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MECHANISMS OF INTERFERON-INDUCED CELL CYCLE ARREST

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1. ABSTRACT

The interferons (IFNs) are a group of cytokines, which in addition to their antiviral activity are capable of modulating a variety of cellular responses. One such prominent effect of IFNs is their potent antimitogenic action, which can be observed both on malignant and non-malignant cells of many different origins. IFNs are also used in the clinic, mainly in malignant and viral diseases, and their cell growth -inhibitory effect has been suggested to be of major importance in their antitumour and antiviral action. The aim of the present review is to provide insight into the molecular mechanisms by which IFNs modulate cell cycle progression in various cell types. With the recent progress in our understanding of how the cell cycle is regulated at the molecular level, it has become possible to delineate intracellular effectors of IFN in this respect. Understanding the antiproliferative effects of IFN may not only help in understanding its antineoplastic and antiviral activities, but may also provide an insight into cell cycle regulation in general and aid in making IFNs a more useful tool in treating disease.

2. INTRODUCTION

In multicellular organisms, cell growth has to be tightly controlled and exquisitely sensitive to changes in the environment. The network of cytokines/growth factors is one of the tools by which the organism obtains balanced growth and tissue homeostasis. To date, an abundance of growth factors have been described, and it was long ago predicted that the large number of positive growth factors would be balanced by an equal abundance of negative growth regulatory cytokines. Until now, however, only a small number of cytokines which act as inhibitors of cell growth have been defined. As many pathological states, including neoplastic and cardiovascular diseases, are at least in part, caused by hyperproliferation, it is of importance to elucidate the molecular mechanisms used by such intrinsic negative growth factors. Understanding the antimitogenic effects of such cytokines will also provide an insight into some aspects of normal cell growth regulation and the regulatory mechanisms which take place in immunity and during hematopoiesis.

The interferons (IFNs) constitute a family of secreted proteins with pleiotropic cellular effects including inhibition of cell proliferation, induction of differentiation, modulation of the immunsystem, and alterations of the cell surface (1). The common denominator for all IFNs is, however, inhibition of viral replication. Two major types of IFNs exist, type I, including IFN-alpha, IFN-beta and IFN-omega, and type II IFN which consist of IFN-gamma (1). Type I and type II interferon's signal through distinct but related pathways (2). The elucidation of the molecular signaling from the IFN receptor has become a role model for cytokine signaling, and is today one of the best characterised pathways.

The binding of IFN to specific receptors trigger signals that are transmitted from the cell surface to the nucleus. IFN receptors lack intrinsic kinase activity and rely on associated Janus family kinases (Jaks) which in turn activate signal transducers and activators of transcription (Stat) proteins by phosphorylation (2). Stimulation with IFN-alpha and beta leads to phosphorylation of Stat1 and

Stat2, which form an oligomeric complex called ISGF3 also containing a third protein, p48. IFN-gamma stimulation on the other hand, leads to phosphorylation of Stat1 and the formation of Stat 1 homodimers. Activated (phosphorylated) Stats translocate to the nucleus as oligomeric complexes and induce transcription of IFN stimulated genes (ISGs) through the binding to so called IFN stimulated responsive elements in the case of ISGF3 and to gamma IFN activated sites (GAS) elements in the case of Stat1 homodimers. The most well characterised ISG's are also part of the antiviral response, acting as inhibitors of protein synthesis and by mediating RNA cleavage. IFN dependent induction of two such genes, 2'-5'-oligoadenylate synthetase (2-5A synthetase) and the dsRNA-dependent protein kinase (PKR) is not sufficient for an anti-viral response, but in the presence of ds-RNA, these enzymes becomes activated and are able to target the viral infection. The Mx-genes are also induced by IFN and protects the cell from infection by inhibiting viral replication (2). Furthermore, less well-characterised genes are highly induced upon IFN stimulation such as ISG15, ISG54 and 6-16 (2). The growing family of transcription factors named Interferon Regulatory Factors (IRFs) play multiple roles in the IFN system. They can be induced by IFNs and viruses, they can themselves regulate IFN induced transcription both in a positive and negative manner, and also play an important role in the transcriptional induction of the IFN genes themselves (3). Apart from this IRFs may regulate proliferation, apoptosis and differentiation in different systems (3).

As early as the 1960s, it was recognized that IFNs could exert potent cell growth -inhibitory effects in some cells. Clinically, IFNs have become the accepted treatment option in several viral diseases, but have also been found to exert potent antitumour effects in a number of malignant conditions. These include hematological disorders such as hairy cell leukaemia and chronic myeloid leukaemia, and also some solid tumors such as mid-gut carcinoid, melanoma and renal cell carcinoma (4). Although the exact mechanism behind IFN's antitumour and antiviral action is still unclear, IFN's cell growth -inhibitory action has been suggested to be of great importance (5, 6).

The early finding that IFN exerts anti-proliferative effects has more recently been shown to be true for both malignant and non-malignant cells of many different origins (7). However, cells differ greatly in their sensitivity to these effects of IFNs. Some malignant cell lines are extremely sensitive in this respect, such as the Daudi Burkitt's lymphoma cell line, whereas in a few instances cell growth can even be stimulated by interferon (6, 8). In different *in vitro* systems, the culture of susceptible cells with IFN has been found to affect various phases of the mitotic cycle. Most commonly, IFN treatment leads to G1 arrest (9), though sometimes slowed growth is due either to a blockage or prolongation of S-phase or a lengthening of all cell cycle phases (10, 11). Only recently has the question of whether G1 arrest may represent an exit into G0 been addressed. It was found that IFN can indeed induce a G0-like state in some neoplastic cells (12, 13), but whether induction of a G0 state is a general phenomenon in cells which are G1-arrested by IFN remains to be determined. Furthermore, in some instances this is clearly not the case as cells may sometimes become arrested at the G1/S border (13).

Until recently, the molecular background of IFN-induced cell cycle arrest was poorly understood. It had been recognized that a number of the genes transcriptionally regulated by IFNs could exert cell growth -inhibitory effects when over-expressed, such as the previously mentioned kinase PKR, 2' 5' oligoandenylate synthetase and the transcription factor IRF-1 (14). It was also well established that IFNs could antagonize the action of certain growth factors, and that they could rapidly reduce the expression of some growth factor -regulated genes, such as c-myc (15, 16).

With current knowledge in the field of cell cycle regulation, it has become possible to determine more directly how IFNs modulate proteins in the core machinery normally regulating the G1/S-phase transition.

3. REGULATION OF THE CELL CYCLE

The commitment of cells to enter S-phase of the cell cycle occurs at the restriction point in late G1 phase, after which further passage through the cycle becomes independent of growth factors (17). In all organisms studied so far, temporal order and continued passage through the cycle is dependent on the sequential formation and activation of a series of serine/threonine protein kinases. These protein complexes consist of a regulatory subunit, termed a cyclin, and a catalytic subunit, the cyclin-dependent kinase (Cdk). From G1, following mitosis, cells may also enter a stable "resting state" called quiescence, or G0. As cells enter the cycle following mitosis (or alternatively from quiescence), the D-type cyclins (D1, D2 and D3) and cyclin E are synthesized sequentially, leading to the formation of cyclin D-Cdk4/6 and cyclin E-Cdk2 complexes (18). Other Cdk-complexes, such as cyclin A-Cdk2, operate in S and G2, whilst cyclin B-Cdk1 functions in G2/M phase (18). To ensure fine-tuning in the regulation of proliferation, the activity of these complexes is regulated at multiple levels, including the synthesis and destruction of the regulatory cyclin, and by phosphorylation and dephosphorylation on specific residues of the kinase subunit by the enzymes CAK and Cdc25A, respectively (19).

Recently, a new level of regulation of these complexes has been elucidated by the identification of two families of low molecular weight cyclin-dependent kinase inhibitors (CKIs) (18). The Ink4 family, including the p15, p16, p18 and p19 proteins, which all contain multiple ankyrin repeat-like sequences, bind specifically to Cdk4 and 6, thereby interfering with cyclin D/Cdk interactions. Recently, an alternative first exon (E1beta) of the p16 gene was described. When the E1beta exon is spliced onto exon 2 of the p16 gene, the original reading frame is shifted, and translation of this so-called beta transcript creates a protein completely unrelated to p16, called p19ARF. Interestingly, this protein seems to have cell growth -inhibitory properties

by enhancing p53-related functions through degradation of an important negative regulator of p53, the MDM2 protein (20).

The Cip/Kip family includes the p21, p27 and p57 proteins, which share no structural homology with the Ink4 proteins (18). The latter group of Cdk inhibitors associates with and inhibits the kinase activity of cyclin E-Cdk2 and cyclin A-Cdk2 complexes, in addition to inhibiting cyclin D-Cdk4/6 complexes. Whether the Cip/Kip proteins inhibit Cdk4 and 6 kinase activity is still a matter of dispute, and it has been proposed that binding of p21/p27 to these kinases rather serves as a latent pool of Cdk2 -inhibitory activity (21). The p21 gene is transcriptionally regulated by the p53 tumour suppressor gene product, thereby mediating p53-induced cell cycle arrest. However, p53-independent induction of p21 has also been described (22, 23). The p27 protein is implicated in the negative regulation of G1 progression in response to a number of anti-proliferative signals, such as TGF-beta, contact inhibition and cyclic AMP (24, 25), and has been shown to be important in keeping cells in a quiescent state (26).

Many proteins have been suggested as substrates for the different cyclin/Cdk complexes. A main substrate of cyclin D-CDK4/6 is the pRb protein that influences the transcription of genes necessary for G1 progression through binding and regulation the activity of members of the E2F family of transcription factors (E2F1-3) as well as histone deacetylases (HDAC:s) (27, 28). pRb-E2F-HDAC complexes may actively repress transcription of genes in quiescent cells and during the first part of the G1 phase. The phosphorylation of pRb by cyclin D-CDK4/6 induces a conformational change, which disrupts its binding to HDAC (28). This change causes de-repression of some genes with E2F sites in their promoters. Further phosphorylation of pRb, and the two other pocket proteins p107 and p130 by cyclin E-CDK2 leads to release of E2F and transactivation of E2F-responsive genes required for S phase progression, such as DNA polymerase alpha, dihydrofolate reductase, thymidine kinase and c-myc (29, 30). The E2F transcription factors consists of a heterodimeric complex, containing a subunit encoded by the E2F gene family and a subunit encoded by the DP family of genes. To date, six different E2F genes (E2F 1-6), and two DP (DP 1-2) genes have been identified (27). Distinctions can be seen in the nature of interaction between the individual pocket protein family members and the specific E2F proteins. pRb interacts mainly with E2F 1-3, p130 with E2F 4-5 and p107 exclusively with E2F4 (27). Finally, there are differences in the expression pattern between these proteins; pRb is expressed in both growing and quiescent cells while the accumulation of p130 and p107 is tightly regulated by the growth state of the cell. The p130 protein being highly expressed in quiescent cells whereas growing cells exhibit low p130 levels, while the inverse is true for p107 (29). Several observations suggest that the phosphorylation of the pRb protein by cyclin/Cdk complexes is a rate limiting step in G1 progression, and is involved in triggering several positive feedback loops, leading to an irreversible progression through late G1-phase and into S-phase (31).

Deregulated growth control in malignant cells has recently been associated with mutations in core cell cycle regulatory genes. This either leads to the over-expression of growth-promoting proteins, such as cyclin D1, Cdk4, Cdk6 and c-myc, or the inactivation of growth inhibitory proteins, for example p16 or pRb (31, 32).

4. MOLECULAR EFFECTS OF IFN ON CELL CYCLE REGULATING PROTEINS; EFFECTS IN MALIGNANT AND NON-MALIGNANT CELLS

4.1. Effects on the core cell cycle machinery components

4.1.1 Pocket proteins

Previous reports established an important link between IFNs and one of the key cell cycle regulatory proteins, the product of retinoblastoma tumour suppressor gene, pRb. In these early studies, IFN was shown to change the phosphorylation state of pRb from the inactive hyper-phosphorylated form to the active, growth-suppressing hypo-phosphorylated form (33). Since then, IFNs have been shown to decrease the phosphorylation state of pRb in various cell types. Recently, IFN-alpha treatment has been found to affect the phosphorylation and expression of not only pRB, but also the other two pocket proteins p107 and p130 (13, 34). Following IFN-alpha -induced G0-arrest in a lymphoma cell line, all three pocket proteins were found to be in their hypo-phosphorylated active state. In addition, the total levels of pRb and p107 were significantly decreased in this cell line, leaving the cells in a state reminiscent of quiescent non-malignant lymphocytes (13, 34). In contrast, a cell line that carries a mutated Rb gene, dephosphorylation of the pocket proteins, p130 and p107, was found to be less pronounced and the total levels of p107 were kept high (13). Interestingly, this pRb-defective cell line was found to accumulate in late G1 phase following IFN-alpha treatment (13). Whether the inability of this latter cell line to enter quiescence is due to the non-functional pRb protein remains to be shown, but this remains a plausible explanation. The effects of IFN-alpha on pocket protein activity have recently been further developed through analysis of transcriptional suppression of the E2F-1 gene. In a recent study, IFN-alpha treatment resulted in the formation of E2F-4/pRb and E2F-4/p130 complexes on the E2F-1 promotor, acting as repressors of E2F-1 mRNA transcription (35).

4.1.2 Cdks

How do IFNs reduce the phosphorylation of the pocket proteins? Since pocket protein phosphorylation is regulated by the Cdk-complexes, several groups have addressed the issue of whether IFN acts by down-regulating Cdk activity. Indeed, both type I and type II IFNs have been shown to strongly repress the activity of the Cdk2 -4 and 6-complexes in a number of different cell types (12, 13, 23, 36, 37, 38). There are several possible explanations for this effect, as there are many ways of regulating Cdk activity (19, 26). First, IFN could act by decreasing the levels of the Cdks or their regulatory cyclin subunits. Second, it could increase the expression of one or several Cdk-inhibitory proteins, or, thirdly, influence the phosphorylation status of the Cdks by affecting CAK-

activity or expression of Cdc25A. Experimental data have shown that, indeed, IFN influences Cdk activity through all of these mechanisms.

In the Burkitt lymphoma cell line Daudi, which is the most extensively investigated model system in this respect, somewhat conflicting data have been described concerning the effects on G1 cyclins. In one study, no immediate reduction in protein levels of the cyclins D3, E or the S-phase specific cyclin-A, could be observed that explain the rapid reduction in Cdk4/6 and Cdk2 activity which is evident after around 8-12 hours of exposure to IFN-alpha. Only after longer incubation times with IFN-alpha (24-48 hours) were expression levels of cyclin D3 and cyclin A found to decrease (13). Moreover, cyclin-E levels were found to accumulate at longer exposure times, a finding that may be explained by the fact that inhibition of Cdk2 activity leads to the increased stability of cyclin E through reduced proteosomal degradation (39). In contrast to these data, other investigators have found a more rapid reduction of cyclin D3 levels in Daudi cells, being evident already after 4 hours of IFN treatment (12). The reason for this discrepancy remains to be shown, but may be due to different Daudi sub-lines. In no case, however, was any major reduction in G1 Cdk levels observed.

As mentioned above, full Cdk activity is only acquired if the kinase subunit is dephosphorylated at certain residues and phosphorylated on others (19). This requires both the phosphatase Cdc25A and the Cdk-complex CAK (Cdk7/cyclin H). With regard to the effects of IFN-alpha on Cdc25A, somewhat conflicting results have been reported. In one study, there was a rapid down-regulation of the phosphatase Cdc25A following IFN-alpha treatment, preceding inhibition of Cdk2 activity (12). In other studies, however, down-regulation of Cdc25A levels seems to occur rather late, or not at all, after IFN-alpha treatment (13, 40). Again, these discrepancies could be due to different clones of Daudi cells used by these groups. The molecular background of this effect of IFN-alpha on Cdc25A remains to be elucidated, but may be associated with the down-regulation of c-myc, as myc activity seems to be closely linked to Cdc25 expression (41). Phosphorylation of Cdk2 by CAK is also affected by IFN-alpha. This is possibly due to increased levels of Cdk-inhibitors (see below) which may block phosphorylation by CAK, as decreased Cdk2 phosphorylation occurs at longer incubation times only, and is not associated with down-regulation of the CAK-kinase itself (13, 42).

4.1.3. CKIs

To date, there are a number of studies that have shown a rapid induction of the CKI p21 following both type I and type II IFN treatment in various cell types (13, 23, 40, 42, 43). In some studies, IFN-alpha also acts as an inducer of the Ink4-inhibitors, p15 and p19 (13, 23, 43). The mechanism behind the upregulation of these CKIs seems to be the result of transcriptional regulation, since their mRNA levels rise rapidly following IFN-alpha treatment (23). Recently, a growing number studies also show an increased expression of the CKI p27 following IFN-treatment (13, 37). The increase in p27 protein levels does not seem to result from transcriptional regulation, as no significant increase in p27 mRNA levels could be observed following IFN-treatment (13, 37). Rather, p27 seems to be regulated by a post-transcriptional mechanism, since the half-life of the protein is significantly increased following IFN-alpha treatment (Sangfelt et al. unpublished data). Furthermore, the enhanced expression of CKIs following IFN-alpha treatment leads to an increased binding of these inhibitory proteins to the Cdks, inhibiting their activity (13). Together, these results suggest that induction of p21/p15 is involved in a primary IFN-alpha response that inhibits G1 Cdk activity, whereas increased p27 expression is part of a second set of events (possibly together with other changes such as decreased cyclin and Cdc25A levels in some cell types) which keep these Cdks in their inactive form (13). These effects on Cdk-inhibitors are in marked similarity with the effects of other negative cell growth signals such as exposure to TGFbeta (24, 25), indicative of a common final pathway for various anti-proliferative signals. Finally, whereas p15 and p19 induction seems to be cell-type restricted, p21 induction seems to be more ubiquitous, occurring in cell lines from various tissues (23, 36, 43, Sangfelt et al. unpublished data), and it has recently also been demonstrated that IFN-alpha upregulates p21 in primary leukemic tumor cells (Szeps et al. unpublished data) Interestingly, IFN-alpha has also been reported to cause a significant upregulation of the p16E1beta transcript in one cell line, but the functional significance of this finding is unclear (23).

4.1.4. Effects on c-myc

The c-myc gene was identified as one of the first downstream targets of IFNs, and it has been reported that IFN-alpha/beta reduces c-myc mRNA levels in several hematopoietic cell lines (44), and that IFN-gamma inhibits the expression of this gene in epithelial cells (45). The reduction in c-myc mRNA mainly seems to result from transcriptional inhibition, and has been shown correlate with an IFN-dependent reduction in the DNA-binding activity of the transcription factor E2F to its recognition sequence within the c-myc promotor (33). From genetic and pharmacological manipulations of growth sensitive cell lines, there is strong support for the existence of multiple pathways, which participate in regulating IFN's growth-inhibitory response. For example, the introduction into IFN-sensitive cells of myc constructs driven by heterologous promoters which fail to be inhibited by IFN, abrogated the prominent type of G0/G1 arrest. Yet this deregulated c-myc expression did not fully restore the proliferative activity of these IFN-treated cells (33). It is noteworthy that myc down-regulation does not seem to be a prerequisite for IFN induced G1 arrest (23, 36, 46). Also, although IFN-alpha and IFN-gamma -induced activation of the Rb protein has been implicated as an important effector in the anti-mitogenic response to IFNs (13, 33, 47), the reduction of c-myc was shown to occur in an pRB-independent manner (33). Similarly, another tumor suppressor gene, p53, with the capacity to transrepress c-myc (48), is not obligatory for IFN-induced G1 arrest (23, 36). Little is known about the signaling events leading to c-myc down-regulation, but depletion of protein kinase C (PKC) does not interfere with the negative effects of IFN

on c-myc mRNA levels in spite of its involvement in the growth response to IFN (49). However, results from other studies functionally place the IFN-induced serine/threonine p68 protein kinase (PKR) upstream of c-myc down-regulation (50). Thus, the exact role of c-myc in IFN-mediated growth suppressive pathways is far from clear, and more mechanistic studies are warranted.

4.2. IFN-stimulated genes

Several components in the IFN system have been suggested as potential mediators of IFNs' growth-suppressive activities. The IFN-induced proteins PKR, 2´,5´-oligoadenylate synthetase (OAS) and 2´,5´-OAS-dependent RNaseL, have all been shown to exert growth-suppressive activity (51, 52, 53). Moreover, the transcription factor IRF-1 demonstrates tumour suppressor activity and inhibits cell growth when over-expressed (14). IRF-1 may function by activating IFN-inducible genes such as PKR, and a catalytically inactive dominant negative PKR mutant has indeed been shown to abolish the anti-proliferative action of IRF-1 (54). Another IFN-induced protein with growth-suppressive properties, the p202 protein, has recently been shown to affect several cell cycle -regulatory proteins and binds to pRB (55), but the exact growth-suppressive mechanism remains to be described.

4.3. The role of IFN signaling

Although the JAK/STAT pathway that mediates IFN-signaling has been the subject of intense investigation, the components directly responsible for IFN-induced cell growth inhibition have yet not been fully established. For example, it is not known whether the promoters of CKIs are directly transcriptionally regulated by IFN-alpha, and if so through what promoter elements/transcription factors. The results from studies using IFN-gamma clearly implicate the direct involvement of STAT1 in the regulation of the CKI p21 (56). In a different study, STAT1 mutations and STAT1/STAT3 domain swaps were tested for their ability to promote the growth-inhibitory effect of both IFN-alpha and IFN-gamma after reconstitution of STAT1-deficient cells. Growth inhibition in response to IFN-alpha was reconstituted with STAT1-beta or STAT1 containing the STAT3 DNA-binding domain, whereas IFN-gamma–induced growth-inhibition required intact STAT1-alpha, which is needed to form active STAT1 homodimers, with its own DNA-binding domain (57). This indicates that IFNs activate different STAT-containing complexes to induce cell growth inhibition. Interestingly, the p21 promoter contains three potential GAS elements which may bind STAT1 homodimers, but no obvious ISRE element, which normally binds the IFN-alpha induced transcription factor ISGF3. Together, these studies suggest a direct involvement of the STAT family of transcription factors in both type I and type II IFN-induced expression of the CKI p21, but the exact combination of STAT proteins that regulate the expression of the p21 gene in response to IFN remains to be shown. Results from IFN signaling transgenic- and knockout -systems using IFN signaling genes other than STAT1 have so far not provided clear evidence for what specific signaling components that mediate IFN-induced cell cycle arrest (58). However, these systems have provided some clear evidence that IFN-gamma induced Stat1 activation can induce apoptosis in some instances, whereas other cells become protected by IFN-gamma against an apoptotic response due to the upregulation of p21 (59, 60). To date, there is no link between IFN signaling and other downstream targets of IFN involved in cell cycle arrest, such as Cdc25A, cyclinD3 and p15. Furthermore, there is accumulating evidence for cross-talk between the JAK/STAT pathway and other signaling cascades, such as the Ras-dependent pathways (61), and the importance of these alternative pathways in IFN-induced G1 arrest can not be excluded.

4.4. Causes for resistance to IFN's anti-proliferative effects

As previously mentioned there is a great variability in the cellular sensitivity to IFN's cell growth inhibitory action, some cell lines being extremely sensitive, others being more or less resistant. With the recent elucidation of the molecular background to IFN's cell growth -inhibitory effects, possible causes for resistance can be addressed.

One cause for resistance could be defects in the signal transduction pathway of IFN, which has been described in both primary tumor cells and established cell lines. However, there are frequent examples of cells that are resistant to the cell growth -inhibitory effects of IFN, although they have a seemingly functional IFN signaling (8). As IFNs act by regulating the key cell cycle regulatory proteins which are also commonly mutated in malignant cells, there exists the possibility that the malignant genotype may be decisive in the cell's sensitivity to IFN. One such example is the previously mentioned abrogation of G0 arrest by forced over-expression of c-myc. In another cell line, which is not growth arrested by IFN, the resistance correlated with an inability to produce the p21 protein, presumably due to a mutation in the p21 gene (23). Furthermore, the fact that cells which either lack Rb expression, or which are unable to downregulate c-myc levels, are still G1-arrested by IFN, does not exclude the possibility that the arrest in cells with these genetic defects may be qualitatively different from cells without these aberrations, for example in their inability to exit into G0. Another possibility is that the sensitivity to IFN may depend on a variable expression of cell cycle -regulating proteins during different stages of differentiation or in different tissues.

4.5. Effects in non-malignant cells

The majority of studies investigating the molecular background of IFN-induced cell cycle arrest have been performed in transformed cell lines, and little is known about how IFNs regulate the cell cycle in a non-malignant context.

In order to understand this issue further, we have investigated the effects of IFN-alpha on the proliferative response of non-malignant T-lymphocytes. This system has the advantage of well-defined *in vitro* stimulation protocols, in combination with previously demonstrated effects on the cell cycle machinery. Peripheral T-lymphocytes are situated in the G0-phase of the cell cycle

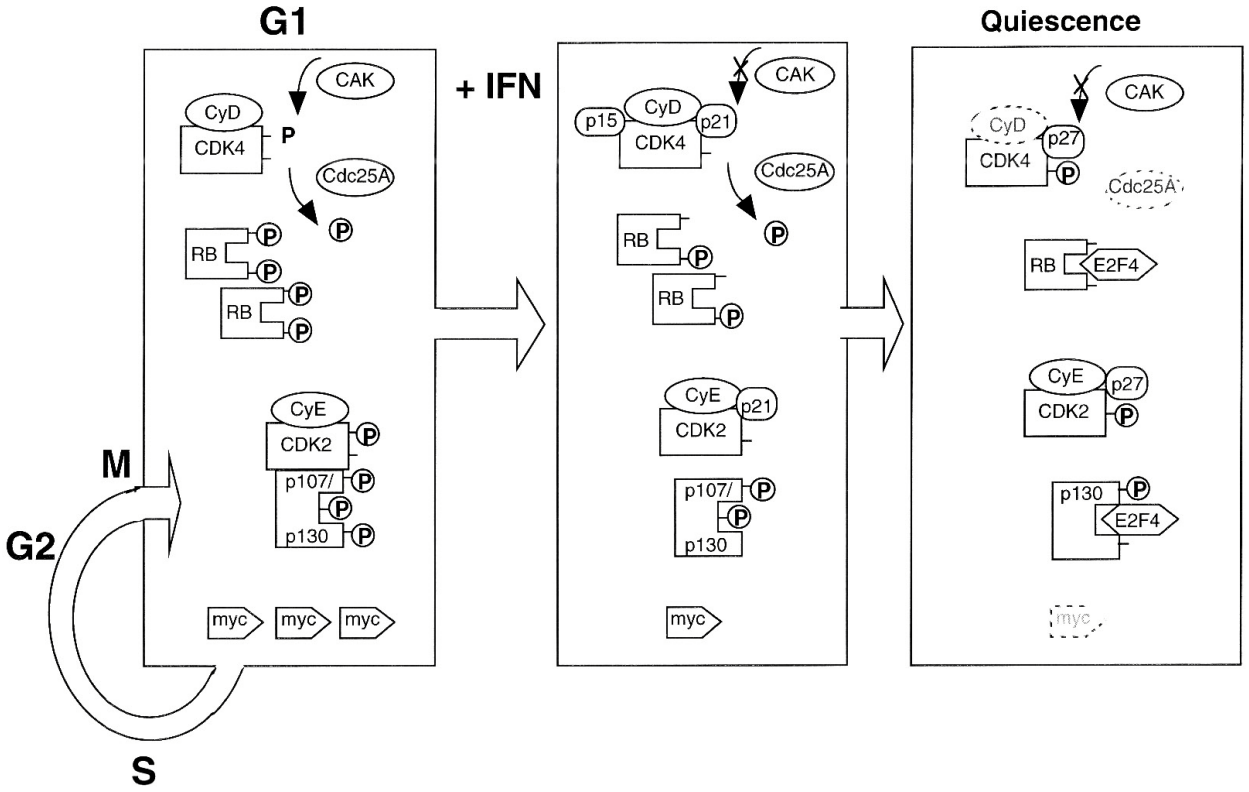

Figure 1. The proposed model for IFN-alpha induced G0-like arrest. Addition of IFN to exponentially growing cells can cause a G0-like arrest. This is associated with a down-regulation of Cdk4 and Cdk2 activity, presumably leading to activation of the pocket proteins. This effect is thought to be brought about through a primary response involving the transcriptional induction of the Cdk inhibitors p15 and p21. This is followed by a secondary response involving a post-translationally regulated increase in p27 levels, down-regulation of cyclins D and A, as well as down-regulation of the Cdk-activating phosphatase Cdc25A. The binding of Cip/Kip inhibitors to the G1 Cdks also leads to a blockage of their phosphorylation by the CAK enzyme. In parallel with the above mentioned processes the levels of c-myc are rapidly downregulated.

until activated by antigen presentation and stimulation by growth-promoting cytokines such as IL-2 (62). The molecular hallmarks of quiescent T cells are high levels of p27, and low levels of the G1 cyclins and Cdks. T-cell receptor stimulation followed by IL-2 addition results in rapid down-regulation of p27 as well as the induction of G1 cyclins adn Cdks, generating Cdk kinase activity and pocket protein phosphorylation leading to entry into S-phase (46, 62, 63)

IFN-alpha was shown to be a potent inhibitor of IL-2 -induced proliferation in peripheral T-lymphocytes. This effect was associated with an almost complete abrogation of the IL-2 -induced effects on the cell cycle proteins mentioned above (46). However, the effect of IFN-alpha on IL-2 signaling was selective, as the induction of growth-promoting genes such as c-myc and Cdc25A was not prevented by IFN-treatment (46).

IL-2 activates multiple signaling pathways necessary for the proliferative response in T-cells, such as activation of phosphatidylinositol 3-kinase (PI3K) as well as the transcription factor STAT5 (64, 65). IFN-alpha was found to target one of these pathways by inhibiting the prolonged IL-2 -induced Stat5 DNA binding, whereas IL-2 -dependent activation of the PI3K pathway seems unaffected by IFN (Erickson et al. Unpublished data). The importance of cross-talk between the IFN signaling pathway and other signal transduction pathways is underlined by the finding that IFN-induced growth inhibition in T-cells requires the association of several components of the T-cell receptor (TCR) with the IFN-alpha receptor signaling complex (66).

5. PERSPECTIVES

IFNs can exert profound anti-mitotic effects in cell cultures. As discussed above, the cell growth-inhibitory effects of IFNs most likely involve multiple molecular pathways. It is clear from these studies that IFNs are potent regulators of several components directly involved in the cell cycle machinery, such as Cdks, their inhibitors (CKIs) and their substrates (the pocket proteins). Based on these findings, a proposed model for the molecular background to IFN mediated G0 arrest in malignant cells may be constructed (Figure 1). However, a more complete picture on how IFNs induce cell cycle arrest will require additional information from several areas of research. First, it should

be of great interest to define the relative importance of the various IFN-induced events described in this article by pharmacologic and/or genetic manipulation of the different pathways. Furthermore, it will be necessary to define in detail the upstream events triggered by IFN's interaction with its receptor, which lead to alterations in cell cycle -regulating proteins.

Another issue is to further define reasons for the variability in cellular sensitivity to the cell growth -inhibitory action of IFNs. It would also be of interest to investigate the effects of IFNs in a larger set of non-malignant tissues, in order to obtain a greater understanding of how IFNs may regulate normal cell growth.

To enhance the clinical usefulness of IFNs, it would also be desirable to investigate the relative importance of IFN's anti-mitotic action in responsive malignant and viral diseases in relation to other IFN-induced effects such as IFN's immunoregulatory and antiangiogenic actions. Studies along these lines will in all likelihood lead to a more efficient use of IFNs in the clinic with regard to patient selection, treatment schedules and combinations with other treatment modalities. Furthermore, a greater understanding of how IFN exerts its antiproliferative activity will probably provide a useful tool to further understand the intricate regulation of the cell cycle.

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6. REFERENCES

1. E. De Mayer & J. De Mayer-Guiginard: Interferons and other regulatory cytokines. John Wiley, New York (1988)

2. Stark, G.R. Kerr, I.M. Williams, B.R. R.H. Silverman, & R.D. Schreiber: How cells respond to interferons. *Annu Rev Biochem* 67, 227-264 (1998)

3. Nguyen, H. J. Hiscott & P.M. Pitha: The growing family of interferon regulatory factors. *Cytokine Growth Factor Rev* 8, 293-312, (1997)

4. Einhorn, S. & H. Strander: Interferon treatment of human malignancies - a short review. *Med Oncol Tumor Pharmacother* 10, 25-29 (1993)

5. Grandér, D. & S. Einhorn: Interferon and malignant disease - How does it work and why doesn't it always. *Acta Oncol* 37, 331-338 (1998)

6. Brenning, G. A. Åhre & K. Nilsson: Correlation between in vitro and in vivo sensitivity to human leukocyte interferon in patients with multiple myeloma. *Scand J Haematol* 35, 543-549 (1985)

7. Taylor-Papadimitriou, J. & E. Rozengurt: Interferons as regulators of cell growth and differentiation. In: Interferons, Their impact In Biology And Medicine. Ed: Taylor-Papadimitriou J, Oxford Medical Publications, Oxford 81-(1985)

8. Heyman, M. Grandér, D. Bröndum-Nielsen, K. Cederblad, B. Liu, Y. B. Xu & S. Einhorn: Interferon system defects in malignant T-cells. *Leukemia* 8, 425-434 (1994)

9. Roos, G. T. Leandersson & E. Lundgren: Interferon-induced cell cycle changes in human hemapoietic cell lines and fresh leukemic cells. *Cancer Res* 44, 5358-5362 (1984)

10. D. Lundblad & E. Lundgren: Block of a glioma cell line in S by interferon. *Int J Cancer* 27, 749-754 (1981)

11. F. Balkwill & J. Taylor-Papadimitriou: Interferons affect both G1 and S+G2 in cells stimulated from quiecence to growth. *Nature* 274, 798-800 (1978)

12. Tiefenbrun, N. Melamed, D. Levy, N. Resnitzky, D. Hoffman, I. S.I. Reed & A. Kimchi: Alpha interferon supresses the cyclin D3 and cdc25A genes, leading to a reversible G0-like arrest. *Mol Cell Biol* 16: 3934-3944 (1996)

13. Sangfelt, O. Erickson, S. Castro, J. Heiden, T. Gustafsson, A. S. Einhorn & D. Grandér: Molecular mechanisms underlying interferon-alpha –induced G0/G1 arrest: CKI-mediated regulation of G1 Cdk-complex and activation of pocket proteins. *Oncogene* 18, 2798-2810 (1999)

14. Yamada, G. Ogawa, M. Akagi, K. Miyamoto, H. Nakano, N. Itoh, S. Miyazaki, I. Nishikawa, S. K. Yamamura & T. Taniguchi: Specific depletion of the B-cell population induced by aberrant expression human interferon regulatory factor 1 gene in transgenic mice. *Proc Natl Acad Sci USA* 88, 532-536 (1991)

15. Einat, M. D. Resnitzky & A. Kimchi: Inhibitory effects of interferon on the expression of genes regulated by platelet-derived growth factor. *Proc Natl Acad Sci USA* 82, 7608-7612 (1985)

16. Einhorn, S. Showe, L. Östlund, L. Juliusson, G. Robert K.H. G. Gahrton & C. Croce: Influence of interferon-alpha on the expression of cellular oncogenes in primary chronic lymphocytic leukemia cells. *Oncogene Res* 3, 39-49 (1988)

17. A.B. Pardee: A restriction point for control of normal animal cell proliferation. *Proc Natl Acad Sci USA* 71, 1286-1290 (1974)

18. X. Graña & E.P. Reddy: Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). *Oncogene* 11, 211-219 (1995)

19. D.O. Morgan: Principles of CDK regulation. *Nature* 374, 131-134 (1995)

20. Zhang, Y. Y. Xiong & W.G.Yarbroung: ARF promotes MDM2 degradation and stabilizes p53: *ARF-INK4a* locus deletion impairs both the Rb and p53 tumor suppressor pathways. *Cell* 92, 725-734 (1998)

21 C.J. Sherr & J.M. Roberts: CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* 13, 1501-1512 (1999)

22. Elbendary, A. Berchuck, A. Davis, P. Havrilesky, L. Bast, R.C. J.D. Iglehart & J.R. Marks: Transforming growth factor-beta1 can induce CIP1/WAF1 expression independent of the p53 pathway in ovarian cancer cells. *Cell Growth Differ* 5, 1301-1307 (1994)

23. Sangfelt, O. Erickson, S. S. Einhorn & D. Grandér: Induction of Cip/Kip and Ink4 cyclin dependent kinase inhibitors by interferon-alpha in hematopoietic cell lines. *Oncogene* 14, 415-423 (1997)

24. Polyak, K.J. Kato, J-Y. Solomon, M.J. Sherr, C.J. Massagué, J. J.M. Roberts & A. Koff: p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes Dev* 8, 9-22 (1994)

25. Kato, J. Matsuoka, M. Polyak, K. J. Massagué & C.J. Sherr: Cyclic AMP-induced G1 phase arrest mediated by an inhibitor (p27Kip1) of cyclin-dependent kinase 4 activation. *Cell* 79, 487-496 (1994)

26. C.J. Sherr & J.M. Roberts: Inhibitors of mammalian G1-cyclin-dependent kinases. *Genes Dev* 9, 1149-1163 (1995)

27. Paggi, M.G. Baldi, A. F. Bonetto, & A. Giordano: Retinoblastoma protein family in cell cycle and cancer: a review. *J Cell Biochem* 62, 418-430 (1996)

28. Harbour, J.W. Luo, R.X. Dei Santi, A. A.A. Postigo & D.C. Dean: Cdk Phosphorylation triggers sequential intramolecular interactions that progressively block Rb Functions as cells move through G1. *Cell* 98,859-869 (1999)

29. Smith, E.J. G. Leone & J.R. Nevins: Distinct mechanisms control the accumulation of the Rb-related p107 and p130 proteins during cell growth. *Cell Growth Differ* 9, 297-303 (1998)

30. J.R. Nevins: Cell cycle targets of the DNA tumor viruses. *Curr Opinion Gen Devel* 4, 130-134 (1994)

31. C.J. Sherr: Cancer cell cycles. *Science* 274, 1672-1677 (1996)

32. I. Palmero & G. Peters: Perturbation of cell cycle regulators in human cancer. *Cancer Surv* 27, 351-367 (1996)

33. A Kimchi: Cytokine triggered molecular pathways that control cell cycle arrest. *J Cell Biochem* 50, 1-9 (1992)

34. Thomas, N.S. Pizzey, A.R. Tiwari, S. C.D. Williams & J. Yang: p130, p107, and pRb are differentially regulated in proliferating cells and during the cell cycle arrest by alpha-interferon. *J Biol Chem* 273, 23659-23667 (1998)

35. Furukawa, Y. Iwase, S. Kikuchi, J. Nakamura, M. H. Yamada & M. Matsuda: Transcriptional repression of the E2F-1 gene by interferon-alpha is mediated through induction of E2F-4/pRb and E2F-4/p130 complexes. *Oncogene* 18, 2003-2014 (1999)

36. Hobeika, A.C. P.S. Subramaniam & H.M. Johnson: IFN alpha induces the expression of the cyclin-dependent kinase inhibitor p21 in human prostate cancer cells. *Oncogene* 14, 1165-1170 (1997)

37. Harvat, B.L. P. Seth & A.M. Jetten: The role of p27Kip1 in gamma interferon-mediated growth arrest of mammary epithelial cells and related defects in mammary carcinoma cells. *Oncogene* 17, 2111-2122 (1997)

38. Matsuoka, M. I. Nishimoto & S. Asano: Interferon-? impairs physiologic downregulation of cyclin-dependent kinase inhibitor, p27Kip1, during G1 phase progression in macrophages. *Exp Hematol* 2, 203-209 (1999)

39. Sheaff, R.J. Groudine, M. Gordon, M. J.M. Roberts & B.E. Clurman: Cyclin E-CDK2 is a regulator of p27Kip1. *Genes Dev* 11, 1464-1478 (1997)

40. Subramaniam, P.S. Cruz, P.E. A.C. Hobeika & H.M. Johnson: Type I interferon induction of the Cdk-inhibitor p21WAF1 is accompanied by ordered G1 arrest, differentiation and apoptosis of the Daudi B-cell line. *Oncogene* 16, 1885-1890 (1998)

41. Galaktionov, K. X. Chen & D. Beach: Cdc25 cell-cycle phosphatase as a target of c-myc. *Nature* 382, 511-517 (1996)

42. Mandal, M. Bandyopadhyay, D. T.M. Goepfert & R. Kumar: Interferon-induces expression of cyclin-dependent kinase inhibitors p21WAF1 and p27KIP1 that prevent activation of cyclin-dependent kinase by CDK-activating kinase (CAK). *Oncogene* 16, 217-225 (1998)

43. Matsoka, M. T. Kenzaburo & A. Shigetaka: Interferon-alpha-induced G1 phase arrest through up-regulated expression of CDK inhibitors p19Ink4D and p21Cip1 in mouse macrophages. *Oncogene* 16, 2075-2086 (1998)

44. Einat, M. D. Resnitzky & A. Kimchi: Close link between reduction of c-myc expression by interferon and G0/G1 arrest. *Nature* 313, 597-600 (1985).

45. A. Yarden & A. Kimchi: Tumor necrosis factor reduces c-myc expression and cooperate with interferon-gamma in HeLa cells. *Science* 243, 1419-1421 (1986)

46. Erickson, S. Sangfelt, O. Castro, J. Heyman, M. S. Einhorn & D. Grandér: Interferon-alpha inhibits proliferation in human T-lymphocytes by abrogation of IL-2 induced changes in cell cycle regulatory proteins. *Cell Growth & Diff* 10, 575-582 (1999)

47. R. Kumar & I. Atlas: Interferon alpha induces the expression of retinoblastoma gene product in human Burkitt lymphoma Daudi cells: Role in growth regulation. *Proc Natl Acad Sci USA* 89, 6599-6603 (1992)

48. Ginsberg, D. Mechta, F. M. Yaniv & M. Oren: Wild-type p53 can down-regulate the activity of various promoters. *Proc Natl Acad Sci USA* 88, 9979-83 (1991)

49. N. Tiefenbrun & A. Kimchi: The involvement of protein kinase C in mediating growth suppressive signals of interferons in hematopoietic cells. *Oncogene* 6, 1001-7 (1991)

50. Raveh, T. Hovanessian, A.G. Meurs, E.F. N. Sonenberg & A. Kimchi: Double-stranded RNA-dependent protein kinase mediates c-myc suppression induced by type I interferons. *J Biol Chem* 271, 25479-25484 (1996)

51. Chong, K.L. Feng, L. Schappert, K. Meurs, E. Donahue T.F. Friesen, J.D. A.G. Hovanessian & B.R. Williams: Human p68 kinase exhibits growht suppression in yeast and homology to the translational regulator GCN2. *EMBO J* 11, 1553-1562 (1992)

52. Salzberg, S. Wreschner, D.H. Oberman, F. A. Panet & M. Bakhanashvili: Isolation and characterization of an interferon-resistant cell line deficient in the induction of (2´,5´) oligoadenylate synthetase activity. *Mol Cell Biol* 3, 1759-1765 (1983)

53. Hassel, B.A. Zhou, A. Sotomayor, C. A. Maran & R.H. Silverman: A dominant negative mutant of 2-5A-dependent RNaseL suppresses antiproliferative and antiviral effects of interferon. *EMBO J* 12, 3297-3304 (1993)

54. Kirchhoff, S. Koromilas, A.E. Schaper, F. Grashoff, M. N. Sonnenberg & H. Hauser: IRF-1 induced cell growth inhibition and interferon induction requires the activity of the protein kinase PKR. *Oncogene* 11, 439-445 (1995)

55. J.U. Gutterman & D. Choubey: Retardation of cell proliferation after expression of p202 accompanies an increase in p21 (WAF1/CIP1). *Cell Growth Diff* 2, 93-100 (1999)

56. Chin, Y.E. Kitagawa, M. Su, W.C. You, Z.H. Y. Iwamoto & X.Y. Fu: Cell growth arrest and induction of cyclin-dependent kinase inhibitor p21 WAF1/CIP1 mediated by STAT1. *Science* 272, 719-722 (1996)

57. Bromberg, J.F. Horvath, C.M. Wen, Z. R.D. Schreiber & J.E. Darnell Jr: Transcriptionally active STAT1 is required for the antiproliferative effects of both interferon alpha and interferon gamma. *Proc Natl Acad Sci USA* 93, 7673-7678 (1996)

58. Ward, A.C. I. Touw. & A. Yoshimura: The Jak-Stat pathways in normal and perturbed hematopoiesis. *Blood* 95, 19-29 (2000)

59. Yamaoka, T. Yano, M. Idehara, C. Yamada, T. Tomonari, S. Moritani, M. Ii, S. Yoshimoto, K. J. Hata & M. Itakura: Apoptosis and remodeling of beta cells by paracrine interferon-gamma without insulitis in transgenic mice. *Diabetologia* 42, 566-573 (1999)

60. Xaus, J. Cardo, M. Valledor, A.S. Soler, C. J. Lloberas & A. Celada: Interferon gamma induces the expression of p21waf-1 and arrests machrophage cell cycle, preventing induction of apoptosis. *Immunity* 11, 103-113 (1999)

61. M.S. Marshall: Ras target proteins in eukaryotic cells. *FASEB J* 9, 1311-1318 (1995)

62. Firpo, J.E. Koff, A. M.J. Solomon & J.M. Roberts: Inactivation of a Cdk2 inhibitor during interleukin 2-induced proliferation of human T lymphocytes. *Mol Cell Biol* 14, 4889-4901 (1994)

63. Nourse, J. Firpo, E. Flanagan, M.W. Coats, S. Polyak, K. Lee, M.H. Massagué, J. G.R. Crabtree & J.M. Roberts: Interleukin-2-mediated elimination of the p27Kip1 cyclin-dependent kinase inhibitor prevented by rapamycin. *Nature* 372, 570-573 (1994)

64. B.H. Nelson & D.M. Willerford: Biology of the interleukin-2 receptor. *Adv Immunol* 70, 1-81 (1998)

65. Brennan, P. Babbage, J.W. Burgering, B.M.T. Groner, B. K. Reif & D.A. Cantrell: Phosphatidylinositol 3-kinase couples the interleukin-2 receptor to the cell cycle regulator E2F. *Immunity* 7, 679-689 (1997)

66. Petricoin, E.F. 3rd. Ito, S. Williams, B.L. Audet, S. Stancato, L.F. Gamero, A. Clouse, K. Grimley, P. Weiss, A. Beeler, J. Finbloom, D.S. Shores, E.W. R. Abraham & A.C. Larner: Antiproliferative action of interferon-alpha requires components of T-cell-receptor signaling. *Nature* 390, 629-632 (1997)

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