as expected. In comparison, cyclin A and Cdk2 levels did not show significant and consistent change. The slight decreases in cyclin A protein levels after Skp2 knockdown in C33A and Saos-2 cells were not detected reproducibly.

At 48 h after transduction when Skp2 was efficiently knocked down, we did not observe any visual effects in all four cell lines (Fig. 1B; data not shown). We did flow cytometry analysis to determine the effects of Skp2 knockdown on cell cycle profile (Fig. 1C). Results showed increases in G_1-phase cell populations of ~10% to 20% with corresponding decreases in S-phase populations in these cells with Saos-2 affected most significantly. Consistent with the lack of cell death upon visual examination, no sub-G_1 cells were observed in any of the four cell lines after Skp2 knockdown. We then determined their proliferation during a course of 4 days as shown in Fig. 1D. We found a 2- to 3-fold reduction in cell numbers in all four cell lines by day 4. These results show that Skp2 knockdown had a general and mild proliferation inhibition effect in these four cancer cell lines with various status of *p53* and *Rb*. Knockdown of Skp2 with a separate target sequence yielded similar results.

Targeting Skp2-Cyclin A Interaction with a Blocking Peptide Inhibits Cyclin A/Cdk2 Kinase Activity in the Presence of p27

We next focused on the cyclin A–binding aspect of Skp2 function. Identification of an 18-residue peptide (the 4060 peptide) that can block Skp2-cyclin A interaction (12) provided us with a means to study the functional significance of this interaction.

We first studied the biochemical effects of the 4060 peptide *in vitro*. Our previous study (12) has determined that the stable interaction between Skp2 N-terminus and cyclin A N-terminus (the 4060 to N-terminal interaction; see Fig. 2A for a cartoon representation) is to protect cyclin A/Cdk2 from inhibition by p27 because Skp2-cyclin A interaction itself does not inhibit or stimulate cyclin A/Cdk2 kinase activity but rather competes with p27 binding to cyclin A/Cdk2. Importantly, whereas Skp2 binding to cyclin A is competitive and mutually exclusive with p27 binding to cyclin A, the 4060 blocking peptide does not interfere with p27-cyclin A interaction, an RxL-HP–type interaction (12). This difference between Skp2 and the 4060 blocking peptide in competing with p27 binding to cyclin A can be most simply explained by the small size of the 4060 peptide, and allowed us to form a working model for the effects of 4060 peptide on cyclin A/Cdk2 kinase in a defined kinase reaction with Skp2 and p27, as presented in cartoon form in Fig. 2A.

Because Skp2 binding to cyclin A did not have inhibitory effects on its associated kinase activity, we predicted that the 4060 peptide should likewise not inhibit cyclin A–associated kinase activity. To test this prediction, we added various peptides to an *in vitro* kinase reaction with purified cyclin A/Cdk2 (~3 nmol/L concentration). We used an E2F1-derived RxL peptide, LDL peptide, for comparison because it could inhibit cyclin A/Cdk2 kinase activity (ref. 14 and see next section). Because we later added a TAT sequence to these peptides for transmembrane delivery (see next section), we used two versions of each peptide (without TAT and with TAT) in these kinase assays. We found that these two versions of peptides yielded identical results and therefore only presented results with one version of the peptides. As shown in Fig. 2B, addition of 1 μmol/L TAT-LDL peptide caused a 10-fold inhibition of cyclin A/Cdk2 kinase activity toward the glutathione *S*-transferase-Rb-COOH terminal substrate, as expected (14). In comparison, the TAT-4060 peptide, like a negative control peptide (TAT-Umt; ref. 14), did not show inhibitory effect at 1 and 10 μmol/L concentrations. Both TAT-4060 and TAT-Umt reproducibly slightly increased the kinase activity although these effects were unlikely to be significant. At 100 μmol/L concentration, both TAT-4060 and TAT-Umt caused significant inhibition of cyclin A/Cdk2 kinase activity, indicating that at very high concentrations, these peptides could nonspecifically inhibit cyclin A/Cdk2 kinase activity. These results reveal a further distinction between Skp2-cyclin A interaction and interactions mediated by the RxL motif.

We then determined the effects of the 4060 peptide on cyclin A/Cdk2 kinase activity in the presence of Skp2 and p27. Consistent with our previous results (12), p27 (5 nmol/L) inhibited cyclin A/Cdk2 (3 nmol/L) kinase activity by 10-fold, which was completely reversed by Skp2N (80 nmol/L), demonstrating the protective effect of Skp2 on cyclin A/Cdk2 against the inhibitory effects of p27 (Fig. 2A and C, *lanes 1–3*). As predicted in the working model, inclusion of the 4060 blocking peptide

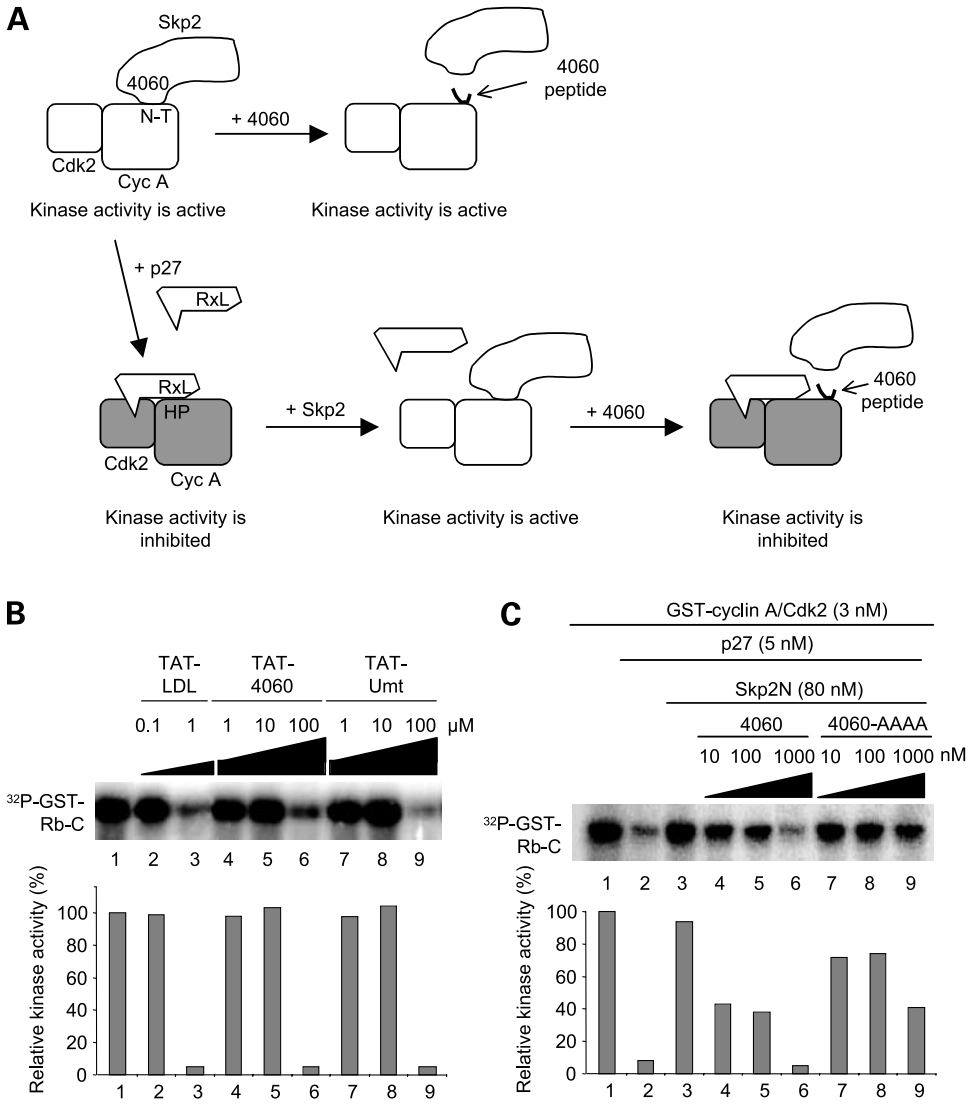

Figure 2. Effects of the 4060 peptide on cyclin A/Cdk2 kinase. **A,** a working model for the action of the 4060 peptide in kinase reactions with purified components. *Cyc A,* cyclin A; *N-T,* N-terminus. **B,** *in vitro* kinase reactions were carried out with purified cyclin A/Cdk2, with inclusion of various peptides at the indicated concentrations. Glutathione *S*-transferase-Rb-COOH terminal (*GST-Rb-C*) was used as substrate. **C,** *in vitro* kinase reactions with purified proteins and indicated peptides at the indicated concentrations. Phosphorylation of glutathione *S*-transferase-Rb-COOH terminal was quantified and plotted.

abolished this protective effect in a dose-dependent manner (*lanes 4–6*). A derivative peptide containing alanine substitutions at four conserved residues and ineffective in blocking Skp2-cyclin A interaction (the 4060AAAA peptide; ref. 12) was much weaker in disruptH_2ing the protective effect of Skp2N (*lanes 7–9*). These results show that the 4060 peptide can inhibit cyclin A/Cdk2 kinase activity when Skp2 and p27 are both present in the reaction. This mode of action of the 4060 peptide represents a distinct mechanism to inhibit cyclin A/Cdk2 than the functional mechanism of RxL peptides (which directly inhibits cyclin A kinase activity by blocking its access to phosphorylation substrates; ref. 14).

Disruption of Skp2-Cyclin A Interaction with Blocking Peptide Induces Cell Death in Cancer Cells but not in Normal Cells

To determine the effects of disrupting Skp2-cyclin A interaction in cancer cells, we fused the HIV-derived cell membrane penetration domain TAT to the 4060 peptide with a glycine linker to generate the TAT-4060 peptide. TAT-Umt and TAT-4060AAAA were used as controls. Nevertheless, the possibility still formally exists that the TAT-4060 peptide may disrupt interactions between the 40 and 60 sequences of Skp2 and other unidentified proteins in the cell that interact with the 40 to 60 sequence in a manner similar to cyclin A.

We confirmed that transmembrane delivery of TAT-4060 peptide resulted in dissociation of interaction between cellular Skp2 and cyclin A/Cdk2 as determined with coimmunoprecipitation with cyclin A (Fig. 3A). The mutant peptide TAT-4060AAAA did not have this effect. The loss of Skp2-cyclin A/Cdk2 interaction was not due to a reduction in levels of these proteins (Fig. 3B). Importantly, cellular p27 protein levels were not affected by the blocking peptide. This result provided further support that cyclin A binding to Skp2 N-terminus is not required for Skp2-mediated p27 ubiquitylation and degradation (12). It also reveals a key biochemical difference

between disruption of Skp2-cyclin A interaction and knockdown of Skp2 (which led to significant increases in p27 protein levels as shown in Fig. 1A). When kinase activities were determined in anti-cyclin A and anti-Cdk2 immune complexes, we found that treatment with TAT-4060 resulted in a clear reduction of the associated kinase activity compared with treatment with TAT-4060AAAA or TAT-Umt (Fig. 3C). Here, it is important to point out that although we used p27 as an inhibitor of cyclin A–associated kinase activity in purified kinase reactions with the 4060 blocking peptide shown in Fig. 2, the effects of blocking Skp2-cyclin A interaction on cyclin A–associated kinase activity in cells is unlikely to be mediated by p27 alone. We were unable to detect a significant interaction between p27 and cyclin A in U2OS cells before and after treatment of TAT-4060. p27 family members p21 and p57, and the pocket proteins p107 and p130 (15), may bind and inhibit cyclin A–associated kinase activity through the RxL-HP interaction mechanism. More studies are needed to determine which of these proteins, or combinations of these proteins, are responsible for inhibition of cyclin A–associated kinase activity when Skp2-cyclin A interaction was blocked by TAT-4060.

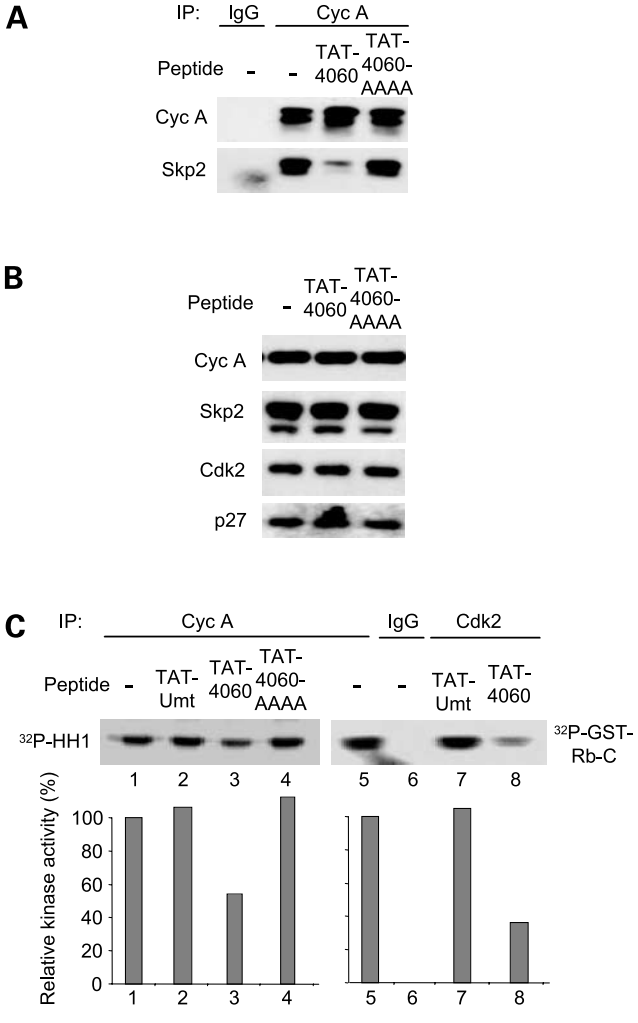

Figure 3. Effects of transmembrane delivery of the 4060 peptide. **A,** U2OS cells were treated with 50 μmol/L of the indicated peptide for 12 h. Cell extracts were prepared for immunoprecipitation and Western blot analysis as indicated. **B,** the same cell extracts were used in Western blots as indicated. **C,** the same extracts were used in immunoprecipitation (*IP*) kinase assays as indicated. The extent of phosphorylation was quantified and plotted.

We next determined the biological effects of TAT-4060. TAT-LDL was used for comparison. Sequence of LDL (PVKRRLDL) is derived from the N-terminus of E2F1 that mediates E2F1-cyclin A interaction. This interaction facilitates phosphorylation of E2F1 by cyclin A/Cdk2, which inhibits DNA binding and transactivation activity of the E2F1/DP1 dimer (16). Because E2F1 can induce apoptosis, this function of cyclin A/Cdk has been shown to play a survival role in cancer cells (17), and TAT-LDL has been shown to induce cancer cell death (18).

As shown in Fig. 4A and C, we found that TAT-4060 induced significant cell death in U2OS cells to similar degrees as TAT-LDL at 24 h after the addition of peptides to cell culture medium. Also, like TAT-LDL, TAT-4060 did not affect human diploid fibroblasts WI-38 (Fig. 4B and D). As a negative control, TAT-Umt did not have cell killing effects in either cell lines at the same concentrations. TAT-4060AAAA also did not have significant cell killing effects (Fig. 4E). These results suggest that the cancer cell killing effects of TAT-4060 were a specific activity of this peptide correlated with its ability to block Skp2-cyclin A interaction. In comparison, both TAT-Umt and TAT-4060 inhibited cyclin A/Cdk2 kinase activity *in vitro* at peptide concentrations of 100 μmol/L (Fig. 2B). It is currently unknown how peptide concentrations in the culture medium translate into intracellular peptide concentrations. We believe that the ability of TAT-4060 to inhibit cyclin A kinase activity *in vitro* at 100 μmol/L concentration was unlikely to be the cause of its cancer cell killing effects because TAT-Umt inhibited cyclin A kinase activity *in vitro* at 100 μmol/L but did not exhibit cell killing effects at 120 μmol/L. TAT-LDL or TAT-4060–treated U2OS cells showed condensed nuclear morphology indicative of apoptosis (data not shown), but the molecular mechanisms of cell killing by TAT-4060 and TAT-LDL remain to be determined. TAT-LDL exhibited higher potency at IC_{50} (7.5 μmol/L for TAT-LDL and 35 μmol/L for TAT-4060). In comparison, TAT-4060 showed higher specificity between U2OS and WI-38 cells (TAT-LDL killed 25% of WI-38 cells at 120 μmol/L, whereas TAT-4060 and TAT-Umt had no effect). These results document that inhibiting Skp2-cyclin A interaction can lead to a significantly different therapeutic effect than knocking down Skp2 in U2OS cells (see Discussion).

TAT-4060 Peptide Can Synergize with TAT-LDL Peptide in Killing Cancer Cells

Combinatorial therapies that target two or more distinct mechanisms of common or related pathways often provide an effective means to increase therapeutic efficacy.

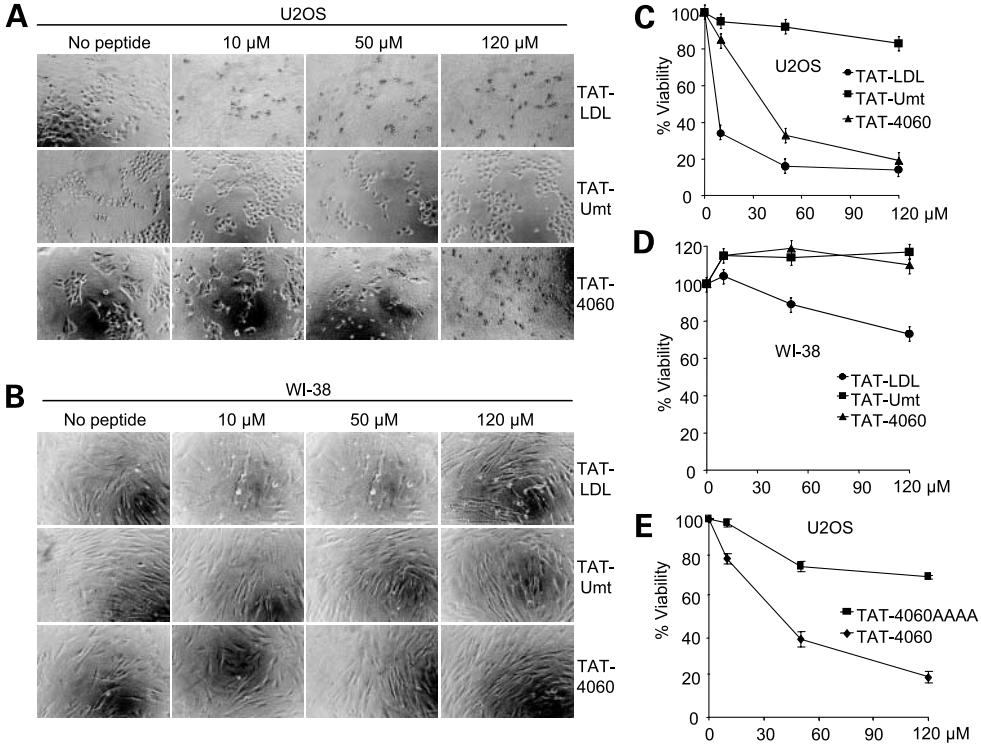

Figure 4. Cancer cell–selective killing by TAT-4060. **A** and **B,** U2OS and WI38 cells were treated with the indicated peptides at the indicated concentrations for 24 h and then photographed at ×10 magnification. **C** and **D,** quantitative analysis of results shown in **A** and **B** by MTS assay in triplicate wells. **E,** the mutant peptide TAT-4060AAAA was tested in the cell killing assay as in **C**.

Identification of a new mechanism to inhibit cyclin A/Cdk2 kinase activity and to kill cancer cells by TAT-4060 prompted us to determine whether there was a synergistic relationship between TAT-4060 and TAT-LDL.

As shown in Fig. 5, when used at 1 or 2 μmol/L concentrations, neither TAT-LDL nor TAT-4060 exhibited any cell killing effects because these concentrations were well below the IC_{50} of these peptides. However, at a combined concentration of 1 μmol/L (0.5 μmol/L TAT-4060 and 0.5 μmol/L TAT-LDL), TAT-4060 and TAT-LDL together already showed clear cell killing effects. At a combined concentration of 2 μmol/L, these two peptides killed >40% of the cells. This significant synergy between TAT-LDL and TAT-4060 is consistent with these two peptides killing U2OS cells through different mechanisms and reveals a significant clinical value for these two potential targeted therapeutics.

IC_{50} of the 4060 Peptide Positively Correlates with Skp2 Protein Abundance in Cancer Cells

Skp2 is frequently overexpressed at various levels in various cancer cells. If TAT-4060 kills cancer cells by disrupting Skp2-cyclin A interaction, higher peptide concentrations should be needed to kill cancer cells with higher levels of Skp2. In comparison, because TAT-LDL is believed to kill cancer cells by blocking access of cyclin A to E2F1, there need not be a direct correlation between effective TAT-LDL concentrations and cellular Skp2 protein levels. To test this prediction, we determined Skp2 protein levels and IC_{50} of both TAT-4060 and TAT-LDL for the panel of four cancer cell lines. As shown in Fig. 6A and B, an unbroken correlation between Skp2 protein levels and IC_{50} was indeed observed for TAT-4060, but not for TAT-LDL. Here, it is important to point out that for cancer cells with high levels of Skp2 such as HeLa cells, cell killing required very high concentrations of TAT-4060 peptide, which may induce nonspecific cytotoxic effects.

We also investigated whether artificial manipulation of Skp2 protein levels in U2OS cells could change the IC_{50} of various peptides. Skp2 protein levels were reduced by knockdown and increased by overexpression from a cytomegalovirus-based expression vector as shown in Fig. 6C. Results shown in Fig. 6D show that artificial decrease in Skp2 protein levels in U2OS cells reduced the concentrations of TAT-4060 peptide to achieve 40% killing from 40 to 20 μmol/L, whereas artificial increase of Skp2 protein levels rendered TAT-4060 nearly as ineffective

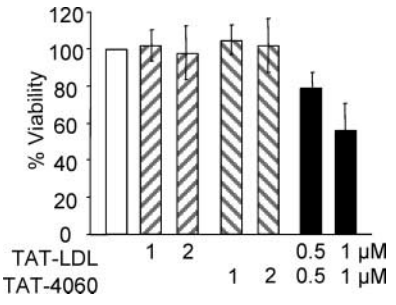

Figure 5. Synergy between TAT-4060 and TAT-LDL. U2OS cells were treated with indicated concentrations of the indicated peptides for 24 h, and MTS assays were done to determine cell viability as in Fig. 4.

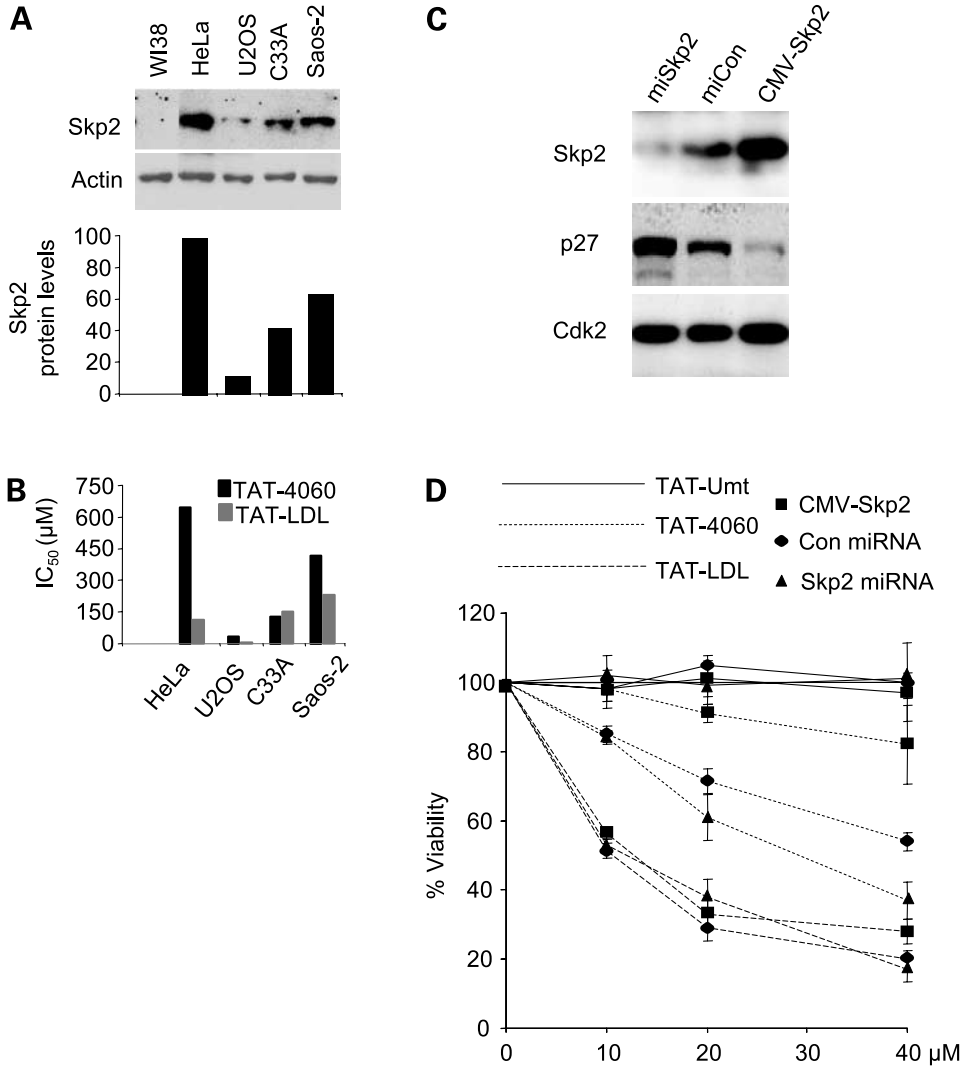

Figure 6. Correlation between Skp2 levels and IC_{50} of TAT-4060. **A,** cellular Skp2 levels were analyzed by Western blot with actin as loading control. The film was scanned and quantified for plotting relative Skp2 protein levels. **B,** IC_{50} of TAT-4060 and TAT-LDL in different cancer cell lines were plotted. **C,** Western blot of U2OS cell lysates 2 d after infection with lentiviruses expressing Skp2 micro-RNA, control micro-RNA, or exogenous Skp2, as indicated. **D,** various U2OS cells were treated with indicated concentrations of indicated peptides for 24 h. Cell viability was determined with MTS assays as in Fig. 4.

as TAT-Umt. In the same experiments, effects of TAT-LDL and TAT-Umt were not affected by Skp2 knockdown or overexpression. Together, these results suggest that the effectiveness of TAT-4060 in killing cancer cells could be predicted by the abundance of its intended target, which is an expected and valuable property of targeted therapeutics.

Discussion

In addition to functioning as a substrate recruiting subunit of SCF^{Skp2}-Roc1 E3 ubiquitin ligase targeting p27 for ubiquitylation, Skp2 forms stable complex with cyclin A/Cdk2 to protect it from inhibition by p27 (12). In this study, we investigated the functional consequences of Skp2-cyclin A interaction using an interaction blocking peptide. In kinase reactions with purified proteins, this blocking peptide (the 4060 peptide) did not itself specifically (at concentrations up to 10 μmol/L) inhibit cyclin A/Cdk2 kinase but could do so when cyclin A/Cdk2 was protected by Skp2 in the presence of p27. Transmembrane delivery of this blocking peptide exhibited cancer cell killing effects, whereas efficient knockdown of Skp2 only had mild inhibitory effects on proliferation for the four cancer cell lines tested. These results show that disrupting Skp2-cyclin A interaction could be a more effective strategy than knocking down Skp2 in targeting Skp2 for therapeutic intervention in the treatment of cancer.

At the same time, these results also raise an obvious question: Why do knockdown of Skp2 and disruption of Skp2-cyclin A interaction have distinctive effects on these cancer cells (because knockdown of Skp2 should also abolish Skp2-cyclin A interaction)? Limitations and differences of the technologies used for these two approaches should first be considered. Because RNA interference–mediated gene knockdown cannot be equated with gene knockout, it is possible that a more complete Skp2 knockout in cancer cells may induce a cell killing effect. It is also possible that the 4060 blocking peptide induced cancer cell killing through mechanisms in addition to

dissociating Skp2-cyclin A interaction. The kinetics of action is also different between RNA interference–mediated gene knockdown and transmembrane delivery of blocking peptides. Although these possibilities remain to be addressed, we believe that results of this study may reflect important functional properties of the Skp2-cyclin A interaction and provide new insights into this aspect of Skp2 function.

A salient feature of Skp2-cyclin A interaction is that Skp2 interacts with sequences in the N-terminus of cyclin A (12) and is therefore specific for cyclin A because sequences in cyclin A N-terminus are not conserved in other cyclins. This contrasts with the RxL-HP–type interaction because HP is present in all cyclins. Because the biochemical significance of Skp2-cyclin A interaction is to protect cyclin A from inhibition by p27 (and possibly by other RxL-type inhibitors), the absolute specificity for cyclin A implies that protecting cyclin A is more important than protecting multiple cyclins together. It follows that specifically inhibiting cyclin A alone may be more detrimental to cancer cells than nonspecifically inhibiting multiple cyclins due to the creation of an imbalance between cyclin A–associated kinase activity and kinase activities associated with other cyclins. This scenario in fact is consistent with a number of previous findings. Elimination of Cdk2, which would inhibit kinase activities associated with cyclin A and cyclin E, did not have cancer cell killing effects (19, 20), whereas elimination of cyclin A/Cdk2 complex could cause cancer cell death (20).

In this respect, it is interesting that the ubiquitylation activity of Skp2 has positive regulatory effects on multiple cyclins because ubiquitylation target p27 (and also p21 and p57 to a lesser degree) inhibits multiple cyclins with the RxL-HP interaction mechanism. This may explain why knockdown of Skp2 and the accompanying increases in p27 did not cause cell death in the four cancer cell lines. A dual role of Skp2 in targeting p27 for degradation and specifically protecting cyclin A–associated kinases may be selected for in tumorigenesis to maximize its oncogenic activity by promoting general proliferation and ensuring cancer cell survival at the same time. Accordingly, specifically inhibiting the cyclin A–binding activity of Skp2 may represent an effective strategy of targeting Skp2, and this study provides the experimental evidence and a drug candidate for further development of this new paradigm of targeting Skp2 for cancer treatment.

Acknowledgments

We thank A. Follenzi and L. Naldini for generous help with the lentivirus system and Ari Melnick for help and advice with peptide studies.

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