p57^Kip2, a glucocorticoid-induced CDK inhibitor, involved in cell proliferation, apoptosis and differentiation.

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Till mina föräldrar
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Abstract

Glucocorticoids (GCs), are steroid hormones with several important physiological implications, of which some are connected with the ability of GCs to inhibit cell proliferation. Although GC treatment normally accumulates cells in the G1 phase of the cell cycle, the mechanisms behind this phenomenon are not well understood. Considering that GCs exert their effects mainly by regulating the expression of genes via binding to and activating a ligand-dependent transcription factor, the glucocorticoid receptor (GR), the main aim of this thesis was to identify novel target genes for the GR, linked to this anti-proliferative effect. Studies presented in this thesis identified the cyclin dependent kinase inhibitor, p57Kip2, to be directly induced by the GR by means of transcriptional induction. Furthermore, p57Kip2 was shown to be sufficient to reconstitute the anti-proliferative effect seen after GC treatment, indicating an important role for this protein in the GC-induced inhibition of cell proliferation. Further investigation also identified a functional glucocorticoid response element (GRE) in the human p57Kip2 promoter, located 5 kb upstream of the transcriptional start site. The GRE was also shown to be well conserved in the mouse both regarding sequence similarity and function, indicating a high biological relevance for this element.

GCs are known for their ability to promote lung maturation in the fetus. Since p57Kip2 has been implicated in mouse embryonal lung development, we hypothesized that GC administration could increase p57Kip2 expression in this tissue. Indeed, GC treatment of pregnant mice increased the expression of p57Kip2 in the proximal pseudostratified columnar epithelial cells of the embryonic lung. p57Kip2 expression and induction was temporally restricted to a period when transition from the pseudoglandular to the canalicular phases occurs during lung development. Furthermore, embryos lacking p57Kip2 presented a phenotype indicating an aberrant differentiation of the distal cuboidal epithelial cells. This study identified a potential in vivo model for GC-induced p57Kip2 expression, and also suggested a novel function for p57Kip2 in distal-proximal airway differentiation.

Apoptosis is a process of programmed cell death involved in various biological events, including tumourigenesis. Interestingly, connections between cell proliferation and apoptosis have been suggested, for instance through the activity of CDK2. CDK2, which is necessary both for progression through the G1-phase of the cell cycle and in
apoptosis, is activated by several apoptotic stimuli, including treatment with the cytotoxic agent staurosporine. Since \( p57^{kip2} \) is a known inhibitor of CDK2, we hypothesized that this protein could affect staurosporine-induced apoptosis in HeLa cells. Results presented in this thesis show that selective expression of \( p57^{kip2} \) potentiates this apoptotic process. The stimulation of staurosporine-induced apoptosis also included increased activation of caspase-3 and a reactivation of CDK2, potentially mediated by a caspase-dependent cleavage of \( p57^{kip2} \). In summary, we suggest a role for \( p57^{kip2} \) in the response of tumour cells to cytotoxic drugs.

Finally, we propose a model, where the GC-induced cyclin dependent kinase inhibitor, \( p57^{kip2} \), participates in the processes of anti-proliferation, differentiation and apoptosis.
Main References

This thesis is based on the following scientific publications, which will be referred to by their roman numerals:

   p57kip2, a Glucocorticoid-Induced Inhibitor of Cell Cycle Progression in HeLa  
   Cells.  
   Mol Endocrinol 13, 1811-1822.

II  Alheim, K., Corness, J., Samuelsson, M.K.R., Murata, T., Nilsson, T., and Okret,  
     S. 2002.  
     Identification of a functional glucocorticoid response element in the promoter  
     of the cyclin dependent kinase inhibitor p57kip2.  
     *Manuscript* under revision for publication in J Mol Endocrinol

   Restricted Temporal Expression and Glucocorticoid Induction of the Cyclin  
   Dependent Kinase Inhibitor p57kip2 during a Critical Period of Mouse Lung  
   Development.  
   *Manuscript* submitted to Pediatr Pulmonol.

   A pro-apoptotic effect of the CDK inhibitor p57kip2 on staurosporine-induced  
   apoptosis in HeLa cells.  
   Biochem Biophys Res Com 296, 702-709.

Abbreviations

aa amino acid
Apaf-1 apoptotic protease-activating factor 1
AP-1 activator protein-1
ATP adenosine triphosphate
BWS Beckwith-Wiedemann Syndrome
Caspase cysteine aspartic acid-specific protease
CDK cyclin dependent kinase
CKI CDK inhibitor
Cip1 CDK interacting protein 1 (also called Waf1 or Sdi1)
CRH corticotropin releasing hormone
Dex dexamethasone
DNA deoxyribonucleic acid
G1-phase gap 1-phase
G2-phase gap 2-phase
GC(s) glucocorticoid(s)
GR glucocorticoid receptor
GRE glucocorticoid response element
HPV human papilloma virus
IGF2 insulin-like growth factor 2
Ink4 inhibitor of kinase 4
Kip1 kinase inhibitory protein 1
Kip2 kinase inhibitory protein 2
M-phase mitosis-phase
Miz-1 Myc-interacting zinc-finger protein 1
NF-kB nuclear factor kappa B
PR progesterone receptor
pRb retinoblastoma protein
PARP poly(ADP)-ribose polymerase
PCNA proliferating cell nuclear antigen
R-point restriction point
RNA ribonucleic acid
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<td>Sdi1</td>
<td>senescent cell-derived inhibitor 1 (also called Cip1 or Waf1)</td>
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<td>SP-A</td>
<td>surfactant protein A</td>
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<td>TGFβ</td>
<td>transforming growth factor beta</td>
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<td>Waf1</td>
<td>wild-type p53 activated fragment (also called Cip1 or Sdi1)</td>
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General Introduction

The capacity of a cell to divide and produce two daughter cells with identical copies of the parental genome is not only fundamental to life but has also given rise to many intriguing scientific questions. For instance, how does a cell in a multicellular organism "know" when to divide and when not to? How is it ascertained that the DNA is completely replicated before the separation into two new daughter cells? These questions are essential for the survival of any multicellular organism and research within these fields led to the discovery of the cell cycle, the process that governs both the fidelity and the speed of one cell division. A cell in a multicellular organism also has "choices" other than cell division. It can either differentiate to become more specialized or die by activation of an intracellular latent suicide program, apoptosis. Recently, the processes of cell division, differentiation and apoptosis have all been implied to interact through the activity of a family of serine/threonine kinases, called Cyclin Dependent Kinases (CDKs). This family of protein kinases is essential for the propagation of the cell cycle and several mechanisms exist to ensure correct regulation of their activity. For instance, there are CDK inhibitors (CKIs), which are proteins that inhibit CDK activity directly by binding to the CDKs. It is well established that these CKIs are important inhibitors of cell proliferation and given the connection between CDK-activity and apoptosis they are also potential modulators of programmed cell death.

Glucocorticoids (GCs) are steroid hormones produced in the adrenal cortex. Although as their name implies they were originally identified as hormones influencing glucose metabolism, several other physiological effects have been discovered. GCs are important modulators of the immunologic and inflammatory responses and are also known to influence blood pressure, fat metabolism and the function of the central nervous system. GCs are widely used in the clinic, often as a potent anti-inflammatory agent. However, there are several adverse clinical effects, where the potential of GCs to inhibit cell division is one important component. Despite this knowledge, the mechanisms behind these anti-proliferative effects have not been fully elucidated. Interestingly, GCs have also been shown to promote differentiation, for instance during lung development, as well as affecting apoptosis of cells in the immune system. Considering the involvement of GCs in these processes as well as the potential
of CKIs to influence cell proliferation, differentiation and apoptosis we hypothesized that GCs might regulate the expression of one or several of these proteins. This thesis describes the induction of the CKI p57\(^{kip2}\) by GC treatment. Results supporting the involvement of this protein in the regulation of cell proliferation, apoptosis and differentiation are also presented, indicating that p57\(^{kip2}\) may be important for deciding the fate of a cell treated with glucocorticoids.
Introduction

Glucocorticoids

Synthesis and physiology

Glucocorticoids (GCs) are steroid hormones, synthesized from cholesterol in the zona fasciculata and zona reticularis of the adrenal cortex by a series of cytochrome P450-catalyzed reactions (Findling et al., 1997). GCs regulate a multitude of physiological processes, exemplified by increased hepatic gluconeogenesis, cardiac output and vascular tone as well as inhibited inflammatory and immunological responses. In addition, GCs affect the central nervous system, increase surfactant production in the fetal lung, and inhibit bone formation. In the clinic GCs are commonly used as anti-inflammatory agents in the treatment of asthma and rheumatoid arthritis. The synthesis and secretion of GCs, in human cortisol and in rodents corticosterone, are mainly regulated by the hypothalamus-pituitary-adrenal axis (HPA-axis). Adrenocorticotropic hormone (ACTH), produced in the anterior pituitary, rapidly induces production and secretion of not only glucocorticoids but also mineralocorticoids and androgenic steroids from the adrenal cortex. The secretion of ACTH is in turn stimulated by corticotropin-releasing hormone (CRH), which is produced in the hypothalamus. Importantly, the HPA-axis is negatively regulated by GCs through a feedback loop mechanism involving GC actions on both the pituitary and hypothalamic levels. Furthermore, the secretion of GCs is also regulated by the circadian rhythm, with the highest levels in the morning. Upon entering the circulation, GCs bind to plasma proteins, such as corticosteroid binding globulin (CBG) and albumin. Following that, the free pool of GCs reaches the target cells, where due to their lipophilic properties, the GCs pass the plasma membrane and bind to the cytoplasmic glucocorticoid receptor (GR) (Fig.1).
Fig. 1. Illustration describing GC binding to GR and subsequent transactivation from a GRE.

The Glucocorticoid Receptor
The glucocorticoid receptor, a member of the nuclear receptor superfamily, is an intracellular ligand-dependent transcription factor that is expressed in most cell types. It belongs to the same subfamily as the mineralocorticoid receptor (MR), progesterone receptor (PR) and androgen receptor (AR) (NRNC, 1999). GR can be divided into three functionally distinct regions (Fig. 2). The N-terminal part of the protein consists of a ligand-independent transactivation domain (τ1/AF-1) (Giguere et al., 1986), whereas the central DNA binding domain (DBD), containing two zinc fingers, participates in homo-dimerization and binding of the receptors to specific DNA sequences (Dahlman-Wright et al., 1991; Luisi et al., 1991), so-called glucocorticoid responsive elements (GREs). In addition, a DBD proximal region is also involved in nuclear localization of the GR (Picard and Yamamoto, 1987). The C-terminal part of the protein constitutes the ligand binding domain (LBD) (Giguere et al., 1986;
Rusconi and Yamamoto, 1987), and also contains a ligand-dependent transactivation domain (α2/AF-2) (Hollenberg and Evans, 1988), as well as sequences involved in nuclear localization (Picard and Yamamoto, 1987), dimerization (Dahlman-Wright et al., 1992) (Bledsoe et al., 2002), and chaperone interactions (Denis et al., 1988). In the absence of ligand, GR is localized to the cytoplasm, where it interacts with multiprotein complexes, containing chaperones such as Hsp 90, which keep the receptor in a conformation capable of binding the ligand with high affinity (Bresnick et al., 1989; Picard et al., 1990). Upon ligand binding GR translocates to the nucleus, where it either binds directly to GREs as a homo-dimer in GC-regulated promoters (Fig.1), or interacts with other transcription factors, for instance AP-1 or NF-κB, in a process called cross-talk. By these mechanisms GR can either activate or repress transcription from the regulated genes. In contrast to the GRE-dependent transactivation of GR, the processes of transrepression of AP-1 (reviewed in (Göttlicher et al., 1998; Herrlich, 2001)) and NF-κB (reviewed in (Dumont et al., 1998)) do not require the DNA binding properties of the GR. The classical GRE, from which GR activates transcription of target genes, is an imperfect palindrome consisting of two hexameric half sites separated by three nucleotides. Although variations in these GREs are common, a consensus site built on base pair conservation in functional response elements reads GGTACANNTGTCT (reviewed in (Beato et al., 1989; Ziliacus et al., 1995)). In some cases transrepression or transactivation by GR is also dependent on interactions with transcription factors binding to neighbouring DNA elements. Examples are the GR-mediated transrepression of the prolactin promoter and transactivation of the phosphoenolpyruvate carboxykinase (PEPCK) promoter (Imai et al., 1990; Subramaniam et al., 1998). Upon binding to the GRE, GR can interact both with chromatin remodelling complexes, such as the Swi/Snf-like BRG1 or hBRM containing complex, and with co-activators, of which some contain histone acetylase activity, for instance SRC1 and p300/CBP (reviewed in (Deroo and Archer, 2001)). The chromatin remodelling and histone acetylation potentially open up the nucleosomal structure of the chromatin, facilitating binding of sequence-specific and general transcription factors. This in turn leads to initiation of RNA polymerase II dependent transcription.
The physiological role of GR has been emphasized not only by clinical studies identifying mutations in the GR associated with GC resistance (reviewed in (Werner and Bronnegard, 1996)) but also through analysis of mice lacking normal GR. The GR−/− mice die postnatally, displaying a lung maturation deficiency, which indicates that GR is necessary for normal embryonal lung development (Cole et al., 1995). However, this phenotype can be reversed by the expression of a dimerization defective GR mutant called GRdim/dim, indicating that the transactivating properties of GR acting through classical GREs is dispensable for normal development and homeostasis in mice (Reichardt et al., 1998). These data do not exclude the possibility that GRdim/dim mice are defective in other physiological settings, for instance during stress responses. An example of this is the impaired hypoxia-induced erythropoiesis observed in these mice (Bauer et al., 1999). Furthermore, it cannot be formally excluded that the results obtained from GRdim/dim mice reflect redundant functions between the transactivating and transrepressive properties of the GR.

**Fig. 2.** Schematic representation of the human GR domain structure. Amino acid numbering and N- and C-terminals are indicated in the figure.
**The cell cycle**

**Basic principles of cell cycle regulation**

The capacity of a cell to replicate its DNA and to divide into two identical daughter cells, a process called mitosis, is regulated by the cell cycle, where one round in the cell cycle represents one mitosis. By the actions of intrinsic and extrinsic factors, this process is essential for decisions regarding initiation, accuracy and time needed for completion of a mitosis.

The cell cycle consists of four active phases (G1, S, G2 and M) as well as one inactive phase (G0) (reviewed in (Nurse, 2000)). The cell enters the cell cycle in the G1-phase and this is the only phase dependent on growth factors. At the restriction-point (R-point), which occurs late in the G1-phase (Gap 1), an important transition from growth factor-dependent to independent propagation of the cell cycle occurs (Pardoe, 1974). Subsequently, DNA-synthesis takes place in the S-phase (Synthesis), followed by the G2-phase (Gap 2) a control phase containing checkpoints for completion of DNA synthesis as well as correct assembly of the mitotic spindle. Finally, the M-phase (Mitosis) completes the cell cycle. This step consists both of the separation of the sister chromatids as well as cytokinesis, producing two identical daughter cells.

Correct propagation of the cell cycle through all phases is dependent on the sequential activation and inactivation of a family of serine-threonine kinases called cyclin dependent kinases (CDKs). The founder of this family of kinases was originally identified by genetic analysis of the cell cycle in yeast (Nurse and Bissett, 1981). Subsequently, by the use of genetic complementation studies in yeast, several human CDKs were identified (Lee and Nurse, 1987; Elledge and Spottswood, 1991; Ninomiya-Tsuji et al., 1991), and this mechanism was found to be a universal hallmark of eukaryotic cell cycles. Regulation of CDK activity occurs by means of three fundamentally different mechanisms: availability of cyclins, activating and inactivating phosphorylations and presence of CDK inhibitors (CKIs), all of which will be discussed in the sections to follow (reviewed in (Obaya and Sedivy, 2002)) (Fig.3).
Fig. 3. Schematic figure illustrating different modes of CDK regulation. Indicated in the figure are phosphorylation events (P), and ubiquitination events (Ub).

An absolute requirement for CDK activity is the direct binding of each CDK molecule to one member of a family of regulatory subunits, called cyclins. These proteins were originally, as indicated by the name, identified as factors synthesized in a periodic manner during embryonal development of marine vertebrates (Evans et al., 1983), and were subsequently isolated in mammalian cells as well (Lew et al., 1991). Each type of CDK can bind to one or a few types of cyclins. Furthermore, different CDK-cyclin combinations are active in different phases of the cell cycle, where the cyclins also influence the substrate specificity of the CDKs. The cyclins have different periodic expression patterns and these patterns are essential for the restricted activity of each CDK during the cell cycle. The oscillations of cyclin expression depend both on altered synthesis as well as ubiquitin-mediated degradation by the 26S proteasome pathway (reviewed in (Yew, 2001)).
There are additional mechanisms regulating CDK-activity. For instance there are both activating and inhibiting phosphorylations of the CDKs themselves. One such important mechanism is the activating phosphorylation of the Thr160 residue on the CDK, which is exerted by cyclinH-CDK7, also called CDK Activating Kinase (CAK) (Fisher and Morgan, 1994). This phosphorylation is necessary for proper activation of CDK-cyclin complexes, as it induces an active conformation of the CDK protein. The opposite reaction also exists, where a phosphatase, cyclin-dependent kinase-associated protein phosphatase (KAP), dephosphorylates CDKs (Poon and Hunter, 1995). Inhibitory phosphorylation events have been best studied for CDK1 in the context of the G2/M-phase transition. Here the cyclinB-CDK1 complex is kept in an inactive state to prevent premature initiation of the M-phase. This inactive state is due to inhibitory phosphorylations on Thr14 and Tyr15 of CDK1 by the protein kinases Wee1 (which mainly phosphorylates Tyr15) (Parker and Piwnica-Worms, 1992; McGowan and Russell, 1993) and Myt1 (which mainly phosphorylates Thr14) (Liu et al., 1997a). At the onset of M-phase the dual specificity phosphatase CDC25C dephosphorylates Thr14 and Tyr15 of CDK1, leading to cyclinB-CDK1 activation (Parker and Piwnica-Worms, 1992). Similar to CDK1, the G1-acting kinases CDK2 and CDK4 are also phosphorylated, on Tyr 15 and 17 respectively, indicating that these inhibitory phosphorylations are of a more general feature (reviewed in (Obaya and Sedivy, 2002)). In agreement with this, the phosphatase CDC25A is also required for induction of S-phase (Hoffmann et al., 1994; Jinno et al., 1994).

The G1-phase
The G1-phase, the only phase of the cell cycle dependent on growth factors, has been extensively studied. This has led to the discovery of mechanisms involving both cell cycle promoting events mediated by the actions of cyclin D-CDK4/6 complexes and cyclin E-CDK2 complexes as well as cell cycle inhibiting events mediated by the actions of the Ink 4 family and the Cip/Kip family of CDK inhibitors (Fig.4).
Fig. 4. Schematic representation of mechanisms regulating the G1-S transition of the cell cycle.

A central component of the machinery regulating G1- to S-phase progression is the retinoblastoma protein (pRb) (Fig. 4). This phosphoprotein exerts its anti-proliferative effects at least partially by binding to and inhibiting proteins necessary for the G1-S transition, such as members of the E2F family of transcription factors (reviewed in Nevins, 2001). One member of the E2F family dimerizes with one member of the DP family and this is the transcriptionally active unit interacting with specific response elements in the promoters of regulated genes (reviewed in (Lavia and Jansen-Durr, 1999)). Among the E2F-activated genes involved in the G1-S transition are cyclin E (Ohtani et al., 1995; Botz et al., 1996), proliferating cell nuclear antigen (PCNA), and DNA polymerase α (DeGregori et al., 1995). The conversion of pRb from a hypophosphorylated to a hyperphosphorylated state inactivates the protein, leading to the release and activation of E2F. Initially, a partial phosphorylation of pRb is carried out by the action of cyclin D-CDK4/6 complexes. The process of pRb inactivation is then completed by sequential phosphorylation performed by cyclin E-
CDK2 complexes (Lundberg and Weinberg, 1998). Interestingly, a molecular basis for the sequential phosphorylation of pRb by CDK4/6 and CDK2 has been presented (Harbour et al., 1999). This model describes the initial phosphorylation step exerted by CDK4/6 to release histone deacetylases from the pRb, thereby blocking the active transcriptional repression by pRb. Subsequently, the second phosphorylation step, exerted by CDK2, prevents the actual binding of pRb to E2F, permitting the transactivation properties of E2F to function.

The sequential activation during the G1-phase, of cyclinD-CDK4/6, followed by cyclinE-CDK2, is initiated by mitogen-mediated induction of cyclin D expression (reviewed in (Sherr, 1995)). Finally, the phosphorylation of pRb leads to a positive feedback on CDK2 activity involving cyclin E expression, since cyclin E is induced by E2F activation (Ohtani et al., 1995; Botz et al., 1996). This positive feedback also includes increased phosphorylation-dependent degradation of p27Kip1, a member of the Cip/Kip family of CKIs (see below), through the direct action of cyclin E-CDK2 (Sheaff et al., 1997; Vlach et al., 1997). The reduced p27Kip1 expression potentially decreases the inhibitory threshold for CDK2 activation. Although cyclin D and cyclin E are both rate-limiting for S-phase entry (Ohtsubo and Roberts, 1993; Quelle et al., 1993), cyclin D is dispensable in pRb negative cells (Lukas et al., 1994; Tam et al., 1994; Ohtsubo et al., 1995) while cyclin E is not (Ohtsubo et al., 1995). This implies that the critical substrate for cyclin D-CDK4/6 is pRb, while cyclin E-CDK2 has other important substrates. Further support for this notion comes from the fact that despite overexpression of E2F, thereby overriding pRb-mediated inhibition of the cell cycle, CDK2 activity is still required for S-phase entry (Alevisopoulos et al., 1997). Indeed, some candidate CDK2 substrates for this other pRb independent function have been suggested. Apart from decreased p27Kip1 stability mediated by phosphorylation by cyclin E-CDK2 (Sheaff et al., 1997; Vlach et al., 1997), CDK2 also phosphorylates HsCdc6 (Jiang et al., 1999), a protein necessary for initiation of DNA replication, and NPAT (Zhao et al., 1998), a protein with potential to induce S-phase entry. Cyclin E-CDK2 also autophosphorylates cyclin E, a mechanism that increases ubiquitin-dependent degradation of the protein as the S-phase progresses (Clurman et al., 1996; Won and Reed, 1996). Interestingly, cyclin E could also compensate for cyclin D1 deficiency in mice where cyclin E was knocked-in instead of cyclin D1 under the
regulation of the cyclin D1 promoter (Geng et al., 1999). One interpretation of these data is that the essential function for cyclin D1 is its regulation acting as a sensor for mitogens finally leading to induction of cyclin E expression, which in turn together with its catalytic subunit CDK2 perform other necessary functions for the G1-S transition. However, this model is challenged by the in vivo finding that overexpression of the D-type of cyclins respectively is more efficient than cyclin E in driving lens fiber cells into S-phase, suggesting that specific effector functions exist for these proteins (Gomez Lahoz et al., 1999).

**CDK inhibitors**

An important and rapidly growing field of research has emerged after the discovery of specific proteins, so-called CDK inhibitors (CKIs), that inhibit the activity of different CDKs by direct protein-protein interactions. These proteins have been shown to be involved in basal cell cycle regulation and also have important roles in processes such as tumourigenesis, embryonal development and apoptosis. Based on structural and functional similarities, these proteins have been divided into two families: the Ink4 family and the Cip/Kip family.

**The Ink4 family of CDK inhibitors**

The inhibitors of kinase 4 (Ink4) family of CDK inhibitors are as the name implies specific inhibitors of CDK 4 and 6. This family has four members, namely p16\(^{INK4a}\), p15\(^{INK4b}\), p18\(^{INK4c}\) and p19\(^{INK4d}\), all of which share a common structural motif-the presence of ankyrin repeats. Another common feature is the competition with cyclin D for binding to the CDK subunit, which is an important mechanism by which these proteins inhibit CDK4/6 activity. The structures of p16\(^{INK4a}\) and p19\(^{INK4d}\) binding to CDK6 have been resolved (Russo et al., 1998), revealing that the Ink4 inhibitors induce structural changes in the CDK6 protein which prevent cyclin binding as well as distort the kinase catalytic cleft. Due to the specificity for inhibition of CDK4/6, the Ink4 proteins are thought to be dependent on the presence of pRb in order to exert their anti-proliferative effects. This is supported by the observation that, in the absence of pRb, p16\(^{INK4a}\) does not inhibit progression into S-phase (Koh et al., 1995; Lukas et al., 1995; Medema et al., 1995). p16\(^{INK4a}\) and p15\(^{INK4b}\) have both been associated with replicative senescence (Hara et al., 1996) (Fuxe et al., 2000), and
p16\textsuperscript{ink4a} is also commonly mutated in human tumours, assuming a role as a tumour suppressor (reviewed in (Rocco and Sidransky, 2001)). On the other hand, p15\textsuperscript{ink4b} mutations are rare in human tumours, confusing its role as a potential tumour suppressor. Members of the Ink4 family have been shown to be induced by various anti-mitogenic stimuli. One example of this is JunB, a cell cycle inhibitory member of the AP-1 family of transcription factors that transcriptionally activates p16\textsuperscript{ink4a} (Passegue and Wagner, 2000). This mechanism is necessary for the anti-proliferative effect of JunB in mouse embryo fibroblasts. Furthermore, p15\textsuperscript{ink4b} is upregulated in response to treatment with the anti-mitogenic cytokine TGFβ (Hannon and Beach, 1994), through a mechanism involving both Smad-mediated transactivation and relief of c-Myc mediated transrepression (Seoane et al., 2001).

**General aspects of the Cip/Kip family of CDK inhibitors**

The second family of CDK inhibitors, the Cip/Kip family, contains three members, p21\textsuperscript{cip1}, p27\textsuperscript{kip1} and p57\textsuperscript{kip2}. Although they bind to and inhibit cyclin D-, E-, and A-dependent kinases in vitro, their main CDK inhibitory function seems to be mediated through cyclin E- and A- dependent CDK2 (reviewed in (Sherr and Roberts, 1999)). These proteins share an homologous N-terminal CDK inhibitory domain with distinct cyclin and CDK binding regions (Fig.5). In contrast to the Ink4 family all the members of the Cip/Kip family bind CDK-cyclin complexes in a cyclin-dependent manner. The structure of p27\textsuperscript{kip1} bound to cyclin A-CDK2 has been resolved (Russo et al., 1996). In this structure p27\textsuperscript{kip1} contacts both cyclin A and CDK2, inserting helix 3\textsubscript{io} into the catalytic cleft of CDK2 mimicking ATP. It has also been suggested that p57\textsuperscript{kip2} differs from p21\textsuperscript{cip1} and p27\textsuperscript{kip1} in that the intact helix 3\textsubscript{io} is necessary for inhibition of cyclin A-CDK2 and cyclin E-CDK2 by p57\textsuperscript{kip2} but is not necessary for inhibition of the same kinases by either p21\textsuperscript{cip1} or p27\textsuperscript{kip1} (Hashimoto et al., 1998). In another model of inhibition of proliferation, both p21\textsuperscript{cip1} and human p57\textsuperscript{kip2} have been shown to bind to and inhibit the activity of the DNA polymerase δ processivity factor, PCNA, thereby preventing DNA replication (Luo et al., 1995; Watanabe et al., 1998). This inhibitory activity, residing in the C-terminus of the proteins (Fig.5) is separable from the CDK inhibitory activities of both p21\textsuperscript{cip1} and hp57\textsuperscript{kip2}, demonstrating a dual function of these CKIs as cell cycle inhibitors. Interestingly, the PCNA inhibitory domain is not conserved in mouse p57\textsuperscript{kip2} or in p27\textsuperscript{kip1}. Cip/Kip proteins not only
inhibit cyclin-CDK activity but in some cases are even required for proper formation of active cyclin-CDK complexes. This is the case for p21\textsuperscript{Cip1} and p27\textsuperscript{Kip1}, which are necessary for formation of cyclin D1 or cyclin D2 complexed to CDK4 (Cheng et al., 1999). Although p21\textsuperscript{Cip1} and p27\textsuperscript{Kip1} inhibit cyclinD-CDK4 activity under certain conditions (Harper et al., 1993; Kato et al., 1994), both proteins can be present in active CDK4 complexes (Zhang et al., 1994; Soos et al., 1996; Blain et al., 1997). A model has even been proposed where cyclinD-CDK4 sequesters the CDK2 dependent kinase inhibitory activity of p27\textsuperscript{Kip1}. Support for this model comes from a study in TGF\textbeta treated cells, where increased p15\textsuperscript{ink4b} expression leads to a reduction in the amount of cyclinD-CDK4 complexes, which in turn release p27\textsuperscript{Kip1} to interact with and inhibit CDK2 complexes (Reynisdottir et al., 1995). In another study consistent with this hypothesis it was also shown that the delayed re-entry into S-phase seen in CDK4 deficient mouse embryonic fibroblasts could be antagonized by p27\textsuperscript{Kip1} deficiency (Tsutsui et al., 1999).
**Fig. 5.** Schematic representation of functional and/or homologous protein domains of the Cip/Kip family of CKIs. The cyclin and CDK binding data are based on the crystal structure of the p27<sup>Kip1</sup>-cyclin A-CDK2 complex (Russo et al., 1996). The alignment is partially adapted from Matsuoka et al. (Matsuoka et al., 1995) and Vlach et al. (Vlach et al., 1997). The domains Proline rich domain, Acidic repeat, QT domain and PAPA repeat refer to sequence properties and not functional properties of the respective domain. The N- and C-terminal ends of the proteins, and the amino acid number are indicated in the figure. Human p57<sup>Kip1</sup> (hp57<sup>Kip1</sup>) and mouse (mp57<sup>Kip1</sup>) are both represented by the 11 amino acids longer (hp57<sup>Kip1</sup>) and 13 amino acids longer (mp57<sup>Kip1</sup>) versions as predicted by alternative translational initiation codons in the cDNA sequences from the respective species (Matsuoka et al., 1995; Tokino et al., 1996). Indicated in the figure are also verified or predicted phosphorylation sites for CDK2 (*). The Thr342 in mp57<sup>Kip2</sup> and Thr311 in hp57<sup>Kip2</sup> are built on amino acid conservation with the established phosphorylation site for CDK2, Thr187, found in hp27<sup>Kip1</sup> (Vlach et al., 1997). Potential or verified cleavage sites for caspases are also indicated in the figure (α, β, γ, δ). β and γ represents verified sites for cleavage by caspase-3, where β is the 13<sup>th</sup>-DPSD<sup>139</sup> site in hp27<sup>Kip1</sup> (Eymin et al., 1999) and γ is the 14<sup>th</sup>DHVD<sup>142</sup> site in hp21<sup>Kip1</sup> (Levkau et al., 1998). α represents a putative cleavage site for caspases in hp57<sup>Kip2</sup>, 80<sup>tevd</sup>, as predicted by the size of a C-terminal caspase dependent cleavage product (see paper IV of this thesis). The necessary C-terminal Asp is also conserved in mp57<sup>Kip2</sup> (8<sup>th</sup>MEVD<sup>88</sup>), as indicated by δ in the figure. Helix 3<sup>10</sup>, which is necessary for CDK2 inhibition by mp57<sup>Kip2</sup> is also marked in the picture (Hashimoto et al., 1998). The sequence of this region is identical in hp57<sup>Kip2</sup>. Amino acid R43 in mp57<sup>Kip2</sup>, which is necessary for the cyclin-CDK binding domains of the protein to interact with MyoD is also indicated in the figure (Reynaud et al., 2000).
p21\textsuperscript{Cip1}

p21\textsuperscript{Cip1}, which is also called p21\textsuperscript{Sdi1} or p21\textsuperscript{Waf1}, was the first CKI identified. The different names of the protein originate from the fact that it was detected using three different approaches: as a CDK2 associated protein in a two hybrid system (Cip; CDK interacting protein) (Harper et al., 1993), as a growth inhibitor from senescent cells (Sdi1; senescent cell-derived inhibitor) (Noda et al., 1994) and from p53 activated cells (Waf1; wild-type p53-activated fragment) (el-Deiry et al., 1993). p21\textsuperscript{Cip1} is mainly regulated on a transcriptional level and is induced for instance in p53 dependent DNA damage induced G1 arrest (el-Deiry et al., 1993; Dulic et al., 1994; el-Deiry et al., 1994), by vitamin D3 during differentiation into monocytes/macrophages (Liu et al., 1996), by STAT1 in IFN-\gamma mediated inhibition of proliferation (Chin et al., 1996), and by the antimitogenic cytokine TGF\(\beta\) (Datto et al., 1995; Reynisdottir et al., 1995). Further emphasizing the importance for p21\textsuperscript{Cip1} in the p53 dependent DNA damage response, mouse embryo fibroblasts lacking p21\textsuperscript{Cip1} displayed an impaired capacity to arrest proliferation in response to DNA damage (Brugarolas et al., 1995; Deng et al., 1995). Despite the fact that induction of p21\textsuperscript{Cip1} by the muscle specific transcription factor MyoD has been implicated in terminal differentiation of skeletal muscle (Halevy et al., 1995; Parker et al., 1995), p21\textsuperscript{Cip1} deficient mice show no major phenotype apart from the impaired DNA damage checkpoint (Deng et al., 1995), indicating that there are redundant mechanisms compensating for lack of p21\textsuperscript{Cip1} during mouse embryonal development. In support of this hypothesis, mice deficient both in p21\textsuperscript{Cip1} and p57\textsuperscript{Kip1} display developmental defects that are more severe than the effects observed in mice lacking either CKI alone (Zhang et al., 1999).

p27\textsuperscript{Kip1}

p27\textsuperscript{Kip1} was originally identified as a CDK inhibitory activity detected in cells arrested by inhibition of Ras, contact inhibition and TGF\(\beta\) treatment, as well as cloned in a screen detecting cyclin D-CDK4 interacting proteins (Koff et al., 1993; Hengst et al., 1994; Polyak et al., 1994a; Polyak et al., 1994b; Slingerland et al., 1994; Toyoshima and Hunter, 1994). Importantly, the amount of p27\textsuperscript{Kip1} has been shown to be reduced on entry into G1-S phase from G0 phase, a process triggered by mitogenic stimulation (Nourse et al., 1994; Coats et al., 1996). By contrast, p27\textsuperscript{Kip1} expression is increased
by many anti-proliferative stimuli such as dioxin-mediated inhibition of proliferation in thymus and hepatoma cells exerted through the Ah receptor (Kolluri et al., 1999), and androgen-repressed proliferation in prostate tumour cells (Kokontis et al., 1998). In contrast to p21Cip1, where the main regulation is on a transcriptional level, regulation of p27Kip1 is more complex, involving not only transcriptional (Kolluri et al., 1999), translational (Hengst and Reed, 1996; Millard et al., 1997), and ubiquitin mediated proteolytic regulation (Pagano et al., 1995; Nguyen et al., 1999) but also changes in the normal nuclear protein localization (Soucek et al., 1998). The ubiquitin mediated degradation of p27Kip1 by the proteasome has been extensively investigated. Interestingly, this pathway is stimulated by phosphorylation of Thr 187 in the murine p27Kip1 exerted by cyclin E-CDK2 (Sheaff et al., 1997; Vlach et al., 1997) (Fig.5). Further studies of this ubiquitin mediated proteasomal degradation identified Skp2, the F-box protein component of an SCF ubiquitin ligase complex, to be involved in this phosphorylation dependent ubiquitination of p27Kip1 (Carrano et al., 1999; Sutterlutty et al., 1999; Tsvetkov et al., 1999). However, there are also data suggesting the existence of other Skp2 independent ubiquitination mechanisms (Hara et al., 2001).

A major in vivo function for p27Kip1 was found in three separate studies of p27Kip1 deficient mice (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). These mice displayed increased body size, multiple organ hyperplasia and an increased cell number, mainly due to increased proliferation. Although other phenotypes were also observed, such as development of pituitary tumors and female sterility, the differentiation of the organs were essentially normal and the mice were viable. In summary, these results indicate that p27Kip1 plays a prominent role in the timely decision to withdraw from the cell cycle during embryonal development.

p57Kip2

The human p57Kip2 gene maps to chromosome 11p15.5 and contains four exons, with the coding sequence present in exons 2 and 3. Furthermore, alternative splicing occurs in the 5'-region with an alternative translation initiation site, which may give rise to an additional 11 aa at the C-terminus of the protein (Tokino et al., 1996). p57Kip2 was originally cloned based on homology with p21Cip1 and p27Kip1 (Lee et al., 1995;
Matsuoka et al., 1995). In addition to N-terminal structures similar to p21Cip1 and p27Kip1 involved in CDK inhibition and a C-terminal sequence homology with p27Kip1, p57Kip2 also contains a central region consisting of proline-alanine repeats in human p57Kip2 and proline-rich acidic repeats in mouse p57Kip2 (Fig.5). Although human polymorphisms in this central region of p57Kip2 have been described (Matsuoka et al., 1996; Tokino et al., 1996), and have been correlated with bone mineral density in clinical cases (Urano et al., 2000), the specific function for this region if any is still unknown. The initial studies of p57Kip2 also revealed that it localizes to the nucleus and that it is a potent inhibitor of G1- and S-phase cyclin-CDK complexes (cyclin E-CDK2, cyclin D2-CDK4 and cyclin A-CDK2) in vitro. Furthermore, p57Kip2 associated with G1 CDK components from a cell line and its overexpression caused cell cycle arrest in the G1 phase. The expression of p57Kip2 transcripts in adult tissues in mouse and human was tissue specific and was mainly detected in skeletal muscle, heart, kidney, and pancreas. In addition, p57Kip2 transcripts were also found to be expressed in differentiated cells in placenta, skeletal muscle, brain, heart, lungs, eye, and cartilage during mouse embryonal development, indicative of a role in development and differentiation (Lee et al., 1995; Matsuoka et al., 1995). Commonly, p57Kip2 protein expression is temporarily restricted during mouse embryonal development, and correlates with cessation of proliferation. This is the case in podocytes of the glomeruli (Hiromura et al., 2001), and hepatocytes during the end of embryonal development (Awad et al., 2000). Furthermore, hypertrophic chondrocytes in the epiphyseal growth plate of bones also express p57Kip2 (Yan et al., 1997; Zhang et al., 1997), suggesting a role in the decreased proliferation observed in these cells. Despite evidence for the involvement of p57Kip2 in cessation of proliferation during differentiation, studies in mouse liver, skeletal muscle and lung showed that p57Kip2 expression was reduced during maturation and aging, suggesting that this protein is not involved in the maintenance of the differentiated and quiescent state in these tissues (Awad et al., 2000; Park and Chung, 2001). p57Kip2 is also expressed in pancreatic β-cells, where loss of expression may be associated with increased proliferation and hyperinsulinism (Kasem et al., 2001). Supporting the hypothesis stating a role in development and differentiation, p57Kip2 is unique amongst the CKIs in that mice deficient in this protein have a varying degree of both embryonal and neonatal lethality. These mice display severe developmental abnormalities, including
abdominal muscle defects, cleft palate, abnormal endochondral bone ossification with incomplete differentiation of hypertrophic chondrocytes, renal medullary dysplasia, adrenal cortical hyperplasia, gastrointestinal defects and lens cell abnormalities (Yan et al., 1997; Zhang et al., 1997). Many of these phenotypes are probably due to the observed alterations in apoptosis, cell proliferation and differentiation. In support of an important function in differentiation, p57^{kip2} expression is temporally and spatially restricted during embryonal development (Westbury et al., 2001), indicative of a role as a switch regulating the onset of different differentiation programs. In line with this, p57^{kip2} has also been shown to induce the activity of the muscle specific transcription factor MyoD, partially by a CDK2 independent mechanism involving direct protein-protein interactions (Reynaud et al., 2000). Interestingly, p57^{kip2} and p21^{cip1} are co-expressed in certain tissues, such as skeletal muscle and cartilage, during embryonal development (Matsuoka et al., 1995). In addition, mice deficient for both p57^{kip2} and p21^{cip1} displayed more severe developmental defects, affecting skeletal muscle, lung, and the skeleton, than observed in mice deficient for either CKI alone (Zhang et al., 1999). This indicates a certain redundancy between these two proteins during development. p57^{kip2} and p27^{kip1} were also partially redundant in the control of cell cycle exit and differentiation of lens fiber cells and placental trophoblasts (Zhang et al., 1998). All in all a central role for p57^{kip2} in embryonal development is suggested, perhaps reflecting its potential to affect the processes of proliferation, differentiation and apoptosis.

p57^{kip2} in Beckwith-Wiedemann syndrome

Together with the function of p57^{kip2} as a CDK inhibitor, the localization of human p57^{kip2} to chromosome 11p15.5 (Matsuoka et al., 1995), a region implicated in sporadic cancers and a familial syndrome of cancer and overgrowth called the Beckwith-Wiedemann syndrome (BWS), gave rise to the hypothesis that p57^{kip2} could play a role in the development of these clinical manifestations. Furthermore, mice deficient in p57^{kip2} displayed some BWS associated phenotypes, such as omphalocele and cleft palate (Yan et al., 1997; Zhang et al., 1997). Like the other genes situated in the same region, for example IGF2 and H19, p57^{kip2} was also found to be imprinted (Hatada and Mukai, 1995; Hatada et al., 1996a; Matsuoka et al., 1996). In contrast to IGF2, a fetal-specific growth factor which is expressed from the paternal allele,
p57\textsuperscript{Kip2} is expressed from the maternal allele, indicative of a complex regulation of imprinting in this region. In addition, there are also some differences between the human and mouse p57\textsuperscript{Kip2} genes regarding the extent of imprinting. Low levels of paternally derived p57\textsuperscript{Kip2} have been detected in humans (Matsuoka et al., 1996), whereas in mice the paternal allele appears to be completely transcriptionally repressed and methylated (Hatada and Mukai, 1995). Although mutations in the p57\textsuperscript{Kip2} gene have been detected, especially in familial cases of BWS (Lam et al., 1999), in sporadic cases of BWS these mutations are rare (Hatada et al., 1996b; O'Keefe et al., 1997). This indicates that BWS is a multifactorial disease potentially involving several genes in the 11p15.5 region, including genetically antagonistic interaction between p57\textsuperscript{Kip2} and IGF2 (Caspari et al., 1999).

**Regulation of p57\textsuperscript{Kip2} expression**

Despite its clear biological relevance, the regulation of p57\textsuperscript{Kip2} was essentially unknown when the studies presented in this thesis were initiated. Following that, some other studies have identified p57\textsuperscript{Kip2} expression to be regulated by different stimuli, for instance both DNA demethylation and inhibition of histone deacetylases have been shown to increase p57\textsuperscript{Kip2} expression from the normally silent paternal allele (El Kharroubi et al., 2001). DNA demethylation also resulted in a heritable expression pattern of p57\textsuperscript{Kip2}, emphasizing the importance for DNA methylation in the process of imprinting the p57\textsuperscript{Kip2} gene. It has also been shown that the p53-related protein p73\(\beta\) increased p57\textsuperscript{Kip2} expression through an unidentified response element involving transcriptional activation (Balint et al., 2002). Interestingly, this effect was only seen in the non-silenced maternal allele and not in the silenced paternal allele, emphasizing the role of imprinting not only in the basal expression but also in the induced expression of p57\textsuperscript{Kip2}. Other studies performed in primary osteoblasts showed that p57\textsuperscript{Kip2} protein expression was increased after vitamin D3 induced differentiation (Urano et al., 2000), and decreased after TGF\(\beta\)1 stimulated proliferation (Urano et al., 1999). In the case of TGF\(\beta\)1 treatment this was mediated through increased ubiquitination and proteasomal degradation of the protein. Hence, p57\textsuperscript{Kip2} can like p27\textsuperscript{kip1} be regulated through the ubiquitin system, although the mechanism responsible for this remains to be identified. With a possible implication for the development of BWS, p57\textsuperscript{Kip2} expression was also reduced by IGF2 in mouse
embryonic fibroblasts and embryos (Grandjean et al., 2000), further suggesting a functional interaction between these proteins. In summary, little is known about the mechanisms governing the expression of p57Kip2 and an increased knowledge may lead to a better understanding of the intricate pathways involved in for instance embryonal development.

**Glucocorticoids and cell proliferation**

Nuclear hormone receptors have important roles in the processes of proliferation, differentiation and apoptosis. Particularly noteworthy are the involvement of the retinoid receptors in embryonal development and the estrogen receptors in the development of mammary tumors (reviewed in (Altucci and Gronemeyer, 2001)). Glucocorticoids (GCs) also have important effects on cell proliferation. Although stimulatory effects of GCs on cell proliferation have been observed in some cases (Guerriero and Florini, 1980; Finlay et al., 1985), the most common effect of GC treatment is inhibition of proliferation. This effect has been observed in a number of different tissues and cell types, including those of lymphoid, fibroblastic and epithelial origin (Miller and Tyrrell, 1995). The anti-proliferative potential of GCs is of clinical importance, since several clinically adverse effects induced by GC treatment are associated with inhibition of proliferation. Examples are the growth suppression observed after high-dose GC therapy in the childhood, affecting the chondrocytes in the epiphyseal growth plate of longitudinal bones (reviewed in (Klaus et al., 2000)), and the cell cycle inhibitory effects of GCs on osteoblasts, which may be involved in osteoporosis (Smith et al., 2000). Conversely, the anti-proliferative effects induced by GC treatment may also have beneficial properties, since GCs are used in the treatment of certain types of cancers, including lymphoproliferative disorders and breast cancer. In line with this, GCs have also been shown to exert potent inhibition of tumorigenesis in the skin, forestomach and lung of rodents (Belman and Troll, 1972; Estensen and Wattenberg, 1993).

In order to delineate the mechanisms behind the anti-proliferative effects of GC treatment, an initial question was to determine the role of the glucocorticoid receptor (GR) in this process. Firestone and his collaborators (Cook et al. 1987) were able to show that rat hepatoma cell clones with reduced expression levels of GR, rendered by
random mutagenesis, were unable to present an anti-proliferative response normally seen after GC treatment of rat hepatoma cells. Furthermore, by introduction of functional GR to these resistant cells, GC-induced suppression of cell division was restored. Although these results stress the fundamental role of GR in the GC-induced anti-proliferative response, mechanisms involving other GC-activated nuclear receptors, such as the mineralocorticoid receptor (MR) (Reul and de Kloet, 1985) or the pregnane X receptor (PXR) (Kliwer et al., 1998), cannot be excluded in other model systems.

Considering the potential of GR to regulate ligand dependent expression of target genes both in a transactivating and a transrepressive mode, investigations regarding the relative importance of these opposite mechanisms in mediating the anti-proliferative effects of GCs are of great importance. Ultimately, the aim of these studies must be not only to obtain a comprehensive view of the mechanisms behind the effects of GCs on cell cycle regulation but also to evaluate the possibility of clinically separating the beneficial effects of GC treatment from the adverse effects. The transactivating properties of GR, mediated by direct GR binding to specific glucocorticoid responsive elements (GREs) on gene promoters, do not explain all actions of glucocorticoids. Instead, several reports demonstrate the capacity of GR to interact with and to repress, or in some cases increase, the activity of other transcription factors, such as AP-1 (reviewed in (Göttlicher et al., 1998; Herrlich, 2001)) and NF-kB (reviewed in (Dumont et al., 1998)).

Several studies have been performed to assess the involvement of the transcriptional activation domains of GR in the anti-proliferative effect of GC treatment. Conflicting data have been presented regarding the importance of the N- and C-terminal transcriptional activation domains in GC-induced inhibition of cell division, some describing both transcriptional activation domains as dispensable (Thompson et al., 1992; Helmberg et al., 1995), and some contending that the N-terminal domain is required (Dieken and Miesfeld, 1992; Chapman et al., 1996). Although these studies have been performed in different cell lines, they all are of lymphocyte origin and hence the anti-proliferative effect described may be further complicated by a paralleling apoptosis. Interestingly, one publication describes the dependence on
transactivation for the GC-mediated anti-proliferative effects in human osteosarcoma cells to exhibit cell type specific differences correlated to alterations in the expression of cell cycle proteins (Rogatsky et al. 1997). Using an N-terminal deletion mutant of GR, GC-mediated inhibition of proliferation was shown to be independent of the transactivation properties of the GR in the pRb expressing cell line U2OS, indicating that repression of transcription may be involved in this anti-proliferative effect. The involvement of transrepressive mechanisms in this response was further supported by the finding that GC treatment of U2OS cells expressing either wt GR or GR deficient in transactivation, reduced the expression of cell cycle activating proteins such as E2F-1, CDK4 and c-Myc. In contrast to the U2OS cells, the Rb-deficient cell line, SAOS2, was reported to be dependent on the transactivation properties of the GR in order to elicit a GC induced anti-proliferative response. Supporting the involvement of transactivation in the inhibition of cell proliferation, SAOS2 cells treated with GCs expressed higher levels of cell cycle inhibiting proteins such as the CDK inhibitors p21Cip1 and p27Kip1. Further complicating the picture, another study suggested different activation domains to be involved in the p21Cip1 associated and the p27Kip1 associated anti-proliferative pathways (Rogatsky et al. 1999). In summary, the importance of the GR transactivating function for the anti-proliferative response induced by GC treatment is clearly context dependent. Furthermore, different trans-activation domains are involved in the regulation of alternative pathways, potentially involving usage of various co-factors.

In the search for signalling pathways involved in the antiproliferative effects of GR the proteins constituting the AP-1 transcription factors have been suggested as possible targets. These factors, which consist of a variety of dimers composed of members from the Fos, Jun and ATF protein families, are important modulators of cell proliferation (reviewed in (Shaulian and Karin, 2002)). In support of this, c-Jun expression is induced in response to growth factors (Quantin and Breathnach, 1988; Ryder and Nathans, 1988; Wu et al., 1989). Furthermore, c-Jun has been shown to be required for progression of the cell cycle from G1 to S phase (Smith and Prochownik, 1992; Schreiber et al., 1999). Several possible target genes that may be responsible for the cell cycle promoting effect of c-Jun have also been identified. For instance, cyclin D1 is transcriptionally induced by c-Jun (Herber et al., 1994; Albanese et al., 1995).
In contrast, c-Jun decreases the expression of the cell cycle inhibitory proteins p16\textsuperscript{ink4a} (Passegue and Wagner, 2000) and p21\textsuperscript{Cip1}, the latter through a mechanism involving down regulation of p53 levels (Schreiber et al., 1999; Shaulian et al., 2000). Considering that earlier studies implicated the transrepressive functions of GR in some of the anti-proliferative effects mediated by GC treatment, GR interacting with and inhibiting the activity of AP-1 is an attractive model for these effects. Indeed, GR and AP-1 have been found to cross-talk, mutually inhibiting the transcriptional activities (Jonat et al., 1990; Lucibello et al., 1990; Schule et al., 1990; Yang-Yen et al., 1990), although in some cases synergistic stimulatory effects have been observed (Diamond et al., 1990). This cross-talk can occur either through a mechanism where only one of the transcription factors binds DNA and the other factor interacts with the DNA binding factor (Jonat et al., 1990; König et al., 1992; Reik et al., 1994) or through a mechanism where both factors bind DNA on neighbouring DNA elements, a so-called composite response element (Diamond et al., 1990). Considering the fact that many AP-1 targets such as cyclin D1, p53 and p16\textsuperscript{ink4a} are commonly deregulated in transformed cells one may speculate that GR interaction with AP-1 is more important for the GC induced anti-proliferative response in non-transformed than in transformed cells.

Interestingly, glucocorticoids have also been shown to inhibit the activity of the extracellular signal-related kinase (ERK)/ mitogen-activated protein kinase (MAPK) (Greenberg et al., 2002). Conversely, GR transcriptional activation is also compromised by the activity of ERK, indicating mutual inhibitory functions of these signalling pathways (Rogatsky et al., 1998). Since the ERK/MAPK pathway has been shown to be important for the G1 to S phase transition (Pages et al., 1993) at least in part through induction of cyclin D1 (Lavoie et al., 1996; Weber et al., 1997), this suggests a potential involvement of this pathway in the anti-proliferative response of GCs.

After discussing the transactivation properties of GR as well as different cross-talk pathways potentially involved in the GC mediated anti-proliferative effects, it is also of great importance to consider the cell cycle modulators on which these signalling pathways directly or indirectly impinge. One model states that GC treatment
p57Kip2, a glucocorticoid-induced CDK inhibitor
decreases the expression of different mitogens and thereby indirectly inhibits cell proliferation in an autocrine or paracrine fashion. Support for this model comes from the GC-mediated decrease in expression of platelet derived growth factor (PDGF) in smooth muscle cells (Nakano et al., 1993), and the inhibited expression of vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF)-I in epiphyseal growth plate chondrocytes after GC treatment (Jux et al., 1998; Koedam et al., 2002). The latter effect has been suggested to be important for the anti-proliferative effects observed in growth plate chondrocytes after GC treatment (reviewed in (Klaus et al., 2000)).

An alternative model for the anti-proliferative effects of GC treatment is the direct effect of GCs on the expression of proteins regulating the cell cycle without intermediate synthesis of various growth factors. This can be achieved either by decreased expression of cell cycle promoting factors or by increased expression of cell cycle inhibiting factors. In line with the observations that in a multitude of cell lines from different origins glucocorticoids exert their anti-proliferative effects mainly in the G1-phase of the cell cycle (Grove and Cristofalo, 1977; Harman et al., 1979; Osman et al., 1985; Forsthoefel and Thompson, 1987; Goya et al., 1993; Sanchez et al., 1993; Frost et al., 1994; Greenberg et al., 2002), several reports describe alterations in the expression levels of proteins involved in the regulation of this phase after GC treatment. For instance, GCs decrease the expression of cell cycle promoting factors such as cyclin D3 and c-Myc. In lymphoma cells, this downregulation was necessary for the anti-proliferative effect observed after GC treatment, since overexpression of cyclin D3 and c-Myc together alleviated this effect (Rhee et al., 1995). As negative regulators of cell cycle progression, the Cip/Kip family of CDK inhibitors, p21Cip1, p27Kip1 and p57Kip2, must also be considered potential targets for regulation by glucocorticoids. Indeed, expression of both p21Cip1 and p27Kip1 have been shown to be increased on mRNA or protein levels after GC treatment (Corroyer et al., 1997; Ramalingam et al., 1997; Rogatsky et al., 1997). Although transcriptional activation of p21Cip1 (Ramalingam et al., 1997), and increased mRNA stability of p27Kip1 (Baghdassarian et al., 1999) have been implicated the exact mechanisms behind GC-regulation of the members of the Cip/Kip-family have not been thoroughly investigated. One exception is the GC-mediated induction of p21Cip1 in rat
hepatoma cells, where the GR-dependent stimulation of this gene is not mediated through a consensus GRE, but rather a mechanism involving several regulatory elements including a response element for C/EBPα (Cha et al., 1998; Cram et al., 1998). It has also been suggested that GR and C/EBPα can interact on C/EBPα response elements, which may account for some of the anti-proliferative effects of GCs (Rudiger et al., 2002).

Another interesting aspect regarding the interplay between GR and cell proliferation is the influence of cell cycle regulation on the activity of the GR. Early studies have suggested that GR is particularly sensitive to GCs in late G1 and S phases but relatively insensitive in the G2, M and early G1 phases (Griffin and Ber, 1969; Martin et al., 1969; Fanger et al., 1986; Hsu et al., 1992). One proposed mechanism for these observations is altered phosphorylation of GR. Increased phosphorylation in response to agonists is observed during late G1- and S- phases but not during the rest of the cell cycle (Hu et al., 1994). Furthermore, GR is phosphorylated in the τ1 domain by CDKs in vitro and deficiency in CDK activity reduced receptor dependent transcriptional activation in a reconstituted GR signalling system in yeast (Krstic et al., 1997). However, the effect of phosphorylation on the transactivating capacity of GR is a matter of controversy and may be context dependent. Within the context of the cell cycle affecting GR activity also lies the observation that the retinoblastoma protein (pRb) enhances transcriptional potentiation of GR by the chromatin remodelling protein hBRM (Singh et al., 1995).

In summary, the anti-proliferative effects of GCs can be mediated by GR actions on several levels, including direct transcriptional activation, interference with other transcription factors/signalling pathways, and secondary effects through decreased expression of growth factors. The consensus must be that there is as yet no consensus. Instead the mechanisms seem to be highly context dependent, reflecting perhaps a high degree of redundancy as well as different levels of differentiation and/or transformation in the cell systems investigated.
Glucocorticoids and lung development

Several studies suggest that GCs exert important functions during lung development. Apart from the lethal lung phenotypes observed in GR−/− mice (Cole et al., 1995), and CRH−/− mice from CRH-deficient mothers (Muglia et al., 1995; Muglia et al., 1999), GCs are also of clinical use in stimulating human lung maturation. Importantly, antenatal glucocorticoids given to mothers at risk of preterm delivery have positive effects on fetal lung maturation, including stimulation of surfactant production (reviewed in (Bolt et al., 2001)). Surfactant, produced by type II pneumocytes in the airway epithelium, is composed of phospholipids and surfactant-associated proteins (SP): SP-A, SP-B, SP-C and SP-D. The function of surfactant is to decrease surface tension at the air-liquid interface of the alveoli, thus stabilizing the lungs at respiration. However, studies investigating morphological defects in GR−/− mice and CRH−/− mice have shown that GCs are likely to exert other functions during lung maturation. Morphological changes in GR−/− mice were detected from E15.5 (Cole et al., 1995), and in CRH−/− mice from CRH−/− mothers from E17.5 (Muglia et al., 1999). The phenotypes of the CRH−/− mice from CRH−/− mothers include delayed induction of SP-A and SP-B transcripts, probably reflecting an effect on type II pneumocyte differentiation, delayed Clara cell maturation, and failure of septal thinning due to continued cell proliferation.

Among the described effects of GCs on lung maturation are effects on lung tissue growth factors, enhanced differentiation of lung epithelial cells, and inhibition of cell proliferation (reviewed in (Bolt et al., 2001)), some of which have been verified in the CRH−/− mouse model system as well (Muglia et al., 1999). All these phenotypes may directly or indirectly relate to regulation of the cell cycle and hence cell cycle proteins may be involved in this process. This has been further implied in the study of the p21Cip1 and p57Kip2 double knockout mouse model, where the embryonal lungs at E16.5 display severely impaired formation of primitive alveoli (Zhang et al., 1999). A common phenotype of all the three knockout mouse models described above, GR−/−, CRH−/− and p21Cip1/p57Kip2 double knockout, is the presence of atelactic lungs. These results are compatible with a model where these proteins exist in a common regulatory pathway. In support of a role for members of the Cip/Kip family of CKIs in the GC-induced lung maturation, one study shows that GC treatment of newborn rats
causes both accelerated lung maturation as well as increased p21\(^{Cip1}\) and p27\(^{Kip1}\) expression in cells of the alveolar septa and in epithelial cells of bronchioli (Corroyer et al., 2002). Although additional research is needed to fully elucidate the mechanisms behind the effects of GCs on pre-natal and post-natal lung maturation, the involvement of cell cycle regulators in this process, for instance CKIs of the Cip/Kip family, may prove to be important.

**Apoptosis**

**Basic principles of apoptosis**

Apoptosis is a form of programmed cell death elicited by diverse stimuli, such as growth factor deprivation, cytotoxic agents, cytokines, and hormones. This process includes certain characteristic morphological and biochemical changes in the dying cells. Examples of these changes are condensation and fragmentation of nuclear chromatin, decreased cell volume and alterations in the plasma membrane leading to recognition and phagocytosis of the apoptotic cells. This process efficiently removes specific cells without eliciting an inflammatory response. Apoptosis is an important mechanism involved in several biological processes, such as embryonic development (reviewed in (Raff, 1996)), regulation of the immune system (reviewed in (Osborne, 1996)), and normal homeostasis of organs (reviewed in (Thompson, 1995)). In addition, mechanisms interfering with this process have been shown to be important in tumorigenesis (reviewed in (Lowe and Lin, 2000)), since a compensatory apoptosis is a normal response to deregulated proliferation. The orderly chain of events which occurs during apoptosis involves sequential activation of members of a family of aspartate-specific cysteine proteases, so-called caspases (reviewed in (Cohen, 1997)). These proteases are important effectors of apoptosis and are responsible for proteolytic cleavages at specific recognition sites of a limited number of proteins, mainly inactivating but in some cases activating the function of the target protein. Proteins cleaved by caspases include proteins involved in maintaining structural components in cytoplasm and nucleus such as Lamin A (Oberhammer et al., 1994; Lazebnik et al., 1995), DNA repair such as poly(ADP-ribose) polymerase (PARP) (Kauffman et al., 1993; Lazebnik et al., 1994), inhibition of DNA degradation such as caspase-activated deoxyribonuclease (CAD) inhibitor (Sakahira et al., 1998), and
proteins involved in cell cycle regulation such as the retinoblastoma protein (pRb) (An and Dou, 1996).

The identification of caspases as important executors of apoptosis was originally carried out in the nematode Caenorhabditis elegans, where the ced-3 and ced-4 genes were shown to be necessary for apoptosis during normal development, while the ced-9 gene antagonized the apoptotic process. The proteins encoded by these genes were subsequently found to be related to mammalian proteins. Ced-9 is related to the Bcl2 family of pro- and anti-apoptotic proteins (Hengartner and Horvitz, 1994), ced-3 is related to caspases (Yuan et al., 1993), and ced-4 to Apaf-1 (Zou et al., 1997), an adapter protein important for activation of caspases in the so-called apoptosome. Caspases are normally present as inactive proenzymes and require proteolytic processing in order to be activated. This processing is carried out either by autocatalytic cleavage or by the action of other caspases. In many forms of apoptosis perturbation of the mitochondria is essential for proper caspase activation. In one such model, the initiator caspase-9 is activated by autocleavage, a process that involves the adapter protein Apaf-1 and cytochrome c (Fig.6). Release of cytochrome c from the mitochondria provides the initial signal for the assembly of these proteins into the apoptosome, activating caspase-9 (reviewed in (Cain et al., 2002)). The mitochondrial release of cytochrome c is either stimulated by pro-apoptotic members of the Bcl2 family, such as Bax, or inhibited by anti-apoptotic members of the same family, for instance Bcl2 and Bcl-xL (reviewed in (Bratton and Cohen, 2001)). Subsequently, activation of the effector caspases 3 and 7 by recruitment and processing by the apoptosome leads to the downstream caspase cascade and cleavage of critical cellular proteins, which manifests the apoptotic phenotype.
**Fig. 6.** Schematic illustration of intrinsic (p53 dependent DNA damage response, cytotoxic drugs and cell cycle perturbations) and extrinsic (death receptors responding to stimuli such as TNFα and CD95L) pathways activating apoptosis in mitochondria-dependent and independent manners (gray arrows). Indicated in the picture are also pro- and anti-apoptotic members of the Bcl-2 family, stimulating or inhibiting cytochrome-c release from the mitochondrion (black arrows). The potential localization of CDK2 activation and cleavage of p21\(^{Cip1}\) and p27\(^{Kip1}\) are also indicated in the figure.

**Cell cycle regulators and apoptosis**

Several studies emphasize the connection between cell cycle regulation and apoptosis. A common response to a deregulated cell cycle is initiation of apoptosis and elucidation of the interplay between these processes is important for a complete understanding of for instance cancer development. Considering this interconnection, it is reasonable to assume the existence of factors capable of regulating both the cell cycle and apoptosis (reviewed in (Sears and Nevins, 2002)). One example of this is E2F-1, a member of the E2F family of transcription factors, which not only has a promoting role in the G1-S transition of the cell cycle (DeGregori et al., 1997), but can also induce apoptosis, presumably by induction of pro-apoptotic proteins such as
p19\textsuperscript{ARF}, Apaf-1 and p73 (DeGregori et al., 1997; Stiewe and Putzer, 2000; Moroni et al., 2001). Further support for this model comes from observations in mice lacking E2F-1, where tumor induction and atrophy is observed in different tissues (Yamasaki et al., 1996).

Similar to E2F-1, the c-Myc transcription factor has the capacity to both stimulate proliferation and induce apoptosis. The pro-apoptotic effect occurs during conditions where survival growth factors are limiting (reviewed in (Prendergast, 1999)). The biological significance for connecting c-Myc to apoptosis is emphasized in prevention of tumour formation \textit{in vivo}, where lesions in the p53 pathway or overexpression of anti-apoptotic Bcl2 family members collaborate with c-Myc in tumour formation (Strasser et al., 1990; Elson et al., 1995).

Another protein involved in both cell cycle regulation and apoptosis is CDK2. CDK2 activity is necessary for staurosporine-induced apoptosis in HeLa cells, and TGF\beta1-induced apoptosis in gastric cancer cells. Interestingly, CDK2 activation was found to occur as a downstream effector of caspase activation. Despite activation of caspase-3, cells with inhibited CDK2 did not display any staurosporine or TGF\beta1 induced apoptosis, confirming CDK2 as critical for caspase-mediated apoptosis (Harvey et al., 2000; Kim et al., 2001). There are however reports from other model systems, which describe an early CDK2 activation upstream of both mitochondrial perturbations and the caspase cascade. This CDK2 activation is necessary for the apoptotic process induced by many different stimuli in thymocytes (Hakem et al., 1999; Williams et al., 2000). However, contradictory data exist whether this CDK2 activity acts upstream or downstream of the Bcl-2 family of proteins (Gil-Gomez et al., 1998; Hakem et al., 1999). In summary, these data indicate that the exact mechanism by which CDK2 participates in apoptosis may be dependent both on cell type and type of apoptotic stimuli. Furthermore, the hypothesis suggesting that CDK2 is an activity necessary for apoptosis downstream of caspases also confuses the identification of a point-of-no-return in the apoptotic process.

Considering the critical involvement of CDK2 in the apoptotic responses induced by many stimuli, one could hypothesize that regulators of CDK2 activity may be
involved in this process as well. Indeed, both p21\(^{C\text{ip}}\) and p27\(^{K\text{ip}}\) have been reported to regulate apoptosis. One example of this is their capacity to protect HeLa cells from staurosporine-induced apoptosis (Harvey et al., 2000). On the other hand, overexpression of p27\(^{K\text{ip}}\) alone induces apoptosis in several transformed cell lines, an effect that was not observed when overexpressing p21\(^{C\text{ip}}\) alone (Katayose et al., 1997; Wang et al., 1997; Kudo et al., 2000). Interestingly, the pro-apoptotic effect of p27\(^{K\text{ip}}\) has been shown to be blocked by ectopic expression of Bcl-2 and is associated with increased caspase-3 activity, indicative of a mechanism involving mitochondrial activation of the apoptosome. In line with the hypothesis suggesting an involvement of p21\(^{C\text{ip}}\) and p27\(^{K\text{ip}}\) in apoptotic responses, both proteins have been identified as substrates for caspases. p21\(^{C\text{ip}}\) and p27\(^{K\text{ip}}\) are C-terminally cleaved by caspase-3 in response to growth factor withdrawal in human endothelial cells and during TGF-\(\beta\) induced apoptosis in human gastric cancer cells (Fig.5 and 6) (Levkau et al., 1998; Kim et al., 2001). This cleavage is associated with decreased binding of p21\(^{C\text{ip}}\) and p27\(^{K\text{ip}}\) to CDK2 as well as increased CDK2 activity. Furthermore, overexpression of an uncleavable p21\(^{C\text{ip}}\) mutant suppressed the apoptotic effect, indicating an important function for this mechanism (Levkau et al., 1998). Noticeably, caspase-3 mediated cleavage of p27\(^{K\text{ip}}\) has also been shown to generate N-terminal cleavage products that inhibit apoptosis induced by cytotoxic stimuli in leukemic cell lines. During this process CDK2 was not activated. Moreover, ectopic expression of cleavage resistant p27\(^{K\text{ip}}\) mutants sensitized the cells to the apoptotic stimuli, indicating that in this system caspase-mediated proteolysis of p27\(^{K\text{ip}}\) mediates the anti-apoptotic function of the protein (Eymin et al., 1999). Despite the potential impact of p21\(^{C\text{ip}}\) and p27\(^{K\text{ip}}\) as downstream effectors of caspases in apoptosis, little is known about the role of the third member of the Cip/Kip family, p57\(^{K\text{ip}}\), in this process. However, increased apoptosis is observed in some tissues, such as the gastro-intestinal tract and the lens of the eye, during embryonal development in mice deficient in p57\(^{K\text{ip}}\) (Yan et al., 1997; Zhang et al., 1997). Whether this increased apoptosis is a direct effect or an indirect effect due to aberrations in cell proliferation and differentiation is not known. The conclusion from these studies must be that CDK2 activation is important for many types of apoptotic processes. The mechanism responsible for this is unknown but most likely involves phosphorylation of either known or novel CDK2 substrates with functions within apoptosis. In addition, caspase-mediated cleavage, especially of
p21\textsuperscript{cip1} and also to some extent p27\textsuperscript{kip1}, is important for the activation of CDK2 as well as the downstream apoptotic events. Under certain conditions caspase-mediated cleavage of p27\textsuperscript{kip1} can also inhibit apoptosis and perhaps this reflects the capacity of p27\textsuperscript{kip1} to act either as an anti-apoptotic or a pro-apoptotic protein.

In general, two different models have been proposed for the action of proteins stimulating proliferation and apoptosis. Either they affect pathways impinging on proliferation and apoptosis separately, as is probably the case for E2F-1 and c-Myc, or apoptosis is a consequence of conflicting proliferative and anti-proliferative signals (reviewed in Prendergast, 1999). It is not clear which model, if any of these, is applicable for the apoptotic function of CDK2. It has however been suggested that apoptosis is not simply an out-of-phase mitotic catastrophe, since cyclinB/CDK1 complexes do not participate in the increased CDK activity observed during GC-induced apoptosis in T-cells (Harvey et al., 1998). Clearly, further research is necessary in order to delineate the connection between apoptosis and cell cycle regulation.

**Glucocorticoids and apoptosis**

A major function of GCs is the ability to regulate apoptosis. One example of this is the strong apoptotic effect observed in cells of the immune system, for instance thymocytes. Furthermore, data suggest that the transactivating capacity of the GR is necessary for this process, since mice expressing a dimerization defective GR (GRdim) are resistant to GC-induced apoptosis in these cells (Reichardt et al., 1998). However, the complexity of the system was revealed when peripheral T-cells were suggested to be independent of the GR transactivating properties for GC-mediated apoptosis (commented in Herrlich, 2001), indicating that different mechanisms may apply to the different model systems. Although the mechanisms behind these effects have not been fully elucidated, the critical involvement of CDK2 activation has recently been proposed (Gil-Gomez et al., 1998; Hakem et al., 1999). Although not sufficient for the CDK2 activation, degradation of p27\textsuperscript{kip1} correlated to the increased CDK2 activity (Gil-Gomez et al., 1998). In addition, GC-induced apoptosis is influenced by Bcl-2, since Bcl-2 deficiency sensitizes thymocytes to GC-induced apoptosis, and Bcl-2 overexpression inhibits the same process (Sentman et al., 1991;
Veis et al., 1993). The capacity of Bcl-2 to modulate GC-induced apoptosis suggests that mitochondrial perturbations are involved in this process. Indeed, alterations of mitochondrial membrane potential have been observed during this apoptotic response (Castedo et al., 1995). Furthermore, thymocytes from caspase-9 deficient mice display a reduction in GC-mediated apoptosis (Kuida et al., 1998), supporting the importance of the mitochondrial activated apoptosome in this process.

Under certain conditions GCs can also inhibit apoptosis. This is the case for apoptosis evoked by serum depletion of T-cells overexpressing Bcl-2 (Huang and Cidlowski, 1999), and for apoptosis induced by cytotoxic substances in glioma cells (Gorman et al., 2000). The latter effect was correlated with induction of Bcl-xL, an anti-apoptotic member of the Bcl-2 family.

Clearly, GCs are potent regulators of apoptosis, with pro- or anti-apoptotic properties under different conditions. The mechanisms behind these processes are not fully elucidated but involve activation of CDK2 and the apoptosome.

**CDK inhibitors and tumourigenesis**

Deregulation of the cell cycle and inactivation of the apoptotic machinery are two important components in the process of tumourigenesis. Considering their traditional effects on inhibition of cell cycle progression as well as their more recently discovered involvement in regulation of apoptosis, several CDK inhibitors are potentially involved in inhibition of tumourigenesis (reviewed in (Lee and Yang, 2001)).

Of the Ink4 family proteins only p16INK4A has been identified as a tumour suppressor and is commonly mutated in human cancer. Another mechanism involved in inactivation of the p16INK4A gene is inappropriate methylation of the promoter region during tumourigenesis. Indeed, demethylation by chemical agents leads to re-expression of p16INK4A as well as G1 cell cycle arrest (Merlo et al., 1995). The location of another potential tumour suppressor, (p14ARF in human and p19ARF in mouse) which results from alternative splicing and altered reading frame in the same locus as
p16
tk, may explain the common alterations of this locus in tumours (reviewed in (Rocco and Sidransky, 2001)).

Among the Cip/Kip family of CKIs only p27
ki is identified as a tumour suppressor. Defined as a haplo-insufficient tumor suppressor, where mice nullizygous or heterozygous for p27
ki display predisposition to tumours when challenged with different carcinogenic treatments (Fero et al., 1998). However, with the exception of pituitary tumourigenesis, p27
ki knockout mice are not more susceptible to development of de novo malignancies (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). p27
ki expression is also commonly reduced in human tumours. Furthermore, decreased p27
ki expression correlates with cancer development, being a prognostic marker for poor survival (reviewed in (Slingeland and Pagano, 2000)). Increased degradation of p27
ki (Loda et al., 1997) and methylation of the promoter region (Worm et al., 2000) are suggested mechanisms by which p27
ki expression is reduced in tumours. p27
ki is also inactivated upon binding to DNA tumour virus oncoproteins, such as the Adenoviral oncoprotein E1a and the Human Papilloma Virus oncoprotein E7 (Mal et al., 1996; Zerfass-Thome et al., 1996). Furthermore, the tumour suppressor PTEN stabilizes the p27
ki protein by reducing SKP2 levels (Mamillapalli et al., 2001), and the transcription factor encoded by the proto-oncogene c-myc both transcriptionally represses the expression of p27
ki, by a mechanism involving the Zn-finger containing transcription factor Miz-1 (Yang et al., 2001), and sequesters p27
ki away from cyclinE-CDK2 by induction of cyclins D1 and D2 (Bouchard et al., 1999; Perez-Roger et al., 1999). Despite the function of c-Myc in regulating p27
ki expression/function, loss of p27
ki synergized with c-Myc overexpression in promoting tumour growth, indicating distinct roles for these proteins in this process (Martins and Berns, 2002). All in all, these data emphasize the importance of p27
ki in tumourigenesis.

In contrast to p27
ki, which has a role as a tumour suppressor, the involvement of p21
Cip and p57
Kip2 in the process of tumourigenesis is more obscure. Despite the observation that overexpression of p21
Cip suppresses the growth of several types of cancers (Eastham et al., 1995; Joshi et al., 1998; Shibata et al., 2001), the role of p21
Cip in tumourigenesis is unclear (reviewed in (Lee and Yang, 2001)). Although
p21\(^{Cip1}\) deficiency in mice did not decrease survival during retroviral induction of lymphomas, its absence was conditional for the haplo-insufficient phenotype of p27\(^{Kip1}\) in the manifestation of these tumours (Martins and Berns, 2002). In several biological settings, p21\(^{Cip1}\) expression is, like p27\(^{Kip1}\), transcriptionally repressed by c-Myc through a mechanism involving inhibition of Miz-1 (Claassen and Hann, 2000; Herold et al., 2002). This suggests that overlapping pathways for regulation of these CKIs may occur, indicating that p21\(^{Cip1}\) in some cases may compensate for p27\(^{Kip1}\), and therefore protect against development of tumours.

Although mutations of p57\(^{Kip2}\) in human cancers are rare (Orlow et al., 1996; Reid et al., 1996), decreased expression of p57\(^{Kip2}\) has been found in bladder carcinomas (Oya and Schulz, 2000), gastric cancer (Shin et al., 2000a), lung tumours (Kondo et al., 1996), adrenocortical carcinomas (Liu et al., 1997b), and hepatocellular carcinomas (Ito et al., 2001a). In addition, it was also shown that expression of this CKI was reduced in poorly differentiated hepatocellular carcinomas with high biological aggressiveness, indicative of a role in the progression of these carcinomas (Ito et al., 2001a). The decreased p57\(^{Kip2}\) expression in human tumours in some cases correlates to selective loss of the expressing maternal allele (Kondo et al., 1996), but may in other cases be due to increased methylation of the p57\(^{Kip2}\) promoter. Recently, this was shown to be the case in some human primary tumours, where the proximal p57\(^{Kip2}\) promoter was hypermethylated (Kikuchi et al., 2002). In addition, treatment of gastric cancer cells with a methylation inhibitor re-activated p57\(^{Kip2}\) expression, suggesting hypermethylation of the promoter to play an important role in the repression of p57\(^{Kip2}\) expression (Shin et al., 2000b). Hypermethylation of the human p57\(^{Kip2}\) promoter was also associated with histone deacetylation, further indicating a transcriptionally repressed state of the promoter (Kikuchi et al., 2002). In contrast to the data described above, p57\(^{Kip2}\) expression was increased in human pancreatic adenocarcinomas as compared to normal tissues (Ito et al., 2001b). However, p57\(^{Kip2}\) levels were reduced in pancreatic adenocarcinomas with high biological aggressiveness as compared to less aggressive carcinomas, still supporting a role for this CKI at least in the progression of these tumours. Mice deficient in p57\(^{Kip2}\) that reach adulthood display no higher frequency of tumours than wt animals (Yan et al., 1997), arguing against the involvement of p57\(^{Kip2}\) in antagonizing de novo tumourigenesis. On the other hand,
these mice also escape the lethal developmental defects observed at a high frequency, which obscures the interpretation of these data. Therefore, the generation of temporal knockout mice may prove to be a useful strategy to elucidate whether p57Kip2 has the capacity to act as a tumour suppressor, preventing de novo or carcinogenically induced tumourigenesis.
Aims of this Thesis

The main aim of this thesis has been to identify novel mechanisms responsible for the anti-proliferative effects exerted by glucocorticoids (GCs). During these investigations we identified the cyclin dependent kinase inhibitor p57^Kip2 as a GC-induced protein able to reconstitute most parts of the anti-proliferative effect seen after GC treatment in HeLa cells (Paper I). In order to further study the mechanism behind the induction of p57^Kip2, as well as the implications of this pathway for differentiation and apoptosis in vivo and in vitro, the following aims were also stated:

- To identify and characterize potential GREs in the human p57^Kip2 promoter (Paper II).
- To find in vivo models for GC induction of p57^Kip2 in an attempt to clarify its potential role for instance in mouse embryonal lung development (Paper III).
- To investigate the role of p57^Kip2 in apoptosis, both spontaneous and induced by cytotoxic stimuli (Paper IV).
Comments on methodology and model systems

The methods and model systems that have been used in the process of generating this thesis are described in detail in the individual manuscripts. However, a few comments on these issues need to be made:

Run-on transcription assay is a method utilizing radioactive labelling during the in vitro elongation of RNA transcripts that have already been initiated in a cell system. After hybridization of the labelled RNA to a plasmid encoding the cDNA of interest, the RNA that has been transcribed from the gene can be detected. This is in contrast to Northern blotting, where only the steady-state level of the transcript is detected.

The CDK2 assay is based on immuno-precipitation of CDK2 from lysates of cells treated with different stimuli. Kinase activity is subsequently detected in vitro by the use of radioactive labelled phosphorylation of Histone H1, which is a commercially available substrate for CDK2. It should be noted that it is impossible to separate the cyclin A and cyclin E associated CDK2 activities from each other since the selection is for CDK2. Another level of specificity is the choice of substrate, where Histone H1 is not a good substrate for instance for CDK4/6.

Cell proliferation was assessed by counting HeLa cells in a Bürker chamber at different time points. A colorimetric method (EZ4U, Biomedica, Vienna, Austria) using reduction of a colourless tetrazolium salt to its coloured derivative was also evaluated. However, values obtained from this assay did not correspond well to the amount of cells in each well after GC treatment of HeLa cells. We speculate that this could be due to the increased size of the observed cells after adding GCs, as theoretically every cell would then convert more of the substrate.

HeLa cells are human cervical carcinoma cells that have been reported to be positive for human papilloma virus type 18 (HPV 18), and thus to express the HPV18 E7 protein (Seedorf et al., 1987). The E7 protein binds to and inactivates pRb as part of its transforming capacity (reviewed in (McMurray et al., 2001)). One implication of this is that anti-proliferative effects impinging on HeLa cells do not involve the Ink4-cyclin D-CDK4/6-pRb pathway as an important component. In addition, HeLa cells
respond with an anti-proliferative phenotype when treated with GCs, making them a suitable model system for these studies (Cavenee and Melnykovych, 1979).

A549 cells are human pulmonary carcinoma cells with some characteristics of type II pneumocytes, such as phospholipid biosynthesis and secretion (Shapiro et al., 1978; Nardone and Andrews, 1979). Furthermore, GC treatment of these cells induces an anti-proliferative response (Croxtall and Flower, 1992), indicating that these cells may be a good model system for studying GC effects on proliferation in cells with a lung epithelial phenotype.
**Results and Discussion**

**Paper I**

Glucocorticoids have earlier been shown to inhibit proliferation in several mammalian tissues and cell lines, including those of lymphoid, fibroblastic, epithelial and bone origin. Although cells normally arrest in the G1-phase of the cell cycle after glucocorticoid treatment (Rogatsky et al., 1997) (Sanchez et al., 1993) (Rhee et al., 1995) (Frost et al., 1994) (Corroyer et al., 1997), the exact molecular mechanism responsible for this effect remain unclear. Given the fact that the glucocorticoid receptor (GR) is a ligand activated transcription factor that can either increase or decrease the transcription of specific genes, we hypothesized that glucocorticoids either induced expression of factors inhibiting progression of the G1-phase or reduced expression of factors promoting progression of the G1-phase. Indeed, such mechanisms are described in the literature. For instance, glucocorticoids have been shown to decrease the expression of the cell cycle promoting factors cyclin D3 and c-myc (Rhee et al., 1995), as well as increase the expression of the cell cycle inhibiting cyclin dependent kinase inhibitors (CKIs) p21\(^{Cip1}\) (Corroyer et al., 1997) (Ramalingam et al., 1997) (Rogatsky et al., 1997) (Cha et al., 1998) and p27\(^{Kip1}\) (Rogatsky et al., 1997). The aim of paper I was to identify novel mechanisms for the antiproliferative effects of glucocorticoids, using the human cervical carcinoma cell line HeLa as a model system.

By the use of immunoblot analysis we were able to identify a novel antiproliferative mechanism exerted by glucocorticoids. This mechanism involves a time- and concentration-dependent induction of the CKI p57\(^{Kip2}\) after treatment of HeLa cells with the synthetic glucocorticoid dexamethasone (DEX). The induction of p57\(^{Kip2}\) protein levels was antagonized by adding the anti-glucocorticoid RU486, suggesting a GR-mediated effect. Furthermore, northern blot analysis in the absence of *de novo* protein synthesis and run-on analysis indicated that the p57\(^{Kip2}\) gene is subject to direct transcriptional activation by GR. Selective ectopic expression of p57\(^{Kip2}\) by a tetracyclin-inducible system was also sufficient to reconstitute the effects seen after DEX treatment, i.e. accumulation of cells in the G1-phase, inhibition of cell proliferation and CDK2 activity. These data imply that induction of p57\(^{Kip2}\) expression
is sufficient for the observed GC-mediated anti-proliferative effect. By further use of transcriptional induction of p57^Kip2^ by the tetracyclin-inducible system, we also compared the p57^Kip2^ protein stability induced with this system with the protein stability after DEX treatment (unpublished data). The results indicate that GC treatment does not affect the stability of p57^Kip2^, arguing against a possible mechanism through the ubiquitin system. In summary, paper I identifies a novel mechanism by which GCs can inhibit cell cycle progression. Furthermore, transcriptional regulation of the CKI p57^Kip2^ was unknown before this study, suggesting a new role for this protein.

**Paper II**

In order to further investigate the mechanisms behind the GC induction of p57^Kip2^ expression (see paper I), a promoter study of the human p57^Kip2^ gene was performed. The aim of this study was to identify and characterize potential GREs in this promoter.

Results presented in paper II identify a functional GRE located 5 kb upstream of the transcriptional start site in the human p57^Kip2^ promoter. The functionality of this element was tested by transient transfections of A549, a human lung carcinoma cell line. By deletion analysis, starting with a 6.4 kb fragment of the human p57^Kip2^ promoter coupled to a luciferase reporter gene, a 446 bp fragment (-5461 bp to -5015 bp) was found to be stimulated by GC treatment. Furthermore, a 40 bp synthetic element spanning a putative GRE (-5082 bp to -5042 bp) was also shown to induce luciferase activity following GC administration, indicating that a GRE is present in this region. This was further confirmed by mutation of the GRE in context of both the 40 bp and 446 bp fragments, showing loss of ability to induce luciferase expression after GC treatment. GR interaction with the p57^Kip2^ GRE was also detected as investigated by the capacity of the p57^Kip2^ GRE to compete with a well-known GRE (from the tyrosine aminotransferase promoter) for GR binding in electromobility shift assays (EMSA). Interestingly, a putative Sp-1 binding site was identified by computational analysis, located immediately downstream of the GRE in the human p57^Kip2^ promoter. This was of particular interest, since the progesterone receptor (PR) had earlier been shown to induce expression of the p21^Cip1^ promoter by interactions
p57Kip2, a glucocorticoid-induced CDK inhibitor

with Sp-1 on Sp-1 binding sites (Owen et al., 1998). However, the hypothesis stating that an intact Sp-1 site, present proximal to the GRE in the p57Kip2 promoter, is important for the GC-mediated induction was found to be false, since mutations of this site actually augmented the inducibility of the promoter. By computational analysis, the GRE in the human p57Kip2 promoter was found to be conserved in the mouse promoter. Only one nucleotide differed between the species and transfection experiments of this putative mouse GRE showed it to be functional.

In summary, paper II identifies a functional GRE in the human p57Kip2 promoter, responsible at least in part for the induction of p57Kip2 expression seen after GC treatment. In addition, the binding of GR to this GRE further emphasizes that p57Kip2 is a gene directly responsive to GC treatment. Furthermore, the evolutionary and functional conservation of the GRE in the p57Kip2 promoter between human and mouse implies a high biological relevance for the GC mediated induction of p57Kip2 expression. Despite the fact that the anti-proliferative effects induced by GCs have been known for a long time, remarkably few cell cycle regulators have been identified as direct targets for the GR. Paper II identifies the CKI p57Kip2 to have those properties in at least some model systems.

Paper III
Glucocorticoids are widely used to stimulate lung maturation when premature birth is expected (for review see Bolt et al., 2001). Increased production of lung surfactant has been suggested to be one mechanism contributing to this effect. However, another described mechanism is GC mediated inhibition of proliferation and stimulation of differentiation. Furthermore, it has previously been shown by gene deletion experiments in mice that both GR (Cole et al., 1995) and corticotropin releasing hormone (CRH) (Muglia et al., 1995) (Muglia et al., 1999) are necessary for normal embryonic lung development, indicating an important role for GC mediated regulation of transcription in this process. It has also been shown that p57Kip2 is necessary for normal embryonic development of several mouse tissues such as bone, muscle and gastro-intestinal tract (Yan et al., 1997) (Zhang et al., 1997) (Takahashi et al., 2000). Although no distinct lung phenotype was reported in the mice lacking p57Kip2 alone, an important role for this protein in lung development was detected in
mice deficient in both p57kip2 and p21cip1 (Zhang et al., 1999), indicating a certain redundancy in the actions of these CDK inhibitors. Considering the GC induced expression of p57kip2 described in papers I and II, we hypothesized that maternal administration of GC could induce p57kip2 expression in mouse embryonic lung.

By the use of immunohistochemical methods, results presented in paper III show that p57kip2 expression is both spatially and temporally restricted. p57kip2 was expressed during day E14.5 to E15.5 of mouse fetal lung development and was limited to the proximal pseudostratified columnar epithelial cells. Furthermore, maternally administered Dex treatment increased the p57kip2 levels in the columnar epithelial cells as seen at E15.5. Interestingly, this temporal expression of p57kip2 occurs during the late pseudoglandular period of lung development, which is a period important for the proximal-distal differentiation or subdivision between conducting and respiratory airways. In order to investigate the involvement of p57kip2 in the development of the mouse embryonal lung epithelium, an immunohistochemical study detecting expression of surfactant protein A (SP-A) was performed in mouse embryos deficient in p57kip2. In wild type embryos SP-A was expressed in the distal single-layered cuboidal epithelial cells. SP-A was also detected in cuboidal epithelial cells in the p57kip2-/- embryos. However, the morphology of the SP-A stained cuboidal cells in the p57kip2 deficient animals displayed a pseudostratified pattern, suggesting an aberration in the differentiation of these cells.

In summary, paper III describes an in vivo model for induction of p57kip2 expression after GC treatment in the mouse embryonal lung epithelium. Although this spatially and temporarily restricted induction of p57kip2 in the mouse lung occurs earlier than any known beneficial effects of GC treatment on human embryos, this mechanism may still affect lung differentiation. Indeed, mouse embryos deficient in p57kip2 have an altered pattern of SP-A expression, indicating an aberrant differentiation process from pseudostratified columnar cells in the proximal airway epithelium to monolayered cuboidal cells in the distal airway epithelium.
Paper IV

Apoptosis is a process of programmed cell death involved in a multitude of biological processes, including embryonic development (for review see Raff, 1996), regulation of the immune system (for review see Osborne, 1996), normal homeostasis of organ systems (for review see Thompson, 1995) and tumourigenesis (for review see Lowe and Lin, 2000). Interestingly, the mechanisms behind cell proliferation and apoptosis have recently been shown to have several features in common. Activation of CDK2 is not only necessary for the G1-S phase transition in the cell cycle but is also involved in apoptosis induced by the protein kinase C inhibitor staurosporine (Harvey et al., 2000). Furthermore, p21\(^{Cip1}\) and p27\(^{Kip1}\), which are CDK inhibitors of the Cip/Kip family, have been shown to have a role in apoptosis. For instance, p21\(^{Cip1}\) and p27\(^{Kip1}\) both protect cells from staurosporine-induced apoptosis in HeLa cells (Harvey et al., 2000). The aim of paper IV was to investigate the role of the third member of the Cip/Kip family, p57\(^{Kip2}\), in both spontaneous and staurosporine-induced apoptosis in HeLa cells. The effect of glucocorticoid treatment on staurosporine-induced apoptosis was also investigated, since GC treatment induces p57\(^{Kip2}\) expression in the same cell line as well as affects apoptosis in several model systems.

Results presented in paper IV show that selective tetracyclin-induced p57\(^{Kip2}\) expression potentiates staurosporine-induced apoptosis in HeLa cells. However, p57\(^{Kip2}\) alone has only a minor apoptotic effect. These results are in contrast to those observed regarding p21\(^{Cip1}\) and p27\(^{Kip1}\), which both protect HeLa cells from staurosporine-induced apoptosis (Harvey et al., 2000). Furthermore, the increased staurosporine-induced apoptosis observed after ectopic p57\(^{Kip2}\) expression correlates with increased caspase 3 activity, suggesting an involvement of this downstream caspase in the execution of this process. As an apparent contradiction, ectopic p57\(^{Kip2}\) expression initially inhibits CDK2 activity despite the fact that CDK2 activity had earlier been shown to be necessary for the execution of staurosporine-induced apoptosis. According to the results presented in this study the initial CDK2 activity does not correlate with the degree of apoptosis. On the other hand, the re-activation of CDK2, as seen during apoptosis after staurosporine treatment of cells expressing ectopic p57\(^{Kip2}\), may be important to this apoptotic process. Earlier studies had shown that CDK2 activation occurs downstream of caspase activity (Harvey et al., 2000).
addition, both p21<sup>Cip1</sup> and p27<sup>Kip1</sup> are known substrates for caspases, their cleavage leading either to activation of CDK2 and apoptosis or to protection from apoptosis in different model systems (Levkau et al., 1998) (Eymin et al., 1999). Indeed, our results show that ectopically induced p57<sup>Kip2</sup> expression declines during the staurosporine-induced apoptosis, correlating with the re-activation of CDK2. Interestingly, a caspase-dependent 42kDa C-terminal cleavage product of p57<sup>Kip2</sup> appears early during the decline of p57<sup>Kip2</sup> expression. These data suggest that a caspase-mediated cleavage of p57<sup>Kip2</sup> may be involved in the re-activation of CDK2. In contrast to selective ectopic expression of p57<sup>Kip2</sup>, DEX treatment of the same cell line inhibited staurosporine-induced apoptosis. Since DEX treatment induced p57<sup>Kip2</sup> expression to a similar level to that seen after treating the cells with tetracyclin, we hypothesized that a protein antagonizing the pro-apoptotic effect of p57<sup>Kip2</sup> was induced by GC treatment. Indeed, Bcl-x<sub>i</sub>, an anti-apoptotic protein of the Bcl2 family (for review see (Bratton and Cohen, 2001)), was induced by DEX treatment. This may explain the anti-apoptotic effect seen after addition of DEX.

In summary, the sensitizing effects exerted by p57<sup>Kip2</sup> on staurosporine-induced apoptosis suggest a role for this CDK inhibitor in the response of tumour cells to cytotoxic drugs. The question remains whether this effect is secondary to inhibition of cell proliferation and accumulation of cells in the G1 phase or represents a novel pathway independent of cell cycle regulation. A recent report however indicates that HeLa cells enter apoptosis from the G2/M phase after staurosporine treatment (Bernard et al., 2001), arguing against an effect secondary to an accumulation in the G1 phase. Although p57<sup>Kip2</sup> on its own has only a small apoptotic-inducing effect, an involvement of this protein in tumourigenesis cannot be excluded. Several lines of evidence associate the expression levels of members of the Cip/Kip family of CDK inhibitors with tumour development. An example of this is the identification of p27<sup>Kip1</sup> as a haplo-insufficient tumoursuppressor, correlating p27<sup>Kip1</sup> degradation to an aggressive tumor phenotype (for review see (Slingerland and Pagano, 2000). Interestingly, it has also been reported that p57<sup>Kip2</sup> expression is reduced in some human malignancies (Kondo et al., 1996) (Liu et al., 1997b) (Oya and Schulz, 2000) (Shin et al., 2000a) (Ito et al., 2001a). Further research is needed to clarify the potential involvement of the different members of the Cip/Kip family of CDK
inhibitors in tumourigenesis and to assess the roles of altered proliferation, differentiation and apoptosis in this process.
Conclusions and Future Perspectives

The investigations presented in this thesis establish the following findings:

- Glucocorticoid treatment of HeLa cells induces p57Kip2 mRNA and protein expression. The induction is at a transcriptional level and does not require de novo protein synthesis. Increased p57Kip2 expression is sufficient to inhibit cell proliferation, accumulate cells in the G1-phase of the cell cycle and inhibit CDK2 activity, mimicking the response seen after glucocorticoid treatment.

- A functional glucocorticoid response element is present 5 kb upstream of the transcriptional start site in the human p57Kip2 promoter. This response element is capable of binding GR and is responsible for the glucocorticoid inducibility seen when transferring a 6.4 kb upstream fragment of the human p57Kip2 promoter to a luciferase reporter gene.

- p57Kip2 protein is temporally and spatially expressed in the mouse embryonal lung. Glucocorticoid treatment during the window of p57Kip2 expression increases p57Kip2 levels, suggesting the embryonal lung to be an in vivo model for GC induced p57Kip2 expression. Furthermore, p57Kip2-/- embryos display an altered morphology of SP-A expressing cells, indicating an aberrant lung differentiation.

- Ectopic expression of p57Kip2 potentiates the apoptotic response seen after staurosporine treatment of HeLa cells. Glucocorticoids, on the other hand, despite inducing p57Kip2, inhibit staurosporine-induced apoptosis. This anti-apoptotic effect exerted by glucocorticoids may be explained by a concomitant increase in Bcl-xL expression.

To conclude, we would like to propose a model for the GC-induced expression of p57Kip2 in which this mechanism participates in the processes of inhibition of proliferation, differentiation and apoptosis (Fig.7). Future experiments including in vivo footprinting and chromatin immunoprecipitations would reveal any in vivo
occupance of GR and Sp-1 on the identified GRE. Further research is also necessary in order to elucidate the involvement of p57Kip2 in differentiation and apoptosis. For instance, is the CDK inhibitory function of p57Kip2 necessary for these mechanisms or are there alternative ways to execute these functions? An indication for the existence of both pathways is the ability of p57Kip2 to induce MyoD activity during muscle differentiation in a partially CDK2 independent fashion. This mechanism involves both stabilization of MyoD by inhibition of CDK2 activity as well as direct protein–protein interactions between MyoD and p57Kip2 (Reynaud et al., 1999; Reynaud et al., 2000). Furthermore, investigations regarding the expression of p57Kip2 in tumour cells and their responsiveness to cytotoxic drugs may provide important information as to whether the pro-apoptotic effect of p57Kip2 is of any clinical relevance. The mechanisms behind this pro-apoptotic effect also remain to be investigated. p57Kip2 potentiates staurosporine-induced caspase-3 activity. At the same time this CKI is a potential substrate for caspases. Where in the apoptotic process, upstream and/or down-stream of the mitochondrial effects, does it act? Furthermore, is a potential caspase-mediated cleavage of p57Kip2 important in such a mechanism? In addition, we hypothesize that the potential cleavage site for caspases is located in the N-terminal of human p57Kip2 (Fig.5). Although the size of the caspase dependent C-terminal cleavage product (42kD) corresponds to a cleavage site in this region, it still remains to be tested experimentally. We also know that p57Kip2 is sufficient to reconstitute many of the anti-proliferative effects of GC treatment in our model system. Therefore, it would be of interest to investigate, using anti-sense techniques, if p57Kip2 is necessary for the anti-proliferative effects observed in GC-treated cells.

Another important task is to identify in which in vivo systems the GC-mediated induction of p57Kip2 occurs. Apart from the already investigated induction during lung development, one possible candidate is the epiphyseal growth plate in the bones, a tissue known to be mito-inhibited by GCs (reviewed in (Klaus et al., 2000)). In this tissue the non-proliferating hypertrophic chondrocytes have been shown to express p57Kip2 (Yan et al., 1997; Zhang et al., 1997). Furthermore, p57Kip2−/− mice display increased proliferation in the chondrocytes of the epiphyseal growth plate, which is associated with a delayed differentiation and altered endochondral bone formation. Indeed, unpublished data from primary human ear chondrocytes indicate that GCs
transiently induce p57Kip2 protein expression (unpublished data). This induction may well be associated with an early switch in proliferation and differentiation of these cells. Considering the lethal developmental defects in the p57Kip2 deficient mice (Yan et al., 1997) (Zhang et al., 1997) (Takahashi et al., 2000), it would also be of great importance to produce temporally and spatially specific p57Kip2 knock out mice, in order to elucidate the effects in individual tissues as well as potential effects in the adult life.

**Fig.7.** Proposed model for the GC-induced expression of p57Kip2 and effects of this CKI on several biological processes. Dashed arrows indicate effects where the mechanisms have not been fully elucidated. Indicated in the figure are the transcriptional induction of p57Kip2 after GC treatment (Paper I and II), and the stimulatory effect of p57Kip2 on apoptosis induced by cytotoxic stimuli (Paper IV). The involvement of p57Kip2 in differentiation has also been implicated both through inhibition of CDK2 and through direct effects, for instance by modulating the expression levels of MyoD during differentiation of muscle cells (Reyna et al., 1999; Reyna et al., 2000). In line with this, results presented in this thesis show that GCs induce p57Kip2 expression in the embryonal lung, and further implicate this protein to be involved in differentiation of the distal lung epithelium (Paper III).
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p57Kip2: a glucocorticoid-induced CDK inhibitor


