The Tyrosphosphin AG1024 Accelerates the Degradation of Phosphorylated Forms of Retinoblastoma Protein (pRb) and Restores pRb Tumor Suppressive Function in Melanoma Cells

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ABSTRACT

Constitutive cell surface receptor kinase signaling and persistent phosphorylation/inactivation of the retinoblastoma (pRb) family of proteins (pRb, p107 and p130, known as pocket proteins) have been implicated in conferring uncontrolled growth to melanoma cells. However, the signals linking receptor kinase activity to neutralization of pocket proteins have not yet been fully elucidated. We therefore used specific chemical inhibitors to examine pRb regulation in melanoma cells. The most efficient agent, AG1024, known as an inhibitor of insulin-like growth factor 1 receptor and insulin receptor, arrested melanoma cell growth in vitro at nanomolar concentrations within 24 h of application. AG1024 inhibited pRb mediated phosphorylation activity of insulin-like growth factor receptor and insulin receptor, arrested melanoma cell growth in vitro at nanomolar concentrations within 24 h of application. AG1024 inhibited the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway and restored pRb tumor suppressive function. The latter was observed by the reduction in the phosphorylated forms of pRb, p107 and p130, and the formation of growth suppressive DNA binding complexes consisting of pRb and E2F1 or E2F3. The loss of phosphorylated forms of pRb at early time points after AG1024 application was not associated with suppression of cyclin-dependent kinases 2 and 4 activity but rather with proapoptotic and proinflammatory degradation. Thus, inhibition of melanoma cell proliferation by AG1024 is mediated by inhibition of mitogen-activated protein kinase/extracellular signal-regulated kinase 2 signaling and activation of pRb by a mechanism involving protein degradation.

INTRODUCTION

A hallmark of melanoma cells is their ability to proliferate and resist apoptosis regardless of environmental cues that control normal melanocyte (1). Release from dependency on external growth factors is conferred to melanoma cells by aberrant expression of growth factor receptors that create and enhance autocrine and paracrine loops, respectively (2–9). Among critical receptors activated in melanomas are FGFR1, IGF-1R, and melanoma growth stimulatory activity/growth-regulated gene class II interleukin 8 receptor. Blocking the activity of each of these receptors individually impedes melanoma cell growth in vitro and in vivo (10–16). This observation suggests that the activities of multiple receptors act in synergy and converge on a shared pathway that can be targeted to restrain melanoma tumor growth. Indeed, continuous MAPK activity, a common pathway downstream of activated receptor kinases (17), has been reported to operate in melanoma cells (1, 18).

Activation of the MAPK kinase cascade leads to pRb inactivation by sustaining the levels of cyclins and consequently activating CDKs (reviewed in Refs. 19–21). One of the underlying mechanisms for autonomous growth in melanomas is sustained inactivation of pRb family of proteins (pRb, p107, and p130, collectively known as pocket proteins) by hyperphosphorylation and elevated free E2F transcriptional activity (22–26). CDK2 and CDK4/6 are constitutively active in melanoma cells driven in large part by the continuous high levels of cyclins (cyclin D1, cyclin E, and cyclin A) and the frequent suppression of the CDK inhibitors p16INK4a and p27KIP1 (22, 23, 26–28). In most cases, persistent cyclin levels are because of unscheduled gene expression. Gene amplifications were identified thus far only in the case of cyclin D1 in 10% of metastatic melanoma cases (29, 30) and in 44% acral melanoma (31), but high expression was observed even in the absence of gene amplification (26, 31). Although cyclin activation is likely mediated by the MAPK/Erk pathway, which is activated in response to elevated receptor kinases, the specific signals in melanoma cells that maintain constitutive levels of cyclins have yet to be identified.

We therefore set out to investigate the signal transduction pathway linking receptor kinases to G1-S cell cycle checkpoint control by using kinase inhibitors. Among the several agents tested, the tyrosphosphin AG1024 was the most efficient inhibitor of melanoma cell proliferation, rapidly inducing pRb dephosphorylation, and the formation of growth suppressive pRb-E2F complexes. Intriguingly, pRb activation occurred in the apparent absence of any reduction in CDK activity. Instead, the results suggest that AG1024 enhanced the degradation of ubiquitinated and possibly nonubiquitinated pRb. Although AG1024 was reported to be an inhibitor of IGF-1R and the IR in other cell types (32), these two receptors were not inhibited in melanoma cells, suggesting that this tyrosphosphin mediates its effect through inhibition of another tyrosine kinase. Taken together, this report demonstrates that growth suppression of melanoma cells by AG1024 is mediated by inhibition of Erk2 signaling and activation of pRb via a mechanism that involves protein degradation.

MATERIALS AND METHODS

Cell Culture and Proliferation Assay. Normal human melanocytes were dissociated from newborn foreskins and maintained in Ham’s F-10 medium (Life Technologies, Inc., Invitrogen Corporation, Grand Island, NY) supplemented with 10% fetal bovine serum (10% FBS), penicillin-streptomycin (100 units/ml and 100 µg/ml, respectively) and 1% L-glutamine (all from Gemini Bio-Products, Woodland, CA), termed basa medium, which was additionally enriched with several ingredients required for optimal proliferation. They included 12-0-tetradecanoyl phorbol-13-acetate (85 nM), IBMX (0.1 mM), Cholera toxin (2.5 µM), Na3VO4 (1 µM), and N6,2′-O-dibutyryladenosine 3′-5′-cyclic monophosphate (cAMP) (10 µM), fos-sensitive 

Received 8/30/02; accepted 1/16/03.

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1 This work was supported by fellowships from the Yale Cancer Center and the Institute for Cancer Research scholarship (to E. Z.).

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3 The abbreviations used are: FGFR1, fibroblast growth factor receptor 1; GFP, green fluorescent protein; CDK, cyclin-dependent kinase; CHAPS, 3-(3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate; EMSA, electrophoretic mobility shift assay; ET-1, endothelin 1; Erk, extracellular signal-regulated kinase/mitogen-activated protein kinase; FGF2, fibroblasts growth factor 2; GST, glutathione S-transferase; HDAC, histone deacetylase; HGF/SF, hepatocyte growth factor/scatter factor; IMX3, 3-isobutyl-1-methyl xanthine; IGF-1R, insulin-like growth factor-1 receptor; IR, insulin receptor; M/SCF, mast/stem cell factor; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; Mek, MAP/ERK kinase; MG-132, N-carboxybenzoyl-Leu-Leu-leucinal; pRb, retinoblastoma protein; pTyr, phosphotyrosine; TICVA, 12-O-tetradecanoyl phorbol-13-acetate; IBMX, Cholera toxin, Na3VO4, and N6,2′-O-dibutyryladenosine 3′-5′-cyclic monophosphate; PBS, phosphate-buffered saline.

1420

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(0.1 mm), all from Sigma-Aldrich Co. (St. Louis, MO), termed TICVA (33). All tests were done with primary cultures of melanocytes (first passage) pooled from three to six donors (~4 weeks in culture). In deproteinization/stimulation experiments, the cells were deprived for 3 or 20 h in Opti-MEM (Life Technologies, Inc.) plus IBMX, followed by stimulation with FGF2 (10 ng/ml), HGF/SF (40 ng/ml), M/SCF (100 ng/ml), ET-1 (1 × 10⁻⁸ M) or combination thereof, where indicated. The pigment cells were highly differentiated as judged by the production of melanin, expression of melanocyte specific proteins (tyrosinase, Tyrp1/gp75/TRP1, Dct/TRP2), and dendritic morphology.

The human primary melanoma cells WW165 (WW) were grown in basal medium supplemented with IBMX (0.1 mm). The metastatic melanomas 501, YUGEN8, YUSAC2, YUSIT1, YUBSM14, YUFIS15, YUDAM, and WM9 were maintained in basal medium (33, 34).

Cell proliferation was evaluated by the standard [³H]thymidine assay and by counting cells at different time points. For [³H]thymidine incorporation, melanoma cells seeded in 24-well plates were serum-starved overnight, treated with experimental media for 24 h, and then exposed to MEMs (Life Technologies, Inc.) containing 5 μCi/ml [³H]thymidine (New England Nuclear, Bedford, MA) for 1 h (24). Each data point is an average of triplicate wells ± SD. Data were normalized to [³H]thymidine incorporation in 50,000 cells/h, and parallel wells were used for cell number determination with the Coulter Counter. Alternatively, melanoma cells were seeded in 6-well plates in Opti-MEM with no serum, treated with or without 1 μM AG1024 and counted with the Coulter Counter over a period of 6 days.

**Inhibitors.** The tyrosphostin AG1024 was a gift from Dr. Alexander Levitzki (Department of Biological Chemistry, The Hebrew University of Jerusalem, Israel) or purchased from Calbiochem (San Diego, CA). SU5402, the FGR1 inhibitor, was from Sugen (San Francisco, CA), the Mek inhibitor PD98059 was from Biomol Research Labs, Inc. (Plymouth Meeting, PA), the Src inhibitor PP2, the p38 inhibitor SB203580, and the proteosome inhibitor MG-132 were from Calbiochem, leupeptin pepstatin A, thiol, and acid protease inhibitors, respectively (neither affect the proteasome) were from Sigma (St. Louis, MO), leupeptin from Pierce (Rockford, IL), and phosphatase inhibitors (100 mM NaF, 10 mM Na4P2O7, and 1 mM Na3VO4). After 10 min on ice, the particulate fraction was spun down in a refrigerated microcentrifuge for 10 min at 12,000 rpm, and the supernatant was used for analyses.

FGF2 levels were measured by an ELISA assay (Quantikine HS FGF basic Immunoassay kit, R&D Systems, Minneapolis, MN) following the manufacturer instructions.

Total cell extracts (20 or 35 μg proteins/lane), measured by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) for fractionation/stimulation experiments or deproteinization of 6, 8, or 10% precast polyacrylamide Tris-Glycine or 10% precast polyacrylamide NuPAGE Bis-Tris precast gels (Novex, San Diego, CA) and Western blotted according to standard protocols (35).

For reciprocal immunoprecipitation, cell extracts (300–350 μg protein/assay) were incubated with the indicated antibody on ice for 6 h or overnight, and then antibody/antigen complexes were captured on bead-bound protein G (Amersham Pharmacia Biotech AB, Uppsala, Sweden) or protein A (Bio-Rad Laboratories) for 1 h at 4°C under constant rotation. Bead-bound immune complexes were eluted with 3× SDS-samples buffer, heated for 5 min at 100°C, and subjected to Western blotting.

Antibodies used for Western blotting and/or immunoprecipitation were anti-pRb mAb (IF8, sc-102), goat polyclonal (M-15, sc-1538), or rabbit polyclonal (C-15, sc-050), anti-ubiquitin mAb (P4D1, sc-8017), anti-IRß (sc-711), anti-IGF-1Rß (sc-713), anti-p70 S6 (sc-317), anti-p107 (sc-318), anti-cyclin D1 (sc-753), and HA-probe (Y-11) G (sc-805-G; all from Santa Cruz Biotechnology, Santa Cruz, CA). We also used rabbit polyclonal antibodies against phospho-pRb (pSer68, pSer780, pSer795, or pSer807/811), phospho-Erk2 (pThr202/Tyr204, 9101), and phospho-Mek1/2 (pSer217/221; all from Cell Signaling Technology, Beverly, MA), p16INK4a (PharMingen, San Diego, CA), p27KIP1 (Transduction Laboratories, Lexington, KY), actin (Sigma Immunochemicals), anti-pTyr mAb (4G10; Upstate Biotechnology, Lake Placid, NY), and control goat serum (Life Technologies, Inc.). Erk2 was identified with rabbit polyclonal antibody 691 (36). The antibodies were used at 1 μg/ml for Western blotting and 2 μg/precipitation or at the dilution recommended by the manufacturer.

**Immune Complex Kinase Assay.** Immune complex kinase assays were performed with slight modifications as described (37). Cells were collected by scraping, washed in cold PBS supplemented with 1 mM Na3VO4, lysed in 2% CHAPS buffer (supplemented with the protease and the phosphatase inhibitors as above), and slightly sonicated. After centrifugation (as above), aliquots of the supernatants (200–500 μg/assay) were incubated with goat polyclonal antibodies against CDK2 (M2-G, sc-163), or CDK4-G (H-22-G, sc-601; both from Santa Cruz Biotechnology), using goat IgG as a control, for 2 h on ice. Antibody/antigen complexes were captured on a 40-μl slurry of protein G-Sepharose for 30 min in the cold.

The bead-bound immune complexes were washed successively three times with lysis buffer and once with kinase buffer (30 mM HEPES (pH 7.5), 10 mM MgCl2, 1.0 mM DTT, and 5 mM benzamidine) and then incubated with 15 μl of kinase buffer supplemented with 5 μM ATP and 5 μCi of [γ-³²P]ATP (3000 Ci/mmol; Perkin-Elmer Life Sciences, Boston, MA) plus 2.5 μg of histone (the chromatography) or 2.5 μg of pRb-GST (773–928) at 30°C or 37°C for 30 min. The reactions were terminated with 10 μl of 3X SDS sample buffer and 5 μl heating at 95°C, the supernatants were fractionated by SDS-PAGE electrophoresis, and dried gels were autoradiographed. Protein bands were excised from the gels, and radioactivity was measured in a scintillation counter. In all cases, Western blotting analysis also confirmed the presence of CDK in immune complexes.

**EMSAs.** Melanoma cells were seeded in 150-cm² Petri dishes at ~80% confluence and were exposed the following day to the experimental medium for the indicated duration. DNA binding assays were performed with 6 μg of nuclear proteins, prepared as previously described (38), and end-labeled double-stranded DNA fragment (1.5–2 × 10⁵ cpm/assay) containing a single E2F consensus binding site derived from the dihydrofolate reductase promoter (sc-2507, Santa Cruz Biotechnology; Ref. 24), termed E2FRE. For competition studies, the DNA binding assays included also 10 ng of unlabeled E2FRE or mutant E2FRE double-stranded oligonucleotide with a CG to AT substitution at the E2F binding motif (E2FREmut; sc-2508). To identify the proteins in complex with the E2F consensus site, extracts were preincubated with rabbit polyclonal antibodies (0.5–2 μg each) to E2F1 (sc-193 X), E2F2 (sc-633 X), E2F3 (sc-878 X or sc-879 X), E2F4 (sc-866 X), E2F5 (sc-1083 X or sc-999 X), E2F6 (sc-8175), pRb (sc-050 X), p107 (sc-318 X), and p130 (sc-317 X), all from Santa Cruz Biotechnology. Reaction mixtures were loaded onto precast 6% polyacrylamide DNA retardation gels (Novex), and dried gels were exposed to Reflection film (Kodak BioMax MR, Eastman Kodak Co., Rochester, NY) at room temperature for 1–2 days.

**Metabolic Labeling.** Pulse-chase experiments were performed as described previously (38). Briefly, normal human or malignant melanocytes were pulse-labeled for 3 h with [³H]Met/Cys (0.2 μCi/ml; Easy Tag Express, Perkin-Elmer Life Sciences, Boston, MA) in methionine/cysteine-free RPMI medium (Life Technologies, Inc.) and either collected immediately or after chase incubation with nonradioactive basal (Ham’s F-12) or TICVA-supplemented medium (for melanoma and normal melanocytes, respectively) for an increasing period of time. When indicated, the medium was supplemented with AG1024 (1 μM), MG-132 (50 μM) plus leupeptin (10 μg/ml) plus pepstatin A (25 μg/ml), or all four inhibitors for 1 h as indicated. Protease inhibitors were added 10 min before AG1024. Cell lysates with equal amounts of radioactivity incorporated in proteins (20 or 50 × 10⁶ cpm/assay in trichloroacetic acid precipitated material determined on 1 μl aliquots) were subjected to immunoprecipitation with anti-pRb mAb IF8. After extensive washing with rapid
immunoprecipitation assay buffer, eluted proteins were fractionated in SDS-PAGE and dried gels were analyzed by autoradiography.

**Plasmids and Transfection.** HA-tagged mouse wild-type and two pRb variants lacking 8 and 11 Ser/Thr CDK consensus phosphorylation sites (Table 1) cloned in pcDNA3, termed RbΔBB/X wt, RbΔp34, and RbΔK11, respectively (40–43), were transiently expressed in melanoma cells (501 mel). Mouse and human pRb are 92% identical, and 15 of 16 CDK consensus sites are preserved (Table 1). The purified pRb encoding plasmids were introduced into the melanoma cells using FuGene 6 transfection reagent following the manufacturer’s instructions (Roche Diagnostic Corporation, Indianapolis, IN). A plasmid encoding GFP was used as a negative control. Expression cells were harvested 2 days after transfection, and cell lysates (700 μg protein/assay) were subjected to immunoprecipitation with goat anti-HA antibodies (HA-Probe), followed by successive Western blotting, first with anti-ubiquitin mAb and then with anti-pRb rabbit polyclonal antibody.

**RESULTS**

**Growth Inhibition by AG1024.** We chose to test the relative efficiency of inhibitors directed against receptor tyrosine kinases known to exert a growth advantage to melanoma cells, such as FGFR1, IGF-1R and Src (10, 44, 45). Previous studies demonstrated that aberrant expression of FGF2 (also known as basic FGF) in melanomas generates an autocrine loop because ligand and cognate receptor are expressed (46, 47). The growth promoting effect of activated FGFR1 in melanomas was validated by the suppressive effect of ectopically expressed dominant negative FGFR1 in vitro and in vivo, an effect that was mediated through inactivation of a Src-like kinase (10, 48). Likewise, IGF-1R is overexpressed in melanoma cells (49), and IGF-1R antisense inhibited melanoma tumor growth in nude mice (50). In our panel, FGFL was aberrantly expressed in six of seven melanoma cell strains (Fig. 1A). IGF-1R was overexpressed to various degrees in all of the seven melanoma cell strains tested, and the IR was overexpressed in four of these melanoma cell strains, compared with normal melanocytes (Fig. 1B).

The FGFR1 inhibitor SU5402 and the Src-inhibitor PP2 caused a modest (~60%) reduction in the rate of DNA synthesis at the high 20–50 mM range after 24 h incubation (Fig. 2A and B, respectively). In contrast, the tyrphostin AG1024, a specific inhibitor of IGF-1R and IR (32), was highly efficient at low concentrations (Fig. 2C). Nucleolar concentrations were sufficient to reduce [3H]thymidine incorporation to almost nondetectable levels when supplemented in Ham’s F-10 medium without serum (IC50, the concentration that caused a 50% inhibition, <50 mM; Fig. 2C). However, higher doses of AG1024 were required to produce similar levels of inhibition when supplied in Opti-MEM (containing insulin and transferrin and possibly other undisclosed ingredients; data not shown) or in the presence of serum (Fig. 2D). Sequestration of AG1024 by albumin was the likely cause for reduced efficiency, because the addition of BSA to Opti-MEM abolished inhibition (Fig. 2E). Cell counting confirmed that AG1024 inhibited the proliferation of several melanoma cell lines (Fig. 2F).

AG1024 Suppressed the Level of Several Tyrosyl-phosphorylated Proteins, including Erk2. Suppression of receptor tyrosine kinase activity by AG1024 was further confirmed by its effect on tyrosyl phosphorylated proteins. Anti-pTyr Western blotting showed reduction in the phosphorylation level of proteins of Mr 180,000, Mr 150,000, Mr 130,000, Mr 90,000, and Mr 44,000 in response to AG1024 (Fig. 3A). The Mr 44,000 AG1024-sensitive phosphoprotein was identified as Erk2 by virtue of its comigration with the upper protein band of the Erk2 doublet (data not shown) and reactivity with antiphospho-Erk2 antibodies recognizing the activated form of Erk2 (Fig. 3B). Treatment of melanoma cells with AG1024 reduced Erk2 phosphorylation levels in total cell lysates as well as in nuclear fractions within 30 min of application (Fig. 3B), suggesting that AG1024 suppressed the constitutive signal transduction that leads to activation of Erk2 in melanoma cells.

The other targets of AG1024 (Fig. 3A, indicated by arrows) are phosphoproteins not yet identified. Immunoprecipitation/immuno-blots experiments ruled out that the Mr 180,000 and the Mr 150,000 proteins were the oncoprotein/receptor tyrosine kinases Neu/ErbB2 and KIT, respectively. The Neu/ErbB2 was expressed, at equally low levels in normal melanocytes and three melanoma cell lines tested, but it was not tyrosyl phosphorylated, indicating that Neu/ErbB2 was not constitutively active in these cells (data not shown). The Mr 180,000 protein is also not likely to be the platelet-derived growth factor

### Table 1 Summary of pRb phosphorylation site mutants

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Fig. 2. Suppression of melanoma cell proliferation in response to kinase inhibitors. Melanoma cells were preincubated in serum-free Ham’s F-10 medium for 24 h and then subjected to proliferation assays in either serum-free Ham’s F-10 medium (A–C) or Opti-MEM (D–F). The rate of DNA synthesis was assessed by 1 h [3H]thymidine (3HdT) incorporation at the end of 24 h of treatment. Data are expressed as percentage of control. A and B, growth responses of YUGEN8 (●, 100% = 2400 cpm) and YUSAC2 (○, 100% = 4000 cpm) cells to increasing doses of SUS402 and PP2, respectively. C, dose response of YUGEN8 (●, 100% = 2840 cpm), YUSAC2 (○, 100% = 21,000 cpm), and 501 mel (□, 100% = 18,170 cpm) cells to AG1024. D, serum-dependent suppression of AG1024 activity. Data represents the concentration of AG1024 required to inhibit 90% DNA synthesis of melanoma cell (501 mel) in the presence of increasing amounts of serum. E, neutralization of AG1024 activity by BSA. Growth response of melanoma cells (YUGEN8) incubated without (-) or with AG1024 (2 μM; +), in the absence (-) or presence (+) of 0.5% BSA (100% = 130,000 cpm). Data are means of triplicate wells. F, suppression of cell proliferation in response to AG1024. Melanoma cells (YUSAC2, YUST1, and 501 mel) were incubated without [●] or with [○] AG1024 (1 μM) for 3 days. Data are means of cell numbers from duplicate wells, represented as percentage of cells on day 0 (YUSAC2 2700 cells; YUST1 1800 cells; and 501 mel 700 cells). Bars indicate SD of the mean.

Fig. 3. AG1024 reduced the level of tyrosyl-phosphorylated proteins, including Erk2. A, anti-p-Tyr Western blot of whole cell lysate derived from metastatic melanoma cells (501 mel). Proteins affected by AG1024 are marked on the left side. B, Western blot of whole cell lysate (WCL) or nuclear extracts (nuclei) with anti-phospho-Erk2 (p-Erk2) and anti-Erk2 antibodies. Serum deprived melanoma cells (501 mel) were equilibrated with Opti-MEM, followed by incubation without (-) or with (+) AG1024 (4 μM) for 30 min. C, anti-p-Tyr Western blot of cell extracts derived from normal human melanocytes incubated in Opti-MEM medium containing insulin but no other growth factors (3 h; none) or were treated for 30 min with peptide growth factors FGF2, HGF/SF, or M/SCF in the absence (-) or presence (+) of AG1024 (4 μM). The position of activated receptor tyrosine kinases (RTKs such as FGFR1, Met, and Kit), Erk2, and additional tyrosine phosphorylated proteins are indicated. D, phospho-Erk2 (p-Erk2) Western blot of cell extracts derived from deprived normal human melanocytes (3 h) that have been treated without (-) or with (+) synergetic growth factors (FGF2, HGF/SF, and EGF) in the absence (-) or presence (+) of AG1024 (4 μM), PD98059 (PD, 30 μM) or SB203580 (SB, 1 μM) for 30 min. Protein loading is indicated by a spurious band (control). E, phospho-Mek (p-Mek) Western blot of cell extracts derived from normal human melanocytes (NM) or melanoma cells (501 mel) normalized to actin. Treatments with growth factors and AG1024 were as in D.

Kit, the receptors for HGF/SF and M/SCF, respectively, possessing similar migration properties in SDS-PAGE. The growth factor-induced Erk2 phosphorylation was suppressed by AG1024 to the same extent as by the Mek inhibitor PD98059, whereas the p38 inhibitor SB203580 had no effect (Fig. 3D). Furthermore, the phosphorylation of Mek, the Erk2 kinase, was also suppressed by AG1024 in either growth factor-stimulated normal melanocytes or 501 mel cells (Fig. 3E) in agreement with the expected inhibition of a target upstream of Mek, the IGF1-R/IR kinases.

Several experiments failed to show any direct effect of AG1024 on the IGF-1R and IR in melanoma cells. Although the IGF-1R and IR were active and phosphorylated in at least one melanoma cell strain, treatment with AG1024 at the growth inhibitory concentration (1 or 2 μM) did not affect these kinases (data not shown). AG1024 did not inhibit the in vitro IGF-1R kinase activity toward its natural substrate IRS-1, and autophosphorylation of IGF-1R or IR was not affected because the level of tyrosyl phosphorylation of the two receptors was not diminished after treatment with AG1024 (data not shown). The conclusion that AG1024 may target other kinase(s) was confirmed using mouse fibroblasts overexpressing IGF-1R or null for this receptor. AG1024 did not suppress the tyrosine phosphorylation levels of IGF-1R in IGF-1R-overexpressing fibroblasts, and it inhibited the growth of IGF-1R-negative fibroblasts (data not shown). Therefore, it

receptor because this receptor is not expressed by most melanomas (51, 52). Likewise, KIT was not constitutively phosphorylated in 501 mel cells (34), and the M, 150,000 AG1024-responsive protein was also present in melanoma cells not expressing KIT (data not shown).

Suppression of receptor-mediated signal transduction by AG1024 was also apparent in normal human melanocytes. Stimulation of these cells with insulin alone or with insulin and FGF2, HGF/SF, or M/SCF enhanced the levels of several phosphotyrosyl-containing proteins (Fig. 3C). In each case, AG1024 diminished the induced phosphorylation of the Mr 44,000/Erk2-protein band (Fig. 3, C and D). The reduction in Erk2 activation in response to AG1024 in the presence of insulin and an additional synergistic mitogen (FGF2, HGF/SF, or M/SCF) is expected because human melanocytes require costimulatory signals to sustain Erk2 activity (1). As indicated above, the AG1024-responsive M, 150,000 phosphoprotein band is not Met or
Rapid suppression of pRb phosphorylated forms by AG1024 was not limited to YUSAC2 and 501 mel melanoma cells but included normal human melanocytes and the melanoma cell strain YUSIT1 (Fig. 4C, lanes marked NM and SIT, compared with SAC and 501). The immunoblot shows that in each case, the most affected phosphorylation sites were Ser\textsuperscript{780}, Ser\textsuperscript{608}, and to a lesser extent Ser\textsuperscript{795}/Ser\textsuperscript{811} (Fig. 4C). Ser\textsuperscript{780} was not affected in any of the cell types tested (data not shown). The results presented in Fig. 4C also confirmed our previous observations that contrary to expected, melanoma cells display higher levels of phosphorylated and underphosphorylated pRb proteins compared with proliferating normal melanocytes (Fig. 4C, compare NM to melanoma cells in all panels; Ref. 26). Interestingly, the phosphorylated pRb forms in these cells appear as a smear spanning the top of the gel down to the main pRb protein band, a characteristic shared with polyubiquitinnated proteins (see below).

**Interaction of pRb with E2F.** To test whether the reduction in pRb phosphorylation species resulted in the accumulation of pRb/E2F complexes, we performed a gel-shift analysis (EMSA). As shown in Fig. 5, AG1024 enhanced the formation of slow migrating E2F/DNA complexes with concomitant suppression of the fast migrating forms (Fig. 5). The slow migrating DNA complexes consisted mostly of pRb/E2F1 or pRb/E2F3 because the addition of specific pRb, E2F1, and E2F3 antibodies disrupted these complexes, whereas antibodies to other E2F transcription factors and pocket proteins had no effect (Fig. 5, compare Lanes 15, 17, and 20 to Lanes 12 and 14). Excess unlabeled oligonucleotide encoding E2FRE but not E2FREmut competed out the radioactive probe, indicating that the binding activity was due to E2F (Fig. 5, compare Lane 2 to Lanes 1 and 3, and Lane 13 to Lanes 12 and 14). These results suggest that the AG1024-induced dephosphorylation of pocket proteins also restores the tumor suppressive function to pRb.

**AG1024 Did not Affect CDK Activity.** CDKs are the major kinases that directly phosphorylate the pRb family of proteins (53, 54). Because CDK2 and CDK4 are known to be constitutively active in melanoma cells (22) and the AG1024 affected site Ser\textsuperscript{608} is preferentially phosphorylated by cyclin D1/CDK4 and Cyclin A/CDK2 (53), whereas Ser\textsuperscript{780} is a substrate for cyclin D1/CDK4 complex (55), we performed immune complex kinase assays to assess the effect of AG1024 on this class of kinases. The results show that
CDK2 and CDK4 activities were not suppressed even after 4-h incubation with AG1024 (Fig. 6A). The slight increase in CDK activities at the 0.5-h time point was not observed when the experiment was repeated. Furthermore, the protein level of cyclin D1 was not altered (Fig. 6B), whereas the levels of three CDK inhibitors p16INK4a, p21CIP1, and p27KIP2 decreased within 2 h of AG1024 addition (Fig. 6C), consistent with the lack of CDK2 and CDK4 inhibition. The reduction in the levels of p16INK4a, p21CIP1, and p27KIP2 by AG1024 was in response to enhanced proteasomal degradation because MG-132, a proteasome-specific inhibitor (56), restored their normal levels (data not shown).

**AG1024-mediated pRB Degradation.** The data presented in Fig. 6 showing lack of CDK inhibition and increased degradation of CDK inhibitors prompted us to explore the possibility that pRB is also degraded in response to AG1024, leading to its apparent reduced levels. Indeed, the addition of MG-132 (50 μM), or to a lesser extent leupeptin plus pepstatin, restored Ser608 and Ser780 pRB phosphorylated forms, as well as total pRB to AG1024-treated melanoma cells (Fig. 7A, compare Lane 3 to Lanes 1, 4, and 6 in each panel). In contrast, long-term incubation of melanoma cells with MG-132 alone reduced the levels of Ser780 pRB phosphorylation (Fig. 7B), suggesting suppression of CDK activity because of stabilization of p21CIP1 (33). Taken together, these results are consistent with the notion that AG1024 down-regulated pRB levels by enhancing degradation.

Radioactive pulse-chase experiments were then performed to determine whether AG1024 accelerated the degradation of newly synthesized pRB or a mature form that was allowed to undergo full modification by CDKs. Because pRB is a relatively stable protein with a half-life of 10 h (57), we monitored pRB stability after short chase periods (1 and 2 h) to that after 18-h chase in nonradioactive medium (Fig. 8, A and B). The data show that only mature but not newly synthesized pRB was affected by brief exposure (1 h) to AG1024 (Fig. 8A compare Lanes 4 and 5 to Lanes 2 and 3 and Fig. 8B Lane 2 to 3). The addition of all three protease inhibitors, MG-132, leupeptin, and pepstatin, restored normal levels of pRB to AG1024-treated cells (Fig. 8B compare Lane 5 to Lanes 2 and 3). Taken together, the radioactive metabolic-labeling experiments confirmed that AG1024 accelerated the degradation of mature forms of pRB but did not affect the newly synthesized protein.

**pRB Is a Highly Labile Protein in Normal Melanocytes.** The overall increase in pRB levels in melanoma cells versus normal melanocytes and the apparent high molecular weight smear of pRB (Fig. 4C) prompted us to determine the stability of this tumor suppressor in normal and tumor cells. Normal human melanocytes and melanoma cells were metabolically radiolabeled for 3 h, then incubated for increasing periods of time in normal medium, and radioactively labeled precipitated material was analyzed by autoradiography as described above. Fig. 8C demonstrates rapid loss of pRB in normal human melanocytes with a half-life of about 4 h, reaching almost undetectable levels at the end of the 18-h chase. In contrast, equal levels of pRB were maintained in melanoma cells during the 18-h chase period. These results support the unexpected conclusion that pRB is stabilized in melanoma.

**pRB Is Ubiquitinated in Normal and Malignant Melanocytes.** The observations described above (Figs. 4 and 7) suggest that pRB might be covalently modified by ubiquitination, a required step for targeting proteins to degradation by the proteasome. This prediction was tested by immunoblotting pRB precipitates with anti-ubiquitin mAb (Ubi-1; Fig. 9A). The results clearly demonstrate that pRB is ubiquitinated in melanoma (501 mel) cells and normal human melanocytes. Furthermore, the levels of conjugated ubiquitin-pRB increased after treatment with MG-132 in the presence or absence of AG1024 (Fig. 9A, compare Ubi-1 Western blot ± MG-134). Ubiquitination was detected in total pRB, as well as in its phosphorylated forms as revealed by immunoprecipitation with goat anti-pRB or anti-Ser807/Ser811 antibodies and probing with anti-ubiquitin mAb (Fig. 9A, Lanes 2, 3, 5, 6, 9, and 10). The high molecular weight Ubi-1 reactive proteins were pRB specific because none or minute amounts were discernable in samples precipitated with control goat or rabbit serum (Fig. 9A, Lanes 7, 8, and 11 and data not shown).

Because ubiquitin was detected in total pRB precipitates as well as in phospho-pRB, we next asked whether pRB ubiquitination requires proteasomal degradation. The results clearly demonstrated that pRB is ubiquitinated in melanoma cells with the high molecular weight smear of pRB (Fig. 4C) and the apparent high molecular weight smear of pRB (Fig. 4C) prompted us to determine the stability of this tumor suppressor in normal and tumor cells. Normal human melanocytes and melanoma cells were metabolically radiolabeled for 3 h, then incubated for increasing periods of time in normal medium, and radioactively labeled precipitated material was analyzed by autoradiography as described above. Fig. 8C demonstrates rapid loss of pRB in normal human melanocytes with a half-life of about 4 h, reaching almost undetectable levels at the end of the 18-h chase. In contrast, equal levels of pRB were maintained in melanoma cells during the 18-h chase period. These results support the unexpected conclusion that pRB is stabilized in melanoma.
in phospho-pRb specific (Ser<sup>807</sup>/Ser<sup>811</sup>) antibodies precipitates, we went on to determine whether modification by CDK is required for ubiquitination. This question was addressed by probing the levels of ubiquitin in ectopically expressed wild-type mouse pRb (RbΔB/Xwt) and two mutants lacking 8 and 11 CDK phosphorylation sites (RbΔp34 and RbΔK11, Table 1). Fig. 9B shows that all three pRb constructs possessed conjugated ubiquitin that was specific to the ectopically expressed proteins precipitated by HA-probe because none was detected in cells transfected with a plasmid encoding GFP. These results show that the presence of these 11 phosphorylation sites, mutated in RbΔK11, are not required for ubiquitination but does not rule out a role for other sites.

**DISCUSSION**

In this study, we show that treatment of melanoma cells with AG1024 suppressed in vitro proliferation within 24 h at nanomolar concentrations. Within 1 h of application, AG1024 caused Erk2 inhibition, down-regulation of phosphorylated forms of pRb, p107 and p130, and the accumulation of pRb/E2F DNA binding activity. The identity of the tyrosine kinase affected by this tyrphostin is not clear. Although AG1024 is an inhibitor of the IGF-1R/IR kinase (32, 58, 59), we could not demonstrate an inhibition of this receptor system. However, the results are consistent with suppression of the MAPK cascade in melanoma cells under conditions that lead to growth arrest. How-ever, the results are consistent with suppression of the MAPK cascade upstream of Mek.

Our studies show a novel mode of kinase-mediated regulation of pRb and probably also the two other members in this family. The early decrease in pRb, p107, and p130 high molecular weight forms in response to AG1024 occurred before any detectable changes in CDK2 and CDK4 kinase activities. Rather, the activity of these two pRb kinases remained intact even 4 h after exposure to AG1024, supporting the notion that they were not responsible for the immediate effect of AG1024 on pocket proteins. Instead, AG1024 accelerated the degradation of pRb, as well as the CDK inhibitors p16<sup>ink4a</sup>, p21<sup>cip1</sup>, and p27<sup>kip1</sup>, suggesting that it released constraints on proteolytic degradation of multiple proteins. A reduction in CDK activity is likely to occur at a later time because of a decline in cyclins, the CDK activators, in response to E2F/pRb transcriptional suppression (60–62).

The reduction in pRb levels was because of released activity of proteosomal and nonproteosomal enzymes. The proteosomal degradation was confirmed by the presence of ubiquitinated pRb. Both normal and malignant melanocytes possess pRb with conjugated polyubiquitin. The levels of polyubiquitinated pRb increased after treatment with the proteosomal inhibitor MG-132, consistent with the notion that these forms of pRb are otherwise targeted to degradation by the proteasome and that AG1024 accelerated this process.

AG1024 was effective at reducing the abundance of phosphorylated and hypophosphorylated forms of pRb, as demonstrated by the use of pRb phospho-specific antibodies and antibodies to total protein. This effect is similar to that induced by growth factor deprivation in normal human melanocytes as the cells become quiescent (22). The observations that free forms of E2F (E2F1 and E2F3) are concomitantly suppressed and pRb bound forms accumulate in AG1024 treated melanoma cells and in deprived normal melanocytes support the idea that elimination of certain phosphorylated forms of pRb shifts the balance from growth promoting to growth suppressive pRb complexes.

We propose three models to accommodate these results. First, it is possible that degradation of partially phosphorylated pRb or ubiquitinated pRb may facilitate the entry of unphosphorylated pRb into

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**Fig. 8. pRb turnover in melanoma cells and normal melanocytes.** Cultures of melanoma cells (501 mel, in A–C) and normal melanocytes (normal, C) were incubated with radioactive medium (35S) for 3 h and were harvested immediately (lanes marked 0) or after a chase with regular medium for the duration of 1, 2, 4, 6, 10, or 18 h, as indicated above each lane. AG1024 (1 μM) and protease inhibitors (MG-132, leupeptin, and pepstatin) were added (+) during the last 1 h of incubation. Radioactive cell extracts were subjected to immunoprecipitation (IP) with anti-pRb (IP8) or control matched mAb (C), and captured proteins were visualized by autoradiography.

**Fig. 9. pRb is ubiquitinated in normal and malignant melanocytes.** A, serum-starved (2 days) 501 mel cells or growth factor stimulated (TICVA) normal human melanocytes (NM) were treated for 1 h with MG-132 (50 μM) or AG1024 (2 μM), as indicated. Cell extracts (350 μg of melanoma cells and 300 μg normal human melanocytes/precipitate) were subjected to precipitation (IP) with anti-pRb (M-15, goat polyclonal antibodies), anti-phospho-pRb Ser<sup>807</sup>/Ser<sup>811</sup> (rabbit polyclonal), or goat serum (control or C). Eluted immunoprecipitates were fractionated in 8% gels and Western blotted successively with anti-ubiquitin (Ubi-1) mAb (Lanes 1–11), followed by anti-pRb (IP8), as indicated on the right hand of each panel. Similar results were obtained with 1-day starved 501 mel cells. B, phosphorylated and hypophosphorylated forms of pRb are ubiquitinated in melanoma cells. Melanoma cells (501 mel) were transfected with plasmids encoding wild-type and phosphorylation-deficient HA-tagged pRb (RbΔB/X, RbΔp34, and RbΔK11, respectively) or GFP. Cells were harvested 2 days later, and lysates were subjected to immunoprecipitation (IP) with anti-HA (HA-probe) and immunoblotting (IB) first with anti-ubiquitin mAb (Ubi-1) and then with anti-pRb (rabbit polyclonal C-15). Solid arrow indicates pRb and empty arrowhead a nonspecific reactive protein (NS).
stable HDAC-pRb-E2F complexes. Thus, during cell cycle progression, pRb is phosphorylated at mid-G1 by cyclin D1-CDK4/6 on a cluster of seven phosphoacceptor sites in exon 23. This induces intramolecular interaction between the negatively charged-phosphorylated Ser/Thr and a group of positively charged lysine residues that flank the LxCxE binding domain in the pRb small pocket (63). As a result of this intramolecular interaction, proteins that bind the LxCxE binding domain such as HDAC are expelled and cannot silence transcription. However, pRb remains bound to E2F/DP on a promoter and represses the transcription domain of E2F. Subsequently, pRb is phosphorylated on Ser667 by cyclin E-CDK2, and this leads to complete loss of pRb-E2F interaction and transcriptional derepression. Degradation of pRb species, phosphorylated on the Ser/Thr sites in exon 23, may allow unphosphorylated pRb species to bind the promoter and recruit HDAC, hence silence transcription, without going through M phase. Similarly, although the effect of ubiquitination of pRb on its activity has not yet been determined, it is possible that this modification interferes with its function and the degradation of ubiquitinated-pRb, accelerated by AG1024, allows unconjugated pRb species to form functional complexes with E2F. In agreement with this model, we observed an increase in active pRb-E2F1-DNA complexes after AG1024 treatment, although the level of total pRb is reduced (Figs. 4 and 5).

Second, despite the lack of obvious reduction in CDK2 and CDK4 kinase activity. AG1024 might inhibit the kinases as well as induce degradation. As a result, pRb might become dephosphorylated, resulting in inhibition of cell proliferation. At the same time, other proteins are degraded by the proteasome, including the CDK inhibitors p27, p21, and p16 (as well as pRb), and this might counteract the reduced kinase activity, hence the failure of the in vitro assay to register any net affect on the kinases (Fig. 6). However, in this model, by the time the CDK inhibitors are degraded, cell proliferation is already irreversibly suppressed by active pRb. These effects may occur sequentially and might be difficult to resolve in nonsynchronized population in which individual cells may respond at different time points depending on whether they have passed he restriction point in late G1 at the time of treatment. According to this model, degradation of phosphorylated pRb is a byproduct of the effect of AG1024 on the proteasome; the apparent persistence of kinase activity merely reflects the conflicting effects on the kinases and their inhibitors.

Third, in addition to its negative effect on cell proliferation, pRb also functions as a survival factor as evident from the massive cell death observed in pRb-deficient mice in tissues where pRb is normally highly expressed (Ref. 64; reviewed in Ref. 65). Positive role in in vivo carcinogenesis is demonstrated by the induction of mammary glands carcinoma in mice transiently expressing the constitutively active pRb alleles RbΔp34 and pRbΔK11 (43). A positive role for pRb in malignant transformation is also implied in human colon cancer. The incidence of pRb-positive cells is increased during multistage colorectal carcinogenesis, and elimination of pRb by antisense technology inhibited growth of colorectal carcinoma HCT116 cells and induced apoptosis (66). Thus, the inhibition of DNA synthesis observed after AG1024 treatment might reflect induction of apoptosis attributable to degradation of pRb. This model is consistent with the elevated levels and stability of pRb in melanoma cells compared with normal melanocytes, the increased degradation of total pRb in response to AG1024, and the apparent persistent kinase activity after drug treatment. Additional analysis will be required to discern between these and other possible models.

Only a score of published reports implicate pRb ubiquitination and degradation in cancer cells. In human leukemic cells HL-60, pRb is a relatively stable protein with a half-life of 10 h (57). In human papillomavirus-containing cervical tumor cells, the human papilloma-virus oncoprotein E7 mediates pRb polyubiquitination and proteasomal degradation (67–70). In hepatocellular carcinoma, the highly expressed gankyrin binds pRb and enhances its degradation, presumably by targeting it to the S6 ATPase of the 26S proteasome (71). Finally, growth stimulation of a prostate cancer cell line with androgen was mediated by enhanced degradation of pRb (72, 73). However, all these cases, in contrast to our results, reported growth advantage by reduction in pRb. Only in one study, degradation of pocket protein p107 was observed in response to growth inhibition (72, 73).

Several proteins that function in the pathway of ubiquitination or deubiquitination were reported to exist in complex with pRb. For example, the deubiquitinating enzyme Unp physically associates with pRb, p107, and p130 in vivo and in vitro (74, 75). Unp cleaves the ubiquitin-proline bond in ubiquitin fusion proteins, thus removing the polyubiquitin chain from the protein and preventing degradation by the proteasome. In addition, MDM2, a protein with E3 ubiquitin-ligase activity, interacts physically and functionally with pRb (76). MDM2 binds specifically to the COOH-terminus of pRb (amino acids 792–928) and stimulates endogenous E2F activity (76). However, phosphorylation of pRb by cyclin E/CDK2 reduces its binding to MDM2, and MDM2 does not bind to the analogous p107 pocket protein (76). These observations may cast doubt on the participation of MDM2 in pRb ubiquitination in melanoma because in these cells the phospholylated forms of pRb are subject to ubiquitination and degradation, and the levels of phosphorylated forms of p107 and p130 are also reduced after AG1024 stimulation.

More recently, it was shown that the decrease in protein stability of the pocket protein p130 as growth arrested cells reenter the cell cycle was mediated by proteasomal degradation (77). The increased protein turnover was dependent on Cdk4/6-specific phosphorylation of p130 on Ser672. In vitro reconstitution assays and in vivo transfection experiments demonstrated that the activity of the ubiquitin ligase complex SCF{sup KIP1}-Cul1/Cdc53-F-box and the proteasome were necessary for p130 degradation (77). It is not clear whether the same complex is involved in pRb ubiquitination in melanoma cells. Our analysis with phosphorylation-resistant mutant pRb alleles suggests that both phosphorylated and underphosphorylated pRb species may be ubiquitinated (Fig. 9). Thus either RbAK11 ubiquitination is not regulated by phosphorylation as is the case with p130 or that the phosphorylation of other phosphoacceptor sites not protected in RbAK11 induce ubiquitination of pRb.

Other observations may shed light on the receptor-mediated signaling directed at pRb degradation. Down-regulation of p27{sup KIP1}, a protein also affected by AG1024, has been reported to involve ubiquitination mediated by the ubiquitin ligase SCF{sup Kip1}- complex (Refs. 78, 79; reviewed in Refs. 80, 81). More recently, Grb2 was shown to serve as the link between receptor tyrosine kinase signal transduction and p27{sup KIP1} degradation (82). Grb2, an SH2/SH3 adaptor protein that mediates receptor activation to Ras signaling, accelerated Jab1/CSN5-mediated degradation of p27{sup KIP1}, whereas its alternatively spliced form Grb3-3 suppressed degradation. A p27{sup KIP1} mutant unable to bind Grb2 is refractory to subsequent degradation. It was suggested that Grb proteins mediate interaction between p27{sup KIP1} and an unknown factor that contains activity for ubiquitination or proteolysis.

Melanomas are known to acquire drug resistance, and development of new, mechanism-based therapies is currently being pursued by several investigators and pharmaceutical companies. As our results show, AG1024 is highly efficient at inhibiting melanoma cell growth in vitro. However, because AG1024 is inactivated by serum albumin, higher concentrations are needed for effective therapy in vivo. Recently, it was shown that AG1024 induced apoptosis and inhibited the proliferation of the breast cancer cell line MCF-7 (83). Moreover, AG1024 enhanced the sensitivity of MCF-7 cells to ionizing radiation,
suggesting that it has a potential synergistic effect in combination therapy. Derivatives of AG1024 that are refractory to albumin might prove potent inhibitors of melanoma and certain other types of cancer.

ACKNOWLEDGMENTS

We thank Dr. Alexander Levitzki (Department of Biological Chemistry, The Hebrew University of Jerusalem, Israel) for the generous gift of AG1024; Dr. Meenhard Herlyn for the WM49 melanoma cells; Dr. Paolootto (Harvard Medical School, Boston, MA) for the pGrk plasmid; and Donna LaCivita, in charge of the Cell Culture Core facility at the Yale School of Medicine Research Center for the normal human melanocytes.

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