

# Interleukin-6-Resistant Melanoma Cells Exhibit Reduced Activation of STAT3 and Lack of Inhibition of Cyclin E-Associated Kinase Activity

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Development of cytokine resistance is an important feature of melanoma cells during tumor progression. To study the mechanisms of interleukin-6 resistance, we examined an interleukin-6 sensitive (WM35) and an interleukin-6 unresponsive cell line (WM9). Interleukin-6 treatment resulted in rapid inhibition of cyclin-dependent kinase 2/cyclin E activity and accumulation of the hypophosphorylated retinoblastoma protein in WM35 but not in WM9 cells. In contrast to previous reports, no differences in the expression of the cyclin-dependent kinase 2 inhibitor p21<sup>Cip1/WAF1</sup> upon interleukin-6 treatment were found in both cell lines. Interleukin-6-induced inhibition of cyclin-dependent kinase 2 was also not due to changes in protein expression of cyclin-dependent kinase 2, cyclin E, p27<sup>Kip1</sup> and cdc25A, a phosphatase positively regulating cyclin-dependent kinase 2 activity. As it is established that interleukin-6 resistance of WM9 cells is not caused by differential interleukin-6 receptor expression, we studied whether this is due to defective interleukin-6 signaling in which acti-

vation of signal transducer and activator of transcription 3 is a critical step. WM9 cells showed reduced tyrosine phosphorylation, DNA binding, and delayed nuclear translocation of signal transducer and activator of transcription 3 as compared with WM35 cells. The kinase upstream of signal transducer and activator of transcription 3, Janus kinase 1, was constitutively tyrosine-phosphorylated in WM9 cells and did not respond to interleukin-6 with increased phosphorylation. As compared with WM35 cells, interleukin-6 treatment of WM9 cells was not paralleled by reduced activity of the mitogen-activated protein kinase kinase-1, which suppresses activation of signal transducer and activator of transcription 3. Our data suggest that resistance of advanced melanoma cells to interleukin-6 is associated with reduced inhibition of cyclin-dependent kinase 2, which appears to be a consequence of a complex alteration in interleukin-6 signal transduction. **Key words:** cell cycle/interleukin-6/melanoma/signal transduction/signal transducer and activator of transcription 3. *J Invest Dermatol* 117:132–140, 2001

**T**ransformation of normal melanocytes to aggressive metastatic melanoma cells is a multistep process defined by distinct stages *in situ* and *in vitro*. According to the tumor model proposed by Clark (1991a) and Elder *et al* (1993), melanomas develop from precursor lesions, i.e., benign nevi (congenital or common acquired), or from normal melanocytes to become so-called dysplastic lesions that are capable of malignant transformation. These premalignant lesions can progress into *in situ* melanomas with

melanocytic cells strictly confined to the epidermis, and subsequently to radial growth phase (RGP) melanomas, which have the same features as *in situ* melanomas except that individual tumor cells have invaded the dermis. Surgical excision of RGP melanomas is mostly curative (Clark, 1991b), indicating a reduced metastatic potential of the infiltrating cells. If left untreated, RGP melanomas become invasive by undergoing a vertical growth phase in which the tumor cells acquire the capability to metastasize.

In the last years distinct biologic features have also been elaborated *in vitro* between normal human melanocytes and melanoma cell lines derived from different stages of disease progression. Whereas normal human melanocytes *in vitro* stringently require the synergistic action of distinct growth factors such as basic fibroblast growth factor (bFGF), mast cell growth factor, hepatocyte growth factor, and endothelin-1, in order to proliferate (Böhm *et al*, 1995), melanoma cell lines established from later stages gradually and become increasingly independent of exogenous mitogens (Halaban, 1996).

In parallel to gradual independence of exogenous mitogens, melanoma cell lines derived from different stages progressively lose their sensitivity towards certain growth-inhibitory cytokines. This

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Reprint requests to: Dr. Markus Böhm, Ludwig Boltzmann Institute for Cell Biology and Immunobiology of the Skin, Department of Dermatology, University of Münster, Von Esmarch-Str. 56, D-48149 Münster, Germany. Email: bohmm@uni-muenster.de Abbreviations: bFGF, basic fibroblast growth factor; cdk, cyclin-dependent kinase; HH1, histone H1; IL, interleukin; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; RGP, radial growth phase; RB, retinoblastoma susceptibility gene product; STAT3, signal transducer and activator of transcription.

<sup>1</sup>This work is part of the PhD thesis of U. Schulte.

phenomenon was coined "multicytokine resistance" (Kerbel, 1992) and has been substantiated for a number of cytokines, including interleukin (IL) -1, IL-6, transforming growth factor- $\beta$ , and oncostatin M (Cornil *et al.*, 1991; Lu *et al.*, 1992, 1993; Lu and Kerbel, 1993; Rodeck *et al.*, 1994). The significance of growth inhibitors has also been demonstrated *in vivo*. For example, mouse melanocytes transduced by dominantly acting oncogenes such as *E1A*, *neu*, or *ras*, or those ectopically expressing cDNA for bFGF, are inhibited in their growth by keratinocytes after cografing (Dotto *et al.*, 1989). Fibroblasts or conditioned medium obtained from these cells contain a soluble factor capable of inhibiting the growth of melanoma cells, which was later identified as IL-6 (Cornil *et al.*, 1991; Lu *et al.*, 1992). IL-6 suppresses the growth of melanoma cells derived from early stages by  $G_1/G_0$  arrest, whereas a large proportion of melanoma cell lines derived from advanced stages are resistant (Lu *et al.*, 1992). Subsequent studies have revealed no differences in the expression of the IL-6R $\alpha$  subunit (gp80) and the signal converting molecule gp130 between IL-6 sensitive and resistant cell lines (Lu and Kerbel, 1993). These findings suggest disturbances within the IL-6 signal transduction and/or cell cycle machinery to be responsible for the stunted response of advanced melanoma cell lines towards IL-6.

In order to elucidate the molecular base for the IL-6 resistance of advanced stage-derived melanoma cells, we examined critical cell cycle regulators implicated in  $G_1$  arrest and the signal transduction of IL-6. We utilized two melanoma cell lines, WM35, derived from a RGP early melanoma and arrested by IL-6, and WM9, established from a late (metastatic) lesion and insensitive to IL-6 (Lu *et al.*, 1992). These cell lines have been previously shown not to differ with respect to IL-6R $\alpha$  and gp130 expression as well as their IL-6 binding affinity, thus representing a suitable model for our studies. Treatment of WM35 melanoma cells with IL-6 leads to inhibition of cyclin E-associated kinase activity and accumulation of hypophosphorylated retinoblastoma protein (RB), whereas in WM9 cells these parameters are only marginally affected. Examination of the upstream signaling molecules of these IL-6-mediated effects disclosed markedly reduced activation of signal transducer and activator of transcription 3 (STAT3) in WM9 melanoma cells. Our findings provide a link between the STAT3 signal transduction and the mechanism of  $G_1$  arrest mediated by IL-6 and give insight into the alterations of these pathways in human melanoma as a function of tumor progression.

## MATERIALS AND METHODS

**Cell culture** The human melanoma cell lines WM35 and WM9 were obtained from Dr. M. Herlyn (The Wistar Institute, Philadelphia, PA). WM35 is derived from an early stage primary tumor (RGP), whereas WM9 was established from a late (metastatic) stage lesion (Herlyn *et al.*, 1989; Herlyn, 1990). Cells were maintained in RPMI 1640 medium with all supplements as described previously (Böhm *et al.*, 1994).

**Fluorescence-activated cell sorter and DNA content analysis** For fluorescence-activated cell sorter analysis, cells were harvested by gentle scraping into phosphate-buffered saline with a rubber policeman, centrifuged and resuspended in 100  $\mu$ l of phosphate-buffered saline containing 1% bovine serum albumin. After incubation with 1  $\mu$ g per 100  $\mu$ l anti-human IL-6R antibody (UBI, Lake Placid, NY) for 1 h at 4°C, unbound antibody was washed off and cells were incubated with a fluorescein isothiocyanate-conjugated anti-rabbit antibody for 30 min. After washing and resuspension in phosphate-buffered saline, cells were analyzed in the presence of propidium iodide in an EPICS VL flow cytometer (Coulter, Miami, FL). For DNA analysis, cells were seeded into 100 mm dishes in regular culture medium and switched to RPMI 1640 containing 2% fetal bovine serum after adherence. Recombinant human IL-6 (30 ng per ml) was added and cells were harvested by trypsinization 36 h later. DNA content analysis was determined by flow cytometry as described previously (Herzinger *et al.*, 1995).

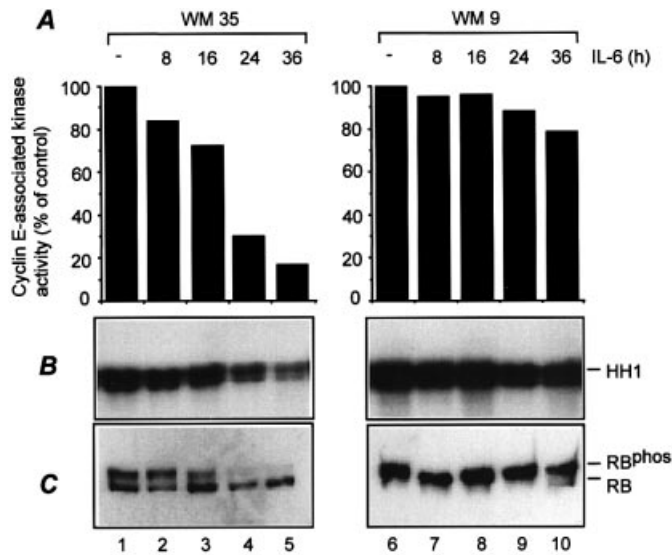
**Immunoprecipitation and immunoblot analysis** Melanoma cells seeded into 100 mm Petri dishes were kept in RPMI 1640 without fetal bovine serum for at least 24 h prior to stimulation with recombinant human IL-6 (30 ng per ml). Immunoprecipitation and immunoblot

procedures were the same as described before (Böhm *et al.*, 1995; Funk *et al.*, 1997). For Western immunoblotting of total cell extracts, identical amounts of lysate protein (usually 20  $\mu$ g per lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), or alternatively by the NuPAGE system from Novex (Invitrogen, Groningen, the Netherlands). For immunoprecipitation, identical amounts of lysate proteins (500  $\mu$ g) in lysis buffer were incubated with the indicated antibodies overnight at 4°C followed by the addition of protein A/G agarose. After 1 additional hour and extensive washing with lysis buffer, immune complexes were eluted with 35  $\mu$ l 2  $\times$  SDS sample buffer for 5 min at 95°C, followed by SDS-PAGE and western blotting. PVDF membranes were probed with the respective antibodies and signals were generated with the ECL detection system and horseradish peroxidase-coupled secondary antibodies (Amersham, Piscataway, NJ). The antibodies were: anti-phospho-Tyr705-STAT3 (New England Biolabs, Beverly, MA); anti-phospho-Tyr1022/1023-Janus kinase (JAK) 1 (Biosource International, Camarillo, CA); anti-cyclin E, anti-p27<sup>Kip1</sup>, anti-cdc25A and anti-STAT3 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-p21<sup>Cip1/WAF1</sup> and anti- $\alpha$ -tubulin (Calbiochem, San Diego, CA); anti-phosphotyrosine, anti-cyclin-dependent kinase (cdk) 2, anti-JAK2, anti-RB, anti-JAK1, and Tyk2 (PharMingen, San Diego, CA). In general, immunoblots were repeated at least twice. In some experiments, the first immunoblot was stripped off as described previously (Böhm *et al.*, 1995).

**Immunofluorescence studies** Melanoma cells seeded on to polystyrene tissue chamber slides were kept in serum-free RPMI 1640 medium for 24 h before stimulation with IL-6. After fixation with 4% paraformaldehyde and aldehyde quenching with 50 mM NH<sub>4</sub>Cl, cells were permeabilized with methanol for 10 min at -20°C. Unspecific binding was blocked with 5% goat/donkey serum and cells were incubated for 2 h at room temperature with an anti-STAT3 polyclonal antibody (1:100, Santa Cruz) and a mouse monoclonal antibody against protein disulfide isomerase (1:100, Dako, Hamburg, Germany). After extensive washing, bound antibodies were visualized by secondary donkey anti-rabbit antibodies conjugated with Texas red (1:100, Dianova, Hamburg, Germany) and goat anti-mouse antibodies coupled to fluorescein isothiocyanate (1:100, Dako). Slides were mounted and examined by confocal laser-scanning microscopy. Subcellular localization of STAT3 was assessed by viewing three independent microscopic fields with 100 cells per experiment. Experiments were performed in triplicate. Statistical significance between WM35 and WM9 cells was calculated by the Student's t test.

**Electrophoretic mobility shift assay** After stimulation with IL-6, cells were swollen in hypotonic buffer as described (Aragane *et al.*, 1998). After addition of Nonidet P-40, cell suspensions were minifuged and pellets containing the nuclear proteins resuspended in hypertonic buffer (hypotonic buffer supplemented with 0.4 M NaCl), incubated for 15 min on ice, and centrifuged. Protein concentrations were measured by the Bio-Rad protein assay (Bio-Rad, München, Germany) and electrophoretic mobility shift assay were performed as described previously (Gerhartz *et al.*, 1996). The  $\gamma$ -<sup>32</sup>P-labeled mutated double-stranded oligonucleotide corresponding to sis-induced element of the *fos* promoter (m67: 5'-GATCCGGGAGGGATTACGGGGAAATGCTG-3') was used as a probe (Wagner *et al.*, 1990). DNA-protein complexes were separated on a 4.5% polyacrylamide gel, fixed, dried, and autoradiographed. Signals were quantified with a phosphorimager. For supershift assays, nuclear extracts were preincubated with 2  $\mu$ l of anti-STAT3 $\alpha$  anti-serum (kindly provided by Dr. Werner Müller-Esterl, Frankfurt, Germany) for 15 min on ice.

**Histone H1 (HH1) kinase assays** Preparation of cell extracts was identical to that described before (Herzinger *et al.*, 1995). Identical amounts of lysate proteins (100  $\mu$ g per assay) were adjusted to a volume of 700  $\mu$ l with lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 2.5 mM ethyleneglycol-bis-( $\beta$ -aminoethyl)-N,N,N',N'-tetraacetic acid, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 M NaF, 10 mM glycerophosphate, 10% glycerol, 0.1% Tween, and freshly added protease inhibitors) and anti-cdk2 or cyclin E antibody was added. Mixtures were rotated overnight at 4°C and immune complexes collected by binding to protein A agarose beads followed by three washes with lysis buffer. The beads were washed in kinase buffer (20 mM MgCl<sub>2</sub>, 10 mM ethyleneglycol-bis-( $\beta$ -aminoethyl)-N,N,N',N'-tetraacetic acid, 40 mM HEPES, pH 7.0). Kinase reactions were performed in 20  $\mu$ l kinase buffer containing 0.5  $\mu$ l of  $\gamma$ -<sup>32</sup>P-adenosine triphosphate (300 Ci per mmol) and 1  $\mu$ g of HH1 (Boehringer, Mannheim, Germany) at 30°C for 20 min and stopped by addition of 20  $\mu$ l of 2  $\times$  SDS sample buffer. After SDS-PAGE, signals were analyzed by autoradiography and quantified with a phosphorimager.

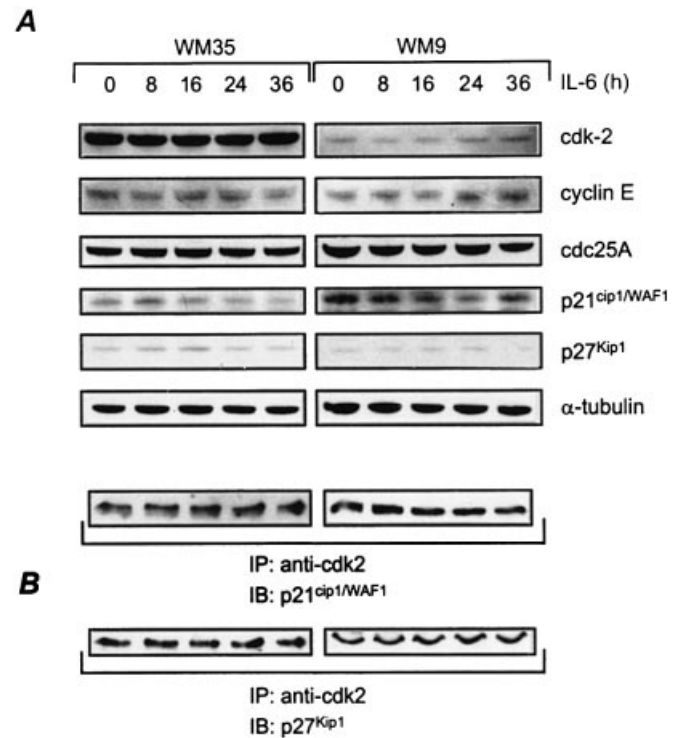


**Figure 1. IL-6 regulates cyclin E-associated kinase activity and phosphorylation of RB in WM35 but not in WM9 melanoma cells.** Phosphorimage analysis of cyclin E-associated kinase activity in both cell lines after treatment with IL-6 for various time periods as indicated (A). After treatment with IL-6, cells were lysed and subjected to immunoprecipitation with antibodies against cyclin E. Purified immune complexes were subsequently incubated for 20 min in kinase buffer containing  $\gamma$ - $^{32}$ P-adenosine triphosphate and HH1 as a substrate. Phosphorylated HH1 was separated by 12% SDS-PAGE followed by autoradiography (B). Total cellular extracts (20  $\mu$ g protein per lane) were separated by 6% SDS-PAGE followed by western immunoblotting with an antibody that recognizes both the hyperphosphorylated (upper migrating) and hypophosphorylated (lower migrating) form of RB (C).

**MEK1 assay** A commercially available immunoprecipitation kinase assay (UBI) was used for determining MEK1 activity. The test is based on MEK1-dependent activation of MAPK2/ERK2. In short, 100  $\mu$ l of protein A/G agarose slurry was preincubated with 10  $\mu$ l of an anti-MEK1 polyclonal antibody (UBI) overnight, washed in phosphate-buffered saline and incubated with 1 mg of whole cell lysate (prepared as described above and normalized for protein content), 1.0 U of recombinant human active MEK1 (positive control) or, with cell lysate without the MEK1 antibody (negative control). After immunoprecipitation, 20  $\mu$ l of assay dilution buffer, magnesium/adenosine triphosphate cocktail containing  $\gamma$ - $^{32}$ P-adenosine triphosphate and 1.6  $\mu$ l of inactive recombinant glutathione-S-transferase-MAPK2 were added and incubated with the protein A agarose immunocomplex for 30 min at 30°C. Four microliters of the supernatant was incubated with myelin basic protein, magnesium/adenosine triphosphate cocktail for further 10 min. The reaction was stopped by transferring 25  $\mu$ l of the reaction mixture on to P81 phosphocellulose paper, followed by thorough washing and liquid scintillation. Specific enzyme activity was measured against recombinant MEK1 minus negative control and statistical significance was calculated by the Student's t test. To monitor equal amounts of immunoprecipitated MEK1, supernatants were carefully removed from the agarose beads. MEK1 was eluted by adding 25  $\mu$ l 2  $\times$  SDS sample buffer followed by western immunoblotting with an anti-MEK1 monoclonal antibody (Transduction Laboratories, Lexington, KY).

## RESULTS

**IL-6 affects cyclin E-associated kinase activity and phosphorylation of RB in WM35 and WM9 cells differently** In order to study the comparative effect of IL-6 on the activity of distinct cell cycle regulators implicated in G<sub>1</sub> arrest, we first reconfirmed the differential growth-inhibitory effect of IL-6 on WM35 and WM9 melanoma cells (Lu *et al*, 1992). IL-6 (30 ng per ml) increased in WM35 cells the percentage of cells in G<sub>1</sub> after 36 h, whereas no significant changes were detected in WM9 cells (data not shown). In addition, fluorescence-activated

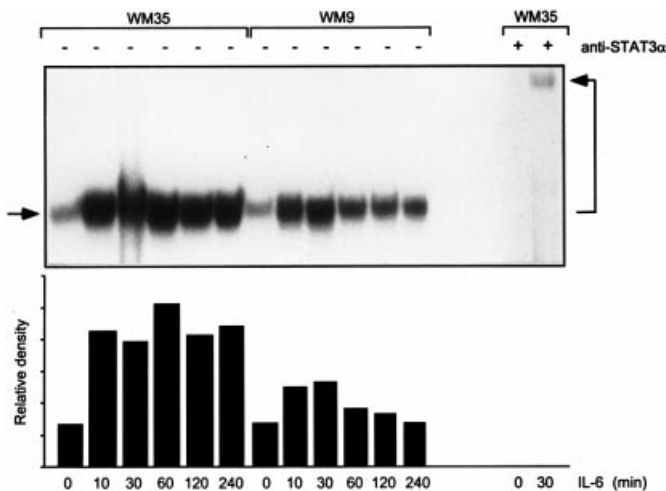


**Figure 2. IL-6 does not affect expression of cdk2, cyclin E, p21<sup>Cip1/WAF1</sup>, p27<sup>Kip1</sup> and cdc25A.** After stimulation with IL-6 (30 ng per ml), 20  $\mu$ g protein derived from whole cell extracts were separated by 4–12% gradient gels using the NuPAGE system followed by western blotting and sequential probing with the indicated antibodies (A). Amounts of p21<sup>Cip1/WAF1</sup> and p27<sup>Kip1</sup> bound to cdk2 after IL-6 treatment as assessed by coimmunoprecipitation (IP). Cell lysates (300  $\mu$ g protein) were incubated with an anti-cdk2 antibody and immunoblotted (IB) with anti-p21<sup>Cip1/WAF1</sup> and anti-p27<sup>Kip1</sup> antibodies (B).

cell sorter analysis using IL-6R antibodies showed an identical mean fluorescence intensity for WM35 and WM9 cells suggesting no differences in IL-6R expression (data not shown). This is in accordance with previous findings detecting no differences in IL-6 binding affinity and expression of both the IL-6R $\alpha$  subunit (gp80) and the signal converting subunit gp130 (IL-6R $\beta$ ) between IL-6 resistant and sensitive melanoma cell lines (Lu and Kerbel, 1993).

Passage through G<sub>1</sub> into S phase is governed by a family of cdk whose activity is regulated by binding to cyclins, phosphorylation/dephosphorylation, and association with low-molecular weight cdk inhibitors. Transition through the G<sub>1</sub> phase is sequentially governed by cdk4/6-cyclin D complexes and by cdk2-cyclin E complexes. Cdk2/cyclin E activity peaks at the G<sub>1</sub> to S transition and constitutes one prerequisite signal for entry into S phase (Morgan, 1995; Sherr and Roberts, 1995; Funk and Galloway, 1998). We therefore examined the effect of IL-6 in WM35 and WM9 melanoma cells on the activity of cdk2/cyclin E. Immunoprecipitation of cyclin E and *in vitro* kinase assays using HH1 as a substrate revealed pronounced inhibition of cyclin E-associated kinase activity as early as 8 h after stimulation with IL-6 in WM35 cells (Fig 1A, B). After 36 h, cyclin E-associated kinase activity was suppressed to less than 25% compared with cells not treated with IL-6. In contrast, cyclin E-associated kinase activity was only marginally affected by IL-6 in WM9 melanoma cells (Fig 1A, B). Equivalent results were obtained when cdk2 was immunoprecipitated and assayed for its kinase activity using HH1 (data not shown).

One substrate for G<sub>1</sub> cyclin-cdk complexes, including cyclin E/cdk2 is RB. RB becomes increasingly phosphorylated in late G<sub>1</sub> (Weinberg, 1995; Bartek *et al*, 1996). Only the hypophosphorylated RB can bind to and thereby inactivate the transcription factor



**Figure 3. STAT3-DNA complex formation in WM35 and WM9 cells upon IL-6 treatment.** Cells were kept in serum-free medium for 24 h and were then treated with IL-6 (30 ng per ml). Nuclear extracts were prepared at the indicated time points and were incubated with the  $\gamma$ - $^{32}$ P-labeled double-stranded oligonucleotide m67 corresponding to the sis-induced element of the *fos* promoter. DNA-protein complexes were separated on a 4.5% polyacrylamide gel and autoradiographed. Intensity of the bands was also quantified by densitometric analysis. Specificity of the DNA-protein complexes was confirmed by supershift assays in which nuclear extracts were incubated with 1  $\mu$ l of anti-STAT3 $\alpha$  antiserum. Data represent one set of two independent experiments with similar results.

E2F whose activity is required for the S phase. Treatment of WM35 melanoma cells with IL-6 resulted in the accumulation of hypophosphorylated RB as demonstrated by its faster migration in low percentage SDS-PAGE and western immunoblotting (Fig 1C). The shift from the phosphorylated to the nonphosphorylated form was observed in WM35 cells 16 h after IL-6 treatment. At 24 and 36 h only dephosphorylated RB was detectable in WM35 cells. In contrast, in WM9 cells levels of phosphorylated RB remained constant after IL-6 treatment at all time points. Thus, the RB changes in the phosphorylation status parallel the regulation of cdk2-cyclin E activity.

**IL-6 does not affect expression of cdk2, cyclin E, cdc25A, p21<sup>Cip1/WAF1</sup> and p27<sup>Kip1</sup>** To elucidate further the mechanism of IL-6-mediated inhibition of cyclin E-associated kinase activity and to check if IL-6 unresponsive WM9 cells display differences in cdk2 regulation, we first examined the expression of cyclin E and cdk2 in whole cell extracts. Western blot analysis revealed that the protein levels of cyclin E and cdk2 in both cell lines did not change upon treatment with IL-6 (Fig 2A). Protein levels of cdk2, however, were consistently lower in WM9 cells compared with WM35 cells. In addition, the protein levels of cdc25A, a dual-specific phosphatase activating cdk2 by dephosphorylating cdk2 at Thr14 and Tyr15 (Hoffmann *et al*, 1994), remained constant in total cell extracts of WM35 and WM9 cells, respectively (Fig 2A).

Next, we analyzed expression and binding of the KIP family members p21<sup>Cip1/WAF1</sup> and p27<sup>Kip1</sup>. It is known that a number of growth arrest signals such as DNA damaging agents or transforming growth factor- $\beta$  lead to transcriptional activation and increased protein expression of these low-molecular weight cdk2 inhibitors (Massague and Polyak, 1995; Sherr and Roberts, 1995). Whereas the KIP family members inhibit a wide range of cdk, members of the INK family preferentially inhibit cyclin D-associated kinases, i.e., cdk4 and cdk6. As WM35 melanoma cells, however, lack expression of p16<sup>INK4A</sup> and p15<sup>INK4B</sup> (Bani *et al*, 1996; Florenes *et al*, 1996; our own unpublished findings), we examined the expression of p21<sup>Cip1/WAF1</sup> and p27<sup>Kip1</sup>. Expression of these cdk

inhibitors was low on the protein level in both cell lines albeit p21<sup>Cip1/WAF1</sup> was expressed at higher levels in WM9 cells as compared with WM35 cells (Fig 2A). Treatment of WM35 and WM9 cells with IL-6, however, did not result in any significant changes in the protein levels of p21<sup>Cip1/WAF1</sup> and p27<sup>Kip1</sup>. To ascertain that identical amounts of lysate proteins were analyzed in all of these experiments, the membranes were finally re-probed with an antibody against  $\alpha$ -tubulin (Fig 2A).

As the total amount of p21<sup>Cip1/WAF1</sup> and p27<sup>Kip1</sup> found in whole cellular extracts does not necessarily correlate with the fraction bound to cdk2, co-immunoprecipitation experiments were performed. Immunoprecipitation of cdk2 in both cell lines and immunoblotting with antibodies against p21<sup>Cip1/WAF1</sup> and p27<sup>Kip1</sup>, however, did not reveal any differences in the amount of these cdk inhibitors bound to cdk2 (Fig 2B).

**DNA binding of STAT3 is significantly impaired in WM9 melanoma cells** As no differential regulation of cdk2 by CKI in WM35 and WM9 cells after IL-6 treatment could be detected, we wondered if changes upstream of the cell cycle machinery, namely within the signal transduction of IL-6 are responsible for the IL-6 resistance of WM9 cells. Elicitation of the diversity of biologic effects by IL-6 on target cells is stringently dependent on activation of STAT3, a latent transcription factor that becomes activated by tyrosine phosphorylation after the binding of IL-6 to its receptor (Darnell, 1997; Heinrich *et al*, 1998). STAT3 subsequently dimerizes, translocates to the nucleus where it binds to response elements consisting of the palindromic consensus oligonucleotide sequence TTCC(C/G)GGGAA.

Thus, we prepared nuclear extracts of WM35 and WM9 melanoma cells after stimulation with IL-6 and analyzed DNA binding of STAT3 using a high-affinity sis-inducible element of the *c-fos* promoter as a probe in the electrophoretic mobility shift assay (Wagner *et al*, 1990). A weak constitutive DNA-protein complex formation was detectable in both cell lines (Fig 3, left). This basal DNA-protein complex formation was not due to suboptimal fetal bovine serum deprivation as both cell lines had been kept in serum-free medium for at least 24 h. Stimulation with IL-6 (30 ng per ml) resulted in a rapid increase in DNA-protein complex formation. Densitometric analysis revealed that this DNA-protein complex formation peaked between 30 and 60 min after stimulation with IL-6 in both cell lines (Fig 3, left). DNA-protein complex formation in WM35 cells, however, was markedly enhanced and prolonged compared with WM9 cells. Whereas the DNA-protein complex formation in WM35 melanoma cells remained elevated as long as 240 min after IL-6 treatment, it returned in WM9 cells almost to the basal level at this time point (Fig 3, left).

In order to confirm the specificity of the detected DNA-protein complexes, "supershift" experiments with a STAT3 $\alpha$ -specific antibody were performed (Fig 3, right). Addition of the antibody to nuclear extracts of IL-6-stimulated cells resulted in a specific shift. In unstimulated cells, no supershift was observed (Fig 3, right), although constitutive DNA-protein complex formation was present (Fig 3, left). This is explained by the nature of the antibody that does not only bind to the STAT3 protein but also interferes with the transcription factor-STAT3 oligonucleotide probe interaction, as observed previously (Haan *et al*, 2000). In accordance with this finding, the "supershift" band for the untreated cells is virtually invisible as would be expected from the much lower level of DNA binding of STAT3 in nuclear extracts of these cells.

**Differential subcellular localization of STAT3 in WM35 and WM9 melanoma cells** One possible explanation for impaired nuclear binding of STAT3 in WM9 melanoma cells could be reduced nuclear translocation upon stimulation with IL-6. Thus, we examined the subcellular distribution, nuclear translocation, and redistribution of STAT3 following stimulation with IL-6 by double immunofluorescence using a monoclonal antibody against protein disulfide isomerase, which served as a cytoplasmic marker (Munro and Pelham, 1986; Vaux *et al*, 1990). STAT3 was detected by a polyclonal antibody. The percentage of cells displaying a diffuse,

**Table I. Differential subcellular localization of STAT3 in WM35 and WM9 cells after IL-6 treatment**

IL-6 <sup>a</sup> (min)	Localization in percentage <sup>b</sup>		
	Diffuse	Perinuclear	Nuclear
WM35			
–	91.6 ± 3.1*	2.3 ± 1.5**	6.0 ± 1.7**
30	15.0 ± 4.4**	17.7 ± 2.5**	67.0 ± 6.8*
60	11.0 ± 3.6**	11.7 ± 2.5**	77.3 ± 1.2*
240	25.0 ± 4.5*	18.6 ± 1.5**	56.0 ± 4.4*
WM9			
–	77.3 ± 3.5*	12.0 ± 2.0**	10.6 ± 2.9**
30	34.3 ± 6.7**	51.3 ± 5.0**	14.3 ± 2.5*
60	7.7 ± 4.0**	24.7 ± 4.5**	67.7 ± 2.5*
240	53.0 ± 5.3*	29.0 ± 4.0**	18.0 ± 2.0*

<sup>a</sup>Cells were stimulated with IL-6 (30 ng per ml) and localization of STAT3 was determined by double immunofluorescence with a monoclonal anti-STAT3 antibody and a polyclonal antibody against protein disulfide isomerase, a cytoplasmic marker. Bound antibodies were visualized by secondary antibodies conjugated to Texas red and fluorescein isothiocyanate, respectively.

<sup>b</sup>Data represent the mean ± SD from viewing three independent microscopic areas, each with 100 cells per experiment. Experiments were performed in triplicate. Significance of STAT3 localization per time point between WM35 and WM9,

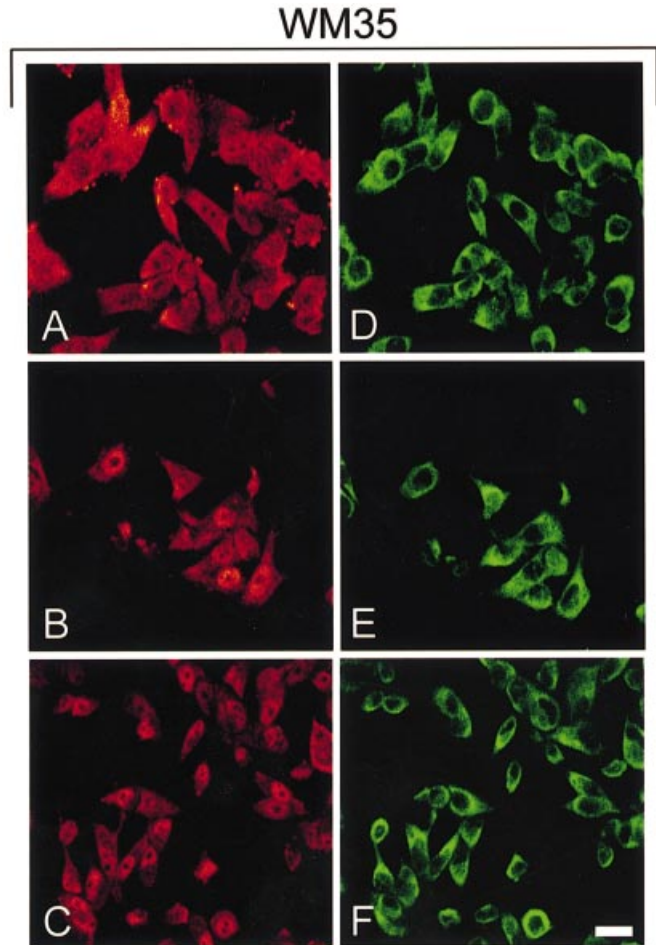
\**p* < 0.01;

\*\**p* < 0.05.

perinuclear, or nuclear staining was evaluated at different time points after IL-6 treatment (Table I).

Both cell lines exhibited diffuse immunostaining in which STAT3 was localized in the cytoplasm and to a lesser degree in the nucleus prior to stimulation with IL-6 (Figs 4 and 5, for representative fields). Within 30 min after IL-6 treatment, STAT3 was found mainly in the nuclei of WM35 cells, whereas in WM9 cells most of the STAT3 was still distributed diffusely or confined to the perinuclear region (Figs 4 and 5). Sixty minutes after IL-6 exposure, most of the STAT3 was translocated to the nucleus in both cell lines, although WM35 still contained statistically more STAT3 in the nuclei. This difference was again further pronounced 240 min after IL-6 exposure, at which most of the STAT3 was already relocated to the cytoplasm in WM9 cells, whereas more than 50% of the nuclei of WM35 melanoma cells still contained STAT3 and presented a mixed cytoplasmic/nuclear staining almost indistinguishable from the staining pattern obtained 30 min after stimulation (Figs 4 and 5). Protein disulfide isomerase as the cytoplasmic marker was always confined to the rough endoplasmic reticulum (Munro and Pelham, 1986; Vaux *et al*, 1990) (Figs 4 and 5). These observations show that STAT3 indeed is translocated to the nucleus in WM9 melanoma cells after stimulation with IL-6. In comparison with WM35 cells, however, quantitative differences exist with respect to the proportion of STAT3 being transferred to the nucleus at a given time period after IL-6 treatment and the speed of cytoplasmic relocation.

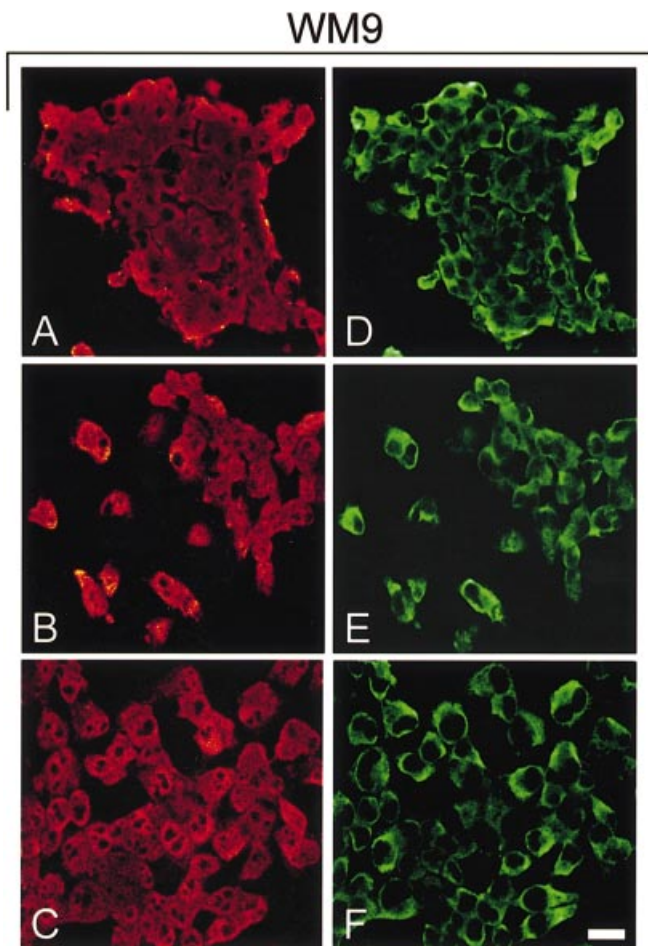
**Reduced tyrosine phosphorylation of STAT3 in WM9 melanoma cells** Regular function and transcriptional activity of STAT3 has been linked to two critical phosphorylation sites located at the carboxy-terminal end of the molecule. Whereas phosphorylation at Tyr705 has been clearly shown to correlate positively with DNA binding, dimerization, and nuclear translocation of STAT3 (Darnell, 1997; Heinrich *et al*, 1998), the role of Ser727 phosphorylation remains controversial (Zhang *et al*, 1995; Wen and Darnell, 1997; Wen *et al*, 1995). Immunoprecipitation of STAT3 from WM35 and WM9 melanoma cell extracts and immunoprobings with anti-STAT3 antibodies showed identical levels of STAT3 protein in unstimulated and IL-6-treated cell lines (Fig 6A, C). Thus, impaired DNA binding of STAT3 in WM9 cells is not due to reduced protein expression. When STAT3 was immunoprecipitated and the membrane probed with a site-



**Figure 4. Subcellular distribution of STAT3 in WM35 cells after treatment with IL-6 (30 ng per ml).** Cells were fixed with methanol and double stained for STAT3 (red fluorescence) and a marker for the endoplasmic reticulum (green fluorescence). Cells were left untreated (A, D) or were stimulated with IL-6 for 30 min (B, E) and 240 min (C, F). Note nuclear staining of STAT3 after IL-6 treatment for 30 min. Pictures are representative fields of three independent experiments. Scale bar: 20  $\mu$ m.

specific anti-phospho-Tyr705-STAT3 antibody (or, an anti-phosphotyrosine antibody), both cell lines showed weak basal phosphorylation of STAT3 without IL-6 treatment (Fig 6B, C). The constitutive tyrosine phosphorylation of STAT3 was in accordance with the observed constitutive DNA binding as shown above and was not due to suboptimal deprivation as both cell lines had been deprived under serum-free conditions for at least 24 h. Upon IL-6 treatment, WM9 cells displayed markedly reduced phosphorylation compared with WM35 cells (Fig 6B, C), suggesting that the IL-6 resistance of advanced melanoma cell lines is due to a defect in STAT3 tyrosine phosphorylation.

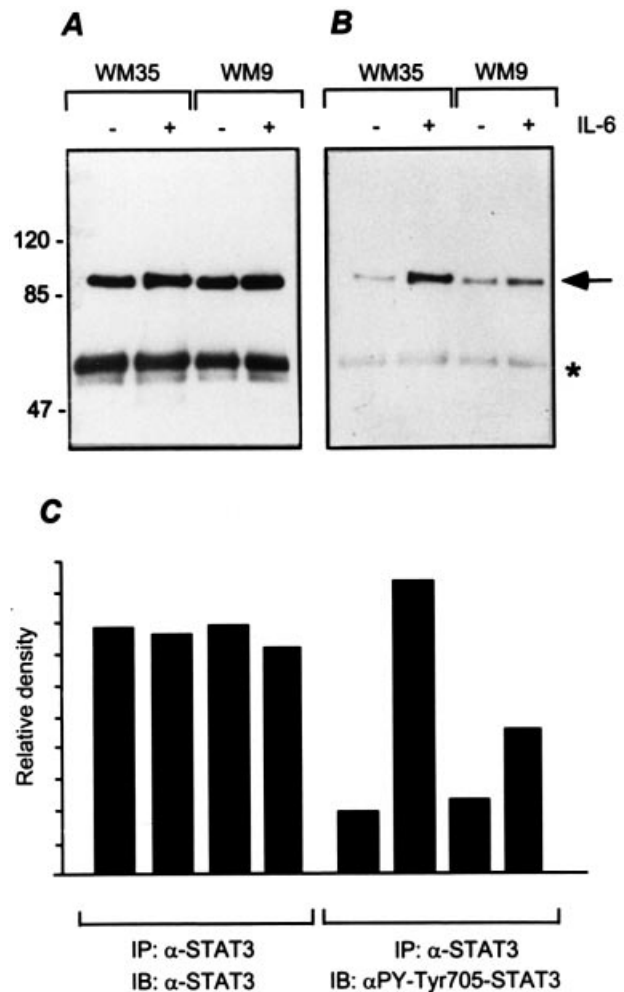
**Loss of IL-6-induced activation of JAK1 in WM9 melanoma cells** IL-6 leads to the activation of a number of nonreceptor tyrosine kinases known as the Janus kinases (JAK1, JAK2, and Tyk2). Extent and pattern of activation of these kinases following IL-6 treatment appears to be cell-type specific (Lütticken *et al*, 1994; Stahl *et al*, 1994). As the stunted response of WM9 cells to IL-6 could be due to the downregulation of JAK, we first examined the expression of JAK in WM35 and WM9 cells. Western blot analysis of total cell lysates with specific antibodies against JAK1, JAK2, and Tyk2 revealed similar amounts of protein in both cell lines (Fig 7A).



**Figure 5. Subcellular distribution of STAT3 in WM9 cells after treatment with IL-6 (30 ng per ml).** Cells were fixed with methanol and double stained for STAT3 (red fluorescence) and a marker for the endoplasmic reticulum (green fluorescence). Cells were left untreated (*A, D*) or were stimulated with IL-6 for 30 min (*B, E*) and 240 min (*C, F*). Note prominent diffuse staining of STAT3 in cells stimulated with IL-6 for 30 min as well as accelerated cytoplasmic relocation of STAT3 after stimulation with IL-6 for 240 min. Pictures are representative fields of three independent experiments. Scale bar: 20  $\mu$ m.

We next addressed the question whether differences in the extent of tyrosine phosphorylation of JAK may account for the impaired activation of STAT3 in WM9 cells. As it was demonstrated that STAT3 activation crucially depends on the presence and proper activation of JAK1 only (Guschin *et al*, 1995), we checked the tyrosine phosphorylation of JAK1 in WM35 and WM9 cells lines upon stimulation with IL-6 (30 ng per ml). Immunoblot analysis of crude cell extracts with an antibody that specifically recognizes JAK1 phosphorylated on Tyr1022 and Tyr1023 confirmed phosphorylation within 5 min (**Fig 7B**, left) in WM35 cells. WM9 cells, in contrast, displayed prominent constitutive tyrosine phosphorylation of JAK1 with no increment upon IL-6 treatment (**Fig 7B**, right). Prolonged treatment of WM9 cells with IL-6 for up to 30 min did not result in any increment in tyrosine phosphorylation of JAK1 (data not shown). These findings suggest that the defect in STAT3 activation in WM9 cells is linked to the defective activation of JAK1.

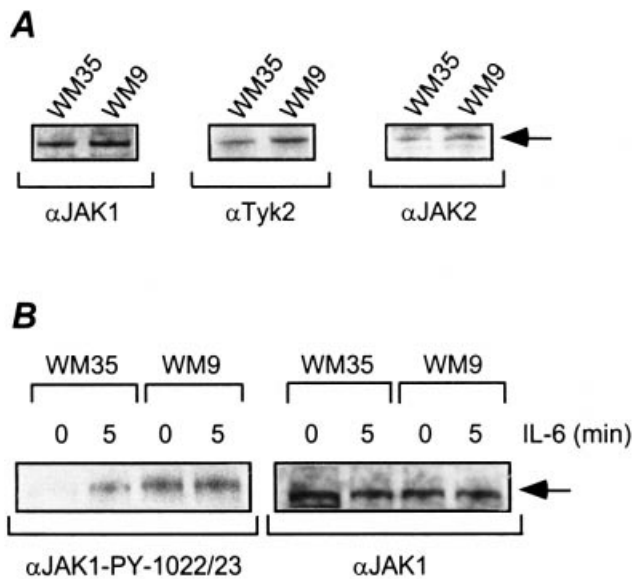
**Differential activity of MEK1 in WM35 and WM9 melanoma cells** The Ras-Raf-MAPKK-MAPK (mitogen-activated protein kinase) pathway has been shown to become synergistically activated in response to distinct melanocyte peptide growth factors (Böhm *et al*, 1995) among which bFGF was identified as an autocrine growth



**Figure 6. Reduced tyrosine phosphorylation of STAT3 in WM9 cells after IL-6 treatment.** Cells were kept in serum-free medium for 24 h followed by stimulation with IL-6 (30 ng per ml) for 30 min. Cells lysates were subjected to immunoprecipitation (IP) with an anti-STAT3 antibody and 8% SDS-PAGE. Membranes were immunoblotted (IB) with a phospho-specific antibody that specifically recognizes STAT3 at Tyr705. Membranes were subsequently stripped and reprobed with an anti-STAT3 antibody. Tyrosine phosphorylation of STAT3 is shown in (*A*), STAT3 protein expression in (*B*). Bands were evaluated densitometrically (*C*). \*Antibody band derived from cross-reaction of the secondary antibody with the antibody used for the IP.

factor for melanoma. As the MAPK signaling cascade was shown to suppress STAT3 and JAK1 tyrosine phosphorylation (Ceresa *et al*, 1997; Jain *et al*, 1998; Sengupta *et al*, 1998) we thus examined the expression and activity of MAPK kinase 1 (MAPKK1 or MEK1) in WM35 and WM9 cells.

Unexpectedly, MEK1 kinase activity in nonstimulated WM9 cells was significantly lower than in WM35 cells (**Fig 8A**). Upon stimulation with IL-6 (30 ng per ml) for 30 min MEK1 activity in WM35 cells declined but rose to the level of unstimulated cells within 36 h. In contrast, no changes in MEK1 activity could be detected after IL-6 treatment in WM9 cells. The differential MEK1 activity between WM35 and WM9 cells was not due to the reduced expression of MEK1 protein as the amount of MEK1 eluted from the immune complexes was similar (**Fig 8B**). These data demonstrate that MEK1 activity in WM9 cells is not increased and do not support the concept that the reduction of IL-6-induced STAT3 activation in WM9 cells is related to increased MEK1 activity.

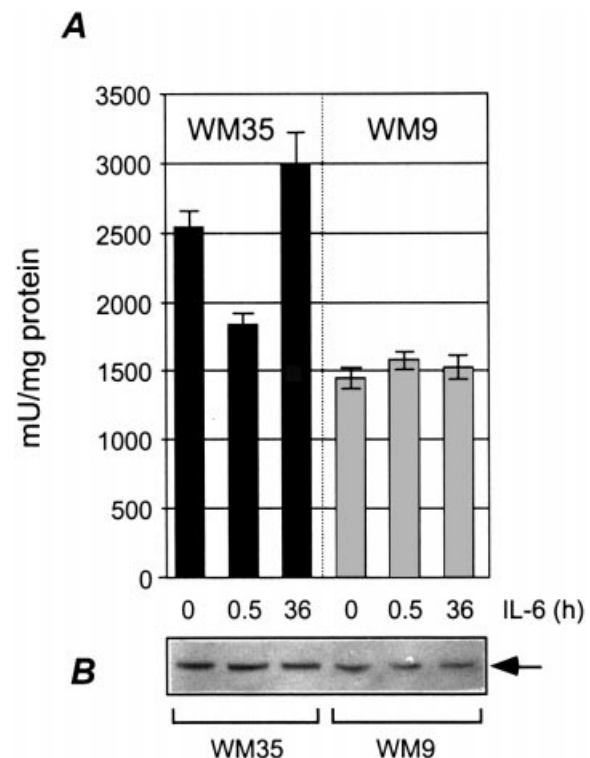


**Figure 7. WM9 cells display constitutive JAK1 tyrosine phosphorylation and do not respond to IL-6 treatment with JAK1 activation.** Identical amounts of protein in total cell lysates (30  $\mu$ g per lane) were separated by 4–12% NuPAGE gradient gels followed by immunoblotting with the indicated antibodies (A). For phosphorylation experiments, cells were stimulated with IL-6 (30 ng per ml) for 5 min and were harvested directly in boiling sample buffer, followed by 8% SDS-PAGE (100  $\mu$ g protein per lane) and immunoblotting with an antibody that specifically recognizes phosphorylated JAK1 on Tyr1022/1023. The membrane was subsequently stripped and reprobed with an anti-JAK1 antibody to assure identical amounts of protein (B).

## DISCUSSION

We have examined comparatively the effect of IL-6 on the signal transduction and regulation of distinct cell cycle players in the human melanoma cell lines WM35 and WM9 in order to decipher the molecular mechanism by which melanoma cells derived from advanced stages become resistant to IL-6. With regard to the cell cycle, our findings show that the cyclin E/cdk2 complex as well as RB are both target molecules for the growth-inhibitory action of IL-6. The observed inhibition of cdk2 and the accumulation of the hypophosphorylated (active) form of RB in WM35 cells upon IL-6 treatment are in accordance with recent findings from others (Florenes *et al*, 1999). In contrast to the latter investigators we did not detect an inductive effect on the p21<sup>Cip1/WAF1</sup> protein expression that could account for the rapid inhibition of cdk2/cyclin E activity in WM35 cells upon IL-6 treatment. This finding was confirmed by repetitive immunoblot analysis of whole cell extracts as well as by co-immunoprecipitation experiments in which the actual amount of p21<sup>Cip1/WAF1</sup> bound to cdk2 was evaluated at various time points after IL-6 treatment. The discrepancies between our data and the findings from Florenes *et al* (1999) with regard to p21<sup>Cip1/WAF1</sup> may be related to differences in culture conditions. In our study cells were treated with higher IL-6 doses (30 ng per ml) and in the presence of higher amounts of fetal bovine serum (2%) as compared with Florenes *et al* (1999) (10 ng per ml IL-6, 1% fetal bovine serum). The WM35 melanoma cell line was originally established by Herlyn *et al* (1989) more than 10 y ago. Thus, it is also possible that different clones of WM35 cells with a differential response to IL-6-induced p21<sup>Cip1/WAF1</sup> expression have originated under long-term culture in the various laboratories using this cell line.

Interestingly, the amounts of cdk2 and p27<sup>Kip1</sup> at the protein level, especially those of cdk2, were higher in WM35 than in WM9 cells. The stronger expression of p21<sup>Cip1/WAF1</sup> in WM9 cells is in accordance with findings from others, who demonstrated that



**Figure 8. Differential activity of MEK1 in WM35 and WM9.** Cells were stimulated with IL-6 (30 ng per ml) and MEK1 was immunoprecipitated from 1 mg of cell lysate. Immunoprecipitates were incubated with recombinant MAPK as a substrate and MAPK activity was assessed by *in vitro* phosphorylation of myelin basic protein. Enzyme activity of MEK1 in cell lysates was compared with 1.0 U of recombinant active human MEK1 in three independent experiments; data represent mean  $\pm$  SD,  $p < 0.001$  (A). MEK1 protein expression was monitored by eluting the immune complexes with SDS buffer, separation on 4–12% gradient gels and immunoblotting with an anti-MEK1 antibody (B).

invasive primary melanomas and metastatic melanomas *in situ* express high levels of this low-molecular cdk inhibitor (Bales *et al*, 1999). The observed cell line-specific differences in p27<sup>Kip1</sup> and cdk2 expression, however, do not explain the effect of IL-6 on cdk2 activity in WM35 cells. Recently, inhibition of cdk2 activity in the absence of altered levels of p21<sup>Cip1/WAF1</sup>, p27<sup>Kip1</sup>, p57<sup>Kip2</sup>, p15<sup>INK4b</sup>, and cdc25A has been reported in HepG2 cells treated with transforming growth factor- $\beta$ . The inactivation of cyclin E/cdk2 complexes were caused by the absence of the Thr160 phosphorylation on cdk2 and associated with the inhibition of Thr160 cdk activating kinase activity (Nagahara *et al*, 1999). It will be interesting to check if an analogous mechanism of cdk2 inhibition also occurs in our WM35 cells. As IL-6-induced growth arrest of another human melanoma cell line, A375, is associated with the induction of p27<sup>Kip1</sup> (Kortylewski *et al*, 1999), different cell cycle players appear to be targeted by IL-6 depending on the examined cell type and the experimental conditions.

Our comparative studies on the IL-6 signaling pathway, on the other hand, disclosed a complex alteration in WM9 cells as compared with WM35 cells. First, the functional state of STAT3 (as determined by DNA binding, tyrosine phosphorylation, and the rate of nuclear translocation and cytoplasmic relocation) was altered in WM9 cells. Second, JAK1, the upstream kinase of STAT3, did not respond to IL-6 treatment in WM9 cells with the phosphorylation at Tyr1022/1023 but displayed constitutive phosphorylation. Third, differences were found in both cell lines with respect to basal and IL-6-regulated MEK1 activity. These findings suggest that the loss of IL-6 response and the subsequent abnormalities in cell cycle

regulation at least in WM9 cells are a direct consequence of a profound disturbance in the IL-6 signal transduction pathway. The critical role of STAT3 as a key IL-6 signal intermediate has been confirmed by various studies, e.g., in myeloid leukemia M1 leukemia cells that respond to IL-6 with terminal differentiation and growth arrest. Cell lines constitutively expressing dominant negative forms of STAT3 do not respond any longer to IL-6 (Minami *et al*, 1996). As WM9 cells are known to secrete bioactive IL-6 (Lu and Kerbel, 1993; Silvani *et al*, 1995) it has been suggested that constitutive secretion may desensitize melanoma cells to IL-6; however, although autonomous IL-6 secretion by WM9 cells may lead to constitutive JAK1 tyrosine phosphorylation, the concomitant reduction in STAT3 activation is not conceivable with a simple feed-forward model. One explanation for these puzzling findings is that several components of the IL-6 signal transduction pathway (as well as components of "cross-talking" signaling pathways) may be altered simultaneously. Candidates that mediate downregulation of the IL-6-induced activation of STAT3 are the suppressors of cytokine signaling (Endo *et al*, 1997; Naka *et al*, 1997; Starr *et al*, 1997; Nicholson and Hilton, 1998) and PIAS3 (Chung *et al*, 1997). Accordingly, abnormal expression of one of these molecules in WM9 cells may lead to reduced activation of STAT3 by IL-6, whereas JAK1 phosphorylation is maintained by autonomous IL-6 production. Whether suppressors of cytokine signaling or PIAS3 are actually involved in the abnormal response of WM9 cells towards IL-6 is currently under investigation in our laboratory (Böhm *et al*, unpublished findings).

Our studies on MEK1 as a potential downregulator of STAT3 activation did not reveal an enhanced enzyme activity in WM9 cells as compared with WM35 cells. In fact, MEK1 activity in WM9 cells was even lower than in WM35 cells, which is in accordance with preliminary data from us showing a reduced tyrosine phosphorylation and activity of MAPK1/2 in WM9 cells. On the other hand, the MEK1 activity was significantly inhibited by IL-6 in WM35 cells in a transient fashion. This finding raises the question as to whether the downregulation of MEK1 is required for growth inhibition by IL-6. It is known that the melanocyte peptide growth factors bFGF, hepatocyte growth factor, endothelin-1, and mast cell growth factor activate the MAPK cascade in normal human melanocytes (Böhm *et al*, 1995). The synergistic action of these growth factors on proliferation was paralleled by an enhanced and prolonged *in vivo* phosphorylation of MAPK2 and Raf. In addition, the *in vitro* kinase activity of Raf using a glutathione-S-transferase-MEK fusion protein as a substrate was synergistically enhanced and sustained by the above mitogens. Preliminary data from us have further shown (Böhm *et al*, unpublished findings) that proliferation of WM35 and WM9 cells is strongly inhibited by PD 098059, a specific MEK inhibitor (Alessi *et al*, 1995). Thus, resistance of WM9 cells to IL-6 may possibly be linked to a deregulated function of MEK1. To confirm the potential role of MEK1 in IL-6-mediated growth inhibition and in IL-6 resistance of advanced melanoma cells, further studies are needed. In accordance with an important role of the MAPK cascade in the development of cytokine resistance of melanoma are the recent findings by Shellman *et al* (2000). These authors demonstrated that ectopic expression of activated N-ras in WM35 cells abrogates growth inhibition by transforming growth factor- $\beta$  and inhibits the accumulation of hypophosphorylated RB.

In summary, we have shown here that IL-6 resistant WM9 melanoma cells exhibit a complex alteration in the IL-6 signal transduction pathway that is associated with an altered response to MEK1. Our findings point to a significance part for STAT3 as a molecular correlate of melanoma progression whose targeting may also offer potential therapeutic strategies for melanoma (Niu *et al*, 1999).

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