Interferon-γ-Mediated Growth Regulation of Melanoma Cells: Involvement of STAT1-Dependent and STAT1-Independent Signals

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Interferon-γ, a known inhibitor of tumor cell growth, has been used in several protocols for the treatment of melanoma. We have studied the molecular events underlying interferon-γ-induced G_0/G_1 arrest in four metastatic melanoma cell lines with different responsiveness to interferon-γ. The growth arrest did not result from enhanced expression of cyclin-dependent kinase inhibitors p21 and p27. Instead, it correlated with downregulation of cyclin E and cyclin A and inhibition of their associated kinase activities. We show that interferon-γ-induced growth inhibition could be abrogated by overexpression of dominant negative STAT1 (signal transducer and activator of transcription 1) in the melanoma cell line A375, suggesting that STAT1 plays a crucial part for the anti-proliferative effect. Erythropoietin stimulation of a chimeric receptor led to a concentration-dependent STAT1 activation and concomitant growth arrest when it contained the STAT recruitment motif Y440 of the interferon-γ receptor 1. In contrast, dose–response studies for interferon-γ revealed a discrepancy between levels of STAT1 activation and the extent of growth inhibition; whereas STAT1 was activated by low doses of interferon-γ (10 U per mL), growth inhibitory effects were only visible with 100-fold higher concentrations. Our results suggest the presence of additional signals emanating from the interferon-γ receptor, which may counteract the anti-proliferative function of STAT1.

Key words: cell cycle/chimeric receptor/cytokine resistance/cytokines/signal transduction.
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Interferons (IFN), initially identified as anti-viral agents, are implicated in a variety of biologic functions concerning anti-proliferative effects, induction of cell differentiation, and modulation of the immune response. They are divided into two classes: type I IFN (comprising IFN-α and IFN-β) and type II IFN (represented by IFN-γ). Each class is characterized by distinct intracellular signaling mechanisms. IFN-γ, produced by activated T lymphocytes and natural killer cells, exerts its pleiotropic effects via a receptor complex consisting of two chains, the ligand-specific IFNGR1 and IFNGR2, expressed on nearly all cell types (Stark *et al*, 1998). Binding of IFN-γ leads to the oligomerization of the receptor subunits and initiates signal transduction. Jak1 and Jak2 protein tyrosine kinases of the Janus family that are constitutively associated with IFNGR1 and IFNGR2 receptor subunits, respectively, become activated and in turn are thought to phosphorylate tyrosine residue 440 of the cytoplasmic part of IFNGR1 among other cellular substrates. The phosphotyrosine pY440 within the sequence Y_{440}DKPH plays a major part in receptor activity as it serves as a docking site for the transcription factor STAT1 (signal transducer and activator of transcription 1). Subsequently, STAT1 also undergoes tyrosine phosphorylation, dissociates from the receptor, forms dimers, and translocates to the nucleus where it regulates transcription of target genes (Darnell, 1997; Stark *et al*, 1998). In addition to the ubiquitous way of IFN-γ-responsive STAT1-mediated signaling, cell-type-specific activation of two other STAT factors was recently reported. Coactivation of STAT1 and STAT3 after IFN-γ stimulation has been described in adipocytes (Stephens *et al*, 1998) and hematopoietic cells (Sato *et al*, 1997). IFN-γ-induced monocyte or macrophage differentiation was shown to coincide with the activation of STAT5 but not STAT1 (Meinke *et al*, 1996), which is also mediated by Y440 of the IFNGR1 (Woldman *et al*, 2001). Moreover, activation of several other signaling pathways has been described, including the extracellular signal-regulated kinase (Erk) pathway induced via protein tyrosine kinase Pyk2 (Takaoka *et al*, 1999) or via the serine/threonine kinase Raf-1 (David *et al*, 1995; Stancato *et al*, 1998), the p38 mitogen-activated protein kinase (MAPK) pathway and

Abbreviations: cdk, cyclin-dependent kinase; EPO, erythropoietin; EMSA, electrophoretic mobility shift assay; IFNGR, interferon-γ receptor; STAT, signal transducer and activator of transcription; XTT, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide.

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the recently reported C3G/Rap1 pathway, a small G protein signaling cascade (Alsayed *et al*, 2000).

Both types of IFN are known for strong anti-proliferative and immunomodulatory effects on melanoma cells. IFN-γ was also reported to induce expression of ISGF3 components, including STAT1, which significantly enhances responsiveness to IFN-α/β of primed cells (Carson, 1998; Wong *et al*, 1998).

As the downstream mechanisms of IFN-γ-mediated effects on growth and survival of human melanoma cells are largely unknown we investigated the molecular events underlying the anti-proliferative effect of IFN-γ on human melanoma cells of metastatic origin. Cell cycle analysis revealed that the growth arrest does not result from accumulation of cyclin-dependent kinase inhibitors. Rather, growth inhibition was correlated with the complex downregulation of several proteins necessary for cell cycle progression, primarily cyclin E, cyclin A, and the kinases cdk4 and cdk2. In this study, we demonstrate that the activity of IFN-γ on melanoma cells is STAT1 dependent. Most interestingly, under conditions of comparable STAT1 activation, a chimeric receptor containing the isolated tyrosine motif Y440 is much more effective at mediating growth inhibition than the endogenous IFN-γ receptor. Our results imply that divergent signals may emanate from the IFN-γ receptor, which could contribute to IFN-γ resistance.

Results

IFN-γ induces a G_0/G_1 arrest in human melanoma cells involving a downregulation of cyclins and cyclin-dependent kinases To study the anti-proliferative effect of IFN-γ on human melanoma cells, four cell lines derived from metastatic tumor stages were chosen. After 4 d of incubation in the presence of IFN-γ, a concentration-dependent growth inhibition was observed (Fig 1*A*). In three of the tested cell lines this inhibition was evident when concentrations of IFN-γ exceeded 1000 U per mL. A375 cells exhibited the greatest inhibition in growth, whereas the effect on WM239 and Mel-Im cells was more moderate. In contrast, WM9 cells were relatively resistant to IFN-γ, reacting only at doses greater than 10,000 U per mL. These findings were confirmed by cell cycle analyses. After 2 d of treatment with IFN-γ, the number of A375, WM239, and Mel-Im cells in G_0/G_1 phase was increased, whereas WM9 cells remained unaffected (Fig 1*B*). This G_0/G_1 arrest in A375 and WM239 cells was paralleled by a marginal increase in apoptotic cells; however, the percentage of counted sub-G_1 events did not exceed 4% of total counts (data not shown).

A known mechanism underlying the IFN-γ-induced G_0/G_1 arrest in many cells is the upregulation of cyclin-dependent kinase inhibitors (Yamada *et al*, 1995; Chin *et al*, 1996; Mandal *et al*, 1998). None of the cell lines whose growth was inhibited by IFN-γ (A375, Mel-Im, WM239), however, showed an upregulation of p21/Waf-1/Cip-1, p27/Kip-1, or p18/INK4c. In fact, a decrease in the protein level of p21 and, to a lesser extent, of p27 and p18 was observed (Fig 2). It is also noteworthy that the basal levels of cyclin-dependent kinase inhibitor, particularly p21, were not consistent in the cell lines studied (Fig 2) and did not correlate with their normal growth rate in cell culture (not shown).

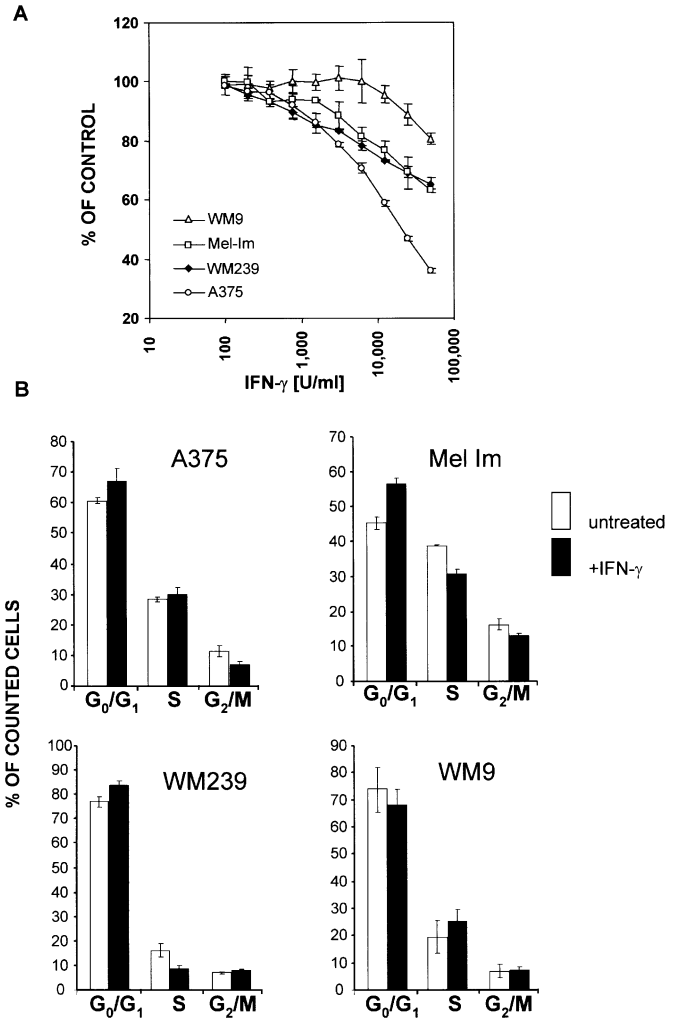

Figure 1
Effect of IFN-γ on the growth of human melanoma cells. (*A*) A375 (circles), Mel-Im (squares), WM239 (rhombs), and WM9 cells (triangles) were cultured for 4 d in the presence of IFN-γ at different concentrations. Growth was assessed by an XTT test. Values were recalculated according to untreated controls. Error bars represent the SD of triplicate samples. (*B*) For cell cycle analysis A375, Mel-Im, WM239, and WM9 cells were incubated in medium with or without IFN-γ (5000 U per mL) for 48 h. After DNA staining with propidium iodide, fluorescence was measured with a FACScalibur (Becton Dickinson), and the data were further quantified using the ModFit 2.0 software (Verity Software). Sub-G_1 values were excluded from the calculation. Error bars represent the SD of three independent experiments.

Growth arrest can also be mediated by the downregulation of positive regulators of the cell cycle. Therefore, we analyzed the protein expression of cyclins D1, E, and A, and the cyclin-dependent kinases cdk4 and cdk2. Cyclin D1 expression did not significantly change after stimulation with IFN-γ in any of the melanoma cell lines (Fig 2). Protein levels of cyclin E or cyclin A, however, were downregulated in all melanoma cell lines after IFN-γ treatment with the exception of the IFN-γ-resistant WM9 cells. We also observed a reduction in the expression of the cyclin-dependent kinases cdk4 and cdk2 in the IFN-γ-sensitive A375, Mel-Im, and WM239 cells but not in the IFN-γ-resistant WM9 cells.

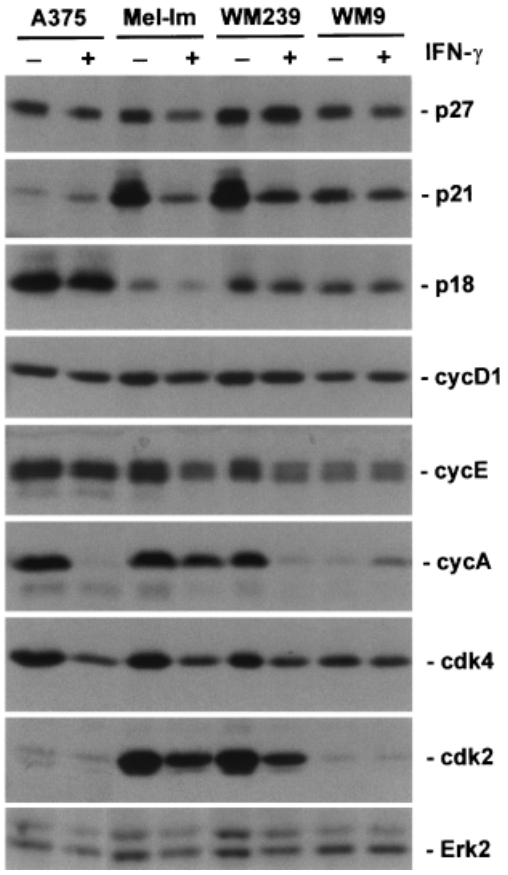

Figure 2
IFN-γ leads to complex downregulation of G_1/S cyclins and cyclin-dependent kinases. A375, Mel-Im, WM239, and WM9 cells were stimulated with IFN-γ (5000 U per mL) for 48 h. Equal amounts of total cell lysates (30 μg protein) were separated on a 15% SDS polyacrylamide gel followed by western blot analysis with antibodies to the indicated proteins.

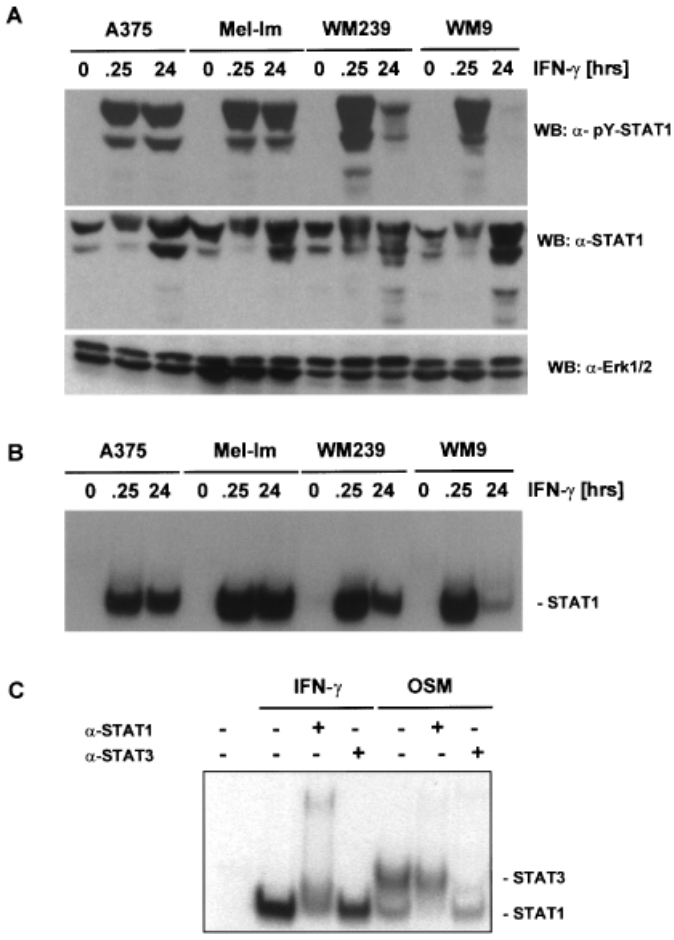

Figure 3
Activation of STAT1 signaling in IFN-γ-treated melanoma cells. (*A*) Phosphorylation and expression of STAT1 in A375, Mel-Im, WM239, and WM9 cells stimulated with IFN-γ (5000 U per mL) for 15 min and 24 h. Equal amounts of total cell lysates (30 μg protein) were separated on a 7.5% SDS polyacrylamide gel followed by western blot analysis using antibodies against phospho-STAT1, STAT1, and Erk1/2. (*B*) A375, Mel-Im, WM239, and WM9 cells were stimulated with IFN-γ (5000 U per mL) for the indicated times. Nuclear extracts containing 5 μg of protein were used for an EMSA with the SIEm67 probe. The position of homodimers of STAT1 is indicated. (*C*) IFN-γ does not activate STAT3. Nuclear extracts were prepared from A375 cells stimulated with IFN-γ (500 U per mL) or with OSM (10 ng per mL) for 15 min. Equal amounts of nuclear extracts (2.5 μg) was used for an EMSA with the SIEm67 probe, if indicated after preincubation (15 min at room temperature) with 3 μg anti-STAT1 or anti-STAT3 antibodies.

These findings suggest that IFN-γ inhibits growth of melanoma cells by a mechanism that does not involve cyclin-dependent kinase inhibitor upregulation, but rather entails a complex downregulation of G_1/S cyclins and cyclin-dependent kinases.

Defects in STAT1 expression do not explain differences in IFN-γ responsiveness We next examined STAT1 expression in the melanoma cell lines, because reduced responsiveness to IFN may result from a lack of STAT1 expression (Wong *et al*, 1997). The basal protein levels of STAT1 in untreated cells were comparable and did not correlate with their responsiveness to IFN-γ (Fig 3*A*). Moreover, all the cell lines, including WM9 cells, exhibited strong upregulation of STAT1 protein expression within 24 h after stimulation, whereas Erk levels remained unchanged.

In all the cell lines tested, including WM9 cells, IFN-γ induced strong phosphorylation of STAT1 after 15 min of stimulation. STAT1 phosphorylation was still pronounced in A375 and Mel-Im cells after 24 h of stimulation, whereas it was considerably reduced in WM239 and WM9 cells (Fig 3*A*). STAT1 phosphorylation was paralleled by the appearance of corresponding DNA-binding activities in EMSA analysis using the SIE-oligo (Fig 3*B*). This probe binds both STAT1 and STAT3. The presence of anti-STAT1 antibodies but not of anti-STAT3 antibodies significantly reduced the binding activity in nuclear extracts of IFN-γ-treated A375-cells (Fig 3*C*). Moreover, the mobility of the IFN-γ-induced DNA-binding activity was clearly different from the STAT3 signals observed upon OSM stimulation (Fig 3*C*).

STAT1 activation is crucial for IFN-γ-induced growth inhibition of A375 cells To study the role of STAT1 in IFN-γ induced growth inhibition we employed tripartite chimeric receptor constructs (Gerhartz *et al*, 1996; Strobl *et al*, 2001). These constructs contain the extracellular region of the mouse erythropoietin receptor (EPOR) fused to the transmembrane and truncated membrane-proximal box1/box2 region (i.e., the Jak binding region) of the interleukin-6 signal transducer gp130, without or with the seven amino acid

STAT1 recruitment motif (Y440) from the IFNGR1 (EGΔB and EGΔBY440, respectively, Fig 4*A*,*B*, left upper panels). Thus, as the endogenous IFN-γ receptor, EGΔBY440 has the ability to activate STAT1. Except for the STAT recruitment motif, however, both receptors are different in sequence.

Stably transfected A375 clones with similar levels of surface receptor expression were isolated (Fig 4*A*,*B*, left lower panels). As previously reported (Kortylewski *et al*, 1999), clones expressing EGΔB did not exhibit STAT activation after stimulation with EPO (Fig 4*A*, right upper panel) and proliferated unrestrained (Fig 4*A*, right lower panel). In contrast, clones expressing EGΔBY440 (Fig 4*B*, left panels) demonstrated a strong EPO-induced STAT1 activation (Fig 4*B*, right upper panel). In addition, growth of these clones was efficiently growth inhibited by EPO (Fig 4*B*, right lower panel) as a result of a G_0/G_1 arrest (data not shown).

Like IFN-γ, EPO decreased the expression of cyclin E, cyclin A and, to a lesser extent, of cdk4 and cdk2 within 24 h of treatment in EGΔBY440 cells (Fig 5). Whereas p21 and p18 protein levels remained relatively stable, levels of p27 decreased. The downregulation of cyclin E and cyclin A, together with their kinase partner cdk2, was also evident by reduced levels of kinase activity of the respective cyclin/cdk complexes (data not shown). We conclude that the presence of the STAT1 recruitment module Y440 within the chimeric receptor was sufficient to mediate an IFN-γ-like growth arrest of A375 cells.

In a separate attempt to elucidate the role of STAT1 in IFN-γ-induced growth inhibition, we examined stable A375 transfectants expressing STAT1F, a dominant negative form of STAT1 in which the tyrosine residue Y701, necessary for STAT1 phosphorylation and dimerization, is replaced by phenylalanine (Nakajima *et al*, 1996; Kortylewski *et al*, 1999). As presented in Fig 6, A375 cells overexpressing STAT1F were almost completely resistant to IFN-γ when compared with mock transfectants, suggesting that STAT1 is a crucial mediator for the growth arrest elicited by IFN-γ.

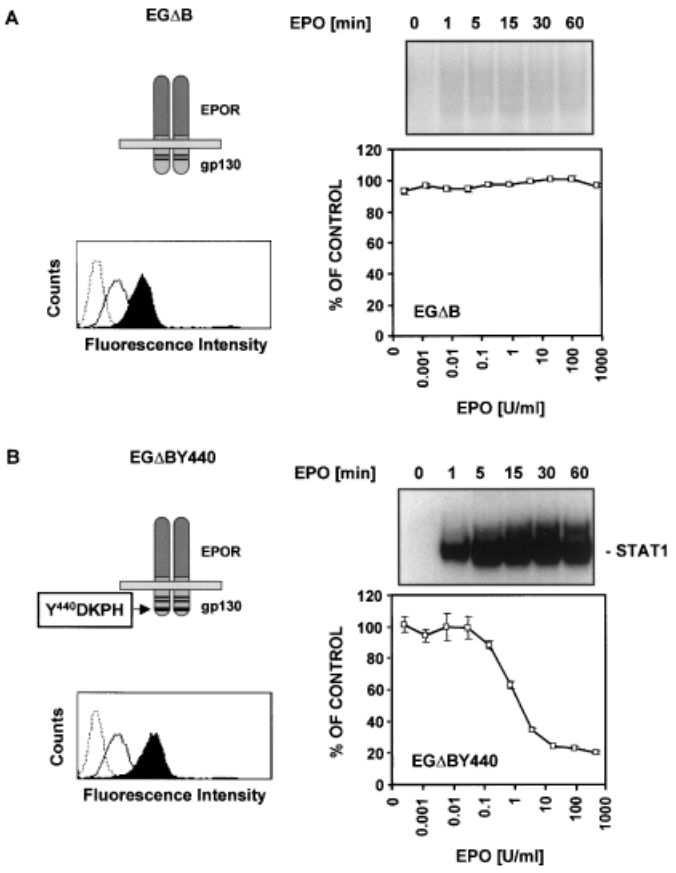

Figure 4
STAT1 activation is sufficient to confer a growth inhibitory effect on A375 cells. Comparison of two hybrid receptors: EGΔB (*A*) and EGΔBY440 (*B*). Upper left panels: schematic representation of receptor chimeras expressed in A375 cells. Lower left panels: receptor expression on the surface of A375 transfectants. Cells were incubated with a polyclonal rabbit-anti-mouse EPOR anti-serum followed by staining with phycoerythrin-conjugated donkey-anti-rabbit IgG anti-serum and measured by cytofluorometry. Each panel contains the histograms of the indicated transfectants (black) in comparison with those of transfectants incubated with secondary antibody alone (dotted line) and of parental A375 cells (solid line) to show unspecific binding of the polyclonal anti-sera. Upper right panels: STAT1 activation in cells stimulated with EPO (10 U per mL) for the times indicated. Nuclear extracts containing 5 μg of protein were used for an EMSA with the SIEm67 probe. The position of STAT1 homodimers is indicated. Lower right panels: Effect of EPO on the growth of transfectants. Cells were cultured for 4 d in the presence of EPO at different concentrations. Growth was assessed by an XTT test, values were recalculated according to untreated controls. Error bars represent the SD of triplicate samples.

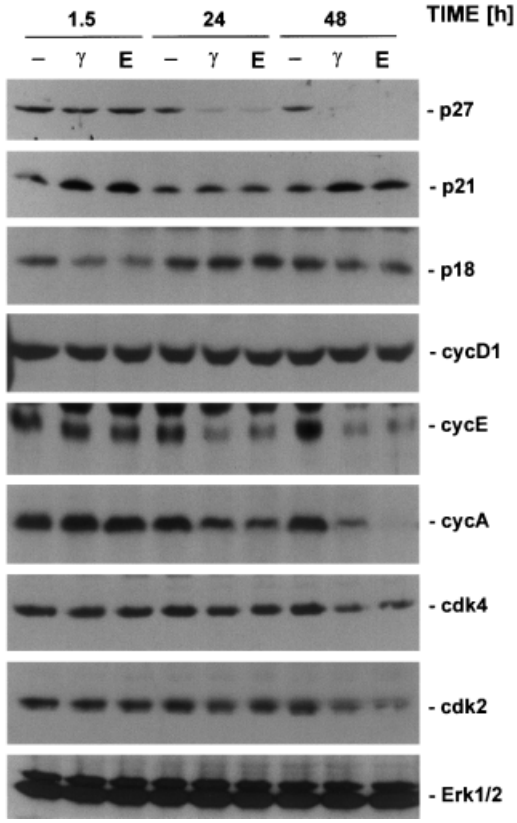

Figure 5
Similar effects on the expression of cell cycle regulatory molecules mediated by the IFN-γ receptor and the chimeric receptor EGΔBY440. A375 cells stably expressing the EGΔBY440 receptor were stimulated with IFN-γ (5000 U per mL) or EPO (10 U per mL) for the indicated times. Equal amounts of total cell lysates (30 μg protein) were separated on a 15% SDS polyacrylamide gel followed by western blot analysis.

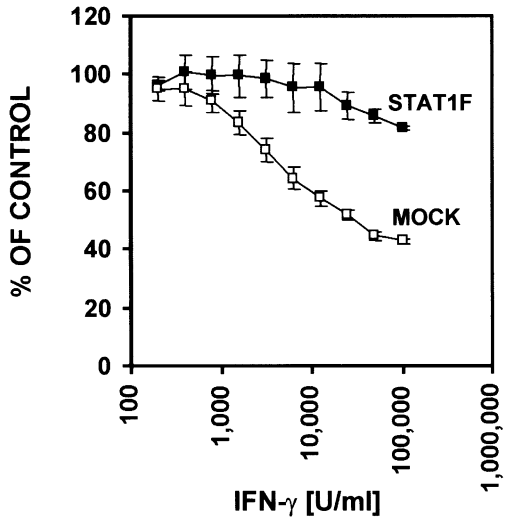

Figure 6
Dominant negative STAT1F abrogates IFN-γ-mediated growth inhibition. A375 cells transfected with the pCAGGS control vector (open squares) or with an expression construct for STAT1F (closed squares) were incubated for 4 d with IFN-γ at different concentrations. Cell growth was assessed by an XTT test, and values were recalculated according to untreated controls. Error bars represent the SD of triplicate samples.

A discrepancy in the level of activated STAT1 and the extent of IFN-γ-induced growth inhibitory effects While examining the responsiveness of the stable transfectants expressing EGΔBY440 in more detail, we observed a striking difference between the signal transduction of IFN-γ (via the endogenous receptor) and EPO (via the transfected receptor): stimulation of EGΔBY440 transfectants with increasing doses of EPO induced DNA-binding activity of STAT1, even at concentrations as low as 0.1 U per mL (Fig 7*A*, right panels). This increase in STAT1 DNA binding activity was paralleled by a proportionally enhanced inhibition in the growth of transfectants (Fig 7*A*, lower panel on the right). Similar to the parental A375 cells (Fig 1), IFN-γ-induced growth inhibition of EGΔBY440 transfectants was detectable at concentrations as low as 1000 U per mL. Gel shift assays indicated that, although the activation of STAT1 by IFN-γ was evident at doses as low as 10 U per mL and was near maximal at 100 U per mL, this dose was too low to affect cell growth (Fig 7*A*, left panels). The induction of STAT1 protein expression by both EPO and IFN-γ paralleled the respective dose–response of STAT1 activity to these factors (Fig 7*B*).

To study further this apparent discrepancy between IFN-γ-induced STAT1 activity and growth inhibition, we examined in detail the kinetics of STAT1 activation with respect to its tyrosine and serine phosphorylation and DNA binding. Doses of IFN-γ and EPO known to induce similar STAT1 activity but affecting cell growth with minimal (1000 U per mL IFN-γ) or maximal (10 U per mL EPO) efficiency were compared.

The short-term kinetics of STAT1 tyrosine and serine phosphorylation were comparable for both stimuli (Fig 7*C*). Two longer-term assays also showed no differences between IFN-γ and EPO stimulation: addition of IFN-γ and EPO resulted in comparable levels of GAS-element-mediated reporter gene activity after 18 h; stimulation of gene activity was not additive, as the addition of both IFN-γ and EPO did not further increase gene activity (Fig 7*D*). Moreover, both agents induced similar STAT1 DNA-binding activities at time points from 14 to 48 h (Fig 7*E*). Therefore, factors other than STAT1 are likely to account for the differential response of EGΔBY440 cells to the two cytokines.

No difference could be observed for STAT5 and IRF-1 as monitored by EMSA using probes for the respective transcription factors (Fig 7*E*). The time course of STAT5 activation paralleled the observed STAT1 activation, whereas DNA binding of IRF-1 was prominent 14 h after stimulation and slowly declined afterwards. C/EBPβ and c-myc, two other transcription factors known to be induced by IFN-γ in several cell types, were slightly downregulated by both stimuli after 24 h (Fig 7*F*), whereas STAT1 was dramatically upregulated.

Discussion

The growth inhibitory effect of IFN-γ on melanoma cells has long been known (Brown *et al*, 1987; Garbe and Krasagakis, 1993). IFN-γ has been used in adjuvant cytokine therapy studies (Agarwala and Kirkwood, 1996) and in gene therapeutic approaches in mouse models (Yu and Thomas-Tikhonenko, 2001) as well as in clinical trials (Nemunaitis *et al*, 1999). The mechanism of IFN-γ-induced growth arrest of melanoma cells, however, has not been examined in greater detail.

IFN-γ-induced growth arrest correlates with a complex downregulation of G_1/S cyclins and cyclin-dependent kinases This study indicated that IFN-γ leads to G_0/G_1 growth arrest in A375, WM239, and Mel-Im cells, whereas WM9 cells are resistant to IFN-γ. This growth arrest correlated with lower levels of certain cyclins (cyclin A and cyclin E) and cyclin-dependent kinases (cdk2 and cdk4). Levels of cyclin-dependent kinase inhibitors, however, were not affected or even drastically decreased (e.g., p21 in Mel-Im and WM239 cells, Fig 2). This finding was somewhat unexpected as IFN-γ induces p21 in a number of cell types, including multiple myeloma (Urashima *et al*, 1997), glioblastoma (Kominsky *et al*, 1998), colon adenocarcinoma (Hobeika *et al*, 1999), and breast cancer cells (Gooch *et al*, 2000), as well as in normal bone marrow macrophages (Xaus *et al*, 1999). Upregulation could be attributed to direct STAT1-mediated activation of the p21 gene promoter (Chin *et al*, 1996). An upregulation of p27 by IFN-γ was observed together with an increase in protein levels of other inhibitors, such as p21 (Mandal *et al*, 1998), p18/INK4c (Yamada *et al*, 1995), or with a concomitant rapid downregulation of positive cell cycle regulators, such as cyclin A, cdk2, cdc2, and c-myc (Harvat and Jetten, 1996; Harvat *et al*, 1997). It should be noted that p27 and p21 inhibitors are upregulated after treatment with interleukin-6 or upon γ-irradiation at least in A375 and WM239 cells (Kortylewski *et al*, 1999), and data not shown). IFN-γ-mediated cell cycle arrest without concomitant upregulation of p21 and p27, however, is not without precedent: in human mesothelioma cells IFN-γ appears to be able to induce a G_0/G_1 block

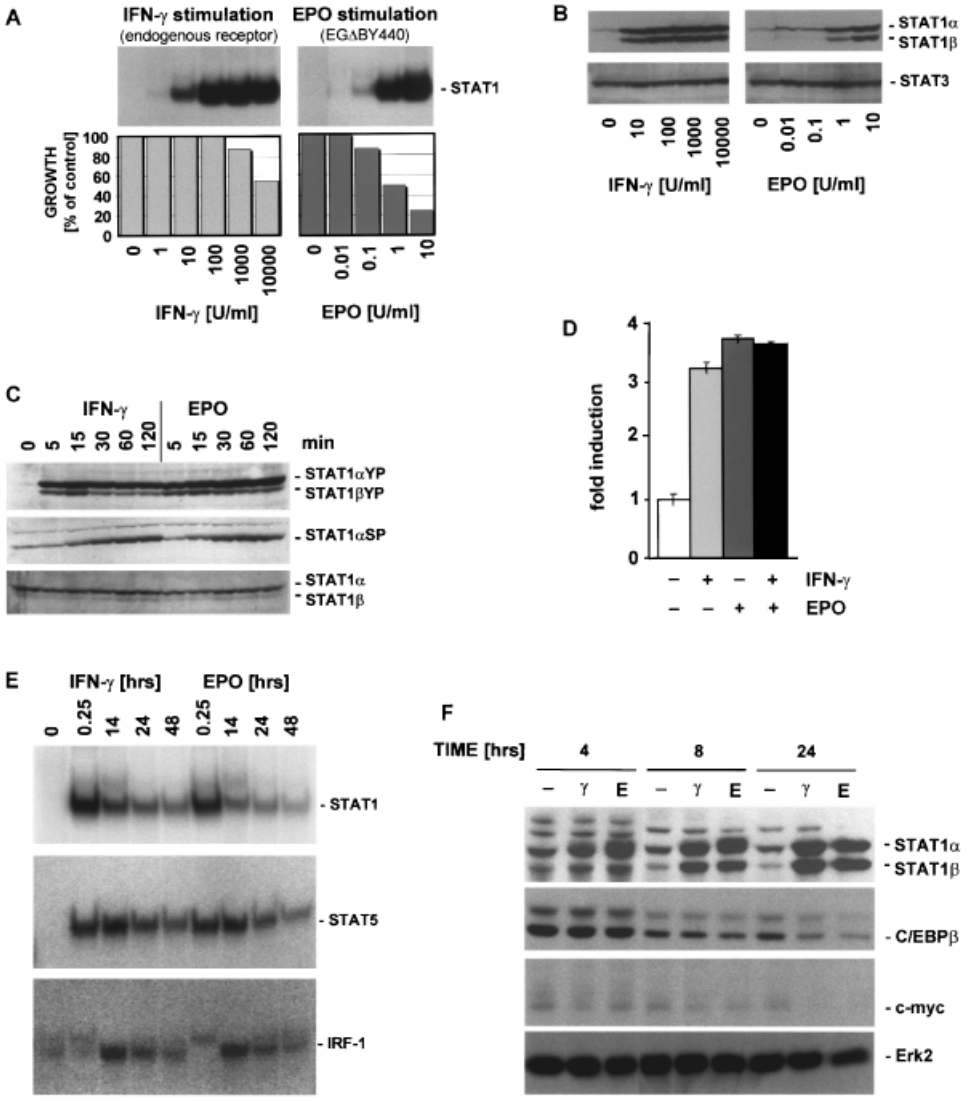

Figure 7
A discrepancy in the level of activated STAT1 and the extent of IFN-γ-induced growth inhibitory effects. (*A*) Upper panel: EGΔBY440 transfectants were stimulated for 15 min. Nuclear extracts containing 5 μg of protein were used for an EMSA with the SIEm67 probe. The position of homodimers of STAT1 is shown. Lower panel: effects of IFN-γ and EPO on growth of transfectants. Cells were cultured for 4 d in the presence of EPO at different concentrations. Growth was assessed by an XTT test. Means of triplicate values were recalculated according to untreated controls and extrapolated for the shown concentrations. (*B*) Cells were stimulated for 24 h with doses of IFN-γ or EPO as indicated. Equal amounts of total cell lysates (30 μg protein) were separated on a 7.5% SDS polyacrylamide gel followed by western blot analysis with antibodies against STAT1 and STAT3. (*C*) Cells were stimulated for the indicated times with IFN-γ (1000 U per mL) and EPO (10 U per mL). Equal amounts of total cell lysates (30 μg protein) were separated on a 6% SDS polyacrylamide gel followed by western blot analysis with antibodies directed against phosphotyrosine-STAT1, phosphoserine-STAT1, and STAT1. (*D*) Activation of a pIRF-1(GAS)-luc transcriptional reporter plasmid in A375 transfectants treated with IFN-γ (1000 U per mL) or EPO (10 U per mL) for 18 h. (*E*) EGΔBY440 transfectants were stimulated with IFN-γ (1000 U per mL) or EPO (3.5 U per mL) for the indicated periods of time. EMSA analysis of the DNA binding activity of STAT1, STAT5, and IRF-1. (*F*) EGΔBY440 transfectants were stimulated with IFN-γ (1000 U per mL) or EPO (3.5 U per mL) for the indicated periods of time. Equal amounts of nuclear extracts (30 μg protein) were separated on a 6% SDS polyacrylamide gel followed by western blot analysis using antibodies directed against STAT1, C/EBP and c-myc and Erk2.

independent of p21 and p27 inhibitors, with a concurrent reduction of cyclin A expression and its associated kinase activity (Vivo *et al*, 2001).

STAT1 deficiency does not account for IFN-γ resistance of WM9 cells Cytokine resistance of melanoma cells is often correlated with diminished Jak/STAT signaling (Wong *et al*, 1997, 1998; Pansky *et al*, 2000; Böhm *et al*, 2001). Wong *et al*, 1997 proposed that a deficiency in ISGF3 components may be responsible for the resistance of melanoma cells to IFN. Indeed, reduced levels of STAT1 were found in certain IFN-resistant cells (Wong *et al*, 1997; Pansky *et al*, 2000), and the restoration of normal STAT1 levels by IFN-γ induction or by overexpression or STAT1 or IRF-1 is sufficient to augment the responsiveness to IFN (Wong *et al*, 1997, 1998, 2002). As shown in this study, however, differences in the sensitivity of melanoma cells to IFN-γ cannot be explained entirely by differential STAT1 expression, as all cells expressed functional STAT1 and STAT1 protein levels substantially increased during IFN-γ treatment. Similarly, no correlation between the resistance of melanoma cells to IFN-α2 and IFN-β and STAT1 expression or activation was found in a recent study (Chawla-Sarkar *et al*, 2002).

Crucial role for STAT1 in IFN-γ-mediated growth inhibition of A375 cells The importance of STAT1 in IFN-γ-mediated growth inhibition of A375 cells was demonstrated by two different approaches: (1) a chimeric receptor was able to mediate growth inhibition of A375 transfectants after addition of the STAT activating tyrosine module Y440 from the IFNGR1, and (2) overexpression of a dominant negative form of STAT1 in A375 cells resulted in abrogation of the growth inhibitory effect of IFN-γ. We conclude that STAT1 is an essential mediator of IFN-γ-dependent G_0/G_1 growth arrest as has been reported for other tumor types (Bromberg *et al*, 1996; Urashima *et al*, 1997; Kominsky *et al*, 1998; Hobeika *et al*, 1999; Xaus *et al*, 1999; Gooch *et al*, 2000).

Evidence for divergent signals emanating from the IFN-γ receptor Interestingly, STAT1 activation by the chimeric receptor in EGΔBY440 transfectants induced a much more pronounced growth inhibition when compared with activation via the endogenous IFN-γ-R. EGΔBY440 contains the same STAT recruitment motif as the IFN-γ receptor. In the chimera, however, Jak activation is mediated by the membrane-proximal region of gp130, the signal transducing chain of interleukin-6-type cytokines. It could be possible that the gp130 part of the receptor might convey growth inhibiting signals, in addition to those mediated by STAT1. This explanation is unlikely, however, as the shorter chimeric receptor EGΔB (which lacks the STAT1 recruitment module) was not able to activate STAT nor did it affect the growth of A375 cells (Kortylewski *et al*, 1999) (Fig 4*A*). This observation is supported by the finding that EGΔB (in contrast to EGΔBY440) lacks the ability to upregulate the expression of typical IFN-inducible mRNA in fibrosarcoma cells (Strobl *et al*, 2001). The kinetics of STAT1 activation after stimulation of EGΔBY440 transfectants with EPO or IFN-γ were similar, precluding a differential sensitivity of the two receptor complexes, e.g., against feedback inhibitors. Therefore, we hypothesize that the activity of STAT1 as a mediator of growth inhibition may be counteracted by growth promoting signals elicited by the IFN-γ receptor.

Our observations are consistent with recent reports providing evidence for the existence of STAT1-independent pathways elicited by the IFN-γ receptor (Ramana *et al*, 2000, 2001; Gil *et al*, 2001). Stimulation of an IFN-γ receptor with a phenylalanine mutation of Y440 substantially enhanced serum-induced proliferation of mouse fibroblast cells, whereas the wild-type receptor inhibited this effect (Ramana *et al*, 2001). Moreover, IFN-γ induced survival and/or proliferation of bone marrow cells only in the absence of STAT1 (Gil *et al*, 2001). A series of genes is induced by IFN-γ in cells derived from STAT1–/– mice (Ramana *et al*, 2001).

Simultaneous activation of two apparently contradictory signals from a single receptor is not without precedent. The interleukin-6 signal transducer gp130 can simultaneously induce growth-enhancing and growth-suppressing signals in myeloid M1 and Ba/F03 pro-B cells (Nakajima *et al*, 1996; Fukada *et al*, 1998). The tumor necrosis factor receptor 1 also elicits both pro-apoptotic signals and anti-apoptotic signals (Hsu *et al*, 1996).

In therapeutic approaches involving IFN-γ as growth inhibitor it would be desirable to trigger growth arrest without concomitant activation of growth promoting signals. Therefore, future studies should focus on the further analysis of divergent IFN-γ-induced pathways and on possible mechanisms for their selective modulation.

Materials and Methods

Cell culture and reagents Human A375 melanoma cells were purchased from the American Type Culture Collection (CRL-1619). WM239 and WM9 cells (Herlyn, 1990) were received from Dr R.S. Kerbel (Sunnybrook Health Science Center, Toronto, Canada). Mel-Im cells are described in Jacob *et al* (1995). Cells were grown in RPMI 1640 medium (Gibco Life Technologies, Karlsruhe, Germany) supplemented with 5% fetal bovine serum, 50 μg penicillin per mL, and 100 μg streptomycin per mL. Human recombinant IFN-γ was purchased from PeproTech (Pepro Tech, London, UK). Human recombinant erythropoietin was kindly provided by Drs J. Burg and K.-H. Sellinger (Roche, Penzberg, Germany). Oncostatin M was purchased from PeproTech. All experiments were conducted with the approval of the RWTH Aachen Medical School.

Generation of transfectants The chimeric receptors EGΔB and EGΔBY440, combining the extracellular portion of the murine EPO receptor and the cytoplasmic portion of gp130, were constructed as described before (Gerhartz *et al*, 1996). Receptor EGΔB retains only the membrane proximal box 1/2 region, whereas receptor EGΔBY440 also contains the seven amino acid (Y440) STAT1 recruitment motif from IFNGR1. The wild-type and dominant negative STAT1 constructs in pCAGGS plasmids were gifts from Koichi Nakajima and Toshio Hirano (Osaka, Japan). Stable transfections were performed as described previously. In short, 2×10^6 cells suspended in 0.8 mL medium with 30 μg of the respective plasmid DNA were subjected to a double pulse (3.0 kV/99 μs) using an Electro Square Porator T820 from BTX (San Diego, California). Transfectants were later grown in the presence of 1.2 mg per mL of G418 (Gibco BRL) and clones expressing exogenous protein were selected by flow cytometry. For monitoring the expression of chimeric receptors EGΔB and EGΔBY440, approximately 10^6 cells were stained with polyclonal rabbit anti-serum against the extracellular region of the murine EPO receptor (kindly provided by Dr U. Klingmüller, Freiburg, Germany). As a secondary antibody, a phycoerythrin-conjugated donkey anti-rabbit IgG antibody (Dianova, Hamburg, Germany) was used. Fluorescence data were collected with a FACSCalibur and further analyzed using the CellQuest software (Becton Dickinson, Heidelburg, Germany). Heterologous expression of STAT1 and STAT1F was assessed with a monoclonal antibody directed against the hemagglutinin tag from BAbCO (BabCo, Richmond, CA) as described before (Kortylewski *et al*, 1999). At least two single clones from each transfection were chosen for further experiments and gave similar results.

Growth inhibition assay Viable cells (3×10^3) were seeded in triplicate into 96-microwell plates and incubated with various concentrations of cytokines in medium containing 1% fetal bovine serum. After 4 d of culture an XTT colorimetric assay (Roche) was performed as described previously (Kortylewski *et al*, 1999). The percentage of growth inhibition was calculated in relation to the growth of untreated control cells.

DNA staining and flow cytometry For the analysis of DNA content 5×10^5 cells were stained with propidium iodide using the CycleTEST Plus DNA Reagent Kit (Becton Dickinson) according to the manufacturer's protocol. Fluorescence data were collected with a FACSCalibur (Becton Dickinson) and further analyzed using the ModFit 2.0 software (Verity Software, Topsham, ME).

Electrophoretic mobility shift assays (EMSA) Nuclear extracts were prepared according to Wegenka *et al* (1993) and protein concentrations were measured using the Bio-Rad protein assay (Bio-Rad, München, Germany) Briefly, 10 fmol (10,000 cpm) of an

α-^{32}P-labeled double-stranded oligonucleotide was added to nuclear extracts containing 5 μg of protein and incubated in gel shift incubation buffer: 10 mM HEPES pH 7.8, 1 mM EDTA, 5 mM $MgCl_2$, 10% glycerol, 5 μM dithiothreitol, 0.7 μM phenylmethylsulfonyl fluoride, 0.1 mg poly(dI-dC) per mL, and 1 mg bovine serum albumin per mL for 10 min at room temperature. The protein-DNA complexes were separated on a 4.5% polyacrylamide gel containing 7.5% glycerol in 0.25-fold TBE at 20 V per cm for 4 h. Gels were fixed in a water solution of 10% methanol and 10% acetic acid for 30 min, dried, and autoradiographed. Data were further analyzed using a Personal FX Phosphorimager with the Quantity One software (Bio-Rad). The oligo probes correspond to the c-fos promoter (m67SIE: 5′-GAT CCG GGA GGG ATT TAC GGG AAA TGC TG-3′, binds STAT1 and STAT3) or to the β-casein promoter (5′-TTA GAT TTC TAG GAA TTC AAT C-3′, binds STAT5). The sequence for the IRF-1 binding probe is 5′-GAT CCT CGG GAA AGG GAA ACC GAA ACT GAA GCC-3′. For supershift analysis, nuclear extracts were incubated for 15 min at room temperature in the presence of polyclonal anti-STAT1 (E23) and anti-STAT3 (C20) antibodies (Santa Cruz Biotechnology, Heidelburg, Germany) before the radioactive probe was added.

Immunoblotting analysis Cells were lyzed on the plate in lysis buffer containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 20 mM Tris, pH 7.2, 158 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_2VO_3, 1 mM phenylmethylsulfonyl fluoride, 5 μg aprotinin per mL, and 5 μg leupeptin per mL. Lysates were further cleared by centrifugation at 12,000 × *g* and the protein concentrations were determined with the protein assay reagent from Bio-Rad. Equal amounts of protein were separated by SDS/polyacrylamide gel electrophoresis, transferred to PVDF membrane (GelmanSciences, Ann Arbor, MI), probed with the respective antibodies and detected for signals using the ECL system (Amersham Pharmacia, Freiburg, Germany). The antibodies used were monoclonal anti-p21 (SX118; Pharmingen, Heidelburg, Germany), polyclonal phospho-specific anti-STAT1 antibodies (tyrosine 701 and serine 727; New England Biolabs, Frankfort a.M., Germany) and polyclonal anti-STAT1 (E-23), anti-cdk2 (M2), anti-cdk4 (H-303), anti-cyclin D1 (R-124), anti-cyclin E (C-19), anti-cyclin A (C-19), anti-p18 (N-20), anti-p27 (C-19), anti-Rb (C-15), anti-Erk1 (C-16), and anti-Erk2 (C-14) antibodies (Santa Cruz Biotechnology).

Histone H1 kinase assay Histone H1 kinase assays were performed according to Hermeking *et al* (1995). Briefly, the anti-cyclin E (C-19) or anti-cyclin A (C-19) immunoprecipitates were prepared from 150 μg of lysate protein and were incubated for 20 min at 30°C in 20 μL of kinase buffer (20 mM $MgCl_2$, 10 mM EGTA, 40 mM HEPES pH 7.0) with 1 μg of histone H1 (Roche) and 1 μL of 300 Ci per mmol of [γ^{32}P]-adenosine triphosphate (Amersham). After stopping the reaction with 2 × SDS sample buffer, phosphorylated proteins were resolved by 12% SDS/polyacrylamide gel electrophoresis and signals were analyzed using a Personal FX Phosphorimager with the Quantity One software (Bio-Rad).

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