Proteasome-Mediated Destruction of the Cyclin A/Cyclin-Dependent Kinase 2 Complex Suppresses Tumor Cell Growth *in Vitro* and *in Vivo*

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**ABSTRACT**

Cyclin-dependent kinases (cdks) represent potentially promising molecular targets for cancer therapeutic strategies. To evaluate the antitumor activity of selective cyclin/cdk inhibition, we constructed a chimeric protein composed of a F-box protein (TrCP) fused to a peptide comprising the cyclin/cdk2 binding motif in p21-like cdk inhibitors (TrCP-LFG). We now demonstrate that endogenous cyclin A and its binding substrate, cdk2, can be tethered to β-TrCP, ubiquitinated, and effectively degraded. Degradation of cdk2 and cyclin A together, but not cdk2 alone, results in massive tumor cell apoptosis *in vitro* and *in vivo* in a proteasome-dependent manner with no toxicity to normal tissue. These data demonstrate that cyclin A and/or the cyclin A/cdk2 complex is a promising anticancer target with a high therapeutic index.

**INTRODUCTION**

Most cyclin-dependent kinases (cdks) are involved in the orderly progression of the cell cycle. Dysregulation of cell cycle progression can result in disordered and uncontrolled cell growth, which is characteristic of neoplasia (1–6). A number of cdk (i.e., cdk4/6, cdk2) target the retinoblastoma protein (pRb), leading to its inactivation through phosphorylation (7–12). One critical set of downstream targets of pRb are the E2F family of cell-cycle regulatory transcription factors, E2F (13–15). Inactivation, has the potential as a selective cancer therapeutic strategy. A major pathway used by eukaryotic cells to degrade specific proteins is ubiquitin-dependent proteolysis, which involves a cascade of enzymatic reactions catalyzed by the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzymes, and the E3 ubiquitin-protein ligases. The substrate specificity of the ubiquitin pathway is conferred by the E3. Most cyclin-dependent kinases (cdks) are involved in the orderly progression of the cell cycle, Dysregulation of cell cycle progression can result in disordered and uncontrolled cell growth, which is characteristic of neoplasia (1–6). A number of cdk (i.e., cdk4/6, cdk2) target the retinoblastoma protein (pRb), leading to its inactivation through phosphorylation (7–12). One critical set of downstream targets of pRb are the E2F family of cell-cycle regulatory transcription factors, E2F (13–15). Inactivation, has the potential as a selective cancer therapeutic strategy.

**MATERIALS AND METHODS**

Reagents. *N*-Acetylated-l-leucinyl-l-leucinyl-l-norleucinal (ALLN) were purchased from Sigma (St. Louis, MO). All other reagents were purchased from Pharmingen (San Jose, CA). Cells were cultured in the media as described in the manufacturer’s data sheet. Plasmids and Adenovirus. The Flag-tagged β-TrCP-LFG (TrCP-LFG), Flag-tagged ΔTrCP-LFG (ΔTrCP-LFG), and Flag-tagged TrCP (TrCP) were generated by PCR using TrCP/PcDNA3 or ΔTr-β-TrCP/PcDNA3 (a gift from Dr. Klaus Strebel) as a template. The final PCR fragments were cloned into the *Bgl*II/Not*I* sites of pAdTrack plasmid. All PCR products were verified by automated sequencing at the National Institute of Neurological Disorders and Stroke core facility at the NIH. These plasmids were recombined with pAdEasy1 and transfected into 293 cells to make recombinant adenoviruses (59). Adp73dd was phosphorylation of substrate by cyclin A/cdk2 and cyclin E/cdk2 and preferentially induces apoptosis in transformed cells (24, 50). As a small peptide, however, the clinical use of LFG may be limited by pharmacological constraints, leading to inefficient delivery *in vivo*. Furthermore, a recent study questions whether selective cdk2 inhibition is a useful cancer strategy (51, 52). Additionally, the Barbacid laboratory has shown no cell cycle abnormalities in either a cdk2 null mouse or following acute ablation of cdk2 in primary cells by Cre-loxP-mediated recombination, thereby raising the question of the true importance of cdk2 in cellular proliferation (53). Thus, there is an urgent need for the evaluation of other potentially more efficient strategies aimed at selectively targeting cdk2 and its binding partners in tumor cells.

One of the potential reasons for the inefficiency of the LFG peptide approach may be that the peptide merely competes with substrate for cdk2 binding sites, thus requiring high, continuous intracellular concentration of the peptide. We reasoned that destruction of the cdk2 protein, with or without its binding partners, might represent a more efficient approach. A major pathway used by eukaryotic cells to degrade specific proteins is ubiquitin-dependent proteolysis, which involves a cascade of enzymatic reactions catalyzed by the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzymes, and the E3 ubiquitin-protein ligases. The substrate specificity of the ubiquitin pathway is conferred by the E3. One of the best characterized ubiquitin ligases is the Skp1-Cullin-F-box (SCF) complex (reviewed in Ref. 54). Within the SCF complex, the F-box-containing proteins serve as the receptors for specific substrates (55). The F-box protein contains two essential modular domains: the F-box that is required for binding to Skp1 and a protein-protein interaction domain for binding distinct substrates (56). It has been previously reported that specific proteins can be degraded when a F-box protein is engineered to contain a specific protein interaction domain without affecting degradation of normal endogenous substrates (57, 58).

We now report the construction of a chimeric protein composed of a F-box protein (TrCP) fused to the LFG peptide and demonstrate that endogenous cdk2 and its binding substrate, cyclin A, can be tethered to β-TrCP, ubiquitinated, and degraded. Degradation of cdk2 and cyclin A together, but not cdk2 alone, results in massive tumor cell apoptosis *in vitro* and *in vivo* in a proteasome-dependent manner.

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Note: W. Chen and J. Lee contributed equally to the work.

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Western Blots and Immunoprecipitation. For Western blots, protein extracts of cells were prepared in lysis buffer [20 mM HEPES (pH 7.4), 50 mM β-glycerol phosphate, 2 mM EGTA, 1 mM DTT, 10 mM NaF, 1 mM sodium orthovanadate, 1% Triton X-100, and 10% glycerol with proteinase and phosphatase inhibition proteins]. The protein concentration of the cell lysates was measured by the Bradford assay (Bio-Rad, Hercules, CA), and 20–30 μg of protein/sample (70 μg for p73) were loaded onto SDS/PAGE gels or NuPAGE gels (Invitrogen). Proteins were transferred to polyvinylidene difluoride membranes and immunoblotted with specific antibodies. For immunoprecipitation, cells were lysed using immunoprecipitation buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA (pH 8.0), 0.2 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% NP40]. Anti-Flag or cdk2 antibodies were added to 500-μg lysates and incubated at 4°C for 1 h. Protein A- or G-agarose beads were added to the antigen-antibody mixture and additionally incubated for 30 min at 4°C. Beads were washed twice with immunoprecipitation buffer and subjected to Western blot analysis using anti-cyclin A, cdk2, and Flag antibodies as described above.

Transient Transfection in Hepa Cells. Various plasmids were introduced into Hepa cells using Lipofectamine Plus reagent following manufacturer’s instructions (Invitrogen). Forty-eight h after transfection, cells were harvested, and protein lysates were resolved via gel-electrophoresis and blotted onto polyvinylidene difluoride membrane. The expression of Flag-tagged TrCP-conjugated protein was confirmed by immunoblotting using the anti-Flag monoclonal antibody.

Small Interfering (si)RNA Transfection. The siRNA sequences used for targeted silencing of cdk2 (5′-aagctctgattcctttgatt-3′), cyclin A (5′-caacagtctgccctttgatt-3′), and p27 (5′-aagctctgattcctttgatt-3′) were chosen as described by Elbashir et al. (60) and recommended by the siRNA supplier (Xeragon, Germantown, MD). Human genome database (BLAST) searches were carried out to ensure that the sequences would not target other gene transcripts. siRNAs were introduced to the cells using Oligofectamine according to the protocol of Elbashir et al. (60) and manufacturer’s instructions (Invitrogen). Briefly, two siRNA transfections were sequentially performed at 24 and 48 h after cells were plated. Four h after the second transfection, cells were infected with the recombinant adenovirus, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Roche, Germany) were carried out 96 h after adenovirus treatment. Two days after treatment, cells were harvested for Western blotting analysis.

Apoptosis and Cell Cycle Analysis. Cells (3 × 106/well) were seeded in 6-well plates and infected 16–18 h later with adenoviruses at a multiplicity of infection of 200. Forty-eight h after infection, both attached cells and floating cells were harvested, washed in PBS, and fixed with 70% ethanol. Apoptotic cells were detected by using ApopTag peroxidase in situ apoptosis detection kit (Intergen, Norcross, GA). Cell cycle analysis was prepared by first fixing cells and treating with RNase A, followed by staining with 5 μg/ml propidium iodide in PBS. Cell cycle analysis was performed on a FACS brand flow cytometer (Becton Dickinson, San Jose, CA); the data were analyzed by MULTI-FIT software.

Determination of Cell Growth Rate. Cell growth was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Roche). U87 cells were plated on 96-well plates at a density of 10,000 cells/well; all other cells were plated at a density of 5000 cells/well. One day after plating, cells were treated with adenovirus at a multiplicity of infection of 200–500 or transfected with siRNA. Cell viability was determined by coimmunoprecipitation in that anti-Flag antibodies precipitated cyclin A and TrCP-LFG (Fig. 1B), Flag antibodies were used for immunoprecipitation. Coimmunoprecipitation with anti-cdk2 antibody also showed cyclin A and TrCP-LFG (Fig. 1A). The faster migration with anti-cdk2 antibody also showed cyclin A and TrCP-LFG (Fig. 1A).

Animal Studies. For the s.c. tumor model, U87 cells (1 × 105) were s.c. injected into the right flank of 4-week-old male nude mice (National Cancer Institute-Frederick Cancer Research & Development Center, Frederick, MD). Once tumor size had reached 200 mm3, the mice were treated with adenovirus expressing either green fluorescent protein (GFP) or TrCP-LFG or PBS. The mice were injected with 100 μl of AdCMV-GFP (109 pfu) or AdTrCP-LFG (109 pfu) every 3–4 days for a total of six treatments. Tumor size was measured every 3–4 days, and tumor volume was calculated from the formula (length × width2), where length and width are perpendicular. The student t test was used to assess statistical significance. Ps of <0.05 were considered significant.

RESULTS

The Engineered β-TrCP-LFG Targets Cdk2 for Degradation. To explore the effects of inducing proteasome-mediated degradation of cdk2, we engineered chimeric proteins with the full-length flag-tagged β-TrCP (TrCP) protein fused at the protein-protein interaction domain to the cdk2 binding 8 amino acid peptide LFG (TrCP-LFG; Fig. 1A). As shown in Fig. 1B, expression of TrCP-LFG in Hepa cells resulted in a decrease in the endogenous level of cyclin A and cdk2. The expression of TrCP or F-box-truncated β-TrCP fused to LFG (ΔTrCP-LFG) did not reduce cyclin A protein levels nor cdk2 protein levels. The faster migrating phosphorylated (Thr160) active form of cdk2 (lower band; Refs. 48, 49) shown by coimmunoprecipitation in that anti-Flag antibodies precipitated and coprecipitated cyclin A and cdk2 (Fig. 1D). The faster migrating protein bands were coimmunoprecipitated with anti-cdk2 antibody also showed cyclin A and TrCP-LFG (Fig. 1A).

Cyclins and Tumor Cell Growth in Vivo and in Vitro
**Inhibition of Tumor Cell Growth and Induction of Cell Death by Adenovirus-Mediated Transduction of TrCP-LFG.** Recombinant adenoviral vectors are a commonly used delivery system for cancer gene therapy. We constructed a replication defective recombinant adenovirus that expresses TrCP-LFG (Ad/TrCP-LFG). Western blot analysis was performed to examine the effect of Ad/TrCP-LFG on cyclin A and cdk2 protein level in different cell lines. In all tested cell lines, both cyclin A and cdk2 protein levels were significantly reduced following Ad/TrCP-LFG transduction (Fig. 2A). Consistent with the effects on protein levels, transduction by Ad/TrCP-LFG resulted in significantly reduced intracellular cdk2 activity (Fig. 2B). Ad/TrCP-LFG transduction of various cell lines in vitro mediated a significant cytotoxic effect in most tumor cell lines examined, whereas there were minimal effects on the survival and growth of normal human fibroblasts and astrocytes (Fig. 2, B and C). Control adenoviral vectors expressing TrCP or ΔTrCP-LFG caused no significant change in cdk2 protein level nor cytotoxicity in any of the cell lines (Fig. 2D). These data suggest a differential cytotoxic response between cancer and normal cells to cyclin A/cdk2 down-regulation.

**Induction of Apoptosis in Tumor Cells Transduced with Adenovirus-Expressing TrCP-LFG.** We performed experiments to elucidate the mechanisms responsible for Ad/TrCP-LFG-mediated death in tumor cells. Tumor cells treated with Ad/TrCP-LFG exhibited membrane blebbing and nuclear condensation (data not shown), suggestive of apoptotic cell death. To confirm that apoptosis had occurred in Ad/TrCP-LFG-transduced cells, we performed terminal deoxynucleotidyl transferase dUTP nick end-labeling assay. Two days after Ad/TrCP-LFG transduction, U2OS, SAOS, and U87 tumor cells all demonstrated significant terminal deoxynucleotidyl transferase dUTP nick end-labeling staining, whereas there were little or no terminal deoxynucleotidyl transferase dUTP nick end-labeling positive normal cells (Fig. 3A). We quantitated the degree of apoptosis induced by adenoviral-mediated transduction of TrCP-LFG by flow cytometric analysis (fluorescence-activated cell sorting). The percentage of apoptotic cells (sub-G1 fraction) increased steadily over the first 96 h after Ad/TrCP-LFG transduction in all tumor cell lines, reaching a high of 40–90% in all transduced cells (Fig. 3B). By contrast, Ad/TrCP-LFG transduction caused essentially no apoptosis in normal human fibroblasts or astrocytes (Fig. 3B and data not shown).

**Induction of p73 Is Required for Ad/TrCP-LFG-Induced Cell Death in Rb-Negative Cell Lines.** Cdk2 kinase activity has been shown to be important for phosphorylating and inactivating E2F activity. High E2F activity can induce apoptosis by a p73-dependent mechanism. Thus, one might predict that inactivation of the Cdk2 complex would lead to unopposed E2F activity resulting in apoptosis. To test this hypothesis, we evaluated the levels of p73 in each of our cell lines (Fig. 4A). Basal expression of p73 was much higher in U2OS cells compared with the other cell lines. Ad/TrCP-LFG treatment induced expression of p73 in SAOS cells and, to a much lesser extent, in U87 cells but failed to induce expression in other cell lines, including U2OS (Fig. 4A, top panel). The increased expression level of p73 in the Rb-negative cell line SAOS and Rb-disregulated U87 in response to the treatment of Ad/TrCP-LFG suggested that the induction of p73 might contribute to Ad/TrCP-LFG-mediated cell death. To test this hypothesis, we constructed a recombinant adenoviral vectors expressing a dominant-negative p73 construct (Ad/p73dd). Preinfecting SAOS cells with Ad/p73dd partially protected cells from apoptotic death induced by Ad/TrCP-LFG (from 42.6% cytotoxicity to 11% cytotoxicity; Fig. 4B). Ad/p73dd also partially protected U87 cells (Fig. 4B, from 82.3% cytotoxicity to 37.5% cytotoxicity; P < 0.01). Interestingly, Ad/p73dd also protected against Ad/TrCP-LFG-induced cell death in another Rb-negative cell line, H596 (Fig. 4B, from 62.35% cytotoxicity to 39.3% cytotoxicity; P < 0.01), despite the fact that no p73 induction was noted in these cells after infection by Ad/TrCP-LFG. By contrast, Ad/p73dd offered no protection against Ad/TrCP-LFG-induced cell death in the two Rb-positive cell lines we tested (U2OS and A549; Fig. 4B). These data suggest that p73 may play a preferential role in Ad/TrCP-LFG-induced apoptotic cell death in Rb-negative cell lines. To explore other potential mechanisms involved in TrCP-LFG-mediated cell death, we examined the changes of protein expression of the cdk inhibitors p27 and p21 in response to Ad/TrCP-LFG treatment. Induction of p27 was evident in all cell lines 48 h after transduction by Ad/TrCP-LFG (Fig. 4A). By contrast, p21 levels were either not or only minimally increased after...
Fig. 2. Effects of TrCP-LFG on cell survival. 

A, reduction of cyclin A and cyclin-dependent kinase 2 (cdk2) expression levels in cells treated with Ad/TrCP-LFG. Cells were transduced with Ad/TrCP-LFG or Ad/GFP (control) vectors (multiplicity of infection (m.o.i.) of 50). Cell lysates were generated 48 h after adenoviral transduction. Cyclin A and cdk2 expression was detected by Western blot using anti-cyclin A or cdk2 antibody. β-Actin was shown to ensure equal loading.

B, comparison of the effects of Ad/TrCP-LFG on U87 versus two colon cancer cell lines (HCT116 and SW480). Top panel: U87, HCT116, and SW480 cells were infected with Ad/TrCP-LFG or Ad/GFP. Similar viral transduction efficiency was observed in all cell lines (m.o.i. of 200). Cell survival was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay 96 h after viral treatment. The relative survival for the Ad/TrCP-LFG-treated cells is normalized by the control (Ad/GFP) vector-treated cells. Values shown are the mean ± SD from one representative experiment, n = 6. Bottom panel: analysis of cdk2 activity in cells treated with Ad/TrCP-LFG. Cells (indicated at the bottom of the figure) were treated with either control virus (Ad/GFP) or Ad/TrCP-LFG (m.o.i. of 50). Cell extracts were generated 48 h after viral infection and followed by immunoprecipitation with anti-cdk2 antibody. Kinase assay was performed using Rb-C fusion protein as substrate (see “Materials and Methods”).

C, the effect of Ad/TrCP-LFG on cell survival. Transformed or nontransformed cells were treated with Ad/GFP, Ad/TrCP, or Ad/TrCP-LFG adenoviral vectors (m.o.i. of 200), and cell killing was measured by the MTT assay at indicated times after vector transduction. Survival index was calculated for the Ad/TrCP-LFG treatment group by normalizing to survival in the control (Ad/GFP) adenoviral vector group. The data shown here is the representative of three independent experiments. Values shown are the mean ± SD, n = 6. P was determined using the student t test by comparing experimental group (Ad/TrCP-LFG) to control (Ad/GFP) group at the 96-h time point. Top panel: time course of growth inhibition of U2OS osteosarcoma; SAOS osteosarcoma and U87 glioma induced by Ad/TrCP-LFG (m.o.i. of 200). Bottom panel: time course of survival rate of Hs27 normal human fibroblasts and normal human astrocytes (NHA) treated with either the control or Ad/TrCP-LFG. The transduction efficiency observed in nontransformed (i.e., normal) cells was similar to that in the transformed cell lines. D, the effect of control viral constructs. Top panel: the effect of control viral constructs on cell growth. U2OS, U87, and SAOS cells were infected with various control viral constructs at m.o.i. of 200 (Ad/GFP, Ad/TrCP, and Ad/TrCP-LFG). Cell growth was measured by the MTT assay 96 h after viral infection. Similar results were observed in several independent experiments, n = 6. Bottom panel: the effect of control viral constructs on the expression of cdk2. U2OS was infected by indicated adenoviruses. Forty-eight h after viral infection, cell lysates were generated, and cdk2 expression level was examined by Western blot analysis using anti-cdk2 antibody. The data shown is the representative result from at least three independent experiments.
exposure to TrCP-LFG, except for a large increase seen in SAOS cells. Knockdown of p27 induction by p27 siRNA treatment did not protect the cell death induced by Ad/TrCP-LFG in the Rb-negative cell lines, whereas it afforded some protection from Ad/TrCP-LFG-induced cell death in Rb-positive cells (data not shown). Taken together, these data suggest that the molecular mechanisms responsible for TrCP-LFG-mediated cell death may be different in Rb-positive cells compared with Rb-negative cells.

Both Cyclin A and Cdk2 Degradation Are Necessary for Maximal Tumor Cell Apoptosis. A recent study (51) suggests that cdk2 is not required for tumor cell survival and growth and cdk2-knockout mice and cells do not show abnormality in cell cycle progression. Because TrCP-LFG mediates destruction of both cdk2 and cyclin A, we performed a series of experiments designed to individually evaluate the effects of isolated cdk2 and cyclin A down-regulation. siRNA-mediated down-regulation of cdk2 alone did not result in cytotoxicity in any of the tumor cell lines evaluated (Fig. 5). siRNA down-regulation of cyclin A did, however, have a small but statistically significant cytotoxic effect in U87 glioma cells and HCT116 colon carcinoma cells lines (Fig. 5B). By contrast, simultaneous down-regulation of cyclin A and cdk2 by siRNAs caused a greater effect on cell killing than seen with down-regulation of either protein alone (Fig. 5B). Of note, SW480 were totally resistant to TrCP-LFG-and siRNA-mediated cell killing despite the effective down-regulation of cyclin A and cdk2 protein levels (Fig. 5A) and diminished cdk2 kinase activity to levels seen in all of the other sensitive cell lines we examined (Fig. 2B). These data demonstrate that the cytotoxic effects of simultaneous cdk2/cyclin A down-regulation are more profound than down-regulation of either protein alone in all cell lines tested with one notable exception.

Suppression of Tumor Growth by Intratumoral Injection of Ad/TrCP-LFG. Given the pronounced and selective cytotoxic effects of Ad/TrCP-LFG on tumor cells but not normal cells in vitro, we evaluated the efficacy of Ad/TrCP-LFG administration on glioma tumor growth in vivo. Subcutaneous U87 gliomas were treated with intratumoral injections of Ad/TrCP-LFG or a control adenoviral vector starting day 3 after tumor implantation and then every 3–4 days for a total of six treatments. All tumors in the mice treated with Ad/TrCP-LFG showed significant suppression of growth compared with tumors in PBS-treated and Ad/GFP-treated mice (Fig. 6A). In a separate experiment, we euthanized a subgroup of animals 24 h after viral injection to examine the effects of Ad/TrCP-LFG on tumor cells in vivo. The transduction efficiency of the different adenoviral vectors was roughly equivalent as evidenced by the fact that the intensity and distribution of GFP expression was similar in both the control GFP-expressing and GFP/TrCP-LFG-expressing adenoviral vector-treated tumors. By contrast, there were significantly more terminal deoxynucleotidyl transferase dUTP nick end-labeled apoptotic nuclei in the Ad/TrCP-LFG-treated group compared with the control group (Fig. 6B).

We further assessed the potential of Ad/TrCP-LFG as a therapeutic strategy for human brain cancers using an orthotopic intracranial U87 glioma model. To evaluate the effects of the TrCP-LFG construct on normal cerebral tissue, Ad/TrCP-LFG or the control Ad/GFP virus was stereotactically injected into the right caudate of nontumor-bearing mice. All animals remained clinically well with no signs of neurological toxicity. The brains of the injected animals were examined at 1, 7, and 28 days after viral transduction. There was no histological evidence of neurotoxicity in any of the examined brains with only a minimal mononuclear cell infiltrate within the injected area, characteristic of the effect seen after adenoviral injection into the brain (data not shown).
no difference between the histological appearance of the brains injected with the either the Ad/GFP or Ad/TrCP-LFG virus. We next stereotactically inoculated U87 glioma cells into the right parietal region of the brain of nude mice after 3 days later by stereotactic administration of recombinant adenovirus expressing either GFP or TrCP-LFG. Vector administration was repeated three times at 3–4-day intervals, and animals were followed for survival. Kaplan-Meier survival curves for the control animals and those treated with Ad/TrCP-LFG are shown in Fig. 7A. The median survival of control vector treated animals was 34 days, whereas the median survival of the Ad/TrCP-LFG-treated animals was 50 days (P < 0.01). Log-rank analysis of Kaplan-Meier survival curves demonstrated a significant survival advantage for the Ad/TrCP-LFG treated animals (P < 0.01). More impressively, 50% of the animals receiving Ad/TrCP-LFG were ultimately cured of their tumors (>120-day survival) with no neurological or physical evidence of tumor nor toxicity. The long-term survivors were ultimately euthanized, and microscopic examination of their brains revealed no evidence of either residual tumor or normal tissue toxicity (Fig. 7B).

DISCUSSION

We demonstrate that a cyclin A/cdk2 inhibitor binding peptide fused to a recombinant TrCP protein can selectively direct cyclin A and its binding partner, cdk2, toward proteasome-mediated degradation resulting...
in tumor cell apoptosis \textit{in vitro} and \textit{in vivo}. This strategy may represent a more efficient way of inhibiting the cyclin A/cdk2 pathway than the LFG peptide approach because, instead of only competitively inhibiting the kinase activity, the TrCP-LFG fusion protein targets the kinase complex for destruction.

Not only was the TrCP-LFG strategy highly efficient for cell killing, but the strategy also appears to be specific for targeting the cyclin A/cdk2 complex given that we did not see effects of TrCP-LFG on cyclin B or D protein levels or associated kinase activity (Figs. 1 and 2 and data not shown). Consistent with these observations is the fact that previous work has demonstrated that LFG and similar peptides can inhibit cyclin A-associated kinase activity but not cyclin B or D-associated kinase activity (28, 42, 48–50).

Furthermore, we did not see effects of the transected TrCP-LFG on endogenous substrates for the \(\beta\)-TrCP E3 ligase such as nuclear factor-\(\kappa\)B (data not shown). At first glance, the highly specific nature of the TrCP fusion peptide approach might appear surprising. Given the central role of proteasome-mediated proteolysis for processes as important and sensitive as cell cycle regulation and cell survival, however, E3 ligases are by necessity highly specific for their targeted binding proteins. Thus, it is not all that unexpected that a TrCP-peptide fusion approach might so specific for its targeted protein binding partner.

Much attention has recently focused on interfering RNAs as a revolutionary strategy for uniquely down-regulating-specific target mRNAs. Recent experience, however, has demonstrated siRNAs to be problematic...
relative to intracellular transduction inefficiencies, their unpredictable effectiveness depending on the specific sequence that is used, and the difficulties in efficiently delivering these molecules in vivo. Additionally, recent data has shown that siRNAs may not be as specific as previously thought resulting in interference in a number of off-target mRNAs (64). By contrast, an approach such as our TrCP-LFG strategy that targets the specific protein of interest, rather than an intermediate RNA species, is theoretically attractive. Thus, we believe our data supports the premise that the TrCP-fusion peptide approach represents a potentially powerful strategy for targeting specific proteins both in vitro and in vivo (57).

Given the abundance of data suggesting that cdk2 is a potentially promising cancer therapeutic target, we initially devised the TrCP-LFG strategy with the expectation that a cytotoxic affect would be a consequence of cdk2 down-regulation. McCormick et al. (51), however, have recently published data using principally colon cancer cell lines, questioning the need for cdk2 in tumor cell proliferation and survival. Barbacid et al. (53) also showed that cdk2 may not be necessary for normal cell cycle progression by using cdk2-knockout mice and Cre-loxP-mediated somatic cell knockout. Because cdk2 binds with several different protein partners, however, loss of cdk2 function may not be synonymous with targeted destruction of the cdk2/cyclin complex. Because TrCP-LFG down-regulates both cyclin A and cdk2, it was possible that the observed cytotoxic effects were a consequence of the destruction of either cdk2, cyclin A, or both. We, therefore, performed experiments using siRNAs to specifically target cdk2 and cyclin A mRNA and demonstrate that, indeed, down-regulation of cdk2 alone had no effects on cell viability, whereas cyclin A down-regulation did mediate a cytotoxic effect. Interestingly, however, it appeared that the combination of both cyclin A and cdk2 inhibition led to the most potent cytotoxic effect in sensitive cell lines. Although more in-depth studies will be required to fully understand these observations, it appears that the cyclin A or the cyclin A/cdk2 complex may be a preferable to cdk2 as a therapeutic tumor target.

The downstream mediators of cytotoxicity after TrCP-LFG-mediated down-regulation of cyclin A/cdk2 remain to be fully elucidated, although our data suggest that those mechanisms may differ depending on the pRb status of the cell. TrCP-LFG-treated tumor cells, which have deregulated Rb function, appear to undergo apoptosis in a p73-dependent manner. This is consistent with previous studies (41, 65, 66) demonstrating that unopposed E2F activity results in p73-dependent apoptosis. Because cyclin A/cdk2 phosphorylates E2F-1, thereby inhibiting its activity, TrCP-LFG-mediated down-regulation of cyclin A/cdk2 would be expected to result in even higher E2F activity in cells with baseline deregulated pRb activity compared with those that have intact pRb pathways. Our data does in fact demonstrate that Rb-deregulated cells generate high E2F transcriptional activity after TrCP-LFG-mediated cyclin A/cdk2 down-regulation and that the resultant apoptosis can be partially inhibited by a dominant-negative p73 mutant. By contrast, tumor cells with intact Rb are not rescued by the dominant-negative p73 after TrCP-LFG transduction. Although our siRNA experiments suggest that inhibiting the p27 induction that occurs after TrCP-LFG treatment in pRb intact tumor cells can reduce the amount of apoptotic cell death, these effects are modest and suggest another prominent mechanism for TrCP-LFG-mediated apoptosis in pRb intact cells. Whether this mechanism involves an E2F-mediated, p73-independent apoptotic pathway remains to be elucidated.

The efficiency with which TrCP-LFG-induced tumor cell death appeared to be cell type dependent, although all cell lines tested (n = 10) were sensitive except for SW480. One of the most impressive features of TrCP-LFG-mediated cell death was the apparent tumor cell selectivity. There was no significant toxicity to normal cell lines in vitro or normal brain tissue in vivo after transduction by TrCP-LFG-expressing vectors. The fact that cyclin A/cdk2 inhibition did not cause toxicity to mitotically quiescent normal cells in the brain is perhaps not surprising given the dominant effect of the cdk inhibitors on G1 cyclins, thereby maintaining pRb in a predominantly

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**Fig. 7.** In vivo antitumor effects of Ad/TrCP-LFG on intracranial tumors. A, effect of Ad/TrCP-LFG treatment on survival of mice bearing intracranial U87 glioma. Viral vector treatment (5 x 10⁹ pfu) was started 3 days after intracranial tumor implantation and then repeated for a total of 3 times at 3–4-day intervals. The Kaplan-Meier survival curve is shown for both the Ad/GFP-treated (n = 4) and Ad/TrCP-LFG-treated animals (n = 6). B, photomicrographs show either Ad/GFP (left panel)-treated or Ad/TrCP-LFG-treated (right panel) animal brain. Animal brain specimens were evaluated histologically by H&E staining.
hypophosphorylated state. The fact that cyclin A/cdk2 inhibition did not cause cytotoxicity in normal proliferating cells in vitro, however, is more surprising and may reflect the baseline altered state of E2F activity in tumor versus normal cells. Regardless of the mechanisms, the efficient cell killing and lack of normal tissue toxicity from TrCP-LFG suggests that cyclin A and/or the cyclin A/cdk2 complex may represent a promising molecular target with a high therapeutic index for many types of tumors. Future strategies aimed at targeting other intracellular proteins using the TrCP-peptide binding approach and strategies aimed at further inhibiting the cyclin A or the cyclin A/cdk2 complex appear warranted.

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